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APPLICATION OF ACTIVE PACKAGING FOR INCREASING MOULD-FREE SHELF LIFE OF BREAD

by

Eliana Silva

December, 2016

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Application of active packaging for increasing mould-free shelf life of bread

Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfill the requirements of Master of Science degree in Biotechnology and Innovation

by

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Resumo

Palavras chave: Embalagem ativa, Pão, Etanol, Tempo de vida útil, Extrato de folha de oliveira

A contaminação microbiana e as consequentes quebras, na indústria da panificação, causam grandes prejuízos a nível económico. As embalagens ativas têm vindo a ser desenvolvidas para proteger os alimentos, inibir ou retardar o crescimento microbiano, aumentando o tempo de vida útil dos alimentos. Paralelamente, o interesse em produtos naturais e substâncias não sintéticas, como potencial alternativa para aumentar o tempo de vida útil, tem aumentado devido à demanda dos consumidores. Neste contexto o objetivo desta dissertação é estudar uma embalagem ativa baseada em etanol, para uma receita tradicional de pão fatiado sem crosta, por forma a melhorar o seu tempo de vida útil.

Foi realizada uma revisão do estado-da-arte, definindo os principais fatores que afetam o tempo de vida útil do pão, explorando o uso de etanol como conservante e as potenciais atividades antioxidantes e antimicrobianas do extrato de folha de oliveira.

Foi produzido pão sem conservantes e embalado em sistemas de embalagem ativa baseados em etanol. Num caso o etanol foi aplicado diretamente por pulverização, noutro caso foram utilizados emissores ativos de etanol com duas concentrações diferentes. O pão foi embalado nestes sistemas ativos e armazenado a 23 ºC. O desempenho de cada sistema foi comparado através da monitorização de vários parâmetros físicos, químicos, sensoriais e microbiológicos, durante o armazenamento.

O etanol foi testado com sucesso, revelando um potencial alternativo aos conservantes convencionais na indústria de panificação. Os emissores de etanol demonstraram ser mais eficazes, sobretudo contra os bolores, comparativamente à aplicação direta de etanol por spray. Concluiu-se que os emissores mantêm uma concentração de etanol no *head-space* constante e com tendência para aumentar, enquanto que no caso do sistema da aplicação direta, a concentração é elevada inicialmente, mas decresce ao longo do tempo. Comparativamente às amostras de controlo, os emissores Antimold Mild® grau 20 aumentaram ca. de 70 % o tempo de vida útil do pão, enquanto que os Antimold Mild® grau 10 e o sistema de aplicação direta apenas aumentaram cerca de 57 % (10 e 13 dias, respetivamente).

Abstract

Keywords: Active packaging, Bread, Ethanol, Shelf life, Olive Leaf Extract

Microbial spoilage of bread and the consequent waste problem causes large economic losses for the bakery industry. Active systems of food packaging have been developed not only to protect food products, but also to inhibit or retard microbiological growth, extending food shelf life. At the same time, the interest in natural and non-synthesized substances, as a potential alternative to extend shelf life has increased regarding consumer's demand.

In this context, the objective of this dissertation is to study an active packaging, based on ethanol, for a traditional recipe of sliced bread without crust, in order to improve its shelf-life.

An analysis of the state-of-the-art was performed defining principal factors that affects shelf life of bread, exploring the use of ethanol as food preservative and the potential antioxidant and antimicrobial activities of olive leaf extract.

Bread, without chemical preservatives, was produced in the kitchen lab and packaged in active packaging systems based in ethanol. In one case the ethanol was applied directly by spray, and in the other case sachets with ethanol emitter at two concentrations were used. The bread was packaged in these active systems and stored at 23 ºC. The performance of each system was compared by monitoring several physical, chemical, sensorial and microbiological parameters during the storage time.

The ethanol was successfully tested, showing a potential alternative to conventional preservatives in bread industry. The ethanol emitters were more efficient, particularly against moulds, than the direct application system. It was concluded that the active emitters allowed for an ethanol concentration in the head-space constant and tending to increase, while the direct system showed a higher concentration in the beginning but decreasing ethanol concertation. In comparison with control samples, the Antimold Mild® grade 20 emitters increased bread's shelf life by 70 %, whereas Antimold Mild® grade 10 and ethanol directly applied at a concentration of 0.5 % increased it by 57 % (10 and 13 days, respectively).

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I dedicate this study to my dear parents, my patient boyfriend and my friends, for all their care and motivation.

To the ones that are no longer here, I give you my most heartfelt smile and pride in reaching this goal.

Contents

List of figures

List of tables

Abbreviations

AOAC - Association of Official Analytical Chemists
ATR – Attenuated Total Reflection
BHA - Butylated Hydroxyanisole
BHT - Butylated Hydroxytoluene
CAS - Chemical Abstracts Service
CEF - Food Contact Materials, Enzymes, Flavourings and Processing Aids
CFU – Colony Forming Unit
CTR – Control samples
DATEM - Diacetyl Tartaric Acid Esters of Monoglycerides
DSC - Differential Scanning Calorimetry
EC - European Commission
EE - Ethanol Emitter
EU – European Union
FCM – Food Contact Material
FDA - Food and Drug Administration
FR10 – samples with EE Antimold Mild® Grade 10
FR20 – samples with EE Antimold Mild® Grade 20
EFSA - European Food Safety Authority
EtOH 0,5% - Direct application of ethanol (0,5%) by spraying
EU - European Union
EVA - Ethyl Vinyl acetate
EVOH - Ethylene Vinyl Alcohol Copolymer
FTIR - Fourier Transform Infrared Spectroscopy
GC – Gas Chromatography
GC-FID – Gas Chromatography with Flame Ionization Detector
GRAS – Generally Recognized As Safe
HDPE - **High-Density Polyethylene**
ICMSF - International Commission on Microbiological Specifications for Foods
LDPE - Low density polyethylene
LLDPE - Low linear density polyethylene
MFSL – Mould-Free Shelf Life
NIST - National Institute of Standards and Technology
OA – Oxygen Absorber
OEO - Oregano essential oil
OLE - Olive leaf extract
PA - Polyamide
PE - Polyethylene

PET - Poly(ethylene terephthalate)

PP - Polypropylene

PS - Polystyrene

PVDC - Polyvinylidene chloride

SML – Specific Migration Limit

SSL - Sodium Stearoyl Lactylate

TDI – Tolerable Daily Intake

TBHQ - Tertiary butylhydroquinone

1. Introduction

1.1. Bread

Bread, in various forms, has been a staple in the diet of several population groups for many centuries and its history traces back to about 3000 B.C. The development of cereal foods has proceeded through several stages, from roasted grain to gruels, to flat breads and finally to leavened bread loaves (Nout et al., 2007).

Bread is made by mixing wheat or rye flour, water, leavening agent and salt. Other ingredients, which may be added, include flours of other cereals (e.g., malt flour and soy flour), fat, emulsifiers, milk and milk products, fruit and gluten (Robertson, 2013; Nout et al., 2007).

The function of water (50 to 60% of flour weight) is to hydrate the starch and gluten, enabling mixing and kneading of a viscoelastic dough that retains the carbon dioxide gas formed during fermentation (Nout et al., 2007). The most commonly used leavening agent is baker's yeast, *Saccharomyces cerevisiae* (Jenson, 1998).

The function of salt is to moderate the fermentation rate in order to obtain a steady production of gas that can be adequately retained in the dough (Nout et al., 2007).

After mixing, the individual dough pieces are shaped, expanded through fermentation (the dough get at least double of its volume) and baked in a hot-air or steam oven for 20 to 40 min at temperatures ranging from 180 to 230 ºC (Nout et al., 2007; Robertson, 2013).

Market in European Union (EU) is relatively stable showing a low growth in western countries (Axel et al., 2016). Britain and Ireland have the lowest consumption of bread in Europe with an annual consumption of less than 50 kg per person per year (The Federation of Bakers, 2012). This could be associated to an effort on reducing the total energy intake and to a negative perception of bread on health-related issues, such as wheat allergy and other gastrointestinal problems (O'Connor, 2012). Germans and Austrians consume bread at the highest level of 80 kg bread per person per year (The Federation of Bakers, 2012).

The production of sliced bread is a growing trend in many countries of Europe, probably due to an increasing consumer demand for convenience, which means less time spent on home food preparation and consumption (The Federation of Bakers, 2012).

Additionally, over the last decade, the number of health-concerned consumers has increased, leading to a higher market demand for "natural" and "wholesome" foods, without chemical preservatives and additives (Axel et al., 2016).

Principal spoilage factors in bakery products

According to Smith *et al.* 2004, bakery products can be classified on the basis of their pH, moisture content, and water activity (a_w). These are the main chemical factors that influence the bread shelf life. The availability of water is a key factor on bread's shelf life, more than its total moisture content. The availability of water is measured by the water activity of a food and is defined as the ratio of the vapour pressure of water in food, to the vapour pressure of pure water at the same temperature. Generally, loaf bread is a product with a high water activity (0,95-0,98) and low acidity (5.5-5.7). The movement of water vapour from a food to the surrounding air depends on the moisture content, composition of the food, temperature and humidity of the air (Farkas, 2007).

Bread spoilage consists in any change in the condition of a product that makes it less palatable at the time of consumption (Smith et al., 2004), wherein the most predominant problems are classified in three types:

Physical – humidity loss, staling, among others;

Chemical – lipid oxidation;

Microbiological – mould and yeast growth.

These deterioration problems are described in more detail over the subsections below.

Physic deterioration

The major changes that occur after baking are moisture redistribution, starch retrogradation, increasing firmness, and loss of aroma and flavour (Smith et al., 2004).

The staling process starts in the moment bread is removed from the oven and becomes sensorially detectable in 1-2 days (Szczesniak, 1998). Although it has been studied for more than a century, bread staling has not been eliminated and remains responsible for huge economic losses to both baking industry and the consumer (Gray & Bemiller, 2003). Staling has been defined as "*almost any change, short of microbiological spoilage, that occurs in bread or other products, during the post baking period, making it less acceptable to the consumer*" (Smith et al., 2004).

Bread staling can occur in the crust or in the crumb. Crust staling is generally caused by moisture transfer from the crumb to the crust, resulting in a soft, leathery texture and is generally less objectionable than crumb staling (Robertson, 2013). From the sensorial standpoint, staling reduces the springback and softness of the crumb and increases its crumbliness and dryness in the mouth. From the physical standpoint, staling causes increase in firmness, crumbliness, and starch crystallinity; and decrease in absorption capacity, susceptibility of starch to enzymatic attack and soluble starch content (Kulp and Ponte, 1981 cit. by Szczesniak, 1998).

Different factors influence the staling phenomenon, such as moisture migration, baking time and temperature and storage temperature. However, it is difficult to determine cause-and-effect relationships because of synergies between formulation, processing and storage conditions (Gray & Bemiller, 2003). The degree and rate of crystallization of starch components, specifically of the non-linear amylopectin fraction, is considered to be the main responsible for staling. Complex interaction between starch

polymers, lipids, and flour proteins is thought to inhibit the aggregation of amylose and amylopectin (Smith et al., 2004).

The introduction of antistaling agents as enzymes (proteases, α-amilases, lipoxygenases, lipases, etc.) and emulsifiers like diacetyl tartaric acid esters of monoglycerides (DATEM) and sodium stearoyl lactylate (SSL) among others, has the purpose of maintaining crumb softness over extended periods of storage by retarding the process of staling (Cauvain, 2015; Gray & Bemiller, 2003).

There are other problems like loss or gain of moisture content, particularly when the bread is to be sliced and packaged before sale. During bread cooling, hotter (around 150 ºC) and dryer (1-2% moisture content) crusts will cause water to move outwards, from the crumb to the crust and then to the atmosphere (Robertson, 2013). Excessive drying in cooler or desajusted parameters of temperature and relative humidity result in weight loss and poor crumb quality. If the moisture content of crust rises during cooling, the texture of the crust becomes leathery and tough and the attractive crispness of freshly baked bread is lost (Robertson, 2013).

The loss of crust crispness is an important and beneficial change in sandwich bread types because consumers can only assess freshness by squeezing the loaf, knowing that fresh bread has little resistance to squeezing and will rapidly spring back to its original shape (Cauvain and Young, 2008 cit. by Robertson, 2013). Problems with loss of crust cripness as a result of moisture gain or excessive moisture loss during storage can be controlled by a selection of a less permeable packaging material (Robertson, 2013).

Lipid oxidation

Oxidative rancidity results in the breakdown of unsaturated fatty acids by oxygen through an autolytic free-radical mechanism causing off-flavours. Consequently, malodorous aldehydes, ketones, and short chain fatty acids are formed. These free radicals and peroxides, formed during lipid oxidation, may lead to even more detrimental effects on food quality by bleaching pigments, destroying certain vitamins and protein degradation (Smith et al., 2004). The most evident oxidative changes are related with “aged”, “dusty”, “rancid”, “bitter” and “sickly sweet” flavor and odor characterizations (Gray, 2010).

Microbiological spoilage

While physical and chemical spoilage limits the shelf life of low and intermediate moisture bakery products, microbiological spoilage by bacteria, yeast and molds is the concern in high moisture products (Saranraj & Geetha, 2012). In order to inhibit spoilage microorganisms and consequent production of off-flavors and undesirable changes in color or/and texture, it is important to understand some of the microrganism’s requirements (Otoni et al., 2016). In Table 1 foodborne microrganisms are grouped according their minimal a_w requirements. In the vital range of a_w, decreasing the a_w increases the lag phase, therfore delaying the microrganism growth (Farkas, 2007).

Table 1. Minimal a_w levels required for growth of foodborne microorganisms at 25 ºC (Adapted from Farkas, 2007).

Group of microrganisms	Minimal a_w required
Most bacteria	0,91-0,88
Most yeasts	0,88
"Regular" molds	0,80
Halophilic bacteria	0,75
Xerotolerant molds	0,71
Xerophilic molds and osmophilic yeasts	0,62-0,60

A potencial reduced a_w and pH values, the residual ethanol from yeast fermentation, and the pasteurization effect during baking are factors that contribute to minimize the rapid mould spoilage of bread (Danyluk et al., 2007). Even though molds spores and vegetative microbial cells are killed during baking, the most common source of microbial spoilage of bread is mould growth (Cauvain, 2015; Danyluk et al., 2007). The re-contamination mostly occurs during cooling, slicing, packaging and storage resulting from atmosphere (Cauvain, 2015). The moulds frequently involved are *Penicillium*, *Aspergillus*, *Eurotium* and *Wallemia* species (Dagnas et al. , 2014; Cauvain, 2015). In wheat breads it has also been observed *Cladosporium*, *Mucorales*, *Neurospora* and *Rhizopus (nigricans) stolonifer* which is the common black bread mould (Cauvain, 2015). The most predominant mould characteristics like colour and appearance of colonies in bakery products are described in Table 2.

Table 2. Characteristics of bread moulds (Seiler, 1992 cit. by Cauvain, 2015).

Mould	Colony colour	Colony appearance	Comments
Penicillium spp.	Blue/green	Flat, spreads rather slowly	The most common type of bread mould
Aspergillus niger	Black	Fluffy, spreading with spore heads often clearly visible	Frequently present
Aspergillus flavus	Olive green	-	-
Aspergillus candidus	Cream	-	-
Aspergillus glaucus	Pale green	-	-
Cladosporium spp.	Dark olive green	Flat, spreads slowly	Often present on damp bakery walls, commonly encountered

Mould	Colony colour	Colony appearance	Comments
Neurospora sitophila	Salmon pink	Very fluffy and fast spreading	Will grow very rapidly on moist bread
Rhizopus nigricans	Grey/black	Very fluffy and fast spreading	Will grow very rapidly on moist bread
Mucor spp.	Grey	-	-

Less common, but still causing problems in warm and humid weather, is the bacterial spoilage known as 'rope', caused by growth of *Bacillus species*. The spores easily survive to baking, germinating and growing within 36–48 h inside the loaf, forming a characteristically soft, stringy, brown mass with an odour of ripe pineapple or melon (Cauvain, 2015; Danyluk et al., 2007). The addition of preservatives and the fulfilment of good hygiene and bakery practices turned 'rope' rarer. However, in countries where salt levels are being reduced there is an increased risk of rope growth (Cauvain, 2015).

Besides moulds and bacteria, yeasts can cause surface spoilage of bread, mainly *Pichia burtonii* ("Chalk mould") (Saranraj & Geetha, 2012). Other wild yeast includes *Trichosporon variable*, *Saccharomyces* and *Zygosaccharomyces* (Cauvain, 2015). Bad cleaning practices resulting in physical contact with dirty equipment or infected high-sugar foods (ideal substrate for osmophilic yeasts) is the main source of this type of contamination (Cauvain, 2015).

The yeast problems in bakery products can be divided into two types (Cauvain, 2015; Saranraj & Geetha, 2012):

Visible yeast which grows on the surface of the bread in white or pinkish patches which can readily be confused with mould growth (yeasts produce single cells and reproduce by budding);

Fermentative spoilage associated with alcoholic and essence odours and hence osmophilic yeasts. The spoilage is perceived by the development of an 'alcoholic' or 'estery' off-odour depending on the species of yeast present.

The shelf life increase of bread is of great importance, as it is related to the productivity and profitability of a company (Katsinis, et al., 2008). The industry often uses preservatives like propionic acid (E280), sodium propionate (E281), calcium sorbate (E203), calcium propionate (E282), potassium propionate (E282), among others, to prevent or inhibit microbial spoilage (Cauvain, 2015). Spray of ethanol on the product is used in some baking industries as anti-mould additive. However, recent market demand for high-quality, minimally processed, and synthetic preservative-free products, has led to increasing the attention toward preservation techniques using natural compounds (Ryan et al. 2008 cit. by Passarinho et al., 2014). The industry has to respond with alternatives to promote the extension of bread mould-free shelf life (MFSL). Active packaging stands out as an interesting alternative to traditional techniques and it consists on the incorporation of active agents into the packaging material instead of directly applying into food (Otoni et al., 2016; Passarinho et al., 2014).

1.2. Active packaging

Conventional food packaging represents a useful tool to extend the shelf life, maintain quality, and assure safety of the food product, especially for consumers whose access to fresh bread is limited (Cozmuta et al., 2015; Malhotra et al., 2015).

The environment to which the product is exposed during distribution and storage, the extrinsic factors such as storage temperature and relative humidity, the surrounding gas composition and exposure to light, are key in determining the product shelf-life (Cauvain, 2015 and Pereira de Abreu, et al., 2012). Apart from temperature, those factors may be controlled by packaging, through its barrier properties to water vapour, oxygen and other gases and light.

Active packaging is, according to Regulation (EC) nº 1935/2004, materials and articles that are intended to extend the shelf life, maintaining or improving the condition of packaged food. Active food contact materials are designed to release or absorb substances from the packaged food or the environment surrounding the food. Regulation (EC) nº 450/2009 establishes specific rules for active and intelligent materials and articles, including the definition of terms like "*released active substances*" and "*releasing active materials and articles*". Substances responsible for the active or intelligent function must be evaluated by EFSA before their inclusion into a positive Community list of authorised substances. A review was carried out on EFSA Scientific Opinions regarding active substances for use in active food contact materials (this review is presented on Appendix A, see Table 12).

Active packaging can be used in different configurations, such as sachets containing volatile substances; direct incorporation of substances (volatile or no volatile substances) in polymers; coating or adsorbing onto polymers surfaces; immobilization to polymers (by ion or covalent linkages) (Nerín, 2014; Robertson, 2013).

1.2.1 Antimicrobial packaging systems

Antimicrobial active packaging is developed to reduce, retard or inhibit the growth of targeted microorganisms (Otoni et al., 2016; Sung et al., 2013), and can be classified as (Jideani & Vogt, 2015):

a) Migrating system - those that contain an antimicrobial volatile agent which is released from the packaging film into the package headspace and onto the food surface (Figure 1a).
b) Non-migrating system - those which are effective against surface growth of microorganism without migration. The food is in direct contact with the antimicrobial surface allowing for the non-volatile antimicrobials undertake its antimicrobial function (Figure 1b).

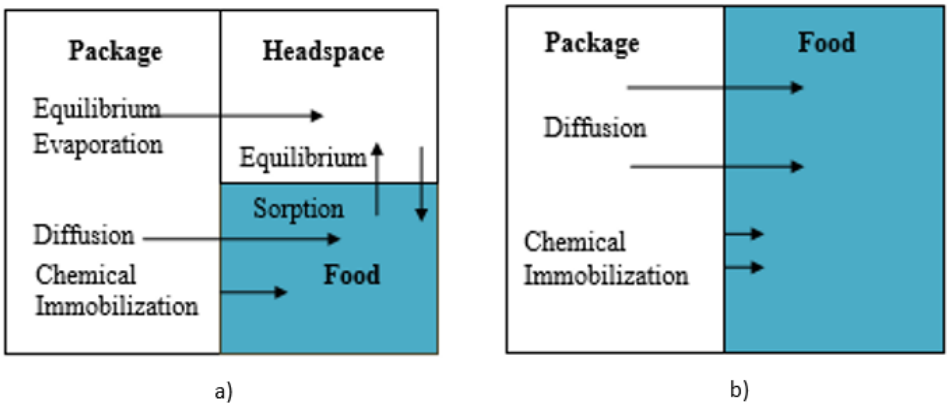

Figure 1. Diffusion of an antimicrobial agent in a migration system (a) and non-migration system (b) (Brody, Strupinsky, & Kline, 2001).

Sachets and pads are one of the most successful applications of active food packaging. The sachets generate antimicrobial compounds *in situ* or carry and release them into the food through the sachet (Otoni et al., 2016).

Many natural substances have been described as antimicrobial and antioxidant activity in food matrices. Table 3 describes three examples of plant/spice extracts and their potential antimicrobial activity.

Several studies have shown that antimicrobial packaging systems can increase the shelf life of packaged food by extending the lag phase and reducing the growth rate of spoilage microorganisms (Appendini & Hotchkiss, 2002).

Table 3. Antimicrobial activity of natural antimicrobial agents.

Antimicrobial agent	**Amount added (%)**	**Packaging material**	**Test type**	**Findings**	**References**
Grape fruit seed extract	0.5–1.5 (w/w)	Biodegradable chitosan-based composite films	Sliced bread	Inhibited the proliferation of fungal growth	(Tan et al., 2015)
Olive leaf extract (OLE)	0.5-2 (w/v)	After treatment, all samples were bacteriologically evaluated, with no packaging process	Raw peeled undeveined shrimp	Reduction of the aerobic and coliforms bacteria at least 1 log cycle at.1%	(Ahmed et al., 2014)

Antimicrobial agent	**Amount added (%)**	**Packaging material**	**Test type**	**Findings**	**References**
Antimicrobial sachet (4 g of resin previously incorporated with OEO)	5 - 15% (v/w)	Metallized polypropylene bags	Preservative-free sliced bread	Efficient in controlling *Penicillium sp.* and fungal spoilage	(Passarinho et al., 2014)

The use of natural extracts in plastic films, to avoid microbial food spoilage, is an attractive option for both packaging manufacturers and demanding consumers (Gutiérrez et al., 2009). These extracts, such as essential oils and their constituents, are categorized as "flavourings" by EU and as Generally Recognized As Safe (GRAS) by US Food and Drug Administration (FDA).

Essential oils (EOs) are characterised by their volatile nature, due to a high content of aromatic constituents of low molecular weight. Their antimicrobial activity is attributed to their major phenolic compounds present in concentrations of as much as 85%. Garlic, cinnamon, lemongrass and oregano are the most common sources of EOs and have been used more recently in food preservation (Otoni et al., 2016).

Olive leaf extract has raised great interest mainly due to three different factors: low-cost source to obtain bioactive compounds, exploitation of residues (essentially from oil industry), antioxidant activity and corresponding health effects (Martín-Vertedor et al., 2016). Olive leaves contain a large variety of phenolic derivatives which mainly consist of simple phenols (tyrosol, hydrotyrosol, caffeic acid, ρ-coumaric acid, vanillic acid, etc.), flavonoids (kaempferol, quercetin, luteolin, apignin, etc.) and secoiricoids like oleuropein, oleuropein glucoside and ligstroside (Martín-Vertedor et al., 2016). Oleuropein has been described as a potent antioxidant with anti-inflammatory properties, aiding in cardiac disease prevention (Gamli, 2016; Musa & Bertrand, 2016). Hydroxytyrosol, a degradation product of oleuropein, protects against atherosclerosis and prevents diabetic neuropathy. Furthermore, it has antimicrobial, anti-cancer, anti-viral, hypoglycemic, hypolipidemic, hypocholesterolic and free radical-scavenging effects (Gamli, 2016; Musa & Bertrand, 2016). Vasodilation and relaxing properties have been associated with secoiridoid derivatives (Musa & Bertrand, 2016).

Musa and Bertrand (2016) concluded that OLE has antimicrobial activity against some microorganisms responsible for human infections such as *B. cereus*, *B.subtilis*, *S. aureus* (Gram positive), *E. coli*, *P. aeruginosa*, *K. pneumoniae* (Gram negative) bacteria, *C. albicans* and *C. neoformans* (fungi). The oleuropein antimicrobial activity is higher against Gram positive than to Gram negative bacteria (Gamli, 2016).

The antioxidant properties of OLE can improve bread quality, by decreasing lipid oxidation and inhibiting the growth of most common microorganisms related with bread spoilage. This represents an interesting combination that could improve consumer health, product shelf life and functional value.

1.2.2. Use of ethanol as food preservative

Ethanol has a natural occurrence in bread because it is produced in the leavening process (an alcoholic fermentation) together with carbon dioxide. Ethanol is removed from dough during baking and its use as bread preservative is not uncommon (Kalathenos & Russell, 2003; Axel et al., 2016). The antimicrobial activity of ethanol has been well documented for its effective extension of bread shelf life, at concentration levels of 0.5% to 3.5% of loaf (Katsinis et al., 2008). Ethanol vapours have been shown to be effective in controlling moulds species including *Aspergillus* and *Penicillium* species, several bacteria species including *Staphylococcus, Salmonella, E. coli* and some yeast species (Brody et al., 2001; Robertson, 2013; Smith et al., 2004).

Ethanol can be used by directly by spraying or through ethanol emitters (EE). In Portugal's baking industry, it is often used as a solvent for spraying aroma on cakes prior to packaging. Ethanol emitters are used mainly in Japan to extend the shelf life of high-moisture bakery products by up to 20 times. Japanese manufacturers have patented several applications of ethanol sachets, like Antimold-Mild® and Negamold® Tender (Freund Industrial Co., Ltd), that are placed alongside the food products within an enclosed packaging system. Table 4 summarizes results of recent studies performed in different types of bread based where ethanol was applied by surface spraying or emitter addition.

Ethanol is also indirectly used in the bread-making industry, for instance, in gliadin powders (microcapsules of gliadin plus linoleic acid in 60% (v/v) solution of ethanol), to improve loaf expansion and flavour and to increase the linoleic acid content in the consumer's diet (Kalathenos & Russell, 2003).

Table 4. Applications of ethanol on bread preservation.

Antimicrobial packaging system	Microorganisms	Type of bread	Results	References
EE combined with an oxygen absorber	Yeasts and moulds count	Wheat sliced bread	Reduction of from 5.1 to 2.0 log CFU g^{-1}	(Latou et al., 2010)
	B. cereus count		Reduction of from 4.7 to 2.0 log CFU g^{-1}	

Antimicrobial packaging system	Microorganisms	Type of bread	Results	References
Ethanol emitters	*Clostridium botulinum*	English style crumptes	EE with 2g of ethanol delayed toxin production for 10 days while complete inhibition (>21 days) was observed in all crumpets packaged with 4 or 6g of ethanol.	(Daifas et al., 2000)
Ethanol spraying at different concentrations	*C. sitophila and H. burtonii*	Sliced bread	Prevent spoilage by *C. sitophila* even at the lowest concentration tested (0.8%, w/w), while higher concentrations (2.0%, w/w) were needed to prevent spoilage by *H. burtonii*.	(Berni & Scaramuzza, 2013)
EE (LDPE-based sachets containing 3 ml of alcohol gel)	Yeasts and moulds counts	Ciabatta bread	The use of EE is shown to extend shelf life without the need of additional modified atmosphere gas. Acceptable limits for microbial quality were maintained for 16 days.	(Hempel et al., 2013)

Dao and Dantigny (2011) published a review about the effects of ethanol on inhibition of growth and germination of fungi and on inactivation of fungal spores. The effectiveness of ethanol vapour is well described, for example, by retarding apparition of *Penicillium* species like *P. digitatum* and *P. italicum* on oranges, and completely inactivating conidia of *A. niger* and *P. notatum* (exposing 25% of ethanol, during 3 and 1 day, respectively).

Although ethanol vapours benefit the shelf-life due to mould spoilage control and delay staling in bakery products, the potential smell (if the dose is not optimised) and the presence of the sachet in the package headspace can result in consumer resistance (Robertson, 2013; Smith et al., 2004). There are no known restrictions related to ethanol use as a preservative (Axel et al., 2016; Pittia et al., 2006), except in Italy, where the use of ethanol as an alternative preservative in packaged bread is allowed only up to a maximum concentration of 2% on a dry weight basis. Whether ethanol is added directly to the food or incorporated in a sachet, it has to be listed as “ethyl alcohol” or “ethanol” on the ingredients (Axel et al., 2016; Kalathenos & Russell, 2003).

The sachet material play an important role in controlling the diffusion of ethanol to the product. The sachet material should be porous or semipermeable. On the other hand, the external package material is required to be a good barrier to retain ethanol in the headspace (Otoni et al., 2016) and the packages must be well sealed to prevent leakage of the gases (Danyluk et al., 2007). For a packaging containing ethanol emitters the use of materials with ethanol vapour permeability lower than 2 g m^{-2} d^{-1} is recommended (Smith et al., 1995). Typical package films such as LDPE have an ethanol permeability of 20 to 30g m^{-2} day at 30 ºC (Brody et al., 2001). Otoni (2016) summarises information on some packaging materials used in antimicrobial emitting sachets and external packaging. The ethanol emitters are composed by LDPE, LLDPE or Paper/EVA laminate (Hempel et al., 2013; Ma, 2012; Day, 2008). On the contrary, the external packaging should be high barrier to ethanol and can be composed, for example, of PE/nylon laminate; PS/EVOH/PE tray and/or PVDC/nylon/LDPE (Smith et al., 1987; Hempel et al., 2013).

1.3. Main goals

The main goal of this master's thesis is to study an active packaging, based on ethanol, for a traditional recipe of sliced bread without crust, in order to improve the mould-free shelf-life. The following specific objectives were defined:

- Comparison of the performance of ethanol, regarding antimicrobial activity, when applied by two techniques: (1) by direct application in fresh product before packaging and (2) through slow releasing by ethanol emitters;
- Evaluation of the principal spoilage factors among the product's shelf-life;
- Evaluation of sensory characteristics to detect potential drawbacks of ethanol application;
- Test the usefulness of ethanol for its use with a commercial bakery product;
- Evaluating the potential of olive oil extract as a natural alternative to improve bread quality as an antioxidant and antimicrobial substance.

2. Materials and methods

2.1. Bread samples

Bread samples were prepared in the kitchen lab facilities. Bread based in a traditional recipe without preservatives was prepared. The following recipe was applied: 2,5 kg of wheat flour type 55, 500 g of corn flour, 1 L of boiling water, 20 g of salt, 500 ml of semi-skimmed milk, 200 g of butter with salt, 80 g of cream yeast (baker's yeast). Firstly, the salt was dissolved in boiling water, then the corn flour was added. Secondly, the wheat flour was incorporated slowly to help the dough's temperature rise. The baker's yeast was previously dissolved in milk, then mixed alternately with wheat flour in the dough. Lastly, the butter was slightly warmed to be easily spread and incorporated on the dough. The dough was divided in five loaves and introduced into metal baking molds. The dough was fermented for 30 minutes in a water bath (40-45 ºC). The dough temperature during fermentation was ca. 32 ºC. Baking was performed at 220 ºC over 25 minutes in an industrial oven (Zanussi Professional, easySteam, Italy). After baking, the bread was allowed to cool down at room temperature for 2-3 hours, after which the loaves crusts were removed and crumbs were sliced in 10-13 mm thick slices with an electric knife (EM 3062, Clatronic). The ends of each loaf were discarded. The bread was packaged and stored for evaluation according to what is described below.

2.2. Characterisation of bread properties

Nutritional value

According to the Regulation (EU) No 1169/2011, the mandatory nutrition declaration shall include the energy value, amount of fat, saturates, carbohydrate, sugars, protein and salt. The content of the mandatory nutrition declaration may be supplemented with an indication of the amounts of one or more of the following: mono-unsaturates, polyunsaturates, polyols, starch, fibre and/or any of the vitamins. The methods and results of chemical analysis used to determine the nutritional value of bread samples are listed on Table 13 and Table 14 of Appendix B, respectively.

pH

A bread suspension 10 g of ground bread: 100 ml of ultrapure water was homogenized using a magnetic stirrer for 30 minutes. pH was measured after standing for 10 minutes and decanting the suspension (pH & IonMeter GLP 22+ from Crison). Measurements were made in triplicate.

Moisture content

Moisture content determination was based on NP 2966 (1993) – *Norma Portuguesa: Derivados de cereais: Pão. Determinação dos teores de água e de matéria seca*.

Water activity

A water activity meter was used (Aqualab® Model Series 3 TE). The sample were blended and exposed in a disposable cup which is sealed in the equipment's chamber. Measurements were performed in replicate.

Texture

The texture profile analysis was measured in a double compression cycle using a TA.XT*plus* Texture Analyser (Stable Micro System, Godalming, Surrey,UK), equipped with a 36mm radius probe (P/36). The operating conditions included a load cell (5 kg), pre-test speed (1.0 mm/s), test speed (5.0 mm/s), post-test speed (5.0 mm/s), trigger force (5 g) and strain (25 %). Two slices were stacked (about 20-25 mm height) and measurements were made triplicates. The following parameters were determined (TTC Texture Technologies, n.d.):

- Hardness (g) – maximum peak force during the first compression;
- Springiness (mm) is how well a product physically springs back after it has been deformed during the first compression and has been allowed to wait for the target wait time between strokes;
- Cohesiveness – ratio of the positive force during the second compression to that during during the first compression. A bread is considered cohesive when, for example, it can withstand tearing when a cold butter is being spread on it;
- Chewiness (g mm) – product of hardness × springiness × cohesiveness;
- Resilience is how well a product "fights to regain its original height". Resilience is measured on the withdrawal of the first penetration, before the waiting period is started. It is calculated by dividing the upstroke energy of the first compression by the downstroke energy of the first compression).

Microbiological evaluation

For the microbiological analysis of bread, a 10 g sample was diluted with 90 ml of aseptically prepared Ringers solution (BR0052G from OXOID, England) and blended for 120 seconds in a stomacher (Smasher from AES Laboratoire). With this solution, dilutions from 10^{-1} to 10^{-5} were prepared and were plated onto Plate Count Agar (Ref. AEB150702 from bioMérieux S.A., France). The total count agar plates were incubated at 30 ºC and an enumeration of the colonies was made after 72 h. Yeast and mould detection was carried out using 10^{-1} to 10^{-3}dilutions onto Rose-Bengal Chloramphenicol Agar Base (Ref. CM0549 from OXOID, England), incubated at 25 ºC for 5 days. During the study, more dilutions were prepared if necessary, with the maximum used set at 10^{-7} for both cases. The calculation and expression of obtained results are based on ISO 7218:2007/Amd.1:2013(E).

Sensory analysis

A panel composed of 6 trained panelists was used to evaluate the bread quality changes over the storage time. Testing facilities were according to the requirements of ISO 8589: 2007). Each panelist

was presented with 4 samples (CTR, FR10, FR20, EtOH), assigned a random three digit identification code to allow for blind assessment. Panel was asked to rate descriptors comparatively to the control sample, according to a bi-dimensional ten-point scale. The perception of the panel concerning the intensity of the descriptors related to odor, flavor, hardness, springiness and after-taste were evaluated in a scale ranging from -10 (much less intense) to 10 (much more intense), being 0 the equal compared with control sample. To avoid bias from the panelists, the term *ethanol* was omitted from the Questionnaire. To verify if the panel recognized ethanol odor and/or flavor, panelists were asked to identify other relevant descriptors, allowing a differentiation of samples relative to control. This sensory evaluation was carried out after two days of storage (23 ºC and 65% of relative humidity).

The bread slice zone used to sensorial evaluation was similar to all panelists in order to minimize variation between samples, such as hardness, springiness and after-taste. Slices from each sample were cut from the center, in the same shape for all panelists. The survey was carried out in Qualtrics, after a pre-test that allowed a performance evaluation and consequent improvement. Panel scores were analyzed by Wilcoxon test in (IBM SPSS Statistics version 21).

2.3. Characterization of active packaging system materials

2.3.1. Plastic bags characterization

Coextruded unprinted PA/PE (85 µm) pre-formed packages (bags) were provided by a local producer (Alsecus – Comércio e Indústria SA,). The plastic bags were evaluated for their gas permeability at different temperatures.

Thickness analysis

The plastic films thickness was measured by a digital micrometer (Adamel Lhomargy MI20). The measurements were made in triplicate.

Plastic films permeability

The concept of permeability is normally associated with the quantitative evaluation of the barrier properties of a material (Siracusa, 2012). The permeability phenomenon: absorption from environment into the material, diffusion and desorption from the material into the environment, depends on the size, shape, and polarity of the permeant molecule and on the polarity, crystallinity, degree of cross-linking and polymer chain segmental motion of the polymer matrix (Siracusa, 2012). Packages made from thermoplastic polymers are permeable in varying degrees to small molecules such as gases, water vapor, organic vapors, and other low molecular weight compounds (Robertson, 2010). The permeation of gas through a polymer can be described by a diffusion model, using Henry and Fick's laws (Robertson, 2010):

$$Q = \frac{DS(p_1 - p_2)At}{X} \quad (1)$$

Here ***Q*** is the quantity of gas or vapor permeating a polymer of thickness ***X*** and surface area ***A*** in time ***t*** under a pressure gradient of p_1 on one side and p_2 on the other, where $p_1 > p_2$. D is the diffusion coefficient and ***S*** the solubility coefficient of the permeant; the product ***DS*** is referred to as the permeability (Robertson, 2010). The quantity of gas passing through the film is directly proportional to the difference in gas pressure on either side of the film, and inversely proportional to the thickness of the film. It is directly proportional to the time during which the permeation has been occurring, and to the exposed area.

The ethanol vapor transfer rate was measured in five replicates at three different temperatures: 23 ºC (65% relative humidity), 30 ºC and 40 ºC. Packages of 13.5 x 22.5 cm x cm (0.061 m^2) were filled with ca 5 ml of ethanol and sealed. The decrease of packages weight (Metler PM1200) was monitored over time and the slope of the linear portion of the graph was used to determine the permeance values, expressed in g m^{-2} day^{-1}.

The oxygen transmission rate was measured with an Ox-Tran® 2/21 (Mocon, USA) according to the ASTM F1307 Standard Test Method for Oxygen Transmission Rate Through Dry Packages Using a Coulometric Sensor. Measurements at 0% relative humidity and 23 ± 2 ºC were made in replicate. The following conditions were set: Barometric pressure - 753,06 Hg; permeant concentration (O_2) - 100%; cell testing time - 20 minutes; test mode - Convergence by 5 hours; test area: 50 cm^3.

2.3.2. Ethanol emitters characterization

The indirect application of ethanol was performed with ethanol emitter sachets Antimold Mild® grade 10 and grade 20 kindly supplied by a Japanese company (Freund Industrial Co, Ltd). The selection of the grades was based on the weight and water activity of bread samples. The product consists of a powder mixture (containing ethyl alcohol, silicon dioxide, water and a natural flavor) filled in a small sachet made of a material water and oil resistant laminate. The composition of this laminate was paper, polypropylene, ethylene-vinyl acetate copolymer. This materials identification was verified by Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC) whose methods are described in Appendix C. The thickness of the material was also determined. The active powder mixture was also analysed microscopically.

Microscopic visualization

The mixed powder was placed on the cover glass without immersion oil or coverslip to avoid possible dilution and disintegration, respectively. A Zeiss Model AXIO Imager.A1 microscope was used, coupled with a Canon Model Powershoot G9 digital camera.

2.4. Assessment of ethanol effect in packaged bread during storage

Bread packages of PA/PE were prepared with four to five slices with approximately 100 ± 20 g. Sealing was performed in a MULTIVAC A 300/41/42 (Multivac Sepp Haggenmüller KG, Wolfertschwenden). The ethanol treatments were as follows:

- CTR – control samples, with no application of ethanol;
- FR10 – samples with EE Antimold Mild® Grade 10 (one sachet);
- FR20 – samples with EE Antimold Mild® Grade 20 (one sachet);
- EtOH – direct application of ethanol on bread's surface, 0.5 % (w/w) of fermentation's absolute ethanol 99.5 % (AGA, S.A.), immediately before sealing. The amount of ethanol delivered by this manual spraying was quantified. A calibration was performed (see Figure 14, Appendix D).

Eight to ten packages of each treatment were prepared. Control samples (CTR), consisting of packages of same material with the same amount of bread but without the ethanol system or spray were also prepared.

Bread packaged samples were stored in a controlled room (23 ºC and 65% relative humidity) and samples were removed for evaluation at days 0, 3, 7, 10 and 13.

During the storage period, all samples were checked visually through the unopened packages to detect microbiological spoilage. At regular time intervals samples were analysed for: pH, water activity, moisture content, microbiological analysis and quantification of ethanol in package head-space.

2.5. Quantification of ethanol

Ethanol concentration initially applied to bread before package sealing in the EtOH experiment was quantified following a calibration curve prepared for the spraying system. Also, ethanol amount in the package head-space during the storage period for all experiments was measured by gas chromatography. These methods are described in Appendix D.

2.6. GC-MS – analysis of olive leaf extract

A solid-liquid extract of olive leaves (OLE) was performed by maceration of olive leaves during 48h at 23 ºC with 100ml of Sigma Aldrich ethanol (≥99,8%). A GC MS (Bruker Scion TQ) chromatography system was used, with BR5MS column (30m×0.25mm I.D.; Df: 0.25µm). Injector temperature was 300 ºC. Oven temperature was 40 ºC during the first 10 minutes, being gradually increased to 320 ºC at a rate of 5 ºC/min and left at 320 ºC for 10 minutes. Injection volume was 0.1µl (splitless time: 0.5 minutes). The ionization was performed by means of electron impact at 70Ev. Full scan was applied between 45 to 700m/z.

3. Results and Discussion

3.1. Characterization of active packaging materials

The identification of the polymers of each layer of the bread packages performed by DSC and IR analyses was discussed in Appendix C. It was confirmed that the material is composed by a PA layer and a PE inner layer, responsible for the sealing.

Observation under microscope of active powder mixture

The active mixture of the EE's sachet was observed under optical microscope (Figure 2). There were no observed differences between Antimold Mild® grade 10 and grade 20. Apparently, the type of mixture is the same in both sachets grades, only the amount per sachets is different (grade 10 sachets holding half (0.47 g) of the grade 20 sachet – 1.03 g).

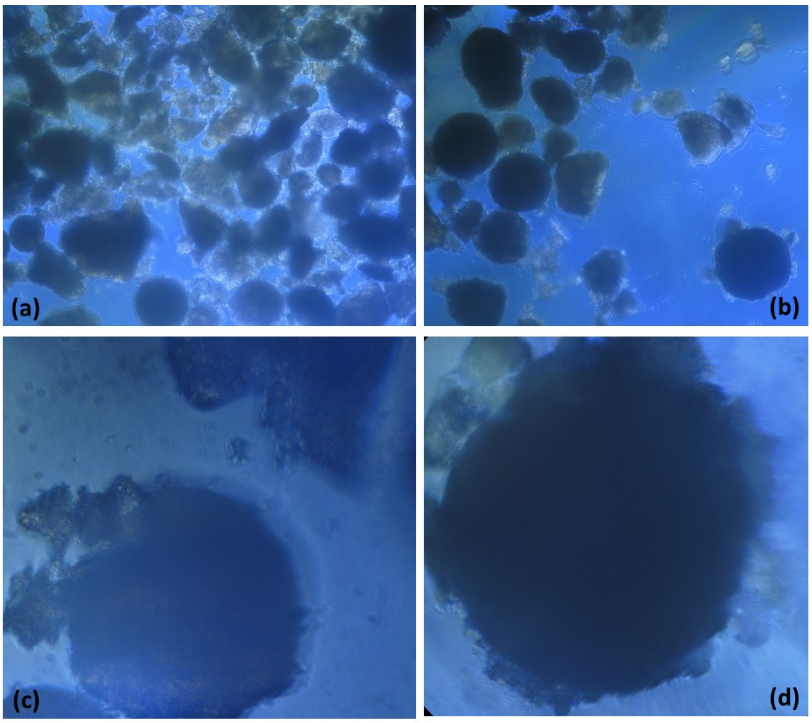

Figure 2. Mixed power of FR10 x10 (a), x40 (c) and FR20 x10 (b), x40 (d) visualized in a optical microscope.

3.2. Plastic films permeability

There is not available a standardised technique for measuring ethanol permeability as in the case of oxygen and water vapour. Therefore, the method used was developed in house adapted from standardised methods recognised for determination of water vapour transmission rate. The method is based on the decrease in mass of a package containing liquid ethanol and stored at constant conditions

of temperature and relative humidity. In this set up, it may be considered that inside the package ethanol is at its saturation pressure. The permeability to ethanol of the bread packaging was measured at three temperatures. The temperature effect on permeability is related to the effect on both solubility and diffusion. Temperature dependence on permeability is typically written in terms of an Arrhenius type relationship (Dury-Brun et al., 2007; Reinas, Oliveira, Pereira, Mahajan, Poças, 2016):

$$P = P_{ref} \cdot exp\left[-\frac{E_a}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \quad (2)$$

The permeability to oxygen was also measured but only at 23 ºC as indicated on the following table for the PA/PE film.

Table 5. Permeability to ethanol and oxygen of the plastic films, at different temperatures.

Material	Thickness (µm)	P to ethanol (g m^{-2} day)			P to O_2 (cc m^{-2} day)
		23 ºC	30 ºC	40 ºC	23 ºC/0% RH
PA/PE	85	2.3 ± 0.05	4.4 ± 0.05	13.4 ± 0.45	72 ± 0,8

There are no easily available published data on ethanol permeability of commercial films that could be used for comparison. The only data available found was for polyolefins based polymers from TOPAS Advanced Polymers (TOPAS, 2012). The results obtained are in good agreement with those available in the commercial reference literature. In accordance to the directions for use from Freund Company, at 40 ºC, a barrier nylon base film with values between 5 – 15 g/m^2/day, is suitable in terms of ethanol permeability. On the other hand, a simple LLDPE would not be suitable for values above 30 g m^{-2} day (40 ºC). It would be reasonable to use a film with no more than 10 g m^{-2} day (40 ºC). During the control days, ethanol odour passing through the plastic film could not be identified.

Regarding the permeability of oxygen a value of 72 ± 0,8 cc / (m^2.day) was found. The barrier to oxygen in this film is mainly due to the PA layer and PE does not contribute much for the oxygen barrier. However, PE has an important role in protecting the PA from moisture as the barrier of PA to oxygen depends a lot on the moisture of the film.

Based on the above results it was decided to use the PA/PE film for the storage experiments.

3.3. Nutritional composition of bread

The results for the nutritional analyses are presented on Table 14 of Appendix B, as the nutritional declaration of bread (see Table 15, Appendix B).

3.4. Evaluation of quality factors during bread storage

3.4.1. Microbiologic quality of bread

Spoilage of bread may easily occur because of the relatively high water activity and moderate pH. The typical microbiological quality of bread has been defined by ICMSF (1980), according to Jenson (1998), and is described on Table 6.

Table 6. Normal microbiological quality parameters for breads.

Criterion	Baked goods (CFU/g)
Moulds	10^1-10^3
Yeasts	10^1-10^3
Standard Plate count	10^1-10^3
Coliforms	0-10^2
E. coli	0-10^1
Coagulase-positive staphylococci	Not detected

Food is considered spoiled when a total microbial count of 10^7 CFU/g is exceeded. Most studies on antimicrobial packaging define a bacteria count of 10^7 CFU/ml (or g or cm^2) as an end-point for shelf-life (Sung et al., 2013).

Visually, it was possible to identify the bread spoilage, especially at day 3 (Figure 3). Most of control samples packages were affected by spoilage at day 3. Additionally, as presented on Figure 3, packages with FR10 and EtOH 0,5% treatments also showed contamination and spoilage at day 3, although not all replicates were affected, and some showed spoilage only at day 4 and 5. FR20 samples showed no mould spoilage, though it was possible to identify a white foam characteristic of yeasts.

Figure 3. Mould spoilage at day 3 in control, EtOH 0.5% and FR10 samples (left to right, respectively).

Table 7 presents the total microorganisms at 30 ºC of bread over storage time for the different ethanol treatments: application of spray and two grades of ethanol emitter active systems.

Table 7. Log10 CFU/g sample for total microbial count at 30 ºC in bread samples held under different packaging treatments.

	Day 0	Day 3	Day 7	Day 10	Day 17
Control	3,95 ± 0,52	7,23 ± 0,34	7,66 ± 0,32	-	-
EtOH 0,5%		5,64 ± 1,18	6,78 ± 0,70	7,43 ± 0,61	-
Freund 10		5,96 ± 0,61	6,73 ± 0,46	8,25 ± 0,05	-
Freund 20		5,08 ± 0,10	6,17 ± 0,15	6,82 ± 0,93	7,06 ± 0

Results indicate that regarding total microorganisms at 30 ºC, all ethanol systems retarded the microbial growth as compared to the control, i.e., the bread without ethanol application. Furthermore, FR 20 was the most effective treatment.

A reference should be made for the initial total count: the process of handling the bread samples (remove crust, slicing and packaging) was optimised to keep the initial bread flora as low as possible. Nevertheless, values of total counts of 10^3-10^4 CFU/g sample were recorded. As presented in Table 7, and considering the standard deviation, results indicate that the shelf life of bread without preservatives is approximately 3 days. A comparison with the control samples, FR20 increased shelf life by 70%, whereas FR10 and EtOH 0.5% increased it by 57% (10 and 13 days, respectively). The analyses were stopped after 7 days of storage for control samples, and after 10 days for EtOH0.5% and FR10 samples. The data is represented in Figure 4, after normalization with the initial total count. It can see that microbial growth was slower on FR20, followed by EtOH 0.5%, FR10 and Control samples. These results are in very good agreement with the concentration of ethanol in headspace (Figure 7): the higher the ethanol concentration, the slower the microorganism's growth, as could be expected. The direct application of ethanol at 0.5% had an effect in bread's spoilage microorganisms similar to that of FR 10 sachets, performing effectively at day 10. The use of fermented doughs as a start-up for new batches of bread could improve these results, by increasing bread shelf life due to its higher alcohol content. The surface spraying with ethanol, without using a preservative, is more effective regarding mould control than single preservatives at concentrations of 0.1% (Katsinis et al., 2008). In this study, ethanol (0.5%) showed to be less effective than ethanol vapour releasing. The evaporation losses occurring during ethanol application and package sealing, has as consequence a decrease in concentration, thus rending this system less efficient. This was confirmed by the chemical analyses for ethanol quantification (see Figure 15, Appendix D).

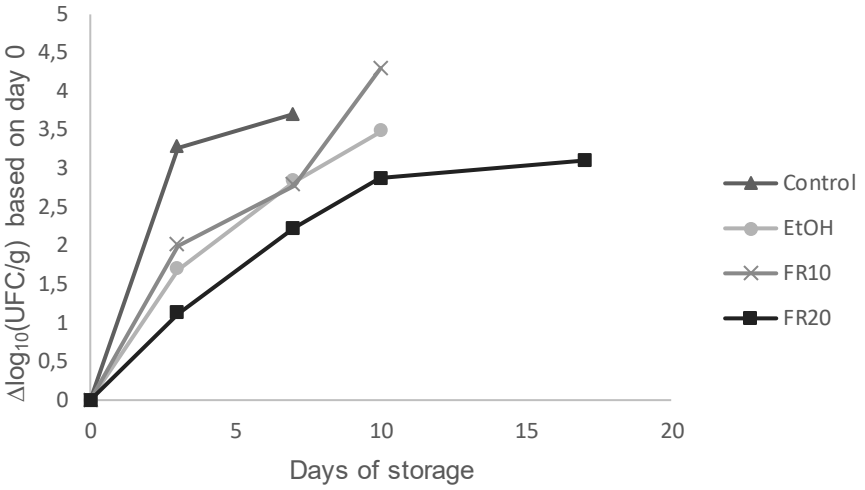

Figure 4. Total microorganisms count (normalised) change with storage time.

It should be noted that the ethanol concentration applied in this system was kept at levels that not effected the sensorial properties of the bread. It would be interesting to determine the ethanol concentration threshold that would be perceived by consumers. At industrial scale, the spraying method should allow for a maximised bread surface area treated without manual contact.

Yeasts and moulds were also quantified in the samples submitted to different treatments and results are presented in Table 8 and 9, respectively.

Table 8. Log10 CFU/g sample for yeasts in bread samples held under different packaging treatments.

	Day 0	Day 3	Day 7	Day 13	Day 17
Control	1,84 ± 0,78	4,95 ± 0,44	< 1	-	-
EtOH 0,5%		3,16 ± 0,04	5,08 ± 0,54	-	-
Freund 10		<1	4,26 ± 0,00	-	-
Freund 20		2,94 ± 0,43	4,91 ± 0,33	6,11 ± 0,15	6,63 ± 0,16

Table 9. Log10 CFU/g sample for moulds in bread samples held under different packaging treatments.

	Day 0	Day 3	Day 7	Day 13	Day 17
Control	0,52 ± 1,53	5,42 ± 0,62	6,80 ± 0,77	-	-
EtOH 0,5%		3,16 ± 0,04	5,08 ± 0,54	-	-
Freund 10		4,24 ± 0,41	5,67 ± 1,02	-	-
Freund 20		< 1	< 1	< 1	< 1

Results indicate that ethanol systems are not so effective in controlling yeast as with controlling moulds. Changes in yeasts and mould counts relative to the initial count during the storage time are presented in Figure 5a and 5b, respectively. The difference in yeasts counts in samples packaged under the different ethanol systems, are not significant, showing for all systems values around 10^5 CFU/g at day 7. An application of an oxygen absorber could control yeast fermentation since the product has a high water activity value.

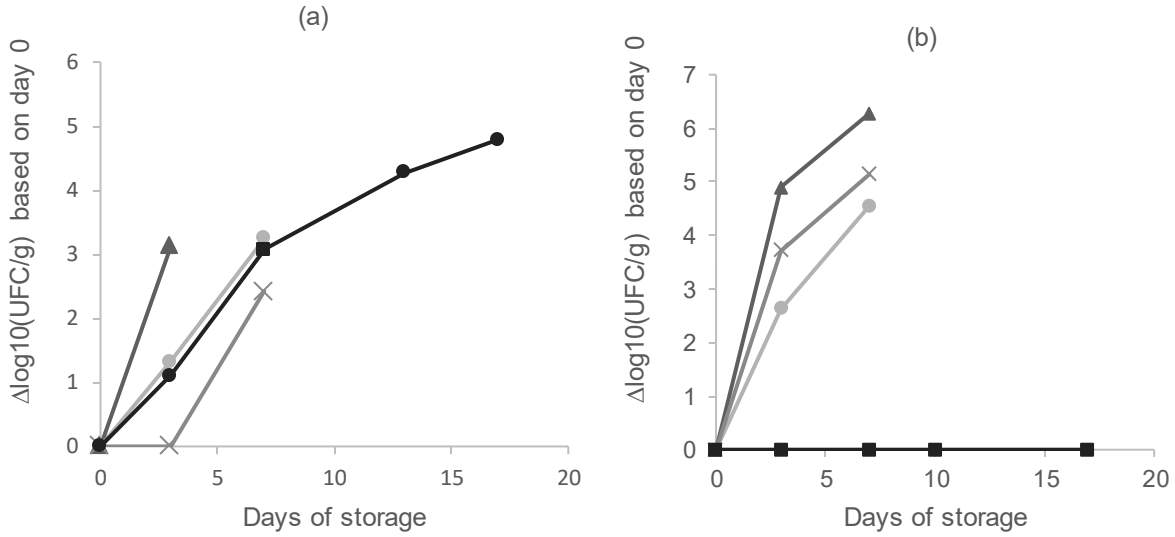

Figure 5. Effect of active packaging (× FR10, ■ FR20, ● EtOH, ▲ CTR) on (a) yeasts and (b) moulds.

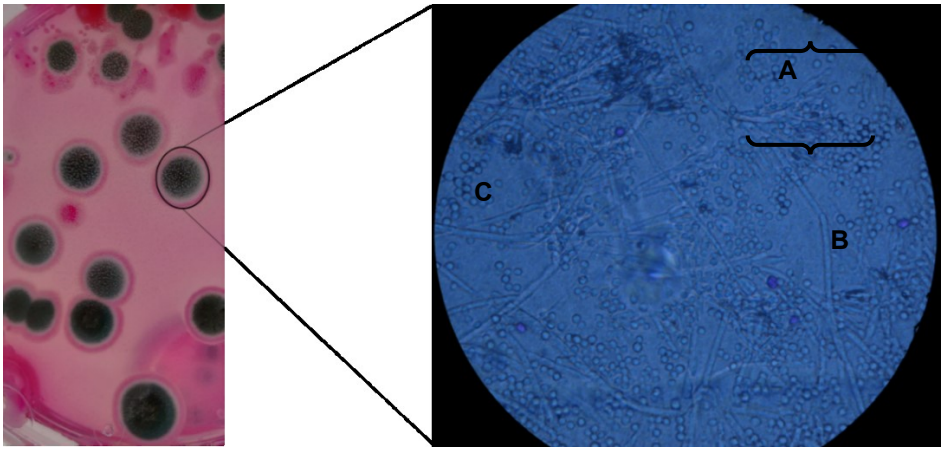

Figure 6. Mould spoilage from FR10 samples at day 3 (first dilution) – (A) coniodophores; (B) hypha; (C) conidias.

A significant mould growth occurred after 3 days of storage and mould counts of 10^6 CFU/g were found after 7 days. The mould spoilage was mostly from *Penicillium spp.* visually identified by its green colour and fast growing nature. The microscopic morphology is represented in Figure 6A where it is possibly

to identify the coniodophores and the hyphas (Figure 6B). The circular structures observed in Figure 6C are the conidias

All systems seems to be efficient in decreasing the growth rate of moulds but Freund 20 was proved to be the most significant as the differences between the control and systems EtOH and FR10 are minor. Nevertheless, Freund 20 samples remained without mould growth until day 17.

Samples handling, even with standardised procedures, and the geometry of the bread contribute to a large variability in the initial flora. To account for this variation the analyses of higher number of replicates would benefit the conclusions. Nevertheless, the present study demonstrates the higher efficiency of ethanol vapour, through active emitters, relative to direct application on surface. In principle, the interaction of ethanol with membranes at the lipid-water interface, weakening the hydrophobic barrier to the point where a free exchange of polar molecules is enabled, affecting the physical state and biological functions of cell membranes (Dao & Dantigny, 2011).

Bread shelf life extension could be more effective with the application of ethanol together with other preservatives, such as potassium sorbate and calcium propionate, than as acting as a single preservative. Additionally, certain ingredients like sugar and salt had influence over this synergic effects of ethanol, when applied with these preservatives (Katsinis et al., 2008).

3.4.2. Physicochemical changes

Headspace gas composition

The ethanol present in the packages headspace is not only from the active systems under study, but also due to the release from bread due to alcoholic fermentation process during baking. This results in a residual peak of ethanol in GC even in the control samples.

Figure 7 presents the ethanol concentration measured by GC in the packages head-space during storage time. Results show a low level mainly constant over time for the control samples. For the system EtOH 0,5% the concentration decreases over time due to permeation through the package. The active systems tend to give results increasing in the initial times corresponding to the gradual release from the emitters that may compensate losses through the packaging. At latter times the ethanol concentration tends to decrease due to permeation through the packaging. The concentration of ethanol in the samples FR20 was found to be higher than for sample FR10 during the whole period, as expected because the amount of emitter mixture in that sachet is double. Furthermore, the initial concentration of the system EtOH 0.5% is higher than that of the sachets, at packaging time, but it decreases to values lower than those of the sachets during the whole storage period. This explains way the active systems are more efficient than the direct application by spray. In the former cases a gradual release occurs that keeps the concentration of ethanol in higher values than that of the spray application. This latter gives higher concentrations in the beginning but ethanol is then lost by permeation through the package.

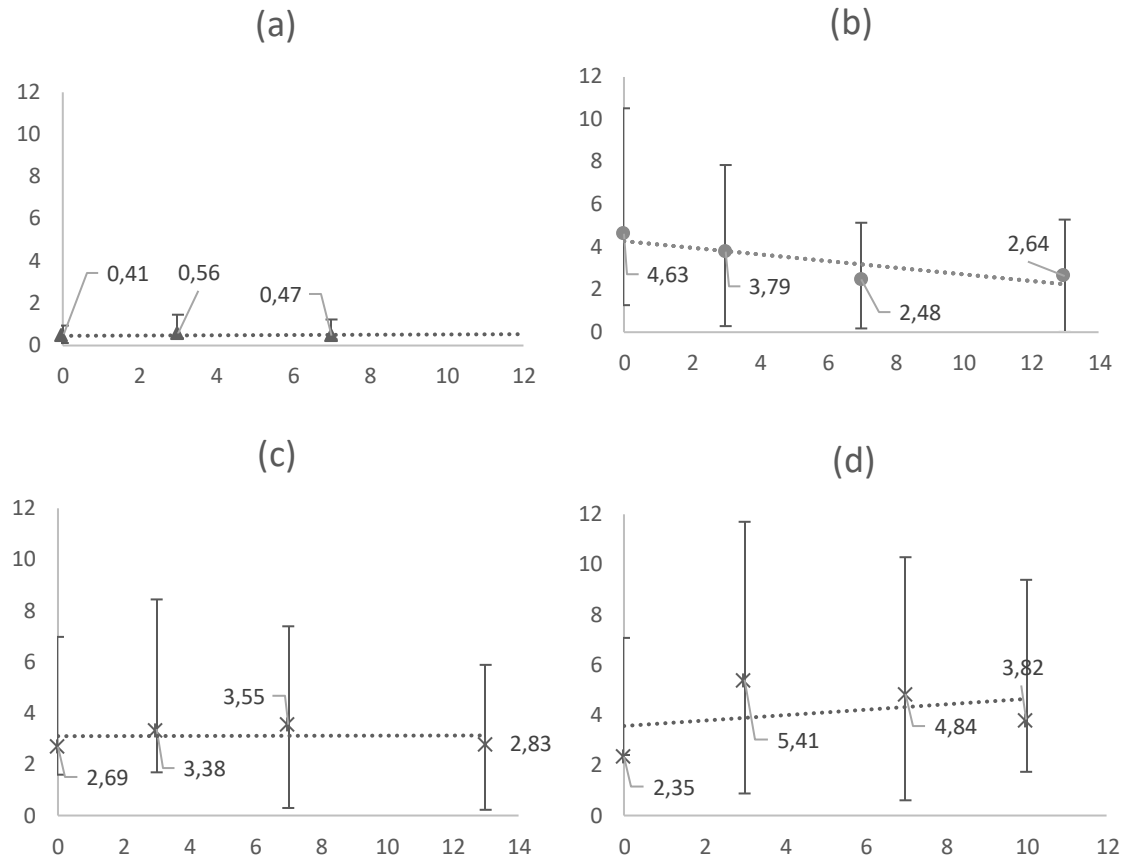

Figure 7. Ethanol concentration of (a) CTR, (b) EtOH 0,5%, (c) FR10 and (d) FR20 packages headspace during storage time.

As all the chromatographic analyses with manual injection and without internal standard addition, results show a high variability between replicates, which is shown in the standard deviation in Figure 7. Nevertheless, the results correlate very well with the results from the microbiological growth: FR20 samples were more efficient in controlling bread spoilage. EtOH 0,5% and FR10 presented a similar behaviour, and in fact Figure 7 shows the systems yield similar concentrations of ethanol.

Ethanol release from the emitters was measured during 12 days within empty packages with the same characteristics as the samples. This way it was possible to estimate the ethanol concentration in bread slices. Comparing FR10 and FR20 samples at day seven, an increase in ethanol concentration was identified when faced with control samples. FR20 had an increase of 5% and FR10 of about 2%. Approximately 97% of ethanol quantity in package headspace was retained in bread slices on FR10 samples, and 95% on FR20 samples.

pH

Bread samples' pH values ranged from 5,80 to 5,98. This change in pH is considered insufficient to have a significant inhibiting effect on microbial activities and the range is within the determination error. So, it can be considered that the pH was constant over the storage time, for all systems.

Water activity and water content

Water activity results ranged between 0,96 and 0,99 and the moisture content ranged from an average 39,0 to 40,3% These values are not considered significantly different given the dispersion between replicates and the method uncertainty.

Texture properties

Figure 8 presents an example of the output relating Force over displacement from which the assessment parameters were calculated.

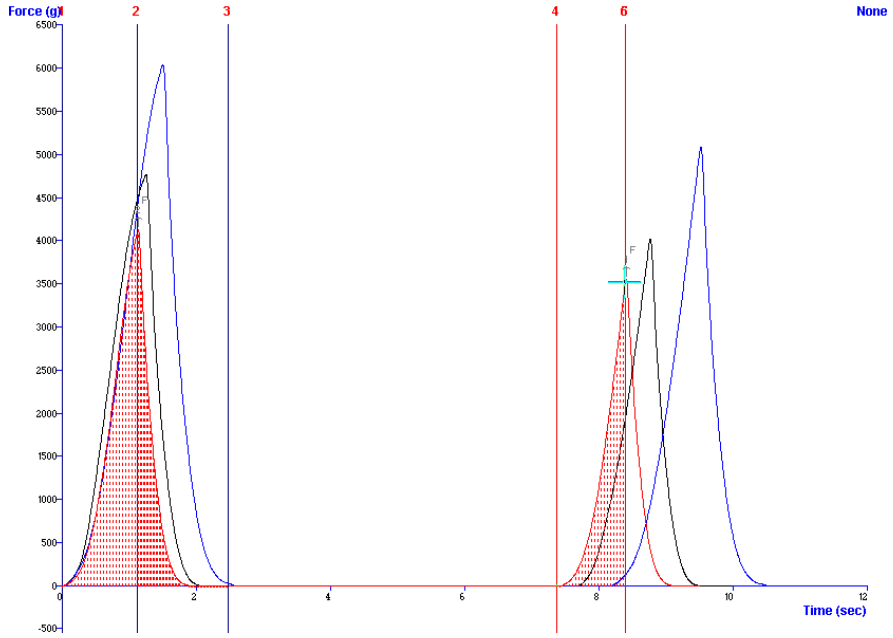

Figure 8. TPA of FR20 (red), FR10 (black) and EtOH 0,5% (blue) at day 3.

From this outputs the several parameters as indicated in the Material and Methods section were calculated. As an example, Figure 8 presents the results for the hardness (first peak represented - felt during the first bite; second peak – felt during the second bite) of all packaging systems used. As can be seen in Figure 9, there was no significant differences between the results from the different systems with ethanol and between those and the control samples, indicating that apparently, ethanol does not affect texture of the bread.

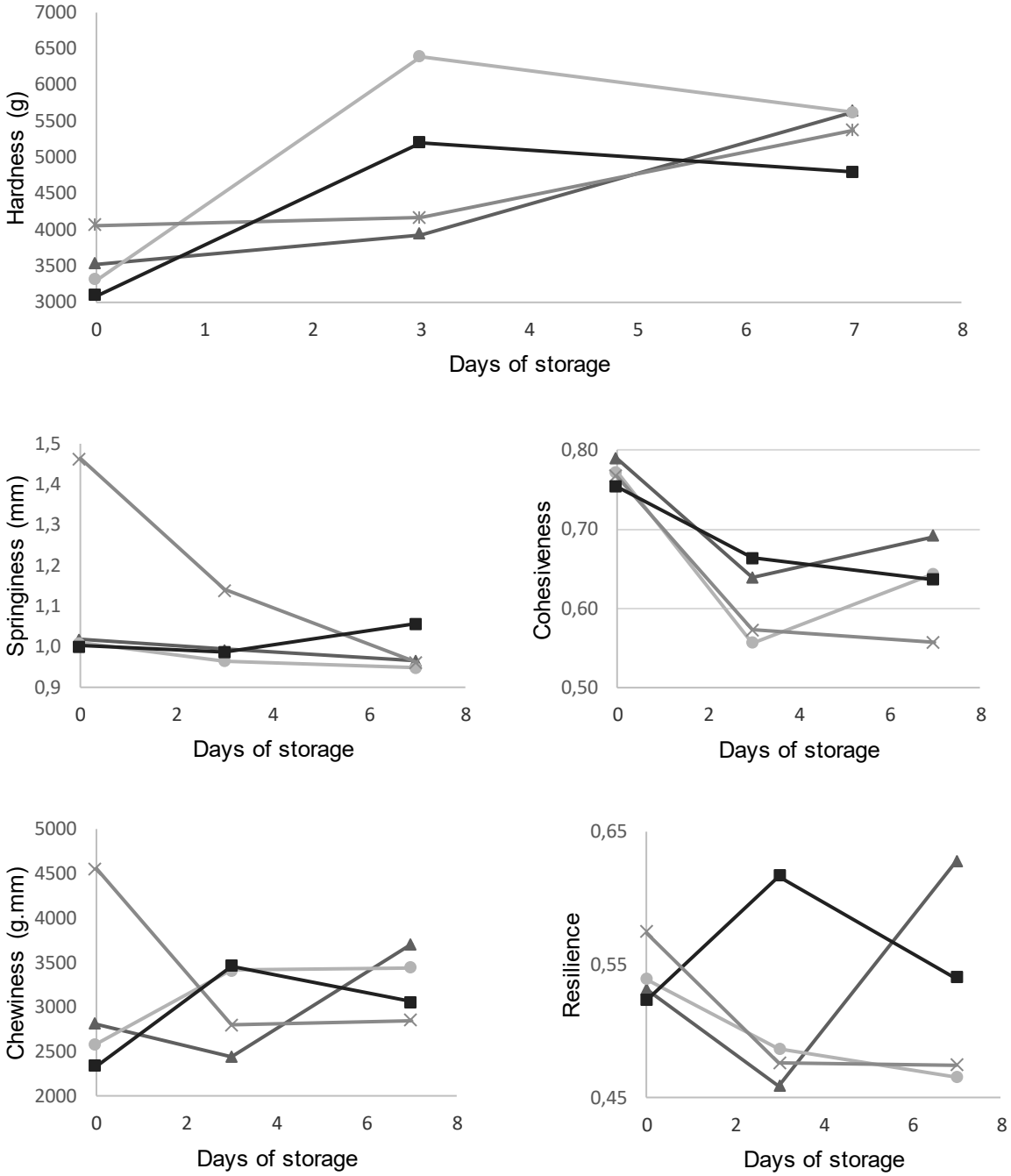

Figure 9. Texture parameters evaluated during storage time in all packaging systems (▲CTR, ● EtOH, ■ FR20, × FR10).

3.5. Sensorial perceptions vs textural profile analysis

Freshness is crucial in consumer acceptability, but its perception is a complex process that involves interaction of sensory sensations and social, demographic and product experiences influences (Heenan et al., 2008). A preliminary sensory analysis was performed for methodology optimisation concerning uniformed slice size, reduced thickness and decision regarding the target area for the results - the centre

of a bread slice. Having implemented the methodology and taking into account the microbiological results, a small batch was produced for sensory analysis only. This analysis was conducted after packaging under hygienic conditions to preserve the health and safety of panellists, on bread stored for 2 days close to the time that corresponded to the highest concentration of ethanol in the packages head-space. Table 10 presents the results from statistical analyses of the panellists answers to the questionnaire.

Table 10. Wilcoxon signed ranks test from different treatments applied to bread samples.

	p value		
	FR10 658	**EtOH 0,5% 329**	**FR20 411**
BREAD ODOUR	0,225	0,075	0,207
INTENSITY OF FERMENTATION ODOUR-	0,684	0,343	0,343
HARDNESS AT TOUCH	0,465	0,588	0,345
SPRINGINESS	0,463	0,345	0,463
HARDNESS AT MOUTH	0,5	**0,043**	0,593
SALTED FLAVOUR	0,317	0,655	0,317
BUTTERED FLAVOUR	0,246	0,339	**0,026**
AFTER-TASTE INTENSITY	1	1	0,593

The panellists did not find significant differences between the different samples and the control sample, relatively to the descriptors identified on Table 10, except for the butter flavour and hardness (felt in mouth) related to FR10 and EtOH 0,5% samples, respectively. There were significant evidences ($p=0,043 < 0,05$) to conclude that EtOH 0,5% samples presented slightly higher hardness (felt in mouth) when compared with control samples (with a mean rank of 3 on a scale of -10 to 10). Moreover, panellists considered that FR10 samples had more butter flavour when compared with control samples ($p= 0,026$). The panellists considered that it was very difficult to discriminate the following descriptors: odour and flavour, hardness and springiness. Furthermore, no other descriptors, essential to the bread's differentiation, were referred by panellists. Very importantly the panel did not identify ethanol odours or flavours in the evaluated samples.

3.6. Olive leaf extract profile

The olive leaf extract was screened to characterise the substances present and evaluate the potential for developing a dual active system, combining the properties of ethanol and of the active substances present in the olive leaf extract. Figure 10 presents the chromatogram obtained for the analyses of the ethanolic extract.

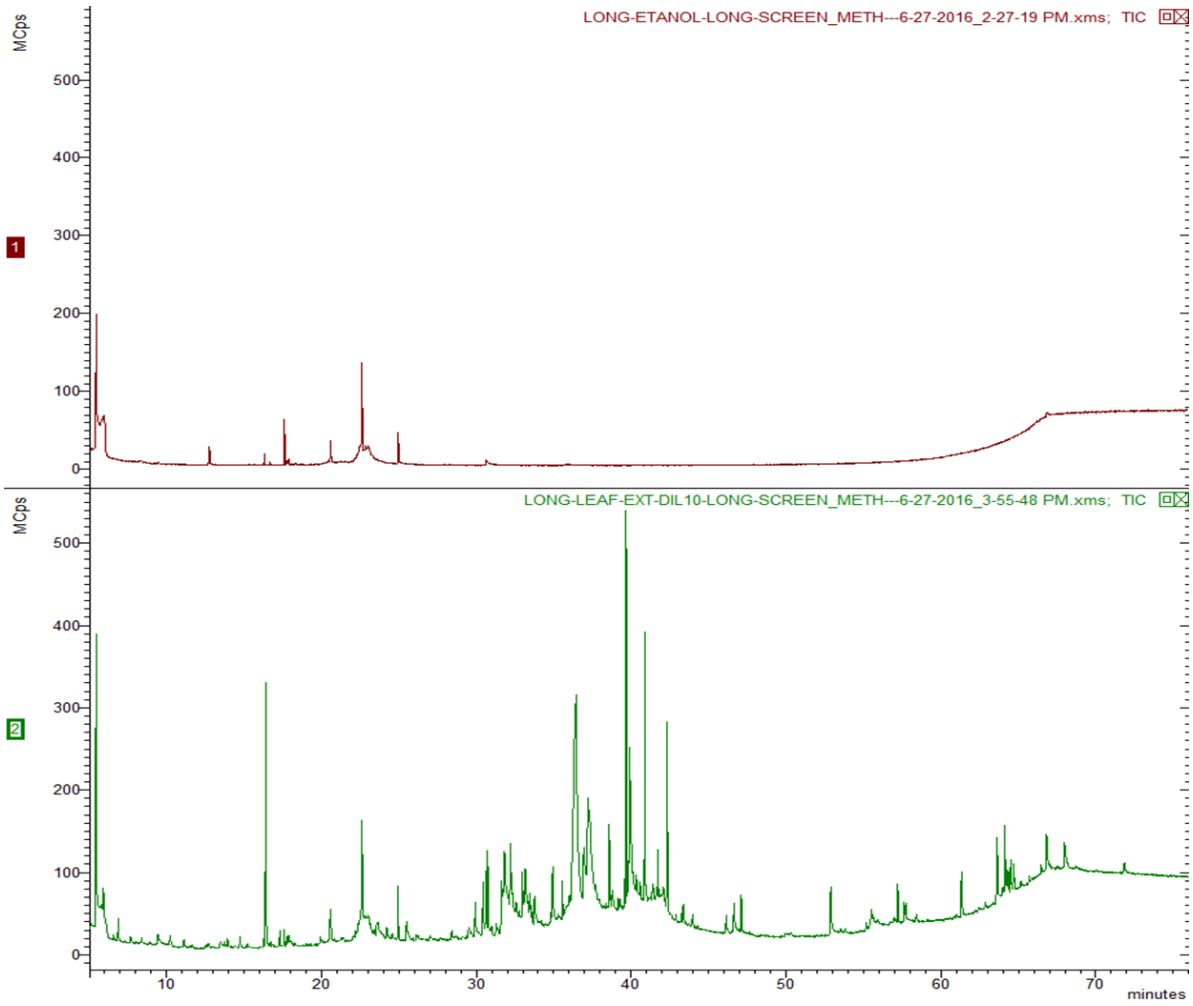

Figure 10. Chromatogram of OLE screening.

The substances corresponding to the most significant peaks were tentatively identified by searching their mass spectra in NIST Library (version 2.2, January 2014). The match between the spectra and the library was used to identify the samples. The software assigned probabilities based on the likelihood of the identification and it is common practice to consider a result above 60% as an acceptable match. Table 11 presents the substances tentatively identified in the OLE. Confirmation of the identification of the substances with standards was not performed.

Talhaoui and co-authors (2015) presents a review of the composition, particularly regarding phenolic compounds in olive leaves and corresponding health benefits. According to these authors: olive leaves contain a large variety of phenolic derivatives, and consist of simple phenols, flavonoids and secoiridoids. Hydroxytyrosol is one of the main components of simple phenols in olive leaves. Flavonoids are one of the most common and widely distributed group of olive leaves polyphenols.

Table 11. Peaks found in the measurement of olive leaf extract.

Retention Time (min.)	Name	CAS	Probability (%)
6,879	Ethyl glycolate	623-50-7	98,1
8,383	Lactic acid, ethyl ester	97-64-3	48,5
9,449	Furfural	98-01-1	52,9
10,219	2-Butenal, 2-ethenyl-	20521-42-0	71,2
11,12	Furfuryl alcohol	98-00-0	55,9
12,497	4-Cyclopentene-1,3-dione	930-60-9	40,4
13,946	2(5H)-Furanone	497-23-4	23
14,735	1,2-Cyclopentanedione	3008-40-0	51,1
15,212	1-Buten-3-one, 1-(2-carboxy-4,4-	N/A	40,9
16,405	Benzaldehyde	100-52-7	73,9
16,753	2H-Pyran-2-one	504-31-4	82
17,322	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-	10230-62-3	95,5
20,844	Diethyl malonate	105-53-3	40,2
23,378	Pyranone	28564-83-2	82,2
24,203	2-Furanacrolein	623-30-3	46,6
25,486	Dianhydromannitol	N/A	72,5
28,423	2-Methoxy-4-vinylphenol	7786-61-0	51,6
29,928	Methyl p-formylbenzoate	1571-08-0	76,9
31,617	Benzeneethanol, 4-hydroxy-	501-94-0	82,8
31,819	Ethyl 4-formylbenzoate	6287-86-1	51
34,923	Tyrosol, acetate	58556-55-1	69,9
36,446	Ethyl α-d-glucopyranoside	19467-01-7	76,9
39,915	2-(2-Hydroxy-2-phenylethyl)-3,5,6-	10130-14-0	46,1
41,751	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	102608-53-7	52,2
43,368	Hexadecanoic acid	57-10-3	45,9
43,973	Ethyl hexadecanoate	628-97-7	42,8
57,213	Squalene	111-02-4	37,8
61,326	dl-α-Tocopherol/ Vitamin E	10191-41-0/59-	43.7/34.4
63,622	β-Sitosterol	83-46-5	68,2
64,136	β-Amyrin	559-70-6	30,2
66,468	Methyl ursolate	32208-45-0	37,4
66,817	Ursolic aldehyde	19132-81-1	36,3
67,973	Uvaol	545-46-0	50,6

They can be present in the aglycone form (quercetin, apigenin, luteolin, diosmetin) or in the glycosylated form (quercetin-7-O-rutinoside, luteolin- 7-O-rutinoside, luteolin-7-O-glucoside, luteolin-5-O-glucoside). However, secoiridoids, are restricted to the Oleaceae family and are the main family of compounds contained in olive leaves. Among them, oleuropein is the main phenolic compound in olive leaves. Phenolic compounds present are very diverse in type and in concentration level. Some of the above mentioned substances or related ones were detected in the analyses performed. Due to time constrains, these results have not been yet further analysed.

A bibliographic search indicates that a great effort has been devoted to the analytical characterisation of phenolic compounds in OLE. However, less has been made regarding the characterisation of the volatile fraction which can be of great interest for application in active packaging with systems where the active substance is not in direct contact with the food product. The analysis performed on the extract should be further evaluated in future work as well, targeting the more volatile compounds.

4. Conclusions

The current application of ethanol in bakery industry is as a fumigant agent to disinfect cool rooms or as a carrier of aromas to be applied on cakes prior to packaging. The presence of ethanol in bread, at low levels, is natural due to the fermentative process, as it was showed by the ethanol concentration in package's headspace of control samples. This study demonstrates the efficiency of an increase of ethanol concentration in the head-space, in retarding microbiological growth rate comparatively to control samples in bread without preservatives

Regarding microbiological analysis, all packaging systems demonstrated to have a beneficial effect against moulds, yeasts and total microorganisms at 30 ºC comparatively to control samples. The shelf life of bread packaged in systems based on ethanol had increased comparatively to control samples. The ethanol emitters were more efficient, mostly against moulds, than direct application systems. The most effective and successful packaging system was FR20, especially in inhibiting moulds in bread.

The textural properties like hardness, chewiness, cohesiveness, springiness and resilience seemed to not be affected by ethanol. Likewise, for the chemical parameters such as pH, a_w, moisture content, were not identified significant differences between different packaging systems and the control samples. Furthermore, the levels of ethanol applied seemed to not affect the bread sensorial properties.

Notwithstanding the efficiency of ethanol had been demonstrated, further testing could be made to ensure its usefulness as bread preservative and optimize it, and to overcome several variability factors recorded during this study.

5. Future work

Following the results obtained, it can be proposed as future work: in order to add value to the bread, first, the olive leaf extract can be used to produce films or coatings with antioxidant and antimicrobial properties. Secondly, it would be interesting to identify how to incorporate OLE in sachets like those used in this study, promoting both effects described above.

Before that, it would be necessary to analyse the antioxidant capacity of the OLE components, and also their antimicrobial activity against the main microorganisms responsible for bread spoilage. This last could be done applying OLE directly in those microorganisms or introducing a small volume (e.g. 2 µl) of innocuous (*Penicillium expansum*, for example) from a previously adjusted fungal suspension, in the middle of a Petri plate with culture medium which is mixed and homogenized with the extract (Nerín, 2014). As had been mentioned, the characterisation of the volatile fraction would be of great interest for application in active packaging with systems where the active substance is not in direct contact with the food product. The antimicrobial effect of these volatile components could be studied to compare with its direct application.

In this respect, the importance of additional studies are becoming increasingly important to solve industries problems and demand consumer trends.

6. References

Ahmed, A. M., Rabii, N. S., Garbaj, A. M., & Abolghait, S. K. (2014). Antibacterial effect of olive (Olea europaea L .) leaves extract in raw peeled undeveined shrimp (Penaeus semisulcatus). *International Journal of Veterinary Science and Medicine*, *2*(1), 53–56. http://doi.org/10.1016/j.ijvsm.2014.04.002

Appendini, P., & Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innovative Food Science and Emerging Technologies*, *3*(2), 113–126. http://doi.org/10.1016/S1466-8564(02)00012-7

Axel, C., Zannini, E., & Arendt, E. K. (2016). Mould spoilage of bread and its biopreservation : A review of current strategies for bread shelf life extension, *8398*(August). http://doi.org/10.1080/10408398.2016.1147417

Berni, E., & Scaramuzza, N. (2013). Effect of ethanol on growth of Chrysonilia sitophila (’the red bread mould') and Hyphopichia burtonii ('the chalky mould') in sliced bread. *Letters in Applied Microbiology*, *57*(4), 344–349. http://doi.org/10.1111/lam.12119

Brody, A. L., Strupinsky, E. R., & Kline, L. R. (2001). *Active Packaging for Food Applications*. CRC Press.

Cauvain, S. (2015). *Technology of Breadmaking*. http://doi.org/10.1007/978-3-319-14687-4

Cozmuta, A. M., Peter, A., Cozmuta, L. M., Nicula, C., Crisan, L., Baia, L., & Turila, A. (2015). Active Packaging System Based on Ag/TiO2 Nanocomposite Used for Extending the Shelf Life of Bread. Chemical and Microbiological Investigations. *Packaging and Technology and Science*, *28*, 271–284. http://doi.org/10.1002/pts

Dagnas, S., Onno, B., & Membré, J. M. (2014). Modeling growth of three bakery product spoilage molds as a function of water activity, temperature and pH. *International Journal of Food Microbiology*, *186*, 95–104. http://doi.org/10.1016/j.ijfoodmicro.2014.06.022

Daifas, D. P., Smith, J. P., Tarte, I., Blanchfield, B., & Austin, J. W. (2000). Effect of Ethanol Vapor on Growth and Toxin Production By Clostridium Botulinum in a High Moisture Bakery Product, *20*.

Danyluk, M. D., Harris, L. J., & Sperber, W. H. (2007). Nuts and Cereals. In M. P. Doyle & L. R. Beuchat (Eds.), *Food Microbiology: Fundamentals and frontiers* (Third Edit, pp. 179–181). Washington, D.C.: ASM Press.

Dao, T., & Dantigny, P. (2011). Control of food spoilage fungi by ethanol. *Food Control*, *22*(3-4), 360–368. http://doi.org/10.1016/j.foodcont.2010.09.019

Dury-Brun, C., Chalier, P., Desobry, S., & Voilley, A. (2007). Multiple Mass Transfers of Small Volatile Molecules Through Flexible Food Packaging. *Food Reviews International*, *23*(3), 199–255. http://doi.org/10.1080/87559120701418319

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2012a). Scientific Opinion on the safety evaluation of the active substance , open-cell expanded polystyrene manufactured with talc and alkyl (C8-C22) sulphonic acid (salts) for use in active food contact materials 1 EFSA Panel on Food Contact Materials , Enz. *EFSA Journal*, *10*(5).

http://doi.org/10.2903/j.efsa.2012.2746.

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2012b). Scientific Opinion on the safety evaluation of the active substance iron (II) modified bentonite as oxygen absorber for use in active food contact. *EFSA Journal*, *10*(10), 1–11. http://doi.org/10.2903/j.efsa.2012.2906.

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2012c). Scientific Opinion on the safety evaluation of the active substances , activated carbon , water , iron powder , kaolin calcined , sulphur and sodium chloride for use as active component in food contact materials 1 EFSA Panel on food contact materials , en. *EFSA Journal*, *10*(3), 1–12. http://doi.org/10.2903/j.efsa.2012.2643.

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2012d). Scientific Opinion on the safety evaluation of the active substances , sodium borohydride and palladium acetate for use in active food contact materials 1 EFSA Panel on food contact materials , enzymes ,. *EFSA Journal*, *10*(3), 1–15. http://doi.org/10.2903/j.efsa.2012.2642.

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2012e). Scientific Opinion on the safety evaluation of the active substances , terephthalic acid , dimethyl ester , polymer with 1 , 4-butanediol , cyclized , polymers with glycidyl methacrylate , hydroxyl-terminated polybutadiene , methyl methacrylate and styren. *EFSA Journal*, *10*(10), 1–11. http://doi.org/10.2903/j.efsa.2012.2905.Available

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2012f). Scientific Opinion on the safety evaluation of the active substances Sodium carboxy methyl cellulose, bentonite, aluminium potassium sulphate for use in active food contact materials. *EFSA Journal*, *10*(10). http://doi.org/10.2903/j.efsa.2012.2904

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2013a). Scientific Opinion on safety assessment of the active substance , iron (0) modified bentonite as oxygen absorber , for use in active food contact EFSA Panel on Food Contact Materials , Enzymes ,. *EFSA Journal*, *11*(10), 1–12. http://doi.org/10.2903/j.efsa.2013.3400

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2013b). Scientific Opinion on safety assessment of the active substance , iron (0) modified kaolinite as oxygen absorber , for use in active food contact EFSA Panel on Food Contact Materials , Enzymes ,. *EFSA Journal*, *11*(10), 1–12. http://doi.org/10.2903/j.efsa.2013.3401

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2013c). Scientific Opinion on the safety assessment of the active substances iron , iron oxides , sodium chloride and calcium hydroxide for use in food contact. *EFSA Journal*, *11*(10), 1–9. http://doi.org/10.2903/j.efsa.2013.3387

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2013d). Scientific Opinion on the safety evaluation of the active substance , acrylic acid , sodium salt , co-polymer with acrylic acid , methyl ester , methacrylic acid , 2 hydroxypropylester , and acrylic acid cross-linked for use in active food contact materia. *EFSA Journal*, *11*(4).

http://doi.org/10.2903/j.efsa.2013.3154.

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2013e). Scientific Opinion on the safety evaluation of the active substance, polyacrylic acid, sodium salt, crosslinked, for use in active food contact materials. *EFSA Journal*, *11*(2). http://doi.org/10.2903/j.efsa.2013.3096

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2013f). Scientific Opinion on the safety evaluation of the active substances , iron , polyethyleneglycol , disodium pyrophosphate , monosodium phosphate and sodium chloride for use in food contact materials 1 EFSA Panel on Food Contact Materials , Enzymes ,. *EFSA Journal*, *11*(6), 1–11. http://doi.org/10.2903/j.efsa.2013.3245

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2013g). Scientific Opinion on the safety evaluation of the active substances , sodium carbonate peroxyhydrate coated with sodium carbonate and sodium silicate , bentonite , sodium chloride , sodium carbonate for use in active food contact materials 1. *EFSA Journal*, *11*(4). http://doi.org/10.2903/j.efsa.2013.3153.

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2013h). Scientific Opinion on the safety evaluation of the active substances citric acid (E330) and sodium hydrogen carbonate (E500ii), used as carbon dioxide generators , together with liquid absorbers cellulose and polyacrylic acid sodium salt crosslinked ,. *EFSA Journal*, *11*(4). http://doi.org/10.2903/j.efsa.2013.3152.

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2013i). Scientific Opinion on the safety evaluation of the active substances iron , sodium chloride , water , silica gel , activated carbon , monosodium glutamate , potassium acid tartrate , powdered cellulose , malic acid , chabazite , hydroxypropyl cellulose ,. *EFSA Journal*, *11*(4). http://doi.org/10.2903/j.efsa.2013.3155.

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2014a). Scientific Opinion on safety assessment of the active substance , polyacrylic acid , sodium salt , crosslinked , for use in active food contact materials 1 EFSA Panel on Food Contact Materials , Enzymes ,. *EFSA Journal*, *12*(5), 1–9. http://doi.org/10.2903/j.efsa.2014.3648

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2014b). Scientific Opinion on safety assessment of the active substance , polyacrylic acid , sodium salt crosslinked , for use in active food contact materials 1. *EFSA Journal*, *12*(7), 1–8. http://doi.org/10.2903/j.efsa.2014.3786

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2014c). Scientific Opinion on the safety assessment of the active substances , palladium metal and hydrogen gas , for use in active food contact materials 1 EFSA Panel on Food Contact Materials , Enzymes ,. *EFSA Journal*, *12*(2), 1–8. http://doi.org/10.2903/j.efsa.2014.3558

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2014d). Scientific Opinion on the safety assessment of the active substances , sodium erythorbate , sodium

carbonate , sodium bicarbonate , iron sulphate , activated carbon , cellulose , calcium hydroxide , calcium chloride and water , for use as active system in. *EFSA Journal*, *12*(2). http://doi.org/10.2903/j.efsa.2014.3571

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2014e). Scientific Opinion on the safety assessment of the active substances iron powder , activated carbon , calcined kaolin , sodium chloride , polyacrylic acid , sodium salt , crosslinked and calcium chloride , for use as active system. *EFSA Journal*, *12*(5). http://doi.org/10.2903/j.efsa.2014.3649

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2016a). Safety assessment of the active substance polyacrylic acid, sodium salt, cross-linked, for use in active food contact materials. *EFSA Journal*, *14*(7). http://doi.org/10.2903/j.efsa.2016.4533

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, F. and P. A. (2016b). Safety assessment of the active substances citric acid and sodium hydrogen carbonate for use in active food contact materials. *EFSA Journal*, *14*(7). http://doi.org/10.2903/j.efsa.2016.4529

Farkas, J. (2007). Physical Methods of food preservation. In M. P. Doyle & L. R. Beuchat (Eds.), *Food Microbiology: Fundamentals and frontiers* (Third Edit, pp. 685–692). Washington, D.C.: ASM Press.

Gamli, Ö. F. (2016). THE HEALTH EFFECTS OF OLEUROPEIN , ONE OF THE MAJOR PHENOLIC COMPOUNDS OF OLIVES ,. *J. Food Sci.*, *28*, 178–190.

Gray, J. A., & Bemiller, J. N. (2003). Bread staling: molecular basis and control. *Comprehensive Reviews in Food Science and Food Safety*, *2*(2), 1–21. http://doi.org/10.1111/j.1541-4337.2003.tb00011.x

Gray, J., & Bemiller, J. (2003). Bread staling: molecular basis and control. *Comprehensive Reviews in Food Science and Food Safety*, *2*(2), 1–21. http://doi.org/10.1111/j.1541-4337.2003.tb00011.x

Gray, N. (2010). Antioxidants may improve bread quality: study. Retrieved 16 August 2016, from http://www.foodnavigator.com/Science/Antioxidants-may-improve-bread-quality-Study

Gutiérrez, L., Sánchez, C., Batlle, R., & Nerín, C. (2009). New antimicrobial active package for bakery products. *Trends in Food Science and Technology*, *20*(2), 92–99. http://doi.org/10.1016/j.tifs.2008.11.003

Heenan, S. P., Dufour, J., Hamid, N., Harvey, W., & Delahunty, C. M. (2008). The sensory quality of fresh bread : Descriptive attributes and consumer perceptions. *Food Research International*, *41*(10), 989–997. http://doi.org/10.1016/j.foodres.2008.08.002

Hempel, A. W., O'Sullivan, M. G., Papkovsky, D. B., & Kerry, J. P. (2013). Use of smart packaging technologies for monitoring and extending the shelf-life quality of modified atmosphere packaged (MAP) bread: Application of intelligent oxygen sensors and active ethanol emitters. *European Food Research and Technology*, *237*(2), 117–124. http://doi.org/10.1007/s00217-013-1968-z

INSA. (2016). Detalhe alimento - pão de milho. Retrieved from http://www.insa.pt/sites/INSA/Portugues/AreasCientificas/AlimentNutricao/AplicacoesOnline/TabelaAlimentos/PesquisaOnline/Paginas/DetalheAlimento.aspx?ID=IS428

Jenson. (1998). *Microbiology of Fermented Foods*. (B. J. B. Wood, Ed.) (Second edi). Blackie Academic and Professional.

Jideani, V. a., & Vogt, K. (2015). Antimicrobial Packaging for Extending the Shelf Life of Bread – A Review. *Critical Reviews in Food Science and Nutrition*, *8398*(June), 00–00. http://doi.org/10.1080/10408398.2013.768198

Kalathenos, P., & Russell, N. J. (2003). Ethanol as a food preservative. In N. J. Russell & G. W. Gould (Eds.), *Food Preservatives* (pp. 196–212). Springer US. http://doi.org/10.1007/978-0-387-30042-9

Katsinis, G., Rigas, F., & Doulia, D. (2008). Synergistic effect of chemical preservatives with ethanol on the microbial shelf life of bread by factorial design. *International Journal of Food Science and Technology*, *43*(2), 208–215. http://doi.org/10.1111/j.1365-2621.2006.01386.x

Klein, R. (2011). Material Properties of Plastics. In *Laser Welding of Plastics* (First Edit, pp. 3–68). Wiley-VCH Verlag GmbH & Co. KGaA.

Latou, E., Mexis, S. F., Badeka, A. V., & Kontominas, M. G. (2010). Shelf life extension of sliced wheat bread using either an ethanol emitter or an ethanol emitter combined with an oxygen absorber as alternatives to chemical preservatives. *Journal of Cereal Science*, *52*(3), 457–465. http://doi.org/10.1016/j.jcs.2010.07.011

Malhotra, B., Keshwani, A., & Kharkwal, H. (2015). Antimicrobial food packaging: potential and pitfalls. *Frontiers in Microbiology*, *6*(June), 611. http://doi.org/10.3389/fmicb.2015.00611

Martín-vertedor, D., Garrido, M., Pariente, J. A., Espino, J., & Delgado-adámez, J. (2016). Bioavailability of Bioactive Molecules from Olive Leaf Extracts and its Functional Value, *1179*(October 2015), 1172–1179.

Musa, M., & Bertrand, Ö. (2016). A review : benefit and bioactive properties of olive (Olea europaea L .) leaves. *European Food Research and Technology*. http://doi.org/10.1007/s00217-016-2726-9

Nerín, S. M. (2014). *Combinación de técnicas analíticas y microbiológicas para el estudio de un envase activo antifúngico, frente a mohos alterantes de alimentos: Tesis Doctoral*. Univesidad de Zaragoza; Escuela de Ingeniería y Arquitectura.

Nout, M. J. R., Sarkar, P. K., & Beuchat, L. R. (2007). Indigenous Fermented Foods. In M. P. Doyle & L. R. Beuchat (Eds.), *Food Microbiology: Fundamentals and frontiers* (Third Edit, pp. 817;821–822). Washington, D.C.: ASM Press.

O'Connor, Á. (2012). Bread consumption in the UK: what are the main attitudinal factors affecting current intake and its place in a healthy diet? *British Nutrition Foundation Nutritional Bulletin*, *37*, 368–379. http://doi.org/10.1111/j.1467-3010.2012.01989.x

Otoni, C. G., Espitia, P. J. P., Avena-bustillos, R. J., & Mchugh, T. H. (2016). Trends in antimicrobial food packaging systems : Emitting sachets and absorbent pads. *FRIN*, *83*, 60–73. http://doi.org/10.1016/j.foodres.2016.02.018

Passarinho, A. T. P., Dias, N. F., Camilloto, G. P., Cruz, R. S., Otoni, C. G., Moraes, A. R. F., & Soares, N. D. F. F. (2014). Sliced bread preservation through oregano essential oil-containing sachet. *Journal of Food Process Engineering*, *37*(1), 53–62. http://doi.org/10.1111/jfpe.12059

Pereira de Abreu, D. a., Cruz, J. M., & Paseiro Losada, P. (2012). Active and Intelligent Packaging for the Food Industry. *Food Reviews International*, *28*(2), 146–187.

http://doi.org/10.1080/87559129.2011.595022

Perkin Elmer. (n.d.). MP, Tg, and Structure of Common Polymers. Retrieved from http://www.perkinelmer.com.cn/CMSResources/Images/46-74863TCH_MPTGAndStructureOfCommonPolymers.pdf

Pittia, P., Anese, M., Marzocco, L., Calligaris, S., Mastrocola, D., & Nicoli, M. C. (2006). Liquid-vapour partition of ethanol in bakery products. *Flavour and Fragrance Journal*, *21*(1), 3–7. http://doi.org/10.1002/ffj.1694

Reinas, I., Oliveira, J., Pereira, J., Mahajan, P., & Poças, F. (2016). A quantitative approach to assess the contribution of seals to the permeability of water vapour and oxygen in thermosealed packages. *Food Packaging and Shelf Life*, *7*, 34–40. http://doi.org/10.1016/j.fpsl.2016.01.003

Robertson, G. L. (2010). *Food Packaging and Shelf Life - A Practical Guide*.

Robertson, G. L. (2013). *Food Packaging: Principles and practice*. (T. & F. Group, Ed.), *Food Packaging: Principles and Practice* (Third Edit, Vol. 1). CRC Press.

Saranraj, P., & Geetha, M. (2012). Microbial Spoilage of Bakery Products and Its Control by Preservatives. *International Journal of Pharmaceutical & Biological Archives*, *3*(1), 38–48.

Smith, J. P., Daifas, D. P., El-Khoury, W., Koukoutsis, J., & El-Khoury, A. (2004). Shelf life and safety concerns of bakery products--a review. *Critical Reviews in Food Science and Nutrition*, *44*(1), 19–55. http://doi.org/10.1080/10408690490263774

Smith, J. P., Hoshino, J., & Abe, Y. (1995). Interactive packaging involving sachet technology. In M. L. Rooney (Ed.), *Active Food Packaging* (pp. 143–173). Glasgow, Scotland: Springer US.

Smith, J. P., Ooraikul, B., Koersen, W. J., van de Voort, F. R., Jackson, E. D., & Lawrence, R. A. (1987). Shelf life extension of a bakery product using ethanol vapor. *Food Microbiology*, *4*, 329–337.

Sung, S.-Y., Sin, L. T., Tee, T.-T., Bee, S.-T., Rahmat, a. R., Rahman, W. a. W. a., … Vikhraman, M. (2013). Antimicrobial agents for food packaging applications. *Trends in Food Science & Technology*, *33*(2), 110–123. http://doi.org/10.1016/j.tifs.2013.08.001

Szczesniak, A. S. (1998). Effect of storage on texture. In I. A. Taub & R. P. Singh (Eds.), *Food Storage Stability* (pp. 193–196). CRC Press.

Tan, Y. M., Lim, S. H., Tay, B. Y., Lee, M. W., & Thian, E. S. (2015). Functional chitosan-based grapefruit seed extract composite films for applications in food packaging technology. *Materials Research Bulletin*, *69*, 142–146. http://doi.org/10.1016/j.materresbull.2014.11.041

The Federation of Bakers. (2012). European Bread Market. Retrieved 16 August 2016, from http://www.bakersfederation.org.uk/the-bread-industry/industry-facts/european-bread-market/

TOPAS. (2012). TOPAS COC - High Barrier for Antiseptic Packaging. Retrieved from http://pt.slideshare.net/TopasAdvancedPolymers/high-alcohol-barrier-films-in-antiseptic-packaging

Appendix A – Review of EFSA Scientific Opinions regarding active substances

Table 12. Review of EFSA Scientific Opinions regarding active substances

The identity of the substance(s)	Description of substances	CAS Number	FCM Substance Number	Authorised as	Function of substance(s)	Action mode of the substance(s)	Material/Article in which the substance or component is added or into which it is incorporated	Types of food in which it is used	The conditions of material's and/or substance(s)'s use	Migration tests	References
Three mixtures comprising activated carbon, water, iron powder, kaolin calcined, sulphur, sodium chloride	Activated carbon	7440-44-0	984	It meets the requirements for activated charcoal, which is authorized as additive for plastic materials and articles in contact with foods with restrictions	Oxygen absorber	Sealed sachets, containing the active mixture, are placed into the headspace of the food packaging to absorb the residual content of oxygen surrounding the product, to scavenge any oxygen enclosed inside the food, and to scavenge any oxygen that enters the pack by permeation through the packaging material.	It is introduced into 2 types of sachets. One is made of porous PET/cellulosic non-woven (NT)/PP laminated film on both sides. PET and PP layers are perforated prior to lamination. The other is made of a porous HDPE (non-woven) film and on the other side of a PET/PE laminated film. PET and PE layer are perforated prior to lamination.	Intended to be used in various food industries such as meat, poultry and their related products, precooked dishes, delicatessen, cheese, bakery, cakes, pastry products which are stored at +4°C. Other applications include room temperature storage of products such as cereals, chocolates, sweets, dry food, cakes and bakery products.	The oxygen absorber components must not be put in direct contact with acid food (pH<4.5) or in contact with a large liquid fraction (liquids or exudates), due to the fact that the oxygen absorption is inhibited under such conditions.	Tests were performed on sachets with the highest weight of active formulation per unit of the sachet surface, by total immersion of sachets in 3% acetic acid, water and 95% ethanol (each for 10 days, at 40°C) and into isooctane (2 days at 20°C). Due to the design of the sachet and the foreseeable uses, sachet must not be placed in contact with a liquid fraction.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2012c)
Terephthalic acid, dimethyl ester, polymer with 1,4-butanediol, cyclized, polymers with glycidyl methacrylate, hydroxyl-terminated polybutadiene, methyl methacrylate and styrene, which is used together with the oxidation catalyst cobalt stearate	-	-	-	-	Oxygen absorber	Active substance: a copolymer made of cyclic oligomers of butylene terephthalate, hydroxyl-terminated polybutadiene and epoxy-functional styrene-acrylate oligomer. The substance is used together with the oxidation catalyst cobalt stearate.	-	-	-	Migration and compositional tests were performed on PET bottles formulated with 0.6% of the active terpolymer and 0.053% of cobalt stearate into 3% acetic acid and 95% ethanol for 2h/70°C, followed by up to 10 days at 60°C.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2012e)

The identity of the substance(s)	Description of substances	CAS Number	FCM Substance Number	Authorised as	Function of substance(s)	Action mode of the substance(s)	Material/Article in which the substance or component is added or into which it is incorporated	Types of food in which it is used	The conditions of material's and/or substance(s)'s use	Migration tests	References
Two mixtures comprising iron, polyethyleneglycol, disodium pyrophosphate, monosodium phosphate and sodium chloride.	Iron	7439-89-6	983	Additive for plastic materials and articles in contact with foods with specific restrictions	Oxygen absorber	The main active ingredient of the oxygen absorber system is iron which reacts with oxygen in presence of water, thereby removing oxygen from the primary packaging. The other chemicals are used to provide a media to facilitate the iron oxidation.	The active mixture is incorporated into PE or PP articles. Final food contact materials are PE or PP films of up to 100 µm thickness and PP trays with a thickness from 300 to 1000 µm.	Intended for contact with various types of oxygen sensitive foods, such as convenience food (e.g. pasta with sauce), meat paste or salads (e.g. tuna salad, various salads), sliced meat and sausage-based snacks.	The contact conditions include long term storage at room temperature and hot fill/pasteurisation for several minutes at 95°C or below. Dried and fatty foods: direct contact with the materials is envisaged (monolayer). Other food types: the foods will be separated from the active material by a layer of PE or PP with a thickness of at least 10 µm (multilayer).	Specific and overall migrations of iron were measured for multilayers and monolayer materials. Two volatile substances were identified as impurities of the polyethylene glycol used to formulate the PE active system.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2013f)
A mixture comprising iron, sodium chloride and calcium hydroxide	Iron	7439-89-6	983	All the substances constituting the OA system have been evaluated and authorised for use as additives in plastic FCM.	Oxygen absorber	The main active ingredient of the OA system is iron, which reacts with oxygen in the presence of water. The other chemicals are used to provide a medium to facilitate the iron oxidation.	The OA formulation is blended with PP, which is used as an inner layer in multilayer materials. The food is separated from the active material by a layer of polyolefin that does not contain the OA mixture and offers a barrier to the diffusion of inorganic species equivalent to at least 10 µm PP.	The OA multilayer materials are intended to be used in packaging materials coming into contact with various types of oxygen-sensitive foods, for long-term storage, at room temperature, with or without in-pack heating of the foods, at up to 125 °C for up to 60 minutes.	-	Specific migration of iron into 3 % acetic acid solution, with contact conditions of four hours at 100°C, and migration of iron into water and 10 % ethanol, under the same test conditions, were measured.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2013c)

The identity of the substance(s)	Description of substances	CAS Number	FCM Substance Number	Authorised as	Function of substance(s)	Action mode of the substance(s)	Material/Article in which the substance or component is added or into which it is incorporated	Types of food in which it is used	The conditions of material's and/or substance(s)'s use	Migration tests	References
Iron (0) modified bentonite made from the starting substances iron (III) chloride anhydrous, bentonite and sodium borohydride.	Iron (0) modified bentonite	-	1036	All starting substances have been evaluated and approved for use as additives in plastic FCM or as food additives.	Oxygen absorber	The active substance is iron (0) modified bentonite, produced from the starting substances iron (III) chloride anhydrous (53 %), bentonite (32 %), sodium borohydride (15 %) and water as solvent. Aluminium is naturally present in bentonite clay. The natural clay is surface modified with the attachment of iron which has oxygen scavenging properties and the process for bentonite modification is a physical blending with iron (III), following reduction to iron (0) at a maximum temperature of 80 °C. Boric acid and sodium chloride are formed in the process.	Intended to be incorporated at level up to 3 % w/w (without compatibilizers) in a polyolefin in contact with any type of foods for long term of storage at room temperature or refrigeration. It may also be used in sachets made of HDPE, which are intended to be placed in the headspace of the primary packaging or in contact with solid foodstuffs, but should not be in contact with liquid foods, or foods with external aqueous liquid phase.	Any type of foods including beverages under conditions of long term of storage at room temperature or refrigeration	Sachets must prevent the physical release of their contents into the food and should not be in direct contact with liquid foods, exudates, or foods with external aqueous liquid phase	Specific migration of iron, boron and aluminium were measured for a HDPE layer containing 10 % w/w iron (0) modified bentonite in direct contact with the food simulants. Long term contact at room temperature was simulated by testing with water, acetic acid 3 % and ethanol 10 % for 10 days at 40 °C. Migration was also conducted into isooctane for 2 days at 20 °C. The overall migration limit tested in the same conditions as used for specific migrations.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2013a)
Iron (0) modified kaolinite, made from the starting substances iron (III) chloride anhydrous , kaolinite and sodium borohydride.	Iron (0) modified kaolinite	-	1037	-	Oxygen absorber	-	Intended to be incorporated at level up to 3 % w/w in a polyolefin or in sachets made of HDPE, which are intended to be placed in the headspace of the primary packaging or in contact with solid foodstuffs, but should not be in contact with liquid	Any type of foods for long term of storage at room temperature or below.	Sachets should not be in direct contact with liquid foods (i.e. dressings, soups, beverages) or foods with external aqueous liquid phase (i.e. sliced fruits, fresh meat and poultry).	Specific migration of iron, boron and aluminium were measured in a HDPE layer containing 10 % iron (0) modified kaolinite in direct contact with the food simulants. Long term contact at room temperature was simulated by testing with water, acetic acid 3 % and ethanol 10 % for 10 days at 40 °C. Migration test was also conducted with isooctane for 2 days at 20 °C. The overall migration limit tested in the same conditions as used for specific migrations.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2013b)

The identity of the substance(s)	Description of substances	CAS Number	FCM Substance Number	Authorised as	Function of substance(s)	Action mode of the substance(s)	Material/Article in which the substance or component is added or into which it is incorporated	Types of food in which it is used	The conditions of material's and/or substance(s)'s use	Migration tests	References
							foods, or foods with external aqueous liquid phase.				
Sodium borohydride and of palladium acetate and its reduction product, palladium	Sodium borohydride	16940-66-2	981	-	Oxygen scavenger	-	Samples tested were composed of two polymer layers, boron being in the layer not in direct contact with food simulant as it is the intended use of the substance.	Liquid food with long term storage at room temperature that may be preceded by hot filling or pasteurisation	The sodium borohydride should only be used behind a plastic layer to prevent direct contact with the packaged food and the palladium species should also be behind a barrier layer or should be incorporated into the plastic of the primary packaging material in order to minimise direct contact of palladium with the food and thereby keep its migration within acceptable levels. The sachet should not intentionally or unintentionally come into direct contact with liquid foods or foods that have an external aqueous liquid phase on the surface such as sliced fruits and fresh meat.	Specific migration tests for palladium and boron were conducted with samples with surface to volume (S/V) ratios corresponding to bottles of 150 ml capacity or larger and liner closures with a S/V ratio up to 0.4dm2/kg. Samples with a formulation similar to the closures, tested for determining the migration of boron and palladium were composed of two plastic layers and the substances were in the layer not in direct contact with the food simulant. The tests were conducted into the simulants 3% acetic acid, 50% and 95% ethanol for 2 hours at 70ºC followed by 30 days at 40ºC, simulating conditions of hot filling or pasteurisation followed by long term storage at room temperature. Migration into olive oil as a fatty food simulant was not determined.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2012d)

The identity of the substance(s)	Description of substances	CAS Number	FCM Substance Number	Authorised as	Function of substance(s)	Action mode of the substance(s)	Material/Article in which the substance or component is added or into which it is incorporated	Types of food in which it is used	The conditions of material's and/or substance(s)'s use	Migration tests	References
Palladium metal and hydrogen gas	Palladium metal	2023568	993	-	Oxygen scavenger	The active component aims extending the products shelf life by scavenging the residual oxygen in the packages headspace. Palladium catalyses the reaction between hydrogen and oxygen, forming water.	The active material containing palladium is inserted in the cap or closure of bottles, and secured as an adhesive label to the lids internal surface of cans, carton containers, heat sealed trays and pouches. The active substance is applied by electron beam deposition, at a loading of 4 to 10 mg/m2, to a passive part of the packaging composed of PET film coated with SiOx. The active material is then placed in the middle of two layers of a nonwoven composite made of PP, polyester and cellulose with an acrylate-based binding aid. This pad is overlaid by a PP layer in contact with the primary packaging, and a laminate layer in contact with the food made of nonwoven spunbonded PE with 15 µm PA.	Palladium metal and hydrogen gas is intended to be used as an OA in packages of fresh fruit juices, carbonated soft drinks, wines and beers, milk formulation powders, cheeses and cream, delicatessen meats, "ready-to-eat" and "ready-to-cook" prepared meal products, at room temperature for long term storage or at refrigeration temperatures for relatively short storage times.	Palladium should not be in direct contact with food and should be incorporated in a passive structure impermeable to liquids which prevents the migration at detectable levels	The specific migration of palladium from a sample composed of nonwoven spunbonded PE/PET/SiOx/Palladium/nonwoven spunbonded PE was determined in 3 % acetic acid, 20 % ethanol, 95 % ethanol and isooctane, at 60 °C for 10 days (surface/volume ratio of 6 dm2/kg).	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2014c)

The identity of the substance(s)	Description of substances	CAS Number	FCM Substance Number	Authorised as	Function of substance(s)	Action mode of the substance(s)	Material/Article in which the substance or component is added or into which it is incorporated	Types of food in which it is used	The conditions of material's and/or substance(s)'s use	Migration tests	References
A mixture comprising sodium erythorbate, sodium carbonate, sodium bicarbonate, iron sulfate, activated carbon, cellulose, calcium hydroxide, calcium chloride and water	Sodium erythorbate	6381-77-7	1042	Food additive (E 316)	Oxygen absorber and carbon dioxide emitter	The active ingredient responsible for the oxygen absorbing function is sodium erythorbate, which reacts with the oxygen present in the primary packaging. The carbon dioxide emitting function is fulfilled by the presence of sodium carbonate or sodium bicarbonate. All the other substances are used to provide adequate media to facilitate both reactions.	The mixture is intended to be introduced into multilayer sachet made from PET/cellulosic non-woven/PP material, and heat sealed after filling. Both PET and PP are perforated prior lamination to allow gas exchanges.	This oxygen absorber/carbon dioxide emitter system is intended to be used in various applications, such as meat and meat products, precooked dishes, delicatessen, cheese, bakery, cakes, pastry products. These foods are generally stored at +4 °C. Shelf-lives vary from several days to several weeks.	The sachets are to be placed in the headspace of the packaging and as such may come into occasional contact with the food. The sachets must not be put in direct contact with acid food (pH < 4.5), with liquid foods or foods with external aqueous liquid fraction (liquids or exudates) to avoid inhibition of the oxygen absorption.	Overall and specific migration tests for iron, sodium, calcium, as well as a screening of volatile byproducts were performed. Measurements were done by total immersion and under more realistic conditions (minced meat). The release of volatile byproducts was analysed by placing seven sachets in one liter sealed plastic bag containing air at 23°C. Samples have been collected after 30 min, 102 min and 1 week.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2014d)
Misture comprising iron powder, activated carbon, calcined kaolin, sodium chloride, polyacrylic acid, sodium salt, crosslinked and calcium chloride	Iron powder	7439-89-6	983	Additive for plastic materials and articles in contact with foods	Oxygen absorber in labels	Mixture which is packed into labels for absorbing oxygen from the headspace surrounding packed food. The active ingredient responsible of the oxygen absorber function is iron which reacts with oxygen, removing the oxygen from the primary packaging. The other chemicals are used to provide adequate media to facilitate the reaction.	The active formulation is deposited on a multilayer film made from porous PET/ nonwoven spunbonded HDPE and covered by a multilayer film PET/PE. Both films are heat-sealed on 4 sides. Labels are stuck inside the packaging.	The labels containing the oxygen absorber system can be used for various foods such as processed-meat products, precooked dishes, delicatessen, cheese, kery, cakes and pastry products. These foods are generally stored at +4 °C.	Labels must not be placed in contact with a liquid fraction neither, intentionally or unintentionally, come into direct contact with liquid foods or foods that have an external aqueous phase on the surface such as sliced fruits. The OA system needs a humid atmosphere (aw > 0.8) to activate chemical reactions but must not be put in contact with liquids or acidic food (pH < 4.5) or entirely covered by food as the system loses its performance.	Overall and specific migration were measured by total immersion of labels. Overall migration was determined in 3 % acetic acid, distilled water and 95 % ethanol (each for 10 days, at 40 °C) and into isooctane (2 days at 20 °C) whereas specific migrations were performed only in 3 % acetic acid and distilled water under same contact conditions.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2014e)

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Iron (II) modified bentonite	Chloride anhydrous	7705-08-0	-	-	Oxygen absorber incorporated without compatibilisers in polyolefin layers of food packages at levels up to 15% w/w	The natural bentonite clay is surface modified by cation exchange between sodium and added iron which has oxygen scavenging properties	The active substance is intended to be incorporated up to 15% in plastic layers in contact with any type of food under conditions of long term of storage at room temperature or refrigeration. It may also be used in sachets placed in the headspace of the primary packaging of solid foodstuffs.	-	Sachets should not be in direct contact with liquid foods, exudates, or foods with external aqueous liquid phase.	Migration tests were performed with samples made of HDPE charged with 10% iron (II) modified bentonite. Migration of iron and aluminium ions into 3% acetic acid which is considered the worst case food simulant for the migration of metal ions.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2012b)
Mixture of coated sodium carbonate peroxyhydrate (approx. 67.5 %), sodium carbonate (approx. 3.5 %), anhydrous sodium chloride (approx. 13 %) and activated calcium bentonite clay (approx. 16 %)	Sodium carbonate peroxyhydrate coated with sodium carbonate and sodium silicate		1009		Oxygen generator and carbon dioxide absorber	The active component contains a powder mixture of coated sodium carbonate peroxyhydrate, sodium carbonate, anhydrous sodium chloride and activated calcium bentonite clay in gas permeable four-side polyethylene sealed sachet separated from food by a pad. In the presence of moisture, coated sodium carbonate peroxyhydrate first decomposes into sodium carbonate and hydrogen peroxide. Then, hydrogen peroxide is transformed into water and oxygen, and sodium carbonate reacts	The powder mixture is placed in a nonwoven, gas permeable four-side sealed PE sachet separated from the food by a pad	Fresh fruits	Sachets should not be in direct contact with food or food exudates. The active mixture has been designed to maintain inside the primary packaging a high oxygen and low carbon dioxide atmosphere. In a typical application 12 g of powder would be used for 500 g of food.	Only hydrogen peroxide may be released which decomposes to water and oxygen with no other potential migration of volatile compounds. Hydrogen peroxide concentration was determined in 3 % acetic acid both by immersion and by placing the sachet between 2 pieces of filter paper saturated with 3 % acetic acid, for 10 days at 40 °C.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2013g)

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						with carbon dioxide to form sodium bicarbonate. Sodium chloride and bentonite clay are moisture access regulators.					
Mixtures comprising active iron, sodium chloride, water, silica gel, activated carbon, monosodium glutamate, potassium acid tartrate, powdered cellulose, malic acid, chabazite, hydroxypropyl cellulose, potassium carbonate, sodium thiosulfate, propylene glycol, glycerin, polyethyleneglycol sorbitan monooleate, sodium propionate and clinoptilolite	Iron	7439-89-6	983	Additive for plastic materials and articles in contact with foods with a specific restriction of 48 mg iron/kg food based on a Provisional Maximum TDI of 0.8 mg/kg bw set by JECFA/WHO (1983) and agreed by the SCF (1990)	Iron based oxygen absorbers	The main active ingredient is iron which reacts with oxygen to form iron hydroxide and iron oxide, thereby removing oxygen from the primary packaging. Activated carbon, powdered cellulose, chabazite, silica gel and clinoptilolite are used as water activity modifiers. Hydroxypropyl cellulose, glycerine, propylene glycol are used as binders. Monosodium glutamate, potassium acid tartrate, potassium carbonate, sodium thiosulfate and malic acid act as buffers to keep the pH at the optimum range to promote the iron oxidation. Sodium chloride and water play the role of electrolyte to facilitate the reaction. Polyethyleneglycol sorbitan monooleate is used to decrease	Sachets, patches, cards constituted by multilayer materials including plastic films and/or paper and adhesive in their structures. Layers are either perforated plastic or non woven substrates in order to allow gas exchanges with the atmosphere surrounding the product. After filling, sachets, patches are heat sealed. For cards both layers between which the active powder is deposited, are stuck together with an adhesive.	Dry foods applications (such as tea/coffee, bakery products, dried fruits, vegetables, spices, flour, pasta) and fat containing foods (such as processed, cured and smoked meats, cheese, nuts and bakery products containing fat, such as cakes and cookies).	Not intended for direct contact with liquid food or food with an external liquid surface such as sliced fruits and fresh meat. The range of contact conditions is from chilled up to ambient temperature for long time storage.	Overall migration was measured by direct contact (total immersion or one side contact) with food simulants. As the active system is based on solid ingredients, only migration through the gas phase is expected.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2013i)

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						the surface tensions and to permit a good dispersion of chemical ingredients. Sodium propionate acts as antimicrobial agent.					
Polyacrylic acid, sodium salt, crosslinked with mixtures of crosslinker 1 and/or crosslinker 2 and/or crosslinker 3 and/or crosslinker 4	Polyacrylic acid, sodium salt	-	-	Additive for plastic materials and articles in contact with food with a group SML of 6 mg/kg food, expressed as acrylic acid.	Liquid absorber	Liquids are absorbed and retained in the absorbent core, even under pressure, by the formation of a hydrogel	It is intended to be used in absorbent pads. The absorbent pads have three layers: - an upper inert plastic or nonwoven layer which is in contact with food. - a bottom nonwoven-based or micro-perforated film layer which is in contact with the bottom of the food tray where the liquids are present. - an inner layer composed of a blend of a compressed cellulosic fluff pulp and the active substance in granular form. The inner layer is confined between the upper and lower layer of the pad, which is sealed on the 4 sides to prevent leakage of the active substance.	Fresh or frozen foods such as meat, poultry, and seafood as well as fresh fruits and vegetables	The absorbent pads must be used only under conditions in which the liquid absorption capacity is not exceeded and direct contact between the substance and the food is excluded. The pads are intended to be used with foods for approximately up to 14 days under refrigerated conditions in case of fresh food and for longer time for frozen food.	The retention capacity of the substance was determined with 0.9 % and 0.2 % saline solution and was 24 g and 40 g of fluid per g of substance, respectively. The amount of the active substance required for the absorption of fluids released from various food types was estimated by the applicant to be up to 2.5 g per kg of food, depending of the exuding nature of the food. Specific migration tests were not performed on the absorbent pads due to the high absorption of liquids by the substance. Since the substance is incorporated into the inner layer of the pads, there is no direct contact possible between the substance and the food.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2014b)

The identity of the substance(s)	Description of substances	CAS Number	FCM Substance Number	Authorised as	Function of substance(s)	Action mode of the substance(s)	Material/Article in which the substance or component is added or into which it is incorporated	Types of food in which it is used	The conditions of material's and/or substance(s)'s use	Migration tests	References
Polyacrylic acid, sodium salt, crosslinked	Polyacrylic acid, sodium salt	-	-	Additive for plastic materials and articles in contact with food with a group SML of 6 mg/kg food, expressed as acrylic acid.	Liquid absorber	Liquids are absorbed and retained in the absorbent core, even under pressure, by the formation of a hydrogel	It is intended to be used in absorbent pads. The absorbent pads have three layers: - an upper inert plastic or nonwoven layer which is in contact with food. - a bottom nonwoven-based or micro-perforated film layer which is in contact with the bottom of the food tray where the liquids are present. - an inner layer composed of a blend of a compressed cellulosic fluff pulp and the active substance in granular form.	Fresh or frozen food such as meat, poultry, and seafood as well as fresh fruits and vegetables	The pads are intended to come into contact with foods for approximately up to 14 days under refrigerated conditions in case of fresh food and for longer time for frozen food. The absorbent pads must be used only under conditions in which the liquid absorption capacity is not exceeded and direct contact between the substance and the food is excluded.	The retention capacity of the substance was determined with 0.9 % and 0.2 % saline solution and was 24 g and 40 g of fluid per g of substance, respectively. The amount of the active substance required for the absorption of fluids released from various food types was estimated by applicant to be up to 2.5 g per kg of food, depending of the exuding nature of the food. Specific migration tests were not performed on the absorbent pads due to the high absorption of liquids by the substance. Since the substance is incorporated into the inner layer of the pads, there is no direct contact possible between the substance and the food.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2014a)
Open-cell expanded polystyrene, manufactured with polystyrene, talc and alkyl(C8-C22) sulphonic acid (salts)	Talc	14807-96-6	615	Food additive (E 553b, colours max 5%) and additive in plastic food contact materials	Liquid absorber	-	-	Fresh meat, poultry and fish	-	Migration test results for trays made of open-cell EPS (Expanded Polystyrene) into water and 3% acetic acid for 10 days at 40°C have been provided and demonstrated the compliance with the OML and the SML for alkyl(C8-C22) sulphonic acid (salts) as established by Commission Regulation (EU) 10/2011.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2012a)

The identity of the substance(s)	Description of substances	CAS Number	FCM Substance Number	Authorised as	Function of substance(s)	Action mode of the substance(s)	Material/Article in which the substance or component is added or into which it is incorporated	Types of food in which it is used	The conditions of material's and/or substance(s)'s use	Migration tests	References
Mixture of cellulose, citric acid (E330), sodium hydrogen carbonate (E500ii) and polyacrylic acid sodium salt crosslinked	Citric acid (E330)	77-92-9	139	Additive and monomer for plastic materials and articles in contact with food; food additive	Liquid absorber and carbon dioxide generator	The active component contains a mixture of citric acid (E 330) and sodium hydrogen carbonate (E 500ii) of food grade quality, incorporated in a liquid absorbent pad. Depending on the absorption capacity needed, pure cellulose or a mixture of cellulose and polyacrylic acid sodium salt crosslinked may be used. In the presence of moisture the active substances react, form sodium citrate, water and release carbon dioxide.	The pads are made of: • a core layer which is composed of a liquid absorber in which citric acid and sodium hydrogen carbonate are incorporated. This layer is covered on both sides by cellulosic tissue layers. • a perforated polyethylene film surrounding the core welded at all 4 sides in order to avoid direct contact with food. The film is designed to let fluids in and carbon dioxide out.	Fresh or frozen meat, poultry, fish, fruits and vegetables	Should be used only under conditions in which the liquid absorption capacity is not exceeded and direct contact between the substance and the food is excluded.	Specific migration tests were not performed on the absorbent pads due to the high absorption of liquids by the substance. The retention capacity of the substance was measured with deionised water and with a 0.9 % saline solution and was 221 g and 37 g of fluid per g of substance, respectively.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2013h)
Acrylic acid, sodium salt, co-polymer with acrylic acid, methyl ester, methacrylic acid, 2 hydroxypropylester, and acrylic acid cross-linked	Acrylic acid	79-10-7	147	Monomer for plastic materials and articles in contact with food	Liquid absorber in the form of fibres in absorbent pads	The absorbed fluid is retained in the cross-linked polymer by transformation into a gel. The capacity of the polymer to absorb liquid was measured for a 0.9 % saline solution. It was found to be 50 g fluid per g polymer.	The polymer is applied in the form of fibres (length 6 mm and diameter 0.025 mm) in nonwoven fabrics in which they are entangled during the manufacturing process with other fibrous material such as cellulosic fluff-pulp what prevents mechanical transfer of the polymeric fibres into the food. The nonwoven fabric	Fresh or frozen meat, poultry, fish, fruits and vegetables		The residual content of methacrylic acid, 2-hydroxypropylester in the polymer was determined by extraction with 95 % ethanol and iso-octane. Specific migration tests by total immersion into aqueous simulants were performed on a sample containing 30 % of the cross-linked polymer.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2013d)

The identity of the substance(s)	Description of substances	CAS Number	FCM Substance Number	Authorised as	Function of substance(s)	Action mode of the substance(s)	Material/Article in which the substance or component is added or into which it is incorporated	Types of food in which it is used	The conditions of material's and/or substance(s)'s use	Migration tests	References
							containing the polymer can be used alone or covered, either on one or on both sides, with plastic film or other nonwoven fabric not containing the polymer. Liquids are absorbed and retained in the absorbent core by the formation of a hydrogel.				
Mixture consisting of sodium carboxymethylcellulose granules (50 to 90%), bentonite clay granules (10 to 30%) and aluminium potassium sulphate (1 to 4%)	Sodium carboxymethylcellulose (E 466)	9004-32-4	-	Food additive and additive for plastic materials and articles in contact with foods	Moisture and liquid absorber	The target function is to absorb moisture and liquids which are released or likely to be exuded from food. After water absorption, a firm gel is formed. The mixture and the gel formed do not come in direct contact with food being separated from it by a permeable non-woven fabric.	The mixture of the active substances is incorporated up to 38% w/w in articles such as pads, trays, containers, boxes, pouches, bags, lids.	Foods with short shelf-life, which are stored and retailed at refrigeration temperature or at room temperature, such as fresh fruit, fresh vegetables, and meat products.	Aluminium potassium sulphate dodecahydrate can be used in formulations with sodium carboxymethylcellulose (50-90% w/w) and bentonite (10-30% w/w) at levels up to 4% w/w. In all cases the absorbers must be placed in components in the food packaging preventing them from being in direct contact with food and the fluid absorption capacity of these absorbers must not be exceeded.	The applicant provided studies on migration of aluminium in water and in 3% acetic acid for 10 days at 40°C, from articles containing the absorbent mixture. The amount of fluid not absorbed was measured to demonstrate that in real conditions of the intended use overload does not occur.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2012f)
Polyacrylic acid, sodium salt, crosslinked	Polyacrylic acid, sodium salt	-	-	Additive for plastic materials and articles in contact with food with a group SML of 6 mg/kg food, expressed as acrylic acid	Absorbent pads (liquid absorber)	Liquids are absorbed and retained in the absorbent core, even under pressure by the formation of a hydrogel	The absorbent pads typically have three layers: 1) an upper inert layer of plastic film (generally PE, PP or polyester), perforated or not, which is in contact with the food; 2) a bottom leach-resistant nonwoven layer	Fresh or frozen meat, poultry, and fish as well as fresh fruits and vegetables	The absorbent pads must be used only under conditions in which the liquid absorption capacity is not exceeded and direct contact between the substance and the food is excluded	Specific migration tests were not performed on the absorbent pads due to the high absorption of liquids by the substance. The substance and its impurities are not volatile and they will not migrate via the vapour phase.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2013e)

The identity of the substance(s)	Description of substances	CAS Number	FCM Substance Number	Authorised as	Function of substance(s)	Action mode of the substance(s)	Material/Article in which the substance or component is added or into which it is incorporated	Types of food in which it is used	The conditions of material's and/or substance(s)'s use	Migration tests	References
							or perforated laminated layer which is in contact with the bottom of the food tray where the liquids are present; 3) an inner layer composed of a blend of compressed cellulosic fluff pulp and the active substance in granular form (core).				
Citric acid	-	77-92-9	139	The active substances are already authorised for use in plastic materials and articles in contact with food and as food additives and are not intended to migrate into the food.	Intended to be used as a carbon dioxide (CO_2) generator in trays for fresh poultry meat at refrigeration temperature for a period of up to 2 weeks.	The substances are activated by contact with meat exudate, whereupon they react together to form sodium citrate and release carbon dioxide into the atmosphere of the pack, which is intended to have an effect on microbial growth in the meat.	The active coating is separated from the meat by a rigid PET perforated layer which is sealed on the four sides to the tray. The exudate drains from the meat by gravity, falling into the bottom of the tray where the active coating is.	Fresh poultry meat at refrigeration temperature	The substances are physically separated from the poultry meat to prevent direct contact. Pack design prevents any return of meat exudate.	-	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2016b)
Sodium hydrogen carbonate	--	144-55-8	21								
Polyacrylic acid, sodium salt, cross-linked	-	-	1015	-	Intended to be used in absorbent pads as liquid absorber	The absorption core consists of the active substance mixed with virgin fluff pulp (cellulose). Liquids are absorbed and retained in the absorbent core, even under pressure, by the formation of a hydrogel.	-	Fresh or frozen foods such as meat, poultry and seafood as well as fresh fruits and vegetables.	Must be used only under conditions in which the liquid absorption capacity is not exceeded and direct contact between the substance and the food is excluded.	The substance is not intended to make direct contact with the food. Migration of the substance and additives and its residual ingredients and impurities is not to be expected, when the substance is used in foreseen conditions and provided the absorption capacity of the pad is not exceeded.	(EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, 2016a)

Appendix B – Methods and results of chemical analysis made to determine the nutritional values

The nutritional composition was determined based on chemical analysis and methods described in Table 13.

Table 13. Chemical analysis and respective methods for nutritional composition determination.

Analysis	Methods and results
Fat content	MSDA 16A/13 (5th edition) reports to MSDA 22C/04 (5th edition) Fat extraction: NF V 03-030:1191 Sample preparation: ISO 14156:2001
Fatty acids	Transesterification procedure: ISO 5509:2000 GC Perkin Elmer Clarus C system was used, with Supelco SP™ – 2560 column (100m×0.25mm I.D.; Df: 0.20µm).
Ash content	AOAC 930.22 (16th edition) reports to 923.03 Sample preparation: AOAC 926.04
Protein content	ISO 1871:2009 Food and feed products - General guidelines for the determination of nitrogen by the Kjeldahl method
Total Sugars, reducing and non-reducing sugars	According NP 1419 (1987) - Munson and Walker technique
Total fibre	Assay procedure based on AOAC 985.29 and AOAC 991.43
Salt	Atomic absorption spectroscopy

Analyses were performed in replicates with checking for the maximum acceptable difference between them (CINATE protocols).

Table 14. Results of chemical analysis to determine nutritional composition.

	Pre drying										
	Sample	**Bread weight (before drying)**	**Bread weight (after drying)**	**Moisture content (%)**	**%Dry matter**	**m_1/m_0**		**m_0-m_1**			
	1	113,80	69,50	38,93	61,07	0,61		44,30			
Moisture content	**Sample**	**m(g) (m_2)**	**m (capsule)**	**m (capsule before drying)**		**m_3**	**%H (Total)**	**Mean**	**Standard deviation**		
	1	5,07	40,08	45,00		4,91	40,83	40,81	0,02		
	2	5,04	32,70	37,59		4,89	40,80				
Ash	**Sample**	**m(g) (m_2)**	**m (capsule)**	**m (capsule before drying)**		**% Ash (Total)**	**Mean**	**Standard deviation**			
	1	3,01	45,16	45,22	1,96	1,19	1,19	0,01			
	2	3,02	45,19	45,25	1,94	1,19					
	Fiber	-0,0091									
Protein	**Sample**	**m(g)**	**V_0**	**V_1**	**ct**	**Nitrogen**	**Conversion factor**	**% Nitrogen**	**Protein content**	**Mean**	**Standard deviation**
	1	0,72	0,10	7,95	0,11	14,01	6,25	1,67	6,36	6,42	0,09
	2	0,72	0,10	8,10	0,11	14,01	6,25	1,70	6,48		

	Fiber	-	0,10	2,35	0,11	14,01	6,25	-	21,36	0,02	

Sugars	Sample	Crucible G4 weight	Crucible G4 weight (before drying)	Cooper oxide	Correlation from the Munson-Walker table			Initial sample weight		Final result (mg/100g of product)	Standard deviation
	1	50,77	50,70	74,7	33,51	67,03	134,06	5,01	2,68	1,64	0,08
	2	49,25	49,17	80,4	36,08	72,16	144,32	5,03	2,87	1,75	

Fat	Sample	m(g) m_2	m (flask)	m (flask before drying)	%	% fat (Total)	Mean	Standard deviation
	1	10,09	127,79	128,27	4,82	2,94	3,0	0,02
	2	10,03	119,96	120,44	4,87	2,97		

Fiber	RI	RII	P	A	B	M_1	M_2	Fiber
	0,06	0,05	0,0	-0,0091	-	1,0229	1,0088	2,73

Nutritional composition of bread

According to Portuguese legislation *(Lei nº 75/2009 de 12 de Agosto*), the value obtained for the salt content is within the maximum allowed in bread after baking (1.4 g per 100 g of bread). Results for total fat indicate this bread does not contain more than 3 g of fat per 100 g of product.

Table 15. Nutritional declaration of bread in study.

	Average value (g) per 100g	% Daily value
Energy	1043kJ/635kcal	12
Fat	3,0	4
Of which		
Saturates	1,8	-
Mono-unsaturates	0,9	-
Polyunsaturates	0,3	-
Carbohydrate	45,9	18
Of which		
Sugars	1,7	2
Fibre	2,7	-
Protein	6,4	13
Salt	0,9	15

The values obtained for the nutritional parameters (Table 15) are in the range of those for wheat bread and corn flour bread taken from INSA database (INSA, 2016). The bread produced has a relative lower fibre content and a relative higher fat content.

Appendix C – Identification of plastic materials by Calorimetry and Infrared analysis

Methods:

Calorimetry

DSC is a method of analysis used to measure phase transitions like glass transition, melting of a crystalline polymer and/or thermal degradation of macromolecular chains of polymers (Klein, 2011). Each polymer film had a characteristic phase transition which is dependent on the molecular structure - for instance, high molecular weight increases phase transition temperature (Klein, 2011). Samples of approximately 10 mg were sealed in aluminum pans and were submitted to two heating/cooling cycles from ambient to 350 ºC at a rate of 10 ºC/min.

Infrared Analyses

FTIR is a spectroscopic method of analysis where IR energy can be related to the vibrational energy of different bonds found within different functional groups in a compound. The FTIR analyses were carried out using a Perkin-Elmer Spectrum 100 FT-IR spectrometer, equipped with Universal ATR accessory. The spectra were obtained from 2 scans and processed by Spectrum software.

Results:

The identification of the polymers of each layer of the bread packages was performed by DSC and IR analyses. Figure 11 presents the DSC thermogram. The transition temperatures indicate that there are two different polymers, polyamide 6 and low density polyethylene. The LDPE shows a double peak at 107.6 ºC and 125.3 ºC and polyamide 6 shows a 213.4 ºC peak (Perkin Elmer, n.d.). These peaks correspond to the melting points of these polymers.

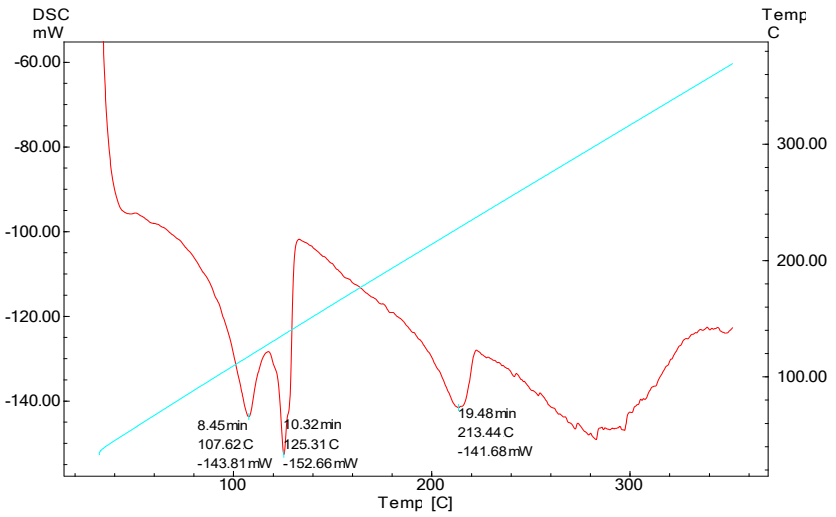


Figure 11. DSC scan measurement of EE's external packaging polymers.

These results were confirmed by the IR analysis. Figure 12 below presents the spectrum obtained for the material of the bread package. The absorption of electromagnetic radiation induces the oscillation of components of macromolecular chains in different ways such as elongation, bending or rotating oscillation (Klein, 2011). Table 16 presents the characteristic bands wavelengths in FT-IR spectra, corresponding to different oscillation/vibration types of atoms and atom groups within a molecule chain that allow for the identification of the polymers LDPE and PA6 (Klein, 2011; Mieth, Hoekstra, & Simoneau, 2016; Perkin Elmer, n.d.). For example, it is possible to identify in Figure 12 the rocking vibration of CH_2 between 600 and 800 cm^{-1} of PE (triangle). In what concerns PA 6, it is possible to identify the plane deformation of NH between 1400 and 1600 cm^{-1}-8 (rectangle). The circle and square identifies the valence or elongation oscillation of C=O and NH, respectively.

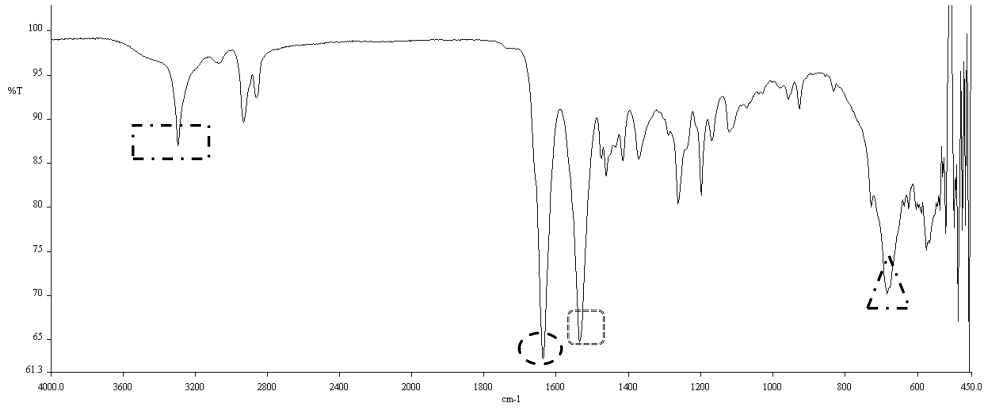
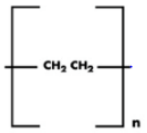
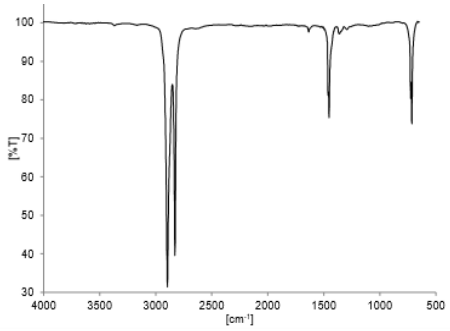
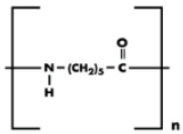
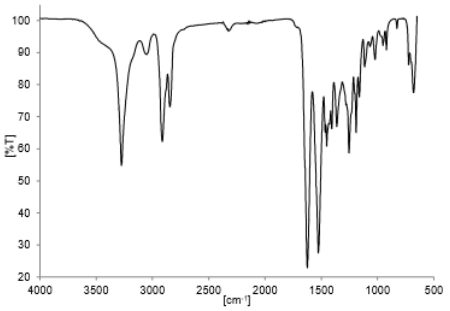


Figure 12. ATR FT-IR spectrum of EE's external packaging material.

Table 16. Characteristic bands in FT-IR spectra (Klein, 2011; Mieth, Hoekstra, & Simoneau, 2016; Perkin Elmer, n.d.)

Polymer	ATR-FTIR spectrum	Oscilation*	Wave number (cm^{-1})
Polyethylene 		$_{va}CH_2$ and $_{vs}CH_2$	3000-2840
		$^{\delta}CH_2$	1463
		$_{\varrho}CH_2$	725
Polyamide 6 		$_{v}NH$	3303
		$_{va}CH_2$	2935
		$_{vs}CH_2$	2860
		$_{v}C=O$	1635
		$^{\delta}NH$ and $_{v}CN$	1539, 1276
		δCH_2	1465
		ωCH_2 and TCH_2	1200
		$_{\gamma}NH$ and $_{\gamma}C=O$	690

: $_{va}$: antisymmetric stretching vibration, $_{vs}$: symmetric stretching vibration, $^{\delta}$: in-plane deformation, $_{\gamma}$: out-of-plane, $_{\varrho}$: rocking vibration, ω: wagging vibration, T: twisting vibration

The FT-IR analysis of the plastic material of the sachet of the EE resulted in the spectrum presented in Figure 13, which presents the absorption band corresponding to valence oscillation of C=O (~1740 cm^{-1}), C(=O)O (~1241 cm^{-1}) and O-C (~1020 cm^{-1}), which are characteristic of EVA polymers. The other absorption bands are similar to EVA and PP polymers. The information from the supplier was thus validated, and the material of the sachets is composed by a paper layer convered by a PP/EVA copolymer.

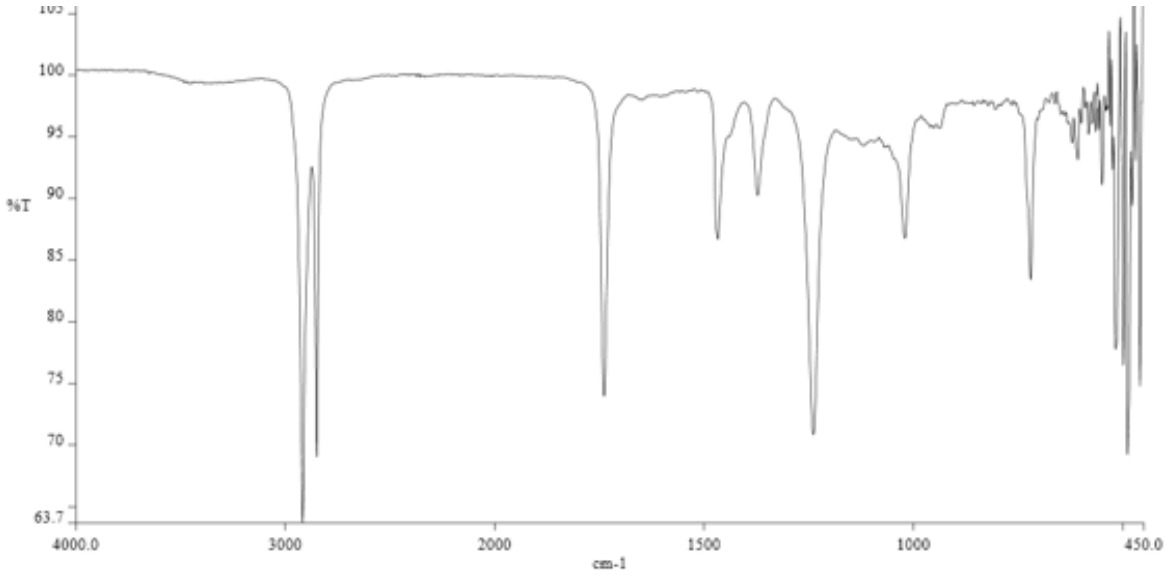


Figure 13. FT-IR spectrum of plastic material of EE sachet.

Appendix D – Calibration curves from the different methods of ethanol quantification

Calibration of spraying system for dispensing ethanol

Absorbent paper to simulate the bread was introduced in GC vials and weighted. Each vial was sprayed with an increasing number of sprays, and immediately closed and weighted. Figure 14 shows the relationship between the number of sprays and the ethanol dispensed. This analysis allowed the determination of the number of sprays required to achieve a 0.5% ethanol concentration in each package with bread.

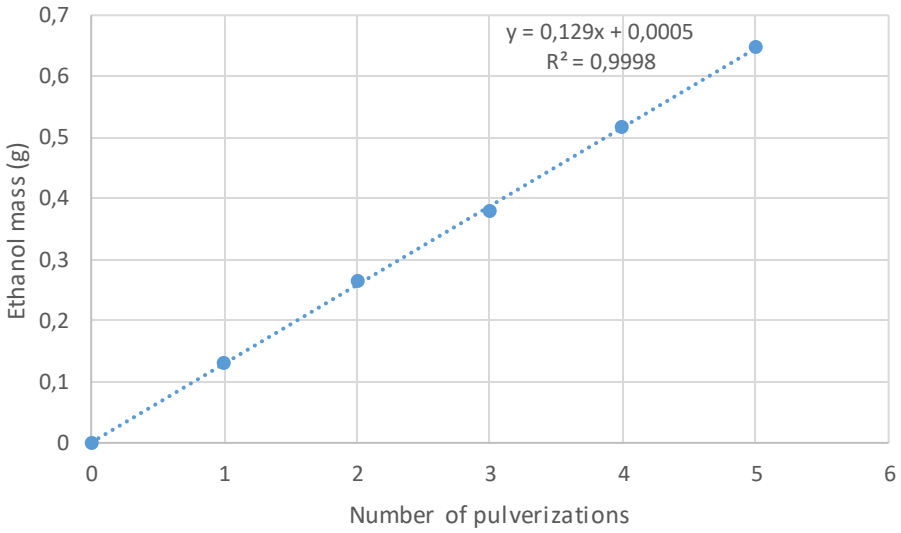


Figure 14. Sprayer calibration.

Quantification of ethanol in the packages headspace

Ethanol concentration in package headspace was monitored using a GC FID (Varian CP3800) chromatography system with VF 624MS column (60 m × 0.32 mm I.D.; Df: 1.8 µm). Injector and detector temperature was 250 ºC. Oven temperature was 60ºC during the first 0.5 minutes, being gradually increased to 200ºC at a rate of 15 ºC/min and left at 200 ºC for 2 minutes. Injection volume was 0.4 ml (split 1:50). The package was perforated with the syringe needle and immediately injected manually in the chromatographic port. Analysis were in replicate and syringe was cleaned between measurements. The area of the chromatogram peak was compared with the area form the calibration curve prepared as follows: bread pieces of same size were placed inside GC vials and spiked with ethanol (10, 15, 20, 25, 30 µl). After equilibration, the vials head-space was sampled and injected. The calibration curve is

shown in Figure 15. The R^2 value was 0,9748. The Limit of detection of the method, calculated from the standard deviation of the intercept of the curve, was 5,83µl and the limit of quantification 19,42µl.

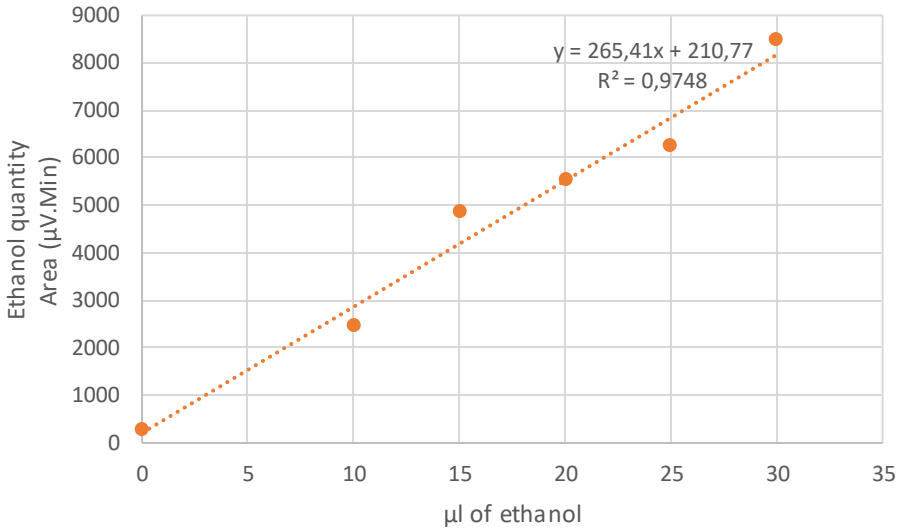


Figure 15. Calibration curve for GC FID – analysis of ethanol in the package headspace.