Universidade do Minho
Escola de Engenharia

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Development of intelligent bio-based packaging to monitor fish quality during storage

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Doctor Joana Martins

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Desenvolvimento de embalagens inteligentes de base biológica para monitorizar a qualidade do pescado durante o armazenamento

RESUMO

A embalagem inteligente é uma tecnologia emergente no sector das embalagens alimentares utilizada como ferramenta para monitorizar a qualidade e segurança dos alimentos através da deteção de alterações no produto alimentar embalado e/ou no seu ambiente circundante. O desenvolvimento de embalagens inteligentes contribui para a diminuição do desperdício alimentar, tem o potencial de melhorar a segurança dos produtos, a logística alimentar e a rastreabilidade, aumentando a eficiência da indústria alimentar.

Neste contexto, o principal objetivo deste trabalho foi desenvolver uma embalagem inteligente de base biológica como indicador de frescura (através de alterações colorimétricas), a fim de monitorizar e fornecer informações relativas à qualidade de pescado utilizando uma técnica não destrutiva, *in situ* e em tempo real. Deste modo, filmes de goma de alfarroba (LBG)/κ-carragenina (κ-car) incorporando extratos de mirtilo (BLE) e/ou beterraba (BEE) foram desenvolvidos. Os filmes foram caracterizados em relação às suas propriedades estruturais, térmicas, de barreira, mecânicas e óticas. Além disso, os filmes BLE e BEE foram avaliados quanto à sua resposta colorimétrica durante o armazenamento de pescado (pescada) a 4 ºC e 25 ºC durante 8 e 3 dias, respetivamente.

Verificou-se que os filmes desenvolvidos sofreram uma mudança colorimétrica de roxo-escuro para azul em paralelo com o aumento da contagem microbiana (4,61 ± 0,36 para 8,61 ± 0,21 log CFU/g), do azoto básico volátil total (TVB-N) (de 10,21 ± 1,97 para 66,78 ± 4,81 mg/100 g), e do pH (de 6,60 ± 0,04 para 8,02 ± 0,03) das amostras de pescado. As propriedades de barreira à água foram melhoradas após a adição dos extratos de mirtilo (solubilidade = 39.66 ± 0.11 %, conteúdo de humidade = 23.54 ± 0.98 %, ângulo de contacto = 41.43 ± 3.32 º).

Os resultados deste estudo são muito promissores, uma vez que foi possível desenvolver um filme inteligente (o filme BLE) indicador colorimétrico de pH consoante o estado de degradação do pescado, de acordo com o objetivo proposto.

Palavras-chave: antocianinas, betalaínas, filme biodegradável, frescura do peixe, indicador de pH

Development of intelligent bio-based packaging to monitor fish quality during storage

ABSTRACT

Intelligent packaging is an emerging technology in the food packaging sector used as a tool to monitor food quality and safety by detecting changes in the packaged food product and/or its surrounding environment. Intelligent packaging development contributes to the food waste reduction, has the potential to improve product safety, food logistics and traceability, increasing food industry efficiency.

In this context, the main goal of this work was to develop a bio-based intelligent packaging as freshness indicator (by colorimetric changes), in order to monitor and provide information regarding food quality using a non-destructive, *in situ* and real-time technique. Locust bean gum (LBG)/κ-carrageenan (κ-car) films incorporating blueberry (BLE) and/or beetroot (BEE) extracts were developed. The films were characterized regarding their structural, thermal, barrier, mechanical and optical properties.

Moreover, BLE and BEE films were evaluated to assess color response during fish (hake) storage under 4 ºC and 25 ºC during 8 and 3 days, respectively.

A visual change in the color of the packaging label from dark purple to blue in parallel with the increase in the microbial count (from 4.61 ± 0.36 to 8.61 ± 0.21 $\log_{10}$ CFU/g), total volatile basic nitrogen (TVB-N) (from 10.21 ± 1.97 to 66.78 ± 4.81 mg/100 g), and pH (from 6.60 ± 0.04 to 8.02 ± 0.03) of the fish samples was detected. The water barrier properties were improved after blueberry extracts addition (WS = 39.66 ± 0.11 %, MC = 23.54 ± 0.98 %, WCA = 41.43 ± 3.32 º).

The results of this study are very promising, since it was possible to develop an intelligent pH-responsive indicator film (BLE film) that changes its color due to fish degradation, according to the proposed thesis aim.

Keywords: anthocyanins, betalains, bio-based film, fish freshness, pH indicator

SCIENTIFIC OUTPUTS

- Faria, C. S., Vicente, A. A. e Martins, J. T. (2022). Embalagens inteligentes de base biológica para aplicação em alimentos. Química - Boletim da Sociedade Portuguesa de Química. Sociedade Portuguesa de Química. Series ii, 46(165), 122-126. doi: 10.52590/M3.P702

- Coelho, L. M., Faria, C., Madalena, D., Genisheva,Z., Martins, J. T., Vicente, A. A. and Pinheiro, A. C. (2022). 'Valorization of Amaranth (Amaranthus cruentus) Grain Extracts for the Development of Alginate-Based Active Films', Molecules, 27,5798. doi: 10.3390/molecules27185798.

INDEX

FIGURES

TABLES

LIST OF ABBREVIATIONS

κ-car – κ-carrageenan

ATR – Attenuated Total Reflectance

BEE – Beetroot extract film

BLE – Blueberry extract film

CFU – Colony Forming Unit

DSC – Differential Scanning Colorimetry

EB – Elongation at Break

FAO – Food and Agriculture Organization

FTIR – Fourier-Transform Infrared

H_2SO_4 – Sulfuric acid

H_3BO_3 – Boric acid

HCl – Hydrochloric Acid

K_2CO_3 – Potassium carbonate

LBG – Locust bean gum

MC – Moisture Content

NaOH – Sodium hydroxide

PCA – Plate Count Agar

RFID – Radio Frequency Identification

SD – Standard Deviation

T – Temperature

t – Time

TS – Tensile Strength

TTI – Time-Temperature Indicators

TVB-N – Total Volatile Basic Nitrogen

TVC – Total Viable Count

UV – Ultraviolet

WCA – Water Contact Angle

WHO – World Health Organization

WPI – Whey Protein Isolate

WS – Water Solubility

WVP – Water Vapor Permeability

WVTR – Water Vapor Transmission Rate

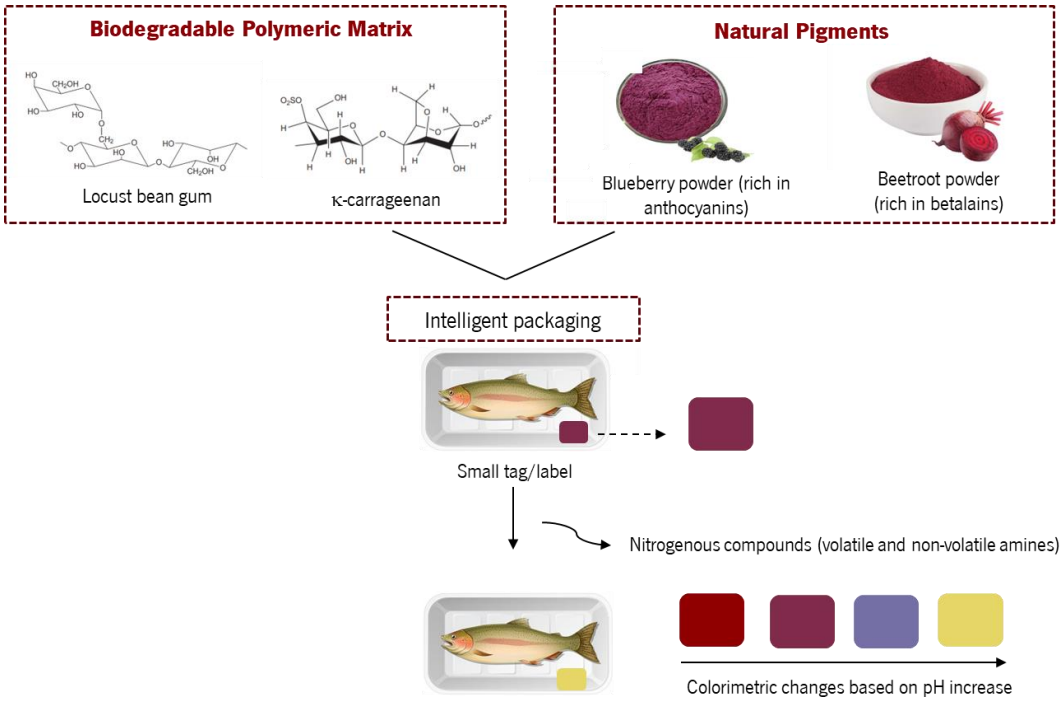
Y_B – Black Standard

Y_w – White Standard

ΔE – Total Color Difference

DISSERTATION STRUCTURE

The dissertation is organized in 4 chapters. Chapter 1 presents a relevant literature review on the dissertation theme topics including bio-based polymers, intelligent packaging technology, and their applications in food area. The main goal and specific aims of thesis are described on Chapter 2. Materials and methodologies used to prepare and characterize bio-based intelligent films, as well as, to evaluate films capacity as fish freshness indicators are described on Chapter 3. Chapter 4 reports the thesis results and a critical discussion regarding the development and characterization of the LBG/κ-car films with blueberry (rich in anthocyanins) and/or beetroot (rich in betalains) extracts incorporated and their validation as freshness indicators. Finally, the main thesis conclusions and suggestions for future work are included on Chapter 5 and 6, respectively. Figure 1 shows an overview of the main topics addressed in this dissertation.



1. INTRODUCTION

Food products that exhibit safety limitations due to the presence of microbial agents (bacteria, viruses and parasites) and/or harmful chemical substances are the cause of more than 200 diseases, including diarrhea and cancer, according to the World Health Organization (WHO). Every year, approximately 600 million people worldwide become ill after eating contaminated food (Zhai *et al.*, 2018). The quality loss of highly perishable fresh products is impossible to eliminate due to their intrinsic properties. Food deterioration as a result of biological, chemical or physical phenomena causes changes, in some cases, not directly recognized by final consumers. However, a small discrepancy from the original product characteristics, whether in color, consistency or flavor, leads to an incorrect assessment of the food quality and, subsequently, avoidable food waste (Heising *et al.*, 2014; Poyatos-Racionero *et al.*, 2018; Müller and Schmid, 2019). Currently, food waste is a problem on a global scale that needs a solution, according to the WHO (Zhai *et al.*, 2018).

Thus, it is important to solve the current challenges of food quality and safety through: 1) the detection of changes in food products, in order to avoid product consumption by final consumer; 2) the identification of potential health risks; and 3) the establishment of strategies to reduce or eliminate the occurrence of the previous mentioned events.

Consumers' increased preference for fresh products have resulted in the development and adoption, by the food industry, of new packaging technologies that can extend food shelf life, effectively improve its quality and final safety, and provide information about the product (Biji *et al.*, 2015).

Packages generally have four basic functions: protection, communication, convenience and containment (Müller and Schmid, 2019). Packages assume a protective role associated to external factors such as light, temperature and contaminants. They are also adjustable to the variable food shape and size and considered easy to use (Biji *et al.*, 2015). Packages contribute to an improvement in food commercialization and distribution, ensuring its safe delivery and preservation (Müller and Schmid, 2019).

In this context, the development of new packaging with specific functions has been observed in recent years, namely active and intelligent packaging. On the one hand, active packaging plays a dynamic role in food preservation through the packaging-food interaction (e.g., compounds' release from the packaging or absorption of compounds released by the food product). Active packages can improve or maintain the conditions of quality and safety of the packaged food (for example,

prevent lipid oxidation and microbial growth), thus extending its shelf life (Fennema, 2000; Realini and Marcos, 2014). On the other hand, intelligent packaging emerged with the aim of detecting, monitoring and informing the consumer about the quality of food throughout the food chain in a non-destructive way, *in situ* and in real time (Lund, 2001; Realini and Marcos, 2014; Zhai *et al.*, 2018; Müller and Schmid, 2019; Qin *et al.*, 2019).

Intelligent packaging is considered an innovative and an emerging technology, presenting main purposes: 1) retains food product integrity; 2) quickly acknowledges consumer regarding food product conditions or its surrounding environment (temperature, pH, gases, etc.) through detection and recording of its physico-chemical changes (Biji *et al.*, 2015; Sohail, Sun and Zhu, 2018); 3) provides information regarding the product itself (e.g., origin, expiration date and composition) and its characteristics during storage conditions (e.g., potential microbial growth and headspace gas composition of packaging system), when arrives to the final consumer (Realini and Marcos, 2014); and 4) confirms product authenticity. However, at this time, it was not described a single intelligent packaging presenting all these features (Kuswandi *et al.*, 2011).

Moreover, intelligent packaging has given an important contribution to avoid unnecessary food waste because it is able to monitor and display the food quality status and, consequently, avoid that food products are thrown away even though they are still suitable for consumption. On the other hand, it has the potential to improve product safety, food logistics and traceability, increasing food industries´ efficiency (Realini and Marcos, 2014; Müller and Schmid, 2019). Intelligent packaging is an attractive target for food industry, but also for the research area, as it is directly related to life quality and human health (Kuswandi *et al.*, 2011; Realini and Marcos, 2014; Sohail, Sun and Zhu, 2018; Müller and Schmid, 2019).

1.1. Types and concepts of intelligent packaging technology

In general, intelligent packaging is classified according to three main types of technology: 1) data carriers, such as barcodes and radio frequency identification (RFID) tags, are used to store and transfer data; 2) sensors, including gas sensors and biosensors; and 3) indicators, namely time-temperature indicators (TTI), freshness indicators and gas indicators (Table 1) (Lund, 2001; Biji *et al.*, 2015; Poyatos-Racionero *et al.*, 2018; Müller and Schmid, 2019). This classification is predicted by 1) the differences in the hardware of each devices; 2) the amount and type of information these can generate and 3) how that information is collected and distributed to the final

consumer (Lund, 2001). However, this type of packaging can also be classified according to the ability to monitor environmental conditions which can lead to changes in the quality characteristics of the food, and the capacity in supporting the information achieved. The packages that monitor environmental conditions deal with possible changes in the surroundings that may cause a quality decrease of the food product, and these systems (e.g., sensors and indicators) may be located inside or outside the packaging. The packages that monitor the food quality characteristics, located inside the package, are used to directly and continuously evaluate the quality attributes of the food product itself, and display the information (Biji *et al.*, 2015; Müller and Schmid, 2019).

Data carriers are devices that help make the information´s flow more easily and efficiently, along the food chain. The functionality of this type of monitoring is based on traceability, automation, anti-theft protection and falsification by food producers. For this purpose, these devices are often placed in tertiary packaging being capable of keep and transmit information about distribution, storage and other parameters. Globally, the most commonly used data carriers are barcode and radio frequency identification (RFID) tags (Table 1) (Lund, 2001; Ghaani *et al.*, 2016; Sohail, Sun and Zhu, 2018; Müller and Schmid, 2019).

Sensor is a device used to detect, locate or quantify energy or matter, emitting a signal for the detection or measurement of a physicochemical property to which the device reacts (Kerry, O'Grady and Hogan, 2006; Biji *et al.*, 2015). It must be able to provide a continuous output signal to be considered a sensor. Most sensors consist of two basic functional units, a receptor (a processing system) and a transducer (a signal display unit) (Neethirajan, Jayas and Sadistap, 2009; Biji *et al.*, 2015; Ghaani *et al.*, 2016). Physicochemical information of the product is transformed, by the receptor, into energy that is measured and translated, by the transducer, into a useful analytical signal (electrical, optical, thermal or chemical) (Kerry, O'Grady and Hogan, 2006; Ghaani *et al.*, 2016; Müller and Schmid, 2019). A sensor should present ideal characteristics, particularly, 1) selectivity and sensitivity to changes in target compounds concentration; 2) give a quick response; 3) increase product shelf life; and 3) present a reduced size allowing its manufacture at low cost (Ghaani *et al.*, 2016). The most developed sensor technologies, which can incorporate intelligent devices in packaging, belong to two main groups, biosensors and gas sensors (Table 1) (Vanderroost *et al.*, 2014; Ghaani *et al.*, 2016).

Table 1. Types and examples of intelligent packaging devices used in food applications and their operation principle and function (Kerry, O'Grady and Hogan, 2006; Kuswandi *et al.*, 2011; Realini and Marcos, 2014; Biji *et al.*, 2015; Ghaani *et al.*, 2016; Poyatos-Racionero *et al.*, 2018; Müller and Schmid, 2019).

	Intelligent Devices	**Commercial Examples**	**Principle/Reagents**	**Functions**
Data Carriers	Barcodes	- Quick response code (QR code)	- One and bidimensional symbology	Product and manufacturer information; stock control; reorganization and check-out
	RFID	- Easy2log; - TempTRIP; - Intelligent box	- Radio waves	Product and manufacturer information; detect cold chain interruptions
Sensors	Biosensors	- Food Sentinel System™	- Biochemical reactions; - Target metabolites and bio-receptor	Food spoilage detection; pathogens identification; Food safety
	Gas Sensors	—	- Dyes sensitive to gaseous analytes	Product spoilage detection; oxygen and carbon dioxide detection
Indicators	TTIs	- Fresh-Check®; - VITSAB® - OnVu™; - FreshCode; - Tempix	- Mechanical reaction; - Chemical reaction; - Enzymatic reaction; - Microbiological reaction	Control storage conditions, exposure to abusive temperatures or interruption of the cold chain during product storage and product quality
	Gas Indicators	- Ageless Eye®; - ECMO Packaging	- Redox dyes; - Reducing components or alkaline compound; - pH dyes	Product quality; oxygen and carbon dioxide detection and monitoring; detection of product spoilage *status*, by colorimetric changes
	Freshness Indicators	- Raflatac; - SensorQ™; - FreshTag®	- Dyes reacting to volatile and non-volatile amines - pH dyes	Detection of microbial growth (spoilage); product quality and freshness

Indicators are substances that indicate the presence or absence of a certain component or the degree of reaction between two or more substances through direct visual changes in indicator characteristics, especially in its color (Table 1). Depending on the indicator, it can be located inside or outside of the package (Kerry, O'Grady and Hogan, 2006; Hogan and Kerry, 2008; Biji *et al.*, 2015). Since the indicators are the type of intelligent packaging to be developed in this project, these systems will be described in more detail on this chapter.

1.1.1. Indicators

Indicators, unlike the sensors, do not need any receptor or transducer components since information are communicated through a direct visual change (Kerry, O'Grady and Hogan, 2006). For this reason, the indicators present the information in a qualitative or semi-quantitative form, depending on its specificity (Balbinot-Alfaro *et al.*, 2019). So, it is considered an important device in intelligent packaging area since it allows risk and cost reduction and losses limitations associated to the replacement and/or repair of damaged products (Barska and Wyrwa, 2017; Balbinot-Alfaro *et al.*, 2019).

Despite the wide variety of indicators, these can be divided into three main categories: TTIs, gas indicators and freshness indicators (Hogan and Kerry, 2008).

1.1.1.1. Time-temperature indicators (TTIs)

TTI can be defined as a device capable of exhibiting a visible, measurable and irreversible change in a physical characteristic, usually color or shape, reflecting the effect of temperature on the food product over time. These are typically small tags or labels that control the time-temperature of a perishable food along the food chain (Kerry, O'Grady and Hogan, 2006; Biji *et al.*, 2015). Depending on the operational principle, TTIs systems detect time-temperature dependent chemical, physicochemical or biological changes of food product, which are manifested as an irreversible or reversible visible color development or a mechanical change in consistency (Pavelková, 2013).

TTIs are distinguished in three categories: 1) critical temperature indicator, which aims to inform whether the food product has been exposed to temperatures above or below the acceptable (critical) temperature; 2) partial history indicator, which communicates whether the product was submitted to temperature changes that put product quality at risk; and, finally, 3) the total history

indicator that records the complete temperature profile of a product along the food chain (Müller and Schmid, 2019).

Some TTIs commercially available are MonitorMark™, Timestrip®, Checkpoint®, Fresh-Check® and OnVu™ (Pavelková, 2013; Gao *et al.*, 2020). Fresh-Check® TTI (Temptime Corp., Morris Plains, NJ, USA) is an indicator based on a solid-state polymerization reaction. The TTI response consists of a measurable change in color (due to a decrease of reflectance), leading to the formation of a highly colored polymer (Figure 2). This indicator may be applied to packages of perishable products (fruit, lettuce, milk and chilled food) to ensure that the product remains fresh and safe for consumption at the point-of-purchase (Pavelková, 2013). OnVu™ (Ciba Specialty Chemicals and FreshPoint, Inc., Basel, Switzerland) is another example of a commercially available TTI system based on photosensitive dye compounds (organic pigments) that change color with time at rates determined by temperature. When TTI is exposed to heat, it gradually changes dye color (dark blue to white) as a function of time and temperature, to alert consumers not to consume or purchase the food product (Gao *et al.*, 2020).

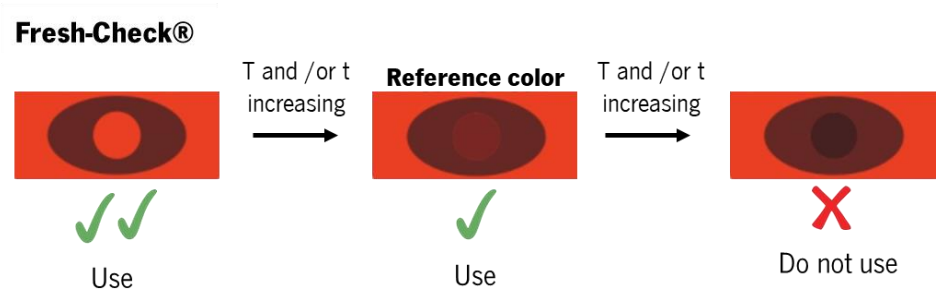


Figure 2. Color changes of Fresh-Check® indicator due to an increase of temperature (T) and/or time (t). The indicator, a small circle, is made of a polymer surrounded by a printed reference ring. If the package has experienced unfavorable temperature exposures, the inner polymer circle darkens, and the color intensity is measured and compared to the reference color scale on the label. Image adapted from Gao *et al.* (2020).

1.1.1.2. Gas Indicators

Gas indicators show product quality *status*, depending on atmosphere composition inside the package. The gas changes inside the package are a consequence of enzymatic and chemical reactions mainly due to gas formation during microbial metabolism or gas transmission through the package which depend on the nature of the packaging and environmental conditions (Müller and Schmid, 2019). The functionality of these devices is based on use of *redox* dyes, reducing

component or alkaline compound (Ghaani *et al.*, 2016). Most of these types of indicators are capable to detect and monitor oxygen and carbon dioxide concentrations since these are strictly correlated with the progress of deterioration in food products (Müller and Schmid, 2019). For instance, researchers from Sejong University (South Korea) developed carbon dioxide indicators, which consist of an aqueous chitosan solution and a whey protein isolate (WPI). The presence of carbon dioxide is detected by changes in the clearness of the indicator due to sensibility of whey to pH changes. Acidification of the WPI aqueous suspension, by dissolution of carbon dioxide, decreased the aqueous system pH towards the pI value of WPI, at which protein molecules lose solubility. WPI aqueous suspension was transparent above pH 6, but became opaque when the pH decreased (Jung, Puligundla and Ko, 2012; Lee and Ko, 2014).

1.1.1.3. Freshness Indicators – pH indicators

Freshness indicators (such as, pH indicators) are devices, usually integrated into the packaging, capable of reacting with metabolites generated by microbial growth and metabolism or able to detect any chemical change, providing visual information about food product quality (Kerry, O'Grady and Hogan, 2006; Kuswandi *et al.*, 2013; Poyatos-Racionero *et al.*, 2018). Differences in metabolite concentration such as glucose, organic acids (lactic acid), ethanol, carbon dioxide, biogenic amines, volatile nitrogen compounds or sulfur derivatives, during storage point out microbial growth (Nopwinyuwong, Trevanich and Suppakul, 2010; Fang *et al.*, 2017; Poyatos-Racionero *et al.*, 2018; Balbinot-Alfaro *et al.*, 2019). For this reason, the presence of any of the mentioned metabolites is considered an opportunity to evaluate the freshness and quality of the food product (Poyatos-Racionero *et al.*, 2018).

Research efforts have been conducted to develop new freshness indicators. For example, Smolander (2003) developed a freshness indicator named Raflatac based on a silver monolayer that reacts with hydrogen sulfide (a product of cysteine decomposition), turning its original brown color to transparent in the presence of this metabolite. Yoshida *et al.* (2014) developed a colorimetric freshness indicator to detect pH changes due to the presence of metabolites derived from microbial growth, such as lactic acid, acetic acid and D-lactate. A commercial example of intelligent packaging that monitor food freshness through metabolite detection is SensorQ™ (its commercialization was discontinued in 2004). This sensitive pH device, based on anthocyanins, is

capable to report the production of microbial biogenic amines by changing its color from orange to brown (Poyatos-Racionero *et al.*, 2018; Müller and Schmid, 2019).

pH indicator systems are capable of providing qualitative information through visual colorimetric changes (e.g., pH indicator reaction with non-neutral compounds) (Zhai *et al.*, 2018). Also, their sensitivity, safety, non-invasive response, low cost and compacted size are also promising characteristics of these type of indicators (Brody *et al.*, 2008; Balbinot-Alfaro *et al.*, 2019). Generally, these indicators involve two components, a solid support and a dye sensitive to pH variations (Pourjavaher *et al.*, 2017; Balbinot-Alfaro *et al.*, 2019). These can be organized into three distinct categories: 1) dyes immobilized on a solid support by physical adsorption; 2) dyes covalently attached to a hydrophilic support such as cellulose or glass; and finally, 3) dyes incorporated in polymeric matrices (Balbinot-Alfaro *et al.*, 2019).

Regarding pH sensitive dyes, synthetic dyes have been applied in the packaging industry, in particular, bromocresol green and purple, chlorophenol, methyl red, among others (Dainelli *et al.*, 2008; Kuswandi *et al.*, 2012). Bromocresol green was previously used as a pH indicator, since a slight increase in the pH value triggers a visible colorimetric change, providing a highly sensitive device. The result is an easily detectable color change from yellow (at pH 3) to blue (pH 5). Additionally, methyl red was used to monitor food freshness status. The food deterioration is noticed, in this case, by the red color change to yellow, at pH above 6 (Kerry, O'Grady and Hogan, 2006; Pacquit *et al.*, 2007; Balbinot-Alfaro *et al.*, 2019). However, the application of these synthetic dyes in packaging applied to the food area has been discontinued due to associated toxicity, becoming difficult to achieve consumer expectations about food safety. As synthetic dyes are considered carcinogenic or mutagenic agents in humans, their application in food packaging is not ideal (Zhai *et al.*, 2018; Balbinot-Alfaro *et al.*, 2019). In attempt to overcome this limitation, more attention has recently been given to alternative use of natural pigments (Silva-Pereira *et al.*, 2015; Zhai *et al.*, 2017, 2018; Luchese, Abdalla, *et al.*, 2018; Balbinot-Alfaro *et al.*, 2019). Natural dyes (pigments) extracted from plants are attractive and relevant options in intelligent packaging field due to low toxicity, easy preparation, renewable and non-polluting properties (Balbinot-Alfaro *et al.*, 2019). Examples of natural dyes, which change their color significantly with pH changes, are curcumin, anthocyanins and betalains (Silva-Pereira *et al.*, 2015; Prietto *et al.*, 2017; Liu *et al.*, 2018; Luchese, Abdalla, *et al.*, 2018; Zhai *et al.*, 2018).

Curcumin, extracted from turmeric, is a hydrophobic polyphenol with a unique structure as is shown in Figure 3. This natural pH dye presents anti-inflammatory and antimicrobial action, and

therapeutic action against various chronic diseases has been demonstrated. Curcumin has also been recognized as a powerful antioxidant agent, being this activity attributed to the existence of its *o*-methoxy groups (Aliabbasi, Fathi and Emam-Djomeh, 2021). Curcumin stability in aqueous solution is pH-dependent, for this reason, yellow color arises between pH 1 to 7 (optimum cut-off point) and turns to reddish orange at pH above 7 (basic pH) (Musso, Salgado and Mauri, 2016). At a pH over 7, the phenolic hydroxyl groups of curcumin react with hydroxyl groups and, as a result, produce phenoxide anion, which leads to further alterations in the color of curcumin.

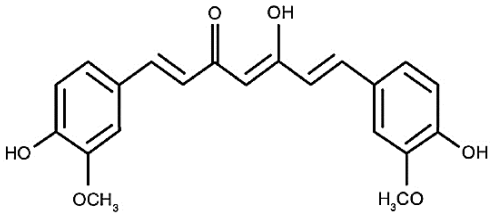


Figure 3. Curcumin chemical structure (Musso, Salgado and Mauri, 2016).

As a result of its natural yellow-orange color, it is mostly used as a spice and food dye. However, recently, it has been used in packaging materials. Musso, Salgado and Mauri (2016) developed an intelligent gelatin packaging incorporating curcumin. All the films were evaluated in the ability to detect pH changes in a liquid or semisolid food or in the food container headspace as food spoilage result. At pH 6, yellow films turned to orange-red when in contact with alkaline gases, liquids, and semisolids. At pH 11, orange-red films turned yellow when in contact with acid media (Musso, Salgado and Mauri, 2017). Furthermore, Liu *et al.* (2018) studied the influence of pH changes in carrageenan films incorporated with curcumin, simulating the conditions experienced during food spoilage. Under alkaline conditions, the yellow films started to show a red-orange color, and under acidic conditions, the red-orange films changed their color to yellow. Films exposed to neutral pH did not change their color. One of curcumin limitations as a natural pH dye is the fact that it presents a wide yellow color range which may not detect possible critical changes in food quality characteristics (such as fish). An alternative to curcumin use are anthocyanins which present a great potential as indicators in intelligent packaging systems, due to their color change in wide pH ranges (Balbinot-Alfaro *et al.*, 2019).

Anthocyanins are phenolic compounds belonging to flavonoids group which structure undergoes pH-dependent reversible transformations. These are water-soluble compounds, highly unstable at

high temperatures, safe and biodegradable. The color expression of anthocyanins depends on several factors, for instance, structure, pH, co-pigmentation, temperature, UV radiation and oxygen presence. For this reason, the color instability of these natural dyes is an important characteristic since it produces useful pigments for observing the quality of packaged foods (Balbinot-Alfaro *et al.*, 2019). Anthocyanins can be found in numerous plant families including *Vitaceae* (grape), *Rosaceae* (cherry, plum, raspberry, strawberry, blackberry, apple, peach), *Solanaceae* (potato), *Saxifragaceae* (black and red currant), *Ericaceae* (blueberry), *Cruciferae* (red cabbage, radish), *Leguminoseae* (pea) and *Gramineae* (cereal seeds) (Krga and Milenkovic, 2019).

There are four main anthocyanins forms: the flavylium cation, the anhydrous quinonoidal base, the pseudo base carbinol and the chalcone (Figure 4) (He and Monica Giusti, 2010). At a pH < 2, anthocyanins predominantly occur in their stable flavylium cation form presenting a red, orange or violet color. An increase on pH values, between 3 and 5, results in cation hydration, which quickly leads to colorless pseudo base carbinol. On the other hand, at pH values 6 to 8, cation deprotonation occurs, through an acid-base reaction, forming the quinonoidal base, causing a color change to a more violet shade. At pH values 9 to 12, additional deprotonation of quinonoidal occurs, causing the formation of quinonoid anions, and it is observed a change of color (to blue) (Torskangerpoll and Andersen, 2005; Choi *et al.*, 2017; Krga and Milenkovic, 2019). Tautomerization of carbinol, at pH 13, leads to retro-chalcone formation responsible for the pale-yellow color (Figure 4). Usually, at high alkaline conditions, anthocyanins will be degraded depending on substituent groups.

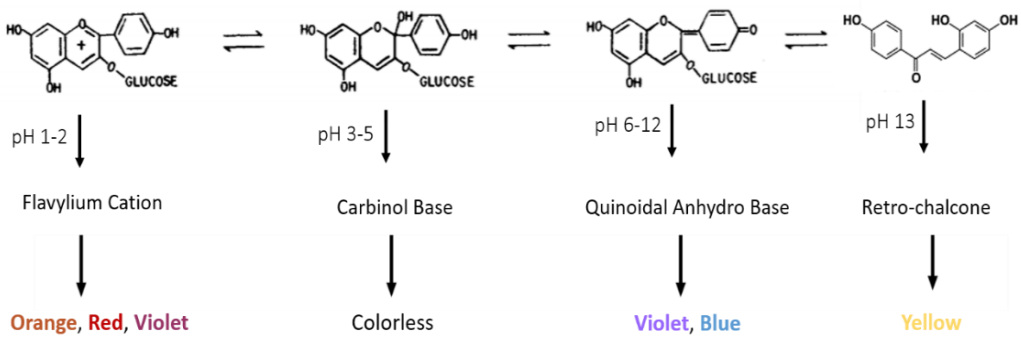


Figure 4. Anthocyanins' structural and colorimetric transformations under different pH conditions. Image adapted from (Wroistad, 1993).

The main challenge in the use of anthocyanins as freshness indicators is their instability to heat during processing and/or storage. Anthocyanins' color tends to fade as temperature increases

above 70 °C (Patras *et al.*, 2010; Valdés *et al.*, 2014; Sui, Bary and Zhou, 2016; Choi *et al.*, 2017; Wu, Yang and Chiang, 2018). García-Viguera *et al.* (1998) reported that storage temperature plays a critical role for anthocyanins stability as they observed a slower anthocyanins' degradation at 20 °C compared to 37 °C. Therefore, as the temperature increases, the degradation rate constant of anthocyanins increases, accompanied by a suddenly decreased half-life value (Huang *et al.*, 2021). Moreover, the anthocyanins content can be affected by a combination of unit operations involving heat (e.g., blanching and steaming). Some studies concluded that blanching, boiling and steaming processes resulted in losses of the total anthocyanin content in red cabbage to 59 %, 41 % and 29 %, respectively (Patras *et al.*, 2010). One of the solutions is to use refrigerated storage conditions, preferably at 4 to 8 °C, in order to ensure that anthocyanins color change depends only on food deterioration caused by chemical and biological changes (e.g., pH and microbial growth) (Wu, Yang and Chiang, 2018). On the other hand, some researchers reported that the anthocyanins' color stabilization may be due to co-pigmentation with other compounds such as betalains (Patras *et al.*, 2010).

Betalain pigments are widely distributed in nature, being easily located in red and yellow beets (*Beta vulgaris L. spp. vulgaris*), colorful chard (*B. vulgaris L. spp. cicla*), amaranth leaves and granules (*Amaranthus sp.)*, cactus (*Opuntia sp.)* and dragon fruit (*Hylocereus sp.)* (Celli and Brooks, 2017). Betalains are poorly explored compared to anthocyanins and information related to the effects of processing on betalains physicochemical properties and stability are still scarce (Celli and Brooks, 2017). Comparing the two natural dyes, betalains are more hydrophilic, stable to pH and temperature changes than anthocyanins. Betalains are relatively stable in a pH range of 3 to 7, unlike anthocyanins which are unstable at pH values above 3 (Stintzing and Carle, 2004; Herbach, Stintzing and Carle, 2006; Slimen, Najar and Abderrabba, 2017). Therefore, the combination of both natural pigments is a good strategy to increase the natural pigments stability and to extend the range of pH values to be detected (Celli and Brooks, 2017).

Betalains are tyrosine-derived pigments composed of a nitrogenous core structure (betalamic acid). Commonly, betalains can be distinguished by their colorimetric and chemical features in two different categories: betacyanins, which exhibit violet coloring; and betaxanthins, which display yellow coloring (Figure 5) (Gandía-Herrero, Escribano and García-Carmona, 2010; W. Yang *et al.*, 2021). At pH values ranging from 4 to 7, betacyanins prevail and, for this reason, the color displayed will be red-violet. The pigment color changes from red to purple when pH < 4 or pH > 7.

At pH values higher than 10, betaxanthins turn to yellow, quickly (Esatbeyoglu *et al.*, 2016; Moreno-Ley *et al.*, 2021).

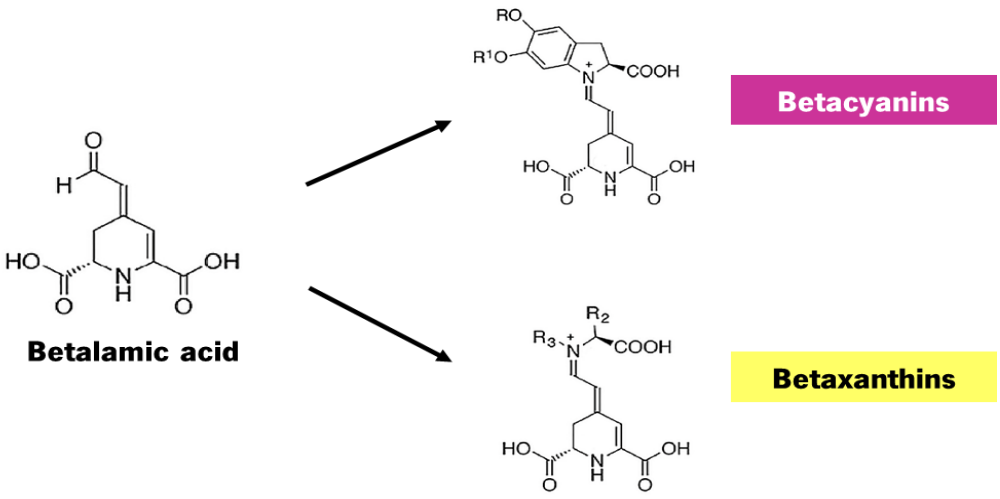


Figure 5. General betalains structure. Betalamic acid, the main structure of betalains, leads to betacyanins and betaxanthins formation, through different pathways. [R, R1] is substituted by glucosyl or derivatives and [R2, R3] by amino acids, amines or derivatives. Image adapted from Moreno-Ley *et al.* (2021).

1.2. Bio-based intelligent packaging

The increase consumer and food industry consciousness related to food safety and environmental pollution, guided researchers to focus on the development of safe, environmentally friendly and biodegradable packaging materials. Thus, the research aim is to replace synthetic, petroleum-based polymers (such as plastics) by eco-friendly materials to be use in food packaging, especially on intelligent packaging (Choi *et al.*, 2017; Qin *et al.*, 2019). In recent years, the development of novel bio-based intelligent packages increased since they provide several advantages such as: 1) control environmental pollution and, consequently, reduce carbon footprint; 2) decrease problems related to waste treatment (such as drop labor and disposal costs); and 3) create more clean food production processes and sustainable supply chain (Luchese, Garrido, *et al.*, 2018; Mellinas *et al.*, 2020).

The potential use of natural biopolymers (such as polysaccharides and proteins) for the development of bio-based intelligent packaging increased scientific interest in these biopolymers due to their high abundance, high availability in nature and in low-value agricultural residues/byproducts (for example, fruit peels and seeds) (Prietto *et al.*, 2017; Martau, Mihai and Vodnar, 2019; Qin *et al.*, 2019). Biopolymers are obtained from natural sources or are totally

biosynthesized by living organisms, presenting exclusive characteristic properties: biodegradability, biocompatibility, flexibility, versatility and absence of toxicity which enable the application of these compounds in multifunctional areas.

In recent years, polysaccharides received significant attention caused by their high abundance and natural availability (Martau, Mihai and Vodnar, 2019). Therefore, they have been explored as raw materials to produce intelligent films to be applied in food products. Polysaccharides are biopolymers formed by a long chain of monosaccharides or disaccharides linked by glycoside bonds. At the structural level, they are formed by a high number of polar groups, such as hydroxyl, capable to bonding to water molecules by hydrogen bonds. Thus, polysaccharides are considered hydrophilic compounds. These biopolymers have the ability to form a film with functional properties namely, thermal stability and low permeability to carbon dioxide, lipids and oxygen, to avoid increasing food deterioration rate. However, the films formed by polysaccharides are highly sensitive to moisture due to hydrophilic nature, forming a weak barrier to water vapor. In addition, the low mechanical resistance of films produced only with polysaccharides is a limitation (Mellinas *et al.*, 2020). Different approaches have been adopted in order to solve these problems such as, incorporation of different functional additives (e.g. essential oils), mixture of different biopolymers, or multilayer films (Valdés *et al.*, 2014; Silva *et al.*, 2018; Wang *et al.*, 2019; Mellinas *et al.*, 2020).

Polysaccharides are frequently used in the food industry as gelling, stabilizers, thickeners and film-forming materials. Polysaccharides can be classified as neutral (e.g. starch, cellulose and locust bean gum - LBG), anionic (e.g. alginate, carrageenan, pectin, xanthan gum and guar gum) or cationic (e.g. chitosan) (Li and Buschle-Diller, 2017). Among these polysaccharides, starch has received considerable attention as a result of its low cost, high abundance and edibility (Qin *et al.*, 2019). Starch is isolated from various plant sources such as cassava, wheat, corn, rice and potatoes (Qin *et al.*, 2019). Starch films provide excellent oxygen barrier properties because of the presence of hydrogen-bonded network structure that involves crystalline and non-crystalline regions, in alternating layers, formed by amylose and amylopectin (Cazón *et al.*, 2017). However, there are limitations to its isolated use in films such as reduced mechanical resistance and water vapor barrier capacity (Qin *et al.*, 2019). In order to improve starch films mechanical and functional properties, the addition of plasticizing agents has been proposed, expanding its application to food packaging (Cazón *et al.*, 2017; Qin *et al.*, 2019). Qin *et al.* (2019) developed a film based on cassava starch with *Lycium ruthenicum Murr* anthocyanins incorporation, in order to monitor the pork meat freshness. A film based on potato starch and *Bougainvillea glabra* betacyanins was

developed in order to observe the pH and ammonia influence on fish freshness (Naghdi, Rezaei and Abdollahi, 2021).

Also, alginate, an indigestible natural polyanionic polymer, is used in multiple applications, including as a film and coating for fresh fruits and vegetables, thickening, gelling, emulsifier and stabilizer agent in food products (Parreidt, Müller and Schmid, 2018; Martau, Mihai and Vodnar, 2019). Its main source is the brown algae (*Phaeophyceae*) (Tapia *et al.*, 2008; Parreidt, Müller and Schmid, 2018). Sodium alginate (a monovalent salt of alginic acid) is commonly used to developing films as it improves the barrier function and the mechanical properties of the film (Parreidt, Müller and Schmid, 2018). Colorimetric films based on sodium alginate, pectin and xanthan gum containing raspberry extract have been developed to monitor the freshness of food products presenting high protein content (J. Yang *et al.*, 2021).

Pectin is a natural and structural polysaccharide present in cell wall of many plant cells. This anionic biopolymer can be extracted and isolated as a biomaterial with different applications, including food packaging. However, pectin films have inadequate moisture barrier, low thermal stability and limited mechanical properties, reasons for pectin be combined with different biopolymers (chitosan and starch) (Martau, Mihai and Vodnar, 2019; Mellinas *et al.*, 2020). A pH-sensitive indicator film incorporating beetroot extract encapsulated in pectin from watermelon peel was prepared to monitor chilled beef quality changes during storage (Guo *et al.*, 2021). Kurek *et al.* (2021) studied the effect of adding red betalains obtained from prickly pear (*Opuntia-ficus indica L.*) to a protective polymer matrix formed of pectin and chitosan.

Chitosan is a non-toxic biopolymer presenting antimicrobial and antioxidant, properties suitable for possible food applications (Souza *et al.*, 2010; Martau, Mihai and Vodnar, 2019). Chitosan is obtained by alkaline N-deacetylation of chitin found in exoskeletons of crustaceans and various insects. For this reason, this polymer is found commercially in abundant renewable sources, mainly on residues from the seafood industry (Kausel, 2010; Cazón *et al.*, 2017). Chitosan-based films usually present excellent oxygen barriers and good mechanical resistance (Souza *et al.*, 2010). However, these are highly permeable to water vapor, which limits their use in food products, since it is an important property for maintaining food quality (Aider, 2010; Cazón *et al.*, 2017). For this reason, some strategies have been developed in order to improve the functional properties of chitosan based films, particularly, the association with other biopolymers (starch and alginate) (Elsabee and Abdou, 2013). Li *et al.* (2021) developed chitosan-based intelligent films incorporating purple tomato anthocyanin in order to check milk and fish freshness/deterioration.

LBG, a galactomannan, is a non-ionic linear polysaccharide composed by a β-(1-4)-manose chain linked by α-(1-6) bonds to the side branch of D-galactopyranosyl units (Figure 6A) (Kalia and Sabaa, 2013). LBG is obtained from seeds' endosperm of some legumes, which belong mainly to *Ceratonia siliqua L.* family found in the Mediterranean regions (Barak and Mudgil, 2014; Sébastien *et al.*, 2014). The water solubility of LBG and its rheological properties are influenced by galactose substitution degree on the mannose chain, the mannose/galactose ratio and molecular weight. Therefore, LBG shows low solubility at room temperature, requiring a thermal treatment in order to obtain a better water binding capacity, assuring complete hydration and maximum viscosity (Dakia *et al.*, 2008; Martins *et al.*, 2012). LBG is considered the first galactomannan to be used as an additive in the paper, textile, pharmaceutical, cosmetic and food industries. The characteristic that makes its use advantageous compared to other gums is its ability, at relatively low concentrations, to form a very viscous aqueous solution, stabilize emulsions and replace fat in many food products (Pollard *et al.*, 2007; T. Li *et al.*, 2021). LBG solutions are not influenced by changes in pH or presence / absence of salts due to its neutral nature. The synergistic combination of LBG with other hydrocolloids, such as carrageenan and xanthan, allows the formation of a film with better elasticity and strength (Pinheiro *et al.*, 2011; Barak and Mudgil, 2014). Intelligent packaging films were developed based on LBG and polyvinyl alcohol containing betacyanins from cockscomb flower to indicate shrimp freshness (Y. Wu *et al.*, 2021).

Carrageenans, derived from red algae, are anionic linear sulfated polysaccharides, which consist in repetition of disaccharide units with alternating bonds of 3-β-D-galactopyranose and 4-α-galactopyranose (Figure 6B) (Souza *et al.*, 2011; Liu *et al.*, 2019). These have been widely used in the food (mainly, in dairy products) and pharmaceutical industries, due to their biocompatible and biodegradable properties. Carrageenans can be classified into three different groups, κ-, ι- and λ-carrageenan. It should be noted that κ-carrageenan (κ-car) contains a negatively charged sulfate and 3,6-anhydro-D-galactopyranose residues in its chain, unlike the other two groups, which give κ-car the ability to develop films (Shahbazi *et al.*, 2016). However, films produced only with κ-car, highly hydrophilic, generally demonstrate ineffective mechanical and barrier properties. These limitations can be overcome through the combination of κ-car with other substances, specifically, glycerol and phenolic compounds (Martins *et al.*, 2012; Liu *et al.*, 2019). He *et al.* (2020) developed a film based on κ-car, gelatin and curcumin, which work as an intelligent indicator for grass carp fillets´ freshness detection. Also, novel films based on κ-car incorporating curcumin were made in order to monitoring pork and shrimp freshness (Liu *et al.*, 2018).

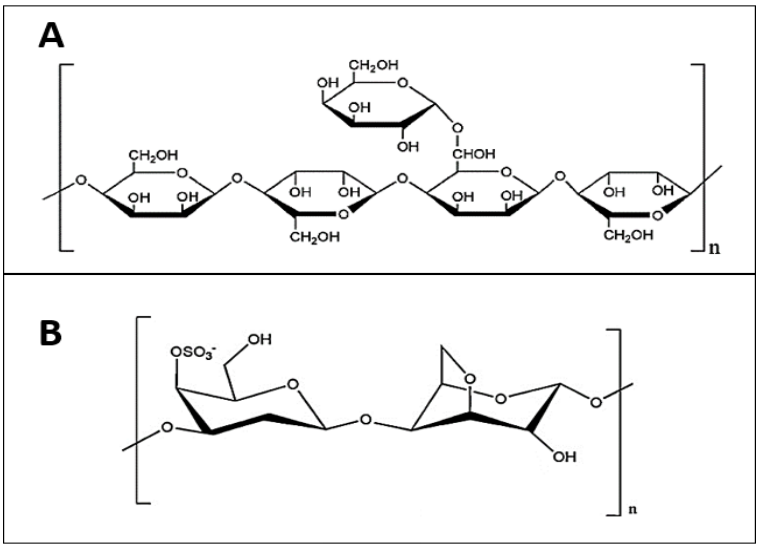


Figure *6.* Chemical structure of locust bean gum (A) and κ-carrageenan (B). Image adapted from Souza *et al.* (2011) and Kalia and Sabaa, (2013).

The biodegradability of these films can be a limitation to their commercialization since bio-based films are normally more predisposal to thermal degradation, present lower mechanical properties than those presented by fossil-based films, and are quite hygroscopic, which can lead to film premature and undesirable deterioration (Musso, Salgado and Mauri, 2016). In order to improve films properties, the synergism between different polysaccharides due to conformational changes and chemical interactions could be explored to develop novel films. In this project, this topic was explored to developing an intelligent film where LBG and κ-car was combined to form a film with desirable properties namely, effective water barrier, good elasticity and transparent (Pinheiro *et al.*, 2011; Martins *et al.*, 2012). Furthermore, other possible solution includes combination with additives (plasticizers and/or surfactants) to improve the biopolymer-based film properties (Musso, Salgado and Mauri, 2016). Plasticizer is a molecule of low volatility that is added to the biopolymeric matrices in order to modify its three-dimensional organization, decrease intermolecular strength and increase chains volume and flexibility (Nur Hanani, Roos and Kerry, 2014). Some of the most commonly used plasticizers are polyols (glycerol, sorbitol and polyethylene glycol), sugars (glucose and sucrose) and lipids (monoglycerides and phospholipids) (Espitia *et al.*, 2014). Plasticizer addition avoids film brittleness as result of the interactions between the different polymer chains (Cazón *et al.*, 2017). Plasticizers increase extensibility, flexibility, elasticity and resistance to break. On the other hand, they decrease deformation tension, hardness, density and viscosity (Vieira *et*

al., 2011; Nur Hanani, Roos and Kerry, 2014; Wang, Qian and Ding, 2018). Moreover, the addition of these components increases film's water permeability and water adsorption capacity (Nur Hanani, Roos and Kerry, 2014). Thus, plasticizer type and quantity contribute to the final film performance.

Surfactants are also important compounds to be added to films as their main function is to stabilize the emulsion solution during film formation process. Without macroscopic separation, the mixture between the two immiscible phases, oil and polymeric phase, is essential to obtain films with desirable properties (Tongnuanchan, Benjakul and Prodpran, 2014; Tongnuanchan *et al.*, 2016). Some examples of surfactants are Tween 80, Tween 20, Brij 56 and Span 20, which are used in order to develop films with improved properties (Wang, Qian and Ding, 2018). Prodpran *et al.* (2007) and Peng *et al.* (2014) used Tween 20 as surfactant in films based on chitosan with lemon, thyme and cinnamon essential oils. The use of Tween 80 as surfactant has been reported in cassava starch films with essential oils (Prodpran, Benjakul and Artharn, 2007; Peng and Li, 2014; Tongnuanchan, Benjakul and Prodpran, 2014).

Thus, polysaccharide-based intelligent films with natural compounds as freshness indicators (e.g., anthocyanins and betalains) has the potential to guarantee food quality and safety, reduce environmental impact and increase product packaged attractiveness to the retailer and the final consumer (Cazón *et al.*, 2017; Choi *et al.*, 2017; Balbinot-Alfaro *et al.*, 2019). However, despite the permanent innovation in food packaging area, research related to this type of bio-based intelligent packaging is still limited.

1.3. Application of polysaccharide-based intelligent packaging to food products

Food quality continuously deteriorates during storage, until the food product becomes uneatable. The metabolites produced by spoilage microorganisms adversely affect food taste, nutritional value, and flavor. A relationship can be established between the food freshness and pH values, since pH changes is a major sign of food product quality state (Prietto *et al.*, 2017). For instance, organic acids (such as, lactic acid, acetic acid and citric acid) concentration changes during fruits and vegetables storage, which lead to pH decrease inside food packages (Gandía-Herrero, Escribano and García-Carmona, 2010; Liu *et al.*, 2018). Also, the decarboxylation of free amino acids in meat by microbial enzymes results on nitrogenous compounds formation and accumulation, which cause a pH increase (Slimen, Najar and Abderrabba, 2017). Similarly, the

volatile basic nitrogen compounds such as dimethylamine (DMA), trimethylamine (TMA), histamine, tyramine and ammonia, are associated with fish deterioration during storage due to microbial growth (Prietto *et al.*, 2017). Therefore, these metabolites can be detected by natural indicators (e.g., natural dyes) based on colorimetric changes triggered by chemical reactions. Most of the results published in the literature focus on real-time quality evaluation of packaged products, such as perishable and high-value food products. Intelligent packaging systems produced with polysaccharides and natural pigments have been developed and applied to several food products, such as fruit, vegetables, meat and fish (Table 2) (Poyatos-Racionero *et al.*, 2018). More information on fish freshness and quality will be next provided since this food product was the focus of the present study.

Table 2. Selected examples of polysaccharide-based intelligent films incorporating freshness natural indicators applied to food products.

Polysaccharide-based matrix	Natural indicator and source	Stimuli responsive factor	Food application	Main findings	Ref.
Agar/potato starch	Anthocyanins from purple sweet potato	pH	Pork meat	Film detected pork meat spoilage state. Initially, pork samples had pH 5.8 and color film was red. After 12 h and 20 h, film was **pink** and **green** (pH 7.5), respectively.	(Choi *et al.*, 2017)
Chitosan/corn starch	Anthocyanins from red cabbage	pH	Fish (fillets)	Film detected fish pH changes. During 72 h at 4 - 7 ºC, no color change was observed. After 72 h, film was blue and 7 days later film was yellow (complete fish spoilage).	(Silva-Pereira *et al.*, 2015)
Chitosan/Gelatin	Betalains from *Amaranthus tricolor L.*	TVB-N	Fish (shrimp)	Film color gradually changed from **pink** to **yellow** after 48 h of fish storage. The color change was in accordance with the increase in TVB-N content (5.2 ± 0.5 to 55.3 ± 1.5 mg/100 g).	(Hu *et al.*, 2020)
Cassava starch	Anthocyanins from blueberry and bayberry	pH, TVB-N	Meat (chicken and pork)	Film detected chicken meat pH and TVB-N changes, which are an indicative of spoilage. During 10 days at 6 ºC, color changes from **pink** to **green**. Film response to changes on TVB-N levels in the pork meat was evaluated during storage at 25 ºC for 48 h. Color changes from **purple** to **green/grey**.	(Luchese, Abdalla, *et al.*, 2018) (Yun *et al.*, 2019)
κ-carrageenan	Curcumin from turmeric (*Curcuma longa*)		Pork meat and shrimp	Pork and shrimp samples were stored at 25 ºC during 3 days. Film color changed from **yellow** (day 0) to **orange/red** (3rd day), being in accordance with pH levels (increase from 3 to 8). Correspondingly, TVB-N levels of pork and shrimp increased from 4.91 to 31.11 mg/ 100 g and from 7.15 to 41.53 mg/ 100 g, respectively.	(Liu *et al.*, 2018)
Potato starch	Betalains from paperflower	pH and TVB-N	Fish (Caspian sprat)	pH and TVB-N values increase, which are indicative of spoilage state, and film color changed from **pink/red** (day 0) to **yellow/green** (day 16).	(Naghdi, Rezaei and Abdollahi, 2021)
Agar	Pigments from *Arnebia euchroma* root extracts	pH and TVB-N	Fish (Wuchang bream)	According to pH, TVC and TVB-N thresholds, the fish spoilage stage was achieved after 12 – 20 h at 25 ºC and 5 – 6th day at 4 ºC, respectively, and film color was **purple**.	(Huang *et al.*, 2019)

1.3.1. Assessment of fish freshness

In recent years, fish consumption and popularity have been increasing due to customers' awareness regarding fish nutritional composition, crucial for human health (Souza *et al.*, 2010; Jasour *et al.*, 2015). Portugal has the third highest fish consumption, *per capita*, in the world, after Japan and Iceland (Figure 7). However, it is the biggest fish consumer in European Union and the main reasons are: 1) geographical location as it has a 942 km coastline and two insular areas; and 2) large cultural and social significance once Portugal has tradition in fishing and fish consumption (Almeida, Karadzic and Vaz, 2015; OECD and FAO, 2021). Presently, the national production supplies only 23 kg per year, when the current consumption is around 62 kg per year (Almeida, Karadzic and Vaz, 2015). The fish and seafood supply per person was 56.84 kg per year, in Portugal, in 2017 (OECD and FAO, 2021). Compared to the world average, which rises every year and grew from 11 kg to 19 kg *per capita*, fish consumption in Portugal is still about twice as high (Almeida, Karadzic and Vaz, 2015). World apparent fish consumption per capita is projected to reach 21.2 kg in 2030, up from an average of 20.5 kg between 2018 and 2020 (OECD and FAO, 2021).

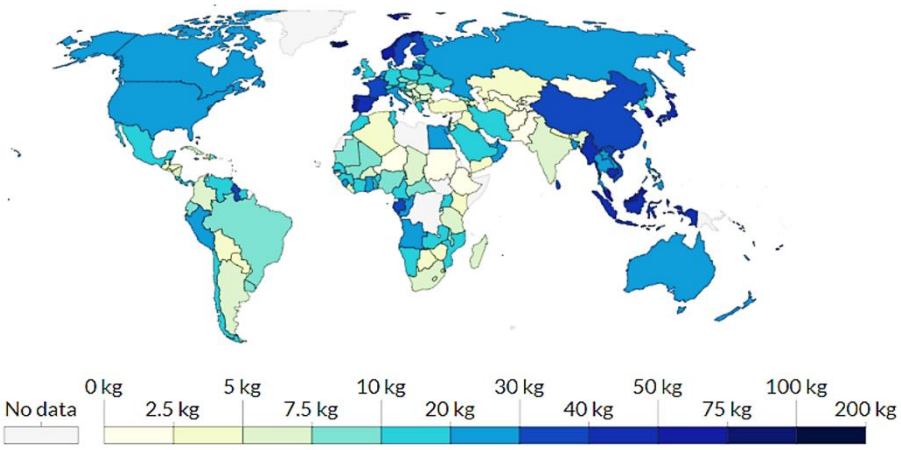


Figure 7. Global fish and major seafood commodities consumption, per capita, in 2017. Source: Food and Agriculture Organization (FAO) (https://ourworldindata.org/grapher/fish-and-seafood-consumption-per-capita?country=~SDN)

In Portugal, soaked cod, canned tuna and hake are the most eaten seafood products. The choice of hake as the model fish studied was based on the fact that it is the third most consumed

(around 6 %), in Portugal, and there is a lack of information about monitoring its quality using bio-based intelligent packaging, which is an opportunity to advance in this research area.

The genus Merluccius is currently represented by 12 species that are widely distributed in the Atlantic, Pacific, and around New Zealand. This genus taxonomy is complex and species (e.g., *Merluccius bilinearis*, silver hake from American Atlantic and *M. capensis*, ray-finned hake from South Africa) identification is hard due to their external morphology similarities. *M. merluccius* is the European and Mediterranean hake, which grows about 1.1 meter long and, in general, it is grey becoming lighter on the sides and silvery white on the belly (Figure 8) (Murua, 2010; Silva-Segundo *et al.*, 2011; Almeida, Karadzic and Vaz, 2015).

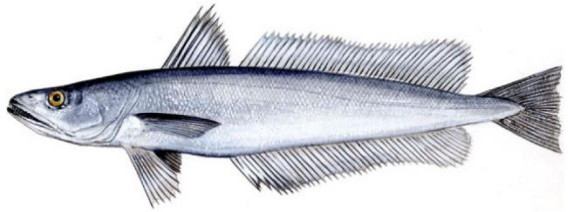


Figure 8. *Merluccius merluccius* (European hake fish) (Murua, 2010).

Fresh fish (83 %) consumption is predominant in Portugal, followed by salted and dried fish (17 %) (namely, cod fish), frozen, smoked and canned fish (11%) (Almeida, Karadzic and Vaz, 2015). Although fresh fish is a more expensive product, consumers are willing to pay a higher price for a better fish product quality (Almeida, Karadzic and Vaz, 2015; Mitra *et al.*, 2021; Witter, Murray and Sumaila, 2021).

However, fresh fish provides ideal conditions for fast microbial and biochemical deterioration mainly due to fish high water content, high concentration of free amino acids, non-nitrogen proteins and unsaturated fatty acids and neutral pH (Souza *et al.*, 2010; Lougovois and Kyrana, 2014; Jasour *et al.*, 2015). After fish death, fish goes through the following phases: pre-rigor, rigor *mortis* and resolution of *mortis*, where autolysis and deterioration occurred throughout these stages (Liu *et al.*, 2013; Yu *et al.*, 2019). The duration of each phase depends on the intrinsic characteristics related to fish species (i.e., endogenous enzymes activity, muscle tissue fragility and initial microbial flora) and the tight relationship to external factors (such as storage temperature). Fish dripping, discoloration, protein degradation, texture changes, nucleotide decomposition, nitrogen

compounds accumulation and lipid oxidation are the most representative signals of significant losses in fish quality during storage (Yu *et al.*, 2019).

Several approaches have been used to determine fish freshness and quality, namely sensorial (e.g., odor, taste and texture), microbiological (e.g., total viable count - TVC), physical (e.g., texture and electrical properties), and chemical (e.g., *K* and *K*1 values, TVB-N level and lipid oxidation) methods. The *K* and *K*1 values reflect ATP degradation process after fish's death and they have often been used as freshness indicators (Pacquit *et al.*, 2006). Also, the TVB-N value affects nutritional, sensorial and safety characteristics of fishery products, being a potential indicator of fish deterioration *status* (Pacquit *et al.*, 2006; Al Bulushi *et al.*, 2009; Liu *et al.*, 2013).

Biogenic amines (mainly histamine, putrescine, tyramine and cadaverine) could be responsible for food poisoning causing serious human health problems when their concentration in fish products is too high. They have been implicated as indicators of fish decomposition since that are generated by microorganisms' enzymatic activity (decarboxylases) (Okuma *et al.*, 2000; Kaniou *et al.*, 2001; Rokka *et al.*, 2004; Kerry, O'Grady and Hogan, 2006; Ruiz-Capillas and Herrero, 2019). Changes in the concentration profiles of above mentioned amines can correlate to several factors such as microbial deterioration, storage conditions (e.g., temperature and storage duration), manufacturing processes and practices (Beyermann and Slemr, 1985; Rokka *et al.*, 2004).

Freshness indicators based on visual (colorimetric) and pH methods have been established to detect the TVB-N content present in fish during its deterioration process (Table 2) (Hurley *et al.*, 2013; Ghaani *et al.*, 2016). Simple devices that permit non-invasive, non-destructive and real-time determination of fish freshness have been described in the literature based on pH changes (Kuswandi *et al.*, 2011). During fish deterioration, a variety of basic volatile and non-volatile amines are gradual release to the packaging headspace, which cause a pH increase and, consequently, lead to dye color change. These changes can be detected through an indicator placed in direct or indirect contact with fish. Thus, depending on the acidic or basic environment to which these indicators are exposed, there will be a visible change on the indicator color, monitoring fish quality level (Pacquit *et al.*, 2006; Nopwinyuwong, Trevanich and Suppakul, 2010; Kuswandi *et al.*, 2011). Recently, Zhai *et al.* (2017) prepared a colorimetric film constituted of starch/PVA incorporated with anthocyanins from rosemary (*Hibiseus sabdariffa L.*) in order to monitor silver carp freshness during storage. This film demonstrated visible changes in color over time due to TVB-N presence. The initial TVB-N value of the fresh fish was 6.61 mg/100 g and the film color was pink (at pH < 5). After 165 h of fish storage at 4 ºC, the TVB-N value increased up to 28.53 mg/100 g and the

film color altered to blue due to basic pH (pH= 8-9). For this reason, it can be considered an intelligent packaging device able to confirm fish freshness using a real time approach (Zhai *et al.*, 2017). More examples of polysaccharide-based intelligent films applied to fish products are reported in Table 2. Some commercial examples of intelligent packaging with systems that monitor fish freshness through metabolites detection have been described. For instance, Innovative Fresh Tag® colorimetric labels was able to detect volatile amines formation during fish and seafood products storage (Hogan and Kerry, 2008; Poyatos-Racionero *et al.*, 2018).

2. AIMS

As mentioned in Chapter 1, the problems associated to consumption of contaminated food products, food waste and environmental impact are current concerns that need an effective solution. Therefore, the main goal of this project was the development and characterization of novel colorimetric bio-based intelligent films incorporating blueberry and/or beetroot extracts as indicators capable of monitoring hake fish (a food model system) freshness during storage.

The specific aims are:

- Development of LBG/κ-car-based film incorporating blueberry and/or beetroot extracts;
- Assessment of the effect of different blueberry and/or beetroot extract contents on the LBG/κ-car films' mechanical, barrier (permeability to water vapor), optical, thermal and structural properties;
- Evaluation of the color response efficiency of the developed LBG/κ-car films to different pH conditions and volatile nitrogen compounds;
- Assessment of the potential use of the colorimetric indicator LBG/κ-car-based film in monitoring hake fish deterioration under refrigerated storage conditions.

3. MATERIALS AND METHODS

3.1. Materials

Polysaccharides, κ-car (Gelcarin® DG 5264) and LBG were obtained by FMC Biopolymer (Philadelphia, United States of America, USA) and Sigma-Aldrich (St. Louis, Missouri, USA), respectively. The glycerol was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Freeze-dried blueberry powder containing anthocyanins (Simplu®) and beetroot extract powder containing betalains (Solgar®) were purchased in Celeiro (Braga, Portugal).

Sodium hydroxide (NaOH), ammonia and Plate Count Agar (PCA) were provided by Merck (Merck SA, Lisbon, Portugal). Boric acid (H_3BO_3), hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) were supplied by Chem-Lab (Chem Lab, Zedelgem, Belgium). Potassium carbonate (K_2CO_3) and methyl red indicator were purchased from Panreac (Panreac AppliChem ITW Companies), Barcelona, Spain). Bromocresol green was supplied by Acros Organic (Acros Organics™, Lisbon, Portugal).

The fresh hake was purchased from local market (Barcelos, Braga).

3.2. Methods

3.2.1. Development of LBG/κ-car film incorporating blueberry and/or beetroot extracts

The development of LBG/κ-car films were based on the methodology presented by Pinheiro *et al.* (2011) and Martins *et al.* (2013). Initially, 60 % LBG and 40 % (w/w) κ-car were dissolved in distilled water under stirring, for 1 h at 25 °C.(Karbowiak *et al.*, 2006) The 60/40 ratio (LBG/κ-car) was choose based on Martins *et al.* (2012) work, since films with this ratio showed the best mechanical and barrier properties. This film-forming solution was used to produce control films (no added blueberry and beetroot extracts), and films incorporating extracts.

Regarding control films development, 30 % (w/w of total polysaccharide content) of glycerol was added as a plasticizer to improve film mechanical properties and processability. To guarantee complete solubilization, the solution was homogenized at 70 °C, under agitation, for 30 min. Then, air bubbles were removed from film-forming solution and, 28 mL of this solution was cast into Petri dishes, which were placed in an oven (WTC Binder) at 35 °C during 22 h, for films drying.

For the development of the colorimetric films, blueberry or beetroot extracts (at 1 to 50 % (w/w)) were, separately, mixed with the LBG/κ-car solution (Martins *et al.*, 2013; Qin *et al.*, 2019). However, the blueberry or beetroot extract content used in the optimized films was 30 % and 50 % (w/w), respectively. This choice was based on preliminary studies results (Figure A1), particularly, color inspection (color homogeneity and color uniformity) of the films and behavior of the sample during handling (e.g., fragile and brittle). Also, blueberry and beetroot extracts were simultaneously added to film forming solutions, at different blueberry/beetroot extract ratios i.e., 5/10, 10/10, 10/20 and 20/30 % (w/w), and tested (Figure A1).

The extracts were added to LBG/κ-car and glycerol solution in the quantities defined for each film. The solution was homogenized at 1,200 rpm for 30 min in a homogenizer at 40 °C. All film solutions incorporating blueberry extract were centrifuged (Hettich® EBA 20) at 5,000 rpm for 2 min, in order to eliminate some residues (e.g., peel) from the extracts. Centrifugation was an added step to the formulation of films incorporating anthocyanins since heterogeneity can be commercially unacceptable due to visual appearance (Luchese, Abdalla, *et al.*, 2018). Then film solutions prepared were divided by Petri dishes for drying in the oven (WTC Binder) with airflow circulation at 35 °C for 22 h.

The films incorporating blueberry or beetroot extracts were denominated BLE and BEE, respectively. The films containing both blueberry and beetroot extracts (optimized film: 10/20 % w/w) were named BLE/BEE (Table 3).

All films produced were conditioned in a desiccator at controlled temperature (20 °C) and relative humidity (54 %), until they are further analyzed (Prietto *et al.*, 2017; Qin *et al.*, 2019).

Table 3. Selected LBG/κ-car film formulations (total polysaccharide concentration = 1 % (w/w)) containing different blueberry and beetroot extract concentrations (i.e., % w/w of total polysaccharide content).

Film formulation	κ-car (% w/w)	LBG (% w/w)	Glycerol (% w/w)	Blueberry extract (% w/w)	Beetroot extract (% w/w)
Control (C)	40	60	30	0	0
BLE				30	0
BEE				0	50
BLE/BEE				10	20

3.2.2. Determination of pH-sensitive property BLE and BEE films

In order to evaluate the effect of the addition of blueberry and/or beetroot extracts on film colorimetric response to pH changes, a drop of different buffer solutions - HCl (0.1 mol/L) aqueous solution (pH 2, 4 and 6), distilled water (pH 7) or NaOH (0.1 mol/L) aqueous solution (pH 10, 12 and 14)- was placed on BEL, BEE and BLE/BEE samples (2 cm x 2 cm) produced in section 3.2.1. for 10 min. The color change of the films occurred instantaneously, however, it was established that colorimetric reading of the samples was performed after a standard interaction period time (10 min) with buffer solutions in order to standardize the color responses, minimizing possible variations (Prietto *et al.*, 2017). The color change was evaluated by comparing color differences between films before and after contact with buffer solutions. The colorimetric parameters (L, a* and b*) were measured in each film using a CR400 colorimeter (Konica, Minolta, Osaka, Japan), according to the method described in section 3.2.5.9. All measurements were taken at three random locations from both sides of each sample (Choi *et al.*, 2017; Luchese, Abdalla, *et al.*, 2018).

3.2.3. Color stability of the BLE and BEE films during storage

The optimized films (BLE and BEE) were stored under light or dark conditions for 60 days at room temperature (25 °C) and under refrigeration (4 °C) (Choi *et al.*, 2017; Pourjavaher *et al.*, 2017; Prietto *et al.*, 2017). The color parameters of the optimized films (Table 3) were analyzed at 2, 5, 10, 15 and 60 days of storage, using the method reported in section 3.2.5.9. The pH-sensitive films were evaluated for their color stability as a function of time and temperature.

3.2.4. BLE and BEE films response to ammonia vapor

The film color-response to volatile ammonia was determined by Qin *et al.* (2020) method. In order to validate the films capacity to volatile compounds detection, BLE, BEE and BLE/BEE film samples (1.5 cm x 1.5 cm) were placed in the headspace of sealed Petri dishes loaded with 0.4 mol L^{-1} ammonia solution, for 120 min at room temperature in the dark (Yun *et al.*, 2019).

The color parameters of the films at different times (every 10 min during 60 min and after 120 min) were determined as described in section 3.2.5.9 (Qin *et al.*, 2020). All measurements were conducted at three random locations from both sides of each sample.

3.2.5. Determination of physico-chemical properties of the developed colorimetric films

Film characterization provides useful information regarding its properties which helps to select the most suitable film to be used on a specific food product. This characterization allowed to choose the more adequate films to monitor fish samples freshness during storage. Thus, the aim of this section is to characterize the films developed in section 3.2.1 regarding their structural, thermal, barrier, mechanical and optical properties.

3.2.5.1. Fourier-Transform Infrared (FTIR) Spectroscopy

This test aims to determine films´ molecular modifications after natural pigments or enzymes addition (Ebrahimi Tirtashi *et al.*, 2019). BLE, BEE and control film (C) samples were dried during 1 h at 35 °C, under vacuum (SVAV2-2 vacuum oven model, MRC - Laboratory Equipment, Israel) at 100 kPa, to remove water molecules which could interfere with FTIR analysis. Chemical groups and bonding arrangement of constituents present in both film samples were determined using a FTIR ALPHA II- Bruker spectrophotometer (Ettlingen, Germany) with a attenuated total reflectance (ATR) cell (Silva-Pereira *et al.*, 2015). All spectra were measured three times for each sample at a 4 cm^{-1} resolution at wavenumber ranging between 4000-400 cm^{-1} and 64 scans per sample were performed (Martins *et al.*, 2012; Pourjavaher *et al.*, 2017).

Additionally, blueberry or beetroot extracts were also analyzed using the same above-mentioned method.

3.2.5.2. Ultraviolet and visible (UV-vis) spectra measurements

The light transmittance of the optimized films (BLE, BEE and BLE/BEE) and control films (C) were measured using an UV-Vis spectrophotometer (V-560 model, Jasco Tokyo, Japan). The film with no-added pigments was used as control. Each film sample (4 cm x 1 cm) was placed directly

in the spectrophotometer cell and measured in the range of 200 to 800 nm. Measurements were performed in triplicate for each film type (Liu *et al.*, 2018; Yun *et al.*, 2019).

3.2.5.3. Thermal stability of the films

BLE, BEE and C films' thermal stability was evaluated by Differential Scanning Colorimetry (DSC) (DSC 6000 model, Perkin Elmer®, Waltham, Massachusetts, EUA). Three samples of each film (5 mg, approximately) were placed into an aluminum DSC pan and sealed with aluminum covers. The measurements were performed at range temperature of 50 °C to 210 °C, at a constant rate of 10 °C min^{-1}, under a nitrogen atmosphere flow of 20 mL min^{-1} (Liu *et al.*, 2018; Kang *et al.*, 2020; Michelin *et al.*, 2020; Zhou *et al.*, 2021).

3.2.5.4. Film thickness

Film thickness was measured using a digital micrometer device (Mitutoyo No. 293-766, Japan), with 0.001 mm accuracy. Ten thickness measurements were randomly taken in different points on three different samples of each film (C, BLE, BEE and BLE/BEE). The mean values obtained were used in water vapor permeability and tensile strength calculations (Casariego *et al.*, 2009; Souza *et al.*, 2009; Huang *et al.*, 2015).

3.2.5.5. Water contact angle (WCA)

The water contact angle (WCA) of the BLE, BEE and C films was measured using an optical contact angle meter (OCA 20, Dataphysics, Germany), through the sensile drop method (Michelin *et al.*, 2020). Three samples of each film were cut into square pieces (1.5 cm × 1.5 cm) and directly placed on the horizontal movable stage fixed with the contact angle analyzer. Afterwards, 5 μL ultra-pure water was dropped onto the film surface using a micro-syringe (Hamilton, Switzerland) with a 0.75 mm diameter needle. Contact angle measurements (using a digital camera) were performed at 0, 5, 10, 20 and 30 s after the droplet placement, on both sides of the water drop to assume symmetry and horizontal level. The average values of three measurements of each sample were presented as the degree of WCA (Huang *et al.*, 2019; Kang *et al.*, 2020; Michelin *et al.*, 2020; Zhou *et al.*, 2021; He *et al.*, 2022).

3.2.5.6. Moisture content (MC) and water solubility (WS)

MC and WS of the films were determined gravimetrically, according to the method report by Casariego *et al.* (2009). For both assays, three replicates were performed for each BLE, BEE and C film sample.

MC was determined by measuring weight loss of the film samples (2 cm of diameter) thoroughly dried at 105 °C, in an oven with airflow circulation (WTC Binder), for 24 h (Zhai *et al.*, 2018; Qin *et al.*, 2020; Zhou *et al.*, 2021). MC (%) was calculated according to the following equation (Eq.) 1:

$$MC = \left(\frac{m_f - m_i}{m_i}\right) \times 100 \quad \text{(Eq. 1)}$$

where m_i is initial sample weight and m_f is final sample weight after drying.

To determine WS, film (free of moisture) samples (2 cm of diameter) were weighed and immersed in 50 mL of deionized water, during 8 h at room temperature, under orbital agitation at 120 rpm (Orbital Shaker Incubator ES-20/80, BOECO, Germany). The water was removed and the film residue was dried, at 105 °C for 24 h, to determine the weight of dry matter which not solubilized in water (Casariego *et al.*, 2009; Huang *et al.*, 2015; Zhou *et al.*, 2021). WS (%) was calculated by Eq. 2:

$$WS = \left(\frac{m_f - m_i}{m_i}\right) \times 100 \quad \text{(Eq. 2)}$$

where m_i is initial sample weight before immersion and m_f is the weight of dry matter that was not solubilized in water.

3.2.5.7. Water Vapor Permeability (WVP)

WVP of BLE, BEE and C films was determined gravimetrically based on ASTM E96-92 method in order to determine water vapor films´ barrier capacity (Mchugh, Avena-Bustillos and Krochta, 1993; Casariego *et al.*, 2009). Three samples were cut from each film and were sealed on a

permeation cup cell containing 50 mL of distilled water (100 % RH; 2337 Pa vapor pressure at 20 °C) and placed in a desiccator containing silica gel (0 % RH; 0 Pa water vapor pressure at 20 °C). Steady-state and uniform water pressure conditions were assumed by keeping the air circulation constant outside of test cup by using a fan inside the desiccator. Periodical cup weightings (every 2 h) were performed during 12 h and the water transferred through the film was determined from weight loss of the permeation cell (Casariego *et al.*, 2009; Liu *et al.*, 2018; Huang *et al.*, 2019; Michelin *et al.*, 2020). Water vapor transmission rate (WVTR) was obtained through the ratio between the slope of the linear regression of weight loss versus time and the film area. WVP (g m^{-1} s^{-1} Pa^{-1}) was calculated according to Eq. 3:

$$WVP = \left(\frac{WVTR \times L}{\Delta P}\right) \quad \text{(Eq. 3)}$$

where WVTR is the water vapor transmission rate (g m^{-2} s^{-1}) through the film, L is the mean film thickness (m), and ΔP is the partial water vapor pressure difference (Pa) across the two sides of the film. All measurements were performed in triplicate.

3.2.5.8. Mechanical Properties

Mechanical properties are a fundamental feature of the film since it reflects film integrity and ability to protect food products. Tensile strength (TS) is the maximum tension that film can resist until it breaks, and elongation-at-break (EB) provides information regarding film flexibility.

TS and EB of the films were measured using a TA-HD plus Texture Analyzer (Serial RS232, Stable Micro Systems, Surrey, UK), with the software Exponent, following the guidelines of ASTM D 882-02 methodology (Martins *et al.*, 2012).

Film specimens (120 mm x 20 mm) from each film sample (BLE, BEE and C) were cut and placed between the tensile grips. The initial grip separation and crosshead speed were set at 30 mm and at 5 mm min^{-1}, respectively. TS and EB were determined from stress-strain curves. Four measurements were performed for each film. (Albuquerque *et al.*, 2017; Huang *et al.*, 2019; Michelin *et al.*, 2020). TS, expressed in MPa, and EB, expressed in %, of each film sample were calculated as follows (Qin *et al.*, 2019):

$$TS = \frac{F}{L \times W} \quad \text{(Eq. 4)}$$

$$EB = \frac{\Delta L}{L_0} \times 100 \quad \text{(Eq. 5)}$$

where F is the maximum load (N), L is the film thickness (mm), and W is the film width (mm); ΔL is the final length at the point of sample rupture and L_0 is the original length (mm) of film sample.

3.2.5.9. Optical properties: color and opacity

The color of BLE, BEE, BLE/BEE and C films was determined with a Minolta colorimeter (CR 300; Minolta, Japan). For instruments´ calibration, a white standard color plate (Y= 93.9; x= 0.3158; y= 0.3321) was used as background for film color measurements (Casariego *et al.*, 2009; Souza *et al.*, 2009; Martins *et al.*, 2012). Lightness – L^* (white to black) – and chromaticity parameters – a^* (red to green) and b^* (yellow to blue) – were determined through reflectance measurements, using the CIELab scale (García *et al.*, 2004). The standard values of a white plate were used as a reference (L_0^*= 89.13, a_0^*= 0.14 and b_0^*= 5.18).

The total color difference (ΔE) of the films was calculated by Eq. 6:

$$\Delta E = \sqrt{(L_1^* - L_0^*)^2 + (a_1^* - a_0^*)^2 + (b_1^* - b_0^*)^2} \quad \text{(Eq. 6)}$$

where L_1^*, a_1^* and b_1^* are the color attributes of the films; L_0^*, a_0^* and b_0^* are the color parameters of the control film.

The opacity, expressed in %, of the samples were determined according to the Hunterlab method, as the relationship between the opacity of each sample on a black standard (Y_B) and on a white standard (Y_W) (Eq. 7) (García *et al.*, 2004). Six measurements were taken on each sample, and three samples of each film were tested.

$$Opacity = \frac{Y_B}{Y_W} \times 100 \quad \text{(Eq. 7)}$$

3.2.6. Evaluation of colorimetric LBG/κ-car-based films as fish freshness indicators

3.2.6.1. Sample preparation and storage conditions

Fresh fish (hake) was purchased from a local market and immediately transported to the laboratory. Fishbones were removed and fish fillet samples (approximately, 5 g each) were prepared.

To validate the concept of colorimetric BLE and BEE films sensitivity pH changes and gas changes, a preliminary test with fish samples stored at 4 °C and 25 °C for 7 and 3 days, respectively, was carried out. The fish storage at 25 °C was reduced to 3 days, since this period was sufficient to detect fish freshness loss (odor and color changes), which is necessary for films validation as freshness indicators. BLE and BEE film samples (2 cm x 2 cm) were fixed to top of sterile polystyrene Petri dish (45 mm diameter) cover (contact to the dish headspace) (Figure 9A) (Silva-Pereira *et al.*, 2015; Choi *et al.*, 2017). Then, fish samples were placed into Petri dishes (Figure 9B) and dish was sealed (Figure 9C). The Petri dish with the film and no-added fish sample was used as control. The color parameters were recorded, as described in Task 3.2.5.9, at different storage time intervals: 0, 1, 2, 3, 6 and 7 days at 4 °C and 0, 1, 2 and 3 days at 25 °C (Zhou *et al.*, 2021).

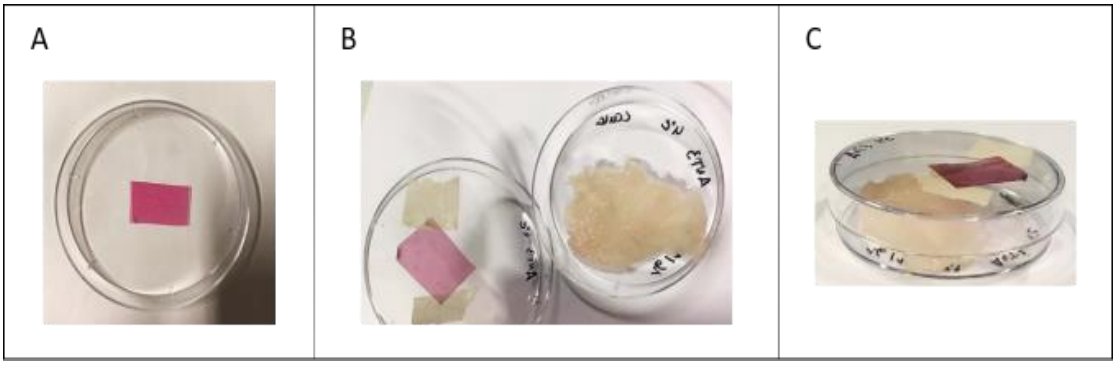


Figure 9. Illustrative example of the experimental set-up used to monitor fish freshness during storage at 4 and 25 ºC. (A) Film sample (2 cm x 2 cm) fixed to Petri dish cover; (B) Petri dishes containing hake samples; (C) Closed system used for monitoring hake freshness status.

Based on the preliminary test results, BLE film was chosen to monitor fish degradation over 7 days at 4 °C. These storage conditions were selected to mimic a real-life situation, since fresh fish is usually stored at 4 °C before consumption.

All samples were stored and analyzed on days 0, 2, 3, 4, 6 and 7 to detect any chemical or microbial changes. For each day of storage, three replicates were performed for each sample. All results obtained over the storage period were compared with the results obtained on day 0.

3.2.6.2. Film color analysis

Film color measurements were conducted according to the method described in section 3.2.5.9. The color change of films during storage (ΔE_s) was determined using the following Eq. 8:

$$\Delta E_S = \sqrt{(L_{S1}{}^* - L_{S0}{}^*)^2 + (a_{S1}{}^* - a_{S0}{}^*)^2 + (b_{S1}{}^* - b_{S0}{}^*)^2} \quad \text{(Eq. 8)}$$

where $L_{S1}{}^*$, $a_{S1}{}^*$ and $b_{S1}{}^*$ are the color parameters of the films at each sampling interval during the storage period; $L_{S0}{}^*$, $a_{S0}{}^*$ and $b_{S0}{}^*$ are the color parameters of the films at $t = 0$ days of storage.

For each day, at least three-color measurements were performed on different film samples to associate the film color changes to the loss of hake freshness, over the storage time.

3.2.6.3. pH measurement of fish samples

The fish samples were blended with 100 mL of distilled water, using a stomacher (Stomacher® 3500, Sweard Medical, UK) in high speed for 4 min. All pH measurements were performed using a digital pH meter (HI 2210, Hanna Instruments, Porto, Portugal), by immersing the glass electrode in homogenized samples (Choi *et al.*, 2017).

3.2.6.4. Determination of total volatile basic nitrogen (TVB-N)

TVB-N measurement was conducted according to the Conway micro diffusion method described by He *et al.* (2020) with some modifications. Initially, hake samples (4 g) were transferred to a stomacher bag and homogenized with 40 mL of distilled water over 8 min, using a stomacher (Stomacher® 3500, Sweard Medical, UK). Then, the mixture was filtered using filter paper (retention range: 5–13 μm) (Rotilabo®-Round filters, type 601A, Carl Roth, Germany). Subsequently, 1 mL of acid boric 0.02 % and one drop of pH indicator (methyl red: bromocresol green = 1:5) were added to the Conway´s dish inner chamber. One mL of saturated potassium

carbonate solution and 1 mL of the filtrate sample were added to the outer dish chamber. The blank sample was prepared as described above but without 1 mL of filtered sample addition.

The lid of the Conway dish was closed, and the resulting mixture was allowed to stand at room temperature for 1.5 h. Finally, the solution in the inner chamber was titrated with hydrochloric acid (0.01 mol/L) until a pale pink color appeared. Analyses were done in triplicate and TVB-N content was presented as mg/100 g of fish sample. The TVB-N content was calculated using the following Eq. 9:

$$TVB - N = (V_1 - V_2) \times c \times 14 \times \frac{100}{m} \times \left(\frac{V}{V_0}\right) \quad \text{(Eq. 9)}$$

where V_1 and V_2 represent the HCl volume (mL) required for titration of the sample and blank (no sample), respectively; c is the HCl concentration (mol L^{-1}); m is the fish sample weight (g); 14 is the molecular weight of nitrogen; 100 is the conversion factor; V is the volume of filtrate sample (mL) and V_0 is the total volume (mL) (Socaciu *et al.*, 2021).

The TVB-N content associates the films color changes to pH variations and volatile compounds release during fish storage (Wu *et al.*, 2018; Ezati *et al.*, 2019).

3.2.6.5. Microbiological analysis

The determination of Total Viable Count (TVC) was estimated and performed according to the procedure reported by He *et al.* (2020) and Huang *et al.* (2019). The peptone water solution and the plate count agar (PCA) medium were prepared according to the manufacturer's recommendations, being sterilized in an autoclave at 121 ºC for 15 min.

Hake samples (4 g) were transferred to a stomacher bag inside a flow chamber (KR-105 Basio, Kojair, Vippula, Finland). Then, 27 mL of sterile 0.1% peptone water as added to the bag and was homogenized for 2 min (this solution is considered the 10^{-1} dilution), using a stomacher (Stomacher® 3500, Sweard Medical, UK). Serial decimal dilutions were prepared (10^{-2} to 10^{-8}) in the flow chamber. One mL of appropriate dilution was placed in a Petri dish and 20 mL of sterile PCA was added to the dish. When the PCA medium solidified at room temperature, the plates were inverted and incubated at 35 ºC for 72 h. The Petri dishes with 30 – 300 colonies were picked and colony forming unit (CFU) was determined by manual counting (Huang *et al.*, 2019; Zhu *et al.*,

2021). Total viable count (TVC) was determined and expressed as $\log_{10}$ colony forming unit per g fish sample (log CFU/g) (Eq. 10). Each sample was cultured in triplicate.

$$TVC = log_{10}\left(\frac{\sum CFU}{V\,(n_1+0{,}1\,n_2)\times d}\right) \quad \text{(Eq. 10)}$$

where $\sum$ CFU is sum of all colonies on plates retained from two successive dilutions, V is the volume in mL of inoculum dispensed on each plate, n_1 is plate number in the 1st dilution retained, n_2 is plate number in the 2nd dilution retained, d is dilution ratio corresponding to the first dilution.

3.2.7. Statistical Analysis

All experiments were performed at least in triplicate, and in some cases more. All data were expressed as mean values ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed, and the significant differences among samples were determined by Tukey HSD test at $p < 0.05$. Statistics were performed on the SigmaPlot Software.

4. RESULTS AND DISCUSSION

4.1. Development of LBG/ κ-car films incorporating blueberry and/or beetroot extracts

Different blueberry and/or beetroot extract concentrations were tested to optimize the LBG/ κ-car films' formulations. As previously mentioned in section Methodology (section 3.2.1), the 60/40 LBG/κ-car ratio and 30% (w/w) of glycerol film formulation was chosen (Figure 10A) since this film formulation showed the best mechanical/handling and gas barrier properties (Martins *et al.*, 2012). Moreover, the optimized films presented 30% (w/w) of blueberry extract concentration or 50% (w/w) of beetroot extract concentration. The optimized extracts' concentrations were essentially based on visual examination of the film, namely the color homogeneity and uniformity throughout the film (Figure 10B and 10C). Also, blueberry and beetroot extracts were simultaneously added to film forming solutions, at different blueberry/beetroot ratio. Among these concentrations, the best formulation was achieved with 10/20 (% w/w) blueberry/beetroot addition. Once again, the choice was based on film color and handling properties (Figure 10 D). Films incorporating blueberry or beetroot extracts showed a violet/pink color.

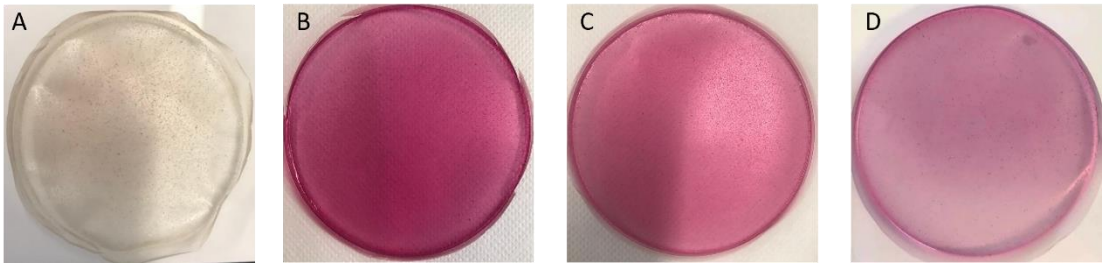


Figure 10. A) Control (C) film; B) C film incorporating 30% blueberry extract (% w/w); C) C film incorporating 50% beetroot extract (% w/w); D) C film incorporating simultaneously 10% blueberry extract (% w/w) and 20% beetroot extract (% w/w).

Thus, the selected films used in subsequent characterization studies were as follows: a) control (C) film - 60/40 (% w/w) LBG/κ-car + 30% (% w/w) glycerol; b) C film incorporating 30% blueberry extract (% w/w) (BLE); c) C film incorporating 50% beetroot extract (% w/w) (BEE) and d) C film incorporating simultaneously 10% blueberry extract (% w/w) and 20% beetroot extract (% w/w) (BLE-BEE) (Figure 10).

4.2. Color response analysis of optimized films

In order to investigate the response of the films to different pH values, droplets of different buffer solutions (pH values ranging from 2 to 14) were placed on BLE, BEE and BLE/BEE film samples. Figure 11 illustrates the visible color changes (ranging from red to green color) of each film sample.

The BLE film presented a red color at pH = 2; light purple color at pH 4 to 7 range; dark purple color at pH =10; blue/green color at pH = 12 and yellow color at pH = 14 (Torskangerpoll and Andersen, 2005; Choi *et al.*, 2017; Krga and Milenkovic, 2019; Qin *et al.*, 2019). The color parameters analysis of BLE films showed that L value increased (39.13 ± 4.04 to 44.63 ± 5.87) and a* values (40.61 ± 3.02 to 26.00 ± 2.23) and b* (26.27 ± 4.16 to 10.36 ± 1.32) significantly decreased ($p < 0.05$), between pH 2 to 4. Therefore, in acidic solutions, the film showed an increase in its luminosity, a decrease in its red color and an increase in its blue color. The values of L, a* and b* parameters remained similar ($p > 0.05$) between pH 4 to 10, which showed that BLE film did not change its color under these pH range. As the pH value increased from 10 to 12, L values increased (45.05 ± 2.29 to 51.05 ± 3.35) and a* (28.66 ± 2.21 to 7.60 ± 5.81) and b* (9.66 ± 1.99 to 3.85 ± 2.39) values dropped sharply. Thus, it can be concluded that BLE film showed a more intense blue color, as can be seen in Figure 11. During pH transition from 12 to 14, L value increased (51.05 ± 3.35 to 62.18 ± 5.03), a* value decrease (7.60 ± 5.81 to 5.03 ± 1.54) and b* value increased (3.85 ± 2.39 to 26.98 ± 3.68). These results demonstrated that the film became more yellow (Figure 11). Research has shown that color stability of the anthocyanins present in blueberry is influenced by external conditions such as pH, storage temperature, incidence of light, among other conditions in their environment (Balbinot-Alfaro *et al.*, 2019). Indeed, anthocyanins are more stable in acidic than in alkaline solutions. Thus, the flavylium cation form of anthocyanins is red and stable under acidic solutions, being its double bonds prone to conjugation (e.g., cation hydration), forming the carbinol basic form (light violet color). At pH 10, anthocyanins are in the quinoidal base form due to cation deprotonation, leading to a color change to violet. At pH 12, a color change to blue – green is observed, since quinoidal anhydro base form occurs by deprotonation from oxygen and carbon ring. Finally, under an alkaline pH (pH 14), the retro-chalcone formation occurs, through carbinol tautomerization, responsible for the appearance of a yellow-green color (Mohd, Khan and Farooqui, 2011). Ma *et al.* (2021) reported similar color changes of polyvinyl alcohol-chitosan-mulberry anthocyanins films. Choi *et al.* (2017) observed color changes from bright red at pH2, to pink at pH 3-4, to brown at pH 5-6 and, finally, to greenish-

brown at pH 9-10 in pH indicator film composed of agar/potato starch and anthocyanin extracts from purple sweet potato.

BEE film exhibited pink-purple colors when the films were in contact with buffer solutions ranging from pH 2 to 10. At this pH´s range, L, a* and b* values did not present statistically significant differences ($p > 0.05$), concluding the BEE film did not present visible colorimetric changes under these conditions. When pH values changed from 10 to 12, L value increased (55.08 ± 1.51 to 60.17 ± 6.27) and a* (44.55 ± 3.37 to 21.97 ± 3.54) and b* (4.94 ± 1.23 to 3.37 ± 1.72) values decreased which means that the BEE film had a higher lightness and showed a green - blue color. At pH 12 to 14, the color film changes to yellow (Figure 11). In this case, the L value (60.17 ± 6.27 to 74.50 ± 2.22) continued to increase, a* decreased dramatically (21.97 ± 3.54 to 1.07 ± 3.26) and b* increased significantly ($p < 0.05$) (3.37 ± 1.72 to 23.06 ± 1.94). Beetroot contains two groups of betalain pigments: red-violet betacyanins and yellow betaxanthins. At pH values ranging from 4 to 10, betacyanins prevails which exhibit a stable violet coloring. At pH values above 10, betaxanthins appears which display yellow – green coloring. Betalains are relatively stable at pH range of 2 to 10, unlike anthocyanins (Stintzing and Carle, 2004; Herbach, Stintzing and Carle, 2006; Gandía-Herrero, Escribano and García-Carmona, 2010; Esatbeyoglu *et al.*, 2015; Slimen, Najar and Abderrabba, 2017; Merz *et al.*, 2020). For this reason, it is not easy to visually distinguish the film color change over a wide range of pH values, being the most visible change between pH 10 and 12. Similar to the results presented in this thesis, Wu *et al.* (2021) described color changes at different buffer solutions of LGB/polyvinyl alcohol and betacyanins (from cockscomb flower) films. The films exhibited stable reddish-purple colors at pH 3 to 8 and turned light brown or light purple at pH 9 to 12. These color changes were related to the structural degradation of betacyanins in alkaline solutions. Furthermore, Guo *et al.* (2021) also observed the color of pectin/ beetroot extract films changed from red to purple, brown and yellow when pH value increased.

The color changes of BLE/BEE films at different pH values were very similar to BLE films: pink (at pH 2) to purple (between pH 4 and 10), and purple to blue - green at pH 12 to 14. Regarding the BLE/BEE film color parameters, L (56.36 ± 3.06 to 62.68 ± 1.52), a* (37.05 ± 2.26 to 28.98 ± 1.29) and b* (8.25 ± 1.05 to -3.37 ± 0.16) values changed between pH 2 and 4; the film presented a less reddish and more bluish color (violet color). Between pH 4 - 10, the values of L, a* and b* parameters were very similar ($p > 0.05$), hence the color change was not different visually, as seen in Figure 11. In the transition from pH 10 to 12, there is a sudden color change

(purple to green), since the a* value decreased (28.79 ± 4.14 to 8.36 ± 0.62) and b* value increased (- 2.84 ± 0.74 to 8.30 ± 0.85) significantly ($p < 0.05$). From pH 12 to 14, a* value continued to decrease (8.36 ± 0.62 to 1.66 ± 1.19) and b* increased (8.30 ± 0,85 to 21.33 ± 2.61), making the BLE/BEE film progressively greenish/yellowish. The aim of simultaneously adding both blueberry and beetroot extracts was to develop a film capable of detecting more easily pH changes, through colorimetric changes to be detected by the human eye, than the extracts added separately to the film. However, no significant changes in film color were detected when compared to BLE films as can be seen in Figure 11.

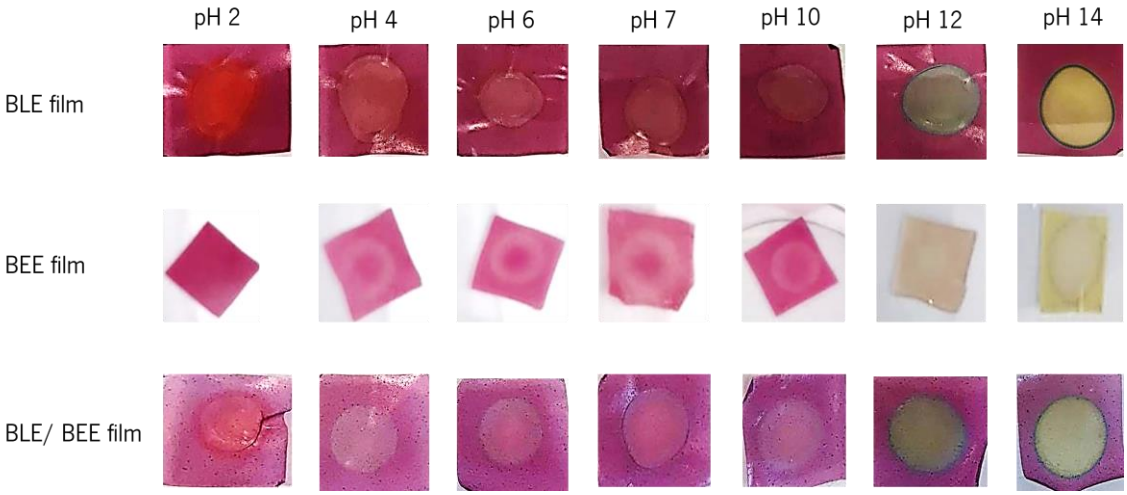


Figure 11. BLE, BEE and BLE/BEE films color changes in contact with different buffer solutions (pH 2 to 14).

To evaluate the film capacity to detect pH changes, through colorimetric modifications, an important parameter to be determined is the ΔE. For consumers to be able to easily visualize the color changes, the ΔE value must be higher than 5 (Jiang *et al.*, 2020; Roy and Rhim, 2021). Figure 12 illustrates the ΔE values for films over the different pH.

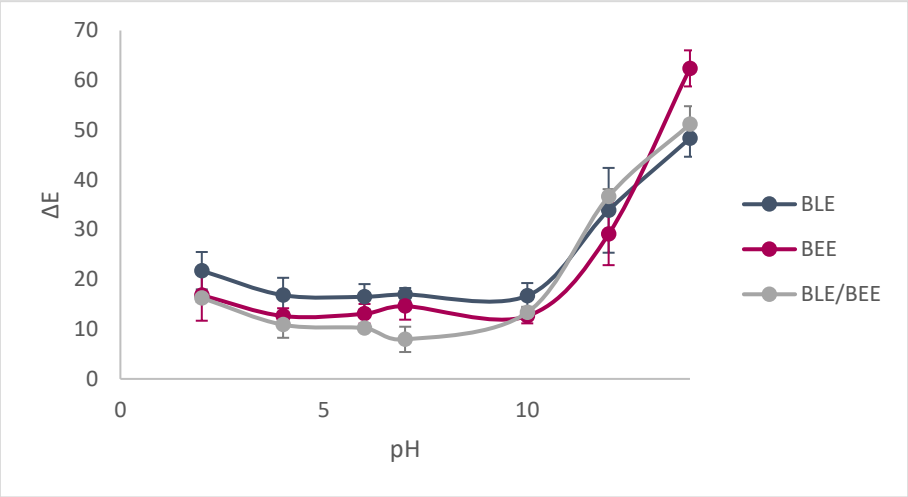


Figure 12. Total color difference (ΔE) of LBG/κ-car films containing blueberry extract (BLE), beetroot extract (BEE) and BLE/BEE.

Throughout pH range, ΔE values greater than 5 were obtained in all films being, for this reason, able to inform consumers through colorimetric changes visibly detectable to the human eye. However, BLE film showed higher ΔE values between pH 2 and 10 when compared with BEE and BLE/BEE films, showing more perceptible color variations. This result agrees with the results presented in Figure 11. The BLE, BEE and BLE/BEE films went through a drastic color change between pH 10 and pH 14, as the ΔE values increased considerably. Thus, it is concluded that the color of all films tested were strongly pH-dependent showing their potential to be applied in intelligent packaging field (Qin *et al.*, 2019; Merz *et al.*, 2020).

4.3. Color stability of the BLE and BEE films during storage

There are still some challenges to be solved for the natural extracts' incorporation in the intelligent packaging applications, including preventing their deterioration under environment conditions such as light, temperature and oxygen. In order to provide reliable visual feedback to consumer, it is necessary to evaluate the influence of temperature and light on produced films. Thus, BLE and BEE films color stability was tested at different temperatures (4 ºC and 25 ºC) and, under light and no light exposure to simulate possible food storage conditions (Zorić *et al.*, 2016; Merz *et al.*, 2020).

As shown in Figure 13, the color of BLE and BEE films were influenced by the incidence of light and temperature during storage. The human eye detects color change when ΔE values are higher than 5 (Tassanawat *et al.*, 2007; Jiang *et al.*, 2020; Roy and Rhim, 2021).

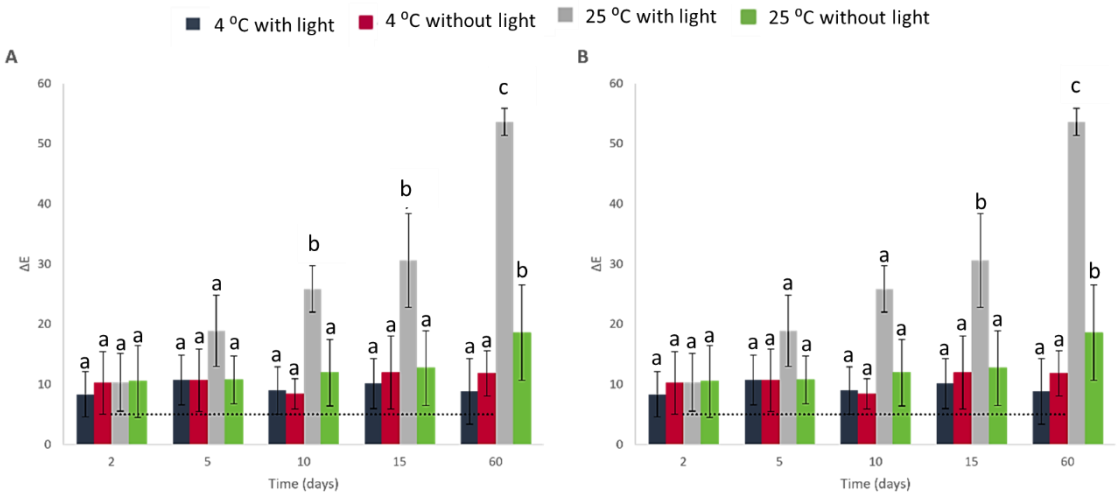


Figure 13. Total color difference (ΔE) of BLE (A) and BEE (B) films during 60 days of storage. The dotted line represents ΔE equal to 5, value at which consumer can visually detect colorimetric changes in the films. [a-c] Values are given as mean ± SD (n = 9). Different letters at the same storage condition over time represent statistically significant differences (Tukey test, $p < 0.05$).

BLE and BEE films, in all tested conditions, present ΔE values higher than 5, suggesting the films color change can be detected by the consumer (Mokrzycki and Tatol, 2011). These changes may be the result of natural pigments such as anthocyanins and betalains photodegradation in the films. However, the ΔE values remain stable in both films with no statistically significant differences when they were stored at 4 °C and protected from light. It can be concluded that, if the films were stored under refrigeration conditions, the eventual pH-indicator films color change will not be directly associated with the photodegradation of incorporated natural pigments present in the extracts. Furthermore, the presence or absence of incident light had no influence on color at 4 ºC, since when comparing the color of the films stored at 4 ºC, with or without light (Figure 14B and 14D), there were no significant differences (L*, a* and b* values remain practically constant over time) ($p > 0.05$). Namely, the colorimetric changes do not depend on light exposure, allowing BLE and BEE films to be applied in food products because film's color change will not be attributed to extract degradation due to light incidence, but rather to changes on food product quality. Etxabide, Kilmartin and Maté (2021) studied color stability of anthocyanins and betalains containing

colorants under different storage conditions for intelligent packaging development. They concluded that the color remained stable under 4 ºC and no light incidence storage conditions for 28 days, although a small color fading was observed during last days.

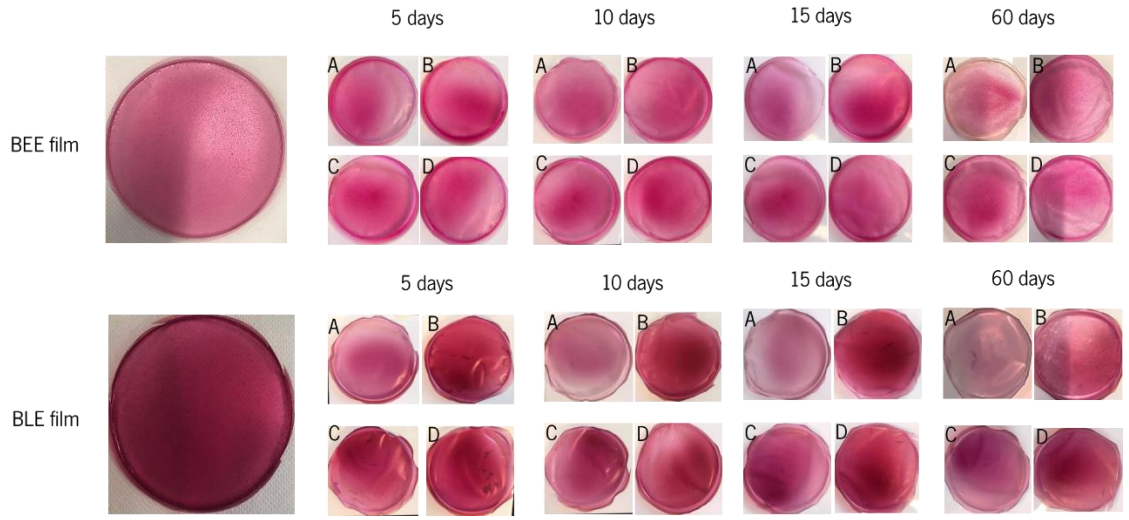


Figure 14. Colorimetric changes of BLE and BEE films during 60 days under different storage conditions: 25 ºC with light incidence (A), 4 ºC with light incidence (B), 25 ºC and no light incidence (C) and 4 ºC and no light incidence (D).

On the other hand, the ΔE results related to storage at 25 °C (under light and no light storage conditions) showed an evident increase of ΔE values possibly due to degradation of the blueberry and beetroot extracts (Figure 13). These ΔE changes were detected more easily when the film was exposed to light during 10 and 15 days of storage of BLE and BEE films, respectively. Also, the BLE and BEE films showed significant differences in ΔE values after 60 days of storage at 25 °C and no light conditions. Probably, the BLE film may present a higher instability to high temperature and light incidence, increasing its possible degradation. Thus, an oscillation of temperature (e.g., during food products transport chain), which could compromise the food quality, can be detected by both films studied. However, as can be observed in Figure 14A and 14C and discussed above, by comparing the BLE and BEE film, when stored at 25 ºC with light incidence, the color change is more noticeable on BLE film. Gao *et al.* (2022) also concluded that ΔE of pH indicator films based on κ-car incorporated with anthocyanins and betalains, increased gradually with the extension of storage time at 25 ºC. The authors stated that the color stability decreased progressively, which may be related to the oxidation and decomposition of anthocyanins and betalains.

4.4. Films response to ammonia vapor

Generally, many protein-rich animal food spoilage can produce a high amount of volatile nitrogenous compounds (e.g., ammonia, dimethylamine and trimethylamine) due to proteins deterioration which directly affect food products pH status (Liu *et al.*, 2018). All films were exposed to ammonia solution for 120 min to simulate the volatile nitrogenous compounds release from food products presenting high protein content. Then, the pH-colorimetric film response was evaluated and color variables (a* and b*) were measured every 10 min (Zhang *et al.*, 2020; Y. Li *et al.*, 2021).

As shown in Table 4, C film color did not change over time and a* and b* values remained nearly constant ($p > 0.05$). As expected, BLE film showed a significant color change from pink/violet to blue after 10 min and pale green/yellow after 60 min when exposed to ammonia vapor. Accordingly, a* and b* values significantly decreased ($p < 0.05$) after 10 min of exposure time, which mean that BLE film became bluer/greener. After 30 min, blue/green color of the film started to fade, and some grey color portions began to appear (Table 4). The a* and b* values increased which, consequently, led to a decrease in the green and blue color. After 60 min, there was a decrease in a* and b* values again, but at 120 min, they increased again, causing another considerable color change in the film (blue to pale green/yellow) (Table 4). The mechanism that may explains these color changes is the fact that ammonia can diffuse through the films, combining with water molecules and, subsequently, hydrolyzing into hydroxyl ions (OH^-), producing an alkaline environment in films. As previously discussed, BLE films, as exposed to increasingly alkaline conditions, change their color from pink/violet to blue and, finally, to green/yellow (Agunos, Mendoza and Rivera, 2020; Jiang *et al.*, 2020; Alizadeh-Sani *et al.*, 2021). Jiang *et al.* (2020) also reported carboxymethyl-cellulose/starch/purple sweet potato anthocyanins film color changed from red to green, after 60 min of exposure time to ammonia. Other research showed a significant color change from reddish/pink to pale green of methylcellulose/chitosan nanofiber/barberry anthocyanins films, after 15 min, and to yellow, after 30 min when exposed to ammonia vapor (Alizadeh-Sani *et al.*, 2021).

Regarding BEE film, the colorimetric changes were not visually significant ($p > 0.05$) as shown in Table 4. The films showed a pink/violet color during 120 min when exposed to ammonia. Overall, a* value decreased and b* value increased during the exposure time, which allowed an increase of green and yellow color of BEE film, despite not being easily visible. The ammonia diffusion on the film surface caused the environment alkalinization, which led to the betacyanins degradation

(present in beetroot), changing the red color into colorless cyclo-dopa-5-O-(malonyl)-β-gucoside and yellow betalamic acid and, consequently, induced minor colorimetric changes in the film (Qin *et al.*, 2020; Jiang *et al.*, 2023). Naghdi, Rezaei and Abdollahi (2021) developed a starch film containing paper flower betacyanin and they reported film sensitivity to ammonia gas as the color changed from pink to yellow after 30 min of exposure. Qin *et al.* (2020) observed that the pink color of polyvinyl alcohol films/starch with red pitaya betalains faded after 10 min of exposure, and yellowness degree gradually intensified after 20 min. Despite these results obtained by other researchers, the BEE film developed did not significantly display the color changes compared to the other films (BLE and BLE/BEE). This can be due to several factors: amount of betalains incorporated in the film, the polymeric base used in the bio-based film development, the time of exposure to ammonia or the ammonia solution concentration used.

BLE/BEE film color changed from pink (at 0 min) to violet (between 10 - 60 min), and, finally, to pale pink/yellow (at 120 min) when exposed to ammonia. In first 10 min, a* and b* values decreased significantly ($p < 0.05$), making BLE/BEE film progressively greenish/blueish. Between 10 min and 60 min, a* and b* values faced minor modifications, showing no significant differences ($p > 0.05$). At 120 min, a* value decreased and b* value underwent into a considerable increase, which caused a greenish/yellowish color in film, an easily detectable colorimetric change.

The real-time, immediate, and sensitive reactions of colorimetric films are important properties of the color and pH-sensing intelligent packaging applications. As a result, BLE film showed the most evident colorimetric differences in a short time of exposure to ammonia and could be applied to monitor protein-rich animal food freshness.

Table 4. Control, BLE, BEE and BLE/BEE films' color response (and corresponding a* and b* values) to ammonia.

Time (min)	Control film	a*	b*	BLE film	a*	b*	BEE film	a*	b*	BLE/BEE film	a*	b*
0		0.25 ± 0.07^{a}	4.32 ± 0.50^{a}		43.49 ± 3.44^{a}	5.42 ± 2.10^{a}		46.09 ± 4.53^{a}	- 4.96 ± 0.52^{a,b}		34.87 ± 1.62^{a}	- 6.98 ± 0.46^{a}
10		0.13 ± 0.05^{a}	4.00 ± 0.39^{a}		3.34 ± 2.31^{b}	-11.59 ± 0.93^{b}		33.61 ± 3.77^{a}	-5.70 ± 1.01^{b}		12.14 ± 2.75^{b}	-11.87 ± 4.08^{b}
20		0.17 ± 0.04^{a}	3.71 ± 0.19^{a}		0.25 ± 0.94^{c}	-6.35 ± 1.21^{b}		27.35 ± 4.51^{b}	-4.45 ± 1.21^{a,b,c}		10.25 ± 1.99^{b}	-9.40 ± 3.94^{b}
30		0.16 ± 0.07^{a}	3.67 ± 0.59^{a}		0.62 ± 0.65^{b,c}	-3.41 ± 1.79^{b,c}		30.50 ± 3.27^{b}	-3.83 ± 0.78^{a,b}		12.87 ± 0.78^{b}	-6.24 ± 2.50^{b}
40		0.16 ± 0.06^{a}	4.00 ± 0.68^{a}		1.54 ±1.77^{b,c,d}	2.22 ± 5.38^{b,c}		28.27 ± 4.12^{b}	-2.24 ± 1.38^{a,d}		9.82 ± 0.70^{b}	-4.30 ± 2.52^{b}
50		0.17 ± 0.06^{a}	4.15 ± 0.46^{a}		1.52 ±1.32^{b,c,d}	5.45 ± 4.33^{c}		29.91 ± 2.61^{b}	-1.82 ± 1.07^{c,d}		10.30 ± 1.20^{b}	-1.58 ± 2.00^{b}
60		0.17 ± 0.05^{a}	4.13 ± 0.31^{a}		0.83 ± 1.07^{b,c,d}	5.02 ± 3.88^{c}		26.85 ± 7.00^{b}	-1.18 ± 0.82^{d}		9.15 ± 0.70^{b}	-0.05 ± 2.46^{b}
120		0.09 ± 0.10^{a}	3.99 ± 0.36^{a}		4.21 ± 1.27^{d}	13.83 ± 4.10^{d}		25.98 ± 5.22^{b}	3.40 ± 2.02^{d}		4.53 ± 2.07^{c}	13.03 ± 3.90^{c}

$^{a-d}$ Values are given as mean ± SD (n = 9). Different letters in the same column indicate significative differences between samples ($p < 0.05$).

4.5. Determination of physicochemical properties of the developed colorimetric films

4.5.1. Fourier-Transform Infrared (FTIR) Spectroscopy

FTIR spectral analysis is a rapid and non-destructive technique that can be used to investigate the functional groups and intermolecular interactions in films. Thus, FTIR has been widely used to characterize different polysaccharide-based films and investigate polymer blend miscibility (Turquois *et al.*, 1996). Absorption bands shifting is one of the changes observed in FTIR spectra, when chemical groups exhibit different physical bonding and interactions at the molecular level (Xu *et al.*, 2007).

The effect of blueberry and beetroot extracts incorporation in LBG/κ-car film was studied by FTIR analysis. A band of great intensity between 3000 and 3600 cm^{-1} was verified on all film samples (Figure 15A e 15B), which was attributed to O-H stretching formed by the hydroxyl group of polysaccharides, water, and phenolic hydroxyl groups (e.g., anthocyanins and betalains present in the extracts (Lawrie *et al.*, 2007; Martins *et al.*, 2012; Huang *et al.*, 2019; Liu *et al.*, 2023). Furthermore, C-H stretching was attributed to broad band around 2800 and 3000 cm^{-1} (Figure 15A) (Cerqueira *et al.*, 2011).

The LBG/κ-car polysaccharides characteristic bands were detected between 700 and 1800 cm^{-1}, so-called carbohydrate fingerprint region (Figure 15A and 15B). The characteristic bands of LBG were detected at 785 cm^{-1} and 860 cm^{-1}, indicating the presence of α-linked D-galactopyranose units and *β*-linked D-mannopyranose units, respectively. The characteristic peaks of κ-car were noticed at: 1243 cm^{-1} which corresponds to the ester sulfate groups; 925 cm^{-1} which is assigned to C-O-C bounds found in 3,6 – anhydrogalactose group and; 818 cm^{-1} which corresponds to 3,6-anhydro-D-galactose-2-sulfate. The bending and asymmetric stretching of C-O and stretching of C-O-C glycosidic bonds were also identified in fingerprint region (Huang *et al.*, 2019).

In addition, FTIR analysis was conducted on blueberry and beetroot extracts as can be seen in Figure 15A. It was concluded that the extracts' addition to the LGB/κ-car film matrix changed the peaks absorption intensity, which indicated possible new interactions between polymer matrix and extracts. Regarding blueberry extract, the peaks corresponding to the C-O stretching shifted from 773 and 843 cm^{-1} to 777 and 872 cm^{-1}, respectively, and the C-O-C stretching peak shifted further to the right (located at 953 cm^{-1}). Regarding beetroot extract, it was observed a sharp increase in

the C-O-C stretching peak absorption, and its displacement to the left. Based on these results, it was concluded that natural pigments incorporation in LBG/κ-car matrix could be recognized since changes occurred in the different molecular groups identified previously.

Blueberry and beetroot extracts' addition to LBG/κ-car films did not lead to significant changes on peak positions of the FTIR spectra. However, regarding intensity, there were some changes in the peaks when anthocyanins was added, leading to an increase of absorbance values in all peaks compared to control film. The FTIR spectra of BLE films showed broad bands at 3310, 2956, 1651, 1418, 1027 cm^{-1} which were attributed to O-H, C-H, aromatic ring C=C, C-O specific angular deformation and C-O-C stretching of anthocyanins present in the blueberry extract (Liu *et al.*, 2023). Huang *et al.* (2019) reported similar results after incorporating *Arnebia euchroma* root in a colorimetric agar-based film indicator. On the other hand, the presence of beetroot extract led to a decrease in peaks intensity (Figure 15B). These changes may be related to the presence of natural pigment extracts (e.g., betalains) in the film, suggesting that beetroot extract have been successfully immobilized in the biopolymeric matrix (Zepon *et al.*, 2019). Regarding FTIR spectra of BEE films, the broad bands at 3325, 2954, 1635, 1420, 1008 were attributed to O-H and N-H, C-H, C=C stretching, C-O specific angular deformation and C-O-C stretching of the betalains present in the beetroot extract (Figure 15B) (Liu *et al.*, 2023). The strong vibration peak, related to the C-O-C stretching, decreased after beetroot extract addition to the LBG/κ-car matrix. This result may indicate the formation of hydrogen bonds between C-O and O-H groups when beetroot extract added to film matrix (Naghdi, Rezaei and Abdollahi, 2021). Hu *et al.* (2020) also obtained similar results after incorporating betalains-rich amaranth extract into quaternary ammonium chitosan/fish gelatin matrix.

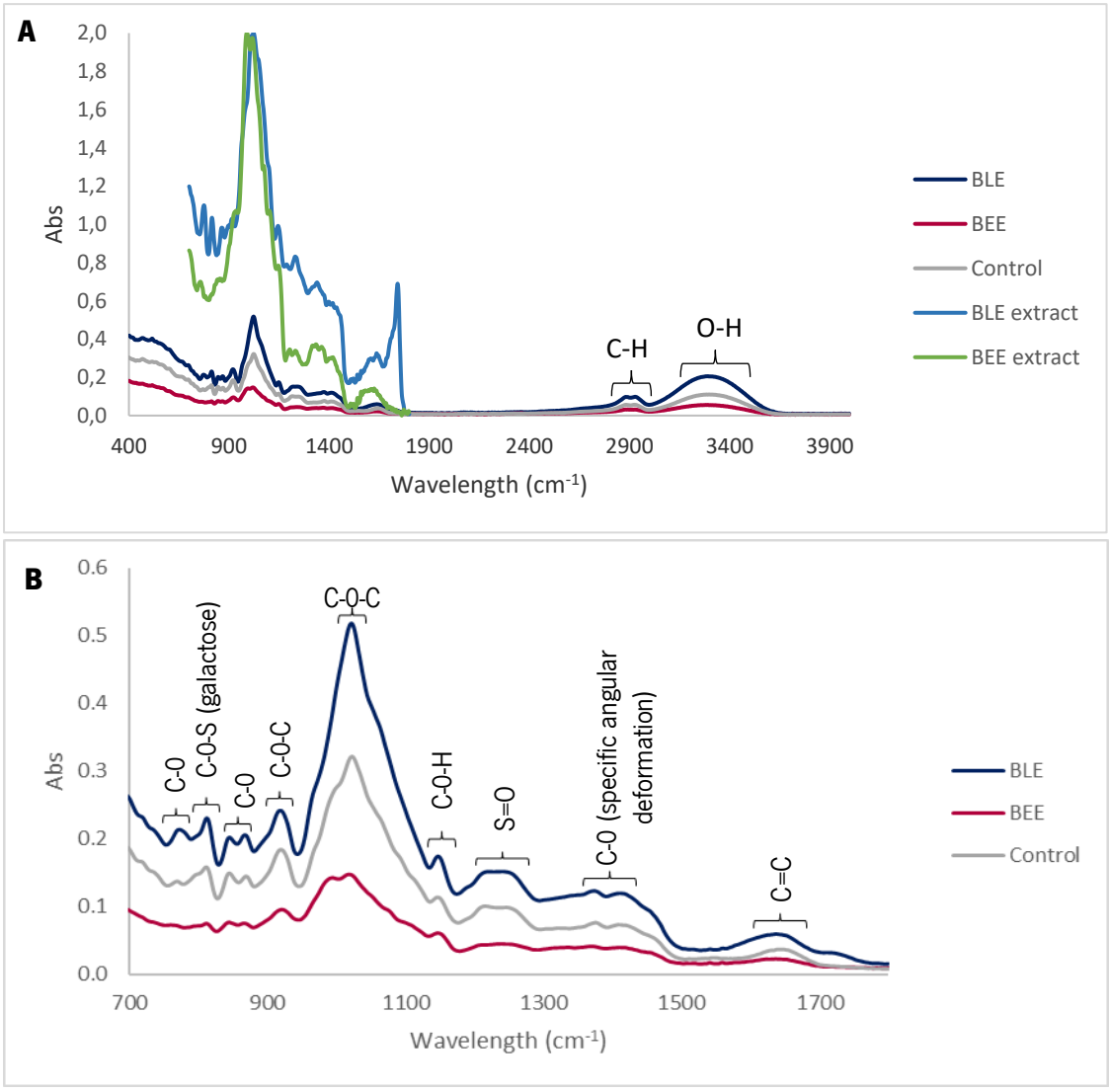


Figure 15. Fourier-transform infrared (FTIR) spectra of LBG/κ-car (Control), BLE and BEE films between 400 and 4000 cm^{-1} (A) and 700 and 1800 cm^{-1} (B). FTIR spectra of BLE and BEE extracts between 700 and 1800 cm^{-1} were presented in A.

4.5.2. UV-vis light barrier properties of the films

Food products exposure to UV-vis light can accelerate oxidative deterioration, leading to nutrients, color and flavor losses which affects food quality (Martins *et al.*, 2012; Yun *et al.*, 2019). The UV-vis spectrophotometric analysis allows to measure the amount of UV-vis that is transmitted through the film sample and, consequently, film barrier ability to UV-vis light (A. De Caro, 2015).

As shown in Figure 16, BLE, BEE and BLE/BEE films showed lower light transmittance in both UV and visible wavelength range when compared to film control (i.e., LBG/κ-car film), suggesting that extracts incorporation into the films increased UV-vis light barrier ability of the films (Yun *et al.*,

2019). The addition of blueberry extract improved the UV light barrier of the film, since its transmittance levels were lower than the other films in the wavelength range of 250 – 300 nm. This feature could be due to the presence of the blueberry extract within the film matrix and the subsequent formation of a stable and dense membrane network structure. Possibly, the presence of double bonds in the aromatic ring of anthocyanins (pigments present in the blueberry extract) could absorb UV radiation and form a compact inner microstructure, reducing the exposure of hydrophilic groups (such as hydrogen bonds between anthocyanins and biopolymers) and improving the light barrier ability BLE films (Aztatzi-Rugerio *et al.*, 2019; Yong and Liu, 2020; Zhao *et al.*, 2022). Yong and Liu (2020) also have shown that UV–vis light barrier ability of the films can be significantly enhanced by incorporating anthocyanins-rich extracts. On the other hand, in visible light range (350 – 700 nm), BLE film transmittance levels increased while those of BEE film decreased. Therefore, it is concluded that BLE films were considered a better barrier to UV light while BEE films had a higher barrier capacity to visible light. The decrease of the light transmittance of the films incorporating extracts was due to the presence of several unsaturated bonds (e.g.: C-C, C-N and C-O of betacyanins) which could absorb UV–vis light (de Mello *et al.*, 2015; Hu *et al.*, 2020; Qin *et al.*, 2020). Akhtar *et al.* (2013) also observed that the addition of beetroot and purple carrot extracts to hydroxypropyl methylcellulose films reduced UV-vis light transmission of the films. Iahnke *et al.* (2016) found that with an increase in beetroot residue powder content, the UV-vis light transmission of gelatin-based films gradually decreased. Furthermore, transmittance of films with both extracts incorporated within the film was studied. It was observed that film with both extracts (BLE/BEE film) showed higher transmittance levels when compared to the BLE and BEE films (Figure 16). Thus, simultaneous addition of the two extracts into LBG/κ-car films did not show advantageous barrier ability to UV-vis light. Accordingly, BLE and BEE films presented promising UV-vis light barrier ability to prevent food lipid oxidation and guarantee its quality and safety.

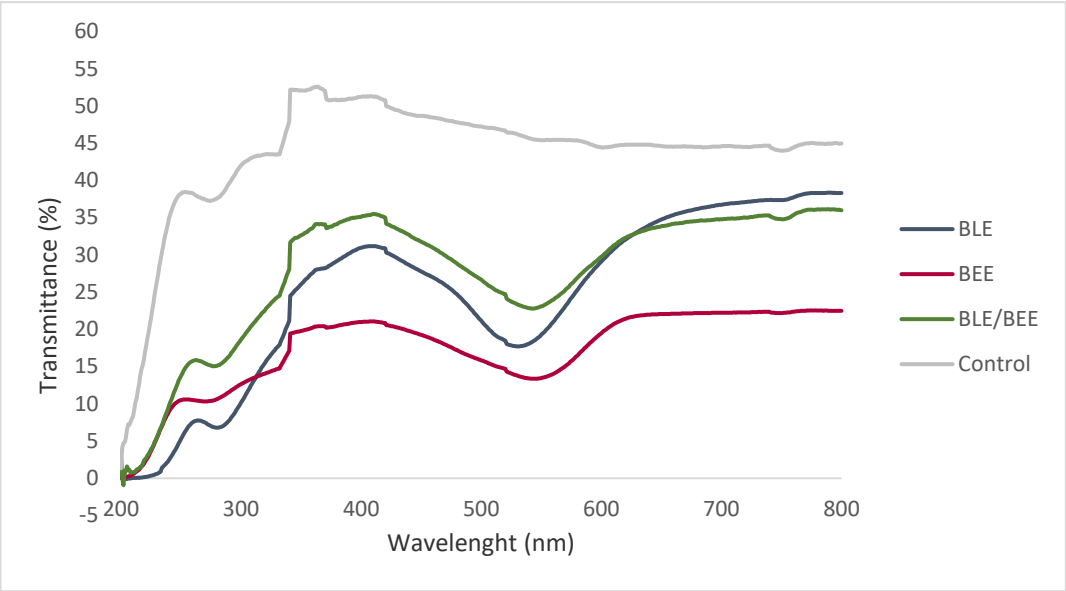


Figure 16. UV–vis light transmittance of BLE, BEE, BLE/BEE and control (no added extracts) films.

4.5.3. Thermal stability of the films

The differential scanning calorimetry (DSC), powerful analytical tool, was used to study films' thermal stability. DSC allows to research the kinetics of the physical processes and chemical reactions that occur during biodegradation through melting and mesomorphic transitions, including entropy and enthalpy changes (Ebert *et al.*, 2017).

The DSC curves of all films showed at least two significant thermal events (Figure 17). The first endothermic peak observed at, approximately, 53 ºC may be attributed to evaporation of bound water molecules, commonly observed in a broad class of hydrated biopolymers. The second stage between 135 ºC and 146 ºC (maximum peak at DSC curve) is usually related to polysaccharide decomposition (Martins *et al.*, 2012; Liu *et al.*, 2018).

The DSC curve of BLE film (Figure 17A) showed an endothermic peak at 134.17 °C corresponding to the anthocyanin-rich blueberry extract degradation, which agrees with the results published by Favaro *et al.* (2018). Also, the endothermic peak at 146.33 °C could be associated to the thermal decomposition of the film matrix. Higher endothermic peak value in BLE film (146 ºC) when compared with control film (144 ºC) can be credited to changes promoted by inter- and intramolecular interactions between anthocyanin-rich blueberry extract and biopolymer chains (Favaro *et al.*, 2018; Luchese, Garrido, *et al.*, 2018; Silva *et al.*, 2019; Zhao *et al.*, 2022). This result indicated that the incorporation of anthocyanin-rich blueberry extract could improve the thermal stability of the film matrix. The BEE film curve presented the maximum peak at temperature

at 135.33 ºC (Figure 17B). The dihydroxylation and separation of betalamic acid from the rest of the molecule lead to betalains decompose around 100-140 ºC, according to Rodríguez-Félix *et al.* (2022). These results indicated that a decreased amount of thermal energy is needed to dissociate interactions between the components in BEE films compared to control films. Moreover, the presence of betalain-rich beetroot extract possibly reduced the LGB/κ-car chain interactions, decreasing the thermal stability of the films.

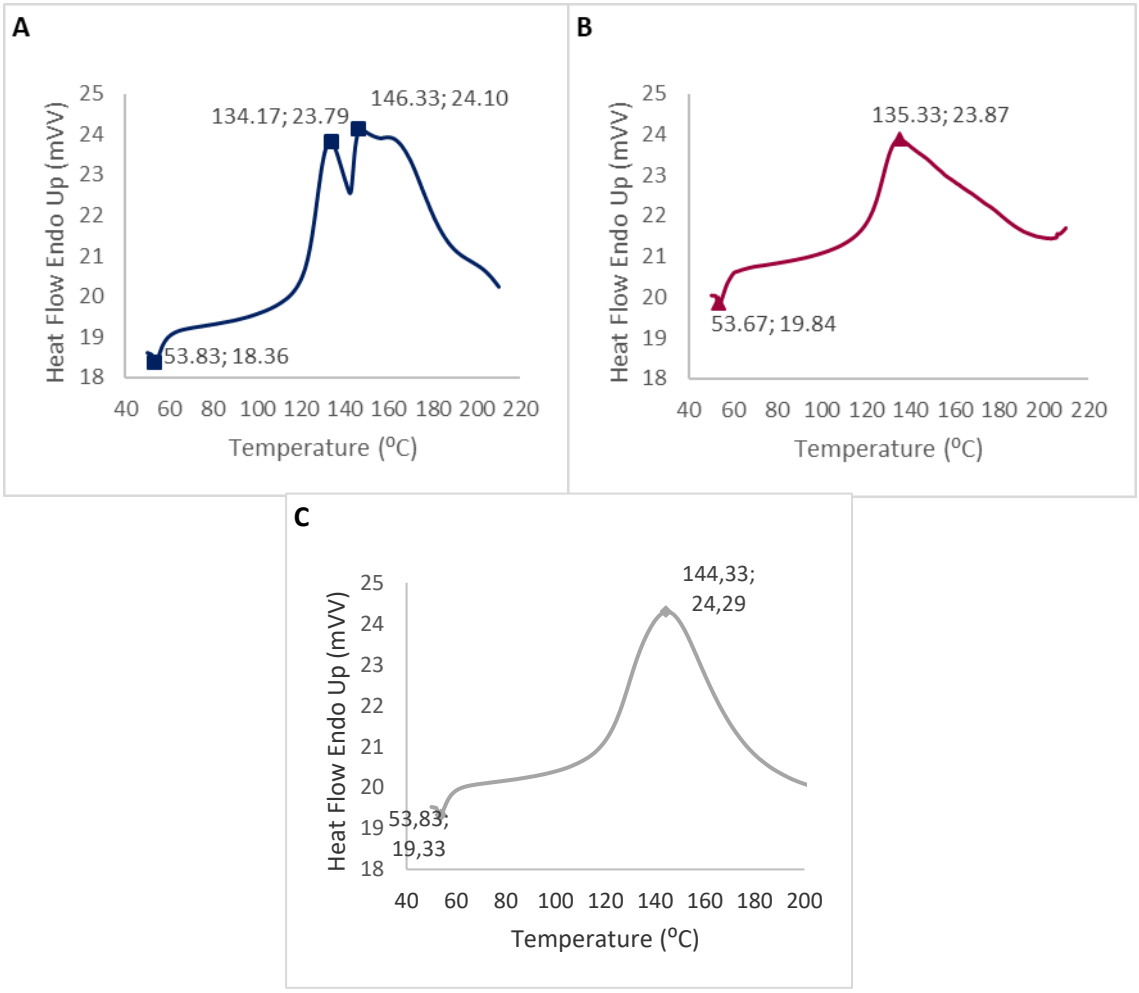


Figure 17. DSC curves of BLE film (A), BEE film (B) and control film (C).

4.5.4. Film thickness

The film thickness has a high influence on mechanical, water vapor barrier and opacity properties of food packaging films (Rigo, 2006).

Table 5 shows that there were no significant thickness differences ($p > 0.05$) between BLE film and control film. This result could occur due to the low concentration of blueberry extract added to biopolymer matrix, causing no significant changes in film thickness. A similar result was previously reported by Moradi *et al.* (2016), which prepared zein films containing *Zataria multiflora Boiss* extract (rich in anthocyanins) and observed similar thickness values for films with added or no-added extract (0.095 mm). Regarding BEE and BLE/BEE films, it was observed that the film thickness increased compared to control and BLE films ($p < 0.05$), since there was an increase of solids content added to the LBG/κ-car formulation (Qin *et al.*, 2019; Hu *et al.*, 2020). According to Benavides, Villalobos-Carvajal and Reyes (2012), film thickness is influenced by the solids content found in the forming solution. Iahnke *et al.* (2016) also showed that beetroot extract addition increased gelatin films thickness from 0.114 mm to 0.124 mm.

Table 5. Thickness (mm) of BLE, BEE, BLE/BEE and control films.

Films	Thickness (mm)
BLE	0.023 ± 0.001^{a}
BEE	0.044 ± 0.001^{b}
BLE/BEE	0.041 ± 0.002^{b}
Control	0.029 ± 0.003^{a}

Values are given as mean ± SD (n = 10). ^{a,b} Different letters in the same column indicate significant differences (Tukey test, $p < 0.05$).

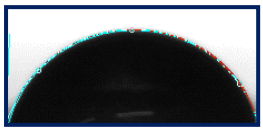
4.5.5. Water contact angle (WCA)

The films' hydrophobicity was assessed through the measurement of the contact angle of water drop on the surface of the films (Table 6). Usually, the WCA increases when surface hydrophobicity increases (Gao *et al.*, 2022).

All WCA measured were less than 90° (Table 6), which indicated that all films' surface had a hydrophilic character (Rodríguez-Félix *et al.*, 2022; Gao *et al.*, 2022). This result could be related to the presence of free hydrophilic groups on the surface of LBG/κ-car film, as shown by FT-IR results, which make the film more hydrophilic (Gao *et al.*, 2022).

Moreover, no significant differences were observed between control film and the other film samples ($p > 0.05$). However, BLE film presented a higher WCA than the BEE film ($p < 0.05$). Therefore, anthocyanins-rich blueberry extract addition to film increased WCA value, indicating that BLE film presented higher hydrophobicity than BEE films (Wu *et al.*, 2021). Wu *et al.* (2021) reported similar results and concluded that polyphenols addition (such as anthocyanins) into polysaccharide-based films led to formation of hydrogen bonds and non-covalent hydrophobic interactions. Luchese *et al.* (2017) also described that adding blueberry residue to starch films increased WCA and, consequently, film hydrophobicity.

Table 6. Water contact angle (WCA) values and photographs of water droplet on surface of BLE, BEE and control films.

Film	WCA (°)	Physical appearance of water droplet on film surface
BLE	41.43 ± 3.32[a]	
BEE	30.62 ± 3.62[b]	
Control	34.50 ± 5.51[a,b]	

Values are given as mean ± SD (n = 3). [a-b] Different letters in the same column indicate significant differences (Tukey test, $p < 0.05$).

4.5.6. Moisture content (MC) and water solubility (WS)

The water solubility (WS) of the films were assessed to evaluate their water resistance and film integrity (Liu *et al.*, 2023). Thus, WS is influenced by the number of free hydroxyl groups available in the polymer matrix as they can form hydrogen bond between polymers. The film solubilization starts with water access into the biopolymeric matrix, followed by a breakdown of hydrogen bonds and Van der Walls forces between the chains of biopolymers (Yoshida *et al.*, 2014). The water solubility and moisture content results of BLE, BEE and Control films are shown in Table 7.

Control and BEE films were immersed in water, and it was possible to observe that films lost their integrity completely after 8 h. As evidenced in FT-IR and WCA results, control films, formed by hydrocolloids, and BEE films were considered hydrophilic. This may be due to the high number

of hydrophilic groups for example, on LBG, κ-car and betalains-rich beetroot extract, which can form strong hydrogen bonds with water molecules, leaving films very hydrated (Segato, 2007). Gao *et al.* (2022) also found that betacyanins from peels of dragon fruits significantly increased the WS of pH-sensing κ-car-based films.

On the other hand, the incorporation of blueberry extract on film led to a significant reduction in WS values obtained ($p < 0.05$). As discussed above, this could be explained by hydrophobic groups present in blueberry extract, which decreased the ability of film to form bonds with water molecules. Thus, interaction between phenolic compounds in blueberry extract with the hydroxyl groups of LBG and κ-car matrix led to a more compact inner-structure formation, with stronger bonds (Nouri *et al.*, 2018; Andretta *et al.*, 2019). Huang *et al.* (2019) reported similar WS behavior of agar-based film incorporating *Arnebia euchroma* root extracts (rich in anthocyanins), concluding that WS reduced significantly due to water-insoluble extract addition. Kanatt (2020) also observed a significant WS decrease of gelatin/polyvinyl alcohol films when *Amaranthus* leaf extract was added to the films.

Table 7. Water solubility (WS, %) and moisture content (MC, %) of the control films, BLE and BEE films.

Films	WS (%)	MC (%)
BLE	39.66 ± 0.11^{a}	23.54 ± 0.98^{a}
BEE	92.90 ± 0.03^{b}	18.23 ± 1.47^{b}
Control	94.41 ± 0.01^{b}	$21.05 \pm 1.30^{a,b}$

Values are given as mean ± SD (n = 3). [a-b] Different letters in the same column indicate significant differences (Tukey test, $p < 0.05$).

The moisture content (MC) influences the structure of the of bio-based films. For instance, a higher MC will suggest a looser microstructure of the film (Kanatt, 2020).

As shown in Table 7, the MC values of BLE and BEE films were not significantly different from MC values of the control film ($p > 0.05$). These results were different from Zhai *et al.* (2017) results. They showed a significant decrease in MC of starch/polyvinyl alcohol–roselle anthocyanins films, which was attributed to the interactions between the film matrix and anthocyanins that decreased the availability of hydroxyl groups in matrix to interact with water molecules. This is an indicative that our films had a tight microstructure which was not significantly affected by blueberry or beetroot extracts addition. Possibly, blueberry and beetroot extracts incorporation into LBG/κ-car had little effect on the interaction between hydrophilic groups and moisture. However, MC of BEE

films were lower than MC of BLE films ($p < 0.05$). Possibly, less hydroxyl group interactions between LBG/κ-car and beetroot extracts occurred comparing to blueberry extract and LBG/κ-car; thereby, the water sorption ability of the BEE films was limited (Qin *et al.*, 2019; Hu *et al.*, 2020). Similarly, Qin *et al.* (2020) noticed a reduction in the MC of films after the addition of betalains from red pitaya. Lately, Jamróz *et al.* (2019) also verified that the MC significantly decreased in furcellaran films by adding beetroot (*Beta vulgaris* L.) extracts.

4.5.7. Water Vapor Permeability (WVP)

Water vapor permeability (WVP) is an important feature in food packaging, which assesses the water barrier properties of the film. Many films used in food industry must have low WVP values (i.e., reduced water permeability), to delay food deterioration process and to extend its shelf life of food products and ensure its quality (Naghdi, Rezaei and Abdollahi, 2021). Furthermore, it has been described that thickness, film matrix integrity and interactions between functional groups of film components can influence films WVP (Qin *et al.*, 2020; Naghdi, Rezaei and Abdollahi, 2021). The results obtained for BLE, BEE, control and BLE/BEE films are shown in Table 8.

The blueberry extract addition to the biopolymer-based matrix did not cause a significant effect on WVP values ($p > 0.05$) of the films. As described in the WCA, WS and MC analysis, the blueberry extract can form intermolecular interactions with LBG and κ-car, which limited the interactions between water vapor and film matrix (Hu *et al.*, 2020). Gao *et al.* (2022) also reported that the addition of anthocyanins-rich purple sweet potatoes extract had no significant effect on the WVP of the κ-car-based film, changing from $2.01 \pm 0.01 \times 10^{-9}$ [g/(h.m.Pa)] to $2.07 \pm 0.18 \times 10^{-9}$ [g/(h.m.Pa)]. On the other hand, when beetroot extract was incorporated into LBG/κ-car matrix, individually or simultaneously with blueberry extract, a significant increase of WVP values were observed ($p < 0.05$). Possibly, the increase of WVP can be attributed to beetroot extract hydrophilicity (in agreement with WCA, WS and MC results) and to the increase of micro-paths in the network microstructure which facilitate the diffusion of water molecules through the matrix. Zamudio-Flores *et al.* (2015) determined that beet extract addition significantly increased WVP of oxidized starch films (from $1.45 \pm 0.58 \times 10^{-10}$ [g/(m.s.Pa)] to $1.85 \pm 1.54 \times 10^{-10}$ [g/(m.s.Pa)]), which also was attributed to hydrophilic property of the betalains present in the extract composition.

Table 8. Water vapor permeability (WVP) of control, BLE, BEE and BLE/BEE films.

Films	WVP * 10^{-10} [g/(m.s.Pa)]
Control	2.09 ± 0.29 [a]
BLE	2.30 ± 0.58 [a]
BEE	3.13 ± 0.46 [b]
BLE/BEE	3.92 ± 0.42 [b]

Values are given as mean ± SD (n = 3). [a-b] Different letters in the same column indicate significant differences (Tukey test, $p < 0.05$).

4.5.8. Mechanical Properties

The packaging film must have enough strength to withstand mechanical stress and retain its integrity during transport and storage. The tensile strength (TS) is a mechanical parameter, which evaluates the film ability to resist traction (under tension) until it breaks. The elongation at break (EB) corresponds to the increase in film length before breaking, measuring the flexibility or ductility of the films (Jamróz *et al.*, 2019; Huang *et al.*, 2019; Naghdi, Rezaei and Abdollahi, 2021; Gao *et al.*, 2022). The mechanical characteristics of the films reflect their ability to protect food products integrity, and for that reason, must be strong and flexible enough to suit their application (Martins *et al.*, 2012). The nature of film-forming matrix and the intensity of intermolecular and electrostatic interactions between components have an effect on mechanical properties (Jamróz *et al.*, 2019). The effect of the extracts' incorporation on mechanical properties (TS and EB) of the biopolymeric matrix are shown in Table 9.

Table 9. Film tensile strength (TS) and elongation at break (EB) of control films (LBG/κ-car films) and films containing blueberry and/or beetroot extracts (BLE, BEE and BLE/BEE).

Films	TS (MPa)	EB (%)
Control	11.70 ± 3.38[a]	16.50 ± 1.48[a]
BLE	40.26 ± 5.79[b]	28.39 ± 1.19[b]
BEE	22.83 ± 2.75[c]	27.76 ± 0.90[b]
BLE/BEE	18.91 ± 2.55[c]	6.01 ± 1.42[c]

Values are given as mean ± SD (n = 4). [a-c] Different letters in the same column indicate significant differences (Tukey test, $p < 0.05$).

The blueberry and beetroot extracts incorporation into film significantly increased TS and EB values compared with control film ($p < 0.05$). This result may be related to the reduction of intermolecular interactions between LBG and κ-car, improving the chain mobility and the overall

film elasticity. Additionally, TS and EB values increase may be associated to the interactions between the phenolic compounds and LBG/κ-car groups. Accordingly, internal structure of film matrix with natural extracts addition changed, becoming more compact and able to withstand mechanical forces (Jamróz *et al.*, 2019; Gao *et al.*, 2022). Zhang *et al.* (2020) significantly increase of TS and EB values in starch/polyvinyl alcohol with anthocyanins-rich purple potato. Qin *et al.* (2020) found a similar trend when betalains from red pitaya peel was incorporated on starch-polyvinyl alcohol matrix. They stated that the increase of film mechanical strength film was due to weakening of intermolecular forces between adjacent macromolecules which facilitated polymer chains motion. On other hand, Iahnke *et al.* (2016) noticed that the addition of betacyanins from beetroot extract reduced the EB of gelatin-based films.

The simultaneous addition of the two natural pigment extracts did not favor mechanical properties since TS did not show significant differences and EB decreased when compared to the control film (Table 9). Thus, as already discussed, no advantages (i.e., improved mechanical properties) were found in combining blueberry and beetroot extracts in the same film.

Films should present good mechanical strength to resist pressure in transportation and to maintain content integrity (Martins *et al.*, 2012). For this reason, it possible to conclude that BLE film had better mechanical properties (i.e., higher TS and EB values) compared to the other studied films and could be used as an intelligent film.

4.5.9. Optical properties: color and opacity

Color is the most intuitive film property, which greatly affects the acceptance degree of consumers. The color parameters (L*, a* and b*) and opacity of the various films developed are shown in Table 10.

Table 10. Color parameters (L*, a* and b*) and opacity of BLE, BEE, control and BLE/BEE films.

Films	L*	a*	b*	Opacity (%)
Control	89.13 ± 2.51^{a}	0.14 ± 0.05^{a}	5.18 ± 0.82^{a}	13.55 ± 1.81^{a}
BLE	41.01 ± 1.46^{b}	40.76 ± 3.20^{b}	4.74 ± 1.90^{b}	37.41 ± 2.73^{b}
BEE	56.80 ± 0.84^{b}	51.58 ± 0.96^{c}	-4.35 ± 0.23^{c}	24.86 ± 1.18^{c}
BLE/BEE	47.19 ± 6.98^{b}	38.54 ± 2.77^{b,c}	-7.06 ± 1.24^{c}	34.17 ± 7.75^{b}

Values are given as mean ± SD (n = 6). $^{a-c}$ Different letters in the same column indicate significant differences (Tukey test, $p < 0.05$)

Control film showed higher transparency and luminosity (i.e., higher L* value) than the other films. The blueberry extract addition led to a decrease in L* and b* parameters ($p < 0.05$), which demonstrated a decrease in film brightness and an increase of blue color of the film (Table 10). On the other hand, there was a significant increase of a* value, compared to control film, which represented an increment in red coloration of the film. This result was in line with the reddish/purple color of the blueberry extract, contributing to a red/purple color of the BLE film. Qin *et al.* (2019) also concluded that the rise of anthocyanins-rich extract (from *Lycium ruthenicum Murr*) content within starch films significantly increased a* value and decreased b* value, indicating a change of film color to red. Merz *et al.* (2020) observed that chitosan and polyvinyl alcohol film color changed to red when anthocyanins from jambolana fruit were added. The addition of beetroot extract also caused a decrease of L* and b* values ($p < 0.05$) of control films (Table 10). Furthermore, a* value of BEE film had a higher increase when compared to BLE film ($p < 0.05$). For this reason, the pink/red color was more visible on BEE film. These changes in color parameters were common after adding red pigments such as betalains from beetroot (Guo *et al.*, 2021; Naghdi, Rezaei and Abdollahi, 2021). Y. Wu *et al.* (2021) showed that films based on LBG/polyvinyl alcohol with betacyanins from cockscomb flower were reddish-purple, which was caused by a* value increase. The similar colors were also observed in pectin-based films incorporated with beetroot powder by Sucheta *et al.* (2019). The BLE/BEE film did not show any significant change in color parameters compared to BLE and BEE films ($p < 0.05$).

Film opacity refers to the film ability to difficult the light path through them. The higher the opacity value, the lower the film transparency.

Control film showed the lowest opacity value confirming its transparency since no colored extract was added (Table 10). The BLE film presented the highest opacity value compared to BEE films and BLE/BEE films. Thus, BLE film reduced film transparency and hindered the passage of light through it which has great potential to prevent oxidative rancidity in foods, caused by exposure to light (Kanatt, 2020). These results were in accordance with those obtained in chapter 4.5.2., since BLE films showed to be a better barrier to UV-Vis light. Other researchers reported similar films opacity results where the increase in film opacity was due to natural extracts incorporation in bio-based intelligent films (Kanatt *et al.*, 2017; Kanatt and Chawla, 2018).

4.6. Monitoring hake fish freshness using the developed colorimetric films

4.6.1. Selection of colorimetric film to be applied on fish freshness study

Based on film characterization results (chapters 4.5.1. – 4.5.9.), only BLE and BEE films were tested as freshness indicators. The BLE and BEE films capacity to act as pH-indicator films when were stored with fish samples were evaluated under different conditions. Figure 18 showed the results of BLE and BEE films color changes during fish storage.

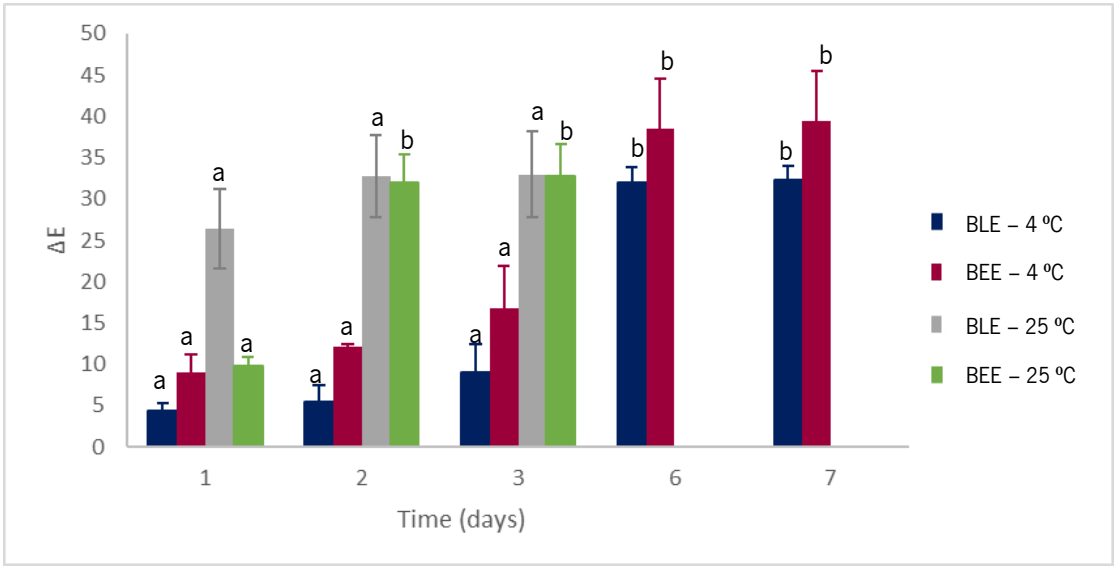


Figure 18. Color changes (ΔE) of the BLE and BEE films during fish storage during 7 days at 4 °C and 25 °C. Values are given as mean ± SD (n = 3). [a,b] Different letters indicate significant differences between the same sample over time (Tukey test, $p < 0.05$).

When evaluating the colorimetric changes of both films under 4 °C, it was concluded that a gradual increase occurred. The ΔE values increased from 0 to 9.01 ± 3.39 and 16.86 ± 5.18 on day 3 in BLE and BEE film, respectively (Figure 18). According to previous reports, a ΔE value higher than 5 could be detected by human eye although only values above 12 represent an absolute difference in color, which is very noticeable even by untrained panelists/consumers (Huang *et al.*, 2019; Kanatt, 2020). Although both ΔE values of BLE and BEE were above 5 and the color changes could be detected by naked eye, they were very faded and difficult to detect (Figure 19). Particularly, BEE film showed ΔE value higher than 12 at day 3; however, color differences were not noticeable, as could be seen in Figure 19. The BEE film showed pink color in this period of time and its color parameters (L*, a* and b*) showed no significant differences ($p > 0.05$). On the other hand, BLE film showed more distinguishable color variations to the naked eye, until day 3 of storage at 4 ºC

when compared to BEE film. The BLE film color changed from pink to dark purple, subsequently to light purple, and finally, to a softer pink/purple beginning to appear a bluish tone at day 3 (Figure 19). These variations agreed with colorimetric parameters obtained since a* decreased (31.16 ± 2.32 to 23.47 ± 2.27) and b* increased (1.00 ± 0.20 to 2.67 ± 0.06), causing a reduction in BLE film red color becoming more greenish and/or yellowish. At day 6, a significant increase in ΔE values ($p < 0.05$) at 4 °C occurred in both films, being colorimetric changes easily detectable (Figure 18 and 19). The BLE and BEE films' a* values decreased and b* values increased abruptly explaining color change from pink to green/yellow. At day 7, films color remained identical to the previous day, with no significant changes in ΔE values.

As a result, BLE and BEE films exhibited a visible color change at 4 ºC at the same time that the fish sample reached the end of shelf life (day 6) (Figure 19). Similarly, Zhai *et al.* (2017) reported that films based on starch/polyvinyl alcohol incorporated with roselle anthocyanins changed its color from initial pink to purple in 3 - 4 days and to green after 6 days of fish storage at 4 ºC. Qin *et al.* (2020) also described that color of starch/polyvinyl alcohol films incorporating betalains from red pitaya changed from purple to yellow after fish storage at 4 ºC for 8 days.

The fish storage condition at 25 ºC was also studied for the two optimized films (i.e., BLE and BEE). On the 3rd day of storage, fish showed quite unpleasant odor and reddish color, signs of advanced deterioration state (Figure 19). Thus, ΔE values of both films were only evaluated for 3 days as the fish reached the end of shelf life (Figure 18). The ΔE value of BLE film underwent a significant increase ($p < 0.05$) in first day of storage (Figure 18). Thus, ΔE value was above 12 (26.34 ± 4.83) on day 1, leading to BLE film colorimetric change which was easily detectable. These results were in line with the results presented in Figure 19, where BLE film color changed from pink to blue color. On the other hand, BEE film showed ΔE value less than 12 (9.80 ± 1.03) on the 1st day of storage at 25 ºC, and its pink coloration was maintained with no significant changes (Figures 18 and 19). On 2nd day, BLE film modified its color from blue to green/yellow and ΔE value showed no significant differences ($p > 0.05$) compared to value of the 1st day. BEE film revealed a significant ΔE value variation (31.91 ± 3.49), occurring a visual perceptible color change from pink to green/yellow (Figure 18 and 19). On 3rd day of storage at 25 ºC, ΔE values of BLE and BEE films remained similar since critical fish deterioration point had already passed. Qin *et al.* (2019) reported similar color changing results in intelligent packaging films based on cassava starch and anthocyanins from *Lycium ruthenicum Murr* when film was applied to monitor pork

freshness during storage at 25 °C. Films presented pink/red/purple color in the first 16 h of storage, grey/dark purple color at 24–32 h and green/yellow color after 32 h.

Both colorimetric films displayed continuous color changes within the fish shelf life, suggesting that they were capable to indicate the real-time fish freshness (Zhai *et al.*, 2017). As expected, fish spoilage was delayed at 4 °C and, in this sense, visible color differences of both tested films were only detected later on (day 6). However, under 25 °C, BLE film detected the quality change of fish earlier (day 1) than BEE film (day 2). This may be caused by anthocyanin-rich blueberry extract ability to detect more easily and react faster with volatile compounds than betalain-rich beetroot extract. Thus, BLE film is a promising fish freshness-indicator film since it presented a good color response in early fish storage stage. These results suggested that BLE film would be more suitable for monitoring real-time fish freshness than BEE film. Based on these results, it was decided to evaluate only BLE film as intelligent freshness-indicator packaging for hake fish samples.

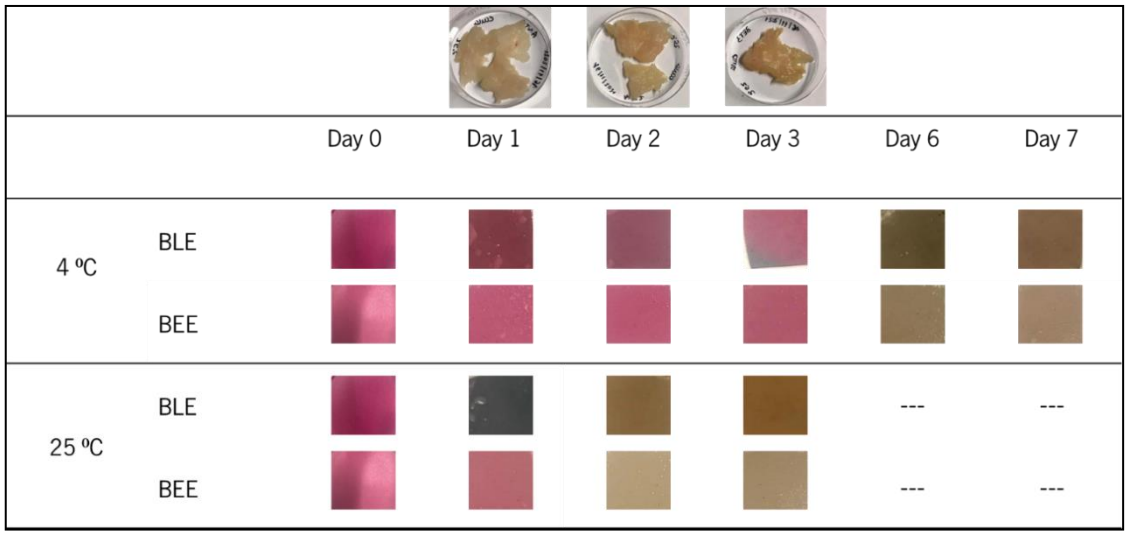


Figure 19. BLE and BEE films color changes for 7 days storage under 4 ºC and 25 ºC conditions. Visual appearance of the fish on day 1, 2, and 3 of storage at 25 ºC.

4.6.2. Relationship between color changes of freshness indicator film and microbiological and physico-chemical variables of fish during storage

Hake fish samples were stored for 7 days at 4 ºC and the following points were evaluated: microbial load, total volatile basic nitrogen (TVB-N), pH of the fish sample, and color change of the colorimetric films during storage.

4.6.2.1. Microbiological analysis

Microbiological spoilage is one of the main factors to monitoring the fish quality during cold storage since seafood are very perishable and susceptible to microbial deterioration. This is mainly due to its higher water activity (fundamental factor for the growth and survival of microorganisms), high fat content (favorable to oxidation) and neutral pH values (useful to the development and growth of most microorganisms), when compared to other food products (Kanatt, 2020; Naghdi, Rezaei and Abdollahi, 2021).

Therefore, microbiological quality of hake samples was assessed as a function of storage time is shown in Figure 20. At the beginning of the storage time, hake samples presented an initial TVC of 4.61 ± 0.36 $\log_{10}$ CFU/g. Possibly, the fish could already be contaminated due to several factors namely, lack of appropriate post-harvest fish handling and processing (e.g., storage and transportation temperature). During storage, the microbial load increased ($p < 0.05$) being equal to 8.61 ± 0.21 $\log_{10}$ CFU/g, on last day (day 7) (Figure 20). As storage time increases, microorganisms generally grow faster and generate various metabolites responsible for off-odors, off-flavors, texture, and color changes resulting in sensorial rejection by the consumer (Zhang *et al.*, 2020; Naghdi, Rezaei and Abdollahi, 2021). Fish is considered acceptable for human consumption if samples microbial load does not exceed 7.0 $\log_{10}$CFU/g, the maximum accepted value for marine species (Horwitz, 1975). As can be observed in Figure 20, this limit was exceeded between day 3 and 4 of storage at 4 ºC, pointing out that hake samples lost their quality/freshness and were no longer suitable for consumption. Huang *et al.* (2019) described novel colorimetric indicator based on agar incorporated with anthocyanins extracts for monitoring Wuchang bream fish freshness under 4 ºC and 25 ºC conditions. They concluded that fish samples stored at 4 ºC were not suitable for consumption (7.11 $\log_{10}$CFU/g) after 5-6 days.

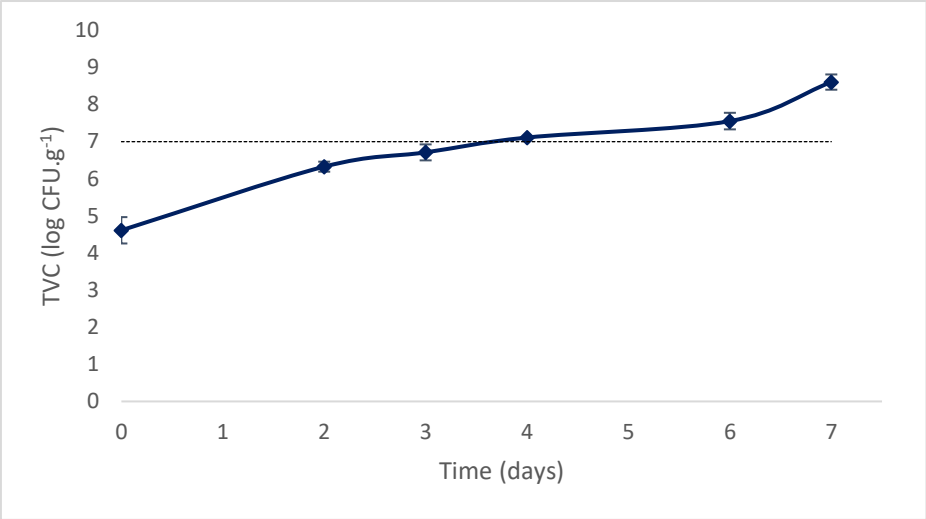


Figure 20. Changes in the total viable count (TVC) of hake samples stored during 7 days at 4 ºC. The maximum admissible intake limit value of 7 log CFU/g is represented by the dashed horizontal line. Values are average ± standard deviation of six experiments.

4.6.2.2. Determination of total volatile basic nitrogen (TVB-N) content

Volatile amines such as ammonia, di- and trimethyl amine, collectively recognized as TVB-N, are produced due to fish proteolysis during storage. For this reason, it is considered an important parameter indicative of muscle tissues degradation and the degree of fish freshness (Kanatt, 2020; Naghdi, Rezaei and Abdollahi, 2021).

Usually, microbiological spoilage leads to an increase of TVB-N content because numerous enzymes produced by microorganism in the fish *post-mortem* phase will degrade protein present in fish muscle tissue, leading to the volatile base nitrogen compounds production, and consequently, an unpleasant fish taste and odor will be developed (Qin *et al.*, 2019).

The average TVB-N values obtained throughout storage of the hake fish samples at 4 ºC are shown in Figure 21.

The initial TVB-N level of hake samples was 10.21 ± 1.97 mg/100 g, which showed the high sample quality (Figure 21). Similar results have been reported by other researchers namely, Naghdi, Rezaei and Abdollahi (2021) who measured a TVB-N level of 7.01 mg/100 g on Caspian spart samples at day 0 of storage at 4 ºC. The TVB-N content increased significantly throughout the storage period ($p < 0.05$), except at time interval between day 2 and 3 when there was a slight decrease. However, this was not relevant since both values (25.52 ± 4.28 mg/100 g and 21.55 ± 0.98 mg/100 g, respectively) did not show statistically significant differences. On last day of

storage, TVB-N values reached 66.78 ± 4.81 mg/100 g. This result coincided with the increase of the microbial growth on the fish samples (TVC values; Figure 20) which is related to the microbial activity and endogenous enzymes' action (Huang *et al.*, 2019; Kanatt, 2020; Qin *et al.*, 2020; Naghdi, Rezaei and Abdollahi, 2021).

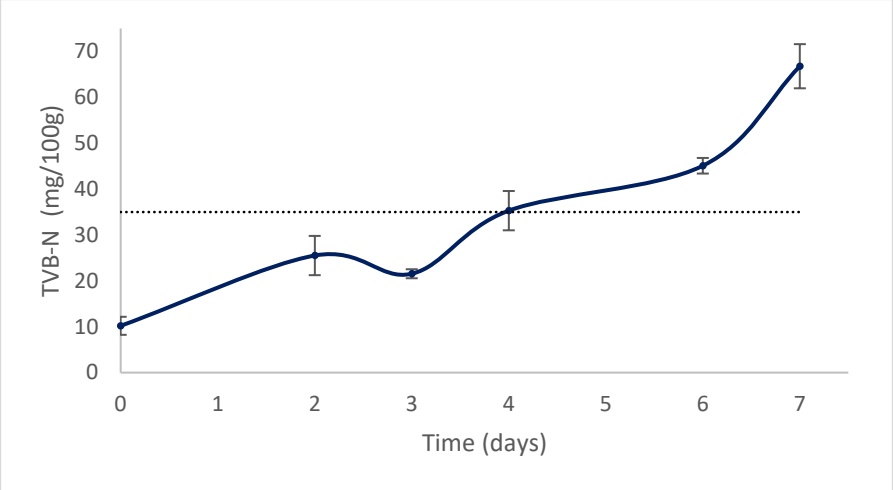


Figure 21. The TVB-N levels of the hake fish samples during storage at 4 ºC. The limit TVB-N value for fish consumption is represented by the dashed horizontal line (the maximum value is 35 mg TVB-N/100 g). Values are average ± SD (n = 3).

According to the Commission Decision 95/149/EC (1995), values of TVB-N greater or equal to 35 mg/100 g in fish, classify it as inappropriate for human consumption (Europeia, 1995). Thus, hake samples exceeded the maximum TVB-N limit of acceptability after 4 days of storage at 4 °C (Figure 21). Furthermore, it was also noticed there was a time difference between lapsed time of reaching the maximum TVB-N limit of acceptability (at day 4) (Figure 21) and TVC threshold (between day 3 and 4) (Figure 20). This lag phenomenon has been previously observed by other researchers who stated that the TVB-N generation follows the increase in microbial population inherently (Huang *et al.*, 2019).

4.6.2.3. pH measurement of fish samples and BLE film color performance

One of the quality indicators for fish spoilage is the change in its pH value, being pH associated with fish freshness and quality. The initial pH value of fresh hake was 6.60 ± 0.04 and then it gradually increased to 8.02 ± 0.03, at day 7 (Table 11). Similarly, Kanatt (2020) reported that fish stored within a packaging containing intelligent *Amaranthus* leaf extract-based film had an initial pH of 5.9, which increased to 8.4 after 12 days of storage at 2 - 4 ºC. The fish pH value is usually

above 6 after being caught. In addition, the high protein and non-protein nitrogen content and the low amount of carbohydrates in the muscle tissue of fresh fish also contributed to a slightly high initial pH (Barros, 2013). During the fish storage, the increase in pH value was correlated to compounds (such as ammonia and amines) generated from protein degradation in hake muscle, resulting from endogenous and microbial proteolytic enzymes activity, and to the TVB-N reabsorbed by fish (Kanatt, 2020; Zhang *et al.*, 2020; Gao *et al.*, 2022).

Hake samples presented a higher pH value on day 4 compared to previous days ($p < 0.05$), achieving the deterioration stage (Table 11). The obtained result showed a good correlation with the microbiological analyses and TVB-N quantification results, since the thresholds of recommended TVC and TVB-N limits for fish freshness were also reached at day 4 of storage (Figures 20 and 21). Therefore, the increase in pH values reflected the increase on microbial population and TVB-N concentration in fish muscle.

Regarding BLE film colorimetric performance, results showed remarkable color changes as the fish samples were degraded during the storage period (Table 11).

The color of BLE film altered during hake storage at 4 ºC. In the initial stage (first 3 days of storage) BLE film color ranged from dark purple color to light purple/pink color. After 4 days of storage, the film color turned to blue/purple color. As the storage time increased to 6 days, the blue color was more intense (Table 11). At 7-day storage, no remarkable color change was observed, and film displayed blue/green color. The film color change from pink/purple to blue/green may be due to pH sensitive characteristic of BLE films where an increase of TVB-N released from fish samples conducted to an increase of pH values, resulting in film color changes.

No significant differences of film brightness (L*) values were observed ($p > 0.05$) during storage (Table 11). Since headspace between the film and the fish sample was slightly wide, it will be necessary to reach a certain number of volatile compounds for anthocyanins to start detecting and interacting with them, and subsequently, BLE film changed it color.

Table 11. Changes in pH values of hake and color parameters (L*, a* and b*) values of BLE film during storage time at 4 °C.

Storage time (days)	pH	L*	a*	b*	Color changes	Freshness/ Spoilage
0	6.60 ± 0.04[a]	62.86 ± 3.00[a]	27.91 ± 1.98[a]	0.58 ± 0.97[a]		Fresh
2	6.87 ± 0.03[b]	50.89 ± 4.04[a]	20.56 ± 1.37[a]	3.09 ± 0.59[a]		Medium fresh
3	6.68 ± 0.06[a]	46.47 ± 1.41[a]	21.59 ± 4.31[a,b]	2.88 ± 0.55[a]		Medium fresh
4	7.04 ± 0.03[c]	55.41 ± 10.75[a]	19.25 ± 6.03[b]	2.69 ± 0.23[a]		Spoiled
6	7.92 ± 0.04[d]	57.40 ± 9.34[a]	1.04 ± 0.44[c]	2.86 ± 2.71[a]		Spoiled
7	8.02 ± 0.03[d]	57.14 ± 5.59[a]	1.11 ± 0.16[c]	3.12 ± 2.93[a]		Spoiled

Values are given as mean ± SD (n = 3). [a-c] Different letters in same column indicate significant differences (Tukey test, $p < 0.05$).

Regarding a* parameter, negative and positive values indicated green and red color tendency, respectively. This could be quite important since developed BLE film had an intense red color due to the color of the added blueberry extract. Based on Table 11 results, it was possible to observe that the a* value decrease significantly ($p < 0.05$) during 7 days, indicating considerable loss of the film red color. As explained above, the reduction of hake freshness due to the microbial growth in muscle tissue (i.e., TVC increased), led to the release of increasing amounts of TVB-N (such as ammonia, trimethylamine and dimethylacetamide) (Yoshida *et al.*, 2014). Possibly, these basic pH compounds were detected by anthocyanins present in blueberry extracts, leading to changes on anthocyanins' chemical structure (i.e., flavylium cation converted into quinoidal bases) and, consequently, decreasing a* values of the films. Regarding b* parameter ranging from blue (negative b* values) to yellow (positive b* values) of the BLE film, there was no significant differences ($p > 0.05$) during the storage (Table 11). Similarly, Qin *et al.* (2019) also described color variation of the films based on cassava starch and anthocyanins from *Lycium ruthenicum Murr*, from purple to green/yellow, in accordance with the change of pH and TVB-N values of pork during storage. Also, a starch/polyvinyl alcohol film incorporated with roselle anthocyanins that changed its color from pink/purple to green when in contact with spoiled fish has been reported (Zhai *et al.*, 2017).

These results suggested that intelligent pH indicator film incorporating blueberry extract would be promising to be applied in a food packaging to monitoring fish freshness in real-time ensuring product quality and safety during transportation and storage. The most noticeable color change for the human eye to recognize occurred on day 4, corresponding to the period when fish was considered unsuitable for consumption (Table 11).

5. CONCLUSIONS

Novel colorimetric LBG/κ-car-based films incorporating blueberry or/and beetroot extracts as freshness indicators were developed in the present work. Moreover, the effect of extracts incorporation on the LBG/κ-car-based film physicochemical properties as well as performance of colorimetric films developed were assessed.

The results showed that extracts were successfully incorporated into bio-based matrix because FT-IR spectra of the colorimetric films displayed newly generated interactions between biopolymers and natural dyes (e.g., C-O, C-O-C, C-H and O-H). Moreover, LBG/κ-car films properties were affected by extracts' addition. The LBG/κ-car films were transparent but after extracts' addition, they became pink/purple. This color change was mostly due to the natural color of the beetroot and blueberry extracts. Consequently, the pink/purple color of the BLE and BEE films promoted the increase of the opacity, which improved the films' barrier to UV and visible light. This feature is advantageous in packaging applied to the food industry since it allows an additional food protection from degradation by light incidence. Also, the mechanical properties (TS and EB) were enhanced in BLE and BEE films due to the reduction of intermolecular interactions between LBG and κ-car, improving the chain mobility and the overall film elasticity. Finally, solubility, moisture content and contact angle results showed that films with blueberry and beetroot extracts became more hydrophobic which could be advantageous since water is an accelerating agent in the food deterioration process. Based on the mentioned features, the film formulation LBG (60%), κ-car (40%), glycerol (30%) and blueberry extract (30%) was chosen in order to evaluate its ability to monitor the hake quality over storage. The fish application trial suggested that the visible color transition point of colorimetric BLE film was consistent with the loss of fish freshness over the storage time as verified by TVC, TVB-N and pH results. In this case, volatile compounds were produced and, pH changes occurred in the package headspace, which induced a color difference of the applied BLE film.

In summary, this work revealed the great potential of the developed LBG/κ-car-based films specially incorporating blueberry extracts as real-time colorimetric indicators to be applied on intelligent packaging systems suitable for visual monitoring of fish freshness.

6. FUTURE WORK

Despite the principal aims of this work have been achieved, some research gaps were identified, and further work can still be done. Following are some recommendations for future work that may be undertaken:

- Application of the developed films to other food products such as other fish (salmon, tuna and shrimp), meat (pork or chicken) or fruit (apple and pear) products in order to assess films' ability to act as indicators of freshness;
- Sensorial analysis studies with aim of determining the consumer's ability to detect the film's colorimetric change when applied to food samples;
- Extraction and quantification of anthocyanins and betalains present in blueberry and beetroot extracts used in this work and development of new LBG/κ-car films incorporating those natural pigments;
- Encapsulation of the anthocyanins and betalains extracted for their protection and consequently, incorporation of those encapsulated pigments into the films, to evaluate their performance response to pH variations;
- Development of new LBG/κ-car films incorporating enzymes (e.g., diamine oxidase and horseradish peroxidase) and a reduced dye (e.g., leuco crystal violet in order to monitor non-volatile amines (such as putrescine) produced by fish during its deterioration process.

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ANNEX

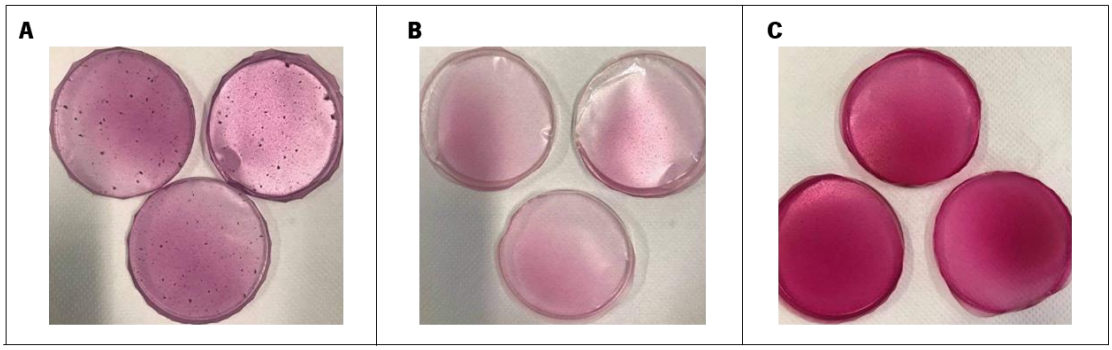

Figure A1. LBG/κ-car film with 10 % (w/w) of blueberry extract (A) showed some residues/peels in the film giving the film poor visual appearance. LBG/κ-car film with 10 % (w/w) of beetroot extract (B) was not homogeneous and presented a very light coloration. LBG/κ-car film with 20 % (w/w) of blueberry extract and 30 (% w/w) of beetroot extract (C) presented a quite strong pink coloration and it would be more difficult to distinguish colorimetric changes of the film.