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Active films based on cocoa extract with antioxidant, antimicrobial and biological applications

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ABSTRACT

Novel films of ethylene–vinyl alcohol copolymer (EVOH) containing flavonoid-rich cocoa were developed. To understand their potential application as active packaging material, antioxidant and antimicrobial properties of the films were determined as well as the antioxidant activity of the release compounds in Caco-2 human epithelial colorectal adenocarcinoma cells. Exposure of the films to aqueous food simulant showed antioxidant capacity. The release of cocoa extract components was dependent on the antioxidant concentration incorporated in the film and on temperature. Cocoa extract and the fraction obtained after *in vitro* gastrointestinal digestion presented antioxidant activity against oxidative stress induced by hydrogen peroxide in Caco-2 cells. Films with 10%, 15%, and 20% cocoa extract produced bactericidal effect against *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enterica*. The application of films to an infant milk formula, previously inoculated with *L. monocytogenes*, inhibited the growth of bacteria 1.5 log units the first day and showed sustained release, inhibiting 0.52 and 0.76 log units, respectively, by the sixth day, while cocoa powder added directly did not produce any effect.

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1. Introduction

The widespread incidence of foodborne diseases associated with microbial pathogens represents a threat to public health, and a challenge for the food industry. Pathogen microorganisms are generally eliminated by standard thermal treatments during processing, but these processes may impair the quality of the product and they do not prevent possible subsequent microbiological contamination. This can cause serious food poisoning, which in the case of children's food can be very dangerous. It is possible to generate additional protection against deterioration and contamination of food with bioactive molecules used as additives. Nowadays there is increasing interest in the replacement of chemical additives with natural products with a low environmental impact. This has encouraged the use of natural antimicrobial and antioxidant compounds such as cocoa extract. Cocoa (*Theobroma cocoa*) has various applications in the food industry, mainly as a flavouring for chocolate manufacturing. Cocoa flavanols have gained additional attention because of their antioxidant capacity and their possible beneficial implications for human health (Katz, Doughty, & Ali, 2011). Because of its high percentage of anthocyanins and flavonoids, cocoa has a protective role against cardiovascular disease, oxygen-derived free radicals, and nitrogen chelation of metals (iron, copper, etc.). *In vitro* studies have shown that cocoa has anti-inflamamatory, antiallergic, antiviral, and anticancer activity (Katz et al., 2011).

The cocoa extract employed in this work was a flavanol-rich cocoa powder developed by a patented industrial process that preserves the major fraction of the original flavanol content (Cienfuegos-Jovellanos, Ibarra, Pasamar, Montanes, & Pons-Andreu, 2008). This product had eight times more epicatechin and procyanidin B2 content than conventional cocoa powder (Tomas-Barberan et al., 2007). Table 1 shows the total polyphenol and flavan-3-ol content of the cocoa powder used in this work (taken from (Quinones et al., 2010).

Novel structures with controllable morphology made from natural materials hold promise for several applications in pharmaceuticals, foods and biotechnology field (Patel, Nijsse, & Velikov, 2011). In recent years antimicrobial release systems have been employed mainly in pharmaceutical applications, while their use in food packaging is very restricted because antimicrobial agents have to be approved for application in direct contact with food. One of the main reasons for packaging of many foods is to prevent surface growth where a large portion of spoilage and contamination occurs (Shakeri, Shahidi, Beiraghi-Toosi, & Bahrami, 2011). Antimicrobial active packaging systems have been proposed to extend the shelf life and improve food safety (Suppakul, Miltz, Sonneveld, & Bigger,

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Table 1
Polyphenol composition (mg/g) of the cocoa extract used in this study.

Polyphenol composition	mg/g
Total polyphenols[a]	162 ± 0.12
Total procyanidins content[b]	42.6 ± 0.25
(+) Catechin	5.18 ± 0.09
(−) Epicatechin	19.4 ± 0.03
Procyanidin B1	1.26 ± 0.07
Procyanidin B2	16.9 ± 0.06

The results are expressed on a dry basis as mean ± SD ($n = 2$).
[a] Spectrophotometric method Folin–Ciocalteu. Results expressed as catechin equivalent.
[b] DAD-HPLC Quinones et al. (2010).

2003). Antimicrobial films can be classified in 2 types: an antimicrobial agent that is effective against surfaces without migration, and an antimicrobial agent that migrates to the surface of the food (Suppakul et al., 2003). Nonmigratory active packaging offers the potential for improving food safety and quality while minimizing the migration of the active agent into food. The aim of release systems intended for food packaging applications is to transfer the bioactive agent from the polymeric matrix to the food in order to keep a predetermined concentration of the active compound in the packed food for a determined period of time (Buonocore et al., 2003).

Several works have studied the antimicrobial activity of cocoa powder (Busta & Speck, 1968; Percival, Devine, Duggal, Chartron, & Marsh, 2006), but few studies have focused on the properties of cocoa as an antimicrobial agent for food application (Pina-Perez, Silva-Angulo, Muguerza-Marquinez, Aliaga, & Lopez, 2009) and it has never been incorporated into films for active packaging to date. Furthermore, there are no studies that combine film production with biological applications such as antimicrobial and antioxidant activity.

In this work, new active films based on ethylene–vinyl alcohol copolymer, EVOH, and flavanol-rich cocoa extract have been developed. EVOH copolymer is a common packaging material mainly characterised by its low permeability to gases and flavours and excellent physical and optical properties. Despite being a synthetic material that comes from oil, this copolymer has the advantage of being biodegradable and biocompatible. It is composed of two different monomers linked to the same polymer chain, combining the hydrophobic nature of ethylene and the hydrophilic behaviour of polyvinyl alcohol. Since the hydroxyl groups (OH) are responsible for the gas barrier property, the greater the number of (OH), the lower the permeability (Catala & Gavara, 2001).

In this study, EVOH 29, containing 29% mol of ethylene, was selected because of its better processability as a coating. The release kinetics of antioxidants from the film into an aqueous media was monitored. The antioxidant effect and cytotoxicity of the compounds released from these films, as well as the gastrointestinal extract obtained after *in vitro* digestion of cocoa extract, were studied against human intestinal Caco-2 cells; a cell culture widely used as an *in vitro* model for the small intestine. Finally, antimicrobial activity of the films was studied against Gram-positive foodborne pathogens (*Staphylococcus aureus*, *Listeria monocytogenes*) and Gram-negative pathogens (*Escherichia coli*, *Salmonella enterica*). In addition, the films were applied to an infant formula in order to reduce the great risk of microbial contamination, reducing its pathogenic flora and also extending its shelf life.

2. Materials and methods

2.1. Chemicals and reagents

An ethylene–vinyl alcohol copolymer with a 29% ethylene molar content (EVOH) was kindly provided by The Nippon Synthetic Chemical Company (Osaka, Japan). Reagent-grade absolute ethanol, 1-propanol, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) were purchased from Sigma (Madrid, Spain).

Flavanol-rich cocoa extract (Table 1) was supplied by Natraceutical Group (Aldaya, Spain). It was developed by a patented industrial process (Cienfuegos-Jovellanos et al., 2008).

Water was obtained from a Milli-Q Plus purification system (Millipore, Molsheim, France).

2.2. Film preparation

EVOH films containing different concentrations of cocoa extract were obtained by a solution–extension–evaporation process (casting). The polymer was dissolved in a 1:1 (v/v) 1-propanol/water mixture at 75 °C and cocoa extract was incorporated at different concentrations: 7.5%, 10%, 15%, and 20% (w/w with respect to polymer weight). Casting was done with a wire wound bar coater on a glass plate. Film drying was accomplished by using a heating tunnel (80 °C) equipped with a 2500 W heat source with ventilation for 3 min, and then transferring to a chamber at 40 °C and 14% relative humidity for 24 h. The thickness of every sample was individually measured before tests using a digital Mitutoyo micrometer (Metrotec, San Sebastian, Spain) with an average value of 25 ± 2 μm.

2.3. Antioxidant release studies

Release studies of the active compounds from the films were carried out by determining the specific migration from the polymer into Milli-Q water at 4 and 40 °C. Double-sided total immersion migration tests were performed as follows: a 3 cm^2 piece of each plastic sample and 5 mL of the simulant (area-to-volume ratio 6 dm^2/L) were placed in Sovirel tubes protected from light. Milli-Q water was deoxygenated by bubbling nitrogen, and a final nitrogen flush was done before closing the cells to reduce the oxygen percentage in the cell headspace. Antioxidant solutions in water using this procedure were stable for 1 month. Periodically, three vials were opened and the concentration of the antioxidant in the aqueous simulant was analysed by indirect measurement with the $ABTS^{\cdot+}$ scavenging activity assay (Sanchez-Moreno, 2002).

The bleaching rate of a stable free cationic radical, $ABTS^{\cdot+}$, was monitored at a characteristic wavelength in the presence of the sample. In its radical form $ABTS^{\cdot+}$ absorbs at 734 nm, but on reduction by an antioxidant or a radical compound its absorption decreases. The percentage inhibition values were calculated using Eq. (1):

$$I\ (\%) = [(\text{Abs control} - \text{Abs sample})/\text{Abs control}] \times 100 \quad (1)$$

Using a calibrated curve of ascorbic acid concentration vs. I (%), the results can easily be expressed as the equivalent ascorbic acid concentration. The antioxidant activity of the cocoa extract was also determined by this method. One gram of ascorbic acid was equivalent to approximately 0.55 ± 0.04 g of aqueous cocoa extract.

2.4. In vitro gastrointestinal digestion of cocoa powder

Samples of cocoa extract (200 μg/mL) were prepared in different matrices: phosphate buffer saline pH = 7.2 (PBS, PAA Laboratories GmbH, Berlin Germany), model drink solution (MD) (Busch Jensen, Lopez-de-Dicastillo Bergamo, Payet, Xueming, & Konczak, 2011), and commercial infant milk. Samples were processed in triplicate and were digested using a simulated digestion (Laparra, Velez, Montoro, Barbera, & Farre, 2003). Enzymes employed for gastrointestinal digestion were purchased from Sigma, (Madrid, Spain).