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EXTRACTION AND CHARACTERIZATION OF POLYSACCHARIDES AND PHENOLIC COMPOUNDS FROM SPENT COFFEE GROUNDS AND THEIR INCORPORATION INTO EDIBLE FILMS/COATINGS FOR FOOD APPLICATIONS

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Tese de Doutoramento em Engenharia Química e Biológica

Trabalho realizado sob a orientação do
Professor Doutor José António Couto Teixeira
e da
Professora Doutora Solange Inês Mussatto Dragone

junho de 2016

DECLARAÇÃO

Lina Fernanda Ballesteros Giraldo

Endereço eletrónico: linafernanda37@gmail.com

Título

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Orientadores:

Professor Doutor José António Couto Teixeira

Professora Doutora Solange Inês Mussatto Dragone

Ano de conclusão: 2016

Designação do Doutoramento:

Programa doutoral em Engenharia Química e Biológica

Universidade do Minho, 28 / 06 / 16 /

Assinatura: Lina Fernanda Ballesteros G.

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

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

University of Minho, 28/06/2016

Full Name: Lina Fernanda Ballesteros Giraldo

Signature: Lina Fernanda Ballesteros G.

"No te rindas
que la vida es eso,
continuar el viaje,
perseguir tus sueños,
destrabar el tiempo,
correr los escombros
y destapar el cielo"

Mario Benedetti

ACKNOWLEDGEMENTS

A PhD is a step of a lot of professional learning, but undoubtedly it is also a great challenge and personal growth. I would like to express my more truthful and special appreciation to every person that made possible the development of this work.

Firstly, I would like to thank my supervisors, Professor Dr. José António Teixeira and Professor Dr. Solange Inês Mussatto for giving me the opportunity to work and learn from them. Thanks to their enormous contribution to my PhD work, all hours sharing and discussing the results. Thanks also for the support in the difficult moments. I really appreciate the freedom, confidence and commitment that they give me all these years to develop my work.

Special thanks to Dr. Miguel Cerqueira for his invaluable and enormous contribution to my PhD work, for sharing his knowledge and enriching my learning process. Thanks also for his commitment with my work, for all hours spent discussing the results and to be always available.

I would like to thank my colleagues and friends from Fermentation laboratory, as well as from other laboratories for making this journey an enjoyable period, and for the brilliant comments, help and suggestions in the process. Thank you also for all motivation and encouraging words.

Special thanks to Dr. Carlos Eduardo Orrego for his comments and suggestions.

I would like also to thank all technicians from Centre of Biological Engineering of the University of Minho that support this research, especially to Engineer Madalena Vieira, Aline Barros, Vitoria Maciel and Mr. Santos. I really appreciate all the availability and help during my PhD.

A special thanks to my husband Sebastián, my great love! Thanks enano for your support, your love, your smile, your complicity and for always pushing me to be a better person. Thanks also for helping me and listening me in the more difficult moments of my PhD, for all the endless nights trying to find a solution when I feel unmotivated. It would not have been possible without you.

ACKNOWLEDGEMENTS

To my family all my love and gratitude. Despite of the distant, I always feel very close and they are in my heart. Thanks to my mom, my daddy, my sister and all others for the sacrifices, love and countless moments of happiness, for contributing in my education and on the way I face life.

I would also like to thank my lifelong friends who support and encourage me to pursuit my goal. Thanks for every moment of joy and complicity.

This thesis was financially supported by a PhD scholarship from Fundação para a Ciência e Tecnologia (Ref.: SFRH/BD/80948/2011), inserted in the Programa Potencial Humano Quadro de Referência Estratégico Nacional (POPH - QREN) Tipologia 4.1 – Formação Avançada. The POPH-QREN is co-financed by Fundo Social Europeu (FSE) and by Ministério da Ciência, Tecnologia e Ensino Superior (MCTES).

ABSTRACT

Coffee has been one of the most popular beverages around the world since ancient times. It is made from a mixture of hot water and coffee powder being consumed for its refreshing and stimulating properties. However, along its production process many wastes are generated. Spent coffee ground is the major waste produced during the soluble coffee preparation. This residue is rich in polysaccharides and polyphenols, making its use interesting as raw material for the production of edible films and coatings for foods. Nowadays, food packaging industries explore edible coatings to replace the synthetic liners, in order to protect the environment and offer consumers a product of high quality, while reducing synthetic chemical preservatives. Therefore, this project aims at the extraction of polysaccharides and phenolic compounds from spent coffee grounds and their incorporation into edible films and coatings for further application on goldenberry fruit *(Physalis peruviana)* in order to increase its shelf-life.

The thesis work is based on a sequence of tasks, starting by the characterization of two coffee residues (i.e. spent coffee grounds (SCG) and coffee silverskin (CS)) in order to have a detailed knowledge of their chemical composition and functional properties and then, choosing the residue with the highest polysaccharide content and antioxidant activity. After having selected SGC as the residue of interest, two different techniques were tested to extract polysaccharides from SCG, including an alkali pretreatment and autohydrolysis, being the later used to extract the phenolic compounds. The polysaccharides and phenolic compounds were characterized in terms of their physicochemical and functional properties, including antioxidant and antimicrobial activities. The extracted phenolic compounds were encapsulated in maltodextrin and gum arabic matrices using freeze-drying and spray-drying processes and then, the encapsulation efficiency was evaluated. On the other hand, different concentrations of the polysaccharides extracted were incorporated in carboxymethyl cellulose (CMC)-based films and their effect on the films properties were evaluated. Finally, the influence of three coatings on physicochemical and microbiological properties and gas exchange rate of goldenberry *(Physalis peruviana)* was determined at different temperatures and relative humidities (RH) of storage. The tested coatings were: coating A (CMC-based coating), coating B (CMC-based coating with a selected polysaccharide concentration in the previous step)

and coating C (CMC-based coating with the selected polysaccharide concentration and phenolic compounds encapsulated).

Results showed that SCG residues are sugar-rich lignocellulosic materials, composed by high levels of insoluble and soluble dietary fibers with interesting functional properties. The methods and conditions used to extract polysaccharides and phenolic compounds from SCG showed to be efficient (39% and 29% (w/w) of recovered polysaccharides by alkali pretreatment and autohydrolysis, respectively, and 41.36 mg/g SCG of phenolic compounds). The most relevant sugar recovered from both methods was galactose, followed by arabinose, mannose and glucose, while chlorogenic acid and flavonoids content were among the recovered phenolic compounds with high antioxidant activity. The results also indicated that the extracted polysaccharides presented good thermal stability. Additionally, these polysaccharides showed high antioxidant activity and antimicrobial against *P. violacea* and *C. cladosporioides*, making them attractive bioactive compounds. Although freeze-drying and spray-drying showed to be appropriated techniques for encapsulation of phenolic compounds, in this case, the use of maltodextrin as wall material and freeze-drying as encapsulation method showed the best encapsulation efficiency. In general, the addition of polysaccharide from SCG in CMC-based films improved and/or maintained the physicochemical properties of the edible films when comparing with CMC-based films without polysaccharides from SCG, reducing the water solubility of the films and acting as a light barrier. The results showed lower gas transfer rates (O_2, CO_2 and ethylene) for the coated fruits in comparison with the uncoated fruits when using a storage temperature of 20 °C and a RH of 65%. Physicochemical properties of goldenberries with or without coatings present significant changes regarding weight loss and the microbiological contamination, being both reduced, in particular when the coating B was applied.

In conclusion, polysaccharides and polyphenols extracted from SCG can be used as raw materials in the production of edible films/coatings for application on goldenberries turning these coatings into a promising way to replace synthetic packaging materials.

RESUMO

Desde a antiguidade que o café tem sido uma das bebidas mais populares em todo o mundo. Esta bebida é obtida a partir da mistura de água quente com café em pó e é consumida essencialmente devido às suas propriedades estimulantes. Contudo, durante o processo de preparação do café solúvel são gerados resíduos (as borras de café) que possuem uma composição química rica em polissacarídeos e polifenois que podem ter potencial como matéria-prima na produção de filmes e revestimentos comestíveis. Este projeto teve como principal objetivo a extração de polissacarídeos e compostos fenólicos da borra de café e a sua posterior incorporação em filmes e revestimentos comestíveis para aplicação em fruta fisális (*Physalis peruviana*),. com o objectivo de aumentar o tempo de prateleira.

O trabalho desenvolvido baseou-se numa série de tarefas que começaram com a caracterização de dois resíduos de café (borra (SCG) e película (CS)) nomeadamente a sua composição química e características funcionais. Estes resultados permitiram selecionar a borra de café como o resíduo com maior conteúdo de polissacarídeos e atividade antioxidante. A extração dos polissacarídeos presentes na borra foi realizada através de duas técnicas: tratamento alcalino e auto-hidrólise, sendo esta última também usada para extrair os compostos fenólicos. Ambos os compostos foram caracterizados em termos de propriedades físico-quimicas e funcionais, tais como a atividade antioxidante e antimicrobiana. Os compostos fenólicos extraídos da borra de café foram encapsulados em matrizes de maltodextrina e goma-arábica usando liofilização e secagem por pulverização, sendo posteriormente avaliada a eficiência de encapsulação. Por outro lado, foram usadas diferentes concentrações dos polissacarídeos extraidos na produção de filmes de carboximetilcelulose (CMC), e posteriormente avaliado o seu efeito nas propriedades dos filmes e revestimentos. Finalmente, foi estudado o impacto da aplicação de três revestimentos nas propriedades físico-químicas e microbiológicas da fruta fisális (*Physalis peruviana*), bem como o seu efeito na taxa de trocas gasosas da fruta quando armazenada a diferentes temperaturas e humidades relativas (RH). Os revestimentos estudados foram: revestimento A (revestimento de CMC), revestimento B (revestimento de CMC com a concentração de polissacarídeos selecionada

na etapa anterior) e revestimento C (revestimento de CMC contendo a concentração de polissacarídeos selecionada e os compostos fenólicos encapsulados).

O trabalho desenvolvido mostrou que as borras de café são materiais lignocelulósicos ricos em açúcares e apresentam propriedades funcionais interessantes. As metodologias e condições experimentais usadas na extração dos polissacarídeos e compostos fenólicos a partir das borras de café revelaram-se eficazes (39% e 29% de polissacáridos recuperados por pelo tratamento alcalino e auto-hidrólise, respectivamente, e 41,36 mg/g SCG de compostos fenólicos). O principal açúcar obtido pelos dois métodos foi galactose, seguido por arabinose, manose e glicose. Por outro lado, a elevada quantidade de compostos fenólicos obtidos mostrou ser constituída, em parte, por ácido clorogénico e flavonóides. Os resultados obtidos também demonstraram que os polissacarídeos extraídos apresentam boa estabilidade térmica. Para além disso, estes polissacarídeos possuem elevada atividade antioxidante e antimicrobiana contra *P. violacea* and *C. cladosporioides*, mostrando ser um composto bioactivo com elevado potencial. A liofilização e a secagem por pulverização mostraram ser técnicas adequadas à encapsulação de compostos fenólicos, no entanto a liofilização e o uso de maltodextrina como material encapsulante, revelaram a melhor eficiência de encapsulação. De um modo geral, a adição de polissacarídeos da borra de café a filmes de CMC melhorou e/ou manteve as propriedades físico-químicas destes filmes, quando comparados com o filme de CMC sem estes compostos, sendo a solubilidade em água, a cor e opacidade as propriedades onde mostraram mais influência. Os resultados mostraram também menores taxas de trocas gasosas (O_2, CO_2 e etileno) para frutas revestidas em comparação com frutas não revestidas quando armazenadas a 20 °C e 65% RH. As frutas revestidas mostram melhorias comparativamente com as não revestidas, apresentando alterações significativas sobre a perda de peso e a contaminação microbiológica, tendo sido ambas reduzidas com a aplicação do revestimento, em particular quando se utilizou o revestimento B.

Em suma, os polissacarídeos e os polifenóis extraídos das borras de café podem ser usados como matéria-prima na produção de filmes/revestimentos comestíveis para aplicação em fruta fisális, podendo estes revestimentos ser apresentados como substitutos para materiais de embalagem sintéticos.

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LIST OF GENERAL NOMENCLATURE

ABBREVIATION	DESCRIPTION
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt
AOAC	Association of Official Analytical Chemists
BHA	Tert-butyl-4-methoxyphenol
BHT	2,6-Di-tert-butyl-4-methylphenol
CFU	Colony forming unit
CMC	Carboxymethyl cellulose
COATING A	CMC-based edible coating
COATING B	CMC-based edible coating with incorporation of PA (0.20 %, w/v)
COATING C	(CMC-based coating containing PA and PE (PA 0.20% + PE 0.20%, w/v)).
CS	Coffee silverskin
DCPIP	2,6-dichlorophenolindophenol
DNS	3,5-dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRBC	Dichloran Rose Bengal Chloramphenicol agar
DSC	Differential scanning calorimetry
EA	Emulsifying activity
EB	Elongation at break
ES	Emulsion stability
Fe(II)	Ferrous equivalent
FLA	Content of flavonoid
FRAP	Ferric reducing antioxidant power
FTIR	Fourier transform infrared spectroscopy
GA	Gum arabic
GAE	Gallic acid equivalents

GLU	Glucose equivalent
HPLC	High performance liquid chromatography
IC_{50}	Inhibition concentration at 50%
ICDD	International Centre for Diffraction Data
ICP-AES	Inductively coupled plasma atomic emission spectrometry
IDF	Insoluble dietary fiber
LM	Lyophilized material
M	Maltodextrin
MIC	Minimal inhibitory concentration
OHC	Oil holding capacity
PA	Polysaccharides obtained by alkali pretreatment
PB	Polysaccharides obtained by autohydrolysis
PC	Phenolic compounds
PCA	Plate Count Agar
PDA	Potato dextrose agar
PE	Phenolic compounds encapsulated in maltodextrin by freeze-drying
QE	Quercetin
RH	Relative humidity
RS	Reducing sugars
SCG	Spent coffee grounds
SDF	Soluble dietary fiber
SEM	Scanning electron microscopy
TAA	Total antioxidant activity
TDF	Total dietary fiber
TE	Trolox equivalents
TGA	Thermogravimetric analyses
TOC	α-tocopherol equivalent
TS	Tensile strength
WHC	Water holding capacity

WVP	Water vapor permeability
WVTR	Water vapor transmission rate
XRD	X-ray diffraction

SYMBOL	**DESCRIPTION**
A_c	Absorbance of the control
A_s	Absorbance of the sample
a_w	Water activity
B	Content of soluble solids
C	Constant of Guggenheim
ρ_{GB}	Density of goldenberry
G	Mean relative deviation modulus
k	Constant of correction
L^*, a^*, b^*	Color parameters
M	Equilibrium moisture content
M_a	Moisture content
M_{DP}	Moisture of the sample after drying process
M_m	Monolayer moisture content
M_p	Predicted moisture content
n	Number of observations
θ	Contact angle
S_{BET}	Specific surface area
R_{CO_2}	CO_2 production rate
R_{ethy}	Ethylene production rate
R_{O_2}	O_2 consumption rate
γ_c	Critical surface tension
γ_{LV}	Liquid-vapor interfacial tension

γ_{SL}	Solid-liquid interfacial tension
γ_{SV}	Solid-vapor interfacial tension
γ_L^p	Polar component of the liquid
γ_L^d	Dispersive component of the liquid
γ_S^p	Polar component of the surface
γ_S^d	Dispersive component of the surface
V_C	Total volume of the container
V_f	Free volume of the container
W_a	Work of adhesion
W_c	Work of cohesion
w_{GB}	Weight of the fruit
W_P	Mass of powder to hydrate
W_s	Spreading coefficient
W_1	Average weight of the sample
W_2	Average final weight of the sample
W_3	Protein weight
W_4	Ash weight
W_5	Blank weight
Y_b	Black standard
Y_w	White standard
Y1	Total yield of the extraction process
Y2	Yield in terms of quantity of sugars extracted during processing
Y3	Yield in terms of quantity of sugars extracted with respect to total sugars existent in SCG

STRUCTURE OF THE THESIS

This thesis is divided in six sections in order to provide a logical sequence of developed work including the characterization of coffee residues (spent coffee grounds and coffee silverskin), the extraction processes of polysaccharides and phenolic compounds, the evaluation of the chemical and functional properties of these compounds and the development of a system for their application in foods. Each section is subdivided in chapters, to a total of ten chapters. Seven of them (from Chapter 3 to Chapter 9) describe the experimental results completed during this study and their respective discussion. They given origin to papers published and submitted in peer-reviewed international journals. In the beginning of each chapter, the reference to the correspondent paper is done.

Section I is formed by Chapter 1 and Chapter 2. The first one corresponds to the motivation and the objectives of this thesis. Chapter 2 presents an overview on the coffee and coffee residues, mainly spent coffee grounds and coffee silverskin, their exploitation and possible uses. Moreover, it describes the polysaccharides and phenolic compounds present in coffee beans, the extraction and encapsulation processes, the relevance of edible coatings/films, their components and applications.

Section II (Chapter 3) consists in the evaluation of the chemical composition, functional properties and structural characteristics of spent coffee grounds and coffee silverskin, in order to obtain more detailed information about these materials and identify potential industrial areas for their reutilization. After this characterization, spent coffee grounds was selected as the most suitable material for this study due to the high hemicellulose content and antioxidant activity.

Section III (Chapter 4 and Chapter 5) is dedicated to the polysaccharides present in spent coffee grounds. Thus, Chapter 4 reports the extraction of polysaccharides from spent coffee grounds by using autohydrolysis technique. Assays were performed using different temperatures, liquid/solid ratios and extraction times and the effects of these operational variables on the extraction yield and antioxidant activity of the recovered polysaccharides were determined. The

polysaccharides obtained under the best autohydrolysis conditions were chemically and structurally characterized. Chapter 5 includes the extraction of polysaccharides from spent coffee grounds by using an alkali pretreatment, followed by the evaluation of their chemical and structural characteristics, as well as the determination of the antioxidant and antimicrobial properties of these polysaccharides.

Section IV (Chapter 6 and Chapter 7) is designated for phenolic compounds. Chapter 6 shows the optimization of process conditions to extract antioxidant phenolic compounds from spent coffee grounds by autohydrolysis. Extractions were performed using different temperatures, liquid/solid ratios and reaction times and the effects of these operational variables on the extraction results were evaluated. Finally, the conditions able to produce a phenolic rich extract with high antioxidant activity were selected. Chapter 7 analyzes freeze-drying and spray-drying as methods to encapsulate phenolic compounds extracted from spent coffee grounds and evaluates the use of maltodextrin and gum arabic as wall materials to encapsulate these bioactive compounds and maintain their antioxidant activity after encapsulation.

Section V (Chapter 8 and Chapter 9) is related to the production of edible films/coatings for food applications. Chapter 8 reports the development of CMC-based films with incorporation of polysaccharide rich extracts obtained by two different methodologies (alkali pretreatment and autohydrolysis, Section III) and evaluates their effect on the physicochemical properties of the films when using different concentration of spent coffee grounds extracts. Chapter 9 evaluates the application of three different coatings (CMC-based coating, CMC-based coating with the selected polysaccharide extract, and CMC-based coating containing the selected polysaccharide extract and the phenolic compounds encapsulated in maltodextrin) on physicochemical and microbiological properties and the gas exchange rate of goldenberry ((*Physalis peruviana*) when subjected at different temperatures and relative humidities.

Finally, Section VI (Chapter 10) presents the main conclusions of the thesis and the future perspectives of this work.

SECTION I

INTRODUCTION

CHAPTER 1

MOTIVATION AND OBJECTIVES

1. Motivation and objectives

1.1. Motivation

During the last years, the use of agro-industrial by-products has gained great interest in the food packaging industry and related areas due to their chemical and functional properties. In this context, this thesis proposes to add value to one of the most abundant residues of coffee industry such as the spent coffee grounds (SCG). This waste is obtained during the processing of coffee powder with hot water to prepare instant coffee. Currently, the market of this kind of coffee around the world is increasing, which in turn generates around 6,000,000 tons/year of SCG (Mussatto, Machado, Martins, & Teixeira, 2011; Tokimoto, Kawasaki, Nakamura, Akutagawa, & Tanada, 2005). This residue has been used to produce fuel for industrial boilers due to its high calorific power of approx. 5000 kcal/kg (Silva, Nebra, Silva, & Sanchez, 1998), as substrate for cultivation of microorganisms (Machado, Rodriguez-Jasso, Teixeira, & Mussatto, 2012), and as raw material to produce fuel ethanol (Mussatto, Machado, Carneiro, & Teixeira, 2012) or a distilled beverage with aroma of coffee (Sampaio et al., 2013), among others. However, SCG is not fully used, making it a dominant source of pollution. A good alternative to utilize SCG might be recovering the polysaccharides and phenolic compounds present in its composition and use them as raw materials for production of edible films and coatings, which could be attractive for food industry, stimulating economy and competitive agro-industrial production.

At this time, food packaging industry is looking for natural films and coatings which can replace synthetic packaging in order to protect the environment and offer to consumers high quality products, reducing synthetic chemical preservatives (Ghanbarzadeh, Almasi, & Entezami, 2010). These packages can be applied in a large number of foods, especially fruits, vegetables, meat, fish, seafood, cereals and nuts among others, which are exported to others countries, requiring a safe protection for an efficient distribution.

Films and coatings are ideal as they can increase the shelf-life of food, reduce microbial contamination and maintain the organoleptic properties (aroma, flavor, color) for a longer time

(Cerqueira, Lima, Teixeira, Moreira, & Vicente, 2009; Durango Villadiego, Soares, & Andrade, 2009; Rojas-Graü, Tapia, & Martín-Belloso, 2008; Vásconez, Flores, Campos, Alvarado, & Gerschenson, 2009). These coatings are good alternatives to preserve the properties of foods that are exported to different countries, as well as those that are consumed within the same country, reducing transport costs and increasing storage time.

Therefore, this work tries to raise some of these possibilities through the extraction and characterization of polysaccharides and phenolic compounds from spent coffee grounds and their incorporation into carboxymethyl cellulose (CMC)-based films that besides improving physical properties of the films could create new functionalities, opening the possible application of this system in food industry. The possible application of the developed coatings in foods will be tested on goldenberries (*Physalis peruviana*). Goldenberry is a fruit that has a short shelf-life without calyx (protective cover enclosing each berry) and that for an optimum preservation requires a continuous cold chain (Puente et al., 2011).

In conclusion, the work developed during this thesis could be of great scientific and technological interest and provide significant advances for the agro-industrial sector and food packaging industries, promoting the use of a biodegradable and/or edible packaging.

1.2. Objectives

The main objective of this thesis was to extract and characterize polysaccharides and phenolic compounds from spent coffee grounds and incorporate them into edible films or coatings for food applications. This incorporation allowed the production and characterization of edible coatings/films that were used to improve the shelf-life of goldenberry (*Physalis peruviana*). To achieve the main objective, this thesis was focused on:

- **Characterization** of chemical, functional and structural properties of coffee residues, including spent coffee grounds and coffee silverskin.

- **Extraction** of polysaccharides from spent coffee grounds by using autohydrolysis technique and an alkali pretreatment.
- **Evaluation** of the chemical, structural, antioxidant and antimicrobial properties of extracted polysaccharides.
- **Extraction** of total phenolic compounds from spent coffee grounds by using autohydrolysis technique and determination of their antioxidant properties.
- **Evaluation** of freeze-drying and spray-drying as methods to encapsulate the phenolic compounds extracted using different coating materials.
- **Incorporation** of the extracted polysaccharides in bio-based edible films and evaluation of their physicochemical properties.
- **Study** of the effect of different coatings on shelf-life parameters of goldenberry *(Physalis peruviana)* during storage.

A schematic summary of the thesis is presented in Figure 1.1.

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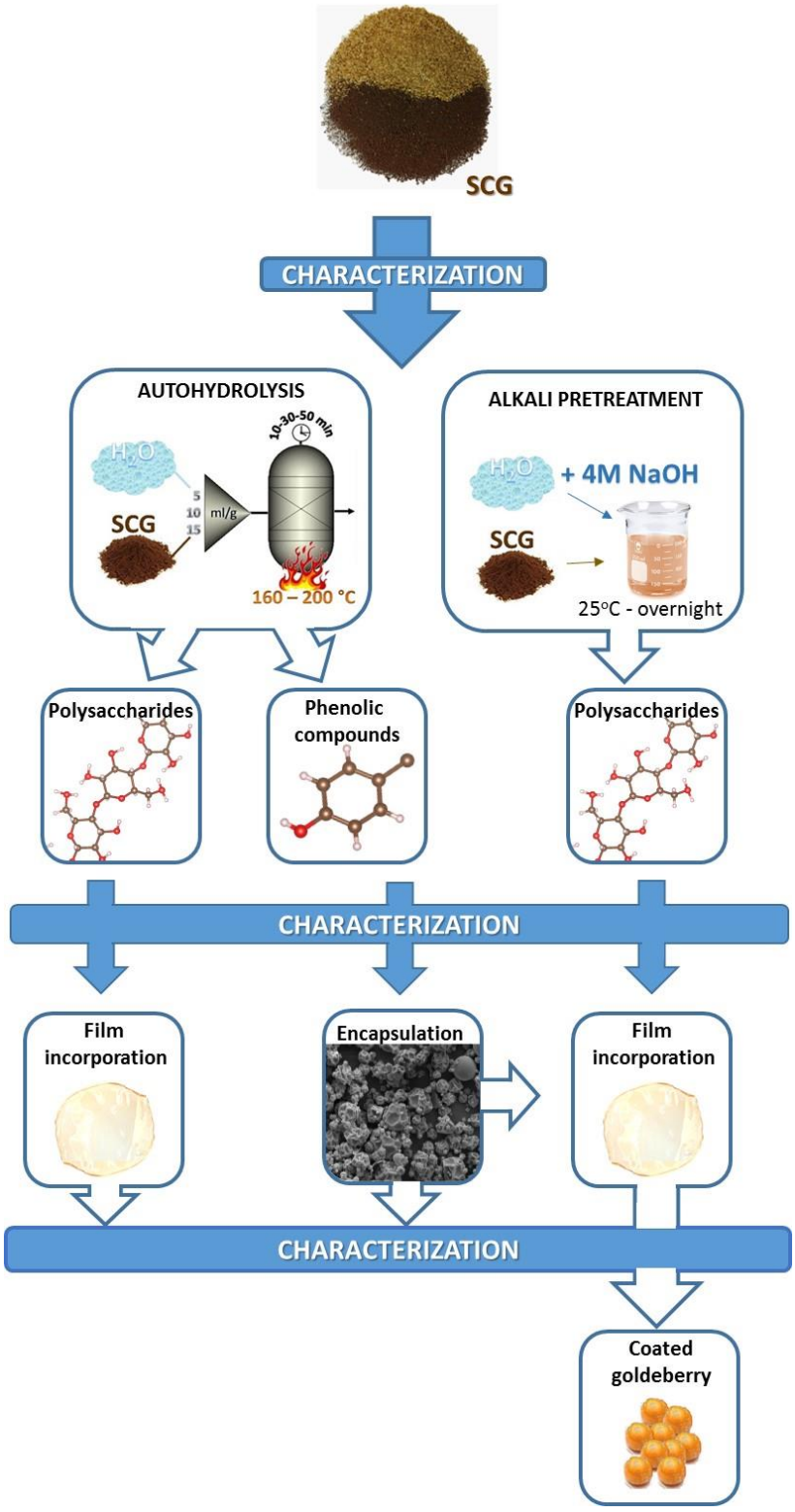

Figure 1.1 Flow-chart of the schematic summary of the thesis

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CHAPTER 2

LITERATURE REVIEW

2. Literature review

2.1. Coffee production

Coffee is one of the most popular and appreciated beverages around the world, being consumed for its stimulating and refreshing properties, which are defined by the green beans composition and changes occurring during the roasting process (Esquivel & Jiménez, 2012; Mussatto, Machado, Martins, & Teixeira, 2011a).

The coffee processing begins with the harvest of red coffee fruit, also known as cherry, produced by the plant of the botanical genus *Coffea*. The two species most commonly grown are *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta). The first of them, is considered the variety with better sensory quality among all coffee plants and corresponds to approximately 75% of the worldwide coffee production (Mussatto & Teixeira, 2013). On the other hand, Robusta is a variety more acid, stronger and hardy, and represents the remaining 25%.

Wet process or dry process are the implemented methods by the coffee industry for the treatment of coffee cherries in order to obtain a green coffee. Dry method is technologically simpler comparing with the wet method and usually is used for Robusta variety (Mussatto, Machado, et al., 2011a). Wet method, generally used for Arabica coffee beans involves several stages including a microbial fermentation, which provides a better aroma quality (Gonzalez-Rios et al., 2007). In spite of the differences between the dry or wet method processing methods, both technologies generate by-products such as coffee pulp, hush, and parchment. Other important residues including coffee silverskin and spent coffee grounds are produce during coffee roasting step and soluble coffee preparation, respectively.

Currently, 56 countries around the world are producers of coffee, and for some of them, coffee is the main agricultural export product. The 10 largest coffee producing countries and their respective production in the last five years (2010 - 2015) are shown in Table 2.1. These countries are responsible for nearly 90% of the total worldwide production. The first, second and third largest coffee producers are Brazil, Vietnam and Colombia, respectively, controlling almost 60% of all world

production (ICO, 2016). According to International Coffee Organization (ICO), the world production of coffee in 2015 increased 7% with respect to the production achieved in 2010 (Table 2.1).

Table 2.1 Annual worldwide coffee production (2010 – 2015)

Countries	Production					
	2010	2011	2012	2013	2014	2015
Brazil	48,095	43,484	50,826	49,152	45,639	43,235
Vietnam	20,000	26,500	25,000	27,500	26,500	27,500
Colombia	8,523	7,652	9,927	12,124	13,333	13,500
Indonesia	9,129	7,288	13,048	11,449	10,365	11,000
Ethiopia	7,500	6,798	6,233	6,527	6,625	6,400
India	5,033	5,233	5,303	5,075	5,450	5,833
Honduras	4,331	5,903	4,537	4,568	5,400	5,750
Mexico	4,001	4,563	4,327	3,916	3,600	3,900
Guatemala	3,950	3,840	3,743	3,159	3,288	3,400
Peru	4,069	5,373	4,453	4,338	2,883	3,200
Nicaragua	1,638	2,193	1,991	1,941	2,050	2,175
Côte d´Ivoire	982	1,966	2,072	2,107	1,750	1,800
Costa Rica	1,392	1,462	1,571	1,444	1,408	1,492
Other countries	15,346	14,667	14,922	13,315	13,085	14,186
Total	133,989	136,922	147,953	146,615	141,376	143,371

Source: ICO (2016).

Values in thousand 60 kg bags.

As a consequence of this big coffee production worldwide, enormous quantities of residues are generated. More than 70% of coffee cherry beans turn into waste materials (Rodríguez & Zambrano, 2013). On the other hand, it is estimated that during the soluble coffee production, 1 ton of green coffee generates approximately 650 Kg (dry matter) of spent coffee grounds (Mussatto, 2015).

2.2. Coffee residues and their applications

As a consequence of the big worldwide coffee production (Table 2.1), coffee industry is responsible to generate large quantities of by-products during the different stages to which coffee beans are subjected. Therefore, husks, pulp, parchment, silverskin and spent coffee grounds

residues appear along the processing of coffee cherry beans (by wet or dry process), their roasting and beverage preparation.

Spent coffee grounds (SCG) and coffee silverskin (CS) are the residues generated in larger amounts. SCG is the residual material obtained during the treatment of coffee powder with hot water or steam for the instant coffee preparation. Almost 50% of the worldwide coffee production is processed for soluble coffee preparation, which generates around 6 million tons of SCG per year (Mussatto, Machado, et al., 2011a; Tokimoto, Kawasaki, Nakamura, Akutagawa, & Tanada, 2005). On the other hand, CS is a thin tegument of the outer layer of green coffee beans obtained as a by-product of the roasting process (Mussatto, Machado, et al., 2011a) and represents about 4.2% (w/w) of fresh coffee beans (Rodríguez & Zambrano, 2013).

Figure 2.1. Generation of spent coffee grounds and coffee silverskin during coffee cherry processing

SCG could be used, for example, to produce fuel for industrial boilers due to its high calorific power of approx. 5000 kcal/kg (Silva, Nebra, Silva, & Sanchez, 1998) and fuel ethanol (Mussatto, Machado, Carneiro, & Teixeira, 2012b) and to produce mannitol (Arya & Rao, 2007), which is a special chemical with a wide variety of uses in the food industry. It has been also used as substrate for cultivation of microorganisms (Machado, Rodriguez-Jasso, Teixeira, & Mussatto, 2012), as support for anaerobic microorganisms in the treatment of wastewater (Hein & Gatzweiler, 2006) and as raw material to produce fuel ethanol or a distilled beverage with aroma of coffee (Sampaio et al., 2013). CS could be used as substrate for cultivation of microorganisms in order to release phenolic compounds (Machado et al., 2012) or to produce enzymes (Mussatto et al., 2013) and fructooligosaccharides (Mussatto et al., 2013; Mussatto & Teixeira, 2010), or as raw material to produce fuel ethanol (Mussatto et al., 2012). Some researchers have explored the use of CS as functional ingredient due to its high content of soluble dietary fiber and marked antioxidant capacity (Borrelli, Esposito, Napolitano, Ritieni, & Fogliano, 2004).

In spite of these possible applications, SCG and CS are still underutilized as valuable material for industrial processes.

2.3. Chemical composition of coffee beans

The main chemical constituents of coffee beans include polysaccharides (cellulose and hemicellulose), lignin, proteins and lipids, as well as phenolic compounds, minerals and caffeine among others. During coffee roasting processing several changes in the chemical composition of coffee beans occur (Table 2.2). Therefore, some of the compounds present in green coffee are transformed, or even destroyed, due to the high temperatures used in the coffee roasting step. Additionally, roasting processing promotes moisture loss and changes in the color, volume, mass, form, pH and density (Mussatto & Teixeira, 2013) and generates the presence of pigments, polyphenols, polypeptides and volatile compounds that significantly improve the organoleptic quality of the final product.

Table 2.2. Chemical composition of green and roasted coffee beans

Chemical component	Green coffee		Roasted coffee	
	Arabica	Robusta	Arabica	Robusta
Polysaccharides	34.0 – 44.0	48.0 – 55.0	31.0 – 33.0	37.0
Sucrose	6.0 – 9.0	0.9 – 4.0	4.2	1.6
Reducing sugars	0.1	0.4	0.3	0.3
Lignin	3.0	3.0	3.0	3.0
Protein	10.0 – 11.0	11.0 – 15.0	7.5 – 10.0	7.5 – 10.0
Lipids	15.0 – 17.0	7.0 – 10.0	17.0	11.0
Chlorogenic acid	4.1 – 7.9	6.1 – 11.3	1.9 – 2.5	3.3 – 3.8
Caffeine	0.9 – 1.3	1.5 – 2.5	1.1 – 1.3	2.4 – 2.5
Trigonelline	0.6 – 2.0	0.6 – 0.7	0.2 – 1.2	0.7 – 0.3
Others compounds	7.7 – 26.3	21.5	27.5 – 33.8	30.5 – 33.2

Source: Adapted from Farah, A. (2012).

Values are expressed in percent dry weigh basis.

The majority of coffee properties have been attributed to the presence of caffeine, i.e. its stimulating characteristic. However, some studies have revealed the great functional potential of other chemical compounds identified in the coffee brew such as polysaccharides (Gniechwitz, Reichardt, Blaut, Steinhart, & Bunzel, 2007; Simões et al., 2009) and phenolic compounds (Farah & Donangelo, 2006). These compounds result very attractive for chemical, pharmaceutical and food industries since they have multiple biological effects and are beneficial to human health.

2.3.1. Polysaccharides

Coffee is considered an important source of polysaccharides, mainly galactomannans, arabinogalactans, and cellulose (Arya & Rao, 2007; Nunes, Domingues, & Coimbra, 2005). They comprise almost 50% of the dry weight of green coffee beans (Wolfrom & Patin, 1965; Farah, 2012). There is not a significant difference in the polysaccharide content from Arabica and Robusta beans. However, the postharvest processing used (wet or dry method) can affect the extractability of water-soluble polysaccharides from both green coffee beans (Tarzia, Dos Santos Scholz & Oliveira Petkowicz, 2010).

Green coffee galactomannans (Figure 2.2) are mainly composed by a backbone of β-(1→4)-linked mannose residues containing single galactose side groups with different degrees of branching (Moreira, Nunes, Domingues, & Coimbra, 2012; Nunes et al., 2005). They are high molecular weight polysaccharides and show low level of branching. Nonetheless, the roasting process influences on the depolymerization and debranching of galactomannans, increasing thus, their extraction and solubility in water (Simões, Maricato, Nunes, Domingues, & Coimbra, 2014). It is well known that the solubility of galactomannans rises when increasing the degree of galactose substitution (Oliveira Petkowicz, 2015).

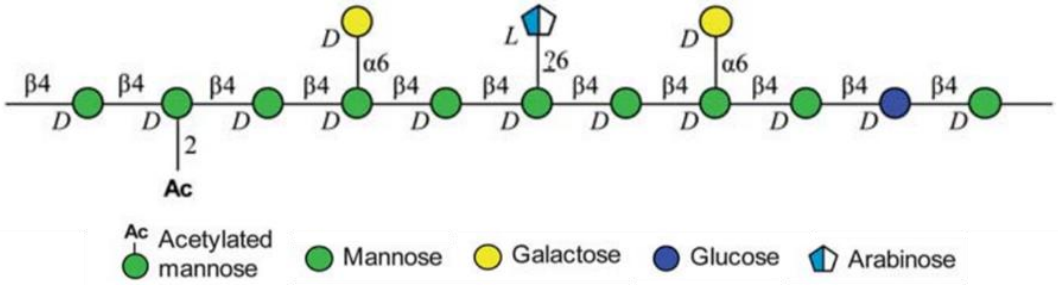

Figure 2.2. Illustration of main structural features of galactomannans isolated by hot water extraction of green coffee beans. Source: Moreira et al. (2012)

On the other hand, green coffee type II arabinogalactans (Figure 2.3) are also high molecular weight polysaccharides, highly branched, mainly composed by a backbone of β-(1→3)-linked galactose residues and side chains of galactose and arabinose residues (Moreira et al., 2012; Passos & Coimbra, 2013). Due to their structure, arabinogalactans are the coffee polysaccharides most exposed to degradation during the roasting and the arabinose side chains are the first to be hydrolyzed (Oosterveld, Harmsen, Voragen, & Schols, 2003). Moreover, type II arabinogalactans are usually linked to proteins (known as arabinogalactan-protein) (Oliveira Petkowicz, 2015).

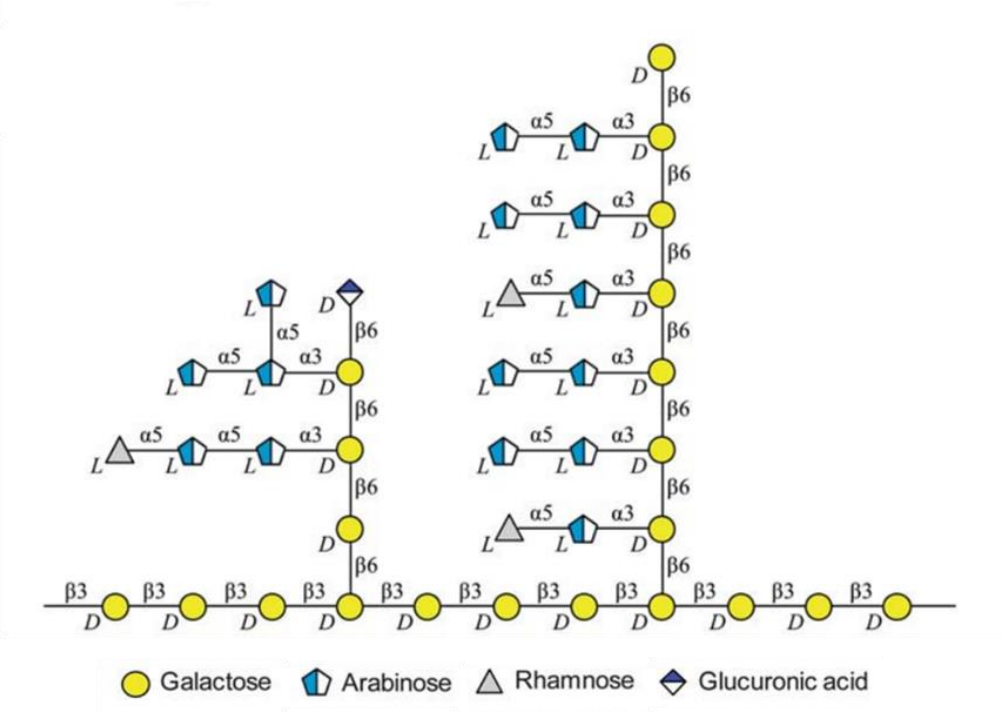

Figure 2.3. Illustration of main structural features of arabinogalactans isolated by hot water extraction of green coffee beans. Source: Moreira et al. (2012)

As already mentioned, during the roasting process, polysaccharides are degraded, releasing monosaccharides and oligosaccharides that may form precursors to flavor compounds. It has been estimated that 20–40% of the carbohydrates from coffee beans are converted into degradation products during roasting (Fischer, Reimann, Trovato, & Redgwell, 2001; Oosterveld et al., 2003). After arabinose degradation, galactose is the second most sensitive sugar, followed by mannose, which is the least sensitive (Oosterveld et al., 2003). Therefore, arabinogalactans are more susceptible to degradation than galactomannans, being degraded up to 60% and 36%, respectively, while cellulose is not degraded, even at longer roasting times (Redgwell, Trovato, Curti, & Fischer, 2002). Both, galactomannans and arabinogalactans strongly affect the quality and properties of the final beverage, being responsible for the retention of coffee volatile substances, stabilization of foam, binding of aroma, formation of sedimentation, and increased viscosity of the extract (Arya & Rao, 2007; Nunes et al., 2005). However, their majority (around 70% of total

polysaccharides from roasted coffee) (Arya & Rao, 2007) remains in the residue, called spent coffee ground, after soluble coffee preparation.

Recently, some researchers have exposed the great potential of polysaccharides presented in coffee, showing that they can provide important functional properties. Some researchers have exposed the great functional potential of polysaccharides presented in coffee. Most of them are not degraded by human digestive enzymes, thus, they reach the colon and potentially serve as substrates for the colonic microbiota supporting the growth of bifidobacteria and other lactic acid bacteria that are considered beneficial for human health (Gniechwitz et al., 2007). Polysaccharides from coffee decrease the cholesterol levels in blood, controlling the blood glucose and insulin response, act against infectious and tumor diseases (Gniechwitz et al., 2007) and have an immunostimulatory capacity (Simões et al., 2009).

Additionally, galactomannans from natural sources have been used as stabilizers and stiffeners of emulsions in different areas including food industry, due to their non-toxic nature (Cerqueira, Lima, Teixeira, Moreira, & Vicente, 2009) and arabinogalactans are also used in food due to their capacity to retain water and form low viscosity emulsions (Dexter & Assoc, 1998).

2.3.2. Phenolic compounds

Phenolic compounds are secondary metabolites synthesized by different plants during their normal development or as a response to environmental stress conditions (Beckman, 2000). These compounds present important functional properties, being therefore, of great interest for chemical, pharmaceutical and food industries. In green coffee, phenolic compounds have been mainly identified as chlorogenic acid (Figure 2.4) and related to substances including caffeoylquinic acid, dicaffeoylquinic acid, feruloylquinic acid, and p-coumaroylquinic acid, (Farah & Donangelo, 2006). Some flavonoids such as kaempferol, quercetin, catechin, epicatechin have been also identified (Mussatto, 2015). Phenolic compounds are partially transformed during the coffee roasting process. These compounds are thermally unstable (Beckman, 2000), and possess low bioavailability and stability after ingestion (Nallamuthu, Devi, & Khanum, 2015).

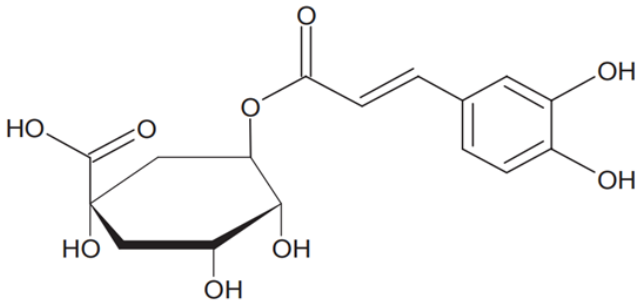

Figure 2.4. Chemical structure of Chlorogenic acid. Source: Mussatto (2015)

Phenolic compounds from coffee have been of great interest due to their enormous benefits for human health. Previous researches have shown that their potential is related to their antioxidant activity (Cho et al., 2010; Mussatto, Ballesteros, Martins, & Teixeira, 2011c). This type of phenolic compounds protects against chronic-degenerative diseases such as cancer (Kasai, Fukada, Yamaizumi, Sugie, & Mori, 2000), cardiovascular diseases, neurodegenerative diseases and diabetes mellitus (Martins et al., 2011; Mussatto, 2015; Prasad et al., 2011). Nonetheless, their properties are not limited to the antioxidant activity. Phenolic compounds, particularly from chlorogenic acid, present anti-obese (Cho et al., 2010), anti-inflammatory and anti-microbial (Shin et al., 2015), anti-diabetic (Karthikesan, Pari, & Menon, 2010) and anti-cancerous properties (Kasai et al., 2000).

Furthermore, phenolic compounds improve the organoleptic properties of vegetable origin food, and can also be used as raw material in the development of functional food or as natural preservatives against food degradation (Ballesteros, Teixeira, & Mussatto, 2014; Rodríguez-Meizoso et al., 2010).

Nowadays, researchers have been focused on identifying natural sources to extract antioxidant compounds that can replace the synthetic antioxidants since it has been proved that they may cause health problems including enlarged liver and conversion of some ingested materials into carcinogenic and toxic substances (Mussatto, 2015), especially when these compounds are incorporated excessively in food.

2.4. Extraction methods

Extraction is an important operation in chemical and food engineering, enabling the recovery of valuable soluble components from raw materials. Nowadays, the solid-liquid extraction methods have been widely used to obtain compounds of interest from natural sources. Lignocellulosic materials, for example, can be subjected to different fractionation steps in order to extract their main constituents in separated fractions including cellulose, hemicellulose and lignin or other secondary compounds such as phenolic compounds.

During the solid-liquid extraction process, the phase liquid, being represented for an organic solvent dissolved in water or simply pure water, has three main objectives: i) isolating a component of interest; ii) removing potential interferents from a matrix; and iii) concentrating the component desired (Lebovka, Vorobiev, & Chemat, 2011). The process consists of mixing solid material with the solvent and then, the mixture is maintained at conditions needed to promote the transference of the solute from the solid to the solvent. The efficiency of the extraction process is affected by several factors such as the type of solvent and its concentration, the solvent/solid ratio, the number of extraction steps, pH, time of contact, temperature, and particle size of the solid matrix (Mussatto, Ballesteros, et al., 2011c), as well as the structure and polymerization degree of molecules and their interaction with proteins and other compounds generated during the extraction.

Additional to the conditions used in the process, the technique employed plays an important role. Techniques such as solid-liquid extraction using organic solvents (Simões et al., 2009), ultrasound-assisted extraction (Yang, Jiang, Zhao, Shi, & Wang, 2008), microwave-assisted extraction (Guoxiang, Dai Jun, Shangwei, & Zaijun, 2009) and autohydrolysis (Rodríguez-Jasso, Mussatto, Pastrana, Aguilar, & Teixeira, 2013) have been applied in order to recover carbohydrates from natural sources. The extraction of polysaccharides from SCG has also been studied through different methods, mainly using chemicals as extraction agents. Sodium hydroxide (Simões et al., 2009; Simões, Nunes, Maria do Rosário, & Coimbra, 2010) and potassium hydroxide (Fischer et al., 2001), for example, have been employed in alkali treatments, while sulfuric acid has been used to recover carbohydrates by dilute acid hydrolysis of SCG (Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011b). Some authors have also studied the extraction of hemicelluloses from SCG by

using microwave-assisted extraction, being considered a more ecofriendly technique to obtain these sugars (Passos & Coimbra, 2013).

Phenolic compound are others compounds commonly extracted from natural sources. Techniques as such solid state fermentation (Machado et al., 2012), solid-liquid extraction using organic solvents (Ballesteros et al., 2014; Martins, Aguilar, Teixeira, & Mussatto, 2012), ultrasound-assisted extraction (Carrera, Ruiz-Rodríguez, Palma, & Barroso, 2012), microwave-assisted extraction (Martins, Aguilar, Garza-Rodriguez, Mussatto, & Teixeira, 2010) have been used to extract phenolic compounds from multiple natural sources. The extraction of these compounds from SCG has been also studied (Murthy & Naidu, 2012; Mussatto, Ballesteros, et al., 2011c; Panusa, Zuorro, Lavecchia, Marrosu, & Petrucci, 2013; Zuorro & Lavecchia, 2012). These findings showed the ability of a conventional solid-liquid extraction method to recover phenolic compounds from SCG using organic solvents such as ethanol (Panusa et al., 2013; Zuorro & Lavecchia, 2012), methanol (Mussatto, Ballesteros, et al., 2011c) and isopropanol (Murthy & Naidu, 2012). However, industry specialists are looking for improved techniques that require less solvents and energy consumption, and are more environmentally-friendly.

The methods evaluated in this study to extract polysaccharides and phenolic compounds from SCG are briefly described below.

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2.4.1. Alkali treatment

Alkali treatment is a suitable method to isolate hemicellulose and lignin from natural sources (Gabrielii, Gatenholm, Glasser, Jain, & Kenne, 2000) as well as the saponification of uronic and acetic esters. Usually, the treatment is based on the use of aqueous solutions of calcium, lithium, barium potassium or sodium hydroxides that act as solvents. The chemicals more utilized in this type of extraction are potassium and sodium hydroxides since the obtained yields are much higher (Lawther, Sun, & Banks, 1996). When the organic solvent is in contact with the solid matrix that contains the compound of interest the solid suffers swelling, causing an increase in the internal surface and a reduction of polymerization grade and crystallinity, as well as a separation between lignin and polysaccharides (Jackson, 1977).

The conditions used during the extraction process may be selective for the compound of interest. The alkali treatment to extract polysaccharides, for example, requires lower temperatures, which does not affect the cellulose and lignin, but causes the solubilization of acetylated hemicelluloses. On the contrary, when temperature is increased breakage of the ester bonds of the lignin and depolymerization of cellulose occurs.

Although alkaline treatment of hemicelluloses requires lower temperature and pressure than acid treatments, this treatment causes environmental concerns, and the costly recovery of reagents could limit its practical potential.

2.4.2. Autohydrolysis

Autohydrolysis is an eco-friendly technology that does not require the use of chemical agents for reaction. This technique has been used to extract polysaccharides from different natural sources such as *Eucalyptus globulus* wood (Romaní, Garrote, López, & Parajó, 2011), *Pinus pinaster* wood and rice husks (Rivas, Conde, Moure, Domínguez, & Parajó, 2013), among others. During autohydrolysis, the mixture (solid matrix together pure water used as solvent) is subjected to a temperature between 160 – 240 °C resulting in both depolymerization of hemicellulose and breakage of lignin-carbohydrate bonds, leading to solubilization of hemicellulose-derived saccharides and some lignin fragments of low molecular weight (Nabarlatz, Ebringerová, & Montané, 2007).

Autohydrolysis is known as an autocatalytic hydrothermal processing. Thus, the process starts with hydronium ions from water auto-ionization, and its progress is favored by the in situ generation of a slightly acid media due to the partial release of organic acids produced from sugar-degradation products, phenolic acids from hemicellulose substituents and acetic acid from acetyl groups (Conde & Mussatto, 2015; Nabarlatz et al., 2007). Along autohydrolysis, water soluble extractives are removed from solid phase, being oligosaccharides, monosaccharides, and sugar degradation products (furfural and hydroxymethylfurfural), as well as cell wall linked phenolic compounds the main solubilized compounds (Felizón, Fernández-Bolaños, Heredia, & Guillén, 2000; Garrote & Parajó, 2002). Additionally, the proteins and amino acids present in the solid

matrix together with the reducing sugars generated during reaction, cause the Maillard reaction (Benjakul, Lertittikul, & Bauer, 2005; Dendy & Crespo, 2004) promoting also the production of volatile compounds, phenolic compounds, pigments, and others compounds of low molecular weight in the extract.

Autohydrolysis is considered an interesting extraction technique since offers several advantages such as elimination of corrosive problems in the equipment due to mild pH of reaction media, reduction of operational costs because no further neutralization is needed and mild operational conditions for selective degradation of the biomass (Carvalheiro, Esteves, Parajó, Pereira, & Gırio, 2004; Conde & Mussatto, 2015; Rodríguez-Jasso et al., 2013).

2.5. Encapsulation of bioactive compounds

Encapsulation is one of the most used techniques in the preservation and stability of different compounds. It is described as a process in which bioactive compounds are encapsulated in a biopolymer in order to protect them of external factors. Phenolic compounds, for example, are very vulnerable to oxidizing environment including the light, oxygen, moisture, among others, due to the existence of unsaturated bonds in the molecular structures. For preserving their properties, phenolic compounds could be encapsulated to enhance their storage stability, making them safer as food ingredients and providing benefits to the consumers. The encapsulation process apart from stabilizing these bioactive compounds in time or during processing, also helps masking unpleasant flavors in food provided by these functional compounds, including bitter taste and astringency of polyphenols (Fang & Bhandari, 2010).

The encapsulated compound can be called active agent, core, fill, internal or payload phase. The biopolymer that encapsulates can be named coating, membrane, cover, carrier material, shell, or wall material, should be generally recognized as safe (GRAS) and must be able to form a barrier between the active agent and its surrounding to ensure the protection (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011).

Forms more commonly obtained during encapsulation process are shown in the Figure 2.5. The first one is a mononuclear capsule, having a single core enveloped by a shell, while the second are aggregates, which have many cores embedded in a matrix (Schrooyen, van der Meer, & De Kruif, 2001). The specific shapes of different systems obtained are mainly influenced by the drying processing technologies and by the active agent and wall materials from which the capsules are made (Fang & Bhandari, 2010).

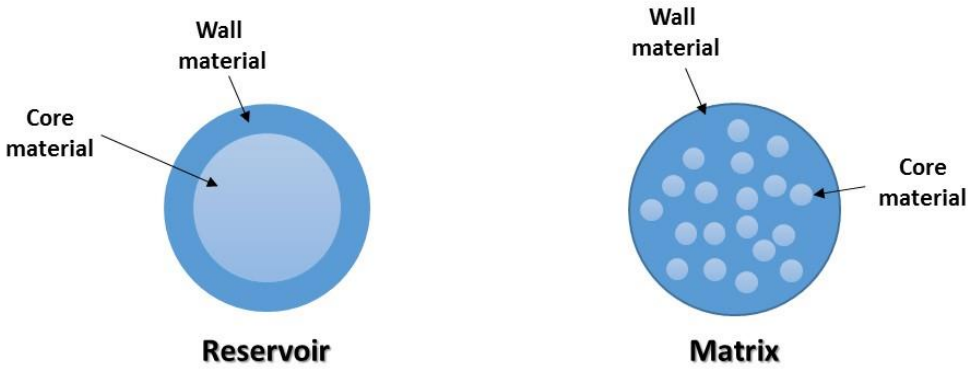

Figure 2.5. Type of capsules obtained during encapsulation process

Bioactive components includes a large number of compounds presenting differences in chemical structure, molecular weight, polarity, solubility, among others, which implies that different encapsulation approaches have to be applied in order to meet the specific physicochemical requirements (Augustin & Hemar, 2009; Kailasapathy, 2002; Ray, Raychaudhuri, & Chakraborty, 2016). Additionally, it has been demonstrated the importance of properly selecting the carriers and encapsulation process to maximize the incorporation and retention of the functional compounds being encapsulated. Several researchers have studied the encapsulation of bioactive compounds such as essential oils (Barros-Fernandes, Borges, & Botrel, 2014), anthocyanins (Flores, Singh, Kerr, Pegg, & Kong, 2014; Khazaei, Jafari, Ghorbani, & Kakhki, 2014), propolis (Silva et al., 2013), cherry pomace phenolic extracts (Cilek, Luca, Hasirci, Sahin, & Sumnu, 2012), among others, demonstrating that the retention capacity is highly dependent on the type of phenolic compound encapsulated and the selection of the composition of the wall material.

2.5.1. Materials used for encapsulation

A large variety of materials can be used for encapsulation in food applications, being polysaccharides such as maltodextrin, gum arabic, hydrophically modified starches and chitosan, as well as different mixtures between them, the most commonly used shell materials (Gouin, 2004; Nedovic et al., 2011; Ray et al., 2016). Additionally, lipids (mono and diglycerides) and proteins (casein, milk serum and gelatin) can also be used as wall materials (Nedovic et al., 2011).

For selecting the encapsulation material, it is very important to take into account some criteria. Coatings must provide maximal protection of the active agent and maintain it active within the capsule structure along processing or storage. Besides, the wall material should not react with the core and must have good rheological characteristics at high concentration, presenting easy work ability during the encapsulation process (Nedovic et al., 2011). Supplementary to the mentioned criteria, the correct choice of the wall material plays a relevant role on the encapsulation efficiency and stability of the encapsulated compound.

Maltodextrin, for example, is relatively low cost polysaccharide with neutral taste and aroma and an effective protection to flavors (Barros-Fernandes et al., 2014). This polysaccharide is obtained from starch hydrolysis, being highly water soluble and presenting low viscosity even when used at high concentrations (Ray et al., 2016). According to the hydrolysis degree, maltodextrin is classified by the dextrose equivalent value (DE), which is measured by the amount of reducing sugars present in a sugar product. DE can be between 3 and 20. The higher the DE value, the shorter the glucose chains, the higher the sweetness, the higher the solubility, and the lower heat resistance (Murugesan & Orsat, 2012; Saénz, Tapia, Chávez, & Robert, 2009).

Maltodextrin has the ability to form a cover for the core, encapsulating aromas and flavors and reducing exposure to oxygen (Santiago-Adame et al., 2015). Additionally, it is the most used material in freeze-drying process for encapsulation stability. Maltodextrin is a powerful barrier against oxidation of core material and protective against undesired physical and chemical changes (Sanchez, Baeza, Galmarini, Zamora, & Chirife, 2013). Maltodextrin is also very utilized to encapsulate products through spray-drying, since it protect cores for long period of time and can release them under digestive conditions (Santiago-Adame et al., 2015).

The greatest limitation of maltodextrin as wall material is its low emulsifying capacity and marginal retention of volatile compounds.

On the other hand, gum arabic also known as acacia gum is a natural polysaccharide obtained from the hardened sap of various acacia tree species. This complex heteropolysaccharide has a highly ramified structure, being the main chain formed by D-galactopyranose units (K. A. Silva, Coelho, Calado, & Rocha-Leão, 2013). Gum arabic has been widely used in food industry due to the nontoxic, odorless and tasteless nature, but sometimes it presents a pronounced effect on taste and flavor of foods.

Gum arabic is the most widely used encapsulating material through spray-drying and freeze-drying due to its good emulsifying and film-forming capacities, as well as its low viscosity in aqueous solution (Silva et al., 2013).

2.5.2. Encapsulation techniques

There are numerous chemical and physical methods to encapsulate bioactive compounds. The physical encapsulation techniques are often based on drying processes due to the liquid nature of the extracts that contain the bioactive compounds. Spray-drying, spray-bed-drying, fluid-bed coating, freeze-drying are included among these technologies, being freeze-drying and spray-drying the most common drying methods to produce encapsulated compounds for food applications.

2.5.2.1. *Freeze-drying*

Freeze-drying, also known as lyophilization, is the most suitable drying process for dehydration of heat sensitive materials, since it conserves almost intact the initial functional properties of the compounds (Ceballos, Giraldo, & Orrego, 2012) and minimizes thermal degradation reactions. This technique is formed by different stages including freezing, sublimation, desorption and product storage, where the sublimation is the most important step. Freeze-drying has been used in the process encapsulation of polyphenols (Fang & Bhandari, 2010) and multiple

substances (Fang & Bhandari, 2010) since it preserves in the long term the biological activity, flavor and taste among others properties of the encapsulated compounds.

Encapsulation by freeze-drying is achieved as the core materials homogenize in a matrix solution (Figure 2.6), usually resulting in uncertain forms (Fang & Bhandari, 2010).

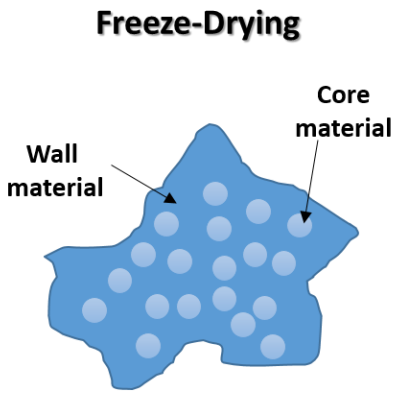

Figure 2.6. Capsules illustration produced by freeze-drying procedure

The major drawbacks of freeze-drying process are the high energy input and long processing time (Ray et al., 2016). Since its utilization is costly, commercial application of freeze-drying is restricted to very high value ingredients such as antioxidants (Augustin & Hemar, 2009).

2.5.2.2. *Spray-drying*

Spray-drying is the oldest and the most widely used encapsulation technique in food industry thanks to its low-cost and flexibility (Fang & Bhandari, 2010). Some studies have highlighted the protection, stabilization, solubility and controlled release of the encapsulated bioactive compounds including phenolic compounds when using spray-drying (Fang & Bhandari, 2010; Nedovic et al., 2011; Ray et al., 2016). This processing involves atomization of a liquid feedstock, being rapidly dehydrated when in contact with hot air, producing thus, a dry powder. The typical shape of spray-dried particles is spherical, with a mean size range of 10-100 μm (Figure 2.7). The physicochemical properties of the final encapsulated product are mainly dependent of

feed rate, viscosity of the liquid, drying air inlet and outlet temperatures, the pressure and type of atomizer (Ramírez, Giraldo, & Orrego, 2015). For instance, when the inlet temperature is very low, it is more difficult to evaporate the water completely in a short time and the encapsulation yield could be compromised. On the contrary, if the temperature is very high, cracking of the microcapsules can occur.

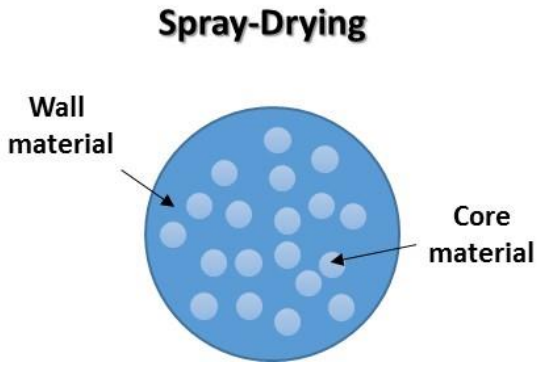

Figure 2.7. Capsules illustration produced by spray-drying procedure

In comparison with others methods, spray-drying can achieve a high encapsulation efficiency. However, one drawback of this technology is the limited number of shell materials available to be treated with this type of drying, since the wall materials used must be soluble in water at an acceptable level (Fang & Bhandari, 2010).

2.6. Coating and Films

Bio-based films or coatings are promising systems to replace the synthetic materials used in the food packaging industry. Nowadays, the textile, pharmaceutical, cosmetic and food industries are looking for new materials from renewable resources that can replace the petroleum-based materials in order to reduce their environmental impact, promoting thus, a new generation of biodegradable packaging with similar properties than synthetics and low cost production (Ghanbarzadeh, Almasi, & Entezami, 2010).

Currently, coatings and films have been used to protect different foodstuffs and play an important role in the quality, safety, transportation, storage and display of a wide range of fresh and processed foods such as meats, nuts, snacks, candies, vegetables and different fruits, among others. Generally, film and coating systems (Figure 2.8) are designed to act as barrier in order to protect the food against physical and mechanical impacts, chemical reactions and microbiological contamination. Thus, films and coatings can provide a barrier against migration of moisture, oxygen, carbon dioxide and volatile compounds, which counteracts the loss weigh, delays the deterioration and prevents the loss of natural aroma of the products and the other components (Lin & Zhao, 2007). They can be used as potential carriers of additives and bioactive compounds to maintain or even improve the nourishing and sensory features of foods (Cerqueira, Lima, Teixeira, Moreira, & Vicente, 2009; Lin & Zhao, 2007). Moreover, edible films and coatings provide a better visual aspect, improve the food quality and safety and simultaneously increasing their shelf-life (Pavlath & Orts, 2009; S Guilbert & N Gontard, 1995).

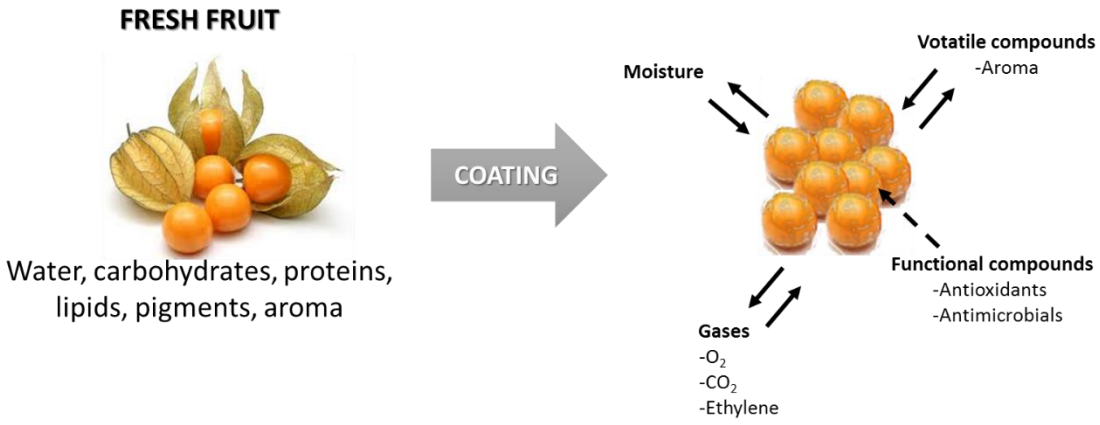

Figure 2.8 Functional properties of edible coatings on fresh fruits

2.6.1. Components of Edible Films and Coatings

Edible films and coatings are defined as continuous matrices that can be prepared from bio-polymers such as polysaccharides, proteins, lipids or waxes and other important components including surfactants and food-grade plasticizers. Additionally, films and coatings may be formed

by heterogeneous polymer materials, or by mixtures of polymer materials. Figure 2.9 shows the most common bio-polymer compounds used for preparation of edible films and coatings.

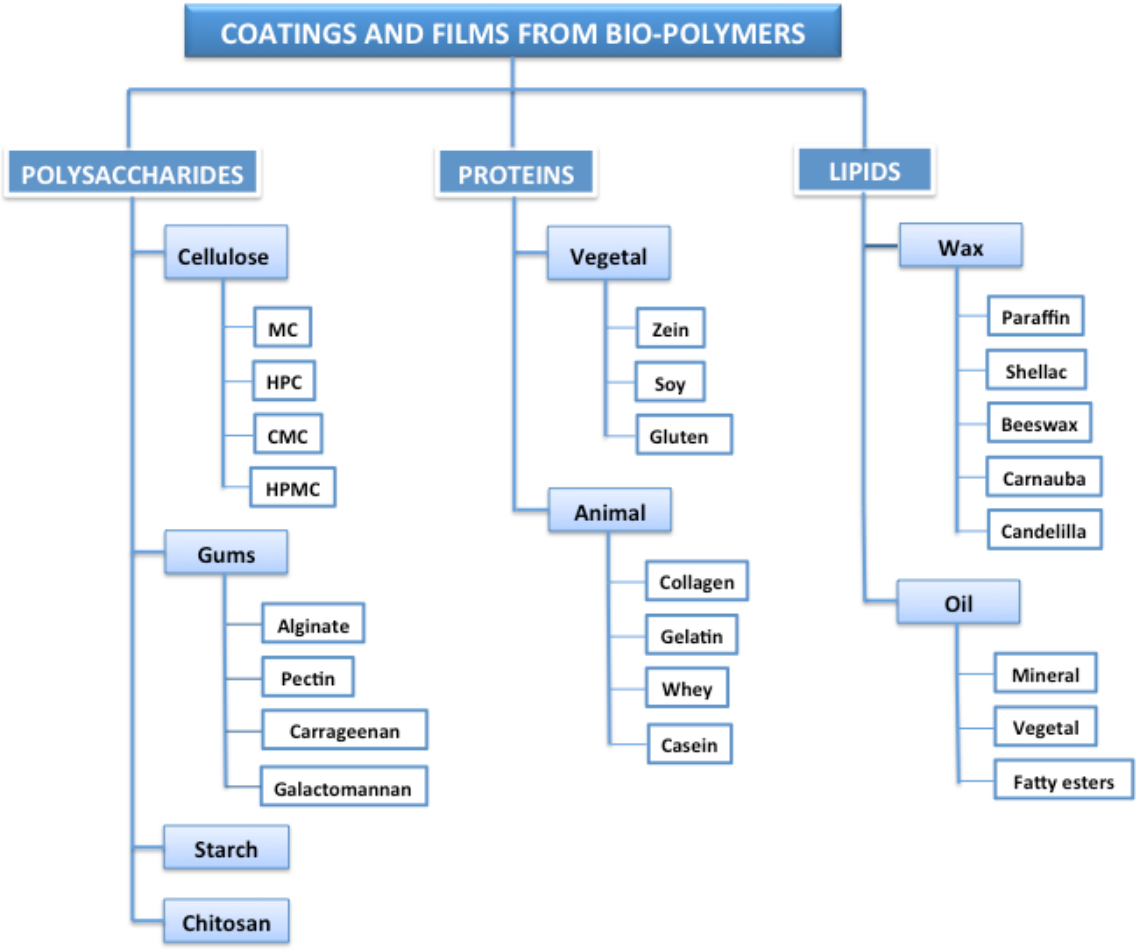

Figure 2.9. Biopolymers used for preparation of films and coatings for food. MC (methyl cellulose), HPC (hydroxypropyl cellulose), CMC (carboxymethyl cellulose), HPMC (hydroxypropylmethyl cellulose)

The origin of biodegradable compounds and their chemical structure, that can be modified depending on the techniques employed during the process extraction, play an important role on the production and features of films and coatings. Thus, the type of molecular linkage and shape, the molecular weight, and the degree of polymerization of the compounds can influence widely the physicochemical properties of the final matrix and affect the synergistic interactions among

materials (Martins et al., 2012; Mikkonen et al., 2007). Moreover, the physical and chemical characteristics of these biopolymers greatly influence the functionality of the produced films and coatings (Sothornvit & Krochta, 2001).

Polysaccharides, proteins and lipids differ widely in their physical and chemical features, and therefore, the attributes that each component provides to overall film and coating properties are also different. Polysaccharides, for instance, are usually used to control oxygen and other gas transport, while proteins provide mechanical stability and fats reduce water transport (Lacroix, 2009).

The selection of coating and films materials is generally based on their water solubility, hydrophilic and hydrophobic nature, easy formation of coatings and films, and sensory properties (Lin & Zhao, 2007). Plasticizers are often added to film-forming solutions aiming to enhance the properties of the final film. These film additives are typically small molecules of low molecular weight and high boiling point, which are highly compatible with the polymers. Common food-grade plasticizers such as sorbitol, glycerol, mannitol, sucrose and polyethylene glycol, decrease brittleness and increase flexibility of the films and coatings, which are important attributes in packaging applications (Pavlath & Orts, 2009; S Guilbert & N Gontard, 1995).

Presently, polysaccharide-based films have attracted great attention among researchers, not only due to their capacity to blend between them, but also with others compounds in order to improve their properties (Cerqueira et al., 2009; Cerqueira, Souza, Teixeira, & Vicente, 2012; Figueiró, Góes, Moreira, & Sombra, 2004; Su, Huang, Yuan, Wang, & Li, 2010). Carboxymethyl cellulose (CMC), which is one of the most important cellulose derivatives, contains a hydrophobic polysaccharide backbone and many hydrophilic carboxyl groups (Su et al., 2010). It is usually used as thickener or viscosity modifier in different fields since is generally recognized as safe (GRAS) (Su et al., 2010) and approved for use in foods. CMC also presents excellent film-forming properties due to its biocompatibility with substances such as: water-soluble polysaccharides, proteins, surfactants and plasticizers (Nisperos-Carriedo, Baldwin, & Shaw, 1991). CMC-based coatings are generally odorless and tasteless, flexible, and are of moderate strength, transparent, resistant to oil and fats, water-soluble, moderate to moisture and oxygen transmission (Lin & Zhao, 2007). This

type of coating has been tested on fruits and vegetables including apples, peaches, lettuce and carrots among others, showing its capacity to retain the original flavor and crispness and reduce the gas exchange rate (Lin & Zhao, 2007).

Some studies have reported the incorporation of polysaccharides extracted from natural sources into edible films or coatings for food applications (Cerqueira et al., 2009; Cerqueira et al., 2011; Ghanbarzadeh et al., 2010; Su et al., 2010; Tongdeesoontorn, Mauer, Wongruong, Sriburi, & Rachtanapun, 2011). In general, coatings based in polysaccharides are ideal to increase the shelf life of food, especially of vegetables, fruits, shellfish and meat products, avoiding the dehydration and reducing the oxidation and the microbial spoilage (Cerqueira et al., 2009; Dang, Singh, & Swinny, 2008). Edible films and coatings with incorporated polysaccharides present emulsifying and gelling agents, are colorless and have an oil free appearance (Cerqueira et al., 2009). They might also influence in the mechanical properties of packing and in the protective, nourishing and sensory features of food, while they are also environmental friendly and biodegradable (Souza et al., 2010). Other important characteristics that make the polysaccharides attractive for incorporation into edible films and coatings are their transport properties (permeability to CO_2, O_2 and water vapor) and the reduction of materials weight loss (Dang et al., 2008). Furthermore, edible coatings may also act as vehicles for additives, antioxidants and antimicrobials agents, nutrients and flavors, improving food quality and increasing its functionality and safety (Cerqueira et al., 2009).

Thus, taking into account the great potential of the polysaccharides from spent coffee grounds, this research opens up the possibility of exploiting this material as a source of bioactive compounds by its incorporation into edible films and coatings while offering consumers a healthy food.

2.7. Goldenberry

A fruit in which these edible coatings and films could be tested is *Physalis peruviana*, also known as goldenberry or cape gooseberry in English speaking countries, and as uchuva in Colombia (Cedeño & Montenegro, 2004; Puente, Pinto-Muñoz, Castro, & Cortés, 2011). It belongs to the

family Solanaceae and genus *Physalis* being found more than 80 varieties in the world (Puente et al., 2011). Goldenberry is able to grow in a wide range of altitudes between 1,500 to 3,300 m above sea level and is native to warm temperate and subtropical regions. The fruit is a juicy orange berry similar in size, shape and structure to a small tomato, but it is completely enclosed in a large papery husk or calyx, that protects it along harvest and postharvest. Shelf-life of goldenberry with calyx is of 30 days, whereas without calyx is around 5 days (Puente et al., 2011). However, at temperature between 3 - 7 °C goldenberry without calyx could have a shelf-life around 45 days, approximately (Castro & Blair, 2010).

Currently, goldenberry is consumed fresh and is used as ornament in meals, salads, desserts and cakes (Cedeño & Montenegro, 2004; Puente et al., 2011), but it can also be transformed in jams, nuts, snacks and candies among others applications (Ramadan & Moersel, 2009). Moreover, goldenberry can be used as preservative for jams and jellies due to its high pectinase content (Ramadan & Moersel, 2009). *Physalis peruviana* contains high amounts of vitamins A, B and C, polyunsaturated fatty acids and minerals as iron and phosphorus. Otherwise, many medicinal properties have been attributed to this fruit such as antispasmodic, antiseptic, diuretic, analgesic, and capacity of eliminating intestinal parasites. Moreover, it helps to fortify the optic nerves, purifies the blood, decreases albumin in kidneys and cleans the cataracts (Cedeño & Montenegro, 2004; Puente et al., 2011).

Colombia is the largest producer of goldenberry, followed by South Africa. Colombia is also the largest exporter worldwide (Puente et al., 2011). Fresh fruit is exported in great quantities mainly to the United States and European Union, requiring the use of modern methods for conservation. Edible films and coatings could give added- value to this fruit since they are good alternatives to preserve the properties of the foods that are exported as well as those that are not, reducing transport costs and storage.

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SECTION II

CHARACTERIZATION OF COFFEE RESIDUES

CHAPTER 3

CHEMICAL, FUNCTIONAL AND STRUCTURAL PROPERTIES OF COFFEE RESIDUES

The following chapter is partially based on the results published in: Lina F. Ballesteros, José A. Teixeira & Solange I. Mussatto (2014). Chemical, functional, and structural properties of spent coffee grounds and coffee silverskin. *Food and Bioprocess Technology, 7*(12), 3493-3503.

3. Introduction

As a result of the big worldwide coffee production, coffee residues including spent coffee grounds (SCG) and coffee silverskin (CS) represent great pollution hazard if discharged into the environment. Nowadays, there is a great political and social pressure to reduce the pollution arising from industrial activities. For that reason, it is necessary to focus on the exploitation of SCG and CS, and their profitable utilization, adding value to these unused materials and decreasing their impact to the environment. Despite that some characteristics of SCG and CS have been recently reported in the literature, to the best of our knowledge, there is not any study that shows a complete characterization of both materials. Such information is of great importance to identify the possible areas for application of these residues. In this sense, the purpose of the present chapter consisted in evaluating the chemical composition, functional properties, and structural characteristics of SCG and CS, in order to obtain more detailed information about these materials and identify potential industrial areas for their reutilization.

3.1. Materials and Methods

3.1.1. Raw materials and chemicals

Spent coffee grounds (SCG) and coffee silverskin (CS), which are derived from mixtures of Arabica and Robusta coffee varieties, were provided by NovaDelta Comércio e Indústria de Cafés, S.A. (Campo Maior, Portugal). As soon as obtained, the materials were dried in an oven at 60 °C until constant weight (6.8% (w/w) moisture). Moisture content in the samples was measured in a moisture analyzer model MAC 50/1/NH (Radwag, Poland). After dried, CS was milled in a Taurus mill. Then, both SCG and CS samples were sieved through a 500 μm mesh screen (obtaining particles ≤ 500 μm) and stored at room temperature for further analyses. All the chemicals used were analytical grade, purchased from Sigma–Aldrich (Sternheim, Germany), Panreac Química (Barcelona, Spain) or Fisher Scientific (Leicestershire, UK). Enzymes were obtained from Sigma-

Aldrich (St. Louis, MO, USA) and ultrapure water from a Milli-Q System (Millipore Inc., USA) was used.

3.1.2. Chemical composition determination

3.1.2.1. *Cellulose, Hemicellulose and Lignin*

Previous cellulose, hemicellulose and lignin determination, the extractives from SCG and CS were removed in a Soxhlet extraction system (Tecator, HT2, Netherlands) using ultrapure water and absolute ethanol as solvents in two sequential stages (Sluiter et al., 2008). The extractive free SCG and CS samples were dried at 60 °C to constant weight to be stored. To determine the cellulose, hemicellulose and lignin (ash-free) contents, the raw material was submitted to a two-steps sequential acid hydrolysis (Sluiter et al., 2010). Sugars in the resulting solution were determined by high performance liquid chromatography (Mussatto et al. 2011b) and were used to calculate the cellulose (as glucose) and hemicellulose (as arabinose, mannose, galactose and xylose) contents (Mussatto and Roberto 2006). The lignin (ash-free) content was also calculated as described by Mussatto and Roberto (2006).

3.1.2.2. *Ashes, Minerals, Fat and Protein*

Ashes were determined by incinerating the samples at 550 °C for 4 h (Horwitz and Latimer Jr, 2005). The mineral content in ashes was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES), as described by Meneses et al. (2013). Fat content was determined using petroleum ether as solvent in a Soxhlet extraction system (Tecator, HT2, Netherlands) during 1 h, according to the official AOAC method nº 920.39 (Horwitz and Latimer Jr, 2005). Nitrogen was determined by combustion using a Thermo Scientific Flash 2000 Elemental Analyzer, and the protein content was estimated by using the $N_2 \times 6.25$ conversion factor.

3.1.2.3. *Total, insoluble and soluble dietary fibers*

The total dietary fiber (TDF) was estimated by enzymatic gravimetric method according to the official AOAC standard procedure n° 985.29 (Horwitz and Latimer Jr, 2005) with some modifications. Briefly, 1 g fat free sample was mixed with 50 ml of phosphate buffer (0.08 M, pH 6) in a flask. Then, 0.1 ml α-amylase (Sigma A-3306) was added to the mixture and the flask was covered and left during 15 min in a boiling water bath with discontinuous agitation. The flask was then cooled to room temperature and the pH of the medium was adjusted to 7.5 by adding a 0.275 N NaOH solution. Later, 0.1 ml protease solution (Sigma P-3910, 50 mg in 1 ml phosphate buffer) was added to the sample, which was covered and heated in a water bath at 60 °C during 30 min with continuous agitation. After that, the sample was left at room temperature and the pH was adjusted to 4 by adding a 0.325 M HCl solution. Additionally, 0.3 ml amyloglucosidase (Sigma A-9913) was mixed with the sample and placed in the water bath at the same conditions used for the protease. After this process, 280 ml of 95% (v/v) ethanol preheated at 60 °C were added to the sample and left at room temperature for 1 h. The sample was filtered through filter paper and washed three times with 20 ml of ethanol at 78% (v/v), twice with 10 ml of ethanol at 95% (v/v), and once with 10 ml of acetone. Finally, the filter paper containing the solid residues was dried overnight at 105 °C, and the final weight of the sample was registered. This methodology was carried out at least twice being one sample used to determine the protein content, while the other sample was employed to estimate the ashes content. Distilled water was used as blank to exclude any contribution from reagents to measurements. The TDF percentage was calculated by using the Eq 3.1, where W_1 is the average weight of the sample (mg) taken, W_2 is the average final weight of the sample, W_3 and W_4 are the protein and ash weights (mg) respectively; and W_5 is the blank weight.

Eq 3.1 $$\text{TDF (\%)} = (W_2 - W_3 - W_4 - W_5) * 100 / W_1$$

Insoluble (IDF) and soluble dietary fibers (SDF) were determined using the same methodology applied for TDF determination, but without adding alcohol in the precipitation stage.

The IDF percentage was calculated by Eq 3.1, and the SDF percentage was obtained by the difference between TDF and IDF values.

3.1.3. Functional Properties

3.1.3.1. *Water holding capacity and Oil holding capacity*

Water holding capacity (WHC) and oil holding capacity (OHC) were determined by mixing 1 g of sample with 10 ml of distilled water or corn oil (Fula, Sovena Portugal; density = 0.92 g/ml). The mixtures were vortexed for 1 min, centrifuged at 2330 *g* for 30 min, and the volume of supernatant was determined. WHC was expressed as gram of water held per gram of sample, while OHC was expressed as gram of oil held per gram of sample (Chau et al., 1997).

3.1.3.2. *Emulsifying activity and Emulsion stability*

Emulsifying activity (EA) and emulsion stability (ES) were determined according to Chau et al. (1997) with some modifications. Firstly, 2 g of sample were mixed with 100 ml of distilled water and homogenized at 6000 rpm for 2 min using an IKA T-25D Ultra-turrax homogenizer. Afterwards, 100 ml of corn oil were added to the sample and the mixture was homogenized for 1 min. The emulsions were centrifuged (1200 *g*, 5 min) and the emulsion volume was determined. EA (in percentage) was calculated by the ratio between the volumes of emulsified layer and total volume used. To determine ES, the prepared emulsions were heated at 80 °C for 30 min, cooled to room temperature and centrifuged (1200 *g*, 5 min). ES (in percentage) was calculated by the ratio between the volumes of remaining emulsified layer and original emulsion volume.

3.1.3.3. *Antioxidant potential*

To determine the antioxidant potential, extracts were prepared by mixing 1 g of SCG or CS with 40 ml of methanol at 60% (v/v). The mixtures were heated during 90 min in a water-bath at 60-65 °C under magnetic stirring. After this time, the extracts were separated by centrifugation (2500 *g*, 20 min), filtered through 0.22 μm filters, and quantified for calculations. The antioxidant

activity of the extracts was determined by two methods: the free radical scavenging activity (DPPH) assay and the ferric reducing antioxidant power (FRAP) as described below.

3.1.3.3.1. Free radical scavenging activity

Free Radical Scavenging Activity (DPPH) assay was determined according to method described by Hidalgo et al. (2010) with some modifications. For the reactions, 10 µl of each duly diluted extract was added to 290 µl of DPPH solution (6×10^{-5} M in methanol and diluted to an absorbance of 0.700 at 517 nm) in a 96-well microplate. The resulting solutions were vortexed and allowed to stand for 30 min in darkness at room temperature. Then the absorbance was measured at 517 nm in a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria) using methanol as blank. The control solution consisted in using methanol instead of the sample. The radical scavenging activity was calculated by using the Eq 3.2, where A_c and A_s are the absorbance of the control solution and the absorbance of the sample solution, respectively. The DPPH values of the each sample were expressed as micromoles of trolox equivalents (TE) per dry weight material (µmol TE/g dry material).

Eq 3.2 $$\% \text{ inhibition of DPPH} = (1 - A_s / A_c) * 100$$

3.1.3.3.2. Ferric reducing antioxidant power

The antioxidant activity by the ferric reducing antioxidant power (FRAP) assay was determined according to the method described by Benzie and Strain (1996) with some modifications. A 10 µl aliquot of the filtered and duly diluted extract was mixed with 290 µl of FRAP reagent in a 96-well microplate, and incubated at 37 °C for 15 min. After that, the absorbance was determined at 593 nm using distilled water as blank. FRAP reagent was freshly prepared by mixing a 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl with a 20 mM $FeCl_3$ solution and 0.3 M acetate buffer (pH 3.6) in a proportion 1:1:10 (v/v/v). A calibration curve was constructed using an aqueous solution of ferrous sulfate ($FeSO_4.7H_2O$ at 200, 400, 600, 800 and 1000 µM). The FRAP values were expressed as millimoles of ferrous equivalent per dry weight material (mmol Fe(II)/g dry material).

3.1.4. Structural Characterization

3.1.4.1. *Morphology and Porosity*

Images of the SCG and CS particles were obtained by scanning electron microscopy (SEM) using an Ultra-high resolution Field Emission Gun Scanning Electron Microscope, Nova 200 Nano SEM, FEI Company. Previous to the analyses, the samples were covered with a very thin film (35 nm) of Au-Pd (80-20 weight %). The images were obtained by applying an acceleration voltage of 10kV, at 200- and 2,000-fold magnifications.

The surface area and porosity of the particles were determined by N_2 adsorption/ desorption isotherms at -196.15 °C using a Quantachrome Instruments Nova 4200e analyzer, as described by Mussatto et al. (2010). The specific surface area (S_{BET}) was determined by the BET method (Brunauer et al., 1938). Total volume of pores was calculated from the N_2 adsorption isotherm at a relative pressure of 0.99. The BJH method (Barrett et al., 1951) was used to evaluate the pore sizes distribution, the mesopore volume, and the specific surface area from adsorption/desorption isotherms.

3.1.4.2. *Thermal behavior*

Differential scanning calorimetry (DSC) and thermogravimetric analyses (TGA) were performed in equipment Shimadzu DSC-50 and Shimadzu TGA-50 (Shimadzu Corporation, Kyoto, Japan), respectively. For the analyses, approx. 10 mg of the sample were placed in an aluminum pan (Al crimp Pan C.201-52943) using an empty pan as reference. The measurements were carried out between 25 and 600 °C with a linear increase of 10 °C per min, under nitrogen atmosphere. TASYS software (Shimadzu Corporation, Kyoto, Japan) and TA Universal Analysis software (TA instruments, universal analysis 2000, USA) were used for data analysis. Enthalpy was calculated using the area of the peaks between the onset and the end set temperatures.

3.1.4.3. *Chemical bonding of constituents*

The chemical groups and bonding arrangement of constituents present in SCG and CS structures were determined by Fourier transform infrared spectroscopy (FTIR) using a Jasco infrared spectrometer (FT/IR-4100) equipped with a diamond-composite attenuated total reflectance (ATR) cell. The measurements were recorded with a wavenumber range from 4000 to 600 cm^{-1} at a resolution of 8 cm^{-1} and 16 scans per sample.

3.1.4.4. *Crystallinity*

Crystalline phases of SGC and CS samples were evaluated by X-ray diffraction (XRD) using a D8 Discover diffractometer (Bruker, corporation) with Cu tube (λ=1.5406 Å). The radiation was generated at 25 mA and 35 kV. The scattering angle of 2θ from 10° to 100° was measured at the step size of 0.04° and 1 s exposure at each step. To analyze and compare the peak positions, a cellulose spectrum from the International Centre for Diffraction Data database (ICDD card no. 00-003-0226) was used.

3.2. Results and Discussion

3.2.1. Chemical composition

3.2.1.1. *Cellulose, Hemicellulose and Lignin*

Polysaccharides are the most abundant components in SCG and CS. In both residues, sugars were polymerized into cellulose and hemicellulose structures, which when summed correspond to 51.50% and 40.45% (w/w) of their composition on a dry weight basis (Table 3.1). Cellulose (as glucose) was more abundant in CS, while hemicellulose was more abundant in SCG. The hemicellulose sugars and their composition significantly differ from one residue to another. Mannose was the main sugar in SCG hemicellulose. On the other hand, xylose that was the main sugar in CS hemicellulose was not present in the SCG composition. In terms of sugars composition, SCG was composed of 37.03% mannose, 31.90% galactose, 24.08% glucose, and 6.99% arabinose; while CS contained 58.76% glucose, 18.81% xylose, 9.29% galactose, 8.75% arabinose and 4.37%

mannose. These values are comparable to other reported in the literature for SCG and CS (Mussatto et al., 2011a; 2011b); some differences could be attributed to the extraction process and variety of coffee beans used. Taking into account that arabinogalactans, galactomannans and cellulose are the most abundant polysaccharides in coffee (Arya and Rao, 2007), it was expected to find at least glucose, galactose, mannose and arabinose sugars in SCG and CS composition.

Lignin was also a fraction present in significant amount in both SCG (23.90% w/w) and CS (28.58% w/w) (Table 3.1). The lignin content in these coffee residues was higher than the values reported for other lignocellulosic materials such as brewer's spent grains (19.40% w/w) (Meneses et al., 2013), sugarcane bagasse (18.93% w/w) (Mesa et al., 2011), rice straw (17.20% w/w) (Roberto et al., 2003) and barley straw (15.50% w/w) (Sun et al., 2002). Lignin is a macromolecule composed by a great variety of functional groups including phenolic hydroxyl, aliphatic hydroxyl, methoxyl, carbonyl, and sulfonates and its structure and composition vary from one raw material to another (Stewart, 2008). Chlorogenic, caffeic, and coumaric acids are the most relevant lignin components in coffee and such compounds play an important role in health due to their antioxidant properties (Maydata, 2002).

3.2.1.2. *Ashes, Minerals, Fat and Protein*

CS presented higher level of ashes (5.36% w/w) than SCG (1.30% w/w) (Table 3.1). A variety of mineral elements including potassium, calcium, magnesium, sulfur, phosphorus, iron, manganese, boron, copper, and others were present in the composition of their ashes (Table 3.2). Potassium was the most abundant mineral element in both, SCG and CS; followed by magnesium and phosphorus in SCG and by calcium and magnesium in CS. The most important minerals present in SCG and SC are considered micronutrients essential for the human health. They regulate multiple metabolic and physiological functions of the human body including hormonal and enzymatic activities, electrolyte balance, and normal growth (Kuan et al., 2011). These minerals also support vital processes such as respiration, digestion and circulation. Thus, the micronutrients found in SCG and CS could be used for the production of nutrient added foods.

Low fat content was present in both residues (2.29% and 3.78% w/w, for SCG and CS respectively). Otherwise, protein was present in more significant amount in these materials (Table 3.1). The protein content in CS (18.69% w/w) was similar to the value reported by Borrelli et al. (2004) for this same material (18.6% w/w); while the protein content in SCG (17.44% w/w) was a little higher than the value reported by Mussatto et al. (2011b) and by Ravindranath et al. (1972) for this coffee residue (about 14% w/w). These differences can also be due to the conditions used for the instant coffee preparation and the variety of coffee beans used. Both SCG and CS residues are rich in polysaccharides, lignin, proteins and minerals, showing their high biotechnological value to be used, for instance, as substrates or solid supports in fermentative processes for the extraction and production of compounds with important applications in the food and pharmaceutical industries (Mussatto et al., 2011a).

Table 3.1 Chemical composition of spent coffee grounds and coffee silverskin

Chemical components	Composition (g/100 g dry material)	
	Spent coffee grounds	Coffee silverskin
Cellulose (Glucose)	12.40 ± 0.79	23.77 ± 0.09
Hemicellulose	39.10 ± 1.94	16.68 ± 1.30
Arabinose	3.60 ± 0.52	3.54 ± 0.29
Mannose	19.07 ± 0.85	1.77 ± 0.06
Galactose	16.43 ± 1.66	3.76 ± 1.27
Xylose	nd	7.61 ± 0.02
Lignin	23.90 ± 1.70	28.58 ± 0.46
Insoluble	17.59 ± 1.56	20.97 ± 0.43
Soluble	6.31 ± 0.37	7.61 ± 0.16
Fat	2.29 ± 0.30	3.78 ± 0.40
Ashes	1.30 ± 0.10	5.36 ± 0.20
Protein	17.44 ± 0.10	18.69 ± 0.10
Nitrogen	2.79 ± 0.10	2.99 ± 0.10
Carbon/nitrogen (C/N ratio)	16.91 ± 0.10	14.41 ± 0.10
Total dietary fiber	60.46 ± 2.19	54.11 ± 0.10
Insoluble	50.78 ± 1.58	45.98 ± 0.18
Soluble	9.68 ± 2.70	8.16 ± 0.90

Results are expressed as mean ± standard deviation; n=3. nd: not detected.

Table 3.2 Mineral composition of spent coffee grounds and coffee silverskin

Mineral element	Composition (mg/kg dry material)	
	Spent coffee grounds	Coffee silverskin
Potassium	11700 ± 0.01	21100 ± 0.00
Calcium	1200 ± 0.00	9400 ± 0.01
Magnesium	1900 ± 0.00	3100 ± 0.00
Sulfur	1600 ± 0.00	2800 ± 0.00
Phosphorus	1800 ± 0.00	1200 ± 0.00
Iron	52.00 ± 0.50	843.30 ± 7.90
Aluminum	22.30 ± 3.50	470.60 ± 13.9
Strontium	5.90 ± 0.00	71.72 ± 0.30
Barium	3.46 ± 0.05	66.26 ± 0.26
Copper	18.66 ± 0.94	63.30 ± 1.00
Sodium	33.70 ± 8.75	57.30 ± 1.10
Manganese	28.80 ± 0.70	50.00 ± 0.60
Boron	8.40 ± 1.10	31.90 ± 1.40
Zinc	8.40 ± 0.20	22.30 ± 0.10
Cobalt	15.18 ± 0.05	21.39 ± 1.04
Iodine	< 0.10	18.30 ± 1.64
Nickel	1.23 ± 0.59	1.64 ± 0.34
Chromium	< 0.54	1.59 ± 0.00
Molybdenum	< 0.08	0.24 ± 0.29
Vanadium	< 0.29	1.01 ± 0.05
Lead	< 1.60	< 1.60
Selenium	< 1.60	< 1.60
Gallium	< 1.47	< 1.47
Tin	< 1.30	< 1.30
Cadmium	< 0.15	< 0.15

Results are expressed as mean ± standard deviation; n=3.

3.2.1.3. *Total, soluble and insoluble dietary fibers*

Dietary fiber including cellulose, hemicellulose, lignin, pectic substances, gums and mucilages, is known as the edible part of plants that is resistant to digestion and absorption in the human small intestine, with complete or partial fermentation in the large intestine (Betancur-Ancona et al., 2004). The content of total dietary fiber (TDF) in SCG (60.46% w/w) was higher than in CS (54.11% w/w). Additionally, insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) were also present in higher amounts in SCG than in CS (Table 3.1).

However, both residues showed similar proportion of IDF and SDF with respect to the total fiber composition, being IDF correspondent to 84% and 85% of the TDF in SCG and CS, respectively; and SDF correspondent to 16% and 15% of the TDF in SCG and CS, respectively. The IDF and SDF contents in CS were similar to the values reported by Borrelli et al. (2004), and by Pourfarzad et al. (2013), who found 86% IDF and 14% SDF in the total dietary fiber (62.2% w/w) present in CS. The higher content of IDF than SDF in the samples is justifiable since cellulose, hemicellulose and lignin are part of the insoluble fibers and significant amounts of these fractions are present in the composition of SCG and CS.

The SDF values in SCG and CS revealed larger soluble fiber potential of these coffee residues when compared to other materials such as Jack bean (*Carnavalia ensiformis*) (6.04%), lima bean (*Phaseolus lunatus*) (2.61%) (Betancur-Ancona et al., 2004), rice husk (2.23%), wheat straw (6.48%) and okara (10.17%) (Kuan and Liong, 2008). It is important emphasizing that each type of fiber (insoluble and soluble) has specific properties. For instance, SDF possess large water retention, promotes the creation of bacterial flora and decreases the absorption of fat and sugars. On the other hand, IDF has low water retention, accelerates the movement of food through the digestive system and promotes stool regularity. SCG and CS are materials with high levels of SDF and IDF, and therefore, they have great potential to be used as raw material in the development of functional foods. In this sense, a previous study demonstrated that CS supports the growth of bifidobacteria in vitro, suggesting the possibility of producing prebiotic foods from CS (Borelli et al., 2004).

3.2.2. Functional properties

3.2.2.1. *Water holding capacity and Oil holding capacity*

Water holding capacity (WHC) and oil holding capacity (OHC) are important properties to be considered in food processing. These properties can be defined as the capacity that a material has to retain water or oil after application of external centrifugal gravity force or compression. SCG showed higher WHC and OHC than CS (Table 3.3). According to some authors, WHC and OHC can be related with the particle size of the material; the holding capacities being increased when smaller particle sizes are used, as a consequence of the highest packing density of smaller particles (Murthy and Naidu., 2012). In the present study, particle sizes with diameter ≤ 500 μm were used because this size is considered ideal to evaluate these functional properties (Betancur-Ancona et al., 2004; Raghavendra et al., 2004) as the contact between the particle surface area and the liquid is enhanced. However, particle structure and its composition can also contribute to the overall distribution of water or oil (Robertson et al., 2000).

Table 3.3 Functional and physiological properties of spent coffee grounds (SCG) and coffee silverskin (CS)

Functional and physiological properties	SCG	CS
WHC (g water/ g dry sample)	5.73 ± 0.10	5.11 ± 0.20
OHC (g oil/ g dry sample)	5.20 ± 0.30	4.72 ± 0.10
Emulsifying activity (%)	54.72 ± 0.90	57.50 ± 0.90
Emulsion stability (%)	92.38 ± 0.90	88.18 ± 1.20
Antioxidant potential		
DPPH (μmol TE/g dry material)	20.04 ± 0.05	21.35 ± 0.39
FRAP (mmol Fe(II)/g dry material)	0.102 ± 0.01	0.045 ± 0.01

Results are expressed as mean ± standard deviation; n=3. WHC: water holding capacity; OHC: oil holding capacity; DPPH: antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl assay; FRAP: antioxidant activity by the ferric reducing antioxidant power assay.

WHC has also been reported to be higher in materials containing more elevated amounts of total dietary fiber (Raghavendra et al., 2004). This is in agreement with the results obtained in the present study, which revealed that SCG presents higher TDF (Table 3.1) and WHC (Table 3.3) than CS. In addition, SCG and CS showed higher WHC than other materials such as rice husk, wheat straw and okara (Kuan and Liong, 2008), which could also be related to the presence of more fibers in SCG and CS than in these materials. The OHC has also been reported to be dependent of some properties of the material sample, including surface properties, thickness, overall charge density and hydrophobic nature (Kuan and Liong, 2008). According to some authors, lignin-richer samples present higher OHC values (Femenia et al., 1997).

The WHC and OHC results obtained for SCG and CS allow concluding that these coffee residues are materials with great swelling capacity, which is one of the most desirable parameters for the functionality of dietary fibers. WHC, for instance, has been considered an important parameter to measure the capacity can have the fibers incorporated in the diet to modify stool weight (Cummings et al., 1978). On the other hand, OHC is fundamental for stabilization of high-fat products and emulsions (Tiwari and Cummins, 2011). Both, WHC and OHC play an important role during preparation, processing and storage of foods. Moreover, they can influence in the nutritional and sensory characteristics of food and its physical behavior (Tiwari and Cummins, 2011).

3.2.2.2. *Emulsifying activity and Emulsion stability*

Emulsifying activity (EA) is the capacity that a compound has to form a homogenous dispersion of two immiscible liquids or emulsions, while emulsifying stability (ES) is the effectiveness of a molecule to maintain a thermodynamically stable emulsion (Sánchez-Zapata et al., 2009). SCG and CS showed similar values of EA (54.72% and 57.50%, respectively), while ES was slightly higher in SCG than in CS (92.38% and 88.18%, respectively) (Table 3.3). Both coffee residues presented higher ES values than other materials such as lima bean (28.25%) (Betancur-Ancona et al., 2004), papaya kernel flour (58%), corncobs (80%) and wheat straw (86.94%) (Kuan et al., 2011). This behavior is directly related to the type of fiber and the percentage of soluble and insoluble fiber in

the material composition. Moreover, the protein fraction present in the residues plays also an important role in anchoring the moiety of fiber to the oil or water interface (Kuan and Liong, 2008).

SCG and CS are then materials with excellent emulsifying activity and emulsion stability, and present therefore great potential to be used as emulsifiers in different food products including beverages, dairy, baking, confectioneries or in products for animal nutrition, which require long emulsion stability.

3.2.2.3. *Antioxidant potential*

In order to evaluate the antioxidant activity of SCG and CS, extracts were produced by solid-liquid extraction using methanol, which has been considered one of the best solvents to extract antioxidant compounds from natural sources duo its polarity, viscosity and ability to promote high extraction yields (Mussatto et al., 2011c). According to the results, SCG and CS showed similar antioxidant potential (20.04 and 21.35 μmol TE/g dry material, respectively) when analyzed by the DDPH assay. However, the FRAP assay revealed a 2.3-fold higher antioxidant potential for SCG when compared to CS (Table 3.3). According to the current literature, different methods can be used to evaluate the antioxidant activity in food and biological systems. However, as each method is based on a different reaction, it is strongly advisable determining the antioxidant potential of a sample by different methods in order to better interpret the results. Such a fact was demonstrated in the present study since the DPPH assay was not able to detect significant differences in the antioxidant potential of the samples, while the FRAP assay was.

Antioxidant compounds have numerous applications in food, cosmetic and pharmaceutical areas, because they can protect against chronic and degenerative diseases such as cancer and diabetes mellitus, and decrease the risk factors of cardiovascular diseases, among others (Ao et al., 2011). These results suggest the possibility of reusing both coffee residues (mainly SCG) to obtain such compounds.

3.2.3. Structural characterization

3.2.3.1. *Morphology and Porosity*

Images obtained by scanning electron microscopy revealed significant morphological differences between SCG and CS. CS particles (Figure 3.1 c and d) presented a denser morphology than SCG particles (Figure 3.1 a and b), and were composed of thin sheets of material that resembles sawdust. In terms of porosity, the N_2 adsorption/desorption isotherms revealed that SCG has higher porosity than CS (Figure 3.2). However, the amount of N_2 adsorbed by both the samples was very low, suggesting that they have poorly developed mesoporosity. It was also verified the absence of micropores in the samples since microporous materials present N_2 adsorption/desorption isotherms with tendency to form a plateau at low relative pressures (Mussatto et al., 2010), which was not verified in the present study for any of the cases. Similar conclusions were obtained by the BET surface area (S_{BET}) results, which revealed that SCG has higher S_{BET} (4.3 m^2/g) than CS (2.1 m^2/g), but the S_{BET} was very low for both the samples, and micropores were not detected in any of them. The total volume of pores was also very close for both residues (0.004 and 0.003 cm^3/g for SCG and CS, respectively).

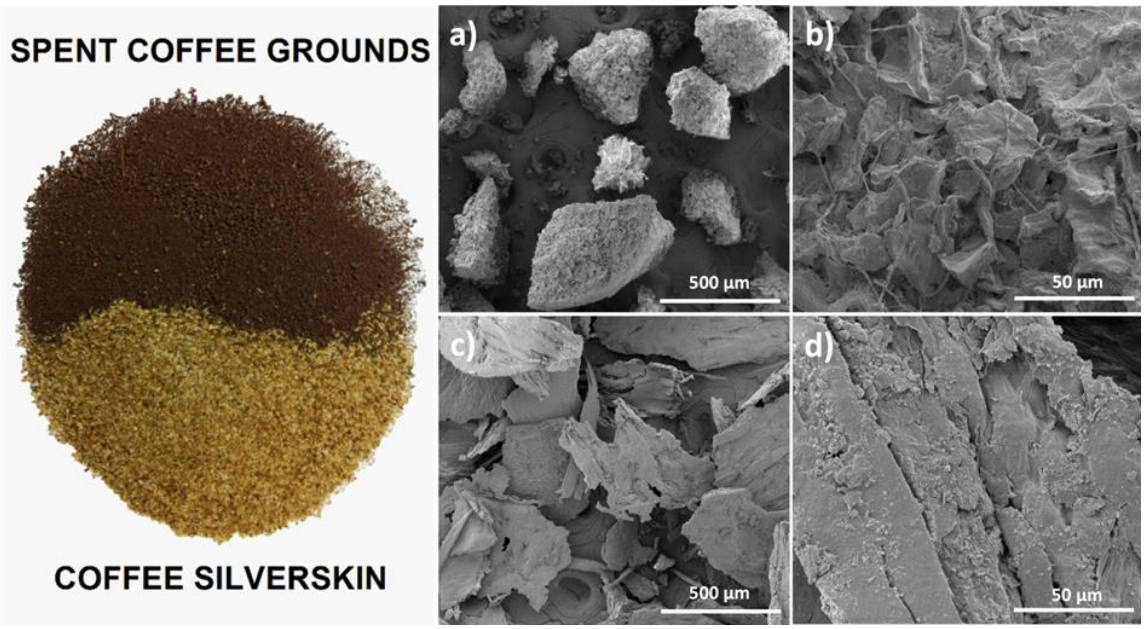

Figure 3.1 Micrographs by scanning electron microscopy (SEM) of spent coffee grounds and coffee silverskin particles. Magnification: 200X (a, c) and 2000X (b, d)

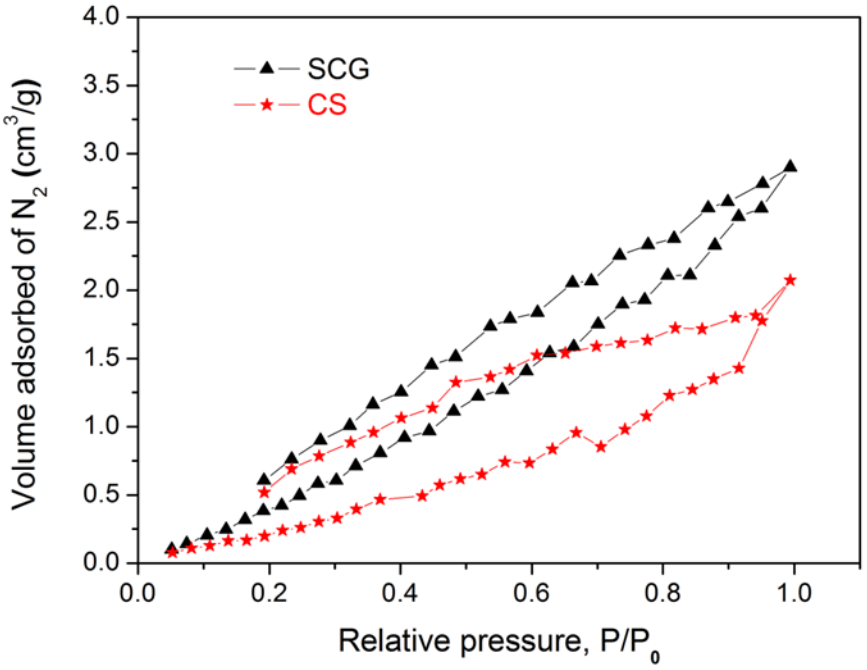

Figure 3.2 N_2 adsorption/desorption isotherms at -196.15 ºC. Volume adsorbed of N_2 as a function of the relative pressure for SCG and CS

Analyses of the mesopore size distribution by the BJH method (Figure 3.3) showed a well-defined profile for both samples with most of the mesopores at around 3 and 12 nm (r = 10 and 60 Å, respectively), and a non-significant amount of mesopores larger than 18 nm. These analyses confirm that SCG and CS are materials with very low porosity, containing mesopores with less than 12 nm, specific surface areas between 2 and 5 m^2/g, and specific pore volumes between 0.003 and 0.004 cm^3/g. The low porosity of these materials can be advantageous depending on the final application. On the other hand, when materials with higher porosity are desired, an alternative to improve the porosity of SCG and CS would be submitting these materials to any treatment in order to promote a total or partial degradation of the cellulose-lignin matrix, which would decrease their crystallinity increasing the porosity as a consequence.

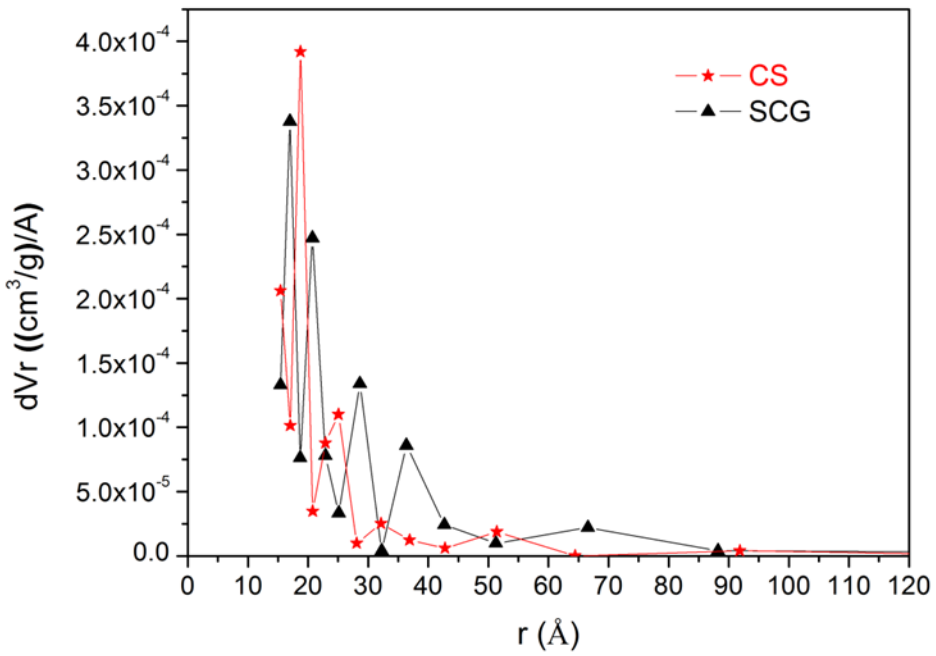

Figure 3.3 Pores size distribution by the BJH method - the derivative of the desorbed volume as a function of the pore radius, which represents the change of volume desorbed by SCG and CS in a pore size range. Standard deviation values were less than 2.5% in all cases

3.2.3.2. *Thermal behavior*

The DSC thermogram (Figure 3.4) shows the thermal transitions of the samples between 25 and 600 °C obtained at a heating rate of 10 °C per min under a constant nitrogen atmosphere. The thermograms obtained for SCG and CS exhibited two events: an initial endothermic phase followed by an exothermic phase. For both, SCG and CS, an early endothermic event was observed with a peak at 76.89 and 74.60 °C, respectively, and an associated enthalpy change of 192.80 J/g and 102.80 J/g. This event was related to the melting transition that occurs over a range of temperature due to the presence of impurities in the samples, the vaporization of water (indicating the presence of hydrophilic groups) and the crystalline nature of the materials. This first event allows concluding that SCG and CS have similar melting point at 76.89 and 74.60 °C, respectively. The second event corresponded to an exothermic transition and was observed at 303.00 and 317.70 °C for SCG and CS, respectively, with an associated enthalpy change of 68.38 J/g and 7.75 J/g.

This transition was related to the thermal depolymerisation and branching of the samples, occurring at temperature ranges varying between 220 and 310 °C (Sperling, 2006).

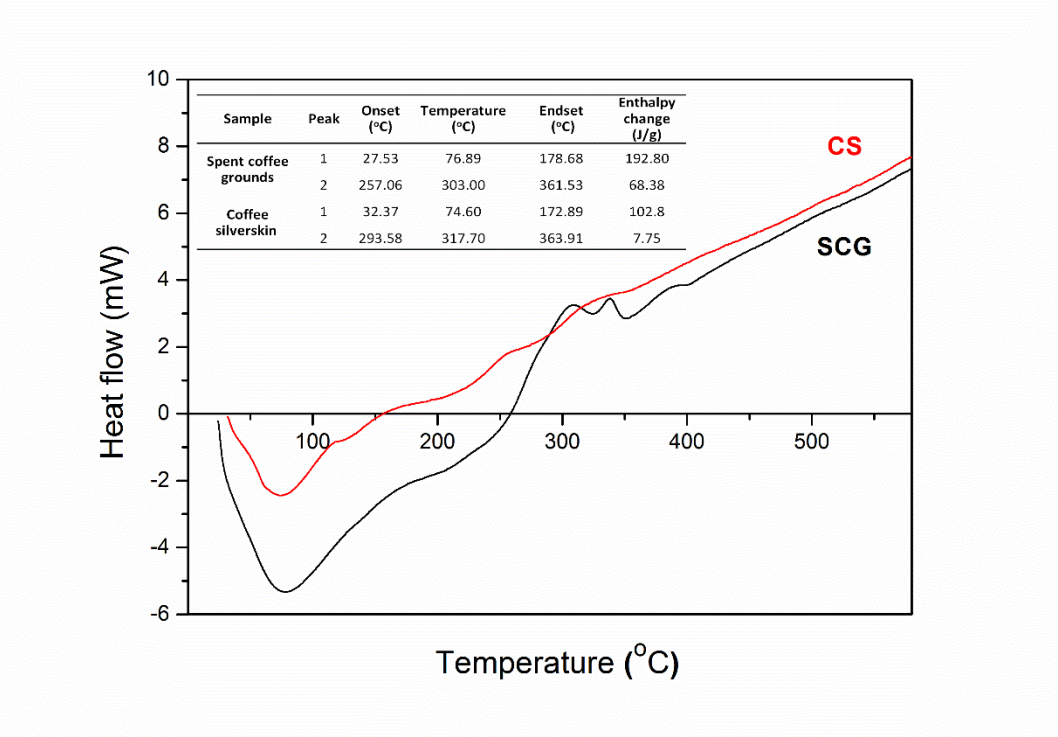

Figure 3.4 DSC curves obtained for spent coffee grounds (SCG) and coffee silverskin (CS)

The TGA curves (Figure 3.5) show the weight losses of the samples when exposed to heating until 600 °C. SCG and CS present similar TGA curves with three defined mass loss stages. The first one started at approx. 60.60 °C and 61.58 °C and corresponded to soft weight losses of about 7.77% and 6.80% for SCG and CS respectively, as a result of the water evaporation (dehydratation of the sample). The greatest transformation and mass loss occurred during the second stage, at approx. 300 °C. At this stage, the depolymerization and decomposition of polysaccharides and some oils present in the sample occur, providing weight losses of 43.50% and 48.01% for SCG and CS, respectively. Finally, the third and last thermal stage related to the decomposition of the samples started at 499.29 °C for SCG and at 457.24 °C for CS and results in weight losses of 33.08% and 34.17%, respectively.

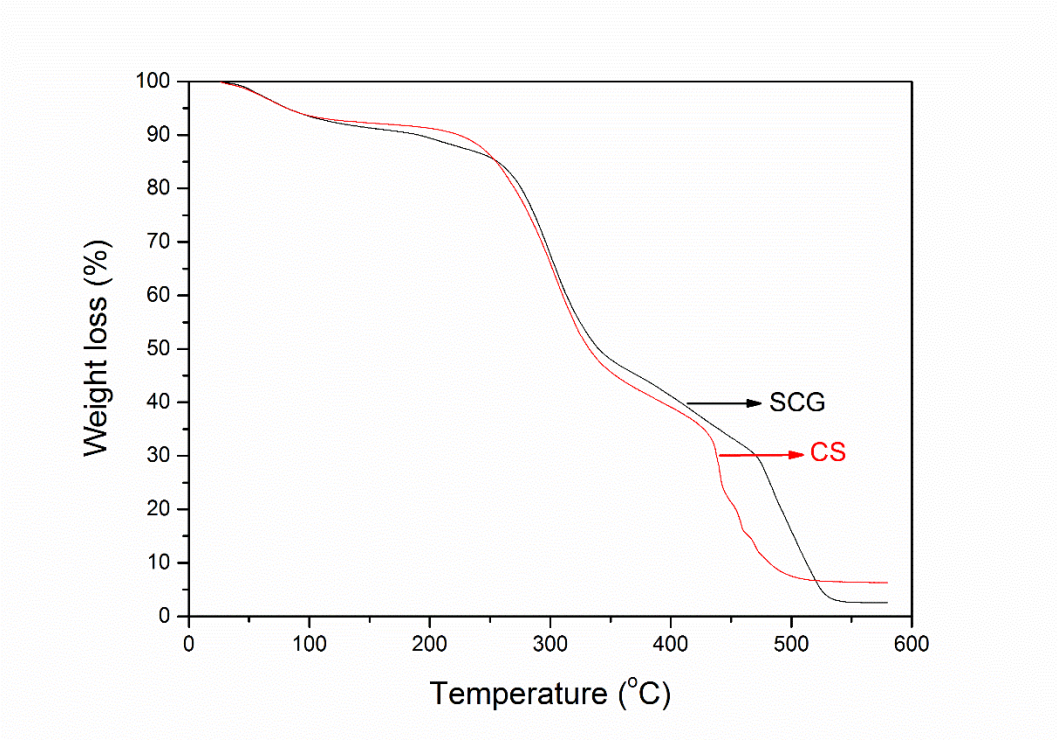

Figure 3.5 TGA curves obtained for spent coffee grounds (SCG) and coffee silverskin (CS)

3.2.3.3. *Chemical groups and bonding arrangement of constituents*

FTIR analyses (Figure 3.6) revealed that SCG and CS have absorption bands typical of lignocellulosic materials, although the magnitude of these bands differs to each residue. The broad peak between 3600 and 3200 cm^{-1} was related to the hydroxyl group of O–H stretching vibration. The region between 3000-2800 cm^{-1}, with two sharp bands at 2923 and 2852 cm^{-1}, was attributed to C-H stretching vibration. These bands have been previously reported in spectra of roasted Arabica and Robusta coffee samples (Kemsley et al., 1995), and roasted coffee husks (Reis et al., 2013). Moreover, studies of FTIR analysis from caffeinated beverages such as tea, coffee and soft drinks have reported peaks at this same region (2882 and 2829 cm^{-1}), which were related to the asymmetric stretching of C-H bonds of methyl ($-CH_3$) group in the caffeine molecule and can be successfully used to develop predictive models for quantitative analysis of caffeine (Paradkar and Irudayaraj, 2002). The band between 1700 and 1600 cm^{-1} was highly associated with chlorogenic acids and caffeine (Ribeiro et al., 2010). Then, the peak at 1654 cm^{-1} can be attributed to the

absorption of these compounds, being the peak more intense when their concentration in the sample increases. The broad band between 1135 and 952 cm^{-1} resulted from the stretching vibration of C–O in C–O–H bonds such as glycosidic bonds, and are related to galactomannans polysaccharide' sugars (Figueiró et al., 2004).

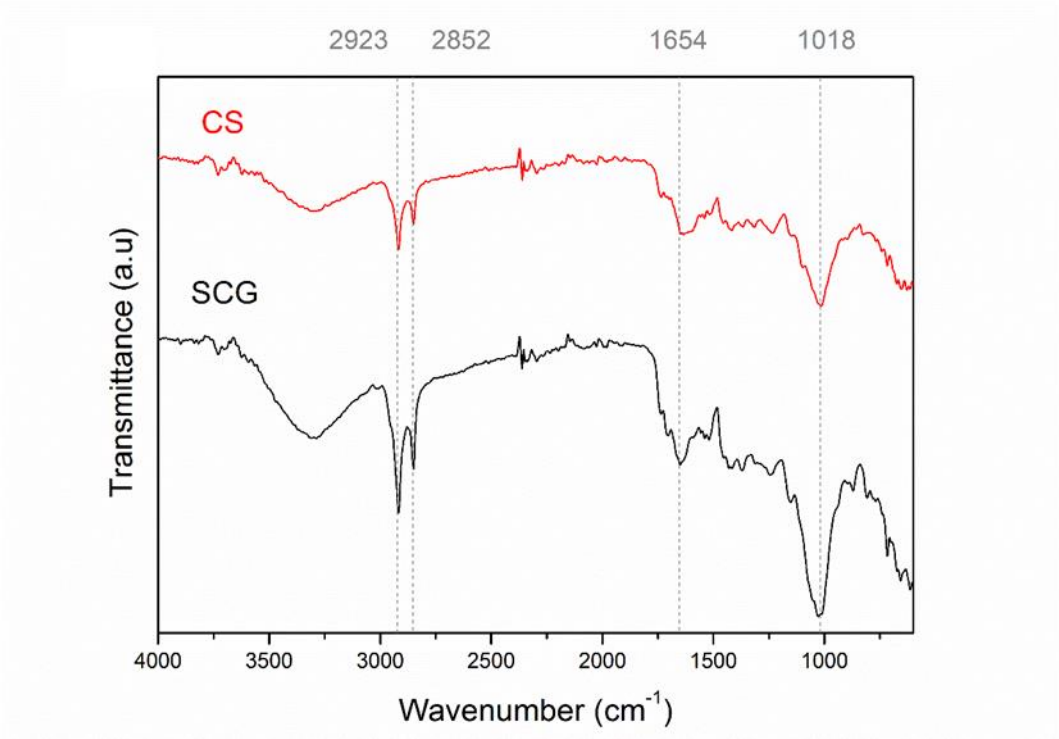

Figure 3.6 FTIR spectra obtained for spent coffee grounds (SCG) and coffee silverskin (CS)

3.2.3.4. *Crystallinity*

In order to evaluate and compare the crystallinity of the coffee residues, a cellulose spectrum taken from the International Centre for Diffraction Data (ICDD) database was used as reference. As can be seen in Figure 3.7, SCG and CS presented similar XRD spectra, which, when compared to the cellulose spectrum used as reference, indicate the existence of crystalline regions in the structure of both coffee residues. The cellulose molecule is known to have crystalline and amorphous regions. Crystalline regions are mostly responsible by a high tensile strength and represent cellulose less accessible to chemical attacks due to hydrogen strong interactions between

the microfibers (Ragauskas and Huang, 2013). In contrast, hemicellulose and other constituents of SCG and CS exhibit an amorphous structure more easily degradable and susceptible to chemical attacks.

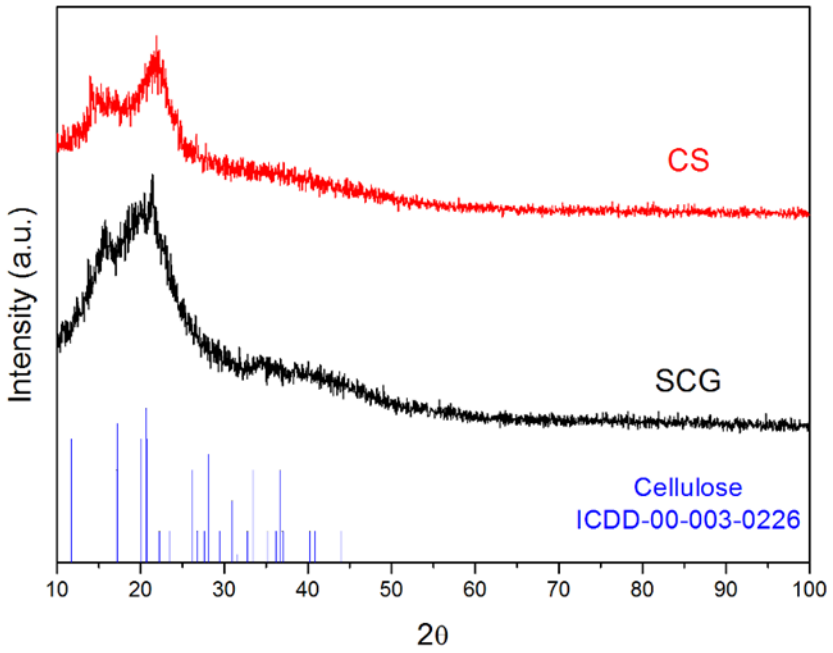

Figure 3.7 XRD diffractograms obtained for spent coffee grounds (SCG) and coffee silverskin (CS). Cellulose peak positions indicated as reference in the XRD diffractograms were obtained from the International Centre for Diffraction Data (ICDD) database (ICDD card no. 00-003-0226)

Although CS showed higher cellulose content than SCG (Table 3.1), the XRD spectra reveal that SCG is more crystalline than CS, suggesting important differences in the cellulosic structure of both materials. According to some authors, the thermal treatment by which the coffee beans are subjected could be responsible for at least a part of the crystallinity observed in SCG structure since this process promotes the elimination of some water molecules incorporated into the crystal fraction, transforming some α-polymorph to β-crystal phase structures (Rivera et al., 2011).

3.3. Conclusions

This study allows concluding that SCG and CS are sugar-rich lignocellulosic materials composed also by high levels of insoluble, soluble and total dietary fibers. Both residues have interesting functional properties including water holding capacity, oil holding capacity, emulsion activity and stability, and antioxidant potential, which open up possibilities for their reutilization in different biotechnological process. They could be used, for example, as preservatives in food formulations, as natural antioxidant sources for application in food and pharmaceutical products, or as raw material to obtain new functional ingredients for food industry. SCG and CS are also thermostable in a large range of temperature, being therefore suitable for application in the manufacture of biomaterials and encapsulation products for several industrial purposes. In brief, the present study allows concluding that SCG and CS have characteristics that make possible their reutilization in different industrial fields. Despite some efforts have recently been done in order to find possible alternatives to reuse these residues, the implementation of industrial processes using SCG or CS as raw material is still a challenge to be surpassed. This study gives support to direct further research and developments in this area.

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SECTION III

POLYSACCHARIDES

CHAPTER 4

EXTRACTION OF POLYSACCHARIDES BY AUTOHYDROLYSIS OF SPENT COFFEE GROUNDS AND THE EVALUATION OF THEIR ANTIOXIDANT PROPERTIES

The following chapter is partially based on the results published in: Lina F. Ballesteros, José A. Teixeira & Solange I. Mussatto. Extraction of galactomannans and arabinogalactans by autohydrolysis of spent coffee grounds and evaluation of their antioxidant activity (*Accepted with revisions in Carbohydrate Polymers)*

4. Introduction

Autohydrolysis is an eco-friendly technology that does not require the use of chemical agents for reaction. This technique has been used to extract polysaccharides from natural sources including *Eucalyptus globulus* wood (Romaní, Garrote, López, & Parajó, 2011), *Pinus pinaster* wood and rice husks (Rivas, Conde, Moure, Domínguez, & Parajó, 2013), among others. During autohydrolysis, a slightly acid media is obtained due to the partial release of acetyl groups from the material structure, providing a selective depolymerization of the hemicellulose (Nabarlatz, Ebringerová, & Montané, 2007). Autohydrolysis of lignocellulosic materials is a complex process since many factors such as the liquid/solid ratio, temperature, particle size of the solid matrix, the extraction time, as well as the structure and polymerization degree of molecules and their interaction with proteins, minerals and phenolic compounds can influence in the reaction efficiency.

Taking these facts into account, the purpose of the present chapter was to evaluate the extraction of polysaccharides from SCG by using the environmentally friendly technique of autohydrolysis. Experimental assays were performed using different temperatures (160 to 200 °C), liquid/solid ratios (5 to 15 ml water/g SCG) and extraction times (10 to 50 min) in order to stablish the conditions that maximize the extraction of polysaccharides with high antioxidant activity. Thus, the effects of these operational variables on the extraction yield and antioxidant activity of the recovered polysaccharides were verified. The polysaccharides obtained under the best autohydrolysis conditions were chemically and structurally characterized.

4.1. Materials and methods

4.1.1. Raw material and chemicals

Spent coffee grounds (SCG) were provided by the Portuguese coffee industry NovaDelta-Comércio e Indústria de Cafés S.A. (Campo Maior, Portugal) and treated as in Section II - Chapter 3. All the chemicals used were analytical grade, purchased from Panreac Química (Barcelona,

Spain), Fisher Scientific (Leicestershire, UK) and Sigma–Aldrich (Chemie GmbH, Steinheim, Germany). Ultrapure water from a Milli-Q System (Millipore Inc., USA) was used.

4.1.2. Autohydrolysis

Autohydrolysis assays were performed under different conditions of temperature (160 to 200 °C), liquid/solid ratio (5 to 15 ml water/g SCG) and extraction time (10 to 50 min), which were combined according to a 2^3 central composite design. For the reactions, ultrapure water and the SCG were poured into 160 - ml cylindrical stainless steel reactors (Parr Instruments Company, Illinois, USA), which were duly closed and placed vertically into an oil-bath with open heating circulator and temperature control (Julabo, Labortechnik GmbH, Seelbach, Germany). The samples were left in the bath, previously heated until desired temperature, during the time required for each reaction. Then, the reactors were removed from the oil-batch and immediately cooled down in an ice-bath for 10 min to stop the reaction. The total content of each reactor was centrifuged (2500 *g*, 20 min) being the supernatant separated and treated to recover the polysaccharides present.

4.1.3. Polysaccharides recovery

In order to recover the polysaccharides present in the liquid fractions obtained after autohydrolysis of SCG, 30 ml of supernatant were mixed with absolute ethanol in a 1:3 (v/v) ratio and the mixture was left over night at 4 °C. The precipitated polysaccharides were recovered by centrifugation (2500 *g*, 20 min), hydrated with 30 ml distilled water and maintained in a shaker during 3 h, at 200 rpm and room temperature. Subsequently, the mixture was again centrifuged and the supernatant was frozen and freeze-dried. Freeze-dried powder was stored at room temperature and protected from light and humidity until further use. The total yield of the extraction process was expressed as milligrams of lyophilized material per gram of SCG (mg LM/g SCG).

4.1.4. Analytical methodology

For evaluating the properties of the polysaccharides recovered from SCG, ultrapure water and the lyophilized material were mixed to obtain 1.5 mg/ml. The samples were vortexed for 1 min, filtered through 0.22 µm filters and then stored for analyses.

4.1.4.1. *Total sugars*

The content of total sugars was determined by the anthrone-sulfuric acid assay. Briefly, a 50 µl aliquot of the sample (LM at 1.5 mg/ml) was mixed with 150 µl of anthrone reagent in a 96-well microplate. Then, the reaction mixture was placed at 4 °C for 10 min and was subsequently incubated at 100 °C during 20 min. After heating, the samples were allowed to cool down at room temperature for 20 min. The absorbance was determined in a spectrophotometer microplate reader (Sunrise Tecan, Grödig, Austria) set at 620 nm and using distilled water as blank. The anthrone reagent was prepared immediately prior to analysis by dissolving 0.1 g of anthrone in 100 ml of concentrated sulfuric acid (98%), protected from light and used within 12 h. A calibration curve was performed using a standard glucose solution (10, 60, 120, 200, 250, 300, 400 and 600 µg/ml). The content of total sugars was expressed as grams glucose equivalent per 100 grams of lyophilized material (g GLU/100 g LM).

4.1.4.2. *Phenolic compounds*

The content of phenolic compounds (PC) was determined by using the Folin-Ciocalteu reagent according to the colorimetric described by Singleton and Rossi (1965), adapted to a 96-well microplate. For the reactions, 5 µl of each filtered and duly diluted extract were mixed with 60 µl of sodium carbonate solution at 7.5% (w/v) and 15 µl of Folin–Ciocalteu reagent. Subsequently, 200 µl of distilled water were added and the solutions were mixed. Thereafter, the samples were heated at 60 °C for 5 min and were allowed to cool at room temperature. The absorbance was then measured by means of a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria) set at 700 nm. A calibration curve was made from gallic acid standard solutions (200, 400, 600, 800, 1000, 2000, 3000 mg/L) and the blank was prepared with distilled water. The total content of

phenolic compounds was expressed as milligrams of gallic acid equivalent per gram of lyophilized material (mg GAE/g LM).

4.1.4.3. *Reducing sugars*

The content of reducing sugars (RS) was estimated by the colorimetric method of DNS (3,5-dinitrosalicylic acid) adapted to a 96-well microplate (Gonçalves, Rodriguez-Jasso, Gomes, Teixeira, & Belo, 2010). Briefly, 25 µl of the sample (LM at 1.5 mg/ml) were mixed with 25 µl of DNS reagent and incubated at 100 °C for 10 min. Thereafter, 250 µl of distilled water were added to each well and the microplate was placed on an ice-bath to stop the reaction. The absorbance was determined in a spectrophotometer microplate reader (Sunrise Tecan, Grödig, Austria) set at 540 nm, using distilled water as blank. DNS reagent was freshly prepared by dissolving 2.5 g of 3,5-dinitrosalicylic acid in 25 ml of distilled water preheated at 80 °C. The solution was cooled at room temperature, and after, 50 ml of a 2 N sodium hydroxide solution and 75 g of potassium sodium tartrate were added being the final volume completed to 250 ml with distilled water. A standard calibration curve was prepared using glucose solution (0.2, 0.4, 0.6, 0.8, 1.0. 1.2, 1.4, 1.6, and 1.8 mg/ml). The content of RS was expressed as milligrams glucose equivalent per gram of lyophilized material (mg GLU/g LM).

4.1.4.4. *Antioxidant activity*

4.1.4.4.1. Total antioxidant activity

The total antioxidant activity (TAA) was estimated as described by Prieto and Aguilar (1999) with some modifications. Briefly, 200 µl of sample was added to a glass tube containing 2 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were covered and maintained during 90 min in a water-bath at 95 °C and then, placed to cool at room temperature. The absorbance was measured at 695 nm using a spectrophotometer V-560 (Jasco, Japan) against a blank of distilled water. A calibration curve was prepared with a standard solution of α-tocopherol (25, 75, 125, 250, 375 e 500 µg/ml). TAA values were expressed as milligrams of α-tocopherol equivalent per gram of lyophilized material (mg α-TOC/g LM).

4.1.4.4.2. Ferric reducing antioxidant power

The antioxidant activity by the ferric reducing antioxidant power (FRAP) assay was determined according to the methodology described in Section II - Chapter 3. The FRAP values were expressed as millimoles of ferrous equivalent per gram of lyophilized material (mmol Fe(II)/g LM).

4.1.4.4.3. Free radical scavenging activity

The DPPH radical scavenging activity was determined using the method described by Fukumoto & Mazza (2000) and Silva et al. (2004) in combination and with some modifications. For each sample, a dilution series (four different concentrations) were prepared. The reaction was carried out in a 96-well microplate containing 25 µl of sample and 200 µl of 150 µM DPPH solution (2,2-diphenyl-1-picrylhydrazyl dissolved in 80% methanol to an absorbance value of 0.700 at 515 nm). The produced solutions were vortexed and allowed to stand for 1 h in the dark at room temperature. Then the absorbance was measured at 515 nm in a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria) using methanol as blank. The control solution consisted in using methanol instead of the sample. The radical scavenging activity was calculated by using the Eq 3.2. A calibration curve was prepared with a standard solution of trolox diluted in methanol (40, 80, 100, 300, 400 and 600 µM). DDPH percent inhibition data were plotted as a function of antioxidant concentration to obtain DPPH inhibition concentration at 50% (IC_{50}). The IC_{50} values were expressed as micromoles of Trolox equivalent per gram of lyophilized material (µmol TE/g LM).

4.1.4.4.4. Radical cation decolorization

The radical cation decolorization (ABTS) assay of polysaccharides extracted from SCG was determined as described by Re et al. (1999) and Ozgen, Reese, Tulio, Scheerens, & Miller (2006) with some modifications. Each sample was diluted to four different concentrations such that the percent inhibition was between 20-80%. Assays were conducted by combining 130 µl of sample with 3 ml of ABTS radical cation solution. The resulting solutions were maintained during 30 min in darkness at room temperature, and the absorbance was then measured at 734 nm using a spectrophotometer V-560 (Jasco, Japan) being distilled water used as control solution instead of

the sample. ABTS radical cation was prepared by mixing 7 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) dissolved in water with a 2.45 mM potassium persulfate solution This mixture was vortexed for 2 min, set in ultrasonic bath during 20 min and then, left in the dark at 4 °C between 12-16 h for achieving a stable oxidative state. After this time, ABTS radical cation solution was diluted in a 20 mM acetate buffer (pH 4.5) solution to an absorbance of 0.70 ± 0.01 at 734 nm. A calibration curve was constructed using a standard solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) diluted in ethanol (50, 100, 200, 250, 300, 400 and 500 μM). The percent inhibition of ABTS radical cation was calculated using the same equation employed in the DPPH radical scavenging. The IC_{50} values were expressed as micromoles of Trolox equivalent per gram of lyophilized material (μmol TE/g LM).

4.1.5. Experimental design and data analysis

The influence of the independent variables, temperature (X_1, °C), liquid/solid ratio (X_2, ml/g) and extraction time (X_3, min), on the extraction of polysaccharides by autohydrolysis of SCG was evaluated through a 2^3 central composite design. The real and coded values of the variables are shown in Table 4.1. Statistical significance of the variables was determined at 5% probability level ($p < 0.05$). The data obtained from the design were fitted to second order polynomial equations, and the models were simplified by elimination of statistically insignificant terms. Statistical significance of the regression coefficients was determined by Student's *t*-test, and the proportion of variance explained by the models were given by the multiple coefficient of determination, R^2. Statistical analysis of the data and the determination of the conditions able to maximize the extraction of polysaccharides with high antioxidant activity were performed using the software Design expert (version 8.0).

4.1.6. Polysaccharide characterization

4.1.6.1. *Sugars composition*

Polysaccharides recovered from SCG were submitted to a dilute acid hydrolysis with sulfuric acid (120 mg H_2SO_4/g LM). The mixture was vortexed and sterilized at 120 °C for 20 min. Then,

sugar concentrations was made by high performance liquid chromatography (HPLC) using an equipment LC-10 A (Jasco, Japan) with a Meta Carb 87P column at 80 °C, ultrapure water previously boiled and degassed in a ultrasonic bath as mobile phase, and a refractive index (RI) detector. The flow rate and the injection volume were adjusted to 0.4 ml/min and 20 µl, respectively. Glucose, arabinose, galactose and mannose were identified and quantified from standard curves made with known concentrations of each compound and expressed as % mol. The response of the RI detector was recorded and integrated using the Star Chromatography Workstation software (Varian).

4.1.6.2. *Structural characterization*

Crystalline phases of SCG polysaccharides were evaluated by X-ray diffraction (XRD) as described in Section II - Chapter 3. The chemical groups and bonding arrangement of constituents present in the polysaccharides were determined by Fourier transform infrared spectroscopy (FTIR) using a Perkin- Elmer 16 PC spectrometer (Boston, USA) equipped with a diamond-composite attenuated total reflectance (ATR) cell. The measurements were recorded with a wavenumber range from 4000 to 400 cm^{-1} and 16 scans per sample.

Differential scanning calorimetry (DSC) was performed in equipment DSC 200 F3 Maia (Netzsch, Germany) and thermogravimetric analyses (TGA) were carried out in equipment SDT 2960 simultaneous DSC-TGA (TA instruments, USA). For the analyses, approx. 5 mg of the sample were placed in an aluminum pan. The measurements were carried out between 25 and 600 °C with a linear increase of 10 °C per min. TA Universal Analysis software (TA instruments, universal analysis 2000, USA) was used for data analysis. Enthalpy was calculated using the area of the peaks between the onset and the end set temperatures.

4.2. Results and discussion

4.2.1. Extraction results

Autohydrolysis technique has been widely used for the extraction of polysaccharides, especially hemicelluloses from natural sources. Although this technique causes a selective depolymerization of hemicellulose chains for oligosaccharides and monosaccharides sugars, other components such as PC (derived from lignin) may also appear in the reaction medium (Clark & Mackie, 1984). Therefore, the present study evaluated the effect of different process variables including temperature, liquid/solid ratio and extraction time on the recovery of polysaccharides from SCG in order to select the conditions that maximize the polysaccharides extraction with high antioxidant powder. The content of PC in the recovered lyophilized material was also quantified.

Table 4.1 shows the experimental conditions used in each assay and the respective results of total sugars, PC, RS, FRAP, DPPH, ABTS, TAA and total yield of the extraction process. As it can be seen, polysaccharides were extracted from SCG in all the studied conditions; however the extraction yield greatly varied according to the conditions used. In terms of composition, the highest amount of total sugars in the LM corresponded to 34.92% (w/w) and was achieved when using a liquid/solid ratio of 15 ml/g SCG during 30 min at 180 °C (assay 14); while the lowest amount of total sugars (15.16% (w/w)) was obtained when using 10 ml/g SCG, at 160 °C and 30 min (assay 9). In general, the results were increased when the values of the variables were raised, but this behavior was observed until a certain limit only. When the temperature was increased to 200 °C, for example, the amount of total sugars was lower than when intermediate conditions were applied. This could be related to the fact that when the highest temperature was used, a stronger hydrolysis of polysaccharides and subsequent degradation of these components might have occurred.

Table 4.1 Experimental conditions and results obtained during the extraction of polysaccharides by autohydrolysis of spent coffee grounds (SCG). Assays according to a 2^3 central composite design

A[a]	Process variables[b] (real and (coded) values)			Responses[c]							
	X_1	X_2	X_3	Total sugars	PC	RS	FRAP	DPPH	ABTS	TAA	Yield
1	160 (-1)	5 (-1)	10 (-1)	25.30 ± 6.28	239.38 ± 2.50	88.45 ± 4.10	0.70 ± 0.02	468.07 ± 26.07	532.86 ± 15.26	268.28 ± 3.31	11.81
2	200 (+1)	5 (-1)	10 (1)	21.79 ± 5.43	214.14 ± 12.08	70.40 ± 4.93	0.61 ± 0.03	501.82 ± 21.37	454.60 ± 7.15	218.21 ± 14.04	20.87
3	160 (-1)	5 (-1)	50 (+1)	21.42 ± 0.80	202.00 ± 3.01	59.90 ± 4.84	0.56 ± 0.03	434.26 ± 6.67	429.23 ± 7.41	187.49 ± 12.78	25.79
4	200 (+1)	5 (-1)	50 (+1)	23.04 ± 1.83	103.54 ± 3.51	20.09 ± 0.81	0.26 ± 0.02	207.35 ± 0.95	202.00 ± 2.39	108.64 ± 4.01	19.32
5	160 (-1)	15 (+1)	10 (-1)	28.56 ± 3.38	234.14 ± 5.30	93.93 ± 4.44	0.68 ± 0.05	515.95 ± 7.00	600.24 ± 12.20	228.46 ± 5.03	35.87
6	200 (+1)	15 (+1)	10 (-1)	25.10 ± 3.45	195.14 ± 12.98	63.26 ± 2.71	0.51 ± 0.02	420.39 ± 7.27	427.74 ± 4.42	185.29 ± 5.92	42.61
7	160 (-1)	15 (+1)	50 (+1)	31.19 ± 5.56	232.95 ± 11.01	76.80 ± 3.62	0.69 ± 0.03	573.93 ± 29.96	529.47 ± 18.05	254.44 ± 3.25	45.15
8	200 (+1)	15 (+1)	50 (+1)	24.60 ± 6.37	82.33 ± 1.63	12.96 ± 0.92	0.20 ± 0.01	155.59 ± 0.58	144.60 ± 0.68	131.52 ± 2.66	32.62
9	160 (-1)	10 (0)	30 (0)	15.16 ± 0.92	239.14 ± 6.07	78.77 ± 2.90	0.61 ± 0.04	504.45 ± 3.25	431.29 ± 0.55	255.38 ± 4.06	35.24
10	200 (+1)	10 (0)	30 (0)	20.70 ± 3.25	132.95 ± 3.25	24.37 ± 2.71	0.30 ± 0.03	208.83 ± 0.50	186.97 ± 0.85	132.25 ± 4.34	80.69
11	180 (0)	10 (0)	10 (-1)	17.73 ± 2.32	254.00 ± 1.86	90.39 ± 1.76	0.65 ± 0.03	596.76 ± 3.24	470.78 ± 8.96	282.67 ± 0.38	31.03
12	180 (0)	10 (0)	50 (+1)	26.77 ± 2.34	156.52 ± 8.57	35.25 ± 3.07	0.43 ± 0.01	344.23 ± 0.05	261.84 ± 2.56	180.53 ± 2.44	82.95
13	180 (0)	5 (-1)	30 (0)	30.32 ± 1.08	169.94 ± 6.12	41.16 ± 2.25	0.47 ± 0.03	349.95 ± 1.66	286.77± 13.18	192.80 ± 4.51	38.96
14	180 (0)	15 (+1)	30 (0)	34.92 ± 3.62	185.81 ± 11.02	51.20 ± 1.39	0.52 ± 0.02	378.84 ± 25.08	317.88 ± 8.93	204.14 ± 3.14	89.50
15	180 (0)	10 (0)	30 (0)	28.47 ± 6.27	175.71 ± 5.29	46.28 ± 1.97	0.45 ± 0.02	374.65 ± 5.64	322.60 ± 3.81	183.49 ± 5.88	56.79
16	180 (0)	10 (0)	30 (0)	31.73 ± 3.93	176.05 ± 2.50	45.78 ± 3.48	0.46 ± 0.01	412.11 ± 9.32	310.85 ± 0.53	197.49 ± 2.03	57.65
17	180 (0)	10 (0)	30 (0)	29.45 ± 3.60	177.48 ± 4.29	45.62 ± 1.14	0.42 ± 0.01	360.95 ± 2.30	347.41 ± 1.63	193.29 ± 4.14	55.71
18	180 (0)	10 (0)	30 (0)	31.29 ± 6.23	179.14 ± 5.71	44.63 ± 2.27	0.50 ± 0.01	429.35 ± 16.59	316.22 ± 18.63	203.01 ± 1.37	57.08
19	180 (0)	10 (0)	30 (0)	28.51 ± 3.36	171.29 ± 1.63	43.81 ± 0.70	0.46 ± 0.02	347.70 ± 1.86	274.72 ± 7.47	183.70 ± 3.29	60.27
20	180 (0)	10 (0)	30 (0)	27.84 ± 5.37	173.75 ± 2.40	43.08 ± 3.54	0.47 ± 0.02	386.55 ± 4.40	386.75 ± 1.53	200.92 ± 1.44	62.79

[a] A: Assay extractions.

[b] X_1: temperature (°C); X_2: liquid/solid ratio (ml/g); X_3: extraction time (min).

[c] Total sugars (g GLU/ 100 g LM); PC: phenolic compounds (mg GAE/g LM); RS: reducing sugars (mg GLU/g LM); FRAP: ferric reducing antioxidant power assay (mmol Fe(II)/g LM); DPPH: antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl assay (μmol TE/g LM); ABTS: antioxidant activity by the 2,2'-azino-ethylbenzothiazoline-6-sulphonic acid assay (μmol TE/g LM); TAA: total antioxidant activity (mg α-TOC/g LM); Yield of extraction process (mg LM/g SCG).

LM: lyophilized material.

The content of PC in the lyophilized material varied between 82.33 ± 1.63 (assay 8) and 254.00 ± 1.86 mg GAE/g LM (assay 11). It is worth highlighting that the highest amount of PC recovered by autohydrolysis of SCG and subsequent precipitation with ethanol was very representative when compared with other methods, indicating autohydrolysis as an efficient technique to extract also PC from SCG.

The content of RS in the lyophilized material was also dependent on the conditions used for autohydrolysis, being observed values in the range between 12.96 ± 0.92 (assay 8) and 93.93 ± 4.44 mg GLU/g LM (assay 5) (Table 4.1). Such results reveal that in some cases a significant part of the recovered polysaccharides were in the form of monosaccharides, as for example in the assays 9 (52%) and 11 (51%). It is important to mention that, previous the precipitation stage, the largest amount of RS had been found in the extract obtained during autohydrolysis at 200 °C, 15 ml/g SCG, 50 min (not presented data). Nonetheless, after precipitation, the greatest amount of RS was recovered in the LM obtained when the lowest conditions of temperature and extraction time where used for autohydrolysis (160 °C, 10 min and 15 ml/g SCG – assay 5). These results suggest that the RS extracted under these conditions had a higher molecular weight, achieving thus, the precipitation with ethanol.

The antioxidant activity of the recovered polysaccharides was also strongly affected by the conditions used for autohydrolysis (Table 4.1). By varying the extraction conditions, the TAA and FRAP results were increased in the order of 2.5-fold and 3.5-fold, respectively. More significant variations were observed for the DPPH and ABTS results, which increased in almost 4-fold. Differences between the results of antioxidant assays could be explained by the fact that the methods differ from each other in terms of reaction mechanisms, oxidant and target/probe species, and reaction conditions (Conde & Mussatto, 2016). Therefore it is of great importance to assess the antioxidant potential by using different methodologies.

All antioxidant activity methods (FRAP, DPPH, ABTS and TAA) showed a highly significant linear correlation to PC and RS (coefficients $R^2 \geq 0.82$), being found the highest correlations to DPPH data correlated with PC ($R^2 = 0.93$) and ABTS data correlated with RS ($R^2 = 0.92$). These

results suggest that the PC and RS present in the lyophilized material contributed significantly to the antioxidant activity of the polysaccharides extracted from SCG. However, the autohydrolysis conditions that extracted the largest amount of polysaccharides from SCG were not the same that generated polysaccharides with the highest antioxidant activity (Table 4.1). For this reason, an optimization of the process conditions is necessary in order to obtain maximum yield of polysaccharides with high antioxidant potential.

4.2.2. Optimization of the autohydrolysis conditions

The Pareto charts in Figure 4.1 show the effect of each operational variable on the different responses. Temperature (X_1) was the most significant variable, followed by the extraction time (X_3) on PC and RS (Figure 4.1a and b), as well as on all the antioxidant activity responses (Figure 4.2a, b, c, d). Both, temperature and extraction time exerted a significant ($p < 0.05$) and negative linear (L) effect on the responses, which means that the extraction of polysaccharides with high antioxidant activity increased when the temperature and reaction time were reduced. However, not only the linear terms, but also the quadratic terms (Q) and interactions between the variables had statistical significance ($p < 0.05$), suggesting that the values of the responses were not always linearly raised when the value of the operational variables was decreased.

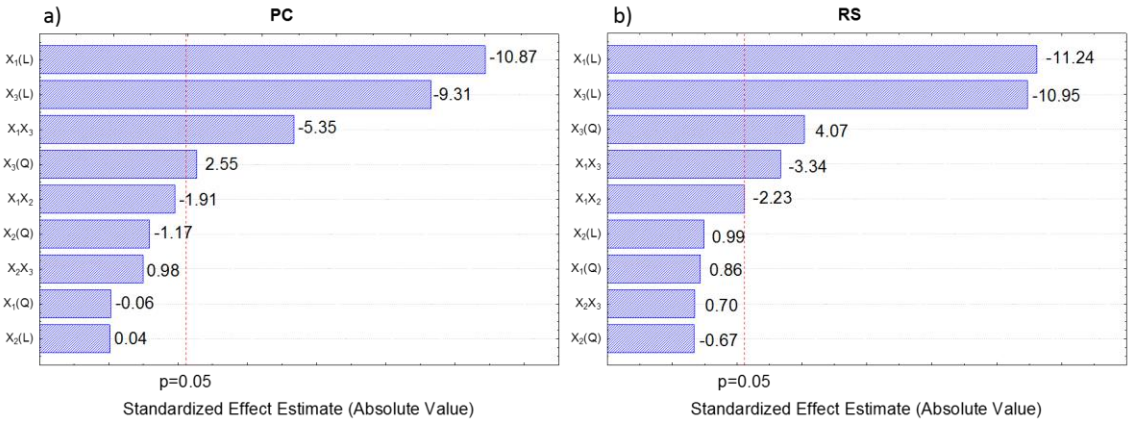

Figure 4.1 Pareto chart for the effects of temperature (X_1), liquid/solid ratio (X_2), extraction time (X_3), and their interactions on the total content of phenolic compounds (PC) (a) and reducing sugars (RS) (b) of the autohydrolysis process for polysaccharides recovery from spent coffee grounds

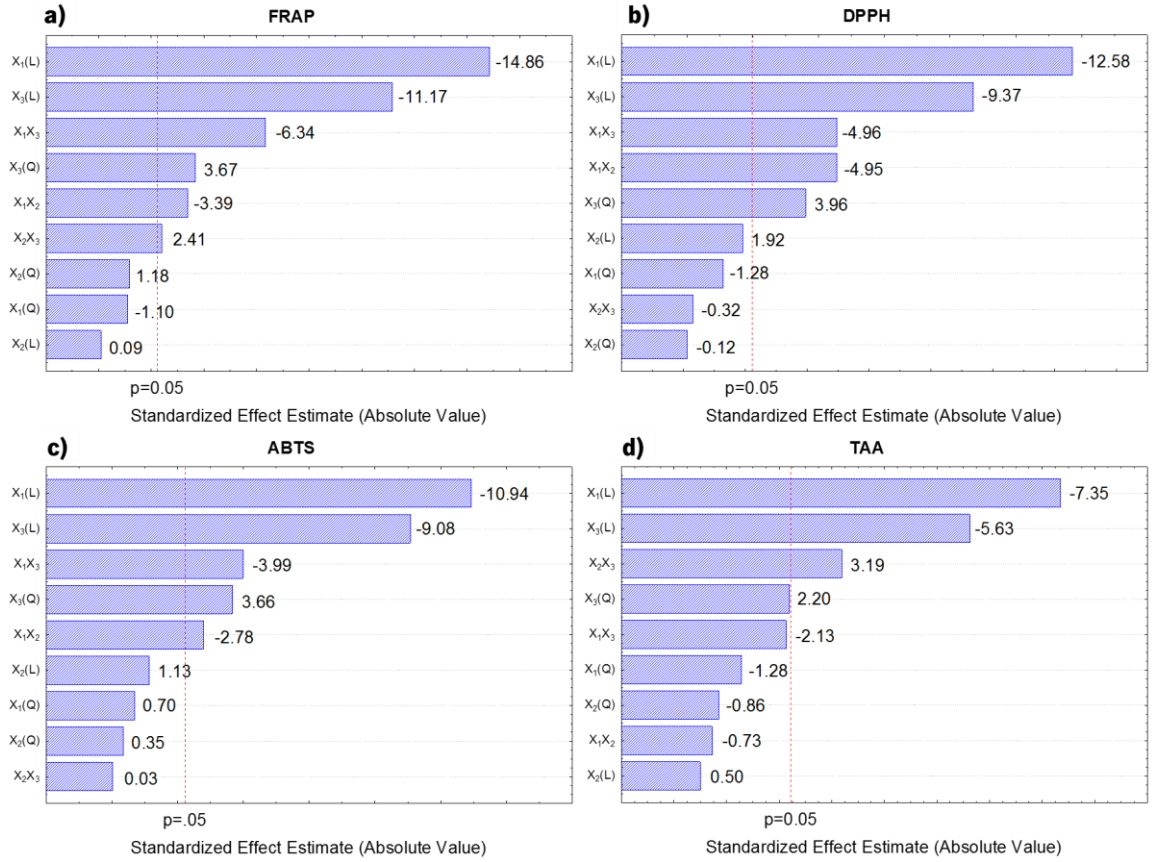

Figure 4.2 Pareto chart for the effects of temperature (X_1), liquid/solid ratio (X_2), extraction time (X_3), and their interactions on the total content of antioxidant activity (FRAP (a), DPPH (b), ABTS (c) and TAA (d) assays) of the autohydrolysis process for polysaccharides recovery from spent coffee grounds

The individual effect of the liquid/solid ratio (X_2) was not significant for any of the responses, but the interaction of this variable with the temperature was significant for the antioxidant activity results. The operational variables did not present significant effects at 95% confidence level for the extraction yield response (Figure 4.3a). However, a mathematical model describing the variations of this response as a function of the process variables could be well-fitted to a second-order polynomial equation (Table 4.2). Second-order mathematical models were also fitted for all the other responses. When possible, the models were simplified by elimination of terms not statistically significant ($p > 0.05$). In other cases, the non-significant variables were kept in the models to minimize the error determination. All the models were established with high coefficient of

determinations R^2, ranging from 0.87 to 0.97, which means a close agreement between the experimental results and those predicted by the equations.

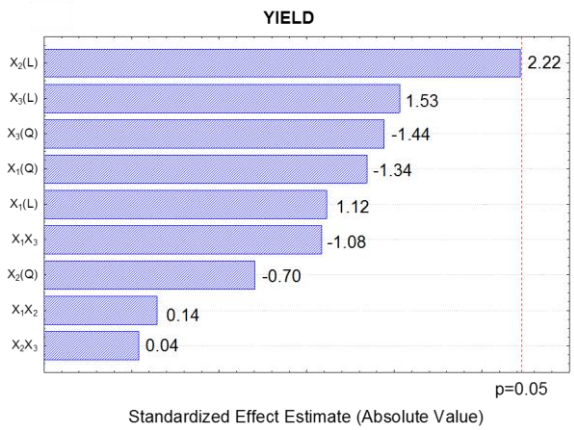

Figure 4.3 Pareto chart for the effects of temperature (X_1), liquid/solid ratio (X_2), extraction time (X_3), and their interactions on the total extraction yield of the autohydrolysis process for polysaccharides recovery from spent coffee grounds

Table 4.2 Quadratic models describing the responses variation as function of the process variables (temperature, liquid/solid ratio and extraction time) and their correspondent R^2 coefficients

Response [a]	**Model equation** *	**R^2**
PC	$PC = 178.13 - 41.95X_1 - 35.95X_3 - 23.11X_1X_3 + 13.29X_3^2$	0.94
RS	$RS = 46.47 - 20.68X_1 + 1.82X_2 - 20.14X_3 - 4.58X_1X_2 - 6.87\ X_1\ X_3 + 14.67X_3^2$	0.96
Yield	$Yield = 56.04 + 0.55X_1 + 10.62X_2 + 2.41X_3 - 4.35X_1X_3 - 12.70X_1^2 - 15.04X_3^2$	0.93
Antioxidant activity		
FRAP	$FRAP = 0.46 - 0.14X_1 + 0.0008X_2 - 0.10X_3 - 0.03X_1X_2 - 0.07X_1X_3 + 0.02\ X_2\ X_3 + 0.07X_3^2$	0.97
DPPH	$DPPH = 375.34 - 100.27X_1 + 8.32X_2 - 78.76X_3 - 40.09X_1\ X_2 - 72.93X_1\ X_3 + 46.50X_3^2$	0.90
ABTS	$ABTS = 319.09 - 110.72X_1 + 11.45X_2 - 91.91X_3 - 31.49X_1\ X_2 - 45.17X_2\ X_3 + 86.25X_3^2$	0.95
TAA	$TAA = 199.60 - 41.81X_1 + 2.84X_2 - 32.03X_3 - 4.65X_1X_2 - 13.57X_1X_3 + 20.32X_2X_3$	0.87

* X_1: temperature; X_2: liquid/solid ratio; X_3: extraction time. Coded values.

[a] PC: phenolic compounds (mg GAE/g LM); RS: reducing sugars (mg GLU/g LM); Yield: yield of extraction process (mg LM/g SCG); FRAP: antioxidant activity by the ferric reducing antioxidant power assay (mmol Fe(II)/g LM); DPPH: antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl assay (μmol TE/g LM); ABTS: antioxidant activity by the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid assay (μmol TE/g LM); TAA: total antioxidant activity. (mg α-TOC/g LM).

LM: lyophilized material.

Contour lines graphs were plotted for all the responses according to the model equations established (Table 4.2). The plots of PC (Figure 4.4a), RS (Figure 4.4b) and antioxidant activities (Figure 4.5a, b, c, d) show a region where the responses can be maximized, which occurs using the lowest the temperature and extraction time and 15 ml/g SCG of liquid/solid ratio. In contrast, the extraction yield of the process (Figure 4.5e) is maximized when using intermediate values of temperature and extraction time in combination with the highest liquid/solid ratio (15 ml/g SCG).

Taking these results into account, a graphical optimization was performed by overlaying the curves of the models and the following criteria were adopted in order to find an extraction condition that simultaneously maximize the contents of PC and RS, as well as the antioxidant activity of the recovered polysaccharides: PC ≥ 220 mg GAE/g LM, RS ≥ 87 mg GLU/g LM, FRAP ≥ 0.65 mmol Fe(II)/g LM, DPPH ≥ 510 μmol TE/g LM, ABTS ≥ 550 μmol TE/g LM, and TAA ≥ 225 mg α-TOC/g LM.

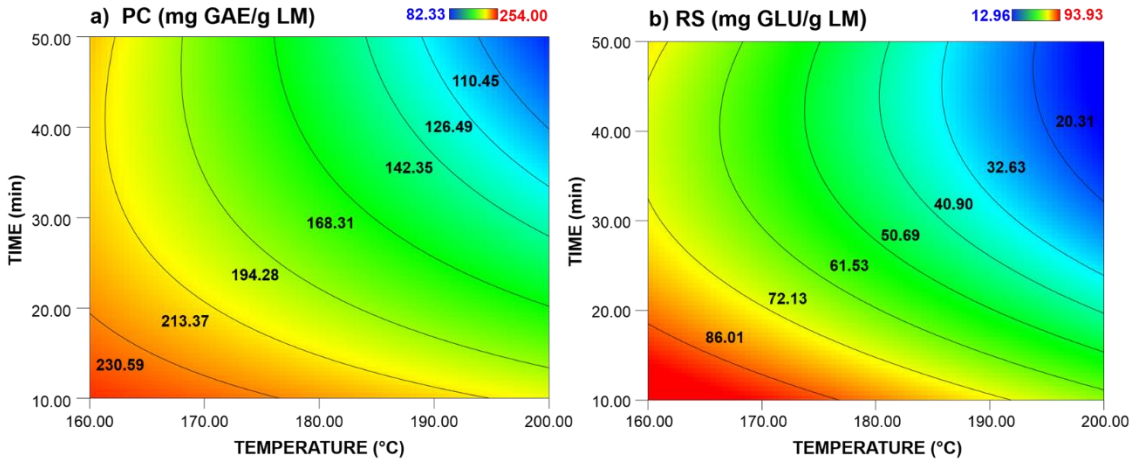

Figure 4.4 Contour line plots representing the total content of phenolic compounds (PC) (a) and reducing sugars (RS) (b) of polysaccharides extracted by autohydrolysis of spent coffee grounds under different conditions of time and temperature

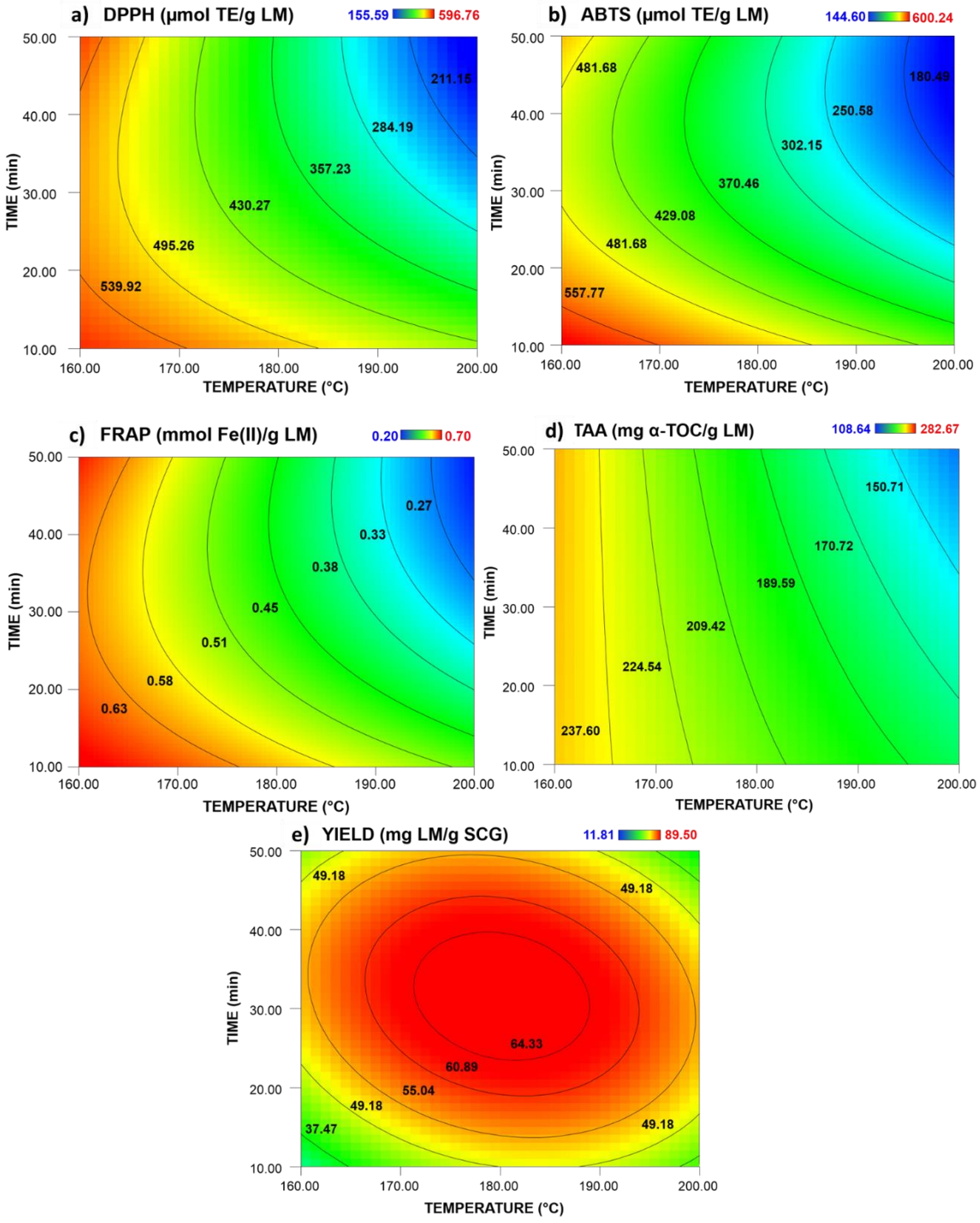

Figure 4.5 Contour line plots representing the total content of antioxidant activity (FRAP (a), DPPH (b), ABTS (c) and TAA (d) assays) and total yield (e) of polysaccharides extracted by autohydrolysis of SCG under different conditions of time and temperature.

The overlaying plot attained (Figure 4.6) shows an area where all the criteria are satisfied (shadow area). A point within this area was assigned as optimum point, which corresponded to the use of 160 °C, 15 ml/g SCG and 10 min. Under these conditions, the model predicts PC and RS results of 246.21 mg GAE/g LM and 101 mg GLU/g LM, as well as antioxidant activity values for FRAP, DPPH, ABTS and TAA of 0.71 mmol Fe(II)/g LM, 576.35 µmol TE/g LM, 605.73 µmol TE/g LM and 247.04 mg α-TOC/g LM, respectively. These values corresponded to one of the conditions previously evaluated experimentally (Table 4.1, conditions -1, +1, -1), being the responses within 5% of relative standard deviation (Figure 4.6 inset). The polysaccharide obtained in this condition as well as the polysaccharide obtained under the condition that gave the best extraction yield (Table 4.1, assay 14) were further evaluated in order to determine their composition and structural and thermal characteristics.

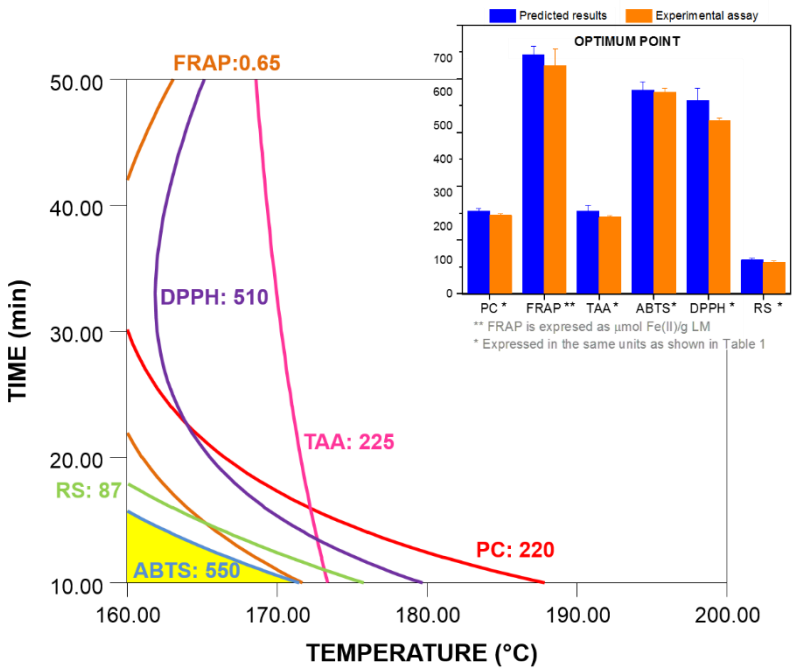

Figure 4.6 Optimum region plot obtained by overlaying the curves of the responses phenolic compounds (PC), reducing sugars (RS) and antioxidant activity by FRAP, DPPH, ABTS and TAA assays as a function of the extraction time and temperature used during the autohydrolysis process, and comparison between the predicted and experimental results (inset figure)

4.2.3. Optimum point characterization

4.2.3.1. *Yield of extraction and sugars composition*

The sugars content in the polysaccharides extracted under the conditions of the optimum point and best yield, as well as the yields obtained for each one of these processes are shown in Table 4.3. In this table, Y1 corresponds to the total yield of extraction (g LM per 100 g SCG); Y2 refers to the quantity of sugars present in LM per 100 g SCG; while Y3 represents the yield of sugars extracted with respect to total sugars existent in SCG (g total sugars in LM/100 g of sugars from SCG).

Table 4.3 Sugars composition and extraction yield of the polysaccharides obtained by autohydrolysis of spent coffee grounds (SCG)

Sample	Yield*			Monosaccharide composition (% mol)				Total Sugars (%)
	Y1	Y2	Y3	Arabinose	Mannose	Galactose	Glucose	
Optimum point	3.59	1.07	2.06	10.02 ± 1.18	31.88 ± 2.08	47.74 ± 0.13	10.35 ± 0.76	29.29 ± 3.47
Best yield	8.95	2.97	5.72	8.05 ± 1.55	16.93 ± 1.47	47.32 ± 1.18	27.68 ± 1.71	33.25 ± 0.34

Results of monosaccharide composition are expressed as mean ± standard deviation; n=3.

* Y1: total yield of the extraction process using autohydrolysis technique, expressed as g of lyophilized material (LM) per 100 g SCG; Y2: yield in terms of quantity of sugars extracted during autohydrolysis, expressed as g of total sugars present in LM per 100 g SCG; Y3: yield in terms of quantity of sugars extracted with respect to total sugars existent in SCG, expressed as g of total sugars in LM per 100 g of sugars from SCG.

The content of total sugars recovered was 29.29% and 33.25% (w/w) for the optimum point and best yield samples, respectively. Although the quantity of sugars in both samples was similar, Y1, Y2 and Y3 were 2-fold higher for the best yield sample, achieving 8.95, 2.97 and 5.72% (w/w). Analysis of the monosaccharide composition (Table 4.3) revealed a structural difference between the polysaccharides samples. However, galactose was the main monosaccharide and arabinose the less representative sugar for both, optimum point and best yield samples. The high content of galactose in both samples (47% mol) allows concluding that polysaccharides recovered under the

optimum point and the best yield conditions include arabinogalactans and galactomannans. The quantity of mannose in the optimum point sample (31.88% mol) was more representative than in the best yield sample, suggesting the presence of higher amount of galactomannans in the optimum point sample. Another structural difference between these two polysaccharides is the percentage of reducing sugars with respect to the percentage of total sugars, being 33% (w/w) and 15% (w/w) for optimum point and the best yield samples, respectively. Thus, the sugars obtained under the best yield conditions are mainly polysaccharides of long chains, while the sugars recovered under optimum point conditions are made, in a great part by oligosaccharides and/or short chain polysaccharides. Taking into account the composition of sugars in SGC described in other studies (Mussatto, Carneiro, et al., 2011; Passos & Coimbra, 2013), it was expected to find mannose, galactose, arabinose and glucose sugars in the lyophilized material. Nevertheless, the efficiency of the extraction depends of different factors including the variety of the coffee beans and their degree of roasting, solid/liquid ratio, solvent, temperature and extraction time, among others. The percentage of total polysaccharides extracted from SCG by using the autohydrolysis technique was slightly lower when compared to the percentage of total sugars extracted from SCG using alkali treatment (Section III – Chapter 5). However, the quantity of mannose extracted (31.88% mol) when using the optimum point conditions was higher than the amount obtained using an alkaline treatment, which shows autohydrolysis as an efficient technique to extract mannose from SCG.

4.2.3.2. *Structural characterization*

Crystallinity of the extracted polysaccharides was evaluated through X-ray diffraction. Figure 4.7a shows the XRD patterns for the optimum point and best yield samples, which were compared with a XRD spectrum of original SCG sample, i.e., not pretreated (Section II - Chapter 3). In general, the optimum point and the best yield polysaccharides samples showed an amorphous behavior, which was expected since the autohydrolysis conditions used are more suitable to extract hemicelluloses. However, the best yield sample presented a broad band, revealing the existence of small crystalline region in its structure, which can be easily observed when comparing to the cellulose region in the SCG spectrum. Cellulose presents both amorphous and crystalline structures

(Park, Baker, Himmel, Parilla, & Johnson, 2010). Thus, the glucose (cellulose) contents shown in Table 4.3 refer to the amorphous structure of cellulose, which, together with hemicellulose (mannose, galactose and arabinose) were more easily susceptible to hydrolysis due to the nature of their structure. Although crystalline cellulose hydrolysis requires the use of stronger temperatures and extraction times, the slight crystallinity observed in Figure 4.7a for the best extraction yield sample suggests that a small part of crystalline cellulose was extracted during the autohydrolysis process when the temperature and extraction time were increased.

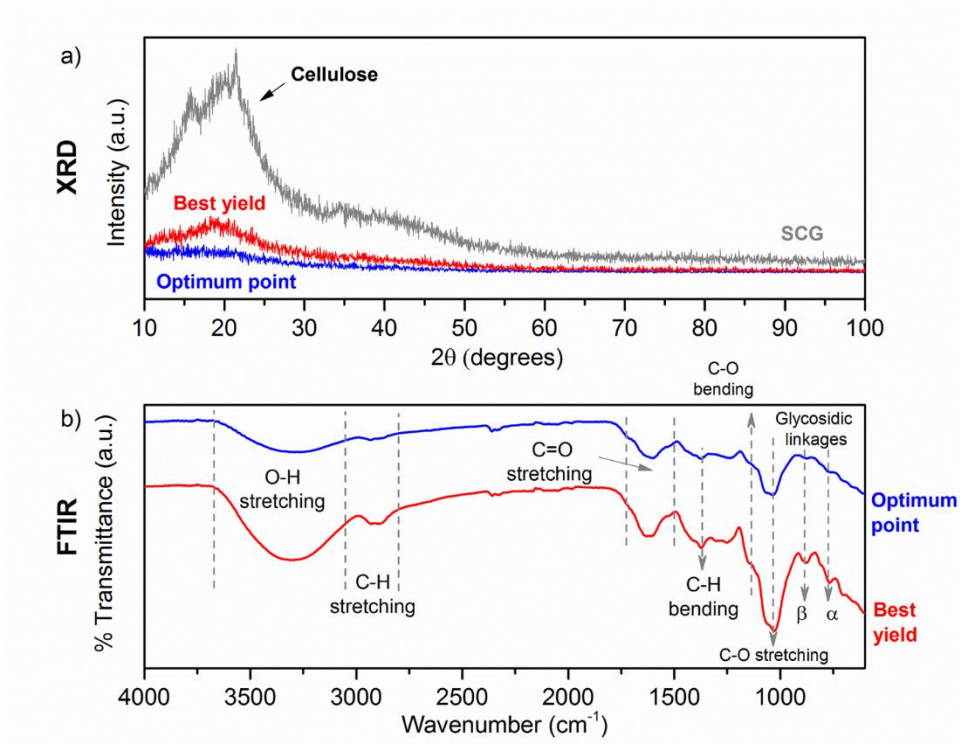

Figure 4.7 XRD diffractograms (a) obtained for spent coffee grounds (SCG) and for the polysaccharides extracted by autohydrolysis of SCG using the optimum point and best yield conditions. FTIR spectra (b) obtained for the polysaccharides extracted using the optimum point and best yield conditions

The polysaccharides samples were also analyzed by FTIR in order to determine the specific absorption bands present in each lyophilized material. When compared to other IR spectra of polysaccharides reported in the literature, the FTIR spectra obtained for both samples (Figure 4.7b) showed a typical carbohydrate pattern behavior (Cerqueira et al., 2011; Ren et al., 2014).

Nevertheless, the magnitude of absorption intensities differed to each sample. The peaks at 778 and 884 cm^{-1} were related with the presence of α- glycosidic and β-glycosidic linkages, attributed to α-D-galactopyranose and β-D-mannopyranose units, respectively (Cerqueira et al., 2011; Figueiró, Góes, Moreira, & Sombra, 2004). The broad band between 1191 and 920 cm^{-1} was related to ring vibrations overlapped with stretching vibrations of (C-OH) side groups and the (C-O-C) glycosidic band vibrations, being specific for polysaccharides. This band showed lower peak intensity for the optimum point when compared to the best yield sample, which was associated to more hydrolyzed sugars (Synytsya & Novak, 2014), confirming shorter polysaccharides chains for the optimum point sample, as previously mentioned. Additionally, the peak belonging to this band located at 1039 cm^{-1} results from C–O stretching (Ren et al., 2014), and the other peak placed at 1140 cm^{-1} were related to bending vibrational modes of C–O existing in the pyranose form (Figueiró et al., 2004). The peak at 1374 cm^{-1} corresponded to C–H in plane bending vibration and deformation in cellulose and hemicellulose (Pandey & Theagarajan, 1997). When the samples show a low crystallinity, there is a decrease or disappearing of some bands in the region of 900 – 1500 cm^{-1} (Synytsya & Novak, 2014). This was evidenced to the optimum point sample, which presents a more amorphous structure when compared to the best yield sample. The region from 1500 to 1700 cm^{-1} was related with carbonyl groups (C=O) asymmetrical and symmetric stretching vibrations (Ren et al., 2014) and to deformation in lignin (Pandey & Theagarajan, 1997). This band was also highly associated with chlorogenic acids and caffeine (Ribeiro, Salva, & Ferreira, 2010). Therefore, the peak at 1600 cm^{-1} could be attributed to a small absorption of these compounds, remaining from the SCG. The region between 2800 and 3000 cm^{-1} was related to C–H stretching vibration and the broad peak between 3200 and 3600 cm^{-1} was attributed to the hydroxyl group of O–H stretching vibration. The significant lowering of the bands in this area indicates the presence of amorphous cellulose (Synytsya & Novak, 2014), being in agreement with the XRD patterns, which revealed that the optimum point sample is less crystalline than the best yield sample (Figure 4.7a).

4.2.3.3. *Thermal properties*

DSC and TGA analyses (Figure 4.8) were carried out in order to evaluate the thermal behavior, chemical changes and weight loss of the polysaccharides extracted from SCG under the optimum point and the best yield autohydrolysis conditions. The DSC curves obtained for both samples exhibited two events. The first event, resulting in an endothermic peak and revealed at 100.90 °C and 99.41 °C for the optimum point and the best yield samples, respectively, was associated to enthalpy changes of 381.32 and 396.40 J/g. This event is related to the presence of impurities in the samples and the vaporization of water (indicating the presence of hydrophilic groups), which occurs over a range of temperature.

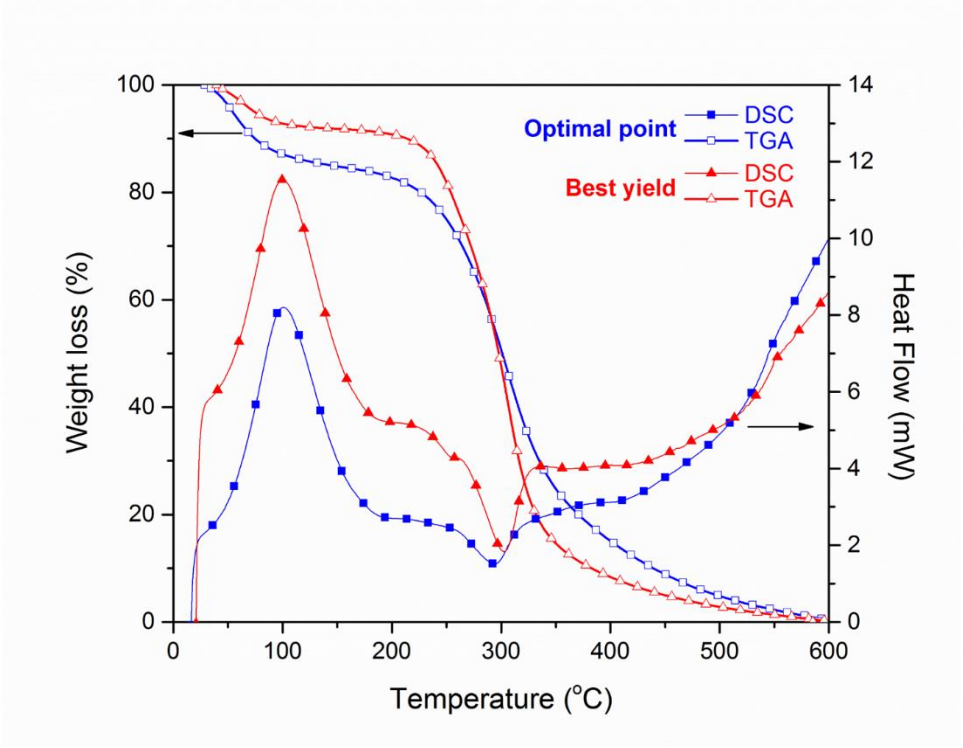

Figure 4.8 TGA and DSC curves showing the thermal behavior, chemical changes and weight loss of the polysaccharides extracted from spent coffee grounds under the optimum point and the best yield autohydrolysis conditions

A second event, corresponding to an exothermic transition was observed at 297.73 °C and 302.60 °C for the optimum point and the best yield samples respectively, was associated to enthalpy changes of 73.06 and 146.5 J/g. This event is related to the thermal decomposition of

the samples, varying at temperature ranges between 220 and 310 °C. During this event, some differences were observed between the samples with respect to enthalpy change, being the value two-fold higher to the best extraction yield condition. This could be correlated to the structure of the polysaccharides, as well as to the molecular weight, degree of polymerization and branching of the samples (Cerqueira et al., 2011).

The TGA curves (Figure 4.8) show the weight losses of the polysaccharides when submitted to severe heating conditions (25 - 600 °C). Both samples showed similar curves, revealing two weight loss stages. The first one, occurring between 80 and 100 °C, resulted from the water evaporation (dehydratation of the sample) and corresponded to weight losses of about 8.74% and 6.24% for the optimum point and the best yield samples, respectively. The greatest transformation and weight losses occurred during the second stage, at approx. 300 °C. At this stage, weight losses of 42.37% and 57.89% were observed for the optimum point and best yield samples, respectively, as a consequence of the depolymerization and decomposition of the samples.

4.3. Conclusions

Autohydrolysis was demonstrated to be an efficient technique to recover polysaccharides with high antioxidant activity from SCG, particularly when applied at 160 °C during 10 min, and using a liquid/solid ratio of 15 ml water/g SCG. Under these conditions, it was possible to obtain a lyophilized material containing 29.29% (w/w) of polysaccharides, from which galactose was the most representative sugar, followed by mannose, glucose and arabinose. Additionally, the lyophilized material contained high content of phenolic compounds (234.14 mg GAE/g LM) and reducing sugars (93.93 mg GLU/g LM), and presented high antioxidant activity, which as confirmed by four different methods. Furthermore, the polysaccharides presented thermostability in a large range of temperature, being therefore of great interest for industrial applications, mainly in the food industry, for encapsulation of additives or as prebiotics, for example, due to their high antioxidant potential and other functional properties.

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CHAPTER 5

CHARACTERIZATION OF POLYSACCHARIDES EXTRACTED FROM SPENT COFFEE GROUNDS BY ALKALI PRETREATMENT

The following chapter is partially based on the results published in: Lina F. Ballesteros, Miguel A. Cerqueira, José A. Teixeira & Solange I. Mussatto (2015). Characterization of polysaccharides extracted from spent coffee grounds by alkali pretreatment. *Carbohydrate Polymers*, *127*, 347–354.

5. Introduction

Arabinogalactan, galactomannan and cellulose are the dominant polysaccharides in coffee beans (Arya & Rao, 2007; Fischer, Reimann, Trovato, & Redgwell, 2001). Arabinogalactans is the most significant group of polysaccharides extracted with hot water from green coffee (Arya & Rao, 2007; Nunes et al., 2005). Nevertheless, after roasting process, galactomannans become the most relevant polysaccharides in roasted coffee infusions. Thus, galactomannans and arabinogalactans are the most important coffee constituents after hot water extraction.

Recently, some researchers have exposed the great potential of polysaccharides presented in coffee, showing that they can provide enormous functional properties (Gniechwitz, Reichardt, Blaut, Steinhart, & Bunzel, 2007; Simões et al., 2009). These properties could be found in the spent coffee ground (SCG), which retains about 70 % of total polysaccharides present in roasted coffee (Arya & Rao, 2007).

The purpose of this Chapter was performed the extraction of polysaccharides from SCG by using an alkali pretreatment with sodium hydroxide at 25 °C, and evaluate the chemical and structural characterization, as well as the antioxidant and antimicrobial properties of the extracted polysaccharides.

5.1. Materials and methods

5.1.1. Raw material

SCG was provided by the Portuguese coffee industry Nova Delta-Comércio e Indústria de Cafés S.A. (Campo Maior, Portugal) and preserved as described in Section II - Chapter 3.

5.1.2. Alkali pretreatment

Polysaccharides extraction from SCG was carried out according to the method described by Simões et al. (Simões, Nunes, Domingues, & Coimbra, 2010) with some modifications. Briefly,

previous to the extraction, the SCG (605 g) were defatted in a Soxhlet extraction system (Tecator, HT2, Netherlands) during 4 h using petroleum ether as solvent (1:5 (w/v)). The fat free SCG were dried at 60 °C until constant weight and stored for the further stages. The alkali pretreatment was then performed for polysaccharides extraction by using 4 M sodium hydroxide (4 L) at 25 °C overnight (0.02 M sodium borohydride was also added to prevent peeling reactions and alkaline oxidation of the polysaccharides). After this time, the produced alkali extract was centrifuged at 9700 *g* for 15 min at 4 °C, filtered through Whatman filter paper and acidified to pH 5.0 with glacial acetic acid. Next, the filtrate was dialyzed at 4 °C with a 8000 Da membrane for 12 days, with several distillated water changes. After dialysis, the retentate into the membrane was centrifuged at the same conditions above mentioned and the supernatant was frozen and freeze-dried. Freeze-dried powder were stored at room temperature and protected from the light and humidity until further use.

5.1.3. Polysaccharide yield

Three different extraction yields of polysaccharides were determined (Y1, Y2, and Y3), which can represent important economic parameters of the process. Y1 represents the total yield of the extraction, expressed as g of lyophilized material (LM) per 100 g SCG; Y2 refers to the quantity of sugars extracted and was expressed as g of total sugars present in LM per 100 g SCG; finally, Y3 represents the yield of the quantity of sugar extracted with respect to total sugars existent in the SCG, which is defined as g of total sugars in LM per 100 of sugars from SCG.

5.1.4. Analytical methodology

5.1.4.1. *Chemical characterization*

The extracted polysaccharides were submitted to a dilute acid hydrolysis with sulfuric acid (120 mg H_2SO_4/g LM) at 120 °C for 20 min. The resulting solution was analyzed by high performance liquid chromatography (HPLC) as defined in Section III - Chapter 4. Glucose, arabinose, galactose and mannose were identified and quantified from standard curves made with known concentrations of each compound and expressed as % mol.

5.1.4.2. *Structural characterization*

Crystalline phases and the chemical groups and bonding arrangement of constituents present in the polysaccharides were evaluated by X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR), respectively, as described in Section II - Chapter 3 and Section III - Chapter 4. Differential scanning calorimetry (DSC) and thermogravimetric analyses (TGA) were carried out as previously described in Section II - Chapter 3.

5.1.4.3. *Antioxidant phenolic compounds characterization*

For the analysis of total phenolic compounds and antioxidant activity of the polysaccharides extracted from SCG by alkali pretreatment, LM was mixed with ultrapure water in a ratio of 1 mg/ml, vortexed for 1 min and then filtered through 0.22 μm filters. Additionally, two commercial antioxidant phenolic compounds (2,6-di-tert-butyl-4-methylphenol and tert-butyl-4-methoxyphenol, known as BHT and BHA, respectively) were used as standards to evaluate the antioxidant capacity of polysaccharides and Tukey's range test was considered to evaluate significant differences ($p < 0.05$) among samples.

5.1.4.3.1. Phenolic compounds

The content of phenolic compounds was determined by using the Folin-Ciocalteu reagent method adapted to a 96-well microplate, described in Section III - Chapter 4. The total content of phenolic compounds was expressed as milligram of gallic acid equivalent per gram of lyophilized material (mg GAE/g LM).

5.1.4.3.2. Antioxidant activity

The antioxidant activity of the polysaccharides was estimated by four different methods: total antioxidant activity (TAA), DPPH radical scavenging activity assay and the radical cation decolorization (ABTS) assay, described in Section III – Chapter 4, as well as the ferric reducing antioxidant power (FRAP) assay, described in Section II – Chapter 3. TAA values were expressed as milligrams of α-tocopherol equivalent per milliliter of extract (mg TOC/ml). DPPH and ABTS data

were plotted as a function of antioxidant concentration to obtain DPPH and ABTS inhibition concentration at 50% (IC_{50}). The IC_{50} values were expressed as milligrams of trolox equivalent (TE) per milliliter of extract (mg TE/ml).The FRAP values were expressed as milligrams of ferrous equivalent per milliliter of extract (mg Fe(II)/ml).

5.1.4.4. *Antimicrobial activity assays*

5.1.4.4.1. Microbial strains

Antimicrobial evaluation was performed against five food pathogenic fungi that drastically influence the quality and safety of postharvest fruits (Jasso de Rodríguez et al., 2011): *Alternaria* sp. MUM 02.42, *Cladosporium cladosporioides* MUM 97.06, *Phoma violacea* MUM 97.08, *Penicillium italicum* MUM 02.25 and *Penicillium expansum* MUM 02.14, being obtained from the collection of the Mycology Laboratory (MUM) of the University of Minho, Portugal. All the strains were cultured into potato dextrose agar (PDA) and incubated at 25 ± 2 °C during 15 days before antimicrobial test.

5.1.4.4.2. Minimal inhibitory concentration

The determination of minimal inhibitory concentration (MIC) of polysaccharides extracted from SCG was performed using the micro-dilution methodology for filamentous fungi described by the Clinical and Laboratory Standards Institute (CLSI, 2002). Briefly, 20 mg of polysaccharides were dissolved in 1 ml of sterile ultrapure water and filtered through a 0.22 µm cellulose membrane. The resulting mixture was serially two-fold diluted in synthetic culture medium RPMI 1640 with glutamine and without sodium bicarbonate buffered with bicarbonate 3-(N-morpholino) propanesulfonic acid (MOPS) to pH 7.0, to obtain samples with the following final concentration after adding the inoculum: 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9 and 1.95 µg/ml. The cell suspension of each fungus tested was also adjusted to achieve a final concentration between $0.4x10^4$ and $5x10^4$ CFU (colony forming unit)/ml when mixed with the sample concentrations.

Experiments were carried out in a sterile 96-well microplate, in which 100 µl of inoculum suspension were added to 100 µl sample. The microplate was incubated at 25 ± 2 °C for 96 h and

the absorbance was measured at 530 nm using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria) at 0, 12, 24, 48, 72 and 96 h to evaluate the behavior of the samples against growth and sterility controls, which consisted in using 100 µl of medium RPMI 1640 plus 100 µl of inoculum suspension as microbial growth control and 200 µl of medium RPMI 1640 as sterility control. Moreover, fluconazole solutions (at concentrations 0.19, 0.39, 0.8, 1.6, 3.1, 46.2, 12.5, 25 50 and 100 µg/ml) were used as standard control. All the assays were performed seven times for each sample against all fungal strains. MIC values were determined as being the lowest sample concentration that prevents visible fungal growth.

5.2. Results and discussion

5.2.1. Yield of extraction and chemical characterization of polysaccharides

Table 5.1 shows the monosaccharide composition and extraction yield of the recovered polysaccharides. SCG is a residue rich in sugars polymerized into cellulose and hemicellulose, which correspond to 51.50% (w/w) of its composition on a dry weight basis as reported in Section II – Chapter 3. In the present chapter, the total sugar content extracted from SCG (lyophilized material) was 39%, while Y1, Y2 and Y3 were 6.05, 2.38 and 4.57% (w/w), respectively. Y1 is in agreement to the values obtained by Simões et al. (2009) when used 4 M NaOH to extract polysaccharides from SGC, in contrast toY2, which was almost 2-fold higher. Y3 was lower taking into account the high amount of polysaccharides present in the SCG.

The chromatogram profile shown in Figure 5.1a revealed glucose, galactose, arabinose and mannose as the only sugars present in SCG polysaccharide. The monosaccharide composition showed galactose (60.27% mol) as the dominant sugar, followed by arabinose (19.93% mol), glucose (15.37% mol) and mannose (4.43% mol). These results are in agreement with others studies which reported that polysaccharides in coffee wall are constituted by galactose, arabinose, mannose and glucose, forming mainly galactomannan, arabinogalactan and cellulose structures (Arya & Rao, 2007; Mussatto, Carneiro, et al., 2011; Simões et al., 2009). However, the obtained sugar percentages revealed differences when compared with those works, but concurred with the

results reported by Simões et al. (2009), who evaluated 4 M NaOH to extract polysaccharides from SGC. As a result, galactose and arabinose were the most representative sugars found in the supernatant, which is in agreement with the presented results. The quantity of mannose extracted in both cases was lower when compared with other methods used to extract polysaccharides from SCG (Mussatto, Carneiro, et al., 2011), which indicates that a large proportion of mannose remains in SCG, suggesting that stronger conditions should be used for their extraction. For instance, mannose from SCG could be subjected to a chemical acetylation process (Simões et al., 2009; Simões et al., 2010) increasing thus the solubility of this sugar in water and other organic solvents, since the solvent plays an important role in the extraction process and should be chosen with respect to the organic compound of interest.

Additionally, the efficiency of the extraction depends of many factors such as solid/liquid ratio, solvent, temperature, extraction time, variety of the beans used and their degree of roasting, among others; parameters that could be optimized but were not the objective of the present study.

Table 5.1 Monosaccharide composition and extraction yield of the polysaccharides from spent coffee grounds

Yield*			Monosaccharide composition (% mol)				Total sugars (%)
Y1	Y2	Y3	Arabinose	Mannose	Galactose	Glucose	
6.05	2.38	4.57	19.93 ± 1.74	4.43 ± 0.16	60.27 ± 0.51	15.37 ± 0.93	39.00 ± 0.19

Results of monosaccharide composition are expressed as mean ± standard deviation; n=3.

* Y1: total yield of the extraction process with 4 M NaOH, expressed as g of lyophilized material per 100 g SCG; Y2: yield of the quantity of sugars extracted with 4 M NaOH, expressed as g of total sugars present in the lyophilized material per 100 g SCG; Y3: yield of the quantity of sugar extracted with respect to total sugars existent in the SCG, expressed as g of total sugars in the lyophilized material per 100 g of sugars from SCG.

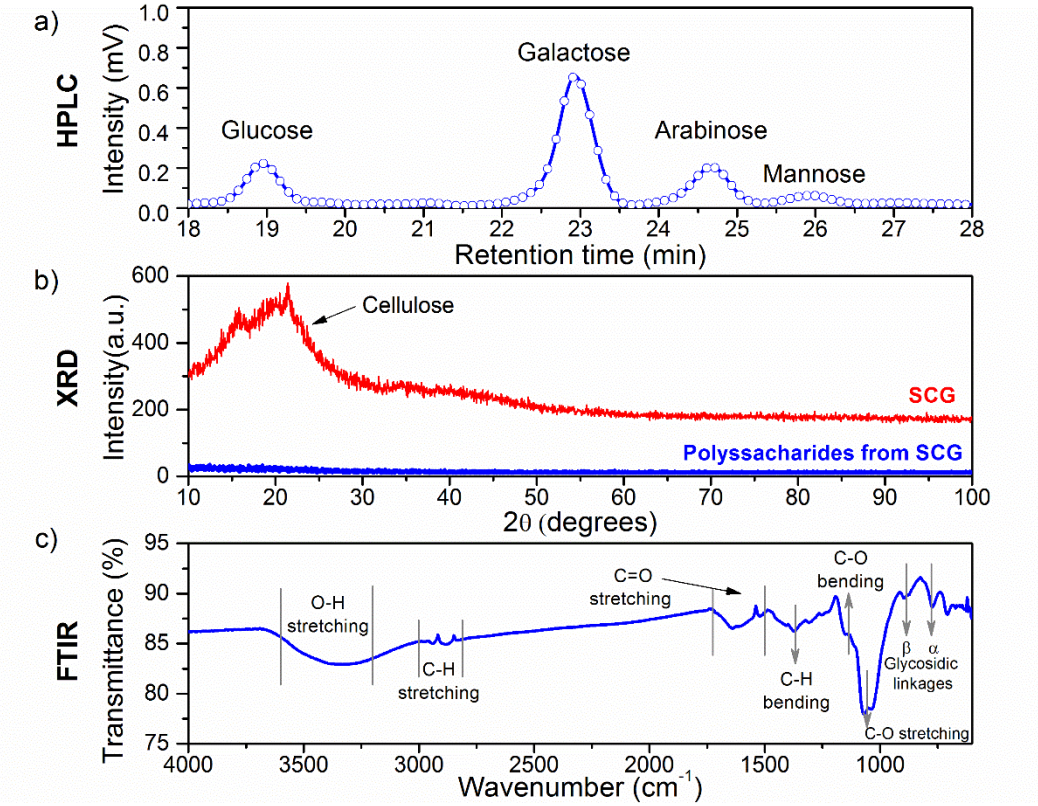

Figure 5.1 Chromatogram profile of sugars solubilized (glucose, galactose, arabinose and mannose) from spent coffee grounds by alkali pretreatment and further acid hydrolysis (a). XRD diffractograms (b) obtained for spent coffee grounds and polysaccharides extracted from this residue. FTIR spectra (c) obtained for the polysaccharides extracted from spent coffee grounds using an alkali pretreatment

5.2.2. Structural characteristics

5.2.2.1. *Crystallinity*

Figure 5.1b displays the XRD patterns for SCG and the polysaccharide extracted from SCG. In order to evaluate the crystallinity of polysaccharides after alkali pretreatment, the XRD spectrum was compared with a XRD spectrum of SCG, obtained in Section II – Chapter 3, in which the SCG did not suffer any chemical pretreatment before the analysis. As it can be seen, the unique crystalline peak in SCG corresponds to the cellulose, while the polysaccharides extracted from SCG

did not present any crystalline region. Although the chemical composition (Table 5.1) revealed glucose (cellulose) into the sugars present in the lyophilized material, the XRD spectra of polysaccharides suggests a glucose with amorphous structure, since it is known to have crystalline and amorphous regions, in contrast with hemicellulose that present an amorphous structure (Ragauskas & Huang, 2013). This result could be related to the fact that alkali pretreatment is more suitable to extract the hemicellulose structure, being more easily degradable and susceptible to chemical attacks than cellulose (Ragauskas & Huang, 2013).

5.2.2.2. *Chemical bonding of constituents*

Figure 5.1c shows the FTIR analysis performed to polysaccharides extracted from SGC. The obtained spectrum corresponds to a typical carbohydrate pattern when compared with others IR spectra of polysaccharides reported in the literature (Cerqueira et al., 2011; Ren et al., 2014; Zeng, Zhang, Gao, Jia, & Chen, 2012). The broad peak between 3600 and 3200 cm^{-1} was related to the hydroxyl group of O–H stretching vibration and the weak band between 3000 and 2800 cm^{-1} was attributed to C-H stretching vibration. The region between 1700 and 1500 cm^{-1} was related with carbonyl groups (C=O) asymmetrical and symmetric stretching vibrations (Ren et al., 2014). This band was also highly associated with chlorogenic acids and caffeine (Ribeiro, Salva, & Ferreira, 2010). Therefore, the peak at 1650 cm^{-1} could be attributed to a small absorption of these compounds, remaining from the SCG. The peak at 1374 cm^{-1} corresponds to C–H in plane bending vibration (Ren et al., 2014). The sharp band between 1194 and 925 cm^{-1} corresponds to stretching vibration of C–O in C–O–H bonds such as glycosidic bonds, and was related to polysaccharide sugars (Figueiró, Góes, Moreira, & Sombra, 2004). The peaks at 1155 and 1080 cm^{-1} resulted from the bending vibrational modes of C–O existing in the pyranose form (Figueiró et al., 2004), while the shoulder at 1024 cm^{-1} was indicated as C–O stretching (Ren et al., 2014). The peaks at 885 and 790 cm^{-1} were related to the presence of β-linked D-mannopyranose units and α-linked D-galactopyranose units, respectively. These glycosidic configurations were reported in most seed galactomannans (Cerqueira et al., 2011; Figueiró et al., 2004).

5.2.2.3. *Thermal properties*

DSC and TGA curves (Figure 5.2) were performed in order to understand the thermal behavior and chemical changes of the polysaccharides extracted from SCG. DSC thermogram exhibited two events: an initial endothermic phase followed by an exothermic phase. Thus, an early endothermic event was detected with a peak at 80.43 °C with an associated enthalpy change of 167.30 J/g. This event was related to the presence of impurities in the sample and the vaporization of water (indicating the presence of hydrophilic groups), which occurs over a range of temperature. Enthalpy change in the first thermal transition was inferior when compared to those obtained for others polysaccharides (Cerqueira et al., 2011), associated to the low content of mannose:galactose ratio (Cerqueira et al., 2011; Chaires-Martínez, Salazar-Montoya, & Ramos-Ramírez, 2008), as reported in Table 5.1. The second event corresponds to an exothermic transition and was observed at 303.60 °C, accompanied with an enthalpy change of 39.96 J/g. This transition was related to the thermal depolymerisation and branching of the polysaccharides, occurring at temperature ranges varying between 220 and 310 °C (Sperling, 2006).

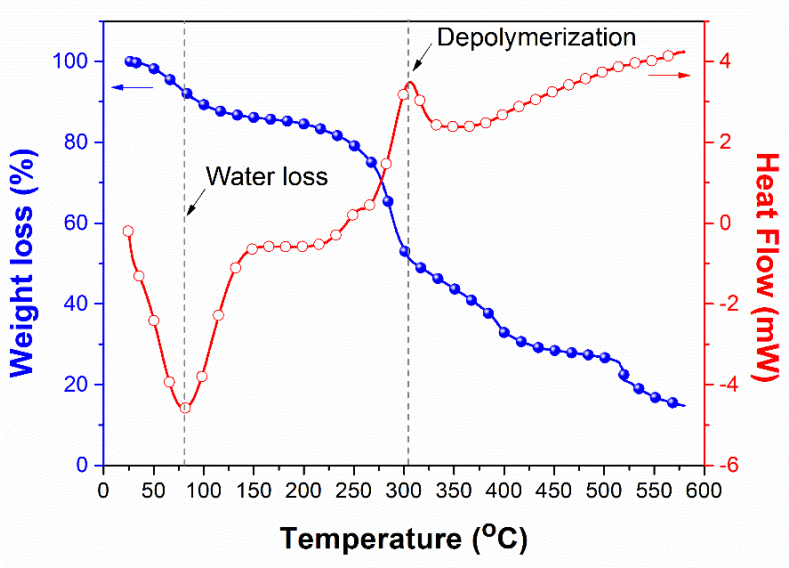

Figure 5.2 TGA and DSC curves showing the thermal behavior, chemical changes and weight loss of the polysaccharides extracted from spent coffee grounds by alkali pretreatment.

The TGA curve (Figure 5.2) shows the weight losses of the polysaccharides when exposed to heating until 580 °C, with four defined mass loss stages. The first one started at approx. 80 °C and corresponded to weight losses of about 12.91%, resulting from the adsorbed and structural water evaporation (dehydration of the sample). The greatest transformation and mass losses occurred during the second stage, at approx. 300 °C. At this stage, the depolymerization and decomposition of polysaccharides occurred, providing weight losses of 37.61%, in agreement with the DSC thermogram. Finally, the third and fourth thermal stages started at approx. 400 °C and 520 °C, respectively, being related with the decomposition of the material and resulting in weight losses of 13.95% and 9.73%.

5.2.3. Antioxidant phenolic compounds

In order to evaluate the phenolic compounds and the antioxidant activity of the polysaccharides extracted from SCG, aqueous extracts were obtained by mixing the lyophilized material with ultrapure water in a relation of 1 mg/ml. The values obtained for the total phenolic compounds and the antioxidant activity determined by different methods are presented in Table 5.2. The content of phenolic compounds (230 mg GAE/g LM) was very closely to the values reported in Section III – Chapter 4 when using the optimum process conditions for extracting polysaccharides by autohydrolysis of SCG, leading to the presence of phenolic compounds in the lyophilized material and achieving values of 234 mg GAE/g LM.

According to the current literature, different methods can be used to evaluate the antioxidant activity in food and biological systems. However, as each method is based on a different reaction, it is strongly advisable determining the antioxidant potential of a sample by different methods in order to better interpret the results. Figure 5.3 shows the antioxidant properties of polysaccharides extracted from SCG, using three different methods.

Table 5.2 Total phenolic compounds and antioxidant capacity of the polysaccharides extracted from spent coffee grounds by alkali pretreatment

Assay method	Response
Total phenolic compounds (mg GAE/g LM)	230.14 ± 1.43
Total antioxidant activity (mg TOC/ml)	0.19 ± 0.01
FRAP (mg Fe(II)/ml)	0.20 ± 0.11
DPPH IC_{50} (mg TE/ml)	0.11 ± 0.00
ABTS IC_{50} (mg TE/ml)	0.08 ± 0.00

Results are expressed as mean ± standard deviation; n=3. FRAP: antioxidant activity by the ferric reducing antioxidant power assay; DPPH: antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl assay; ABTS: antioxidant activity by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt.

The obtained values were compared with standard antioxidant compounds such as BHT and BHA, which were analyzed under the same procedure and concentration than the extracted polysaccharides. Significant differences ($p < 0.05$) were obtained when comparing the values of extracted polysaccharides and the standards. When analyzed by FRAP assay the values of BHT were 3.5-fold higher than the values obtained for extracted polysaccharides. BHA was 15-fold higher in both FRAP and TAA assays, but the polysaccharides showed a similarly antioxidant potential for TAA when compared with BHT, which was 1.2-fold higher. On the other hand, the percent inhibition for all samples (at concentration of 1 mg/ml) when analyzed by DPPH and ABTS methods was much closer to the standards, clearly seen in Figure 5.3. However, it is known that the scavenging activity of compounds is directly related with the concentration, and hence for the polysaccharide concentration showing the IC_{50} (at concentrations of 0.7 and 0.9 mg/ml, for DPPH and ABTS, respectively), BHA and BHT exhibited higher than 50% of inhibition, revealing the stronger antioxidant capabilities of the standards. Although in almost all antioxidant assays the standards showed to have higher values than polysaccharides extracted form SCG, the antioxidant activity obtained by DPPH assay revealed higher free radical scavenging activity compared with other works; e.g. polysaccharides extracted from edible mushrooms species, such as *Pleurotus australis*, *Ileodictyon*

cibarium, *Hericium erinaceum* and *Hericium coralloides* need higher concentration of polysaccharide extract to achieve inhibitions at 50%, being 4.03, 5.78, 5.82 and 7.19 mg/ml, respectively (Ren et al., 2014).

There are several factors that can influence the antioxidant activity of the extracts. For instance, defatting process, which is normally used to remove fatty compounds in coffee before polysaccharides extraction (Bravo, Monente, Juániz, De Peña, & Cid, 2013; Nunes et al., 2005), may influence the antioxidant capacity of the samples since antioxidant compounds could be also removed (Bravo et al., 2013).

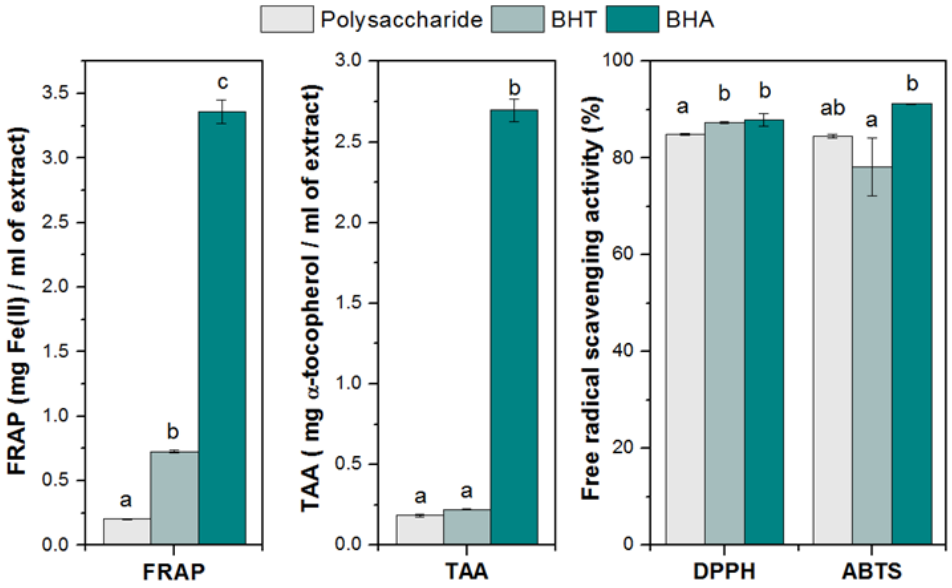

Figure 5.3 Antioxidant activity of the aqueous extracts from SCG polysaccharide and two commercial antioxidant (BHT and BHA) evaluated by different methods including FRAP, TAA, DPPH and ABTS assays. Different letters within each method mean values statistically different at 95% confidence level

5.2.4. Antimicrobial activity

Polysaccharides extracted from SCG were screened for antimicrobial activity against five fungi using the micro-dilution methodology. All strains were evaluated as a function of the incubation time, assessing the growth rate after 24, 48, 72 and 96 h of incubation at 25 ± 2 °C. Figure 5.4

shows the absorbance values at 530 nm obtained for *P. violacea* as an example of the absorbance values at 530 nm for different polysaccharide concentrations.

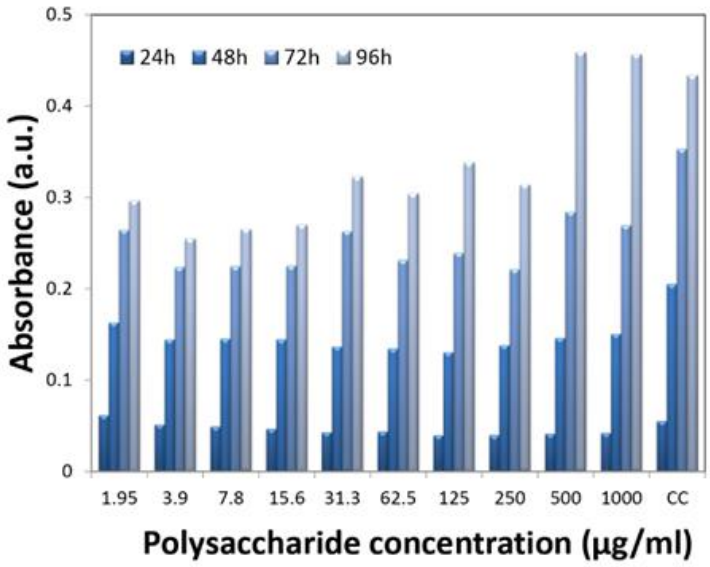

Figure 5.4 Absorbance values at 530 nm for the different polysaccharide concentrations after 24, 48, 72 and 96 h of fungal inoculation with P. violacea (a) as an example of the all fungi behavior

The graph clearly depicts a normal development of *P. violacea* growth control (CC), while clear alterations to the fungi growth are noticed when different concentrations of the polysaccharides are tested. These changes were observed in all fungi tested and were discussed in more detail using the percentage of growth, plotted in Figure 5.5a and Figure 5.5b. The evolution of all microbial strains growth on two different concentrations, 1000 µg/ml (maxima condition) and 1.95µg/ml (minimal condition) are displayed in Figure 5.5a and Figure 5.5b, respectively. Both polysaccharide concentrations properly represented two distinctive behaviors for low and high concentrations against all fungi. Thus, when the polysaccharide concentration was lower than 31.3 µg/ml, the trends of fungi growth with respect to time was similar and was presented in Figure 5.5a, while for higher concentration the growth tendencies were presented in Figure 5.5b.

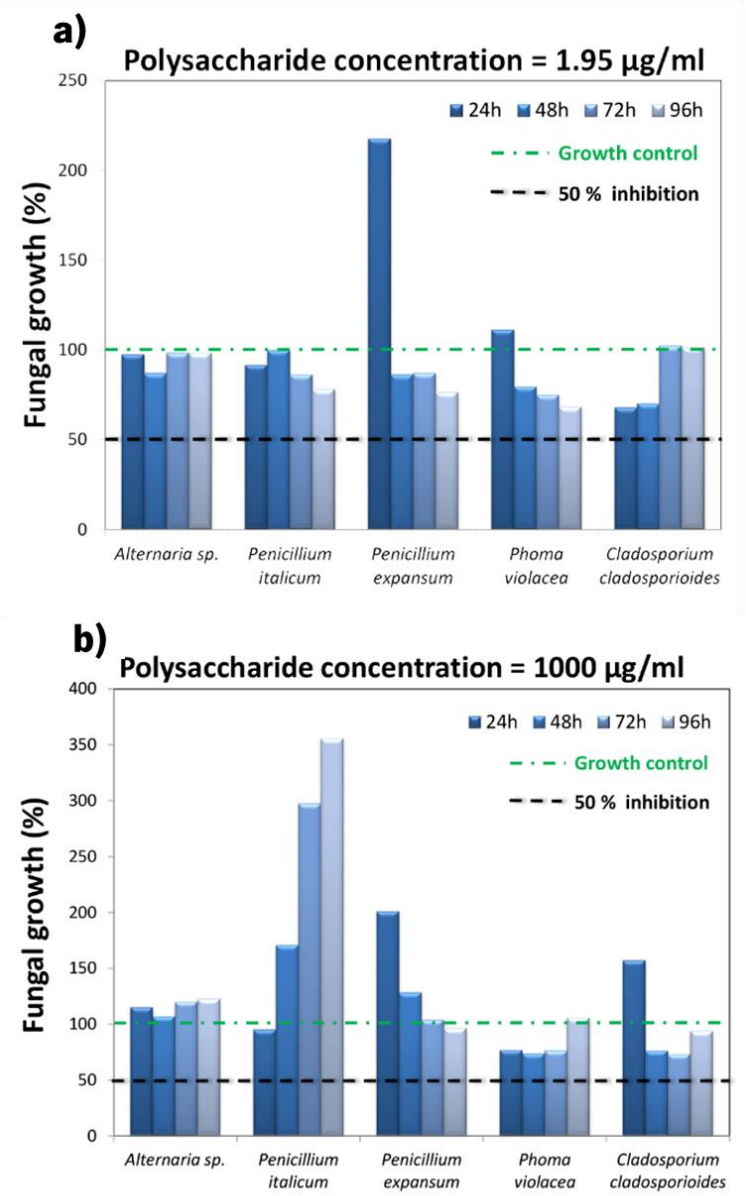

Figure 5.5 Evolution of all microbial strains on two different polysaccharide concentrations, 1000 µg/ml (a) and 1.95 µg/ml (b), being the highest and lowest used concentrations, respectively

Alternaria sp. exhibited very low inhibition growth for all the tested concentrations, reaching values no larger than 20% of inhibition. On the other hand, *P. italicum*, showed a particular behavior when compared to the growth of the control (CC), where higher polysaccharide concentrations

promoted the fungal growth instead of inhibiting, attaining a growth almost 4-fold higher after 96 h of incubation. This behavior suggests that higher polysaccharide concentrations may act as a carbon source, stimulating the *P. italicum* growth in greater proportions than the culture media. On the contrary, for lower concentrations, the fungus growth was slightly inhibited up to value no greater than 25%. *P. expansum* exhibited higher growth rates for the first 24 h compared to the growth control. However, a monotonic reduction of fungus growth was observed when the experiment time increased for all the concentrations, achieving higher inhibition values for lower polysaccharide concentrations. The extracted polysaccharide presented the higher inhibition efficacy for *P. violacea* and *C. cladosporioides* among all the strains tested. At higher polysaccharides doses, the *P. violacea* showed a constant inhibition, reducing to 0 % of inhibition after 96 h of incubation, indicating a short-term inhibition that may be due to the consumption of the inhibitory components present in the extracted polysaccharide. Nonetheless, for lower doses, an increase of the growth inhibition was observed. This behavior was also observed for *C. cladosporioides* at some concentrations, but with less defined tendencies in this particular strain. The differences between the high and low polysaccharide concentrations suggest a competition between the antimicrobial components in the extracts and the increased carbon source that the polysaccharide may offer to the microorganism, limiting the extracts function as an antimicrobial agent to low polysaccharide concentrations.

The previous behavior could be more clearly observed in Figure 5.6, where the growth percentage for all the strains at 96 h is plotted as a function of the polysaccharide concentration. This figure evidences that the increment of the polysaccharide doses reduced the inhibition of growth for the five different fungi strains. As previously mentioned, *Alternaria* sp. did not show significant changes among the concentrations studied, indicating the lack of interaction between the extract and the strain. *C. cladosporioides* exhibited the highest inhibition at 31.3 μg/ml (54.60%), as shown in Table 5.3. Concentrations of 3.9 μg/ml showed high percent of inhibition, being the concentration in which the five strains revealed higher antimicrobial activity.

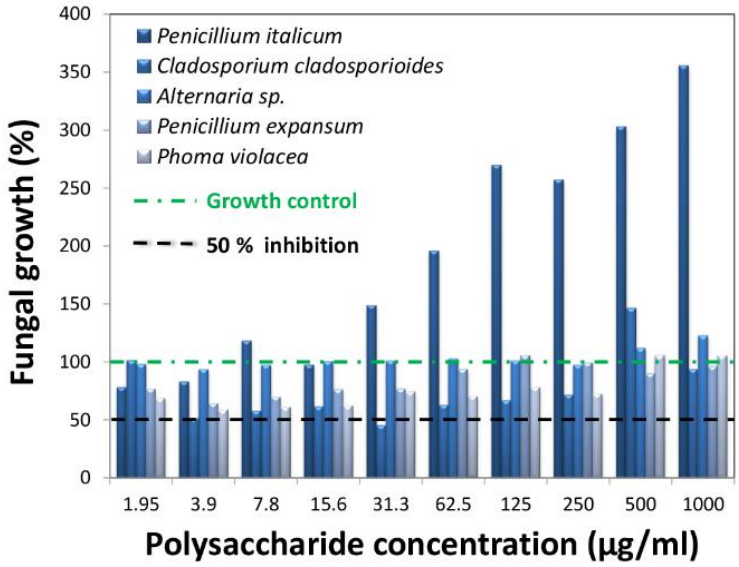

Figure 5.6 Fungal growth as a result of the effect of polysaccharide extracts at different concentrations on P. italicum, C. cladosporioides, Alternaria sp., P. expansum and P. violacea after 96 h of inoculation and incubation at 25 ± 2 ºC, expressing the minimal inhibitory concentration (MIC) of polysaccharides extracts when compared with a growth control

Table 5.3 Optimal conditions and percent inhibition of the polysaccharide extracts on growth of different microbial strains

Microbial strains	Optimal conditions* (µg/ml)	Percent inhibition (%)
Penicillium italicum	1.95	22.04 ± 4.98
	3.9	17.03 ± 4.89
Cladosporium cladosporioides	31.3	54.60 ± 7.06
	3.9	48.63 ± 9.84
Alternaria sp.	3.9	6.62 ± 0.73
	7.8	2.78 ± 0.18
Penicillium expansum	3.9	36.08 ± 5.60
	7.8	30.48 ± 5.75
Phoma violacea	3.9	41.27 ± 6.95
	7.8	38.89 ± 4.49

* Results of the two better concentrations for each fungus. Percent inhibition was expressed as mean ± standard deviation; n=6.

The evolution of the growth inhibition as a function of time, is exposed in the Figure 5.7, confirming the facts previously described. Whereas the optimal conditions and percent inhibition of

the polysaccharide extracts after 96 h of incubation are shown in the Table 5.3 for the different microbial strains.

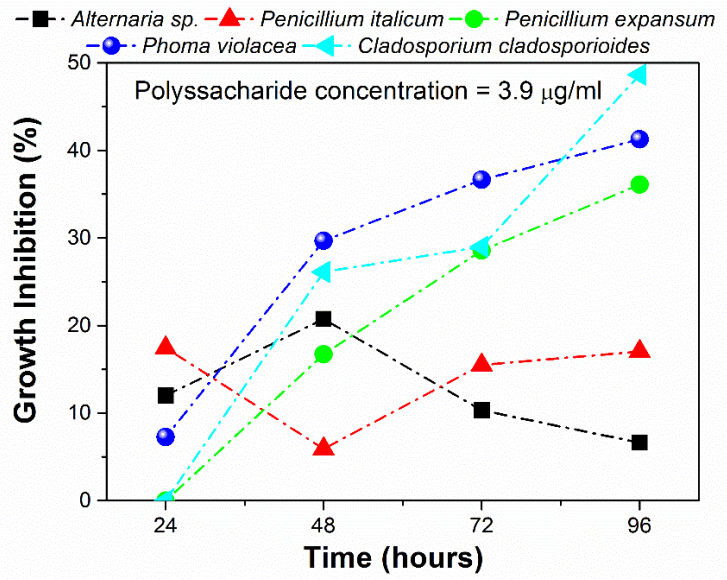

Figure 5.7 Growth inhibition percentage of the polysaccharide concentration at 3.9 µg/ml, as a function of time, revealing higher antimicrobial activity against the five tested stains (Alternaria sp., P. italicum, P. expansum, Phoma violacea and Cladosporium cladosporioides)

Additionally, tests with a known antimicrobial agent (fluconazole) revealed antimicrobial behavior (50% of growth inhibition or more) for concentrations larger than 50 µg/ml for *Alternaria sp* and *C. cladosporioides*, and 100 µg/ml for *P. italicum* and *P. violacea*, without evidence of the antimicrobial effect on the *P. expansusm*, where the growth inhibition was no higher than 30%.

Although the antimicrobial effect of extract rich in polysaccharides is not well understood, some authors have proposed that the polysaccharide may act as an external barrier, blocking the essential nutrients, impeding the microbial growth (Ren et al., 2014). Nevertheless, this barrier behavior should be increased as the polysaccharide concentration is increased, contradicting the results found in this report, where for higher concentration the antimicrobial effect is not evidenced. As a result, probably the antimicrobial effect of the extract may be due to residual components such as phenolic compounds (Jasso de Rodríguez et al., 2011) that are retained in the extract during the process, which compete between the polysaccharide as a carbon source for high concentrations.

5.3. Conclusions

The alkali pretreatment using 4 M NaOH as solvent showed to be a good option for an efficient extraction of polysaccharides from SGC. The most relevant sugars in SCG polysaccharides were galactose, followed by arabinose, glucose and mannose. Polysaccharides were thermostable in a large range of temperature, being therefore suitable for application in the manufacture of biomaterials and encapsulation products for several industrial purposes. Additionally, they revealed good antioxidant activity through different methods and presented high antimicrobial percent inhibition against *P. violacea* and *C. cladosporioides*. These findings open up possibilities to evaluate SGC polysaccharides as bioactive compounds in different food and pharmaceutical applications.

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SECTION IV

PHENOLIC COMPOUNDS

CHAPTER 6

EXTRACTION OF ANTIOXIDANT PHENOLIC COMPOUNDS BY AUTOHYDROLYSIS OF SPENT COFFEE GROUNDS

The following chapter is partially based on the results published in: Lina F. Ballesteros, Mónica J. Ramirez, Carlos E. Orrego, José A. Teixeira & Solange I. Mussatto. Optimization of autohydrolysis conditions to extract antioxidant phenolic compounds from spent coffee grounds *(Submitted in Journal of Food Engineering).*

6. Introduction

In a previous study, autohydrolysis under mild reaction conditions was demonstrated to be a technology with great potential to recover phenolic compounds from spent coffee grounds (SCG) (Conde & Mussatto, 2016). However, the conditions that maximize the extraction of these compounds from SCG were not established yet, and it is well-know that the efficiency of this extraction process is affected by the variables used for reaction, such as the solvent/solid ratio, time of contact, temperature, particle size of the solid matrix, among others. Thus, it is very important to optimize the extraction conditions in order to maximize the extraction efficiency. Optimizing the process conditions is also important because it allows a more suitable and complete exploitation of the feedstock, saving time, manpower, and making the process less expensive, reliable, cleaner and attractive to be implemented at industrial scale. Taking these facts into account, the aim of the present chapter was to optimize the process conditions to extract antioxidant phenolic compounds from SCG by using the eco-friendly technique of autohydrolysis. Extractions were performed using different temperatures (160 to 200 °C), liquid/solid ratios (5 to 15 ml/g SCG) and extraction times (10 to 50 min) in order to determine the conditions that maximize the extraction results. The effects of these operational variables on the extraction results were also verified. Finally, the conditions able to produce a phenolic rich extract with high antioxidant activity were determined. Apart from being a green technology, autohydrolysis under optimized conditions, it was demonstrated to be an efficient method to extract antioxidant phenolic compounds from SCG.

6.1. Materials and methods

6.1.1. Raw material and chemicals

Spent coffee grounds (SCG) were supplied by the Portuguese coffee industry NovaDelta-Comércio e Indústria de Cafés S.A. (Campo Maior, Portugal) and treated as in Section II – Chapter 3. All the chemicals used were analytical grade, purchased from Sigma–Aldrich (Chemie GmbH,

Steinheim, Germany), Panreac Química (Barcelona, Spain) and Fisher Scientific (Leicestershire, UK). Ultrapure water from a Milli-Q System (Millipore Inc., USA) was used.

6.1.2. Autohydrolysis process

Autohydrolysis assays were performed under different conditions of temperature (160 to 200 °C), liquid/solid ratio (5 to 15 ml water/g SCG) and extraction time (10 to 50 min), which were combined according to a 2^3 central composite design. The reactions was carried out as described in Section III – Chapter 4. The total content of each reactor was centrifuged (2500 *g*, 20 min) and the supernatant (SCG extract) was filtered through 0.22 μm filters and stored at -20 °C until further analyses. The volume of extract recovered after each extraction was quantified and used for calculating the extraction yield, being expressed as g recovered extract per 100 g SCG.

6.1.3. Analytical methodology

6.1.3.1. *Phenolic compounds*

The total content of phenolic compounds (PC) in SCG extracts was measured by using the Folin-Ciocalteu reagent method adapted to a 96-well microplate, as previously described in Section III – Chapter 4. The total content of PC was expressed as milligram of gallic acid equivalent per gram of dry weight material (mg GAE/g SCG).

6.1.3.2. *Ferric reducing antioxidant power assay*

The antioxidant activity of SCG extracts according to the ferric reducing antioxidant power (FRAP) assay was determined as described in Section II - Chapter 3. FRAP values were expressed as milligrams of ferrous equivalent per gram of dry weight material (mg Fe (II)/g SCG).

6.1.3.3. *Free radical scavenging activity*

The DPPH radical scavenging activity of SCG extracts was determined as indicated in Section III - Chapter 4. The IC_{50} values were expressed as milligrams of trolox equivalent (TE) per gram of dry weight material (mg TE/g SCG).

6.1.3.4. *Radical cation decolorization assay*

The ABTS radical cation decolorization assay was performed as described in Section III - Chapter 4. The IC_{50} values were expressed as milligrams of trolox equivalent (TE) per gram of dry weight material (mg TE/g SCG).

6.1.3.5. *Total antioxidant activity*

The total antioxidant activity (TAA) of SCG extracts was estimated as described in Section III - Chapter 4. TAA was expressed as milligrams of α-tocopherol equivalent per gram of dry weight material (mg TOC/g SCG).

6.1.3.6. *Flavonoids*

The total content of flavonoids in SCG extracts was estimated by colorimetric assay as previously described by Ballesteros et al. (2014). Briefly, a volume of 30 µl of the filtered and duly diluted extract was sequentially added to 90 µl methanol, 6 µl aluminum chloride at 10% (w/v), 6 µl potassium acetate (1 mol/L), and 170 µl distilled water, in a 96-well microplate. The mixtures were maintained during 30 min in the dark at room temperature, and the absorbance was then measured at 415 nm against a blank of distilled water using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). A calibration curve was prepared with a standard solution of quercetin (25, 50, 100, 150, 200 mg/L). The results was expressed as milligram quercetin equivalent per dry weight material (mg QE/g SCG).

6.1.3.7. *Determination of other compounds in SCG extracts*

Chlorogenic acid, furfural and hydroxymethylfurfural were analyzed by high performance liquid chromatography (HPLC) on an equipment LC-10 A (Jasco, Japan) using a UV detector at 276 nm and a Nucleosil 120-5 C18 5 µm (4.6 mm × 250 mm) column at room temperature. A mixture of acetonitrile and water (ratio 1/8) with 10 g/L of glacial acetic acid and with the final pH adjusted to 2.5 with phosphoric acid was used as mobile phase at a flow rate of 0.9 ml/min. The solvent mixture was degassed in an ultrasonic bath before to be used as mobile phase. The concentration of these components was determined from standard curves made with known concentrations of

each compound. The response of the UV detector was recorded and integrated using the D-7000 HPLC System Manager software (Hitachi).

6.1.4. Statistical analysis

The influence of the variables temperature, liquid/solid ratio and extraction time on the recovery of antioxidant PC by autohydrolysis of SCG was investigated through a 2^3 central composite design. The real and coded values of the variables used in the experimental design are given in Table 6.1. Statistical significance of the variables was determined at 5% probability level ($p < 0.05$). The data obtained from the design were fitted to second order polynomial equations, and the models were simplified by elimination of statistically insignificant terms. Statistical significance of the regression coefficients was determined by Student's *t*-test, and the proportion of variance explained by the models were given by the multiple coefficient of determination, R^2. Statistical analysis of the data as well as the determination of the conditions able to maximize the extraction results were performed using the software Statistica (version 8.0), and Design expert (version 8.0).

6.2. Results and discussion

The variables used for extraction, such as the reaction time, temperature and liquid/solid ratio, usually have great influence both in the kinetics of PC release from the solid matrix as well as in the antioxidant activity of the produced extracts. Therefore, this study evaluated the effect of these three variables on the recovery of PC with high antioxidant activity by autohydrolysis of SCG with the objective of selecting the conditions that maximize the extraction results. The experimental conditions used in each assay and the respective results of PC, FRAP, DPPH, ABTS and TAA are presented in Table 6.1. In the range of values studied in this work, the operational variables exerted great influence on the evaluated responses. The content of PC in the extracts, for example, varied between 6.09 ± 0.07 (assay 1) and 39.29 ± 0.83 mg GAE/g SCG (assay 8) according to the conditions employed for autohydrolysis. The antioxidant activity values increased from 0.03 ± 0.001 (assay 1) to 0.25 ± 0.008 mmol Fe(II)/g SCG (assay 8) by the FRAP assay, from 18.28 ± 0.09

(assay 1) to 119.01 ± 1.60 μmol TE/g SCG (assay 14) by the DPPH assay; from 21.53 ± 1.83 (assay 1) to 124.39 ± 3.21 μmol TE/g SCG (assay 8) by the ABTS assay and from 8.14 ± 0.23 (assay 1) to 64.79 ± 0.98 mg α-TOC/g SCG (assay 8) by the TAA assay. The differences between the results of antioxidant activity for the different assays can be explained by the fact that the methods differ from each other in terms of reaction mechanisms, oxidant and target/probe species, and reaction conditions (Conde & Mussatto, 2016; Karadag, Ozcelik, & Saner, 2009).

The worst values for all the responses were achieved when using the lowest limit to each variable (Table 6.1), while the best results were obtained when using the highest limit (except for DPPH assay). Similarly, the best yield of extraction (26.06 % (w/w)) was achieved with the highest conditions of extraction time, temperature and liquid/solid ratio were used (assay 8, Table 6.1).

The greatest content of PC (39.29 ± 0.83 mg GAE/g SCG) obtained in present study by autohydrolysis of SCG, was significantly higher than those reported in the literature for the recovery of PC from SCG by using organic solvents including isopropanol, ethanol and methanol (Murthy & Naidu, 2012; Mussatto, Ballesteros, et al., 2011; Panusa et al., 2013; Zuorro & Lavecchia, 2012), or by using autohydrolysis under mild process conditions (Conde & Mussatto, 2016). This value was also higher when compared to those reported for autohydrolysis of other natural sources such as corncobs (23.9 mg GAE/g dry matter), eucalypt wood (19.2 mg GAE/g dry matter), almond shells (36.2 mg GAE/g dry matter) and grape pomace (21.6 mg GAE/g dry matter) (Conde, Moure, Domínguez, & Parajó, 2011). The antioxidant activity of SCG extracts was also higher than the values reported to other antioxidant sources including medicinal plants like *Sophora japonica*, *Terminalia chebula*, *Prunella vulgaris* and *Scutellaria barbata* when aqueous extracts were evaluated by ABTS assay (Cai, Luo, Sun, & Corke, 2004), and fruits and grains such as black chokeberry, peach, apricot, hulled buckwheat, oat flakes when assessed by DPPH and FRAP methods (Stratil, Klejdus, & Kubáň, 2007). These results confirm that SCG is a phenolic rich agro-industrial waste with important antioxidant potential, and autohydrolysis is an efficient technique to extract such compounds from SCG.

Table 6.1 Experimental conditions and results obtained during the extraction of antioxidant phenolic compounds by autohydrolysis of spent coffee grounds (SCG). Assays according to a 2^3 central composite design

Assay	Process variables (real and (coded) values)[a]			Responses [b]					
	X_1	X_2	X_3	PC	FRAP	DPPH	ABTS	TAA	Yield
1	160 (-1)	5 (-1)	10 (-1)	6.09 ± 0.07	0.03 ± 0.001	18.28 ± 0.09	21.53 ± 1.83	8.14 ± 0.23	3.00
2	200 (+1)	5 (-1)	10 (-1)	8.59 ± 0.09	0.05 ± 0.002	33.06 ± 0.06	32.08 ± 0.01	12.61 ± 0.27	6.15
3	160 (-1)	5 (-1)	50 (+1)	8.59 ± 0.14	0.06 ± 0.004	35.33 ± 0.73	34.83 ± 0.15	14.15 ± 0.23	7.09
4	200 (+1)	5 (-1)	50 (+1)	10.95 ± 0.24	0.08 ± 0.006	40.01 ± 0.19	41.39 ± 0.65	20.94 ± 0.08	10.91
5	160 (-1)	15 (+1)	10 (-1)	12.63 ± 0.27	0.10 ± 0.004	55.74 ± 0.50	51.94 ± 0.61	26.06 ± 0.26	8.22
6	200 (+1)	15 (+1)	10 (-1)	19.55 ± 0.77	0.15 ± 0.006	78.42 ± 0.04	85.75 ± 0.28	37.38 ± 1.38	11.98
7	160 (-1)	15 (+1)	50 (+1)	17.39 ± 0.30	0.16 ± 0.009	96.16 ± 6.94	88.59 ± 0.47	39.30 ± 1.38	14.66
8	200 (+1)	15 (+1)	50 (+1)	39.29 ± 0.83	0.25 ± 0.008	118.15 ± 0.27	124.39 ± 3.21	64.79 ± 0.98	26.06
9	160 (-1)	10 (0)	30 (0)	23.57 ± 0.47	0.13 ± 0.005	63.73 ± 0.45	61.50 ± 0.78	25.63 ± 0.42	10.51
10	200 (+1)	10 (0)	30 (0)	28.26 ± 0.23	0.19 ± 0.014	84.22 ± 0.37	57.01 ± 0.20	40.29 ± 0.55	21.31
11	180 (0)	10 (0)	10 (-1)	21.42 ± 0.47	0.12 ± 0.007	60.70 ± 0.37	70.34 ± 0.58	22.95 ± 0.18	8.32
12	180 (0)	10 (0)	50 (+1)	27.57 ± 0.32	0.18 ± 0.006	93.97 ± 0.88	92.89 ± 0.05	37.58 ± 1.85	20.16
13	180 (0)	5 (-1)	30 (0)	10.62 ± 0.07	0.07 ± 0.002	40.94 ± 0.19	34.61 ± 0.34	17.08 ± 0.04	8.82
14	180 (0)	15 (+1)	30 (0)	36.88 ± 0.51	0.20 ± 0.002	119.01 ± 1.60	107.98 ± 0.43	45.48 ± 0.36	19.45
15	180 (0)	10 (0)	30 (0)	21.52 ± 0.50	0.14 ± 0.003	80.10 ± 6.65	70.34 ± 0.58	29.80 ± 0.20	11.90
16	180 (0)	10 (0)	30 (0)	21.40 ± 0.52	0.13 ± 0.005	78.31 ± 0.31	69.80 ± 0.17	28.72 ± 0.96	13.03
17	180 (0)	10 (0)	30 (0)	23.21 ± 0.29	0.14 ± 0.003	79.05 ± 1.14	73.45 ± 1.09	31.33 ± 0.88	14.11
18	180 (0)	10 (0)	30 (0)	25.10 ± 0.65	0.14 ± 0.006	79.76 ± 0.38	72.88 ± 0.98	29.12 ± 0.48	14.47
19	180 (0)	10 (0)	30 (0)	23.77 ± 0.13	0.13 ± 0.001	81.64 ± 1.35	84.65 ± 0.87	33.99 ± 1.14	13.78
20	180 (0)	10 (0)	30 (0)	25.95 ± 0.18	0.14 ± 0.003	79.05 ± 0.85	85.94 ± 0.68	32.46 ± 0.84	14.26

[a] X_1: temperature (°C); X_2: liquid/solid ratio (ml/g); X_3: extraction time (min).

[b] PC: phenolic compounds (mg GAE/g SCG); FRAP: antioxidant activity by the ferric reducing antioxidant power assay (mmol Fe(II)/g SCG); DPPH: antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl assay (μmol TE/g SCG); ABTS: antioxidant activity by the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid assay (μmol TE/g SCG); TAA: Total antioxidant activity (mg α-TOC/g SCG); Yield: (% (w/w)).

Some researchers have related the potential of PC with their antioxidant activity (Alothman, Bhat, & Karim, 2009; Ballesteros et al., 2014; Cai et al., 2004, Mussatto, 2015). However, usually the correlation cannot be evidenced for all the antioxidant activity assays due to the fact that each method has different reaction mechanisms, as previously explained. In the present study, the relationship among total PC extracted by autohydrolysis of SCG and the results of antioxidant activity obtained by the different methods (which were based on different reaction mechanisms) was verified. A correlation analysis chart was plotted and revealed that the antioxidant activity by FRAP and ABTS assays was directly proportional to the content of PC present in the SCG extracts, the data being correlated with coefficients R^2= 0.9396 for FRAP assay and R^2= 0.9459 ABTS assay (Figure 6.1). These results suggest that the PC present in the SCG extracts contributed significantly to the antioxidant activity of the extracts when evaluated by both FRAP and ABTS assays.

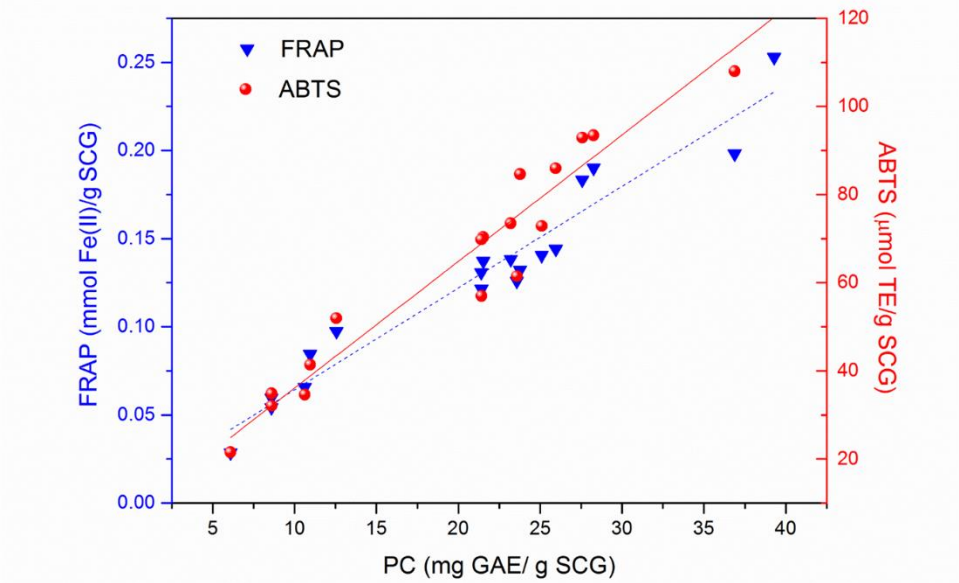

Figure 6.1 Correlation analysis chart for the responses total phenolic compounds (PC) and antioxidant activity (FRAP and ABTS assays) of extracts obtained by autohydrolysis of spent coffee grounds

In order to corroborate the estimated effect of each operational variable used for the autohydrolysis of SCG on the efficiency of the responses, Pareto charts were plotted (Figure 6.2). In this figure, bars extending beyond the vertical line corresponded to the effects statistically significant at 95% confidence level. The length of each bar was proportional to the standardized

effect. The statistical analysis revealed a significant effect ($p < 0.05$) of the three variables on the total PC extraction from SCG through autohydrolysis technique, being the liquid/solid ratio (X_2) the most significant variable, as shown in Figure 6.2a. As a result, similar trends on the antioxidant activity responses including FRAP, DPPH, ABTS and TAA (Figure 6.2b, c, d, e) and the extraction yield (Figure 6.2f) were expected. Although temperature (X_1) was significant on antioxidant activity and yield responses, it had more influence on PC extraction, being the second most important variable, after de liquid/solid ratio, for this response (Figure 6.2a). On the other hand, the reaction time (X_3) affected more significantly the antioxidant activity and yield responses when compared to temperature (Figure 6.2b, c, d, e, f). Similar to the present study, the solvent/solid ratio and temperature have been reported to be the most significant variables during the extraction of antioxidant PC from SCG when using a conventional solid-liquid extraction and ethanol as solvent (Zuorro & Lavecchia, 2012).

Not only the linear terms (L) of the variables, but also the quadratic terms (Q) and interactions had statistical significance on the PC and antioxidant activity responses ($p < 0.05$), as shown in Figure 6.2. These results reveal that the value of the responses was not linearly raised by increasing the value of the operational variables, but there was a maximum point after which the values of the responses decreased. Therefore, all the responses were fitted to second-order polynomial equations, in order to describe the responses variations as a function of the variables in the range of values studied. The non-significant terms at $p < 0.05$ were disregarded in order to improve the fitting and prediction of the model. The equation for each response as a function of the variables (temperature, X_1; liquid/solid ratio, X_2; time, X_3; – coded values) is shown in Table 6.2. None of these models presented lack-of-fit and revealed high coefficient of determination R^2, ranging from 0.84 to 0.98, which means a close agreement between the experimental results and those predicted by the equations. These models could be efficiently employed for a rapid prediction of the extraction results to be achieved when using temperatures, liquid/solid ratios and extraction times in the range of values evaluated in this study.

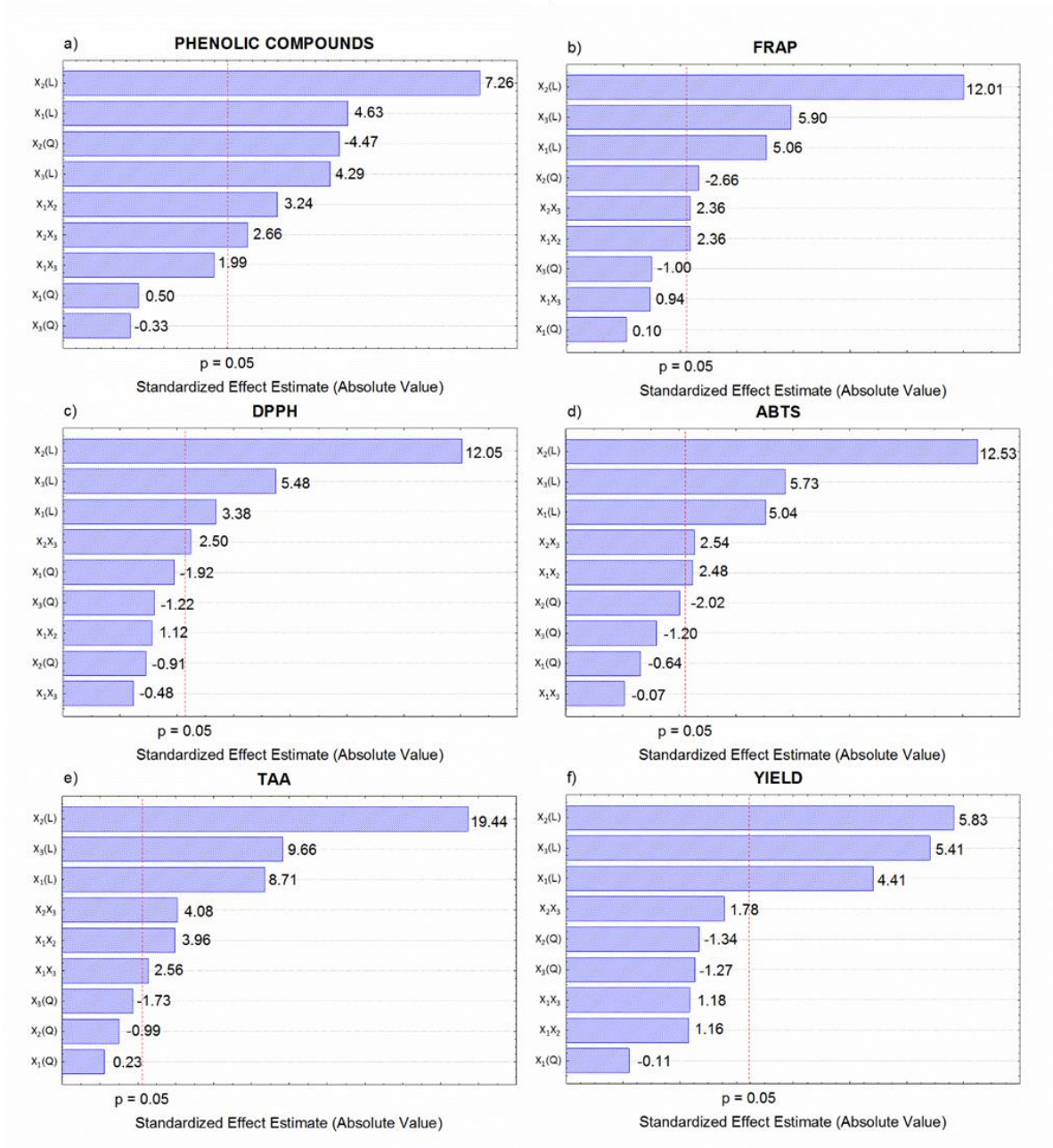

Figure 6.2 Pareto chart for the effects of temperature (X_1), liquid/solid ratio (X_2), extraction time (X_3), and their interactions ($X_1.X_2$, $X_1.X_3$, $X_2.X_3$) during the autohydrolysis of spent coffee grounds, on the total content of phenolic compounds (PC) (a), antioxidant activity (FRAP (b), DPPH (c), ABTS (d) and TAA (e) assays) and yield extraction (f) of the produced extracts. L and Q correspond to the effects at linear and quadratic levels, respectively

Table 6.2 Quadratic models describing the responses variation (total phenolic compounds (PC), antioxidant activity by the FRAP, DPPH, ABTS and TAA methods and extraction yield) as function of the process variables (temperature, liquid/solid ratio and extraction time) and their correspondent R^2 coefficients

Response[a]	Model equation *	R^2
PC	$PC = 24.18 + 3.84X_1 + 6.61X_2 + 3.56X_3 + 3.00X_1X_2 + 2.46X_2X_3 - 8.59X_2^2$	0.93
FRAP	$FRAP = 0.14 + 0.026X_1 + 0.056X_2 + 0.029X_3 + 0.012X_1X_2 + 0.013X_2X_3 - 0.03X_2^2$	0.96
DPPH	$DPPH = 77.20 + 8.46X_1 + 30.21X_2 + 13.75X_3 + 7.02X_2X_3 - 14.89X_1^2$	0.94
ABTS	$ABTS = 76.19 + 11.81X_1 + 29.37X_2 + 13.43X_3 + 6.50X_1X_2 + 6.65X_2X_3 - 13.93X_2^2$	0.95
TAA	$TAA = 31.39 + 6.27X_1 + 14.02X_2 + 6.96X_3 + 3.19X_1X_2 + 2.06X_1X_3 + 3.29X_2X_3 - 3.00X_3^2$	0.98
Yield	$Yield = -14.78 + 3.33X_1 + 4.40X_2 + 4.08X_3 - 3.11X_2^2$	0.84

* X_1: temperature; X_2: liquid/solid ratio; X_3: extraction time. Coded values.

[a] PC: phenolic compounds (mg GAE/g SCG); FRAP: antioxidant activity by the ferric reducing antioxidant power assay (mmol Fe(II)/g SCG); DPPH: antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl assay (μmol TE/g SCG); ABTS: antioxidant activity by the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid assay (μmol TE/g SCG); TAA: total antioxidant activity. (mg α-TOC/g SCG); Yield (% (w/w)).

Based on the previously established model equations, contour lines graphs for PC, antioxidant activity responses and extraction yield were plotted (Figure 6.3). The graphs are presented at constant temperature (200 °C) since it revealed the highest values for all responses studied, and less significant effect in the majority of them when compared to the extraction time and liquid/solvent ratio. This is in agreement with the findings reported by Dorta, Lobo, & Gonzalez (2012), who observed an enhanced diffusion rate and solubility of the compounds in the solvent when the temperature was incremented, improving the extraction process. Figure 6.3 shows the existence of a single region where all the responses are maximized. Additionally, it can be seen in Figure 6.3a that the content of PC increased when the liquid/solid ratio was higher than 10 ml/g, probably, due to the fact that more water could react with the SCG particles while more PC could permeate to the water (Prasad, Yang, Yi, Zhao, & Jiang, 2009). However, the time played a significant role in the PC extraction, since the recovery was maximized between 45-50 min of extraction time.

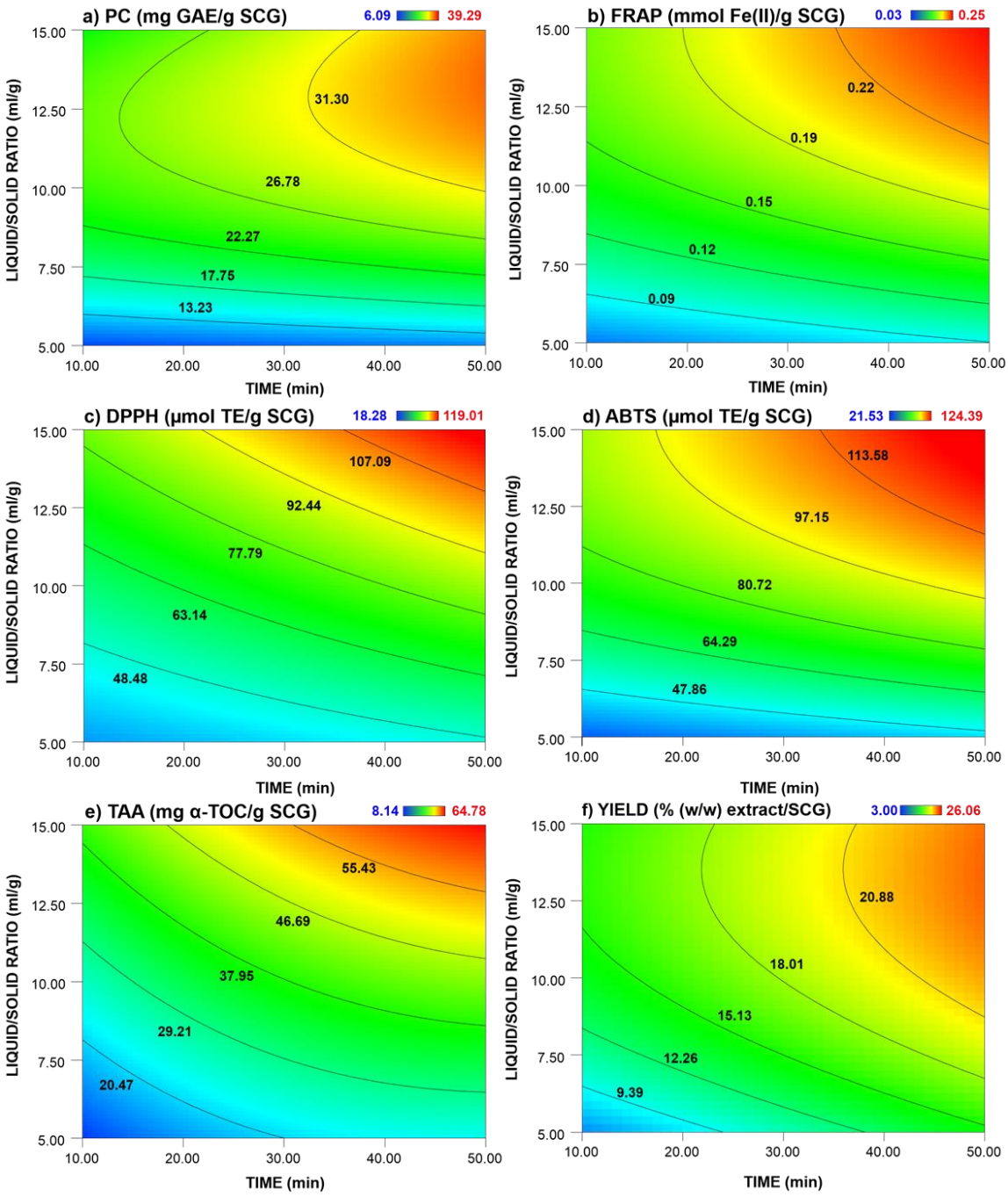

Figure 6.3 Contour line plots representing the total content of phenolic compounds (PC) (a), the antioxidant activity (FRAP (b), DPPH (c), ABTS (d) and TAA (e) assays) and the extraction yield (f) of extracts obtained by autohydrolysis of spent coffee grounds under different conditions of extraction time and liquid/solid ratio

The results of FRAP, DPPH, ABST and TAA assays plotted in Figure 6.3b, c, d, e, respectively, had a similar behavior when compared to the results of PC presented in Figure 6.3a. All the responses were maximized when the liquid/solid ratio was higher than 12.5 g/ml and the extraction time was superior than 40 min. The extraction yield (Figure 6.3f) presented an almost linear behavior to the extraction time, revealing that for a constant temperature and liquid/solid ratio the yield always increases when the time rises. Nonetheless, the liquid/solid ratio was the most significant variable for this response. The quantity of PC and antioxidant activity were maximized when the extraction yield was also increased.

Considering these results, a graphical optimization was carried out in order to determine the extraction conditions able to simultaneously produce an extract with high content of total PC and high antioxidant activity. The optimization process was conducted by overlapping the curves obtained in the models to each response. To determine the optimal extraction conditions, the following criteria was adopted: PC ≥ 35 mg GAE/g SCG, FRAP ≥ 0.23 mmol Fe(II)/g SCG, DPPH ≥ 100 μmol TE/g SCG, ABTS ≥ 110 μmol TE/g SCG, and TAA ≥ 60 mg α-TOC/g SCG. The overlaying plot attained (Figure 6.4) revealed an area in which all these criteria are satisfied (shadow area) and the optimum point (within this area) where the results of the responses were maximized, was then chosen, which corresponded to the use of 200 °C, 15 ml/g SCG and 50 min. Under these conditions, the model predicts a PC extraction of 35.07 mg GAE/g SCG; and antioxidant activity values for FRAP, DPPH, ABTS and TAA of 0.25 mmol Fe(II)/g SCG, 121.75 μmol TE/g SCG, 130.01 μmol TE/g SCG and 64.17 mg α-TOC/g SCG, respectively.

The optimal point was later reproduced to validate the results, obtaining values within 5 % of relative standard deviation (Table 6.3), which demonstrates a good degree of prediction.

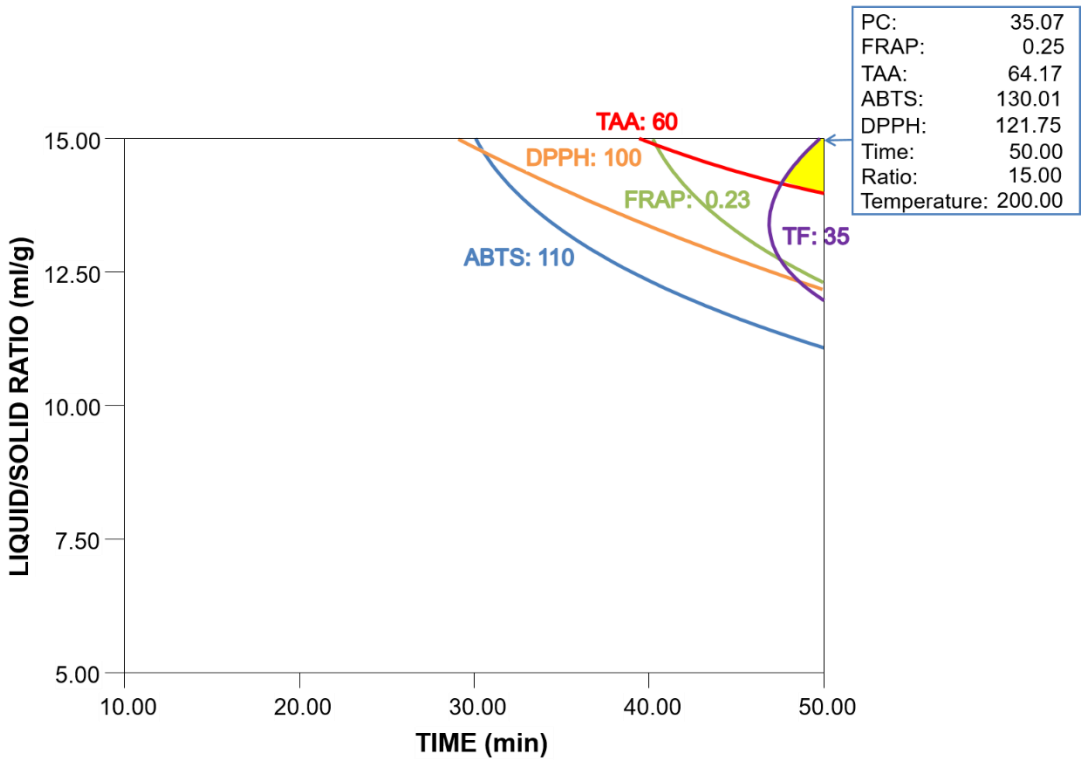

Figure 6.4 Optimum region overlaying the curves of the responses phenolic compounds (PC) and antioxidant activity by FRAP, DPPH, ABTS and TAA assays as a function of the extraction time and liquid/solid ratio used during the extraction process (g). The variables are presented in their original levels

The results of PC obtained in the present study under the optimized autohydrolysis conditions (40.36 mg GAE/g SCG) were significantly higher than the values reported in other studies using conventional solid-liquid extraction to recover PC from SCG. The values were 4-fold higher when compared to those achieved by Murthy & Naidu (2012) using isopropanol 60% as extraction solvent (10.20 mg GAE/g SCG), 2-fold higher when compared to the results reported by Mussatto, Ballesteros, et al. (2011) and Zuorro & Lavecchia (2012) using methanol (18.00 mg GAE/g SCG) and ethanol (19.98 mg GAE/g SCG), respectively, and 1.4-fold higher than those reported by Panusa et al. (2013) also using ethanol as solvent (28.26 mg GAE/g SCG).

Table 6.3 Results obtained in the assays for validation of the conditions optimized for extraction of antioxidant phenolic compounds by autohydrolysis of spent coffee grounds

Optimum point values	Responses [a]				
	PC	FRAP	DPPH	ABTS	TAA
200 °C,	40.43 ± 1.00	0.25 ± 0.01	112.11 ± 1.01	125.28 ± 0.02	66.95 ± 0.35
15 ml/g SCG	41.23 ± 0.79	0.24 ± 0.01	111.03 ± 0.15	126.96 ± 0.07	63.40 ± 0.38
50 min	3 39.36 ± 2.13	0.25 ± 0.01	114.25 ± 0.32	125.80 ± 0.57	68.27 ± 0.38
Experimental average	40.36 ± 0.90	0.25 ± 0.01	112.47 ± 1.64	125.68 ± 1.13	66.21 ± 2.51
Criteria	≥ 35.00	≥ 0.23	≥ 100.00	≥ 110.00	≥ 60.00
Predicted results	35.07	0.25	121.75	130.01	64.17

[a] PC: phenolic compounds (mg GAE/g SCG); FRAP: antioxidant activity by the ferric reducing antioxidant power assay (mmol Fe(II)/g SCG); DPPH: antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl assay (μmol TE/g SCG); ABTS: antioxidant activity by the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid assay (μmol TE/g SCG); TAA: Total antioxidant activity (mg α-TOC/g SCG).

Although organic solvents have been widely used to recover compounds from different natural sources, their toxic nature, mainly for isopropanol and methanol, can cause serious issues for food and pharmaceutical applications. On the contrary, pure water, as used in the present study for autohydrolysis, is more suitable to extract compounds used in these type of applications, besides being able to extract a higher amount of antioxidant PC.

Finally, the extract produced under the optimized process conditions was submitted to HPLC and colorimetric analyses in order to characterize the PC present. As a result, flavonoids and chlorogenic acid were found in the extract in concentrations of 1.87 ± 0.11 (mg QE/g SCG) and 2.25 ± 0.02 (mg/g SCG), respectively. Such compounds have been previously described to have antioxidant capacity and numerous bio-functionalities (Middleton, Kandaswami, & Theoharides, 2000; Shan et al., 2009). Sugar derived compounds including furfural and hydroxymethylfurfural were also identified in the extract in concentrations of 1.40 ± 0.02 and 2.09 ± 0.04 (mg/g SCG), respectively.

6.3. Conclusions

Autohydrolysis, which is an eco-friendly method that employs only water as extraction solvent, was an efficient technology to extract antioxidant phenolic compounds from spent coffee grounds. The total content of phenolic compounds and the antioxidant activity of the produced extract were affected by the variables used in the process, the liquid/solid ratio being the process variable with the highest influence on all the responses. The optimal extraction condition, achieved when using a temperature of 200 °C, liquid/solid ratio of 15 ml/g and extraction time of 50 min, was able to produce an extract containing high content of phenolic compounds (40.36 mg GAE/g SCG), including flavonoids and chlorogenic acid, and high antioxidant activity (FRAP = 0.25 mmol Fe(II)/g SCG, DPPH = 112.47 μmol TE/g SCG, ABTS = 125.68 μmol TE/g SCG and TAA= 66.21 mg α-TOC/g SCG). Such results highlight the great potential of spent coffee grounds for use as raw material on biotechnological processes due to their low cost and availability, and mainly due to their antioxidant capacity and presence of phenolic compounds, which have an outstanding role in health area, and wide applications in food and pharmaceutical products.

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CHAPTER 7

ENCAPSULATION OF ANTIOXIDANT PHENOLIC COMPOUNDS EXTRACTED FROM SPENT COFFEE GROUNDS BY FREEZE-DRYING AND SPRAY-DRYING USING DIFFERENT COATING MATERIALS

The following chapter is partially based on the results published in: Lina F. Ballesteros, Mónica J. Ramirez, Carlos E. Orrego, José A. Teixeira & Solange I. Mussatto. Encapsulation of antioxidant phenolic compounds extracted from spent coffee grounds by freeze-drying and spray-drying using different coating materials *(Submitted in Journal of Functional Foods).*

7. Introduction

Spent coffee grounds (SCG), the main residue of coffee industry obtained from soluble coffee preparation, has been gaining an increasing interest in the scientific community due to their high content of phenolic compounds (Murthy & Naidu, 2012; Mussatto, Ballesteros, Martins, & Teixeira, 2011; Panusa, Zuorro, Lavecchia, Marrosu, & Petrucci, 2013; Zuorro & Lavecchia, 2012). Generally, this type of compounds is known for presenting enormous benefits for the human health. Nevertheless, the phenolic compounds are very vulnerable to oxidizing environment, for example, to the light, oxygen, moisture, among others, due to the existence of unsaturated bonds in the molecular structures. For trying to conserve their properties, the encapsulation process have been considered as a good alternative, being so far proved in the conservation of different bioactive compounds including essential oils (Fernandes, Borges, & Botrel, 2014), anthocyanins (Flores, Singh, Kerr, Pegg, & Kong, 2014) propolis (Silva et al., 2013) among others, but it has never been applied on phenolic compounds extracted from SCG.

In this chapter, the encapsulation of antioxidant phenolic compounds extracted from SCG was studied, focused on comparing two encapsulation processes and evaluating two raw materials as vehicles of the compounds present in the extract. Spray-drying and freeze-drying technologies were utilized to encapsulate the antioxidant phenolic compounds of SCG, extracted by autohydrolysis using the optimum conditions reported in Section IV - Chapter 6, while maltodextrin, gum arabic and a mixture of these wall materials were assessed to retain the bioactive compounds and their antioxidant activity. Scanning electronic microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), dynamic scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were performed, together with determinations of phenolic compounds (PC), flavonoids (FLA) and antioxidant activity evaluated by Ferric reducing antioxidant power (FRAP) and total antioxidant activity (TAA) assays in order to corroborate the encapsulation of compounds and evaluate its efficiency.

7.1. Materials and methods

7.1.1. Raw material and chemicals

Spent coffee grounds (SCG) were provided by the Portuguese coffee industry Nova Delta-Comércio e Indústria de Cafés S.A. (Campo Maior, Portugal) and treated as in Section II - Chapter 3. All the chemicals used were analytical grade and maltodextrin (dextrose equivalent 20 (DE20)) and gum arabic were purchased from Sigma–Aldrich (Chemie GmbH, Steinheim, Germany). Ultrapure water from a Milli-Q System (Millipore Inc., USA) was used.

7.1.2. Extraction procedure

The extraction of antioxidant phenolic compounds from SCG was performed by autohydrolysis using the process conditions optimized in Section IV - Chapter 6 (200 °C, 15 ml water/g SCG and 50 min). The total content of the reactor was centrifuged (2500 *g*, 20 min) and the supernatant (SCG extract) was filtered through 0.22 μm filters and then encapsulated. The volume of extract recovered after centrifugation was quantified and used for data treatment.

In order to evaluate the structural properties of the extracted phenolic compounds, SCG extract was submitted to a reaction for the phenolic compounds precipitation. In brief, the extract was mixed with ethyl acetate (1:3 v/v) and the mixture was kept at room temperature during 24 h, being then centrifuged (2500 *g*, 20 min) and the precipitated dried at 100 °C.

7.1.3. Encapsulation process

Encapsulation of the SCG extract was carried out using maltodextrin and gum arabic as coating materials. For the assays, 100 ml of extract were mixed with 20 g of coating material and the mixture was homogenized at 6000 rpm in an IKA T-25D Ultra-turrax homogenizer until obtaining a good dispersion. Three matrices were evaluated: i) 100% maltodextrin; ii) 100% gum arabic; and iii) a mixture of maltodextrin and gum arabic at ratio 1:1. A blank consisting of distilled water instead of SCG extract was also prepared for each matrix. All the samples were prepared in triplicate and

the total soluble solids (°Brix) were measured using a digital refractometer. Afterward, the samples were subjected to freeze-drying and spray-drying processes. For freeze-drying, the samples were previously frozen and then put into a chamber at -60 °C under pressure of 0.05 bar, being maintained under these conditions during 48 h. A Christ alpha 1-4 LD equipment (SciQuip, UK) was used. Spray-draying was carried out in an equipment mini Buchi model 191 (Büchi Laboratoriums Technik, Switzerland) using a liquid feed volumetric flow rate of 108 ml/h, drying air inlet temperature of 100 °C, nozzle air flowrate, 600 NL (litters at normal conditions)/h and aspiration 75% (28 m3/h).

The moisture content of the dry powders was determined in a moisture analyser model MAC 50/1/NH (Radwag, Poland) and they were stored at room temperature and protected of the light until further analyses.

7.1.4. Analytical methodology

7.1.4.1. *Chemical characterization of SCG extract*

High performance liquid chromatography (HPLC) was used to analyze again the compounds present in the SCG extract as previously defined in Section IV - Chapter 6. Chlorogenic acid, furfural and hydroxymethylfurfural were identified and quantified in this extract and the concentration of these components was determined from standard curves made with known concentrations of each compound. The response of the UV detector was recorded and integrated using the D-7000 HPLC System Manager software (Hitachi).

7.1.4.2. *Structural characterization*

Morphology and crystalline phases of SCG extract and phenolic compounds encapsulated were evaluated by scanning electron microscopy (SEM) and X-ray diffraction (XRD), respectively, as described in Section II - Chapter 3. The chemical groups and bonding arrangement of constituents present in the samples were determined by Fourier transform infrared spectroscopy (FTIR) as

defined in Section III - Chapter 4. Differential scanning calorimetry (DSC) and thermogravimetric analyses (TGA) were carried out as previously described in Section II - Chapter 3.

7.1.4.3. *Antioxidant phenolic compounds characterization*

In order to evaluate the contents of total phenolic compounds and flavonoids, as well as the antioxidant activity of the samples after encapsulation process, the powders obtained by freeze-drying and spray-drying were rehydrated until achieving the same content of soluble solids measured before drying. The rehydration was calculated by using the Eq 7.1, where W_P, is the mass of powder to hydrate; M_{DP}, is the moisture of the sample after drying process; and B, represents the content of total soluble solids (°Brix) that had the sample before drying.

Eq 7.1 $$H_2O_{rehydration} = \frac{W_P\left(1-\frac{M_{DP}}{100}\right)}{B} * 100 - \left(\frac{W_P * M_{DP}}{100}\right)$$

7.1.4.3.1. Phenolic compounds

The total content of phenolic compounds (PC) of encapsulated samples was determined by using the Folin-Ciocalteu reagent according to the colorimetric method described in Section III - Chapter 4. The blank corresponding to each encapsulated was used for correcting the final content of phenolic compounds in the samples. The total content of phenolic compounds was expressed as milligram gallic acid equivalent per 100 milliliters of encapsulated sample (mg GAE/100 ml).

7.1.4.3.2. Flavonoids

The total content of flavonoids (FLA) was estimated by a colorimetric assay as defined in Section IV - Chapter 6. The blank corresponding to each encapsulated was used for correcting the final content of flavonoids in the samples. The content of total flavonoids was expressed as milligram quercetin equivalent per 100 milliliters of encapsulated sample (mg QE/100 ml).

7.1.4.3.3. Ferric reducing antioxidant power assay

The antioxidant activity of encapsulated compounds by the ferric reducing antioxidant power (FRAP) assay was determined as in Section II - Chapter 3. The blanks of the encapsulates were used for correcting the final antioxidant activity of the samples. The FRAP values were expressed as millimoles of ferrous equivalent per 100 milliliters of encapsulated sample (mmol Fe(II)/100 ml).

7.1.4.3.4. Total antioxidant activity

The total antioxidant activity (TAA) of encapsulated compounds was estimated as in Section III - Chapter 4. The blanks of the encapsulates were used for correcting the final TAA in the samples. TAA was expressed as milligrams of α-tocopherol equivalent per 100 milliliters of encapsulated sample (mg TOC/100 ml).

7.1.5. Statistical analysis

Statistical analyses were carried out using GraphPad Prism (version 6.1). One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were performed to determine the significant differences ($p < 0.05$) between the encapsulated samples.

7.2. Results and discussion

7.2.1. Extract characterization

7.2.1.1. *Chemical composition and antioxidant activity*

The contents of phenolic compounds and flavonoids, as well as the antioxidant activity values of the SCG extract before and after encapsulation are shown in Table 7.1. HPLC analyses (Figure 7.1a) revealed also the presence of chlorogenic acid (19.99 ± 3.56 mg/100 ml) and sugar derived compounds, namely hydroxymethylfurfural (18.57 ± 3.32 mg/100 ml extract) and furfural (12.44 ± 2.29 mg/100 ml extract), in SCG extract. Chlorogenic acid, considered the most important phenolic compound in coffee, is known to have antioxidant capacity and numerous biofunctionalities

(Mussatto, 2015; Farah & Donangelo, 2006).. The high content of phenolic compounds (with presence of flavonoids and chlorogenic acid) and the antioxidant activity of SCG extract confirm the great potential of SCG as a natural source of antioxidant phenolic compounds.

Table 7.1 Contents of phenolic compounds, flavonoids and antioxidant activity of the extract produced from spent coffee grounds (SCG) before and after encapsulation into different coating materials by freeze-drying or spray-drying

Drying process	Sample	PC	FLA	FRAP	TAA
	SCG Extract	350.28 ± 11.71	16.51 ± 1.03	2.15 ± 0.03	591.37 ± 12.41
Freeze-drying	M	216.37 ± 10.32	12.14 ± 0.34	1.56 ± 0.09	506.30 ± 14.72
	M + GA	173.57 ± 3.40	11.36 ± 0.93	1.58 ± 0.03	128.90 ± 13.82
	GA	145.32 ± 12.08	5.38 ± 0.33	1.21 ± 0.07	257.84 ± 17.78
Spray-drying	M	174.07 ± 7.27	7.88 ± 0.16	1.67 ± 0.02	380.25 ± 15.49
	M + GA	204.86 ± 13.00	3.60 ± 0.23	1.58 ± 0.05	144.73 ± 17.79
	GA	117.67 ± 12.58	6.72 ± 0.87	1.59 ± 0.03	194.13 ± 11.41

Results are expressed as mean ± standard deviation; n=6.

M: maltodextrin; GA: gum arabic; PC: total phenolic compounds (mg GAE/100 ml); FLA: flavonoids (mg QE/100 ml); FRAP: antioxidant activity by the ferric reducing antioxidant power assay (mmol Fe(II)/100 ml); TAA: antioxidant activity by the total antioxidant activity assay (mg α-TOC/100 ml).

7.2.1.2. *Structural characterization*

The crystallinity and chemical groups and bonding arrangement of constituents present in the SCG extract after precipitation with ethyl acetate were evaluated through X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). The XRD pattern (Figure 7.1b) revealed a mostly amorphous structure. However, around $2\theta = 20°$ a broad band was diffracted, revealing the existence of small crystalline regions in the SCG extract structure. This peak is related with the crystalline cellulose present in SCG when it has not been subjected to any treatment (Section II - Chapter 3). Although the autohydrolysis process is more suitable to extract antioxidant phenolic compounds and hemicelluloses from lignocellulosic materials, the high temperature and extraction time (200 °C, 50 min) used during the process allowed extracting the small part of crystalline cellulose as evidenced in Figure 7.1b.

FTIR spectrum (Figure 7.1c) showed the typical band from 1500 to 1700 cm^{-1} ((C=O) asymmetrical and symmetric stretching vibrations) highly associated with chlorogenic acid and caffeine (Ribeiro, Salva, & Ferreira, 2010) and deformation in lignin (Pandey & Theagarajan, 1997). Thus, the peak at 1654 cm^{-1} can be attributed to the absorption of these compounds, being the peak more intense when their concentration in the sample increases. The peak at 2930 cm^{-1} was assigned to the $C\text{-}H_{2,n}C\text{-}H_3$ stretch, being closely related to aromatic compounds with phenyl bonds similar to those in polyphenolic compounds, such as flavonoids (Mehanna et al., 2014; Santiago-Adame et al., 2015). Supplementary bands were found in the SCG extract, being in agreement with the findings reported in Section II - Chapter 3.

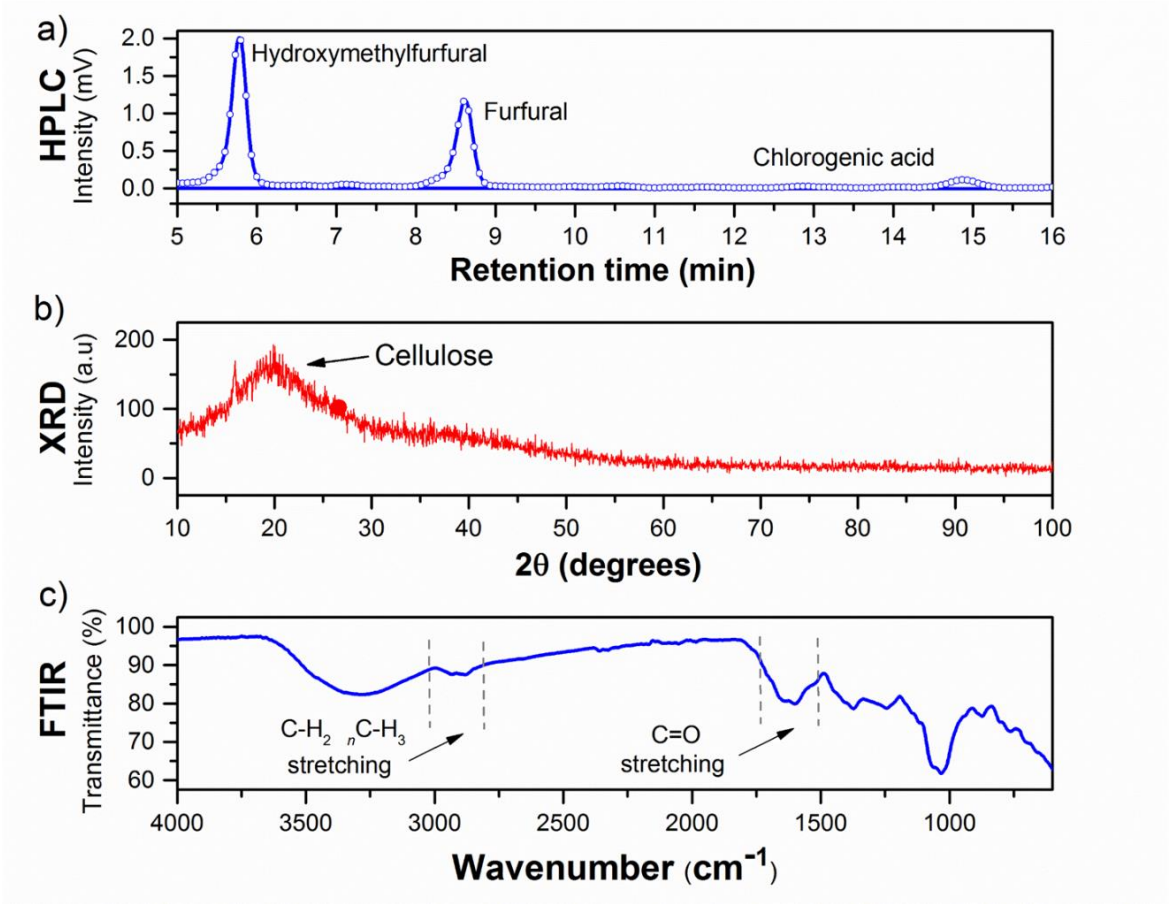

Figure 7.1 Chromatogram profile of the extract obtained by autohydrolysis of spent coffee grounds (SCG) (a). X-ray diffractogram (XRD) (b) and Fourier transform infrared spectra (FTIR) (c) of the extract obtained by autohydrolysis of SCG and then precipitated with ethyl acetate

7.2.1.3. *Thermal behavior*

DSC and TGA curves of extract obtained by autohydrolysis of SCG and subsequently precipited with ethyl acetate are shown in Figure 7.2. When the SCG extract was exposed to 600 °C three events were identified. The first one revealed an endothermic peak at 93.91 °C, being related to the presence of impurities in the sample and the vaporization of water (indicating the presence of hydrophilic groups), which occurs over this range of temperature. The second event corresponded to a broad exothermic transition starting approximately at 180 °C and finishing at 320 °C. In the initial phase (180 – 256 °C) this event was related to the degradation of antioxidant phenolic compounds (Reda, 2011) and in the last phase (256 – 320 °C) it was associated to the depolymerisation and branching of carbohydrates present in the SCG extract (Section II - Chapter 3). Finally, the third stage started over 400 °C and was related to the decomposition of the material.

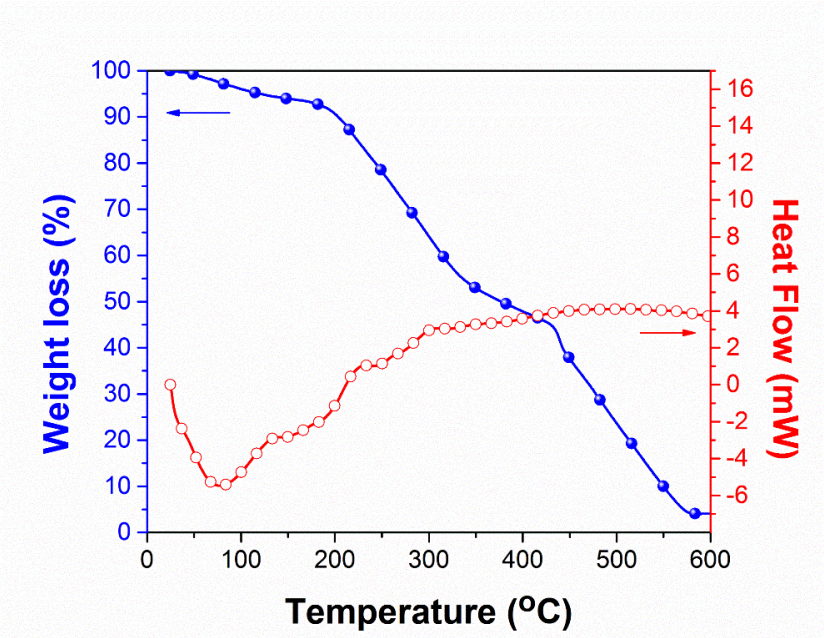

Figure 7.2 Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) curves of the extract obtained by autohydrolysis of SCG and then precipitated with ethyl acetate

7.2.2. Extract encapsulation

7.2.2.1. *Morphology*

Images obtained by scanning electron microscopy (SEM) for the pure coating materials, as well as for the samples encapsulated by freeze-drying and spray-drying and techniques are shown in Figure 7.3. Both coatings, maltodextrin and gum arabic possess similar morphologies. Nevertheless, maltodextrin revealed spheres of around 30 µm of diameter or smaller, while gum arabic showed more irregular particle sizes.

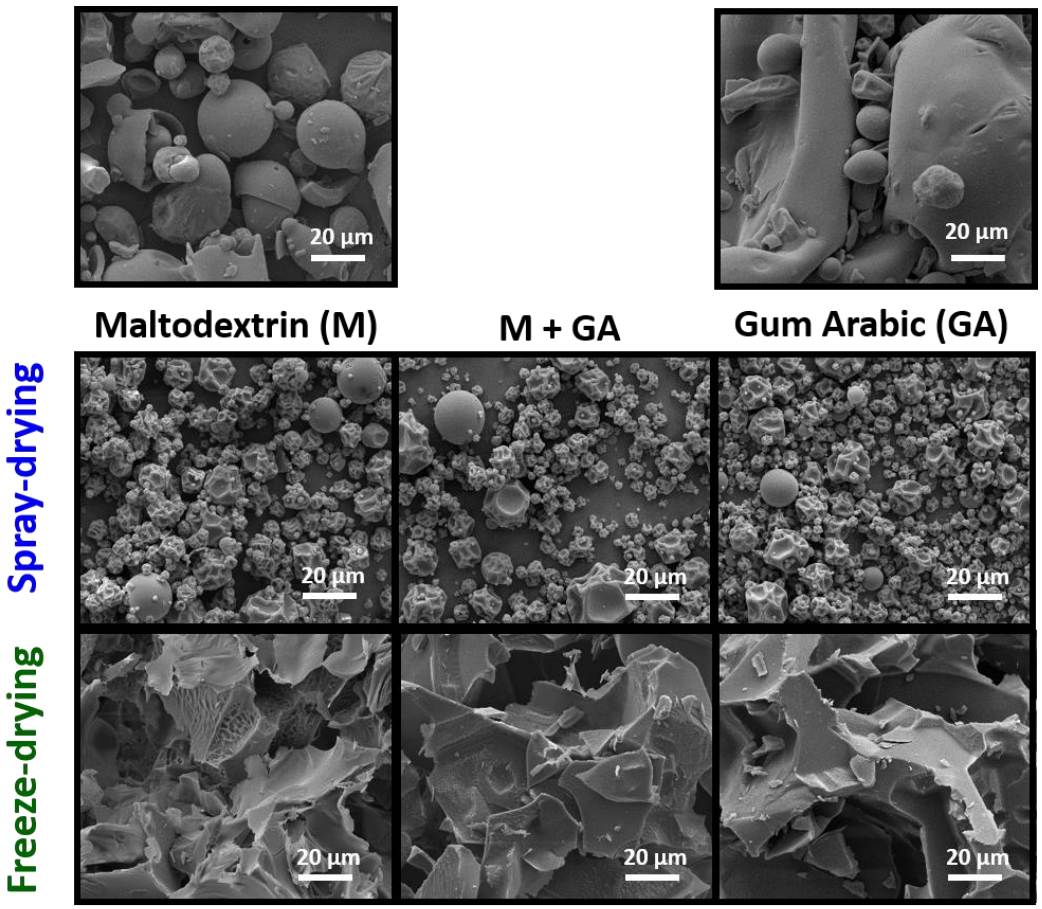

Figure 7.3 Scanning electron micrographs (SEM) micrographs for pure maltodextrin and gum arabic as well as for the phenolic compounds encapsulated and drying by spray-drying and freeze-drying. Magnification, 2500X.

These spherical capsules are used to absorb the extract and, after the drying process, they allow the components to remain in the coating materials. The morphology, shape and size of the capsules were expected to change after the freeze-drying and spray-drying processes, due to the conditions used in each process. For spray-drying, for instance, which utilized a temperature of 100 °C, the maltodextrin and gum arabic maintained the spherical form with very similar sizes (less than 30 μm), but in most of the cases a dehydrated aspect was shown. This morphology has been previously reported for spray-drying process (Santiago-Adame et al., 2015).

The freeze-drying, on the other hand, clearly modified the original morphology of the coating materials, leaving a more sawdust-like morphology, both in maltodextrin and the gum arabic, typical of lyophilization process in these matrices (Mahdavee Khazaei et al., 2014). Such morphological changes are expected to alter the power of encapsulation, due to the variation in the surface area of the coatings that allow more or less degradation of the encapsulated compounds.

7.2.2.2. *Structural characteristics*

7.2.2.2.1. Crystallinity and chemical bonding of constituents

Figure 7.4 displays the X-ray diffraction (XRD) patterns for maltodextrin and gum arabic, as well as the spectra for the SCG extract encapsulated into these matrices dried by freeze-drying and spray-drying. The XRD of the samples revealed a very low degree of crystallinity, evidencing a very broad peak around $2\theta = 18°$ and an amorphous background from the beginning of the spectra to $2\theta = 55°$. Quantifying the degree of crystallinity of a compound is difficult since very small crystalline regions give broad peaks, and larger crystalline regions translate in better defined peaks; however the amount of such regions cannot be directly quantified. As a result, only a tendency regarding the sizes of the crystalline regions can be given. For that purpose, the peaks were fitted using a Voight function and the full width at half maximum (FWHM) was reported in the spectra in order to analyze possible differences between the samples. For larger FWHM, smaller ordered regions were expected and vice versa. Maltodextrin, for instance, showed larger FHWM compared to gum arabic, suggesting a less ordered structure. The same behavior was maintained in the

samples after encapsulating the phenolic compounds regardless of the type of drying, but when a combination of both matrices, maltodextrin and gum arabic (ratio 1:1) was used, intermediate crystalline sizes were observed. This clearly evidences that the used coatings are the main responsible for the final structure of the encapsulated products.

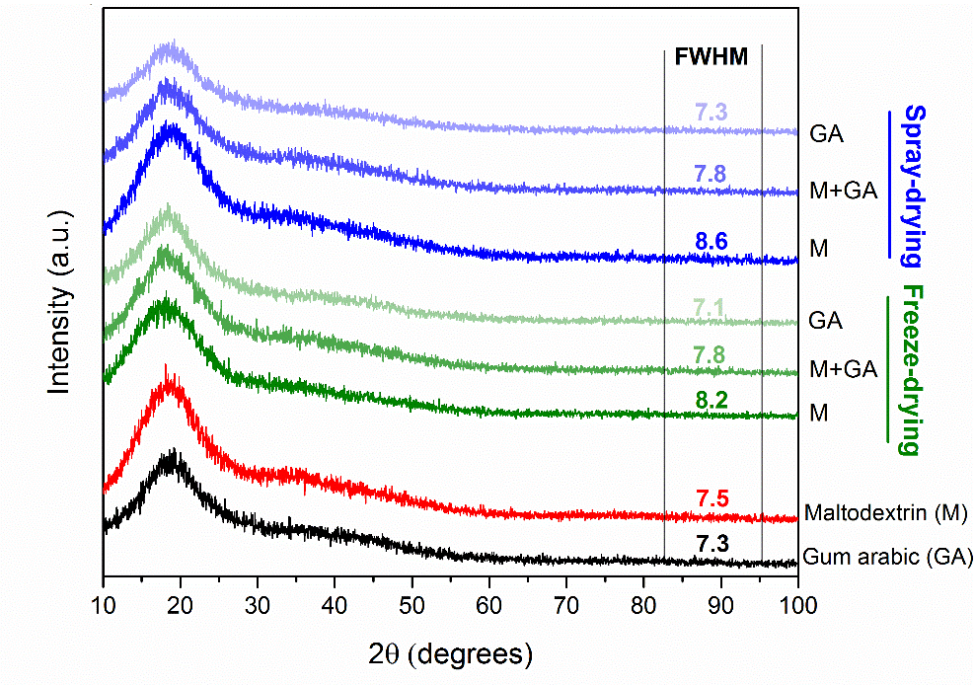

Figure 7.4 X-ray diffractogram (XRD) obtained for pure maltodextrin and gum arabic as well as for the phenolic compounds encapsulated and drying by spray-drying and freeze-drying. FWHM: full width at half maximum

Fourier transform infrared spectroscopy (FTIR) results (Figure 7.5) show the predominant effect of both matrices, maltodextrin and gum arabic in the final sample, since the coating material structures were not affected by the addition of SCG extract. A summary of the absorption bands characteristic for maltodextrin (Castro-Cabado, Casado, & San Román, 2016; Santiago-Adame et al., 2015) and gum arabic (Leonor et al., 2013; Paulino, Guilherme, Mattoso, & Tambourgi, 2010) are shown in Table 7.2. It must be also stressed that the conditions used for the different drying

processes did not alter the structure of the matrices, since independently of the process, no significant changes are observed.

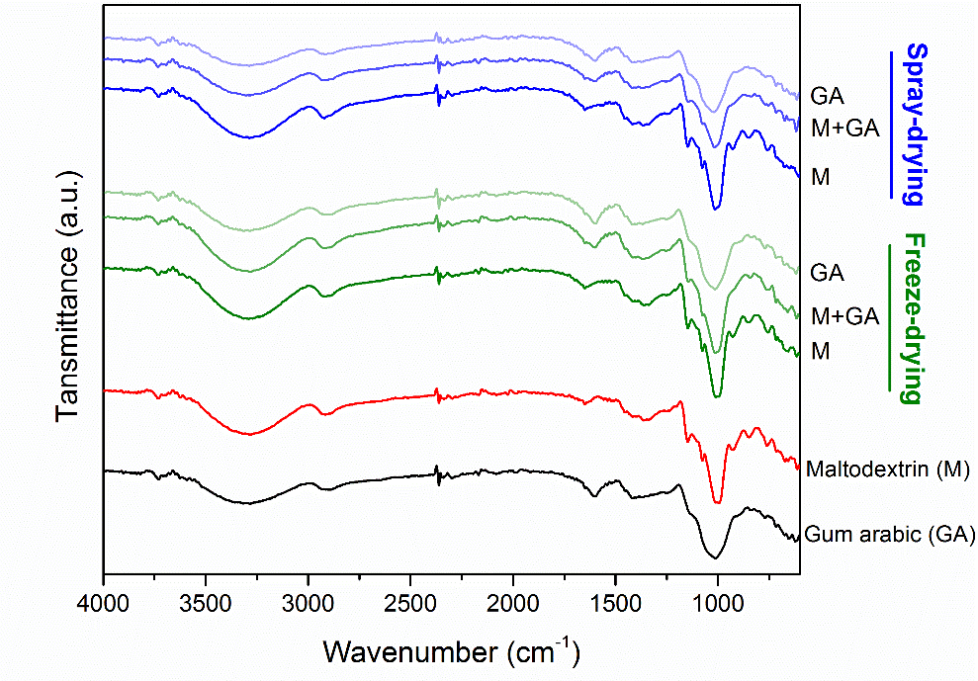

Figure 7.5 Fourier transform infrared spectra (FTIR) obtained for pure maltodextrin and gum arabic as well as for the phenolic compounds encapsulated and drying by spray-drying and freeze-drying

7.2.2.2.2. Thermal stability

DSC and TGA analyses for pure maltodextrin and gum arabic and the samples of SCG extract encapsulated using these coating materials were carried out in order to evaluate the thermal stability of the samples (Figure 7.6). As it can be seen, the structural features exposed in the thermal characterization were largely dependent of the coatings, evidencing thus, that the changes suffered in the samples are directly related with the transition temperatures of the maltodextrin and gum arabic. The first event occurring between 30 – 160 °C revealed an endothermic peak at 80 °C, which was associated to water evaporation and chemisorbed water through hydrogen bonds. This event was observed for all the samples by both, DSC and TGA analyses.

Table 7.2 Infrared (IR) assignments of the main vibrations in the FTIR spectra from maltodextrin and gum arabic

IR region (cm^{-1})	Vibrations (cm^{-1})	Assignments	
		Maltodextrin	**Gum arabic**
3600 - 3000	3300	O–H stretching broad band (hydroxyl group)	O–H stretching broad band (hydroxyl group)
3000 - 2800	2908	$C–H_2$ asymmetric stretching band	$C–H_2$ symmetric stretching band
1700 - 1500	1641	C=O stretching band (free carboxyl groups)	
	1603		C=O stretching band (carboxylic acid group)
1500 - 1200	1418		C–H bending bands
1200 - 650	1150	C–O stretching bands (ether group)	C–O stretching bands (ether group)
	1084	C–O stretching bands	
	1015		C–O stretching bands (pyranose form)
	1005	C–O and ring stretching modes	
	928	C–O stretching bands (ring and skeletal modes)	
	842	C–O–C stretching of glycosidic bonds	
	768	CH_2 out-of-plane bending	CH_2 out-of-plane bending (twisting)

On the other hand, maltodextrin and the samples encapsulated with this carbohydrate presented a double peak between 190 – 350 °C, generating a total weight loss of about 64%. This double transition is in agreement with the results reported by Paini et al. (2015) and Saavedra-Leos, Leyva-Porras, Araujo-Díaz, Toxqui-Terán, and Borrás-Enríquez (2015). However, it has also been shown that the onset of this peak (~ 190 °C) can vary slightly depending on the dextrose equivalent amount that the maltodextrin possesses and the water activity which the coating and the encapsulated samples were stored (Paini et al., 2015; Saavedra-Leos et al., 2015). The second part of the maltodextrin transition coincided with the transition observed for gum arabic and the

samples coated with this wall material revealing an exothermic peak for all the samples at about 300 °C. This transition located between 190 and 370 °C was attributed to the depolymerisation of the materials. Additionally, the samples containing gum arabic, presented a weight loss approximately 55% in this transition.

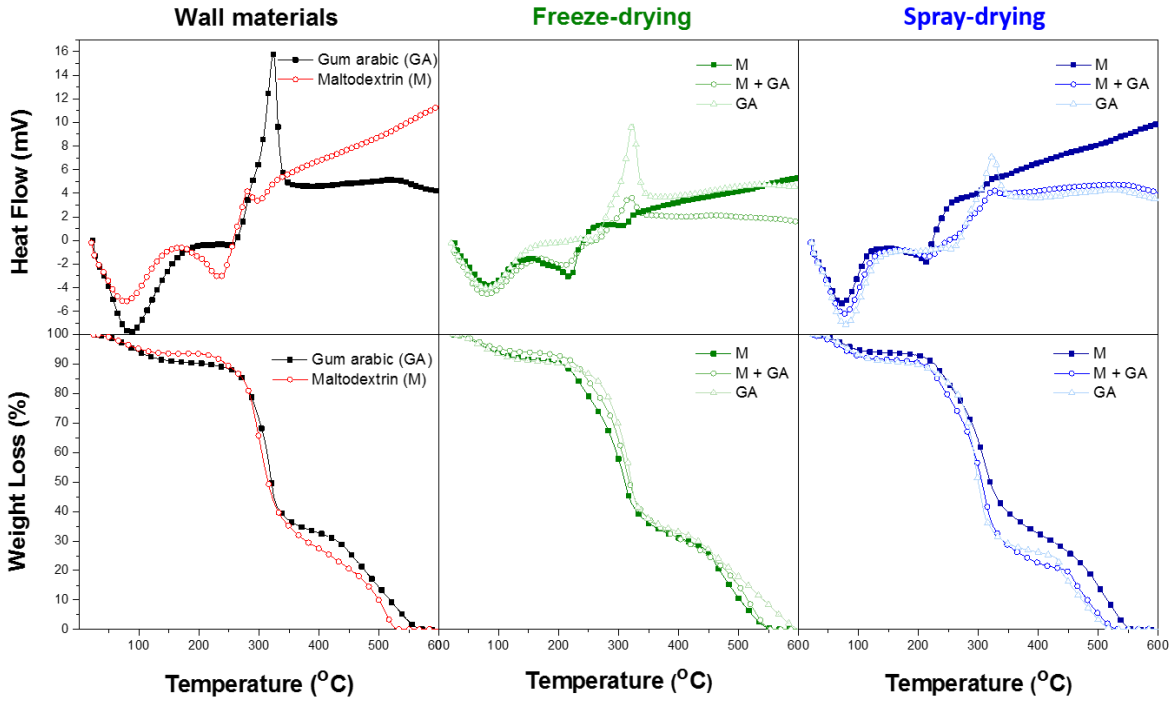

Figure 7.6 Thermogravimetric analyses (TGA) and differential scanning calorimetry (DSC) curves for pure maltodextrin and gum arabic, and for the samples of spent coffee grounds extract encapsulated into these coating materials, dried by freeze-drying and spray-drying

Although the thermal transition indicating the decay of the samples was very close between all of them, a slight increase in the temperature was observed for the samples after encapsulation, when compared to the SCG extract without encapsulating (Figure 7.2), revealing thus more thermally stable samples, mainly those encapsulated with gum arabic. This effect was more marked from the onset temperature in which the thermal degradation of the SCG extract started at lower temperatures (~ 190 °C) than those reported for the encapsulated samples with gum arabic (>

225 °C) and maltodextrin (> 190 °C), confirming again that the thermal stability achieved by the encapsulated samples is provided by the material used as coating.

7.2.2.3. *Encapsulation efficiency*

In this step, the efficiency of the different drying processes (freeze-drying and spray-drying) and coatings to encapsulate the antioxidant phenolic compounds extracted from SCG was evaluated and compared. Figure 7.7 shows the percentage of phenolic compounds and flavonoids retained in the matrix, and the antioxidant activity of the samples after encapsulation, when compared to the initial values present in SCG extract (Table 7.1). The results revealed that the coating used for encapsulation had an important role on the retention of antioxidant phenolic compounds in the matrix. The best results were achieved when using 100% maltodextrin as wall material and freeze-drying as encapsulation technique. Under these conditions, the amount of phenolic compounds and flavonoids retained in the encapsulated sample corresponded to 62% and 73%, respectively. These results are in agreement with those reported by Ramírez, Giraldo, and Orrego (2015), where the highest content of phenolic compounds was attained when the compounds where subjected to freeze-drying and 100% maltodextrin was used as coating material. Gum arabic retained the lowest amount of phenolic compounds independently of the drying process employed. This behavior may be explained by the fact that the encapsulation efficiency is highly dependent on the encapsulated compounds and the coating material used (Rosa et al., 2014). The antioxidant activity was expected to be reduced when compared to the initial antioxidant capacity of the SCG extract, due to the lower amount of phenolic compounds and flavonoids present in the encapsulated sample. Additionally, the reduction percentage of TAA values obtained for the matrices containing 100% maltodextrin and 100% gum arabic, presented a direct correlation with the proportion of phenolic compounds retained, independently of the drying process (linear correlation, R^2 = 0.99). However, the lowest TAA values were observed when maltodextrin and gum arabic were mixed, demonstrating a detrimental effect by combining both matrices with respect to antioxidant activity.

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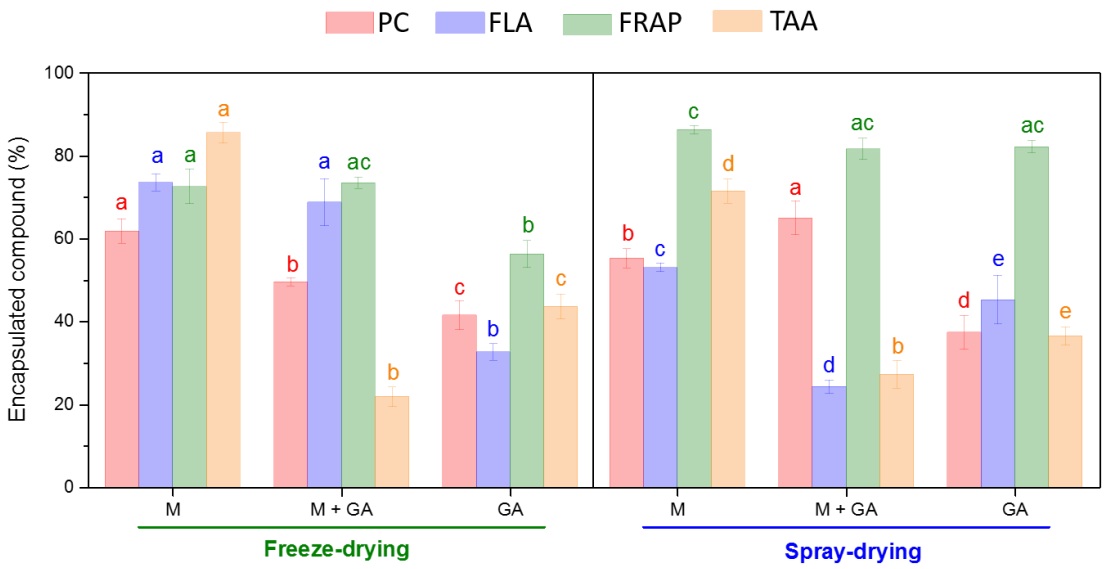

Figure 7.7 Percentage of encapsulated compounds taking into account their initial amount present in SCG extract and their final amount retained in the coating materials, dried by freeze-drying and spray-drying. Different letters within each method (PC: phenolic compounds; FLA: flavonoid content; FRAP: antioxidant activity by the ferric reducing antioxidant power assay; TAA: antioxidant activity by the total antioxidant activity assay) mean values statistically different at 95% confidence level

The drying process demonstrated to be fundamental in the efficacy of encapsulation, being freeze-drying a more effective technique for simultaneous encapsulation of phenolic compounds and flavonoids. This behavior may be partially attributed to the changes in morphology caused by the drying process. For the lyophilization process, the sawdust-like shape creates a lower surface area/volume ratio compared to the microspheres of the spray-drying process, which due to the smaller sizes of the spheres possess larger surface area for the same amount of material, leading to the deterioration of phenolic compounds and flavonoids from the surface to be easily deteriorate

7.3. Conclusions

The technique (freeze-drying and spray-drying) and the coating material (maltodextrin, gum arabic, or a mixture of these components) are factors of great influence on the encapsulation of antioxidant phenolic compounds extracted from spent coffee grounds. Although gum arabic was more thermally stable when compared to maltodextrin, the encapsulation with gum arabic showed a detrimental effect on the retention of phenolic compounds and flavonoids, as well as on the antioxidant activity of the encapsulated sample. The use of maltodextrin as coating material was more appropriate for preserving these components providing the highest retention percentages of phenolic compounds and flavonoids in the matrix and also the best functional properties for the encapsulated samples, especially when freeze-drying was performed. Finally, freeze-drying using maltodextrin as coating material can be considered a good option for encapsulation of antioxidant phenolic compounds extracted from spent coffee grounds since is able to retain 62% and 73% of phenolic compounds and flavonoids, respectively, preserving 73-86% of the antioxidant activity existent in the original extract.

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SECTION V

EDIBLE FILMS/COATINGS FOR FOOD APPLICATIONS

CHAPTER 8

USE OF POLYSACCHARIDE RICH EXTRACTS OBTAINED FROM SPENT COFFEE GROUNDS AS CONSTITUENTS OF CARBOXYMETHYL CELLULOSE-BASED FILMS

The following chapter is partially based on the results published in: Lina F. Ballesteros, Miguel A. Cerqueira, José A. Teixeira & Solange I. Mussatto. Use of polysaccharide rich extracts obtained from spent coffee grounds as constituents of carboxymethyl cellulose-based films (*Submitted in Food Hydrocolloids).*

8. Introduction

Bio-based films or coatings are promising systems to replace the synthetic materials used in the food packaging industry. Nowadays, food industry are looking for new materials from renewable resources that can replace the petroleum-based materials in order to reduce their environmental impact, promoting thus, a new generation of biodegradable packaging with similar properties than synthetics and low cost production (Ghanbarzadeh, Almasi, & Entezami, 2010).

The use of natural polymers such as polysaccharides, proteins and lipids into edible coatings and films have been studied as a possible alternative for food preservation. Likewise, the use of agricultural-residues for the extraction of new functional materials has been extensively studied in last years (Aguedo, Fougnies, Dermience, & Richel, 2014; Costa et al., 2015; Ruiz et al., 2013). As a result, the polysaccharide rich extracts obtained from spent coffee grounds (SCG), by using an alkali pretreatment (PA) and autohydrolysis (PB) reported in previous chapters, were incorporated into carboxymethyl cellulose (CMC)-based films aiming at the development of bio-based films with new functionalities. Different concentrations of PA and PB extracts (0.00, 0.05, 0.10 and 0.20%, w/v) were used and their effect on physicochemical properties of CMC-based films were evaluated. Scanning electronic microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and thermogravimetric analysis (TGA) were performed, together with determinations of optical and mechanical properties, moisture content, solubility, water vapor permeability (WVP), contact angle and sorption isotherms in order to highlight the interactions between the SCG extracts and film matrix.

8.1. Materials and methods

8.1.1. Materials for films production

Extracts from SCG were obtained using two different extraction methods: an alkali pretreatment (Section III - Chapter 5) and ii) autohydrolysis (Section III - Chapter 4), and named as

PA and PB extracts, respectively. Carboxymethylcellulose-CMC (Blanose, 7M65) was obtained from Ashland Inc (Düsseldorf, Germany), analytical reagent grade glycerol 99.5% was purchased from Himedia (Mumbai, India) and ultrapure water from a Milli-Q System (Millipore Inc., USA) was used.

8.1.2. Films production

CMC solutions was prepared by dissolving CMC in ultrapure water at 70 °C, during 4 h at constant agitation (300 rpm) using a magnetic stirrer. Subsequently, glycerol was added and left under agitation one more hour. On the other hand, different concentrations (0.00%, 0.050%, 0.10% and 0.20%, w/v) of extracts obtained by alkali pretreatment (PA) and autohydrolysis (PB) were dissolved in ultrapure water and placed at 20 °C during 3 h with magnetic agitation. Each one of the PA and PB solutions was slowly added to CMC-glycerol solution and maintained for 30 min at 70 °C. The components of films were prepared and mixed taking into account the desired concentrations in the end, which was 1.50% of CMC and 0.50% of glycerol with increasing concentrations of SCG extracts (0.00%, 0.050%, 0.10% and 0.20%, w/v). The concentrations of PA and PB were chosen based on preliminary experiments (data not shown) where six different concentrations were evaluated. Films were produced by casting a constant amount (27 ml) of film-forming solution into a 90 mm diameter Petri dishes, dried at 33 °C for 48 h. Films were stored at 20 °C and 53% RH (desiccator containing a saturated salt solution of $Mg(NO_3)_2$ until further analysis.

8.1.3. Characterization of the films properties

8.1.3.1. *Film thickness*

The films thickness was measured using a digital micrometer (No. 293–561, Mitutoyo, Japan). For each sample, ten measurements were made in different points through the film. The values of thickness were used to calculate water vapor permeabilities and mechanical properties.

8.1.3.2. *Morphology*

Images of PA and PB as well as those of the produced films were analyzed as described in Section II - Chapter 3. The films were examined on the surface and transversely. For the cross-

section analyses, they were fractured using liquid nitrogen and after that, all the samples were covered with a very thin film (10 nm) of Au-Pd (80-20 weight %). The images were obtained by applying an acceleration voltage of 10kV, at 5,000-fold magnifications.

8.1.3.3. *Crystallinity and chemical bonding of constituents*

Crystalline phases of the produced films were evaluated by X-ray diffraction (XRD) using a as described in Section II - Chapter 3. Chemical groups and bonding arrangement of constituents present in the films were determined by Fourier transform infrared spectroscopy (FTIR) as described in Section III - Chapter 4.

8.1.3.4. *Thermal behavior*

Thermogravimetric analyses (TGA) were conducted using an equipment TGA Q500 (TA instruments, USA). Approx. 2 mg of the film sample were placed in an aluminum pan. The measurements were carried out between 25 and 480 °C with an increasing rate of 10 °C per min under nitrogen atmosphere. TA Universal Analysis software (TA instruments, universal analysis 2000, USA) were used for data analysis.

8.1.3.5. *Mechanical properties*

The mechanical properties including tensile strength (TS) and elongation at break (EB) of the films were determined using an Instron Universal Testing Machine (model 4500, Instron Corporation, Canton, USA), according to the ASTM D882-10 Standard test method for tensile properties of thin plastic sheeting as described by Cerqueira, Souza, Teixeira, and Vicente (2012b). Each sample was properly cut and set to an initial grip separation at 100 mm, and a force and deformation speed of 50 mm per min. TS was calculated by dividing maximum load (N) by cross-sectional area of the film (m^2) and expressed in mega Pascal (MPa). EB was calculated as the ratio of the final length at the point of sample rupture to the initial length of the sample and expressed in percentage (%). For each film at least six replicates were performed.

8.1.3.6. *Moisture content and water solubility*

The moisture content of the films were determined by gravimetric analysis, placing approx. 30 mg of each sample at 105 °C during 24 h (until constant weight). The weight loss of the samples was determined, and then, the moisture content was calculated and expressed as percentage of moisture (%).

For the determination of water solubility, films free of moisture with 2 cm diameter were weighed and subsequently immersed in 50 ml of distilled water and then, placed in a shaker at 120 rpm at room temperature during 24 h. The samples were taken out and dried at 105 °C (until constant weight). Solubility was determined by the weight difference between the dry matter that was not solubilized in water and the initial weight before immersion. Three replicates were obtained for each film and the solubility results were expressed as a percentage (%).

8.1.3.7. *Water vapor permeability*

Water vapor permeability (WVP) was performed gravimetrically according to ASTM E96-95 Standard test (Cerqueira et al., 2012b). Briefly, the films were sealed on the top of permeation cells containing 60 ml of distilled water (100% RH and 2,337 Pa vapor pressure at 20 °C) and then, placed into a desiccator with silica gel (0% RH and 0 Pa water vapor pressure at 20 °C). The cells were weighted at 2 h intervals for monitoring the weight loss during 10 h. Steady-state and uniform water pressure conditions were assumed by keeping the air circulation constant outside the test cell by using a miniature fan inside the desiccator. Water vapor transmission rate (WVTR) was determined by dividing the slope of the linear regression of weight loss versus time by the film area (expressed as g/s m^2); afterwards WVTR was multiplied by the film thickness and divided by the vapor partial pressure difference to obtain WVP (expressed as g/m s Pa). Three replicates were made for each film.

8.1.3.8. *Water sorption isotherms*

Water adsorption/desorption isotherms of the films was determined at 25 °C using an AquaLab moisture 4TE analyzer (Decagon Devices, Inc., USA). Previous to analysis, the films were

placed into a desiccator with silica gel (0% RH at 25 °C) for at least 5 days, and then weighted. Water activity (a_w) was varied from 0.11 to 0.97 using different saturated salt solutions such as lithium chloride (LiCl), magnesium chloride ($MgCl_2$), magnesium nitrate ($Mg(NO_3)_2$), sodium chloride (NaCl), and potassium sulfate (K_2SO_4), with a_w of 0.11, 0.33, 0.53, 0.75 and 0.97, respectively. For the measurements, the film sample was left on the top of an especial cup containing 5 ml of each saturated salt solution and placed into the moisture analyzer chamber. When the atmosphere within the chamber reached the equilibrium, the film was quickly removed and weighed again. Adsorption isotherms were obtained by starting the measurements from low a_w to high a_w values, on the inverse way for desorption isotherms. Moisture content was determined at the equilibrium as the difference between the weight before and after the samples were in the presence of the saturated salt solutions. Results were expressed as grams of water per 100 grams of dry film at each a_w (g H_2O/100 g dry film). Guggenheim, Anderson and De Boer (GAB) model was used for fitting the experimentally obtained data (Bizot, 1984) through Eq 8.1, where M is the equilibrium moisture content at a specific a_w (g H_2O/100 g dry film), M_m represents the monolayer moisture content (g H_2O/100 g dry film), C is the Guggenheim constant related to thermal effect and k the corrective constant related to the properties of multilayer water molecules with respect to bulk liquid,

Eq 8.1

$$M = \frac{M_m C k a_w}{[(1-k a_w)(1-k a_w+C k a_w)]}$$

The parameters of the model were estimated with the nonlinear regression procedure. The fit accuracy was evaluated by the mean of the relative percent difference between the experimental and predicted values of moisture content, being defined as the mean relative deviation modulus (G) (Gencturk, Bakshi, Hong, & Labuza, 1986), and determined using Eq 8.2, where n is number of observations, M_a is experimentally determined moisture content (g H_2O/100 g dry film) and M_p is predicted moisture content (g H_2O/100 g dry film).

Eq 8.2

$$G = \frac{100}{n} \sum_{i=1}^{n} \left(\frac{|M_{ai} - M_{pi}|}{M_{ai}} \right)$$

G values lower than 5 correspond to extremely good fit, G values between 5 and 10 show a reasonably good fit and G values greater than 10 are considered a poor fit (Gencturk et al., 1986).

8.1.3.9. *Surface hydrophobicity*

Surface hydrophobicity of the films was evaluated by measuring the contact angle of a water droplet (θ) upon the film surface through an optical contact angle meter (OCA 20, Dataphysics, Germany). The measurements were made according to sessile drop method (Kwok & Neumann, 1999) using a 500 μL syringe (Hamilton, Switzerland) with needle of 0.75 mm diameter containing ultrapure water. The samples were put on a glass and then 2 μL of ultrapure water was placed on the film surface. Measurements were made from 0 to 12 min and the contact angle was determined by using the measuring system OCA 15 Plus and C20 software with CCD video camera (resolution of 752 × 582 pixel) at 24.7 °C. Ten replicates were obtained for each film.

8.1.3.10. *Optical properties - color and opacity*

The color parameters and the opacity of the films were determined with a Minolta colorimeter (CR 400, Minolta, Japan). Briefly, a white standard color plate (Y = 93.9, x = 0.3158, y = 0.3321) was used for the equipment calibration and as background for the color measurements, being the L^*, a^*, b^* values determined by reflectance. In the color system L^* represents the luminosity (ranging from black to white), thus, low L^* values correspond to dark, while high L^* values belong to light. On the other hand, a^* and b^* are the chromatic coordinates, where $+a^*$ and $-a^*$ are in the red and green directions, respectively, while, $+b^*$ is in the yellow direction, and $-b^*$ is in the blue direction. The a^* and b^* values approach zero for neutral colors and increase when the color becomes more chromatic and more saturated (Ke, Changde, Wande, & Xiaoping, 2004).

The opacity of the samples was determined according to the Hunter lab method, as the relationship between the opacity of each sample on a black standard (Y_b) and the opacity of each sample on a white standard (Y_w) (Casariego et al., 2009). For both, color and opacity analyses, ten measurements were made for each film. The solubility was expressed in percentage (%).The color and opacity of the films was simulated using an image software (Photoshop CS6).

8.1.4. Statistical analysis

Statistical analyses were carried out using GraphPad Prism (version 6.1). One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were performed to determine the significant differences ($p < 0.05$) between film samples.

8.2. Results and discussion

8.2.1. Characterization of the polysaccharides present in SCG residues

In the Section III - Chapters 4 and 5, the lyophilized materials obtained from SCG through autohydrolysis and an alkaline pretreatment were characterized in terms of percentage of total carbohydrates and content of total phenolic compounds. Table 8.1 shows again the values obtained in those chapters. It can be seen that the total content of polysaccharides was higher for PA (39%, w/w) than PB (29%, w/w). Additionally, the monosaccharide composition found in both extracts included galactose, arabinose, mannose and glucose, but % mol of these sugars in PA and PB extracts was different. The content of total phenolic compounds and moisture were slightly higher for PB extract (Table 8.1).

Table 8.1 Chemical sugar composition of extracts obtained from SCG by an alkaline pretreatment (PA) and autohydrolysis process (PB)

Components	Extract*	
	PA	PB
Total polysaccharides content (g/100 g lyophilized)	39.00 ± 0.19	29.29 ± 3.47
Arabinose (% mol)	19.93 ± 1.74	10.02 ± 1.18
Mannose (% mol)	4.43 ± 0.16	31.88 ± 2.08
Galactose (% mol)	60.27 ± 0.51	47.74 ± 0.13
Glucose (% mol)	15.37 ± 0.93	10.35 ± 0.76
Phenolic compounds (mg GAE/g lyophilized)	230.14 ± 1.43	234.14 ± 5.30
Moisture	15.50 ± 1.50	17.50 ± 2.10

Results are expressed as mean ± standard deviation; n=3.

8.2.2. Morphology

Images obtained by scanning electron microcopy (SEM) for PA and PB extracts as well as surface and cross sectional images of the produced films are shown in Figure 8.1 and Figure 8.2, respectively. Both, PA and PB revealed significant morphological differences. PA presents a denser morphology, composed of thin sheets that resembles to sawdust, while PB consists of microscopic small grains and resulted in a more porous material (Figure 8.1).

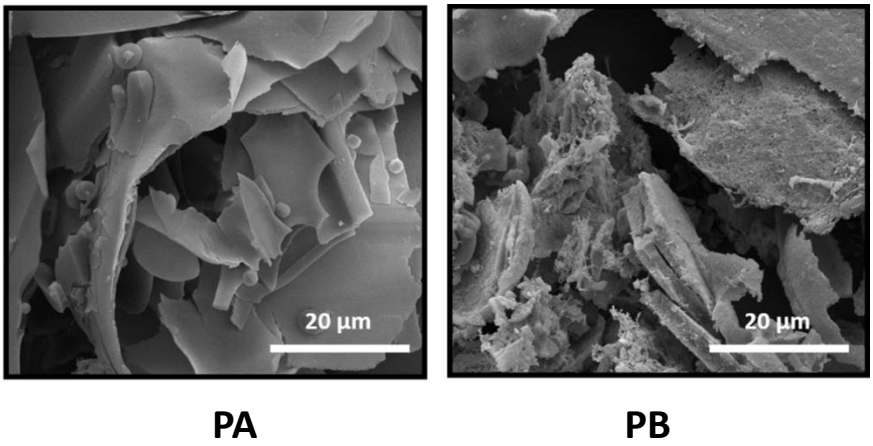

Figure 8.1 SEM micrographs for SCG extracts obtained by an alkali pretreatment (PA) and autohydrolysis process (PB) Magnification, 5000X

On the other hand, when analyzing the CMC-based films without and with incorporation of PA and PB extracts at different concentrations (Figure 8.2), the images showed different characteristics for films with extracts for surface and cross-section images. For films with the incorporation of PA extract there are no evidence of pores or surface features being independent of the concentration of PA extract used (i.e. similar imagens for all concentrations used). Thus, CMC-based films without extract together all PA films exhibited a uniform and compact structure, suggesting a good incorporation of PA into the matrix film. On the contrary, when PB was added into the CMC-based films, a clear increase of the surface defects were observed as the concentration was raised. This behavior was corroborated during the film-forming process, being PB extracts more difficult to dissolve in water than PA extracts. This fact may be related to the

molecular weight and possible structural differences between both extracts (Izydorczyk & Dexter, 2008). Additionally, the morphological differences on the surface of the films containing PA and PB may be due to the processes used to obtain the SCG extracts and the different stages employed in each method, since PA was dialyzed though a membrane of 8000 Da, while PB was not summited to this stage, which could have extended its polymerization degree (Aguedo et al., 2014).

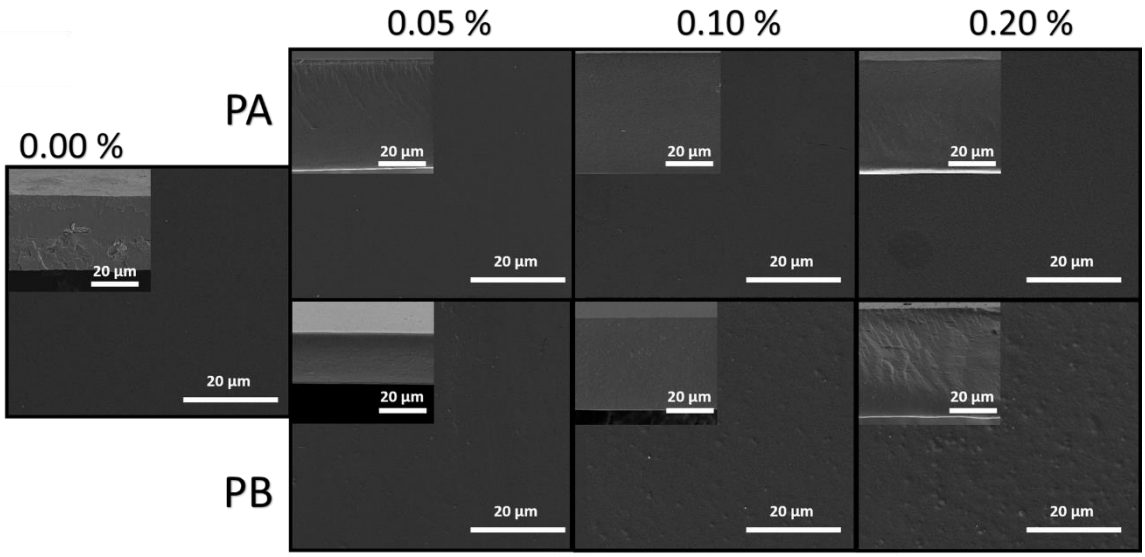

Figure 8.2 SEM micrographs for surface and cross-sectional images of CMC-based films without and with the PA and PB extracts at different concentrations. Magnification, 5000X

8.2.3. Crystallinity and chemical bonding of constituents

Figure 8.3 and Figure 8.4 show the X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) spectra of the samples, respectively. Results show that the incorporation of low concentrations of SCG extracts (0.05 - 0.20 %, w/v) can lead to slight changes in the structural characteristics of the films.

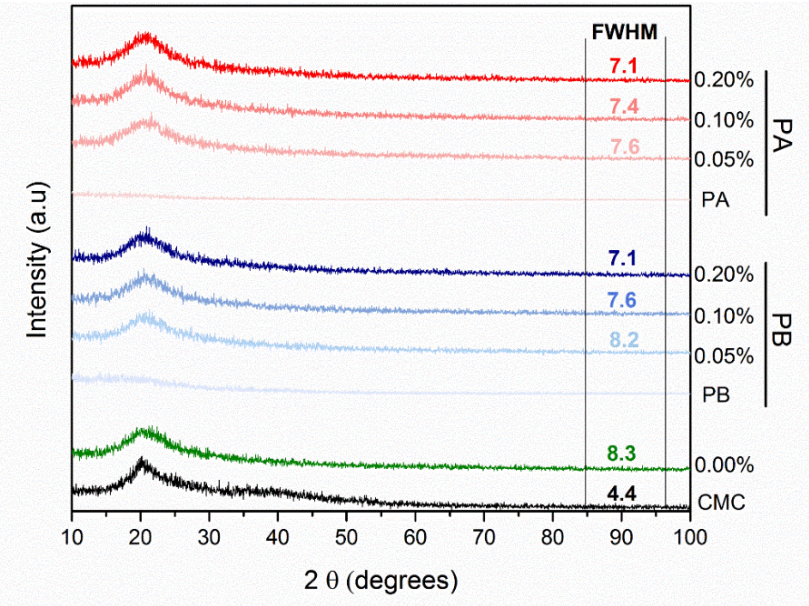

Figure 8.3 XRD diffractograms obtained for the CMC-based films without and with PA and PB extracts at different concentrations

The XRD patterns of the films are shown in Figure 8.3, as well as those for PA, PB and CMC powders for comparison. As can be seen, after the film formation, the CMC suffers a structural change, evidenced in the disappearance of a very weak broad peak located around $2\theta = 36°$, which distinguishes the pure CMC structure as reported (Chai & Isa, 2013; El Sayed, El-Gamal, Morsi, & Mohammed, 2015). The acquired structure was maintained even when PA and PB extracts were added to CMC-based film. All the films revealed a semi-crystalline diffraction peak around $2\theta = 20.6°$ (Figure 8.3), which is a characteristic of cellulose. The full width at half maximum (FWHM) of this peak was calculated in order to analyze possible differences between the samples. The results shown that the crystallinity of pure CMC decreased when CMC was used to film-forming (i.e. higher values of FWHM), since polysaccharides naturally interact with water generating structural transitions (amorphous or crystalline), which plays an important role on the mobility of the molecules and thus on the functional properties of the films (Yakimets et al., 2007). Although the differences among the films with respect to FWHM were very small, it can be seen that the incorporation of PA or PB into CMC-based film raised the crystallinity of the film being influenced

by the increase of extracts concentrations. Hence, higher concentrations of extract increased the crystallinity of the films, suggesting an influence of PA or PB in film matrix.

FTIR spectra (Figure 8.4) evidenced the same structure and chemical bonds for the CMC-based films without and with incorporation of PA and PB extracts. Moreover the same transmission bands of pure CMC were observed in all the films, increasing the intensity of the peaks after formation of the film, that is explained by the physical blends and chemical interactions of the final film matrix with the other compounds (Cerqueira et al., 2012a; Xu, Kim, Hanna, & Nag, 2005). The transmission band between 3000 and 3600 cm^{-1} was assigned to the hydrogen bonding OH stretching vibration, being a characteristic of moist materials which was intensified by the presence of glycerol in films (Cerqueira et al., 2012a). The peak at 2920 cm^{-1} was assigned to the C-H stretch and the band at 1590 cm^{-1} confirmed the presence of COO^- being assigned to stretching of the carboxyl group (Chai & Isa, 2013).

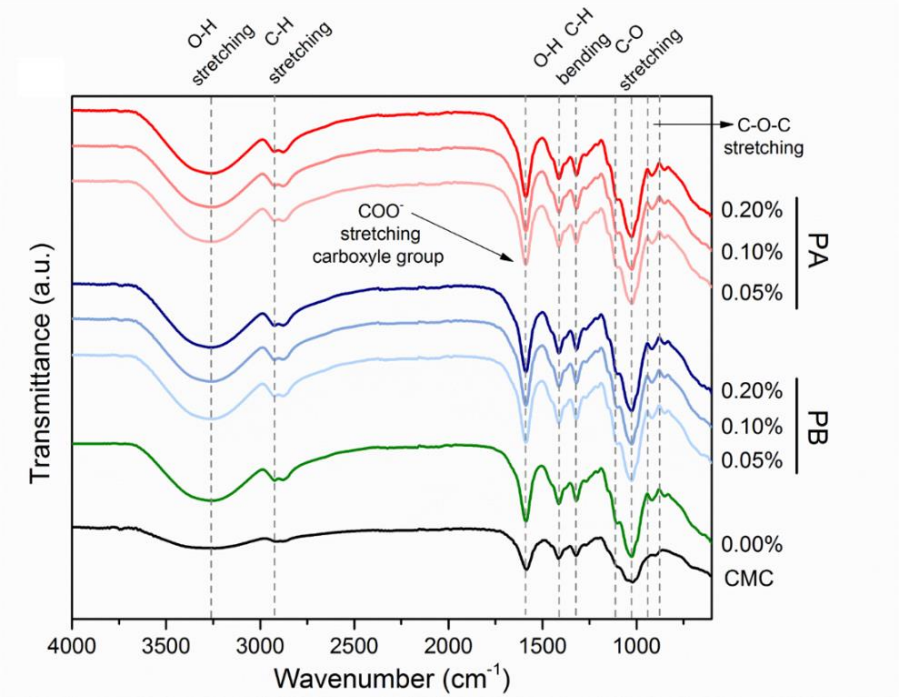

Figure 8.4 FTIR spectra obtained for the CMC-based films without and with PA and PB extracts at different concentrations

The bands at 1410 cm^{-1} and 1320 cm^{-1} were attributed to OH stretching in-plane and C-H stretching in symmetric of CMC (Su et al., 2010). The peaks depicted at 1110 cm^{-1} and 1040 cm^{-1} were characteristic of the C-O stretching on polysaccharide skeleton.

Additionally, CMC-based films containing or not PA and PB extracts showed soft bands at 948 cm^{-1} and 884 cm^{-1} which did not appear in pure CMC and that are justified by the presence of glycerol (Nanda, Yuan, Qin, Poirier, & Chunbao, 2014). These peaks were also reported by (Cerqueira et al., 2012a) to films where glycerol was used as plasticizer corresponding to asymmetric and symmetric stretching vibrations of the alcoxyl group (C-O-C). From FTIR analysis is clear that the incorporation of PA and PB extracts do not change the chemical structure (detectable from CMC) of the CMC-based film.

8.2.4. Thermal behavior

Thermogravimetric analyses (TGA) (Figure 8.5) was carried out in order to evaluate the stability of CMC-based films containing or not PA and PB extracts. When the films were exposed to heating until 480 °C, three weigh loss stages were identified. The first stage (60 – 130 °C) was associated to water evaporation and chemisorbed water through hydrogen bonds. It can be seen that the CMC-based films with 0.10% and 0.20% (w/v) of PA extract were stable up to about 95 °C, while all the others showed stability up 45 – 60 °C, suggesting that the water loss occurs slower to the films containing PA at 0.10% and 0.20% (w/v). This behavior was maintained until start the second stage, where was recognized to maximum rate of mass loss (40-45%) for the all films, took place around 270 °C (maximum peak in the DTG curve) and was related to the presence of glycerol (Cerqueira et al., 2012a). The last stage, around 300 – 350 °C was attributed to degradation of the polysaccharides.

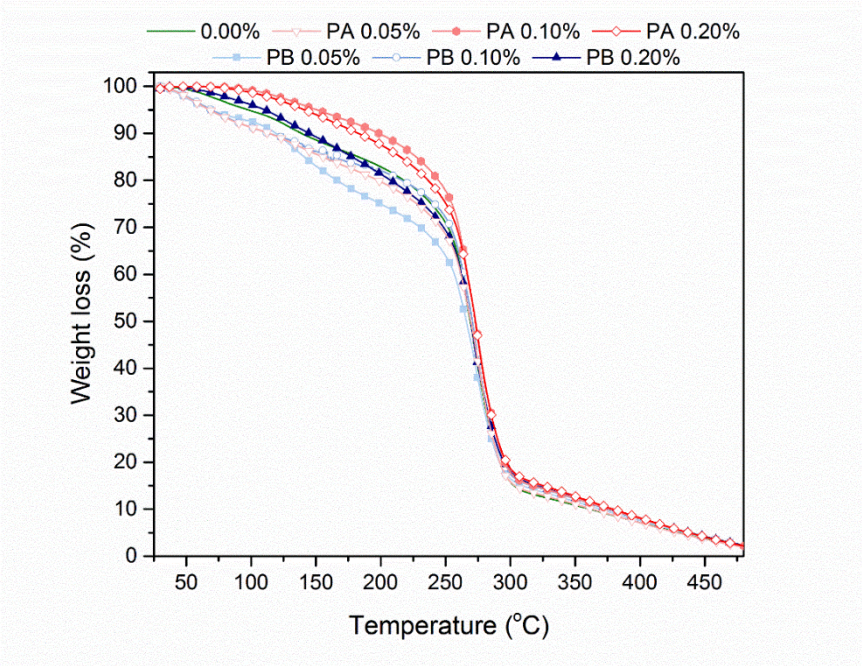

Figure 8.5 TGA curves for the studied CMC-based films

8.2.5. Mechanical properties

Table 8.2 shows the elongation at break (EB) and tensile strength (TS) values obtained for the films. CMC-based film without extracts presented similar EB values to those reported by (Ebrahimzadeh, Ghanbarzadeh, & Hamishehkar, 2016) when used CMC at 1.50% (w/v) (same concentration used in this study). The addition of PA and PB extracts lead to a decrease of the EB values when they are compared with CMC-based films without extracts, showing significant differences ($p < 0.05$) for films with a high concentration of PA extract and for the films with the lowest concentrations of PB. As can be seen, the behavior of the films containing PA and PB extracts was completely opposite with relation to the concentration added. This fact suggests that the interaction between film matrix with PB leads films stiffer and more compact when using the lowest concentration, becoming more extensible as the concentration increased. In contrast, the presence of PA extracts in the film matrix increases the chains mobility and flexibility at lower concentrations, and its stiffness when the PA extracts are used at higher concentrations. These results are directly related with the water solubility data, where the same behavior was observed.

Table 8.2 Elongation at break (EB) and tensile strength (TS) values of the CMC-based films without and with different PA and PB concentrations

Extract (%, w/v)	EB (%)	TS (MPa)
0.00 (CMC)	10.54 ± 0.17^{a}	14.18 ± 3.71^{a}
PA 0.05	8.95 ± 0.65^{ab}	22.86 ± 2.24^{bc}
PA 0.10	7.96 ± 1.38^{ab}	19.59 ± 4.17^{abd}
PA 0.20	6.56 ± 0.86^{bc}	22.33 ± 2.35^{bc}
PB 0.05	4.50 ± 1.99^{c}	16.43 ± 0.14^{ab}
PB 0.10	6.84 ± 0.43^{bc}	26.04 ± 1.29^{c}
PB 0.20	8.22 ± 1.02^{ab}	23.35 ± 0.89^{cd}

Different letters in the same column correspond to statistically different samples for a 95% confidence level.
PA: extracts containing polysaccharides obtained by an alkali pretreatment of SCG; PB extracts containing polysaccharides obtained by autohydrolysis of SCG.

On the other hand, the obtained TS values indicated significant changes ($p < 0.05$) when PA and PB extracts were added to the films, exception made to films with 0.05% (w/v) of PB and 0.10% (w/v) of PA extracts. TS values of CMC-based films containing PA extracts did not suffer significant ($p > 0.05$) modifications when the concentration of PA was changed (TS values were equal when increased PA concentrations). For films with the PB extracts the TS values was raised when the extract concentration increased, obtained thus, TS values higher when using 0.10% and 0.20% (w/v) on contrary to 0.05% (w/v). The obtained results are in agreement with other studies (films with phenolic compounds, gelatin or proteins) (Hoque, Benjakul, & Prodpran, 2011; Mu, Guo, Li, Lin, & Li, 2012), where was demonstrated that the interactions between the matrix components are determined by the chain length of the materials added.

8.2.6. Moisture content

Moisture content of the films provides information about the water affinity of the films and gives indication on how PA and PB could influence their properties. Table 8.3 presents the values of moisture content of the films and shows that the all the films presented very close values between them. Nevertheless, films with PB at 0.20% (w/v) showed a lower moisture content ($p < 0.05$) compared to CMC-based films without the incorporation of the extracts, while for all other films no

differences were observed ($p > 0.05$). For films with PA extracts a difference was noticed when using 0.10% and 0.20% (w/v), presenting the former a high moisture content, while for PB only the higher concentration showed significant difference among PB group. Results show that the incorporation of the extracts can influence the moisture content of the films, but only using the PB extract at higher concentration a significant difference can be observed. Some authors reported that the addition of galactomannans into the film matrix could increase the water-binding capacity, but also a decrease when an high amount of galactomannan is added (Arda, Kara, & Pekcan, 2009; Martins et al., 2012), which is in agreement with the results presented in this study. Finally, no significant differences were found between the two SCG extracts when the lowest and highest concentrations of PA and PB were evaluated; only for concentration of PB 0.10% was observed a small decrease in the moisture content when compared with PA 0.10%.

Table 8.3 Thickness, moisture content, water solubility, water vapor permeability (WVP) and contact angle values of the CMC-based films without and with different PA and PB concentrations

Extract (%, w/v)	Thickness (mm)	Moisture (%)	Solubility (%)	WVP × 10^{-10} (g/ m s Pa)	Contact angle* (θ)
0.00 (CMC)	0.070 ± 0.006^{a}	22.71 ± 0.87^{ab}	75.08 ± 3.37^{a}	3.36 ± 0.19^{a}	54.80 ± 6.29^{a}
PA 0.05	0.068 ± 0.005^{a}	23.36 ± 1.36^{ab}	59.47 ± 0.90^{b}	3.56 ± 0.65^{a}	104.96 ± 2.05^{b}
PA 0.10	0.078 ± 0.004^{b}	25.45 ± 1.97^{a}	54.72 ± 1.17^{bc}	3.66 ± 0.33^{a}	108.54 ± 2.18^{bc}
PA 0.20	0.075 ± 0.006^{bc}	20.97 ± 0.82^{bc}	53.10 ± 0.18^{cd}	3.64 ± 0.10^{a}	111.48 ± 3.38^{c}
PB 0.05	0.068 ± 0.004^{a}	23.55 ± 0.67^{ab}	49.94 ± 1.34^{c}	2.99 ± 0.32^{a}	103.30 ± 4.86^{b}
PB 0.10	0.070 ± 0.006^{a}	21.63 ± 0.32^{bc}	50.52 ± 1.53^{c}	3.23 ± 0.45^{a}	107.34 ± 4.12^{bc}
PB 0.20	0.071 ± 0.008^{ac}	19.05 ± 0.14^{c}	58.59 ± 3.51^{bd}	3.46 ± 0.46^{a}	107.40 ± 3.21^{bc}

Different letters in the same column correspond to statistically different samples for a 95% confidence level.
*Measurement at 0 min.
PA: extracts containing polysaccharides obtained by an alkali pretreatment of SCG; PB extracts containing polysaccharides obtained by autohydrolysis of SCG.

8.2.7. Water solubility

Table 8.3 shows the water solubility values obtained for all the films. Results demonstrated that the films with the addition of extracts present lower ($p < 0.05$) solubility values than the CMC-based films without any extract incorporation, showing that the incorporation of the SCG extracts decrease the solubility of CMC-based films, independently of the concentration or the type of extract (PA or PB) used. Such behavior was visually corroborated after the test, since the films with the

incorporation of PA and PB preserved the integrity, while the films without extract only presented some fragments in the water. On the other hand, the films with the incorporation of extracts had an opposite behavior, revealing a significant reduction of the solubility values with the increase of PA concentrations, whereas a significant increase was observed for higher concentrations of PB. These differences can be explained by the fact that PA showed to be better incorporated into the film matrix than PB, as demonstrated by SEM images (Figure 8.2).

When comparing the water solubility between the films containing PA and PB extracts at the same concentration, only a significant difference ($p < 0.05$) were observed for the lowest concentration (0.05%, w/v), being obtained a lower value for the films with PB extracts. Results suggest that the differences between the molecular structure of the matrix, including the presence semi-crystalline fraction (changes shown in XRD section) could determine the solubility of the edible films in water.

8.2.8. Water vapor permeability

Water vapor permeability (WVP) being the most widely property studied of the films, allows to understand the influence of the components present in the final matrix on features such as solubility, sorption and diffusion of water molecules (Cerqueira, Costa, Fuciños, Pastrana, & Vicente, 2014). Results revealed similar values for all studied films ($p > 0.05$), showing that in the range of concentrations used for PA and PB extracts, the transport properties of CMC-based films are not changed (Table 8.3). The value obtained for CMC-based film (3.36×10^{-10} g/ m s Pa) was higher than the values found by Bifani et al. (2007), who reported 7.14×10^{-11} g/ m s Pa when using CMC at 2.00% (w/v) and glycerol and sunflower oil as plasticizers (0.50% and 0.40%, v/w, respectively). However, it was lower than the VWP value (1.62×10^{-8} g/ m s Pa) achieved using CMC at 1.50% (w/v) and glycerol 0.90% (w/v) (Ebrahimzadeh et al., 2016). The difference may be due to the type and amount of plasticizer used, the presence of sunflower oil, the CMC concentration and the process for the film production.

8.2.9. Water sorption isotherms

Water sorption is closely related to the matrix microstructure of films and will depends of the environmental relative humidity. Figure 8.6 shows the adsorption isotherm profile of the studied films, presenting the variation of the moisture content with respect to the water activity (a_w). Some authors have mentioned that the moisture sorption isotherms represent the combined hygroscopic properties of the individual components in the films (Kim & Ustunol, 2001). All studied films presented similar behavior when exposed to different relative humidity, with exception of CMC-based film with addition of PA at 0.10% (w/v), which showed a rise over the others, suggesting that it is the most hygroscopic film. This greater water association was confirmed by the C value obtained, higher for these films. As can be seen, the equilibrium moisture content (g H_2O/100 g dry film) increased almost linearly until an a_w range of 0.70 – 0.80, and after, it increased exponentially. This type of nonlinear sorption profile is typical of hydrophilic films and has been reported to others CMC-based films (Kibar & Us, 2013). The general curve experimentally obtained for the all films was fitted to the GAB moisture sorption model, which evaluates a_w ranges between 0.1 and 0.9 being widely used in food. The parameters were determined (Figure 8.6) and showed G values lower than 5 for PA extracts at 0.20%, and 0.10% (w/v), indicating an extremely good fit.

The films containing 0.05% (w/v) of PA and PB corresponded to a reasonably good fit (G between 5-10), while CMC-base film control, and the films with 0.10% and 0.20% (w/v) of PB showed values greater than 10, being considered a poor fit. On the other hand, M_m values were reported between 6.79 and 8.40 (g H_2O/100 g dry film), indicating the number active sites available to the water adsorption (Inchuen, Narkrugsa, & Pornchaloempong, 2009), which is strongly related to the presence of glycerol. The lower M_m value was obtained for CMC-films with 0.2% of PB extracts that is in agreement with the moisture content results where this film was also the one with a lower moisture content.

The desorption isotherms showed a similar to the adsorption indicating absence of hysteresis (results not shown).

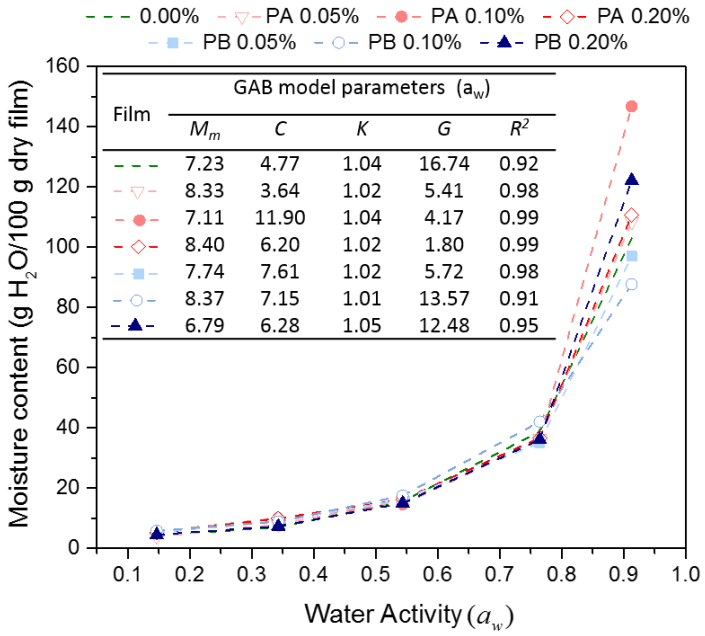

Figure 8.6 Water adsorption isotherms of the CMC-based films without and with the PA and PB extracts at different concentrations (measurements were performed at 25 °C). M_m represents the monolayer moisture content (g H_2O/100 g dry film), C is the Guggenheim constant related to thermal effect and k the constant related to the properties of multilayer water molecules with respect to bulk liquid, G is the mean relative deviation modulus and R^2 the coefficient of regression

8.2.10. Surface hydrophobicity

Surface hydrophobicity was evaluated by measuring the water contact angle on the surface of the films. Instantaneous contact angle measurements (0 s) revealed an increase on the hydrophobic behavior of the film surface after the incorporation of the SCG extracts (Table 8.3). CMC-based films (0.00%, w/v) showed a $\theta = 54.80°$ while, both PA and PB modified this value, reaching to values ranged between 103° and 111°. θ values of films containing PA and PB were not statistically different ($p > 0.05$) with exception to films with PA extracts where the contact angle values increased from 104.96° to 111.48 when the concentration was raised from 0.05% to 0.20%. The behavior of the water drop on the upper surface of the films was also evaluated as a function of time (Figure 8.7). The results revealed that the contact angle decreased through time for all the films, but the statistic difference ($p < 0.05$) between the CMC-based film and those elaborated with PA and PB was maintained, confirming the initial hydrophobic behavior of the films with SCG extracts approximately during the firsts 5 min. However, at 12 min the film with the highest contact

angle (19°) was PA when using an extract concentration of 0.20% (w/v), being statistically different of the others films, except to PA 0.05% (w/v) (data not shown), which is in agreement with the values reported for the film solubility. The reduction of contact angle is supported by the fact that the water was spread out through the film surface or adsorbed (Phan, Debeaufort, Luu, & Voilley, 2005).

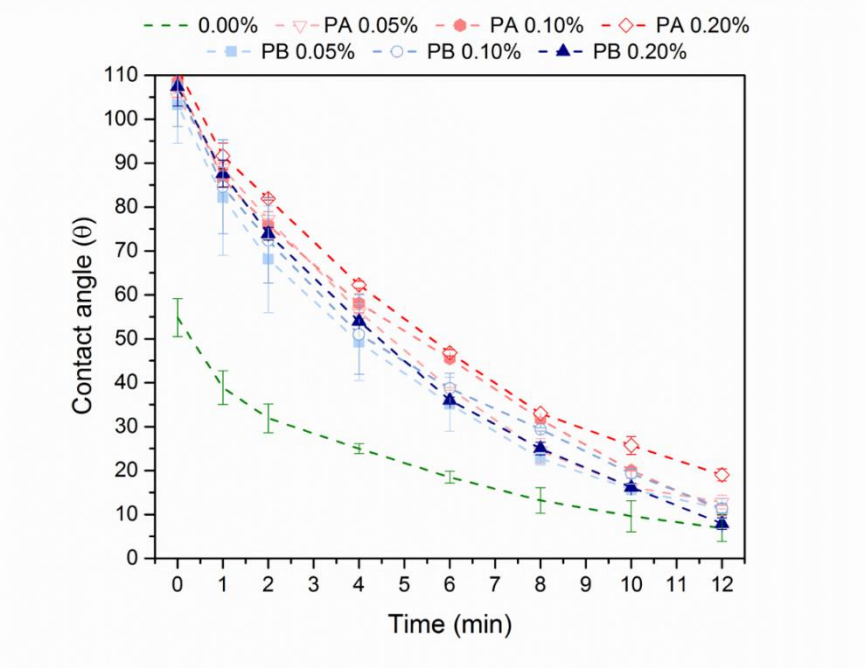

Figure 8.7 Changes of contact angle measurement for CMC-based films without and with the incorporation of PA and PB extracts at different concentrations as a function of time after the drop deposition

8.2.11. Optical properties - color and opacity

The results for color and opacity measurements are presented in Table 8.24. As can be seen, when the extracts from SCG were incorporated into the films an evident change in color was noticed. Thus, the color of films was changed from a very light transparent material (CMC film) to a brownish color, characteristic of the SCG extracts, which results of the Maillard reaction that occur during coffee roasting process (Mastrocola, Munari, Cioroi, & Lerici, 2000). Moreover, films color is highly dependent of the amount of extract added to matrix, being observed a darker brown color when the concentration was increased. In general, the a^* values of the films containing PA and PB

suggested a trend to reddish, while b^* values indicated a yellower appearance. L^* values decreased when the SGC extract concentrations was higher, while the opacity increased (Figure 8.8a), being the highest value obtained for films with 0.20% (w/v) of PB.

Table 8.4 Color parameters and opacity values of the CMC-based films without and with different PA and PB concentrations

Extract (%, w/v)	L^*	a^*	b^*	Opacity (%)
0.00 (CMC)	96.63 ± 0.44^{a}	0.14 ± 0.05^{a}	2.74 ± 0.42^{a}	9.39 ± 0.76^{a}
PA 0.05	81.01 ± 1.17^{b}	4.74 ± 0.30^{b}	27.08 ± 0.81^{b}	12.38 ± 1.40^{ad}
PA 0.10	63.52 ± 0.84^{c}	13.95 ± 0.39^{c}	41.30 ± 0.39^{c}	17.99 ± 0.74^{be}
PA 0.20	51.27 ± 1.96^{d}	20.10 ± 0.93^{d}	39.19 ± 0.75^{d}	26.66 ± 3.82^{c}
PB 0.05	71.91 ± 0.70^{e}	8.38 ± 0.27^{e}	31.69 ± 0.60^{e}	15.47 ± 1.48^{bd}
PB 0.10	55.01 ± 2.21^{f}	15.81 ± 0.77^{f}	36.08 ± 0.80^{f}	21.17 ± 2.22^{e}
PB 0.20	33.86 ± 2.55^{g}	17.92 ± 1.69^{g}	20.54 ± 2.69^{g}	53.00 ± 8.78^{f}

Different letters in the same column correspond to statistically different samples for a 95% confidence level.
PA: extracts containing polysaccharides obtained by an alkali pretreatment of SCG; PB extracts containing polysaccharides obtained by autohydrolysis of SCG.

Although transparency is an appreciated feature in films since often it influences in the consumer choice, opacity is also an important attribute once films with this characteristic can be used to protect the food, acting as a barrier to light. The color and opacity simulation of the films is shown in Figure 8.8b. In order to distinguish the film color, the opacity parameter was kept constant (100%) in the left column of the graph considering only the L^*, a^* and b^* parameters, while the measured opacity was used in the right column to simulate the real color of the film. PA when used at different concentrations provides a film with a less opaque color compared to PB. This is probably related with the amount of phenolic compounds and the interactions between they and polysaccharides present in the materials used in the production of the films (Gómez-Estaca, Giménez, Montero, & Gómez-Guillén, 2009). Both, PA and PB extracts possess a high quantity of phenolic compounds (Section III - Chapter 5 and 4, respectively), however, the PB extract was subjected to an extra Maillard reaction during autohydrolysis process, where can be generated additional pigments that greatly influence in the opacity of the films.

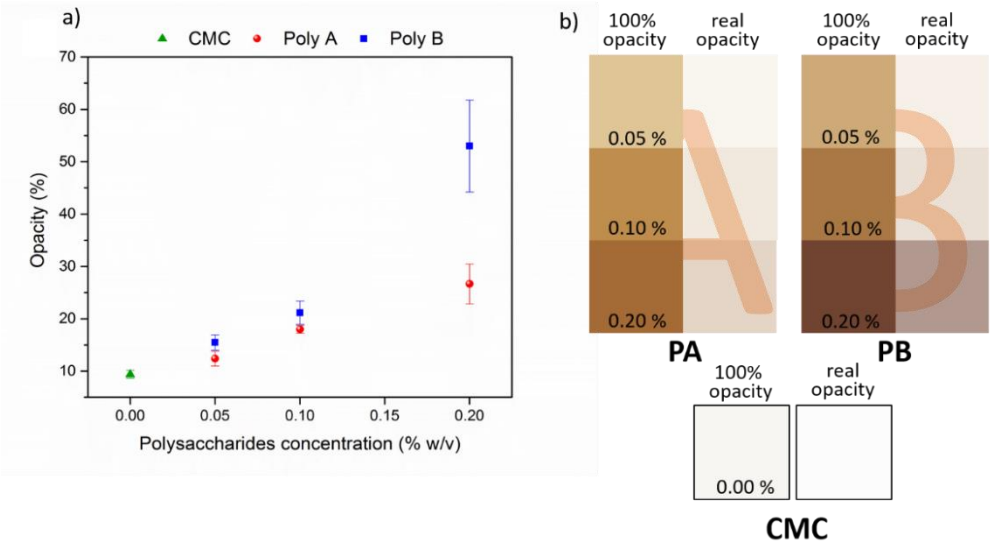

Figure 8.8 Opacity values of films with increasing extract concentrations (a), evaluation of the film color when the opacity parameter is kept constant at 100% (left column) and when the real opacity is used to simulate the real color of the film (right column) (b)

8.3. Conclusions

In general, the addition polysaccharides rich extracts obtained from SCG by alkali pretreatment (PA) and autohydrolysis (PB) improved or preserved the physicochemical properties of the edible films with respect to the control film. SEM images, TGA and XRD patterns showed changes on the films containing PA and PB at different concentrations, being confirmed by the results obtained from tensile strength, contact angle, water solubility, color and opacity, showing important changes when comparing to the control film (CMC-based film). Results suggest that the addition of SCG extracts affect the films matrix and change its properties. Color and opacity, for example, were the most affected properties when PA and PB were incorporated, significantly improving the light barrier of the films. Besides the improvement of the physicochemical properties, the incorporation of PA and PB into CMC-based films, can give important functional properties to the films, such as antioxidant and antimicrobial activities (corroborated in Section III - Chapter 4 and 5), increasing the possibilities of applications of these bio-based films on foods.

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CHAPTER 9

EFFECT OF CARBOXYMETHYL CELLULOSE-BASED COATINGS ON THE SHELF-LIFE PARAMETERS OF FRESH GOLDENBERRIES

9. Introduction

The consumption of fresh fruits has been always related to multiple health benefits. However, after harvesting the quality of fruits can be affected since they can present a short shelf-life becoming an obstacle for the commercialization of some fruits. On the other hand, customers are looking for foods with high quality and as far as possible, products free of synthetic preservatives and chemical additives. Packaging plays an important role in the food preservation and is very important if the commercialization of the food requires high storage periods. Edible coatings obtained from natural sources have showed to be a good alternative to protect and increase the shelf-life of foods, especially fruits (Dotto, Vieira, & Pinto, 2015; Li et al., 2009; Souza et al., 2015), avoiding dehydration, reducing microbial contamination and maintaining the organoleptic and nutritional properties safe for a longer time. Additionally, edible coatings can be used as vehicles for bioactive compounds.

Physalis peruviana, also known as goldenberry, is a fruit with high amount of vitamins A, B and C, polyunsaturated fatty acids and minerals as iron and phosphorus. It is a juicy orange berry similar in size, shape and structure to a small tomato, but that is completely enclosed in a large papery husk or calyx. Although the calix protects the goldenberry along harvest and postharvest, it becomes a disadvantage when the fruit is storage due to the high volume that occupies. Shelf-life of goldenberry with calyx is of 30 days, whereas without calyx is around 5 days at room temperature (Puente, Pinto-Muñoz, Castro, & Cortés, 2011). However, at temperature between 3 - 7 °C goldenberry without calyx could have a shelf-life of approximately 45 days (Castro & Blair, 2010).

This chapter evaluated the possibility of using functional polysaccharides and phenolic compounds encapsulated, extracted from spent coffee grounds, into carboxymethyl cellulose (CMC)-based coatings in order to increase the shelf-life of goldenberries. The coatings tested on the fruit were chosen by evaluating different film forming solutions containing polysaccharide rich extracts (solutions produced in Section V - Chapter 8) and phenolic compounds encapsulated (obtained from the Section IV - Chapter 7 when using maltodextrin and freeze-drying). Thus, wettability and antimicrobial tests were carried out to select the two best film forming solutions with

respect to these characteristics (one of them containing polysaccharides and the other with phenolic compounds encapsulated). The selected coatings were applied on goldenberries and the physicochemical and microbiological properties as well as the gas exchange rate of the fruits when subjected at different temperatures and relative humidities were evaluated.

9.1. Materials and methods

9.1.1. Raw material and chemicals

Polysaccharide rich extracts from SCG were obtained using the extraction methods previously described in Section III - Chapter 5 (alkali pretreatment) and Chapter 4 (autohydrolysis treatment) and named as PA and PB extracts, respectively. Phenolic compound extract was obtained by autohydrolysis of SCG using the optimum process conditions reported in Section IV - Chapter 6 and subsequently encapsulated in maltodextrin and dried by freeze-drying (as defined in Chapter 7) and named as PE extract. Carboxymethylcellulose-CMC (Blanose, 7M65) was obtained from Ashland Inc. (Düsseldorf, Germany), analytical reagent grade glycerol 99.5% was purchased from Himedia (Mumbai, India) and ultrapure water from a Milli-Q System (Millipore Inc., USA) was used. Goldenberries (*Physalis peruviana*), being produced in Colombia, were purchased from a Portuguese company Nativa, sabores de outro mundo, having the same date of packaging.

All the chemicals used were analytical grade, purchased from Sigma–Aldrich (Chemie GmbH, Steinheim, Germany), Panreac Química (Barcelona, Spain), Merck (Darmstadt, Germany) and Fisher Scientific (Leicestershire, UK).

9.1.2. Coating production

CMC film forming solutions were prepared using the metodology proposed in Section V - Chapter 8. Briedly, CMC was dissolved in ultrapure water at 70 °C during 4 h at constant agitation (300 rpm) using a magnetic stirrer. Subsequently, glycerol was added and left under agitation one

more hour. On the other hand, different concentrations of PA, PB and PE extracts (0.00%, 0.050%, 0.10% and 0.20%, w/v) were dissolved in ultrapure water and placed at 20 °C during 3 h with magnetic agitation. Each one of the solutions was slowly added to CMC-glycerol solution and maintained for 30 min at 70 °C. The components of coatings were prepared and mixed taking into account the desired concentrations in each final solution. Thus, the film forming solutions with the polysaccharide rich extracts were composed by 1.50% of CMC and 0.50% of glycerol with increasing concentrations of PA/PB extracts (0.00%, 0.050%, 0.10% and 0.20%, w/v), while the film forming solutions containing phenolic compounds encapsulated were composed by 1.50% of CMC and 0.50% of glycerol with increasing concentrations of PE extract (0.00%, 0.050%, 0.10% and 0.20%, w/v). Additional film forming solutions were carried out being composed for 1.50% of CMC, 0.50% of glycerol and 0.20% of PA with increasing concentrations of PE extract (0.050%, 0.10% and 0.20%, w/v). After production, all solutions were stored at 4 °C until further use.

9.1.3. Selection of coating solutions

Surface tension, wettability and antimicrobial tests were carried out to select a CMC-based coating solutions, one containing polysaccharide rich extract and other with encapsulated phenolic compounds. The selected coatings were applied on goldenberry fruit and their effect on shelf-life parameters of the fruit evaluated.

9.1.3.1. *Goldenberry surface and Wettability*

9.1.3.1.1. Surface tension of goldenberry

The Surface tension of goldenberry skin were determined using the Young-Dupré equation (Eq 9.1) according to (Van Oss, Chaudhury, & Good, 1988). For a pure liquid, if polar $\left(\gamma_L^p\right)$ and dispersive $\left(\gamma_L^d\right)$ interactions are known, and if θ is the contact angle between that liquid and a solid, the interaction can be described in terms of the reversible work of adhesion (W_a), as showed in Eq 9.1, where γ_S^p and γ_S^d represent the polar and dispersive contributions of the surface of the solid studied.

Eq 9.1 $$W_a = W_a^d + W_a^p \Leftrightarrow W_a = 2\left(\sqrt{\gamma_s^d \cdot \gamma_L^d} + \sqrt{\gamma_s^p \cdot \gamma_L^p}\right) = \gamma_L(1 + \cos\theta)$$

Rearranging Eq 9.1, results the Eq 9.2:

Eq 9.2 $$\frac{1+\cos\theta}{2} \cdot \frac{\gamma_L}{\sqrt{\gamma_L^d}} = \sqrt{\gamma_s^p} \cdot \sqrt{\frac{\gamma_L^p}{\gamma_L^d}} + \sqrt{\gamma_s^d}$$

The contact angle (θ) formed on the surface of the fruit (goldenberry skin) was evaluated using three pure liquid compounds, including bromonaphthalene, formamide and ultrapure water. All measurement were performed at 20.5 ± 0.5 °C with 10 replicates for each pure liquid used. The obtained contact angles combined with the values of each dispersive and polar component values of the pure liquids were used to calculate the variables of the Eq 9.2.

9.1.3.1.2. Critical surface tension

Critical surface tension (γ_c) was also estimated according to Cerqueira et al. (2009) using the Zisman plot extrapolation (Zisman, 1964), which is used to characterize the wettability of low-energy surfaces. In systems where the surface tension is lower than 100 mN/m (low-energy surfaces), the contact angle formed by a drop of liquid on a solid surface is considered as a linear function of the surface tension of the liquid, (γ_{LV}), where phase *V* is air saturated with the vapor of liquid, *L*.

Zisman plot was obtained by plotting the cosine of the contact angle of the pure liquid compounds evaluated on the surface of the fruit (goldenberry skin) against the surface tension of each compound. The intercept of this curve with cos θ = 1 is known as the critical surface tension (γ_c). The critical surface tension (γ_c) is defined in Eq 9.3.

Eq 9.3 $$\gamma_c = \lim \gamma_{LV} \; as \; \theta \rightarrow 0$$

9.1.3.1.3. Wettability surface tension of the coating solutions

The wettability of the film forming solutions on goldenberry skin was studied by determining the values of spreading coefficient (W_s), and work of adhesion (W_a) and cohesion (W_c), according to Cerqueira et al. (2009). The adhesive forces promote the liquid spreading in a solid surface and the cohesive forces promote their contraction. The wetting behavior of the solutions mainly depend on the balance between these forces.

The contact angle (θ) of a liquid drop on a solid surface is defined by the mechanical equilibrium of the drop under the action of three interfacial tensions: solid-vapor (γ_{SV}), solid-liquid (γ_{SL}), and liquid-vapor (γ_{LV}). The spreading coefficient (W_s) is defined by Eq 9.4 (Rulon & Robert, 1993) and can only be negative or zero.

Eq 9.4
$$W_s = W_a - W_c = \gamma_{SV} - \gamma_{LV} - \gamma_{SL}$$

Where W_a and W_c are defined by Eq 9.5 and Eq 9.6, respectively.

Eq 9.5
$$W_a = \gamma_{LV} + \gamma_{SV} - \gamma_{SL}$$

Eq 9.6
$$W_C = 2\gamma_{LV}$$

The surface tension of the coating solutions (γ_{LV}) was measured using a tensiometer (Force tensiometer – K20, krüss, Switzerland) at 20.5 ± 0.5 °C. Five replicates were made for each coating solution. On the other hand, the contact angle of the coating solutions on the goldenberry surface was carried out according to sessile drop method (Kwok & Neumann, 1999) as previously described in Section V - Chapter 8. Measurements were made in less than 5 s at 20.5 ± 0.5 °C and ten replicates were obtained for each coating solution.

9.1.3.2. *Antimicrobial activity assays*

Antimicrobial evaluation was performed against six food pathogenic such as: *Alternaria sp.* MUM 02.42, *Cladosporium cladosporioides* MUM 97.06, *Phoma violacea* MUM 97.08, *Botrytis cinerea* MUM 97.08, *Fusarium culmorum* MUM 97.01 and *Penicillium expansum* MUM 02.14 were obtained from the collection of the Mycology Laboratory (MUM) of the University of Minho, Portugal and cultured as described in Section III - Chapter 5.

Antimicrobial test was carried out by using the agar diffusion method as reported by Hili, Evans, and Veness (1997) and Scorzoni et al. (2007) in combination with some modifications. Briefly, 100 µl of inoculum suspension ($1x10^6$ - $5x10^6$ CFU/ml) were spread with sterile swabs on Petri dishes (90 mm) containing approx. 25 ml of potato dextrose agar (PDA), and then, a 25 µl drop of the coating solution was placed on contaminated agar and incubated at 25 °C for 48 h. After this time, the growth inhibition was evaluated by the naked eye. Natamycin and Fluconazol were used as positive controls and distilled water as negative control. For each microbial strain and each coating solution, a minimum of six replicates was made.

9.1.4. Goldenberry coating

Firstly, the papery husk or calyx that covered the goldenberries was removed. The fruits were duly selected, being discarded those that presented injuries or ripeness very different when assessed by the naked eye. Once selected, goldenberries were not subjected to any proceeding before the application of the coating. 4 different treatments were performed and named as: uncoated (fruit without coating), coating A (CMC-based edible coating), coating B and coating C, which represent the film forming solutions that after testing on goldenberries were selected and will be described in the results and discussion section.

The coating application was performed by spray. Briefly, the fruits were placed on a plastic mesh, duly separated. The film forming solution was applied on the goldenberries using a spray equipped with compressed air and later, the fruits were dried at 33 °C for 2 min in a drying

chamber. After that, goldenberries were again sprayed, repeating the spraying process two more times for the coated fruits.

For physicochemical and microbiological analysis, each treatment was subdivided in aluminum containers (about 40 g per container) which were placed inside a plastic container. The plastic containers were closed and stored in chambers at two controlled conditions. Temperature and relative humidity (RH) at 4 °C and 95% as well as at 20 °C and 65%, respectively, were used in order to evaluate the influence of these variables on coated and uncoated goldenberries.

9.1.5. Evaluation of Goldenberry

9.1.5.1. *O_2, CO_2 and Ethylene exchange rates*

Measurements of O_2, CO_2 and ethylene exchange rates of goldenberries (coated and uncoated) were carried out using the closed system method with air as initial atmosphere according to Cerqueira et al. (2009) with some modifications. Briefly, saturated salt solutions including sodium nitrite (RH = 65% at 20 °C) or potassium sulfate (RH = 95% at 4 °C) were put in the bottom of a glass container of 2 L in order to achieve the desired RH. Later, 136 g of fruit were placed inside the container, being separated of the saturated salt solution by a mesh. The system was closed and storage at 4 °C and 95% RH as well as at 20 °C and 65% RH. The concentrations of O_2, CO_2 and ethylene inside the container were measured by drawing gas samples with a 500 µl syringe, suitable for gas chromatography (Hamilton, Switzerland) through a silicone septum fitted in the container lid.

The ethylene content in the glass containers was determined using a gas chromatograph (Varian Star 3400 CX, USA) equipped with a flame ionization detector (FID) at 280 °C, a non-polar column Varian and Helium (1 ml/min) as carrier gas. A standard ethylene sample (500 ppm) was used for calibration. On the other hand, O_2 and CO_2 contents in the glass containers was performed employing a gas chromatograph (Bruker Scion 456, Canada), equipped with a thermal conductivity detector (TCD) at 130 °C and two channels to separate O_2 and CO_2. Thus, Molsieve column and Argon (30 ml/min) as carrier gas were used to separated O_2, while CO_2 was separate through a

Poraplot column and Helium (15 ml/min) as carrier gas. A mixture containing 10% CO_2, 20% O_2, and 70% N_2 was used as standard sample for calibration.

Two containers were performed for each fruit treatment and three injection samples were taken from each container. The content of gases (ethylene, O_2 and CO_2) in the containers were measured daily until it was kept constant.

The O_2 consumption rate was calculated through Eq 9.7, while CO_2 and ethylene production rates were determined applying Eq 9.8 and Eq 9.9, respectively (Salvador, Jaime, & Oria, 2002). These models were developed for a closed system impermeable to gases, where R_{O_2}, R_{CO_2} and R_{ethy} represent the O_2 consumption rate and CO_2 and ethylene production rates (cm^3/Kg h), respectively, w_{GB} is the weight of the fruit (Kg), and V_f represents the free volume of the container (ml).

Eq 9.7 $$dy_{O_2} = -R_{O_2}\frac{w_{GB}}{V_f}dt$$

Eq 9.8 $$dy_{CO_2} = R_{CO_2}\frac{w_{GB}}{V_f}dt$$

Eq 9.9 $$dy_{ethy} = R_{ethy}\frac{w_{GB}}{V_f}dt$$

The free volume was calculated by the Eq 9.10, where V_P is the total volume of the container (ml), w_{GB} is the weigh of the fruit (kg) and ρ_{GB}, is the density of goldenberry.

Eq 9.10 $$V_f = V_P - \frac{w}{\rho_{GB}}$$

The graphs of O_2 consumed and CO_2 and ethylene produced against time were used to calculate the slopes, which correspond to the derivatives, dy/dt of each gas.

9.1.5.2. *Physicochemical analysis*

Physicochemical analysis such as weight loss, pH, acidity, total soluble solids, browning, ascorbic acid, total phenolic, flavonoid content were determined along of the storage time. Coated and uncoated goldenberries stored at 20 °C and 65% RH were evaluated at 0, 2, 4, 6, 9, 12 days of storage, while the coated and uncoated fruits stored at 4 °C and 95% RH were analyzed at 0, 3, 7, 11, 15, 22, 28 days of storage. Two homogenates were prepared from each treatment in each time of analyses. For obtaining the homogenates, 40 g of goldenberries, from each aluminum container, were put into a plastic bag and then crushed using a rolling pin. Later the homogenates were reserved in a falcon tubes until further analyses.

9.1.5.2.1. Weight loss

Weight loss was determined using an analytical balance (Kern ABS-N/ABJ-NM, Germany). Goldenberries (coated and uncoated) were weighted at the beginning of the experiment and during the days that fruits were evaluated. Weight loss was expressed in percentage (%).

9.1.5.2.2. pH

The determination of pH of goldenberries (coated and uncoated) was carried out using a pH-meter (Hanna Instruments HI 2221 digital, Hungary), where 15 - 20 g of fruit homogenate were placed into a beaker and pH was directly measured. Two replicates were performed for each homogenate.

9.1.5.2.3. Acidity

For titratable acidity measurement of goldenberries (coated and uncoated), 5 g of fruit homogenate were dilute in 50 ml of distilled water. The mixture was vortexed and then centrifuged at 4000 rpm during 15 min. The supernatant volume recovered was measured and used for titration. Afterwards, the sample (containing 3 drops of phenolphthalein) was titrated with 0.1 N NaOH solution until change of color was observed (faint pink) and pH value achieved 8.2. A standard sample of citric acid (2 mg of citric acid per ml distilled water) was freshly prepared each day in

which the titratable acidity was evaluated in order to determine of citric acid factor. The results were expressed as milligrams of citric acid per 100 grams of fruit (mg citric acid/100 fruit). Two replicates were obtained for each homogenate.

9.1.5.2.4. Total soluble solids

Total soluble solids of goldenberries (coated and uncoated) were determined using a digital refractometer (Hanna Instruments HI 96801, Hungary). In brief, 2 g of fruit homogenate were centrifuged during 5 min at 4000 rpm and then, 200 µl of supernatant were collected and measured in the refractometer. Total soluble solids were expressed as °Brix (g fructose/100 g fruit juice). Three replicates were obtained for each homogenate.

9.1.5.2.5. Browning

The browning of goldenberries (coated and uncoated) was measured using a colorimetric method according to Li et al. (2009). Briefly, 2 g of fruit homogenate were mixed with 5 ml of ethanol at 95%, homogenized. The mixture was vortexed and then, centrifuged during 20 min at 4000 rpm and room temperature. The supernatant was collected, filtrated through 0.22 µm filters and the absorbance was measured at 420 nm using a spectrophotometer V-560 (Jasco, Japan) against a blank of distilled water to assess browning rate The browning values were expressed as the absorbance at 420 nm. Two replicates were obtained for each homogenate.

9.1.5.2.6. Vitamin C

The ascorbic acid present in goldenberries (coated and uncoated) was measured by 2,6-dichlorophenolindophenol (DCPIP) titration. Briefly, 5 ml of oxalic acid at 4% (w/v) were mixed with 2 ml of fruit juice (previously centrifuged) and 2 ml of distilled water. The mixture was vortexed and then titrated to a permanent pink color by using a DCPIP solution (0.24 mg DCPIP per ml of distilled water). A standard sample of ascorbic acid (0.2 mg of ascorbic acid per ml of distilled water) was freshly prepared each day in which vitamin C was evaluated in order to determine of ascorbic acid factor. The results were expressed as milligrams of ascorbic acid per 100 milliliters of fruit juice (mg ascorbic acid/100 ml fruit juice). Two replicates were obtained from each homogenate.

9.1.5.2.7. Phenolic compounds and Flavonoids

To determine the total phenolic compounds (PC) and content of flavonoids (FLA), was carried out a sequential methanolic extraction according to Giovanelli, Limbo, and Buratti (2014). In brief, 5 g of fruit homogenate were put into a centrifuge tube and mixed with 15 ml of acidic methanol (methanol:HCl, 99:1, v/v). The mixture was stirred for 1 h in the dark and centrifuged at 9500 rpm during 10 min at 10 °C. The supernatant was stored and the solid part was mixed two more times with 15 and 10 ml of the organic solvent for 15 min with shaking in the dark, and then centrifuged using the same conditions above mentioned. Finally, the extracts were made up to 50 ml with acidic methanol and filtered through Whatman filter paper.

PC was determined as described in Section III - Chapter 4 and expressed as milligrams of gallic acid equivalent per grams of fruit (mg GAE/g fruit). Ten replicates were obtained from each homogenate. On the other hand, FLA was performed as defined in Section IV - Chapter 6 and expressed as milligram quercetin equivalent per grams of fruit (mg QE/g fruit). Eight replicates were obtained for each homogenate.

9.1.5.3. *Microbiological analysis*

Microbiological analysis were performed by the determination of the total mesophilic count and total mould and yeast growth (FDA, 1998). Goldenberries samples (about 8 g) were transferred for a sterile plastic bag where they were crushed using a rolling pin and after vigorously stirred. Subsequently, 1 ml of sample was mixed with 9 ml of peptone water (0.1%, w/v) and then vortexed. Appropriate dilutions of the samples were prepared in duplicate (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). Later, 1 m of each dilution was transferred to a Petri dish and the selective media (between 45 and 50 °C) were added to the dish. Thus, Plate Count Agar (PCA) was added for evaluating the total mesophilic count and Dichloran Rose Bengal Chloramphenicol agar (DRBC) for determining the total mould and yeast growth from coated and uncoated goldenberries. The samples were mixed immediately after pouring by rotating the Petri dish sufficiently to obtain evenly dispersed colonies after incubation. After complete solidification, the plates were closed with parafilm, inverted and

incubated. PCA plates were incubated at 35 °C for 2 days and DRBC plates were incubated at 25 °C between 5-7 days.

Two homogenates were prepared for each treatment. The results were expressed in Log colony forming units per milliliters of fruit juice (Log CFU/ml fruit juice).

9.1.5.4. *Sensorial analysis*

The sensorial analysis was carried out using a Triangle sensory test, which is a discriminative method very used in food industry to determine if there is a sensory difference between two products. In brief, three goldenberries sets (Set1, Set 2 and Set 3) were presented to the panelist group conforming by 25 people. Each set was composed for three goldenberries, with one goldenberry exposed to a different treatment (coating A, coating B or coating C), while the other two samples of each set were uncoated goldenberries. The panelists, based on appearance, aroma, taste and texture of the samples, selected one goldenberry from each set as the sample with different treatment. Results were analyzed with a significance level of 95%, using the appropriate interpretation table for Triangle sensory test (for 25 panelist, the number of correct answers to establish a significant difference should be ≥13). For the analysis, the fresh goldenberries (uncoated or coated) were previously stored at 4 °C and 95% RH during 15 days.

9.1.5.5. *Statistical analysis*

Statistical analyses were carried out using GraphPad Prism (version 6.1). One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were performed to determine the significant differences ($p < 0.05$) between the treatments evaluated in the same storage day.

9.2. Results and discussion

9.2.1. Selection of the coatings

9.2.1.1. *Surface and critical surface tension*

The surface tension and critical surface tension were determined in order to characterize the goldenberry skin surface. Goldenberries presented a surface tension of 30.78 ± 2.81 mN/m, while for critical surface tension the value was 24.95 ± 1.37 mN/m. The fruit surface, being a low-energy surface (<100 mN/m), presented a high dispersive component (29.26 ± 1.39 mN/m), which indicates the ability of the surface to participate in non-polar interactions; while presents a very low polar component (1.52 ± 0.4 mN/m). A surface with these characteristics interacts with liquid mostly by dispersion forces, influencing the effective spreading of the film forming solution on the goldenberry skin.

9.2.1.2. *Wettability and antimicrobial activity*

Wettability and antimicrobial tests were carried out to evaluate different CMC-based coating solutions containing PA, PB and PE in order to select the edible coatings that were tested on fresh goldenberries.

Wettability is one of the most important properties when evaluating the capacity of a solution to coat a designed surface. In practical terms, the closer the W_s values are to zero, the better a surface will be coated. Table 9.1 presents the W_s values obtained for each coating solution tested on goldenberries. The bets W_s values for the CMC-based coating solutions with PA or PB extracts were obtained when PA and PB were used at 0.20% and at 0.10% (w/v), respectively, being statistically different from the other samples ($p < 0.05$) with polysaccharide extract. CMC-based coating solutions containing PE extracts showed the bets W_s values when using PE at 0.10% (w/v) and also when PE and PA were mixed in the same ratio (PA 0.20% + PE 0.20%, w/v), showing significant changes ($p < 0.05$) when compared with the others solutions produced with phenolic encapsulated extracts (Table 9.1). Thus, two coatings solutions from each group evaluated (solutions with polysaccharide extracts and solutions with phenolic encapsulated extracts) showed

be equally good in terms of wettability (data at bold), being the most suitable for using on goldenberries.

Table 9.1 Spreading coefficient (W_s) obtained for the tested solutions on fresh goldenberry surface

Film forming solutions with polysaccharide extracts		**Film forming solutions with phenolic encapsulated extracts**	
Concentration (%, w/v)	Spreading coefficient (W_S)	Concentration (%, w/v)	Spreading coefficient (W_S)
0.00 (CMC)	-81.69 ± 3.36^{a}	0.00 (CMC)	-81.69 ± 3.36^{d}
PA 0.05	-44.96 ± 4.21^{b}	CMC + PE 0.05	-58.22 ± 3.70^{a}
PA 0.10	-59.81 ± 5.84^{c}	CMC + PE 0.10	**-48.05 ± 3.82**^{bc}
PA 0.20	**-35.72 ± 3.82**^{d}	CMC + PE 0.20	-50.13 ± 6.62^{b}
PB 0.05	-43.87 ± 4.63^{b}	PA 0.20 + PE 0.05	-58.02 ± 2.30^{a}
PB 0.10	**-39.95 ± 4.75**^{bd}	PA 0.20 + PE 0.10	-51.76 ± 4.87^{b}
PB 0.20	-55.99 ± 5.72^{c}	PA 0.20 + PE 0.20	**-44.13 ± 3.94**^{c}

All coatings solutions are produced from 1.50% (w/v) CMC and 0.50% (w/v) glycerol with different concentrations (0.00%, 0.05%, 0.10% and 0.20%, w/v) of PA, PB and PE. Results are expressed as mean ± standard deviation; n=10. Different letters in the same column indicate a statistically significant difference (Tukey test $p < 0.05$). The data at bold represent the best obtained values for each group of solutions.

On the other hand, Table 9.2 shows the obtained results for the antimicrobial tests when CMC film forming solutions containing PA, PB and PE were evaluated against six food pathogenic fungi that drastically influence the quality and safety of postharvest fruits (Jasso de Rodríguez et al., 2011). The results were based on a qualitative test (naked eye) and the X represents the film forming solution that had an antimicrobial effect to a specific microbial strain, delaying thus, the microbial contamination.

All coating solutions with polysaccharide extract presented antimicrobial effect against at least one strain. However, the CMC-based coating solutions containing PA or PB extract at a concentration of 0.20% (w/v) showed antimicrobial effect on all fungi (Table 9.2). Although CMC-based coating solution with 0.10% (w/v) of PB presented the best W_s value for the group of polysaccharides together with CMC-based coating solution containing 0.20% (w/v) of PA (Table

9.1), the antimicrobial effect of 0.10% (w/v) of PB was lower (against two strains) than 0.20% PA (Table 9.2).

Table 9.2 Antimicrobial test of the CMC-based coating solutions containing PA, PB and PE on growth of different microbial strains

Film forming Solutions (% (w/v))	*Alternaria sp*	*Phoma violacea*	*Penicillium expansum*	*Cladosporium cladosporioides*	*Fusarium culmorum*	*Botrytis cinerea*
CMC		X				
PA 0.05		X			X	X
PA 0.10			X			
PA 0.20	X	X	X	X	X	X
PB 0.05	X	X			X	
PB 0.10	X	X				
PB 0.20	X	X	X	X	X	X
CMC + PE 0.05				X		
CMC + PE 0.10		X				
CMC + PE 0.20		X			X	
PA 0.20 + PE 0.05		X			X	
PA 0.20 + PE 0.10		X	X			
PA 0.20 + PE 0.20		X			X	

X: Represents the coating solution that had an antimicrobial effect to a specific microbial strain. All coatings solutions are produced from 1.50% (w/v) CMC and 0.50% (w/v) glycerol with different concentrations (0.00%, 0.05%, 0.10% and 0.20%, w/v) of PA, PB and PE. Results are expressed as mean ± standard deviation; n=8.

The antimicrobial effect of the solutions with phenolic encapsulated extract showed a minor effect against the fungi tested being the CMC-based coating solution with a mixture of PA and PE at ratios of 2:1 (PA 0.20% + PE 0.10%, w/v) and 2:2 (PA 0.20% + PE 0.20%, w/v) the only coatings solutions with antimicrobial effect against two strains (Table 9.2).

Taking into account the obtained results in Section V – Chapter 8 for the films produced with PA and PB extract, as well as the obtained results for wettability and antimicrobial activity of all evaluated solutions, coating B and coating C were selected to be tested on fresh goldenberries. Therefore, the 4 treatments evaluated were: uncoated (fruit without coating), coating A (CMC-based

edible coating), coating B (CMC-based edible coating with incorporation of PA (0.20 %, w/v)); and coating C (CMC-based coating containing PA and PE (PA 0.20% + PE 0.20%, w/v)).

9.2.2. Evaluation of coatings on goldenberry

9.2.2.1. *O_2, CO_2 and ethylene exchange rates*

In order to understand how the selected coatings (coating A, coating B and coating C) can influence in the gases exchange, the concentration of O_2, CO_2 and ethylene gases was measured until it was kept constant into the system and then, the transfer rate of each gas was calculated.

The obtained results for O_2 consumption $\left(R_{O_2}\right)$ and CO_2 $\left(R_{CO_2}\right)$ and ethylene $\left(R_{ethy}\right)$ production are showed in the Figure 9.1a, Figure 9.1b and Figure 9.2, respectively. It can be seen that $R_{O_2,}$ R_{CO_2} and R_{Ethy} values increased considerably when the goldenberries (uncoated and coated) were stored at 20 °C and 65% RH in comparison to the values obtained for goldenberries stored at 4 °C and 95% RH, indicating that the storage conditions clearly affect the respiration rate.

Figure 9.1a shows that at 20 °C and 65% RH, R_{O_2} of goldenberries without coating was significantly higher ($p < 0.05$) than the values obtained for coated goldenberries with coatings A, B and C, while the all coated goldenberries (regardless of treatment used) did not present significant differences in terms of R_{O_2} between them. As can be also seen in Figure 9.1a, R_{O_2} at 4 °C and 95% RH was much low for all treatments, nonetheless, when the coating A and B were used, the $R_{O_2}$was significantly lower ($p < 0.05$) when compared with uncoated and coated with coating C goldeberries.

Although CO_2 production was higher than O_2 consumption for all treatments, the lowest R_{CO_2} at 4 °C and 95% RH (4.18 ± 0.32 cm^3/Kg h) was achieved when the coating B was used, being statistically different to the other treatments (Figure 9.1b). A similar behavior was observed at 20 °C and 65% where R_{CO_2} of goldenberries with coating B was significantly lower ($p < 0.05$) than the reported values for R_{CO_2} of goldenberries treated with coating A or without coating.

However, the coated fruits with coating B did not showed statistical differences in term of R_{CO_2} when compared to the coated goldenberries with coating C stored at 20 °C (Figure 9.1b).

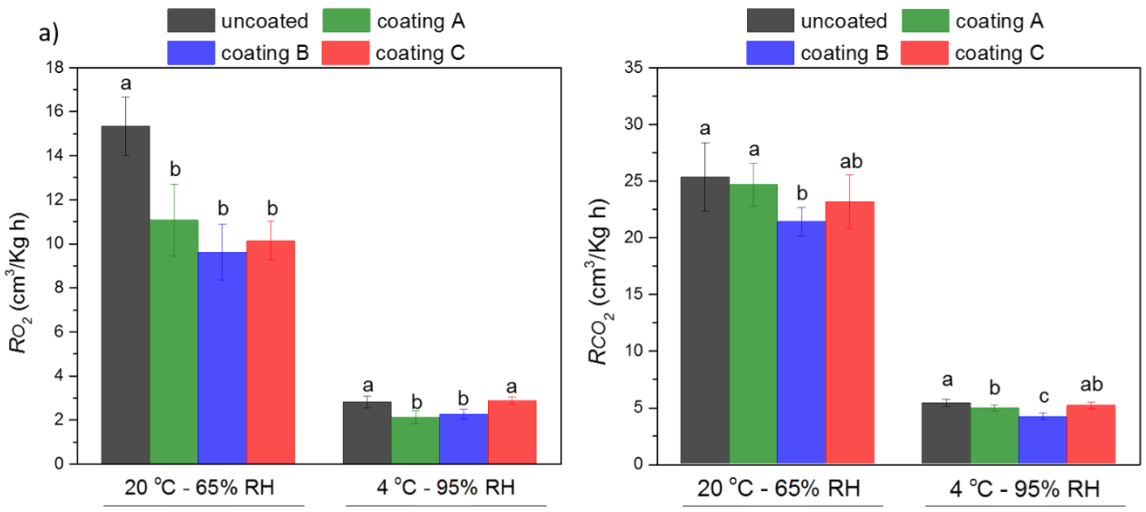

Figure 9.1 O_2 (a) and CO_2 (b) transfer rates (RO_2 and RCO_2) in fresh goldenberries at 20 °C and 65% RH as well as at 4 °C and 95% RH. Results are expressed as mean ± standard deviation (n=6). Different letters within each temperature and RH group mean values statistically different at 95% confidence level

Ethylene production at 20 °C and 65% RH was significant lower ($p < 0.05$) for the all coated goldenberries, especially for those fruits coated with the coating B and C, when compared with the goldenberry without coating. However, at 4 °C any significant difference was noted between the uncoated and coated goldenberries (Figure 9.2).

Goldenberry can be classified as a fruit highly climacteric due to after physiological maturity presents an increased respiratory rate (Gutierrez et al.2008). The obtained values for the ethylene production and respiration rate of goldenberry are in agreement to the reported by Carvalho, Villaño, Moreno, Serrano, and Valero (2015) when the fruits were stored at 20 °C.

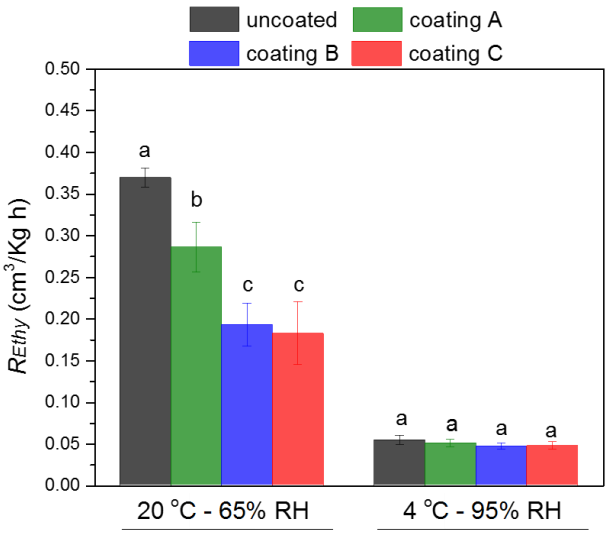

Figure 9.2 Ethylene transfer rate (R_{Ethy}) in fresh goldenberries at 20 °C and 65% RH as well as at 4 °C and 95% RH. Results are expressed as mean ± standard deviation (n=6). Different letters within each temperature and RH group mean values statistically different at 95% confidence level

Overall, results showed that the application of the developed coatings can reduce the gas exchange (O_2, CO_2 and ethylene) of goldenberries at different storage conditions, being more effective at a higher temperature and a lower RH. This can be explained by the fact that CMC-based coatings containing PA/PE increase the skin resistance to gas diffusion by blocking the pores on the fruit surface, resulting in a modified internal atmosphere of relatively high CO_2 and low O_2.

9.2.2.2. *Weight loss*

Weight loss in the fruits is mainly related with the decrease of their water content during the post-harvest storage, leading to changes in texture, flavor and appearance (Lin & Zhao, 2007). Figure 9.3a displays the weight loss for coated and uncoated goldenberries when stored during 12 days at 20 °C and 65% RH, while Figure 9.3b shows the obtained results for goldenberries placed at 4 °C and 95% RH during 28 days of storage. For both storage periods, all coated and uncoated fruits showed weight losses during storage, which increased along storage time, being the higher weigh losses obtained for the uncoated goldenberries ($p < 0.05$), independently of temperature and relative humidity used. Although the uncoated goldenberries were the most affected by weight loss,

all treatments (uncoated, coating A, coating B and coating C) suffered a weight loss higher when stored at 20 °C and 65% RH, as expected, since the fruit quality is greatly affected when the storage temperature is increased.

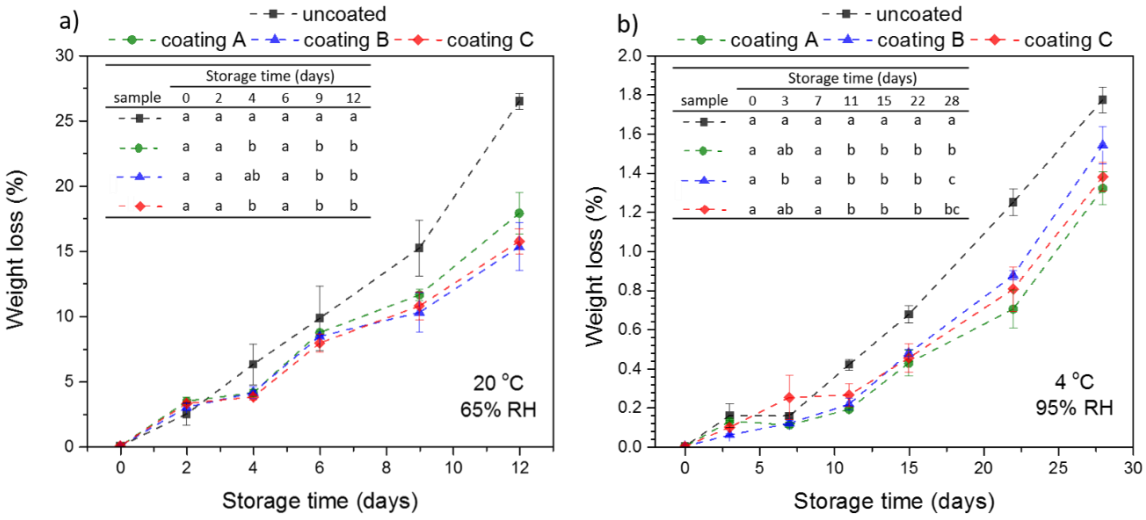

Figure 9.3 Weight loss of uncoated and coated fresh goldenberries as a function of storage time when using 20 °C and 65% RH (a) as well as 4 °C and 95% RH (b). Results are expressed as mean ± standard deviation (n=3). Different letters in the same day (column) indicate values statistically different at 95% confidence level

At the end of the 12th day of storage (Figure 9.3a), the weight loss for uncoated fruits was 26.48%, while for coated goldenberries with the different treatments (coating A, coating B and coating C) were 17.88%, 15.31% and 15.73%, respectively. The fruits stored at 4 °C during 28 day of storage (Figure 9.3b) presented the same behavior, but the weight loss was much lower, with values of 1.77% for uncoated goldenberries and 1.32%, 1.54% and 1.37% for goldenberries using the coating A, coating B and coating C, respectively. These differences can be explained by the water vapor barrier provided by the coatings used on goldenberries, which decrease water loss during storage and, thus, a lower weight loss.

9.2.2.3. *pH and acidity*

The pH values for uncoated and coated goldenberries stored at 20 °C and 65% HR, as well as at 4 °C and 95% HR are shown in Figure 9.4a and Figure 9.4b, respectively. Some variations can be observed with respect to the obtained initial values in each treatment (3.50 – 3.77), indicating differences ($p < 0.05$) in the degree of maturity of the fruits. The pH values of goldenberries (coated and uncoated) placed at 20 °C and 65% RH did not showed significant changes ($p > 0.05$) regardless of treatment from 4th day of storage, however, the pH values of all samples were increased during the storage time. The pH raise is explained by the fruit ripening and decomposition process caused by hydrolysis, oxidation or fermentation that modifies the concentration of hydrogen ions (Souza et al., 2015).

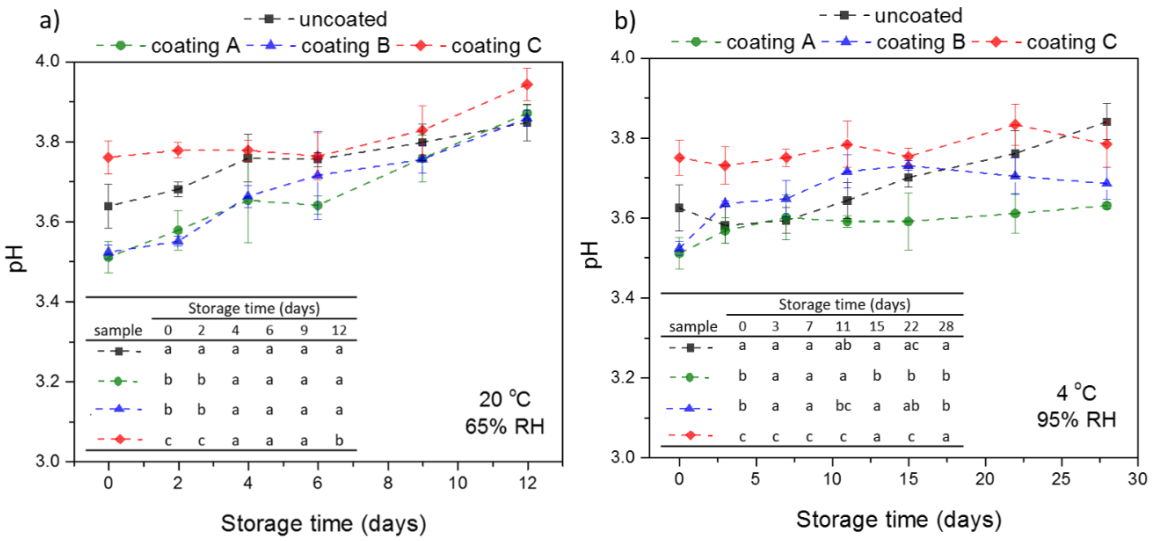

Figure 9.4 pH of uncoated and coated fresh goldenberries as a function of storage time when using 20 °C and 65% RH (a) as well as 4 °C and 95% RH (b). Results are expressed as mean ± standard deviation (n=4). Different letters in the same day (column) indicate values statistically different at 95% confidence level

On the other hand, the fruits stored at 4 °C and 95% RH maintained the pH values constant along storage days, except the uncoated goldenberries, in which the pH values increased. Results suggest that the coatings help to maintain the initial pH values when the fruits were left at 4 °C and

95% RH, during 28 days of storage, delaying the fruit ripening and ensuring a controlled microbial growth. This behavior is explained by the fact that the coated fruits maintained a more acidic pH, which is favorable to inhibit bacterial growth (Tovar, García, & Mata, 2001).

The raise of pH values is directly related to the decrease of acidity occurring in the fruits (Mgaya-Kilima, Remberg, Chove, & Wicklund, 2014). Goldenberry is rich in organic acids, mainly citric acid. During maturity phase this organic acid is usually degraded or consumed, since it is considered a respiratory substrate (Souza et al., 2015) affecting thus, the shelf-life of goldenberry. Figure 9.5a and Figure 9.5b show that the four treatments evaluated (uncoated, coating A, coating B and coating C) presented the same behavior when subjected to different temperatures (20 and 4 °C), relative humidities (65 and 95%) and storage times (12 and 28 days).

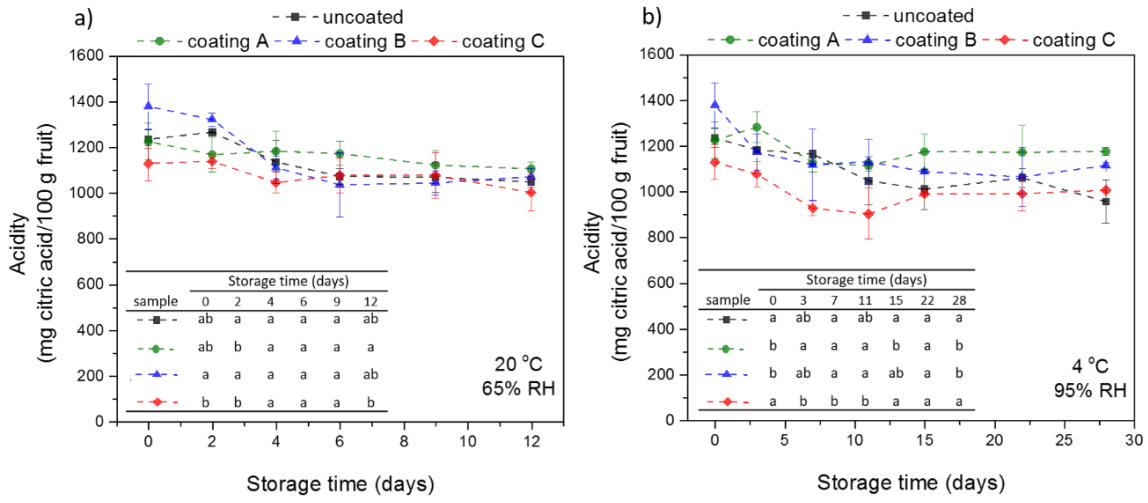

Figure 9.5 Acidity of uncoated and coated fresh goldenberries as a function of storage time when using 20 °C and 65% RH (a) as well as 4 °C and 95% RH (b). Results are expressed as mean ± standard deviation (n=4). Different letters in the same day (column) indicate values statistically different at 95% confidence level

At the beginning of the analyses, the acidity values of all treatment were between 1200–1400 mg acid citric per 100 g fruit and decreased along of the storage time for all the treatments. However, it can be noted that from 15th day of storage at 4 °C and 95% RH (Figure 9.5b) the acidity values were more stable for coated goldenberries than for uncoated goldenberries. Similar results

were reported for goldenberries coated with a alginate-based coating at 2 °C during 21 days of storage (Carvalho et al., 2015).

9.2.2.4. *Total soluble solids and browning*

In the fruits, the total soluble solids represent water-soluble substances such as sugars, acids, and vitamin C, among others. However, this parameter is currently used as an indicator of total sugar content since 90% of the soluble solids present in the fruits correspond to the sugars (Souza et al., 2010). The initial values of total soluble solids for uncoated and coated goldenberries ranged between 14 –15 °Brix. During storage at 20 °C and 65% RH the total soluble solids increased for all treatments (Figure 9.6a) achieving at the end of 12th day of storage a value of appox. 18.5 °Brix without significant differences between the samples ($p > 0.05$).

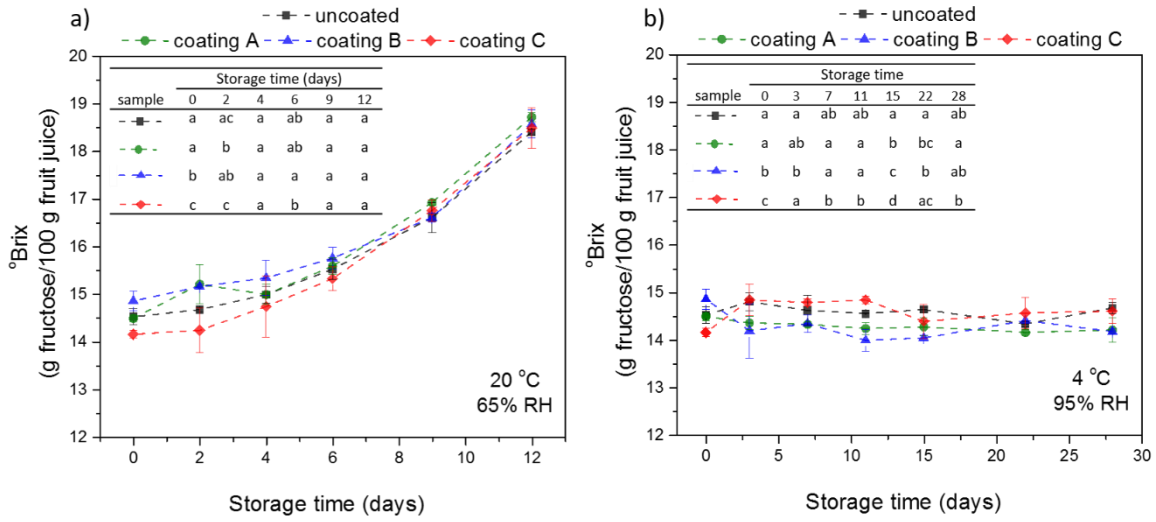

Figure 9.6 Total soluble solids of uncoated and coated fresh goldenberries as a function of storage time when using 20 °C and 65% RH (a) as well as 4 °C and 95% RH (b). Results are expressed as mean ± standard deviation (n=6). Different letters in the same day (column) indicate values statistically different at 95% confidence level

Usually, total soluble solids rise during fruit ripening due to the gradual degradation of starch and cell wall materials (Souza et al., 2015), resulting in an increase of sugar content. On the

contrary, the total soluble solid values of the uncoated and coated goldenberries were maintained constant along 28 days when stored at 4 °C and 95% RH (Figure 9.6b). Although the coated and uncoated goldenberries presented the same behavior without statistical significant changes when stored at the different values of temperatures and relative humidities, it can be noted that fruits placed at 4 °C and 95% RH had a reduction of the metabolic activity, which is in agreement with the results obtained for gases transfer rate.

The browning rate was other important parameter to evaluate goldenberries quality during storage. The obtained results showed that the browning rate of the treatments subjected at different temperatures and relative humidities had the same behavior previously reported for the total soluble solids. As a result, the browning rate of the uncoated and coated fruits stored at 20 °C and 65% RH increased with time (Figure 9.7a), but without significant differences between the samples ($p > 0.05$).

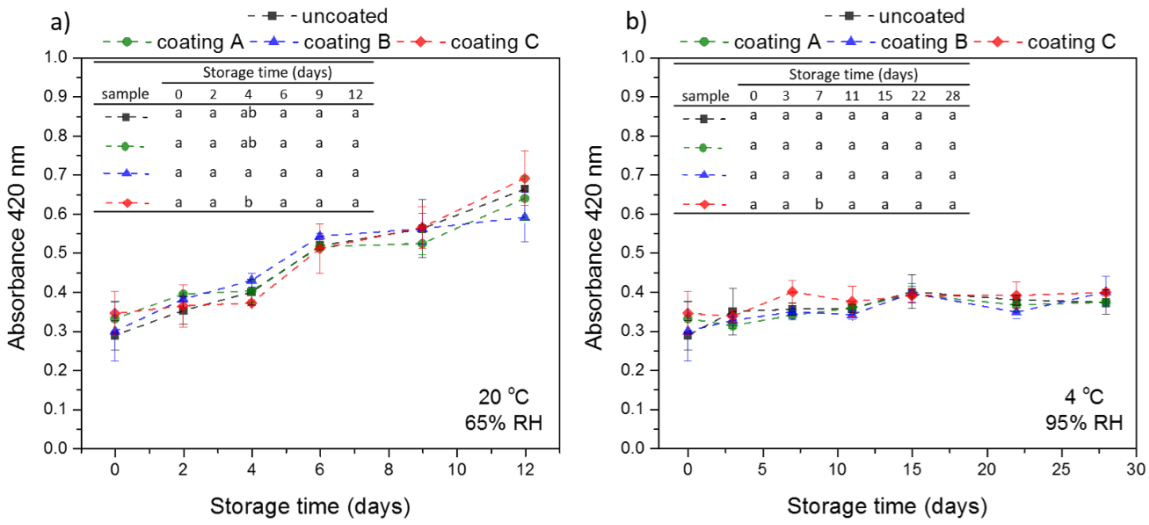

Figure 9.7 Browning rate of uncoated and coated fresh goldenberries as a function of storage time when using 20 °C and 65% RH (a) as well as 4 °C and 95% RH (b). Results are expressed as mean ± standard deviation (n=4). Different letters in the same day (column) indicate values statistically different at 95% confidence level

The browning rate of the samples (uncoated and coated) placed at 4 °C and 95% RH remained constant during all storage time (Figure 9.7b) and neither presented significant statistical differences ($p > 0.05$) between goldenberries with and without coatings. However, the results showed that at 4 °C and 95% RH the browning rate can be prevented, which is possibly related with a lower O_2 transfer rate, preventing thus the browning caused by oxidative or enzymatic processes. (Souza et al., 2015).

9.2.2.5. *Vitamin C*

Vitamin C, also known as ascorbic acid is an important constituent of the fresh fruits and vegetables. It is classified as a hydro-soluble vitamin, being abundant in fruits where the content water exceeds 50% (Gutiérrez et al., 2007). It would explain the high level of ascorbic acid in goldenberry when compared with other fruits since 79% of its composition is water (Repo de Carrasco & Encina Zelada, 2008). The obtained values at 0 days of storage for coated and uncoated goldenberries ranged around 21-23 mg/100 ml of fruit juice, which is in agreement with those values reported by Gutierrez et al. (2008).

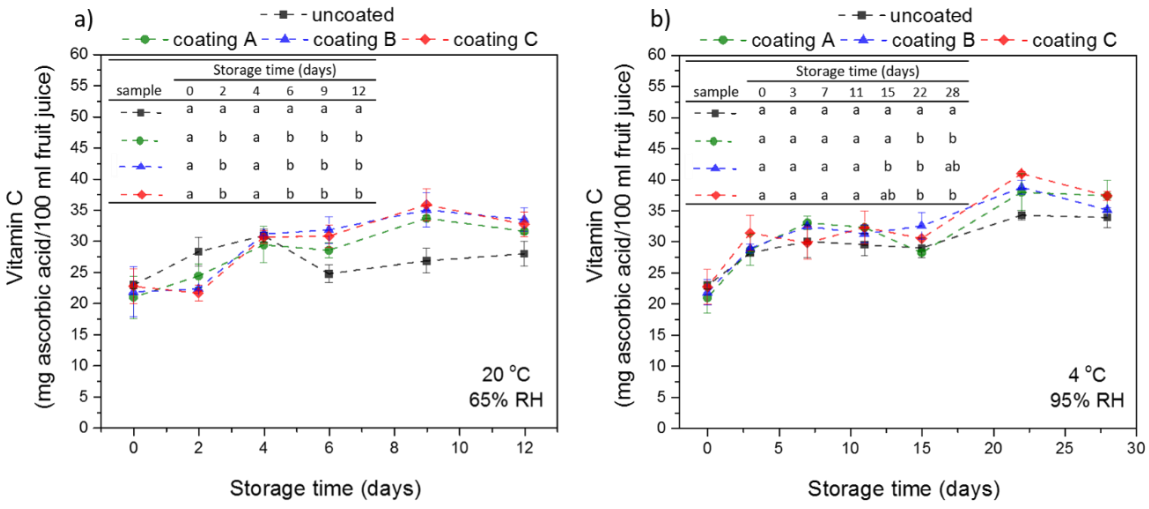

Figure 9.8 Vitamin C content of uncoated and coated fresh goldenberries as a function of storage time when using 20 °C and 65% RH (a) as well as 4 °C and 95% RH (b). Results are expressed as mean ± standard deviation (n=4). Different letters in the same day (column) indicate values statistically different at 95% confidence level

For the goldenberries (uncoated and coated) stored at 20 °C and 65% RH the content of vitamin C increased along time (Figure 9.8a), except for fruits without coating that after 4 days of storage showed a significant reduction in the ascorbic acid content ($p < 0.05$) with respect to the coated fruits. For goldenberries (uncoated and coated) stored at 4 °C and 95% RH the content of ascorbic acid increased for all treatments during storage time (Figure 9.8b). At the end of the storage significant changes ($p < 0.05$) were observed between all coated fruits and those without coatings (day 12) when stored at 20 °C and 65% RH and between the uncoated fruits and those protected with coating A and coated C (day 28) when subjected at 4 °C and 95% RH.

Some studies have reported the decrease of ascorbic acid content in fresh cut mangoes (Souza et al., 2015) and Chinese jujube (Li et al., 2009) during storage time. However, the obtained results in this chapter are in agreement with those reported by Gutierrez et al. (2008) who evaluate the ascorbic acid content in the goldenberry during four different stages of maturity and proved that ascorbic acid raises when the golbenberry becomes more mature. After achieving a total maturity, it is expected that starts vitamin C loss. Therefore, the results suggest that coating A, B and C protect the vitamin C content and delay its loss in goldenberries when stored at 20 °C and 65% RH.

9.2.2.6. *Phenolic compounds and flavonoids content*

Figure 9.9 presents the content of phenolic compounds of goldenberries (uncoated and coated) when stored at 20 °C and 65% RH (Figure 9.9a) as well as at 4 °C and 95% RH (Figure 9.9b). As can be seen, at 0 days of storage the goldenberries with the coating C showed higher phenolic compounds values ($p < 0.05$) than the other samples. It can be explained by the fact that coating C has an extra content of phenolic compounds, which were incorporated during production of film forming solution (0.20%, v/w of PE added coating C). During storage, uncoated and coated goldenberries stored at 20 °C and 65% RH, presented the phenolic compounds values constant (Figure 9.9a). However, the samples covered with the coating C showed higher values, being significantly difference ($p < 0.05$) than the uncoated goldenberries and those coated with the coating

A. At the end of storage time the goldenberries with coating B and C did not present significant differences between them ($p > 0.05$) regarding the phenolic compounds.

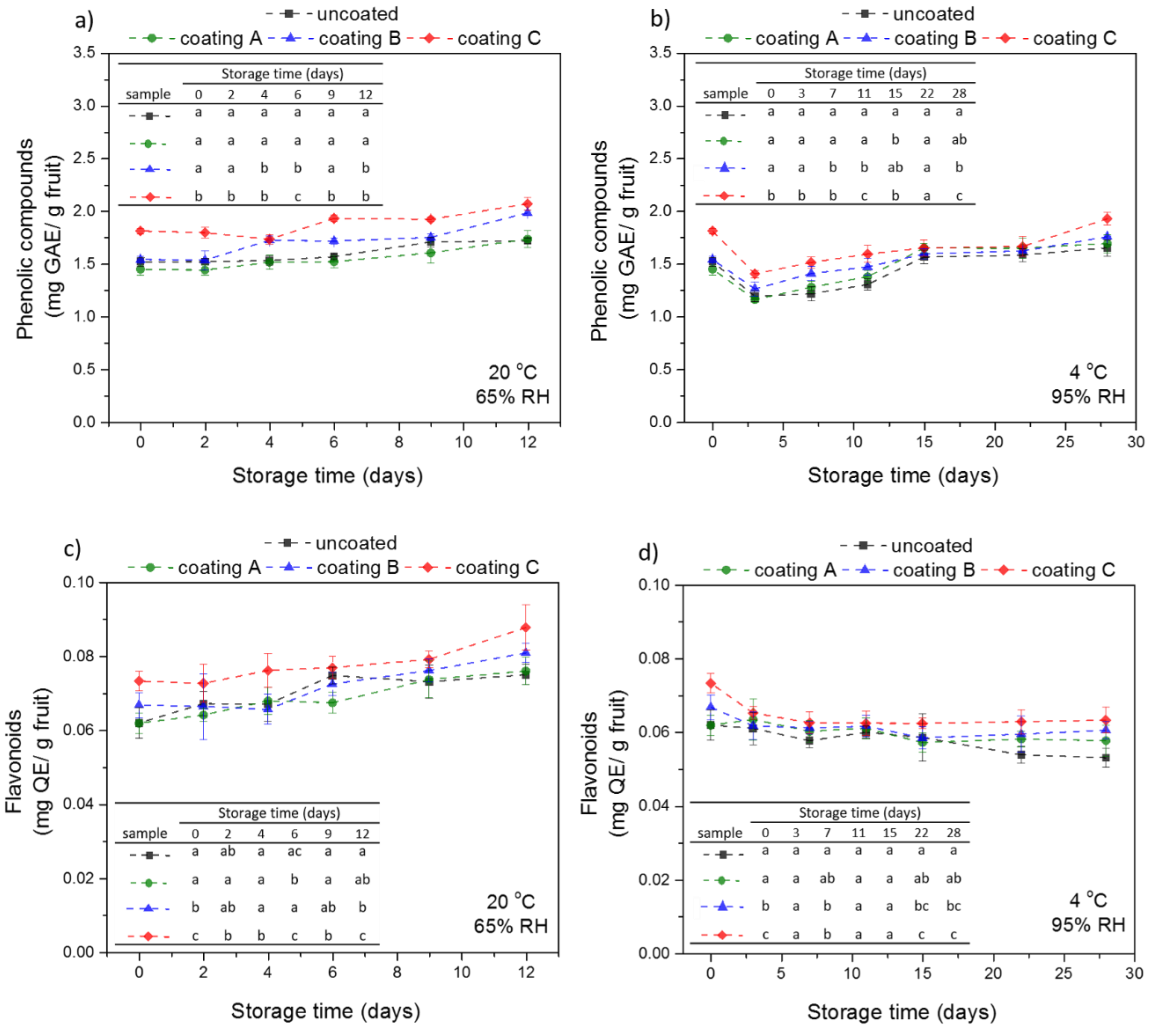

Figure 9.9 Total phenolic compounds (a, b) and flavonoids content (c, d) of uncoated and coated fresh goldenberries as a function of storage time when using 20 °C and 65% RH as well as 4 °C and 95% RH. Results are expressed as mean ± standard deviation (n=10). Different letters in the same day (column) indicate values statistically different at 95% confidence level

When the goldenberries were stored at 4 °C and 95% RH an decrease with respect to the initial content of phenolic compounds was observed for all samples. Some authors have observed this reduction in goldenberries being related to the cold storage (Carvalho et al., 2015; Valdenegro,

Fuentes, Herrera, & Moya-León, 2012). During storage at 4 °C and 95% RH , the content of phenolic compounds in uncoated and coated goldenberries increased until 15th day of storage and after it was maintained constant. Nevertheless, significant differences ($p < 0.05$) between goldenberries coated with coating C and those uncoated or coated with coating A were observed during all storage. The results are in agreement with the other studies reporting that the content of phenolic compounds in the fruits increase during ripening (Amira et al., 2012; Valdenegro, Fuentes, Herrera, & Moya-León, 2012).

The content of phenolic compounds obtained for uncoated goldenberries was higher than the values reported by Carvalho et al. (2015). It can be due to the extraction conditions used in this work (sequential extraction process, solvent, temperature, liquid/solid ratio, and extraction time) and also to the storage temperature to which the fruit was subjected.

Figure 9.9c and Figure 9.9d show the content of flavonoids of uncoated and coated goldenberries. Results showed that flavonoids was increasing in all to the samples placed at 20 °C and 65% RH, while it decreased for the uncoated and coated goldenberries stored at 4 °C and 95% RH. Although all the fruits presented the same behavior when subjected to the different conditions, significant changes ($p < 0.05$) were observed among goldenberries with coating C and the studied others samples, especially at day 0 and in the final days of storage at each condition, being the content of flavonoids higher to coating C.

9.2.2.7. *Microbiological analysis*

Figure 9.10a shows the evolution of mesophilic bacteria and yeast and molds in goldenberries fruits (uncoated and coated) during the storage at different temperature and RH conditions. Figure 9.10a shows that the goldenberries coated with coating B presented the lowest values for Log (CFU/ml fruit juice) of mesophilic bacteria, being statistically different ($p < 0.05$) than uncoated fruits after 2 day of storage at 20 °C and 65% RH. Additionally, the goldenberries with coating A and C, placed at the same conditions, showed significant changes ($p < 0.05$) when compared to the uncoated goldenberries, mainly after 9 day of storage. Although at 4 °C and 95%

RH, goldenberries coated with coating B also presented lower values of mesophilic bacteria counts (Figure 9.10b), the values were not statistically different ($p > 0.05$) compared to the others treatments.

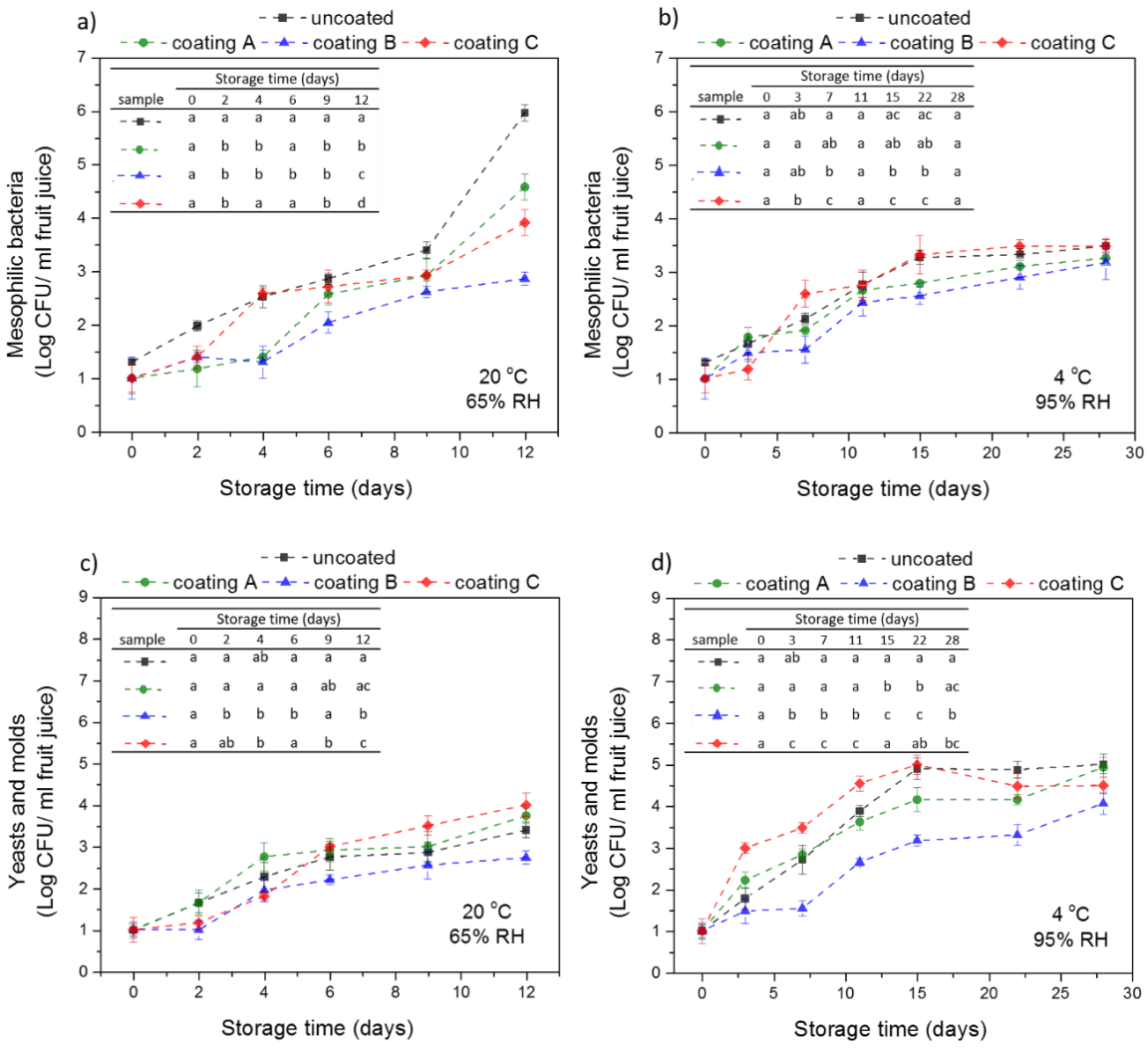

Figure 9.10 Evolution of mesophilic bacteria (a,b) and yeasts and molds (c,d) in uncoated and coated fresh goldenberries during storage time when using 20 °C and 65% RH as well as 4 °C and 95% RH. Results are expressed as mean ± standard deviation (n=4 by each dilution 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). Different letters in the same day (column) indicate values statistically different at 95% confidence level

In the same way, the lower values of yeasts and molds counts at 20 °C and 65% RH were achieved for goldenberries coated with coating B, being significantly different than the other

treatments only at the end of storage (day 12) (Figure 9.10c). However, at 4°C and 95% RH the fruits coated with coating B presented statistically different values ($p < 0.05$) when compared to the uncoated goldenberries and those coated with coating A and C (from 7th to 22th day of storage) (Figure 9.10d).

The results suggest that coating B presents antibacterial effects observed when used at 20 °C and 65% RH and antifungal effects when used on goldenberries at 4 °C and 95% RH, which is in agreement with the antimicrobial tests previously carried out in order to select the coating solutions.

9.2.2.8. *Sensorial analysis*

Sensorial analysis was carried out in order to determine if the coatings A, B or C had any negative influence on the sensorial properties on fresh goldenberries.

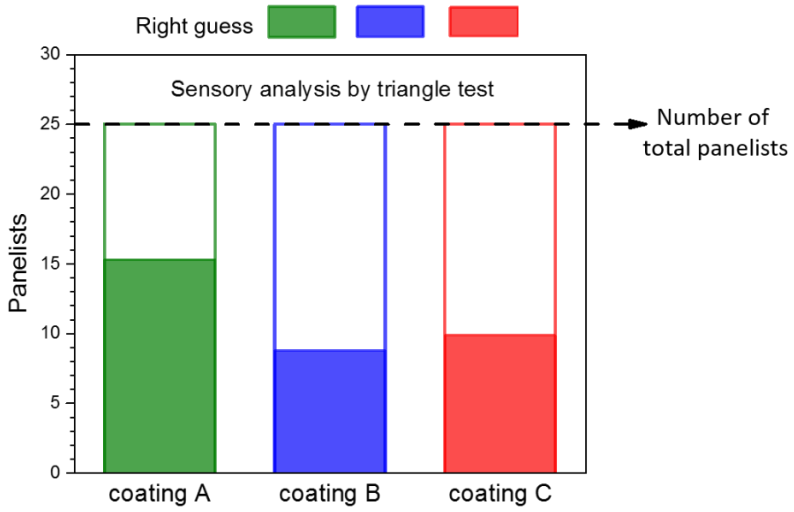

Figure 9.11 Sensory analysis results through triangle test (for 25 panelist, the number of correct answers to establish a significant difference should be ≥13)

Figure 9.11 shows the results obtained during the triangle test performed to 25 panelists, who based on appearance, aroma, taste and texture of three samples (two uncoated goldenberries and one with coating A, B or C), selected the goldenberry that considered with a different treatment. As can be seen, 15, 9 and 10 people made the right choice for coating A, B and C, respectively, indicating that coating B and C did not present significant differences ($p > 0.05$) on the sensorial properties when compared with uncoated goldenberries after 15 days of storage at 4 °C and 95% RH.

9.3. Conclusions

Shelf-life parameters of fresh goldenberries were improved when the fruits were coated with coating B and coating C. Additionally, the temperature and relative humidity used during storage also showed influence on the shelf-life parameters of the fruits. Lower gas transfer rates (O2, CO2 and ethylene) were obtained for fruits coated with coatings B and C in comparison with the uncoated goldenberries when stored at 20 °C and 65% RH. Coating B was better to control the weight loss of goldenberries as well as to delay the microbial growth, while coating C gave an extra content of phenolic compounds to goldenberries. Additionally, the use of coating B and C did not have a negative effect on sensorial properties of goldenberries. These findings show that CMC-based coatings are a good alternative for postharvest handling of fresh goldenberries, maintaining their quality and increasing the storage time.

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CHAPTER 9 - EFFECT OF CARBOXYMETHYL CELLULOSE-BASED COATINGS ON THE SHELF-LIFE PARAMETERS OF FRESH GOLDENBERRIES

SECTION VI

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

10. General conclusions and future perspectives

10.1. Conclusions

The main objective of this thesis was to extract and characterize polysaccharides and phenolic compounds from spent coffee grounds (SCG) and incorporate them into edible films or coatings for food applications. To cover successfully the thesis aims, several strategies were proposed. Firstly, two coffee residues were characterized in order to choose the one presenting higher carbohydrate content and antioxidant activity. Secondly, the extraction and characterization of polysaccharides and phenolic compounds were performed. Thirdly, phenolic compounds were encapsulated in order to preserve their functional properties. Later, the polysaccharides extracted were incorporated in edible coatings and their influence in the films' properties was evaluated. Finally, the developed edible films/coatings containing polysaccharides and phenolic compounds encapsulated were applied on goldenberry fruits and their effect on shelf-life parameters studied. Therefore, the main contribution of this thesis may be summarized as follows:

- Demonstration of spent coffee grounds (SCG) and coffee silverskin (CS) residues as materials with very interesting properties for application in food industry; in this particular case, SCG was selected as the more suitable material to develop this work due to their high hemicellulose content and antioxidant activity when compared to CS.

- Autohydrolysis and alkali pretreatment demonstrated to be efficient techniques to recover polysaccharides with high antioxidant activity from SCG, being possible to obtain a lyophilized material containing 29.29% and 39.00% (w/w) of polysaccharides, respectively. Galactose was the most representative sugar obtained through both methodologies, but mannose, arabinose and glucose were also recovery. Additionally, the lyophilized materials showed a high antioxidant activity, which was confirmed by four different methods, as well as a high antimicrobial activity against *P. violacea* and *C. cladosporioides.*

- Autohydrolysis showed to be an efficient technology to extract antioxidant phenolic compounds from SCG. It was possible to obtain an extract with high content of phenolic compounds (40.36 mg GAE/g SCG), including flavonoids and chlorogenic acid with high antioxidant activity.

- The technique (freeze-drying and spray-drying) and the coating material (maltodextrin, gum arabic, or a mixture of these components) were factors of great influence on the encapsulation of antioxidant phenolic compounds extracted from SCG.

- Freeze-drying using maltodextrin as wall material can be considered a good option for encapsulation of antioxidant phenolic compounds extracted from SCG since it is able to retain 62% and 73% of phenolic compounds and flavonoids, respectively, preserving 73-86% of the antioxidant activity existent in the original extract.

- In general, the addition of different concentrations of polysaccharide rich extracts obtained from SCG by using an alkali pretreatment and autohydrolysis improved or preserved the physicochemical properties of the edible films with respect to the control film.

- Water solubility, color and opacity, for example, were the most affected properties when polysaccharide rich extracts were incorporated, significantly improving the solubility and light barrier of the films.

- Besides the improvement of the physicochemical properties, the incorporation of polysaccharides rich extracts into CMC-based films, can give important functional properties to the films, such as antioxidant and antimicrobial activities (previously corroborated in Section III - Chapters 4 and 5) increasing the advantages of using these bio-based films on foods.

- The results showed lower gas transfer rates (O_2, CO_2 and ethylene) for the coated fruits in comparison with the uncoated fruit when using a storage temperature of 20 °C and 65 RH. Overall, the physicochemical properties did not present significant changes between the goldenberries with or without coating. However, the loss weight and the microbiological contamination were reduced when the coating containing the polysaccharide rich extract was used.

- In general, results showed the great potential of SCG to be used as raw material on biotechnological processes due to their low cost and availability. Due to their high content of polysaccharides and phenolic compounds presenting antioxidant and antimicrobial activities, it is expected a wide number of applications in food and pharmaceutical area.

10.2. Guidelines for future work

Despite the main objectives have been achieved, there are some work that could be done in the future to understand better the properties of polysaccharides and phenolic compounds as well as their effect in the films and/or coatings properties. Based on this some recommendations and guidelines for future work are give:

- Although the alkali treatment and autohydrolysis demonstrated to be efficient methods to recovery polysaccharides and phenolic compounds from SCG, others technologies used to extract these type of compounds could be evaluated including microwave-assisted extraction and ultrasound-assisted extraction among others.

- Using methodologies for the characterization of the polysaccharides extracted; e.g. intrinsic viscosity, methylation and GC/MS analyses in order to know their molecular weight and structure, backbone and how they are branched.

- Consider the possibility of using methods such as injection molding, films blowing or extrusion, which are currently utilized to produce synthetic packaging, in the production of edible films evaluated in this work.

- Applying the selected coating containing the polysaccharide rich extract in a packing house facility, in order to understand the effect of this coating on goldenberry fruit storage on an industrial environment.