Un ménage à trois, understanding the multifaceted interactions between polysaccharides, phenolics and digestive enzymes

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A thesis submitted in partial fulfilment of the requirements for the Doctorate in Philosophy degree in Chemistry

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GENERAL DECLARATION

This doctoral research has generated 7 original research articles published/submitted in international peer reviewed journals and 1 published abstract, of which, 5 original research articles have been utilised in the formulation of this thesis. The motivation for this project was the quest to understand the "importance of considering three-way interactions between polysaccharides, phenolics and digestive enzymes using different food systems".

The research candidate bore the responsibility of conceptualising individual studies, formal analysis, investigation, methodology, validation, visualisation, writing, review, and editing. This research was conducted at the University of Ottawa under the supervision of Professor Nicolas Bordenave. The inclusion of co-authors in the manuscripts of various chapters indicates that there were technical collaborations in the execution of the studies, and their contribution fits within the main subject framework of this study.

I hereby declare that the data and materials contained in this thesis have not previously been published by another author or approved for the award of a diploma or degree by any institution, except where appropriate acknowledgement has been made. Since no animal or human was used in this study, no research ethics approval was required for it or its inclusion.

Signed by: A.S.D

Date: 05/03/2024

ACKNOWLEDGEMENTS

The completion of this doctoral thesis marks a significant milestone in my academic journey. I am profoundly grateful to all those who have contributed to this achievement. This endeavour has been a labour of passion, perseverance, collaboration, and it is only fitting that I express my heartfelt appreciation to those who have made it possible.

First and foremost, I extend my deepest gratitude to my thesis advisor and mentor, Prof. Nicolas Bordenave, whose unwavering support, guidance, humour, wit, and sage like wisdom have been instrumental throughout my research journey. His mentorship has not only enriched my academic understanding but has also inspired me to reach higher and explore new horizons. His dedication to the pursuit of knowledge has been a constant source of motivation, especially when dealing with uncooperative experiments and moody enzymes. I also greatly appreciate the opportunities that were given to me to co-supervise and mentor graduate students: Aden Chen and Mrudav Raval, along with undergraduate students: Billy Golding, Emilie Hamann, Kaden Venugopal, and Radia El Iraki. These mentorship opportunities have helped me hone my collaborative and supervisory skills, which has ultimately helped me get out of my shell and grow as a research lead.

Second, I would also like to express my gratitude to my co-supervisor Prof. Corrie da Costa and the members of my thesis advisory committee; Prof. Chibuke Udenigwe, and Prof. Apollinaire Tsopmo, for their invaluable insights, critical feedback, and enduring encouragement. Their collective expertise has helped broaden the scope of my research, ensured its rigor, academic excellence, and overall has helped me grow as a better scientist.

Third, this PhD would not be possible without the generous contributions, numerous scholarships and TA opportunities from the University of Ottawa and its encompassing faculties (department of Chemistry and Biomolecular sciences/department of Health and Nutritional sciences). They have provided the resources, opportunities, and collaborative atmosphere, which have been instrumental in the developments of this thesis.

Fourth, I would like to thank my former lab mate Dr. Caleb Acquah, who during the first three years of my PhD has been a friendly face, unofficial mentor, and a source of motivation during difficult phases of my PhD journey. The discussions and debates had, have been integral to my research process.

Fifth, I would like to extend my thanks to my family, the Costas and Souzas as well as my newfound family, the Ankenmanns (In-Laws), who despite not having any background in my field of research have been a constant support (both financially and emotionally), a source of motivation, helping me to become the man I am today.

And finally, I can't forget to thank my dearest wife Caroline, whose constant love, encouragement, and daily reminders that giving up is not an option, have been invaluable to me throughout the course of our relationship, my stay in Canada and my tenure at the University of Ottawa.

TABLE OF CONTENTS

ABBREVIATIONS

-OH group: hydroxyl group

ΔE: Predicted binding energy

CA: Caffeic acid

ChA: Chlorogenic acid

cP: Apparent RVA viscosity

DMSO: Dimethyl sulfoxide

DNS: 3,5-dinitrosalicylic acid

DP: Degree of polymerization

DRI: Differential refractive index

EC: Epicatechin

EC50: Effective inhibitory concentration

ECG: Epicatechin gallate

EG: Ethyl gallate (chapter 5)

EGA: Ethyl gallate (chapter 4)

EGC: Epigallocatechin

EGCG: Epigallocatechin gallate

Eq: equation

FA: Ferulic acid

FQ: Fluorescence quenching

FQ_{max}: Final signal attenuation

GA: Gallic acid

H-bonds: Hydrogen bonds

HPAEC: High-performance ion-exchange chromatography

HPSEC: High-performance size exclusion chromatography

HSA: Human salivary amylase

IC50: half inhibition concentration

Log P: oil/water partition coefficient

MALLS: Multi-angle laser light scattering

MANOVA: Multivariate analysis of variance

NMR: Nuclear magnetic resonance

PBS: Phosphate buffer saline

pCA: p-coumaric acid

pNPL: p-nitrophenyl laurate

PPA: Porcine pancreatic amylase

PVDF: Polyvinylidene fluoride

Qu: quercetin

RDS: Rapidly digestible starch

RS: Resistant starch

RVA: Rapid ViscoAnalyzer

SCFA: Short chain fatty acids

SDS: Slowly digestible starch

SEM: standard error mean

SER: Standard error of regression

UV: Ultraviolet

Van: Vanillin

VS: Viscometry

w/w: Weight / Weight

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The subject of this doctoral thesis presents an exploratory study into the complex intricate and multifaceted interactions between bioactive compounds specifically, phenolic compounds and polysaccharides (comprising dietary fibres and starches) in conjunction with digestive enzymes. An enduring paradox exists between the outcomes of *in-vitro* experimentation and clinical evidence regarding the consumption of nutritionally rich bioactive compounds. The principal motivation of this research is to bridge this gap by emphasising the critical importance of considering three-way interactions between phenolics, polysaccharides, and digestive enzymes, instead of focusing solely on pairwise relationships, as has been done in prior research.

The investigation spans five chapters, concluding in the sixth chapter. With there being a significant lack of literature in this field of study, this thesis explored multiple new ways to study three-way interactions between phenolics, polysaccharides, and digestive enzymes, by means of observing multiple potential consequences rather than focusing on any one very specific aspect (on fibre viscosity, on starch viscosity, on enzyme activity, on complexation, etc). These chapters collectively furnish a comprehensive understanding of how these bioactive components could potentially intermingle within the dynamic context of the human digestive system, spanning the entire spectrum from the preparation of dietary constituents to their eventual consumption and subsequent digestion. The findings emerging from this study are anticipated to serve as a stepping stone in the reconciliation of divergent *in-vitro* and *in-vivo* observations, further furnishing invaluable insights into the intricate web of dietary interactions, their impact on the digestion process, and the broader implications they hold for human nutrition.

Chapter 2 provides an in-depth analysis of the interactions between phenolic compounds and digestive enzymes, particularly α-amylases and starch, using tea phenolics as a representative model. The study focuses on understanding binding kinetics and the intricate mechanisms of phenolic amylase inhibition. The outcomes elucidate the distinct binding kinetics exhibited by various phenolic compounds, with structural dissimilarities emerging as a decisive factor in these interactions (phenolics with terminal galloyl moieties showing greater inhibitory capacity). Additionally, this chapter highlights the pivotal role of incubation times when considering the interactions between phenolics and digestive enzymes, as prolonged incubation periods are shown to result in heightened enzyme inhibition levels, an important observation to consider when dealing with less potent α-amylase inhibitors. Notably, the chapter underscores the introduction of a competitive dimension by the presence of starch, necessitating a nuanced approach to understanding these intricate dynamics.

Chapter 3 explores the interplay between potato starch, phenolic compounds from potatoes, and pancreatic lipase. The research delves into an alternative and simplified perspective to consider a static system in which the digestive enzyme doesn't act on one of the components (starches) that is both its substrate and a sequester for phenolic compounds. Lipase is a suitable example in this context, as it primarily works on lipids and not starches, thus starch would maintain its supramolecular structure throughout the assay acting as a dietary fibre. The study further uncovers distinct inhibitory strengths among various phenolic compounds derived from potato starch, shedding light on the influence of both structural differences and solubility. Moreover, the chapter highlights the influence of starch on phenolic inhibition of lipase activity, emphasising the importance of context-specific assessments for understanding these complex interactions.

Chapter 4 focuses on interactions between gallic acid and different starch types, including potato, maize, wheat, and rice, delving into their effects on α-amylase activity and starch digestion. The experimental design modulates the timing of gallic acid introduction, thereby promoting either the formation of starch-GA complexes or competition between starch and enzymes for phenolic binding. The findings indicate that physical effects, such as diffusion kinetics and entrapment, play a role in gallic acid's inhibitory capacity, underscoring the significance of the microenvironment.

The final research chapter, Chapter 5, provides insights into the interactions between phenolics, polysaccharides (including dietary fibres and starches), and digestive enzymes, using oat bran as a food model. The chapter explores the evolution of digestive viscosity development and reveals a significant reduction in viscosity during digestion in the presence of phenolics. This reduction is attributed to both the inhibition of digestive enzymes and the formation of aggregates between phenolics and β-glucans. Moreover, molecular weight disparities among phenolic compounds emerge as a pivotal determinant in shaping these outcomes, further underscoring their profound relevance in this intricate narrative.

In summary, this doctoral thesis enriches our understanding of the intricate interplay between phenolic compounds, polysaccharides, and digestive enzymes, emphasising the necessity of context-specific assessments, considering structural characteristics, incubation times, pH levels, and the immediate microenvironment. The work done here is an intentional step towards a broader, more integrative perspective that recognises the value of multi-way interactions. These findings offer a more comprehensive perspective on the dynamics governing nutritional outcomes in diverse dietary scenarios and underscore the significance of considering three-way

interactions to enhance the reliability and reproducibility of experimental results and help in bridging the gap between *in-vitro* experimentations and *in-vivo* observations.

PUBLICATION DETAILS

This is to acknowledge that the following original research articles published in or submitted to international peer reviewed journals and presented in scientific conferences were used in formulating this thesis. The publications are listed in ascending order of year of publication or submission.

Original Research Articles

D'Costa AS, Bordenave N. Inhibition of starch digestion by flavonoids: Role of flavonoid-amylase binding kinetics. *Food Chemistry*. **2021**; 341:128256.
doi:10.1016/j.foodchem.2020.128256

Northrop G, **D'Costa AS**, Tosh SM, Bordenave N. Viscosity development from oat bran β-glucans through *in vitro* digestion is lowered in the presence of phenolic compounds. *Food Funct*. Published online **2022**: 10.1039. D2FO00162D.
doi:10.1039/D2FO00162D

D'Costa AS, Golding BA, Raval M, Rolland-Sabaté A, Bordenave N. Probing gallic acid-starch interactions through Rapid ViscoAnalyzer in vitro digestion. *Food Research International*. Published online August **2023**:113409.
doi:10.1016/j.foodres.2023.113409

D'Costa AS, Bordenave N. Whole grain chemistry and nutrition from a health perspective: Understanding the fibre-phenolic-starch ménage à trois. *Trends in Food Science & Technology*. **2023**; 141:104196.
doi:10.1016/j.tifs.2023.104196

D'Costa AS, Chen AA, Hamann E, El Iraki R, Venugopal K, Bordenave N. Impact of potato starch on the inhibition of pancreatic lipase by potato phenolic acids. *Food Bioscience*. Published online November 30, **2023**:103414.

doi:10.1016/j.fbio.2023.103414

Conference Presentations

D'Costa AS, Bordenave N. (2020, November 10-12). *A new perspective on inhibition of α-amylase by flavonoids: Time matters* [Conference presentation]. 34th EFFoST International Conference 2020, Online Event.

D'Costa AS, Bordenave N. (2022, July 10-13). *Understanding the interactions between Starch and Phenolics* [Conference presentation]. IFT FIRST Convention 2022, Chicago, IL, United States.

D'Costa AS, Bordenave N. (2022, November 7-9). *Understanding the effects of Phenolic-Starch interactions on Phenolic acids inhibitory properties of α-amylase* [Conference presentation]. 36th EFFoST International Conference 2022, Dublin, Ireland. [1]

Original Research Articles/Published Abstract not included in this thesis

This is to acknowledge that the following original research articles published in or submitted to international peer reviewed journals and presented in scientific conferences were not used in formulating this thesis. The publications are listed in ascending order of year of publication or submission.

[1] Accepted for poster presentation, but not attended due to visa issues.

McMunn LE, **D'Costa AS**, Bordenave N, Ben RN. Probing interactions between small-molecule ice recrystallization inhibitors (IRIs) and water molecules using 1H NMR. *Cryobiology* [Published Abstract]. 2022; 109:15.
doi:10.1016/j.cryobiol.2022.11.049

McMunn LE, **D'Costa AS**, Bordenave N, Ben RN. Probing the Mechanism of Action of Small-Molecule Ice Recrystallization Inhibitors Using Proton Nuclear Magnetic Resonance Relaxation. *J Phys Chem Lett*. 2023;14(26):6043-6050.
doi:10.1021/acs.jpclett.3c00845

Débora Cerdá-Bernad, **Adrian S. D'Costa**, Nicolas Bordenave, María José Frutos. Unveiling the interaction between food thickeners and flavonoids on model beverages of saffron floral by-products model beverages. *Food Research International*. 2023 [Submitted]

DEDICATIONS

Dedicated to my younger self, who decided to go through this challenging life experience, my lovely wife, Caroline D'Costa, my ever-supportive parents, Anthony, and Cecilia D'Costa, my grandparents Agnelo and Carmelina de Souza, Marcelino and Cecilia† da Costa and my wonderful in-laws Douglas and Diane Ankenmann

And finally, to my dearest Snowbell

Chapter 1: Introduction.

Unveiling the significance of tripartite interactions between Fibres-Phenolics-Starches in food systems.

Chapter 1 : Unveiling the significance of tripartite interactions between Fibres-Phenolics-Starches in food systems.

1.1. Published contributions

Adapted from: D'Costa AS, Bordenave N. Whole grain chemistry and nutrition from a health perspective: Understanding the fibre-phenolic-starch ménage à trois. *Trends in Food Science & Technology*. Published online October 4, 2023:104196.
doi:10.1016/j.tifs.2023.104196

- A.S. D'Costa, with the supervision from N. Bordenave, conceptualised the study, collected data and literature and completed the writing up, with review and editing of subsequent versions. Author N. Bordenave obtained funding for this study.

1.2. Introduction

This chapter is primarily centred on gaining insights into the three-way interactions among fibre, phenolics, and starch within whole grain food systems. While these insights could apply to other food systems containing significant amounts of fibre, starch, and phenolics (e.g., potatoes), the choice of whole grain food systems offers an ideal framework for illustrating these intricate relationships.

The term whole grain refers to intact, ground, cracked, or flaked cereal grains that contain all of the naturally occurring components of the grain kernel, which includes the bran, germ and starchy endosperm [1]. The bran and germ are rich in bioactive nutrients, such as dietary fibres (mostly cellulose, arabinoxylans and β-glucans, but resistant starch as well), phenolic acids

(hydroxycinnamic and hydroxybenzoic acids) and polyphenolic compounds [2,3], as well as vitamins, minerals, and other minor phytochemicals, while the endosperm mainly contains starch, along with proteins as well as some vitamins and minerals [4]. Consumption of whole grains has been strongly associated with decrease in cardiovascular diseases, hypertension, stroke, metabolic syndrome, type-2 diabetes and specific types of cancer (such as colorectal, pancreatic, and gastric cancers) [4–6]. The beneficial effects of whole grains highlighted in the literature may be particularly attributed to the bioactivity of dietary fibres and phenolics, whose individual contributions and effects have been largely documented.

The effects of processing on the nutritional properties of whole grains and their individual components have also been documented and reviewed [7,8]. Nonetheless, the causal relationship between the effects of processing on the physico-chemical properties of the whole grains' components on the one hand, and the shift in nutritional properties on the other hand, can be difficult to establish. In this perspective, the individual roles of starch (its physical state, molecular weight, branching patterns, etc.), of dietary fibres (their composition, molecular weight, solubility, etc.), and of phenolic compounds (their structure, their reactivity, etc.) have been widely studied, as mentioned above. However, the role of the two-way or even three-way molecular interactions between these three components (starch, dietary fibres and phenolic compounds) ***(Figure 1-1)*** have received less attention, despite strong evidence of such interactions [9]. For example, a recent review article addressed the effect of phenolic-starch interactions on inhibition of α-amylase by phenolics [10] and another one addressed fibre-phenolic interactions [11]. Whereas both two-way interactions are critical to understand whole grain processing, chemistry and nutrition, there is evidence of another two-way interaction, namely starch-fibres. Additionally, as shown by the works of Giuberti *et al*. (2020) [10] and Quirós-Sauceda *et al*. (2014) [11], the two-way interactions have been addressed independently

thus far in the literature, whereas they may be integrated: there may be enough evidence to study fibres, starch and phenolics as a whole system governed by three-way interactions, which, to the best of our knowledge hasn't been done yet. Furthermore, the potential three-way interactions between whole grain components i.e., polysaccharides (starches and dietary fibres), phenolic compounds and digestive enzymes that could potentially occur *in-vitro* or *in-vivo* cannot be ignored [10,11].

Therefore, this chapter aims to address the molecular basis of these two-way interactions and their consequences on the respective physico-chemical properties and biological activity of the individual compounds, in whole grain products wherever possible, and on other types of foods where relevant. These two-way interactions are discussed in the perspective of potential three-way interactions and their consequences on the nutritional properties of whole grain products, highlighting knowledge gaps and future research directions.

Finally, it must be noted that this chapter focuses on the potential importance of three-way interactions between polysaccharides (fibres and starch), starch digestive enzymes (α-amylase) and phenolic compounds at the molecular level, not on the effect of processing on these interactions. However, it is understood that these interactions can only occur in whole grain that are going through or have undergone some transformation, as fibres, starch and phenolic compounds are segregated and have very limited interactions in native cereal grains (fibres constitute the cell walls, starch is confined in amyloplasts, and phenolic compounds are found in cell vacuoles). Therefore, although this chapter does not address explicitly and directly the effect of processing and processing conditions of whole grains, it aims to provide molecular-level information to understand the fate of fibre-starch-phenolics interactions through processing.

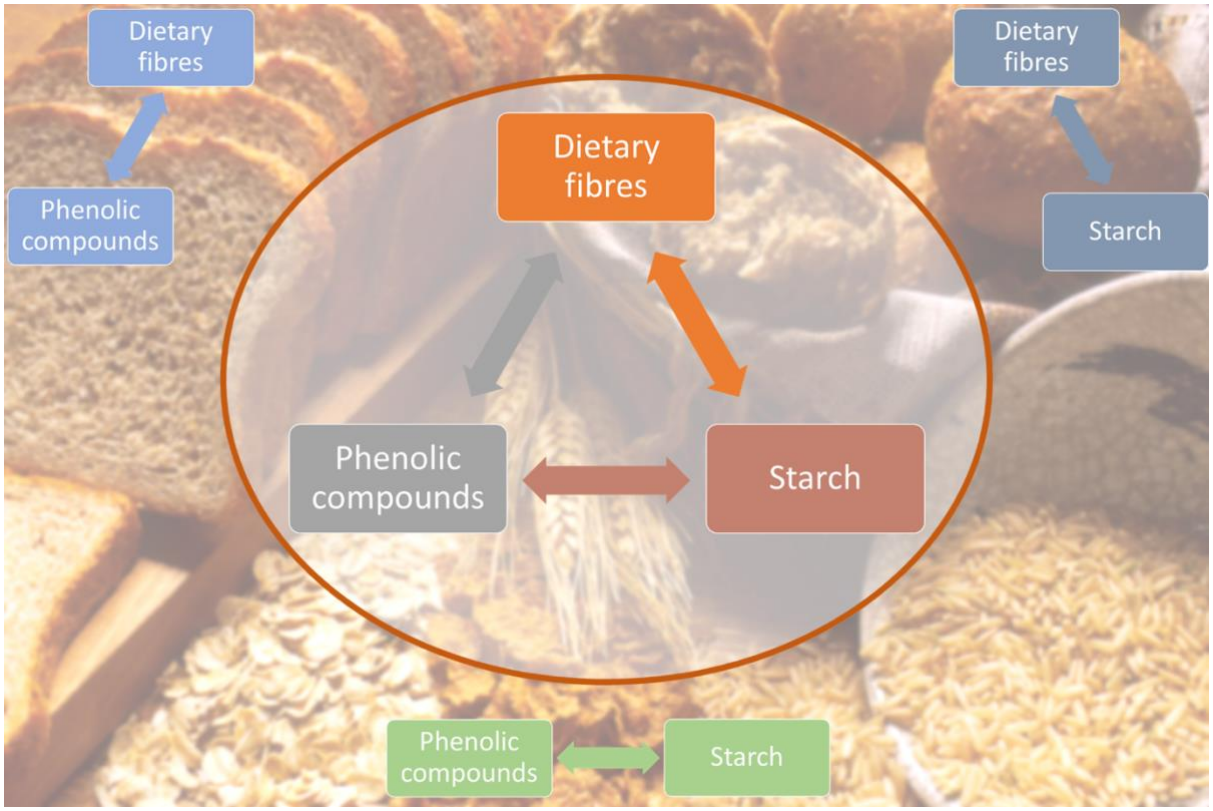

Figure 1-1: Figure of the potential three-way interactions between phenolics, starches and, fibres in whole grain foods. **License notice.** Original image: unknown photographer, National Cancer Institute, Bread and grains. Sketch addition by NB, CC0 1.0.

1.3. Effects of phenolics-fibre interactions on their properties and functionalities

1.3.1. Molecular interactions between phenolics and dietary fibre

Free phenolics compounds (non-covalently bound to other molecules) are known to interact with cell-wall components [12]. The interactions between phenolics and fibres are generally not specific, although they depend on the structure of both compounds ***(Figure 1-2)***. With neutral polysaccharides such as arabinoxylans, β-glucans and cellulose, phenolics may bind through hydrogen bonds involving their respective hydroxyl groups [13] and the glycosidic linkages of the polysaccharides [14], as well as Van der Waals interactions as both polysaccharides and

phenolics possess polarizable functional groups [15] ***(Figure 1-2)***. Hydrophobic interactions may also be at play, involving the phenolics' hydrophobic aromatic ring and hydrophobic regions of the polysaccharides. Finally, free phenolics can also bind with other phenolic groups covalently bound to the polysaccharides such as feruloyl groups on arabinoxylans [16]. Binding between free phenolics and polysaccharides often result from collaborative processes involving several binding modes.

A two-step approach has been proposed to explain this process. First, hydrogen bonds would occur between the hydroxyl groups of the phenolics and of the polysaccharides, bringing the phenolics closer to the polysaccharidic chain [17]. Second, aided by the proximity of the two interacting molecules, Van der Waals interactions would occur, which would further connect them [17,18]. This proximity could also potentially aid in the formation of CH-π bonds between phenolic aromatic rings and the sugar backbone of polysaccharides, while π-π stacking could occur between two phenol rings when the polysaccharide bears covalently bound phenolic groups [19]. These interactions would potentially result in the formation of non-covalent crosslinks between polysaccharides and phenolics, as seen with free ferulic acid and arabinoxylans from wheat [16].

The structure and the supramolecular spatial arrangement of both phenolics and polysaccharides groups significantly influence their intermolecular interactions. The presence of hydroxyl groups (three or fewer) on flavonoids favoured their adsorption onto β-glucans, but decreased the adsorption of those that contained four or more hydroxyl groups [20]. A similar result was obtained by Havlik *et al*. (2020) [21], where phenolic acids with two hydroxyl groups showed a higher affinity to wheat bran dietary fibres than with phenolics that had only one hydroxyl group [21]. Methylation and methoxylation of phenolics acids lowered their ability to

be adsorbed by oat β-glucans and wheat bran [20,21]. The esterification of gallic acid weakened its adsorption onto oat β-glucans. Finally, the position of hydroxyl groups on the phenolic compounds can also play a role in binding processes, as shown by the variable affinity of oat β-glucans with coumaric acids, this affinity being greater with o-coumaric acid than with p- and m-coumaric acids [20].

The interactions between phenolics and dietary fibres are heavily influenced by pH. Phenolic acids, being acidic in nature, can undergo structural changes depending on their local microenvironment. Previous studies have also demonstrated that phenolics stability is directly impacted by the pH, with lower pH values promoting greater stability [22]. Low pH, favouring stability of the phenolics and protonation of all hydroxyl groups, therefore favour these interactions in particular through strong hydrogen bonds [18,23]. This was supported by the observations of Li *et al.* (2019) [23] who found that under acidic conditions (pH 2 to 4), tannins were able to form aggregates that precipitated out of solution when complexed with β-glucans, whereas no such aggregation was observed as the pH was raised to 5 and 6, indicating weaker interactions between phenolics and dietary fibres [23]. Similarly, while not in whole grains, Liu *et al.* (2021) [24] found that fruit pectins associated and aggregated more with procyanidins at lower pH values (pH 2) than at higher values (pH 6), further showing the importance of protonation of the interacting molecules, pectins bearing a significant negative net charge at pH above 4 [24]. Nonetheless, potential degradation of phenolics due to their instability at higher pH should not be overlooked when explaining apparent weaker interactions in such conditions [15]. Considering the low pH conditions in the stomach, these observations are significant and might suggest that phenolics could interact with dietary fibres in the upper digestive tract. Although pH during simple heat and moisture treatments may not play a significant role in affecting the interactions between phenolics and fibres, pH of the microenvironment becomes

important when we examine whole grain formulated products. Furthermore, while formulated whole-grain products contain more phenolics than their equivalent made with refined flours, polysaccharidic additives such as pectins, hemicellulose, xanthan gum, and guar gum are commonly used as texture modifiers, thereby increasing the chances of polysaccharide-free phenolic interactions in these products.

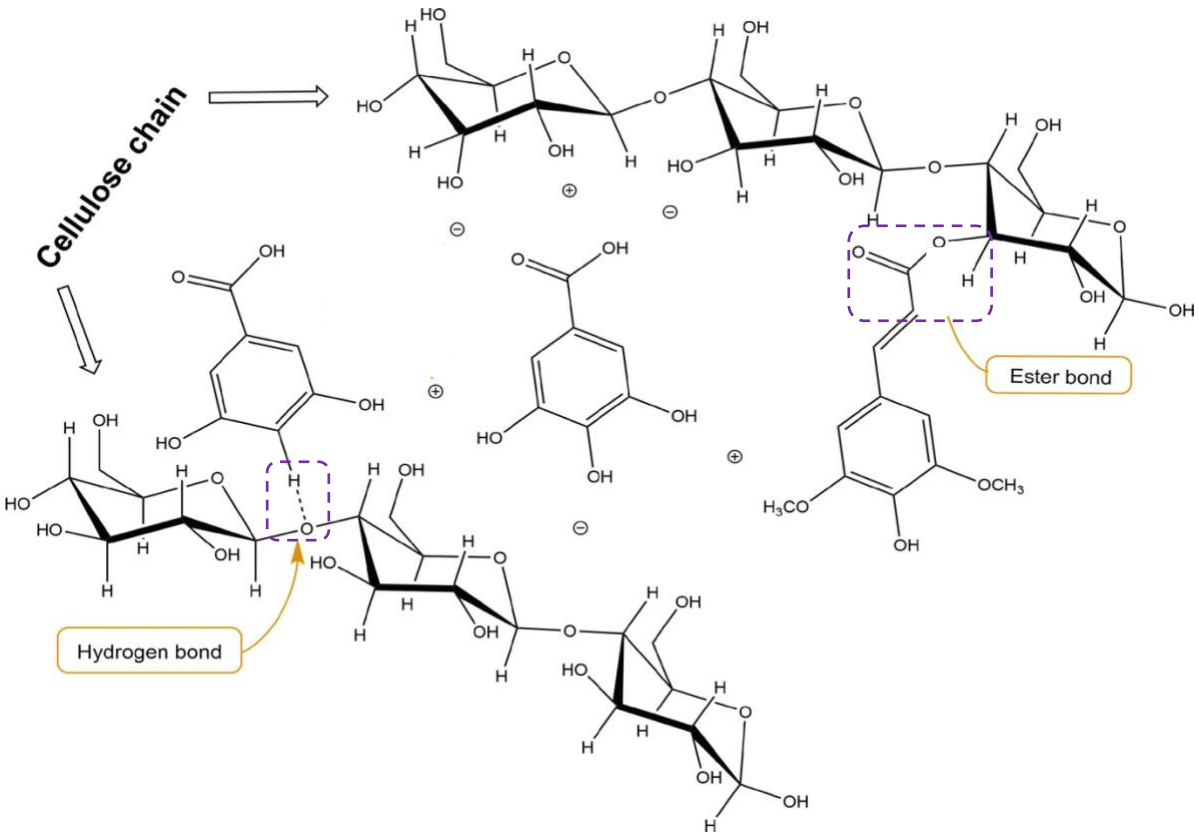

Figure 1-2: Representation of possible chemical interactions between phenolic compounds and dietary fibres (cellulose). Adapted from Guardiola-Márquez (2020) [25] with permission.

Similar to pH, the ionic strength of the local microenvironment can play a role in the interactions between phenolics and dietary fibres. In phenolics containing larger number of hydrophobic aromatic rings, the association between phenolics and dietary fibre could increase with increasing ionic strength [15], whereby the increased hydrophobic interactions between

phenolics and fibres would result in aggregation, ultimately resulting in decreased interactions with the ionic surroundings.

1.3.2. Consequences of phenolic-dietary fibres interactions

1.3.2.1. Effects on the physicochemical properties of dietary fibres

One of the essential nutritional functions of soluble dietary fibres is their ability to enhance satiety by increasing the viscosity of the food bolus. However, studies showed that the addition of phenolic compounds (caffeic acid, ferulic acid, vanillin, tea phenolics) to a solution containing various polysaccharides (guar, xanthan, β-glucans) was able to significantly decrease the viscosity and the pseudo-plastic behaviour of the solutions [26]. Furthermore, the addition of gallic acid significantly reduced the viscosity and significantly increase water mobility of solutions of high viscosity β-glucan [27]. In these β-glucan solutions containing gallic acid, NMR showed that water mobility was closer to that of free water in polysaccharide solutions, suggesting that polysaccharides and phenolics formed aggregates among themselves, thus limiting their interactions with one another, leading to a reduction in their overall viscosity of the system [23,26]. A similar observation was made by Silva-Escalante *et al*. (2015) [28], when the intrinsic viscosity of two varieties of maize differing by their ferulic content was measured [28]. The viscosity of arabinoxylans from vitreous maize containing higher ferulic content (4.72-5.10 μg/mg) was lower when compared with the arabinoxylans from floury maize having a lower ferulic acid content (0.69-0.94 μg/mg). This could potentially be due to the formation of cross links between ferulic acid and arabinoxylans, which was similarly observed by another study with wheat arabinoxylans and ferulic acid [16]. However, the contrary was observed in a study that demonstrated the formation of caffeic acid arabinoxylan esters where Li *et al.*, (2020) [29], found that the formation of caffeic acid-arabinoxylan esters led to an increase in the overall viscosity of the solution and that increasing the concentration of caffeic acid further increased

the viscosity [29]. A similar trend was observed when sinapic acid was esterified with corn bran arabinoxylans. The authors noted that the increased hydrophobic associations led to a higher viscosity and stronger gel behaviour [30]. These contradictions may be due to the effect of phenolic-polysaccharide interactions, leading to either aggregation of the polysaccharides and a reduction in viscosity, or to the formation of junction zones within the polysaccharide network and an increase in viscosity. Whereas there is no clear explanation for these opposite behaviours, it is possible that the effect of phenolic-polysaccharide interactions on polysaccharide's conformation could play a role in the outcome of the association: if the interaction leads to a compaction of the polysaccharides (for example through H-bonds involving distant monosaccharide units), this may lead to a reduction in viscosity; on the contrary, if the interactions occur without affecting on the conformation of the polysaccharides, they may be functionally equivalent to cross-linkages among dispersed polysaccharides, thereby leading to an increase in viscosity.

In these phenomena, concentration of both phenolics and polysaccharides may play an important role. At high phenolics concentrations, polysaccharide may be saturated with phenolics, thus preventing polysaccharide-polysaccharide crosslinking. Indeed, using Isothermal Titration Calorimetry, Tudorache *et al*. (2020) [27] found that β-glucans could host up to 18-23 molecules of gallic acid per 100 glucosyl units, which could be enough to hinder polysaccharide-polysaccharide interactions [27]. Furthermore, bound phenolic acids may bind with more phenolics through π-π stacking [19], potentially resulting in further steric hindrances. Similarly, Dridi and Bordenave (2021) [31] showed that polysaccharides concentration plays a major role in their interactions with phenolics [31]: interactions between various combinations of phenolics and xanthan, guar or β-glucans seemed maximal just around the critical overlap concentration of each polysaccharide, whereas the interactions were not detectable at lower

concentration (maybe due to polysaccharide chain being too far apart to interact) and at higher concentrations (maybe too entangled to engage in interactions with the phenolics).

1.3.2.2. Effects on the biochemical properties on phenolics

Phenolic compounds are known to inhibit the activity of digestive enzymes by binding onto their active site. Considering the binding affinity also existing between phenolics and fibres, there is potential competition between fibres and digestive enzymes to bind free phenolic compounds, which should in turn affect the ability of phenolics to inhibit the activity of the enzymes. This has indeed been observed with different fibre-phenolic systems. For example, the presence of citrus pectin, wheat arabinoxylan and oat β-glucan significantly increased the half inhibition concentration (IC_{50}) of tea polyphenols on porcine pancreatic α-amylase [32]. Similarly, the presence of gum arabic, dextran and citrus pectin increased the IC_{50} of banana condensed tannins onto pancreatic lipase [33], the inhibitory activity of procyanidins onto β-galactosidase was reduced by approximately 50 % by the presence of carboxymethylated β-glucans [34], and the rate of α-amylase inhibition by raspberry phenolics declined with increased concentration of cellulose [35]. Finally, although their study didn't address inhibition of enzyme activity, Gonçalves *et al*. (2011) [36] showed that polygalacturonic acid, gum arabic, pectin, and xanthan gum also competed with trypsin for binding with procyanidin B3 [36], and Brandão *et al*. (2020) [37] showed that grape skin pectic polysaccharides reduced the binding of grape tannins with salivary proteins [37]. Similar effects have been observed with another property of phenolics, namely their ability to inhibit glucose transporters in the small intestine. Indeed, in an orange juice system, the addition of orange pomace (mostly cellulose and pectins) decreased the ability of orange phenolics to inhibit glucose transport in Caco-2 cells [38].

This latter study also highlighted a paradoxical effect of fibres on phenolics. Indeed, it has been shown that plant cell wall polysaccharides bind, stabilise and protect phenolics from degradation in the upper gut [14,21,35,39,40]. To explain these observations, it was hypothesised that when phenolics form complexes with fibres, most of their peripheral hydroxyl groups are engaged in strong H-bonds that decrease the lability of their protons and therefore limit the occurrence of degradative reactions. Simultaneously, fibre-phenolic complexes help incorporate or maintain phenolics in the aqueous/micellarized phase of the digesta due to the hydrophilic nature of the fibres. These two effects led to make phenolics appear as more bio-accessible in presence of dietary fibres [41,42]. However, this bio-accessibility may only be apparent: despite being incorporated and stabilised in the aqueous/micellarized phase of the digesta, the phenolics are sequestered by the fibres and less available than in free form to inhibit digestive enzymes, inhibit glucose transporters, or be absorbed in the small intestine [14,21,35,38–40,43].

Although none of the studies described above address polysaccharide-phenolic systems that are directly relevant to whole grains, it seems reasonable to think that the same can happen in whole grain-based products, between phenolic acids and cellulose, arabinoxylans and other gums potentially used in product formulation. Nevertheless, although fibre-phenolic interactions may seem counterproductive as far as beneficial biochemical functions of phenolics are concerned, these associations may provide functional advantages in the lower gut. Indeed, several of the studies cited previously have found that fibre-phenolic complexation seems to stabilise the phenolics, to prevent their digestive degradation and to make them unavailable for intestinal absorption and for enzyme or transporter inhibition. Therefore, fibres may serve as carrier of free phenolics towards the lower gut and the gut microbiota. This has already been mentioned by Saura-Calixto as early as 2011 [44], although the focus was mainly

on covalently bound phenolics carried by the fibres, and on the antioxidant activity of the phenolics. The studies mentioned above therefore suggest a broader role of the non-covalent interactions between fibres and free phenolics as a carrier system towards the gut microbiota where both can be utilised as substrates. Indeed, the fermentation products of polysaccharides and phenolics themselves can play a major role in the proliferation of certain types of bacteria that are beneficial to human health, through increased production of SCFAs (short chain fatty acids) and changes in microbial enzyme activity, thus promoting the growth of beneficial bacterial species [21,40,45].

1.4. Effects of phenolics-starch interactions on their properties and functionalities

1.4.1. Molecular interactions between phenolics and starches

1.4.1.1. Characterisation of interactions

Notably, the interactions between starch and phenolic compounds are highly dependent on the supramolecular structure and organisation of starch, which can vary dramatically between its native and granular form, and its dispersed forms, either hydrated (pastes, gels, or retrograded gels) or dry and glassy (as found in extruded starchy foods or bread). Whole grains are often consumed in processed form, and thus would have undergone some degree of gelatinisation and dispersion. As such, the scope of this section will primarily focus on starch in non-native, non-granular form. In this case, non-covalent binding of phenolics with starches is very similar to that of phenolics with fibres, owing to the polysaccharidic nature of starch. They occur primarily through hydrogen bonding, hydrophobic interactions and electrostatic interactions [12]. As with other polysaccharides, starch-phenolic interactions often result from cooperative processes between different modes of binding. Beyond dominant hydrogen bonding, hydrophobic interactions can occur by the stacking of aromatic rings against starch's pyranose

rings via weak CH-**π** bonds [46]. Additionally, α-(1,4) glycosidic linkages give starch chains a helicoidal conformation creating a hydrophobic cavity that can host small molecules. Phenolics are able to be trapped within this cavity and interact with amylose thus forming V-type complexes driven by hydrophobic interactions [46–50] ***(Figure 1-3)***. The formation of such complexes was evidenced by the influence of starch's ratio of amylose to amylopectin which plays on phenolic-starch interactions: as V-complexes are formed primarily with amylose chains, increasing amylopectin to amylose ratios led to a decreased ability of starch and phenolics to form V-type inclusion complexes [51–55]. In parallel, while increasing number of hydroxyl groups on phenolics has been found to facilitate increased hydrogen bonding with starch, leading to the formation of non-covalent bonds stronger than the bonds between adjacent chains of starch [56], increasing number of hydroxyl group can also limit specific interactions such as the formation of inclusion V-complexes, due to steric hindrance and the limited size of amylose's inner cavity, and due to an increase in phenolics' polarity that is detrimental to hydrophobic interactions within the hydrophobic cavity. Indeed, while caffeic and ferulic acids were able to form such complexes, gallic acid (bearing one more hydroxyl group than ferulic and caffeic acid) could not [57]. This was confirmed by Diez-Sánchez *et al.* (2021) [13] who showed that that inclusion type complexes would only occur with lower molecular weight phenolics with limited steric hinderance effects to fit within amylose's cavity [13]. Nonetheless, proanthocyanidins of increasing molecular weight and phenolic acids containing long alkyl chains also yielded stronger hydrophobic interactions with starch [47,52,58].

Together, these observations highlight the importance of cooperative mechanisms of binding between phenolics and starch, especially with larger and more hydrophobic phenolics which may interact with starch through hydrophobic interactions but not necessarily happen *via* the formation of V-type inclusion complexes. Only one study seemed to differ with these

widespread observations: in a 2020 study, Han *et al.*, found that increasing concentrations of amylose led to a reduction in the complex formation between caffeic acid and maize starch [59]. The authors suggested that caffeic acid may be too hydrophilic to form inclusion complexes with amylose. It is also possible that the protocols used to assess starch-caffeic complexation may have biased the experimental results in favour of higher amylopectin content as far as complexation was concerned: starch-caffeic acid complexes were formed in hot water, precipitated and dried; they were then redispersed in boiling water at a concentration of about 10 % w/w before recovery, cooling and measurement of iodine binding capacity, the difference in iodine binding capacity between starch-caffeic complexes and starch alone giving a starch-caffeic acid complexation index. However, with high amylose starch at a concentration as high as 10 % w/w starch in water, the redispersion and cooling of the complexes may have favoured the reassociation of amylose chains at the expense of inclusion amylose-caffeic acid V-complexes, which is unlikely with high-amylopectin starch and likelier with increasing ratios of amylose to amylopectin. Consequently, the protocol may have underestimated the degree of complexation of amylose with caffeic acid, thereby leading to results opposite to all other reported observations.

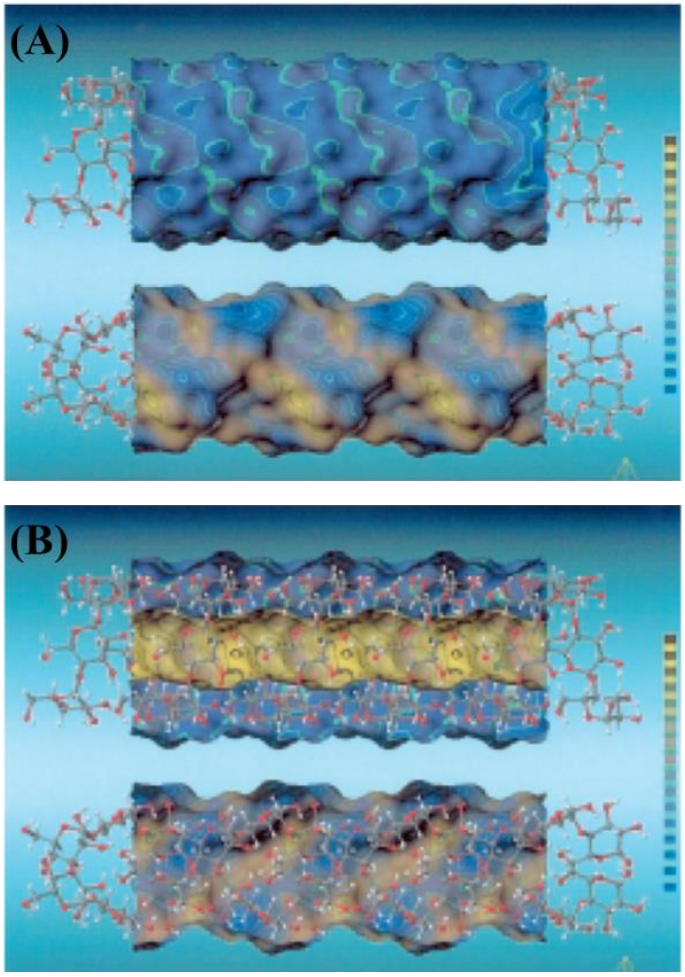

Figure 1-3: Hydrophilic/hydrophobic topography of internal (A) and external (B) surfaces of amylose (blue: hydrophilic regions, yellow: hydrophobic regions) of the single-stranded V-amylose helix (upper structures) and the parallel-stranded double-helical A-form (lower structure). The outside surface area of V-amylose is uniformly hydrophilic, whereas the centre cavity is as distinctly hydrophobic. By contrast, the double-helical A-form of amylose, devoid of a centre channel, exhibits an irregular distribution of hydrophilic and hydrophobic regions over the entire external surface. Adapted from Immel and Lichtenthaler (2000) [60] with permission.

1.4.2. Consequences of phenolic-starch interactions

1.4.2.1. Effects on the physical properties of starch

Starch's physical properties are essentially driven by the interaction of amylose and amylopectin chains among themselves and with water. Engaging starch in complexation with phenolics is therefore very likely to affect these interactions and the physical properties of starch, such as gelatinisation, pasting and retrogradation. Throughout gelatinisation followed by Rapid Visco-Analyzer, gallic acid was found to reduce starch's peak viscosity [61,62] ***(Figure 1-4)***. Following gelatinisation and breakdown, gallic acid and tannins were found to reduce the hot paste viscosity of wheat starch [63] and of rice starch [62]. Several hypotheses have been proposed to explain these observations. Some authors suggested that the interaction of phenolics with water led to restricted water mobility and water availability to complete gelatinisation, and possibly led to decreased peak viscosity [51,64]. Whereas this phenomenon could indeed contribute to altered gelatinisation, limited water availability would also have resulted in an increase in gelatinisation temperature, which was not observed.

An alternate hypothesis suggested that gallic acid may have disrupted hydrogen bonds among amylopectin and amylose chains within the granule and during the gelatinisation process, thereby leading to its premature breakdown [62,65]. However, it must be noted that in most of these studies pH was not controlled and the addition of phenolic acids led to significant decreases of pH, by 2.5 to 4.1 below the pH of starch alone [51,54]. It is very likely that these variations of pH affected gelatinisation and pasting properties of starch as well as it solubility [51,66], a well-known effect of pH on starch pasting properties [67].

Similarly, the potential hydrogen bonding between the hydroxyl groups of phenolics and starch could disrupt starch-starch interactions involved in gel formation and retrogradation, after pasting [65]. For instance, oligomeric procyanidins and phenolic acids were able to limit retrogradation of starch, the effect depending on amylose content of starch [51,59,68]. The reduction in setback values was attributed to the effect of phenolic-amylose complexation limiting the number of linear glucan chains available for engaging in the formation of junction zones in the gels [51,63,64,66]. While the effect of phenolics on starch retrogradation depended on the types and concentrations of starch and phenolics used, it was found that sufficiently high concentrations of phenolics were able to completely inhibit starch retrogradation [54,69–71].

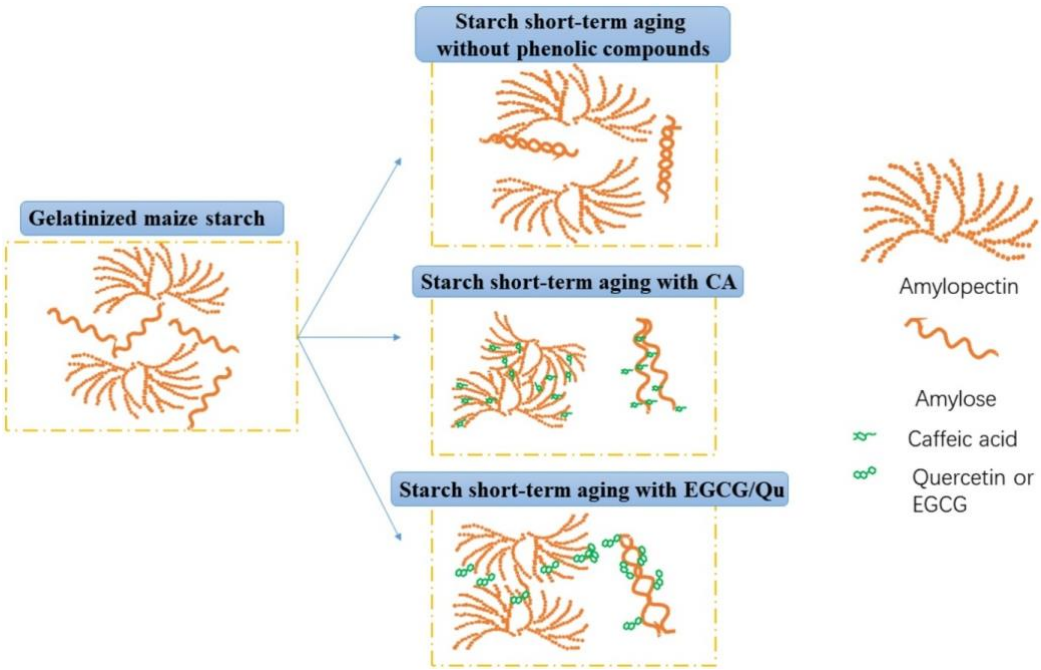

Figure 1-4: Representation of the interference of phenolic compounds namely, caffeic acid (CA), quercetin (Qu) and epigallocatechin gallate (EGCG) with starch retrogradation. Reproduced from B. Liu *et al*. (2020) [61] with permission.

1.4.2.2. Effects on the biochemical properties of phenolics

As seen in section ***1.3.2.2***, phenolic compounds are known to be inhibitors of most digestive enzymes, mainly through binding to the active site of the enzymes, and the effect of phenolic compounds on starch hydrolysis by α-amylase has been reviewed extensively before [72]. Nevertheless, some studies addressed this phenomenon with specific consideration for the effect of starch-phenolic interactions on inhibition of α-amylase by phenolics. For example, Rapidly Digestible Starch (RDS) was converted to Slowly Digestible Starch (SDS) and Resistant Starch (RS) specifically by complexation with ferulic acid [73] or with caffeic acid [59]. In this latter study, RS content increased significantly in normal and waxy maize starches and only slightly in high amylose starch, in presence of caffeic acid, thereby highlighting the role of starch-caffeic acid binding. However, we have seen in section ***1.4.1.1***, that phenolic acids tend to bind more readily with amylose. These results may suggest that the formation of inclusion V-complexes between phenolic acids and amylose may not be a significant factor of resistance to digestion for starch, although such V-complexes are often considered as RS [74]. This is potentially in contradiction with several studies showing that inclusion V-complexes between amylose and proanthocyanidins [75], anthocyanins [76] flavonoids or phenolic acids [77] behave as resistant starch. Whether starch-phenolic inclusion V-complexes are a source of RS is indeed a contemporary and open scientific question [78]. Phenolic compounds may also be able to affect starch digestion through physical effects, for example by decreasing starch's swelling capacity, thus making it resistant to enzymatic hydrolysis [51,55,64,66,79]. Furthermore, since α-amylase activity requires accessibility of starch chains, the formation of starch-phenolic aggregates can slow down starch digestion [52,55,68,75,80–85]. However, this is potentially contradictory with the capacity of phenolics to limit starch retrogradation reported in the previous section, which would limit starch's potential resistance to digestion. Finally, starch, as a polysaccharide, has the potential to bind phenolics similarly to dietary fibres (section

1.3.2.2). Therefore, starch can compete with enzymes for binding with phenolics, leading to potential alteration of phenolics' inhibitory capacity on enzymes. This would also be the case with proteases and lipases, although this hypothesis has never been tested in published work. The case of α-amylase is potentially more complex and more interesting, though. In addition to the effects reported above (formation of inclusion complexes, interference with swelling or retrogradation), evidence of competition between starch and α-amylase to bind phenolics have been reported [86,87]. This competition could potentially decrease phenolics' availability towards α-amylase inhibition ***(Figure 1-5)***. Two particular studies can illustrate this potential effect. Li *et al*., (2020) [73] reported that the rate of starch digestion was follows: starch > starch-phenolic complex > starch-phenolic mixture, showing the effect of potential phenolic sequestration by starch. Chi *et al*., (2019) [62] showed that gallic acid decreased the rate of rice starch digestion through two mechanisms: 1- by increasing starch's molecular order at concentrations of 4.5 and 11.2 mg gallic acid per gram of starch, and 2- by an additional effect of α-amylase inhibition at 13.2 mg gallic acid per gram of starch. Together, these observations may support the idea that phenolics complexed with starch are generally less available for α-amylase inhibition and that α-amylase inhibition may be observed only by increasing phenolic concentration, potentially above starch binding capacity, so that enough free phenolics can engage in inhibiting binding with α-amylase. This would mean that starch itself could paradoxically decrease the capacity of phenolics to inhibit its digestion. Therefore, we may hypothesise here that any measurement of phenolics' inhibitory capacity on α-amylase may be biased by such competitive binding. This could shed a new light on phenolics' capacity to alter starch digestion. Indeed, each type of starch have different binding capacity with phenolics, depending on its fine structure, molecular weight, branching pattern, chain length distribution, amylose to amylopectin ratio, etc. Again, to the best of our knowledge, this hypothesis has never been tested in published work, and filling this knowledge gap may be crucial to

understand more deeply the role of phenolic compounds on starch digestion, glycaemia, and metabolic health.

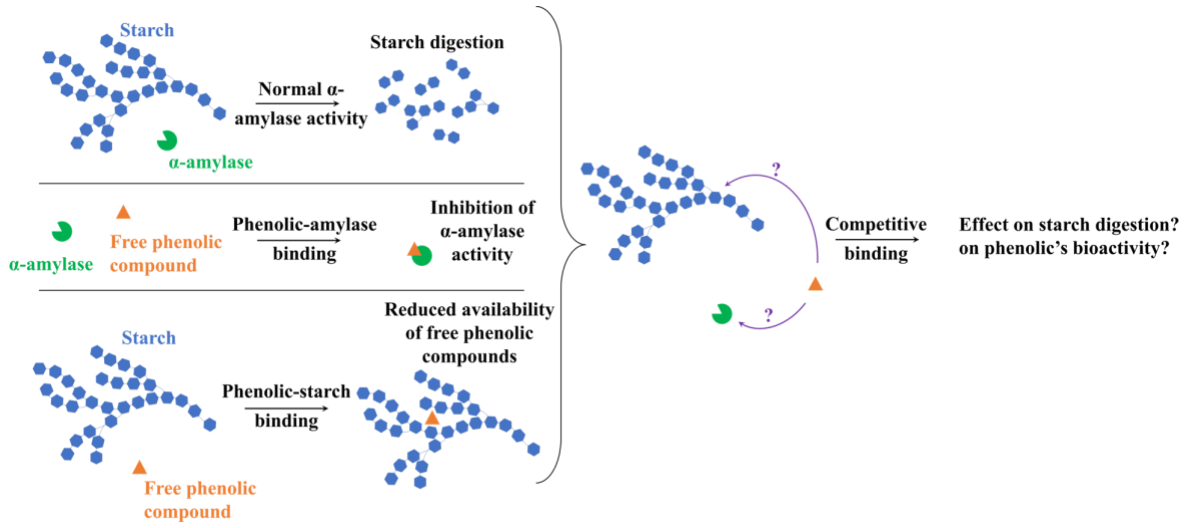

Figure 1-5: Schematic representation of potential competition between starch and digestive enzymes for phenolic compounds.

In parallel to starch limiting phenolics' availability for enzyme inhibition, starch may also affect phenolics' bio-accessibility and bioavailability, similar to what is observed with dietary fibres and phenolics. Indeed, Li *et al.* (2020) [73] showed that starch improved caffeic acid's bio-accessibility, likely because caffeic acid complexed with starch was protected from oxidation and other degradative processes and released progressively upon starch hydrolysis during digestion [73]. Similarly, starch has been shown to prevent oxidative degradations of green tea catechins [49] and of trans-resveratrol [50] through UV and heat treatments. However, due to changes that starch undergoes through digestion while dietary fibres remain intact, it is possible that improved bioaccessibility does not translate into improved bioavailability. This does not

necessarily decrease phenolics' bioaccessibility and bioavailability: most observations on the effect of starch on phenolics' bioavailability are notably inconsistent.

Finally, in cases where phenolics decreased the rate of starch digestibility and increase starch's fraction that is resistant to digestion, starch-phenolic interactions logically favoured the transfer of both phenolics and starch to the colon where they could be utilised by the gut microbiota, thereby shifting fermentation patterns and production of short chain fatty acids [88,89].

1.5. Effects of starch-fibre interactions on their properties and nutritional functionality

1.5.1. Molecular interactions between dietary fibre and starch

The molecular interactions between only starch and hydrocolloids, and the effect of these interactions on the physical properties of starch-based pastes and gels have been reviewed thoroughly before [90]. Here, we focus primarily on arabinoxylans, and β-glucans, which are relevant to whole grain products, as well as pectins, which are relevant to whole grain products formulated with fruits. As mentioned by the BeMiller, (2011) [90], the effects of these hydrocolloids on the physical properties of starch were inconsistent, probably due to the diversity of starches and hydrocolloids (even within hydrocolloid families) used on the studies. Nevertheless, a few general consistent observations could be gathered from the literature. Arabinoxylans had little to no effect on gelatinisation and pasting of starch but favoured its retrogradation. β-glucans increased the viscosity of starch pastes and tended to reduce its retrogradation. Finally, pectins displayed mixed results, with no effect on gelatinisation, no effect or a slight decrease in paste viscosity, and possible synergistic effects on gelling of starch. However, these general results must be taken with caution due to the variability of results induced by the diversity of starches and hydrocolloids as mentioned before, and due to

the variability of environmental conditions during possible product applications (pH, ionic strength, etc.).

1.5.2. Consequences of dietary fibres-starch interactions

In whole grain products, it has been shown in some instances that non-starch polysaccharides could compete with starch molecules for water, thus partially inhibiting starch gelatinisation and slowing down its hydrolysis by α-amylase [91–95]. Soluble viscous dietary fibres can also increase the viscosity of the medium, thereby decreasing the diffusion of α-amylase towards the starch substrate and the diffusion of α-limit dextrin products towards the gut lumen, with the effect of reducing the glycaemic response to food, whether fibres are added or native to the whole grain products, as it is well-known with oat β-glucans [96]. Finally, one study mentioned that the presence of glucose units within the structure of rice soluble dietary fibres could induce binding of α-amylase to the fibres and consequently, reduce the activity of α-amylase onto starch through competitive binding or through partial unfolding and denaturation of the polypeptide chain of the enzyme [97–99]. In any case, apart from the case of β-glucans which are well known to increase viscosity of cereal products and contribute to their nutritional properties (increased satiety, reduced and extended glycaemic response) [96], it seems that hydrocolloids such as arabinoxylans and pectins may have little effect on the nutritional characteristics of starch itself in food products.

1.6. Perspectives on phenolic-starch-fibre interactions on their respective properties and current gaps in literature

Altogether, the three previous sections strongly suggest that the respective properties and bioactivities of dietary fibres, phenolics and starch are interdependent in whole grain products and formulated whole grain products. Therefore, it seems reasonable to hypothesise that the

nutritional properties of such products are in great part the result of the three-way interactions between dietary fibres, phenolics and starch.

1.6.1. Research gaps and project novelty

All studies reported in the previous sections have focused on some variation of a pairwise relationship between these three components. Many other published studies are observational evaluations of the combined effect of dietary fibres and phenolic compounds on the nutritional properties of whole grain products, very often through the rate of starch digestion or colonic fermentation. However, to the best of our knowledge, no published study has attempted to unravel this three-way relationship.

At the centre of these aforementioned interactions is the notion of competitive binding. For example, fibre-phenolic and starch-phenolic binding compete with phenolic-enzyme binding, which is critical to phenolics' enzymatic inhibitory activity. In return, phenolic-enzyme binding and starch-phenolic binding compete with fibre-phenolic binding, the latter having the potential to lead to aggregation and loss of viscosity of the fibres. Similarly, starch-phenolic binding equilibrium, which can alter starch's physical properties and digestibility, could be shifted by competition with fibre-phenolic binding. The lack of understanding of these three-way interactions may therefore represent a considerable gap in the understanding of the nutritional properties of whole grain products, although from an epistemological standpoint, it seems logical to address three-way interactions after all the pairwise interactions are thoroughly understood. Chapters 2-5 aim to address this gap by focusing on understanding these three-way relationships from different perspectives (i.e., their impact on α-amylase activity and starch digestion kinetics and thermodynamic perspectives, their impact on viscosity build-up from fibres, and their impact on lipase activity).

Nonetheless, it must be acknowledged that compositional and structural diversity of starch, fibres, and phenolic compounds that make the study of pairwise interactions difficult, may render the task of studying their three-way interactions even more difficult. Indeed, starch's fine structure in particular is highly variable not only across cereal species and varietals, but also according to the plant's growth conditions. Similarly, of all the phenolics used in the studies reported in the sections above, caffeic, ferulic and gallic were the most common. While gallic acid can be and has been used as a representative molecule for all lower molecular weight phenolic acids, this literature review has showed that even related compound with minor structural differences and spatial arrangements have drastically different relationships with other bioactive molecules. The same could apply to whole grains' dietary fibres (especially the most complex one such as arabinoxylans and pectins) and gums added in formulated products such as xanthan and guar. Additionally, it is notable that very few studies on this topic have taken into account cellulose, which is a significant fraction of dietary fibres present in whole grains [6]. Nonetheless, using these representative molecules that have been widely studied in literature would help in providing a backbone to deciphering the three-way interactions observed, by allowing us to develop better models to study the consequence of their interactions as shown in Chapters 2-5.

It is also notable that many studies use concentrations of phenolic acids that far exceed the amounts one would obtain from normal foods, as such there may be excessive free phenolics present (which would alter normal phenolic behaviour of competing between polysaccharides and digestive enzymes), this increased amount of free phenolics would allow for phenolics to bind with digestive enzyme rather than just interact with starch molecules. This in turn makes the comparison of phenolic-starch/phenolic-fibre interactions with digestive enzymes

convoluted and improbable. The subsequent chapters make use of phenolic, starch, and fibre concentration commonly found in normal consumption of food to address this issue.

In light of the likelihood of three-way interactions between phenolic compounds, starch and dietary fibres; or polysaccharides, phenolics and enzymes, especially in whole grain products and formulated products, it seems critical to deepen the understanding of these interactions in order to better understand the nutritional properties of food groups that are often "encouraged" in public health policies.

1.7. Research Questions

Based on the research gaps highlighted in section ***1.6***, the following research questions helped shape the main idea of this study:

- Would the presence of polysaccharides (starch/fibres) affect digestive enzyme inhibition by phenolics? How would the molecular structure of phenolics affect phenolic-enzyme/phenolic-polysaccharide interactions?
- Given that interactions involving phenolics-polysaccharides, polysaccharides-enzymes, and phenolics-enzymes are influenced not only by their thermodynamic characteristics but also by their kinetics, does the duration of incubation between phenolics and enzymes/polysaccharides matter? Is it possible for milder inhibitors (phenolics) to enhance their inhibitory capacity as the incubation time with digestive enzymes increases?
- Would polysaccharides affect phenolic enzyme interaction if the microenvironment was changed from a hydrophilic one to a hydrophobic one? Does the lipophilicity or hydrophilicity of phenolic compounds influence the choice of their interaction with polysaccharides or enzymes?

- Would promoting phenolic-polysaccharide interaction over phenolic-enzyme interaction permanently sequester phenolics out of solution? Would the now bound phenolics be released upon some hydrolysis of starch?
- How would we promote phenolic starch interactions throughout digestion without the structure of starch being altered through digestion?
- Does the physical state of starch matter? How does uncooked (granular) vs cooked (gelatinised) starch affect starch-enzyme, phenolic-enzyme, and phenolic-starch interactions? How would the molecular structure of starch affect starch-enzyme and starch-phenolic interactions?
- Would changes in viscosity due to cooking of starch/fibres or increase in the concentration of starch/fibres affect diffusion of phenolics and digestive enzymes? Would changes in diffusion of enzymes and phenolics affect accessibility of starch binding sites?

1.8. Aim

The aim of this thesis is to understand the importance of three-way interactions between health beneficial bioactive compounds namely, phenolics, polysaccharides (starch and fibres), and enzymes.

1.8.1. Specific objectives

The specific objectives would make use of different food models/viewpoints to study the three-way interactions between phenolics-polysaccharides-enzymes, the specific objectives are:

- To understand the binding kinetics between phenolics and digestive enzymes in the presence and absence of starch and the impact of time of incubation between phenolics and enzymes on the inhibitory capacity of phenolics.
- To elucidate the interactions between phenolics and starch, as well as phenolics and enzymes, both in the presence and absence of starch within a lipophilic food model, and to examine how the structure, lipophilicity, or hydrophilicity of phenolic compounds may influence their binding with starch and digestive enzymes.
- To investigate the impact of starch structure, source, and concentration variations on interactions involving phenolic compounds, starch, and enzymes, and to assess how the physicochemical properties of starch may influence the binding kinetics between phenolics and enzymes, as well as affect starch digestion.
- To investigate the interplay between phenolic compounds, starch, fibre, and enzymes within a practical food system, with a focus on examining the impact of varying phenolic structures and concentrations, as well as the cooking of starch and fibre.

1.9. Significance of the study

Previous research has predominantly focused on the examination of two-way interactions involving starch, fibres, phenolics; or polysaccharides, phenolics, and digestive enzymes as isolated entities. However, these two-way interactions fall short in elucidating actual physiological effects of foods like fruits, vegetables, and whole grains. To bridge the gap between chemistry and nutrition there is a pressing need to transition to a three-way interaction framework, a domain that has yet to be fully explored. This thesis tackles multiple aspects of this domain by taking a first step in addressing this significant knowledge gap. It further demonstrates the importance of integrating these two-way interactions into a unified system within the context of specific food compositions, thereby revealing their three-way

interdependencies. In essence, the focus on three-way interactions proves to be more meaningful than the exploration of three separate two-way interactions. The study's diverse approach offers valuable insights in the complex relationships between phenolics, polysaccharides, and digestive enzymes across diverse food models and viewpoints, thus contributing significantly to the fields of food science and nutrition.

This research further underscores the critical role of incubation time when studying the interplay between phenolics and enzymes. It reveals that phenolic compounds with rapid binding kinetics, such as ECG and EGCG, achieve maximum α-amylase inhibition with brief incubation periods, while those with slower binding kinetics, like EC and EGC, necessitate longer incubation times. This finding highlights the profound impact of binding kinetics on enzyme inhibition effectiveness. Moreover, the thesis highlights the importance of understanding potential three-way interactions between starch, enzymes and phenolics, by demonstrating starch potentially interfering with phenolic-enzyme binding and inhibition process. This observation accentuates the intricate nature of enzymatic reactions and underscores the importance of considering potential substrate-inhibitor interactions in enzyme inhibition studies.

Furthermore, the research delves into an alternative and simplified perspective to consider a static system in which the digestive enzyme doesn't act on a substance (starches) that is both its substrate and a sequester for phenolic compounds. Lipase is a suitable example in this context, as it primarily works on lipids and not starches, thus starch would maintain its supramolecular structure throughout the assay acting as a dietary fibre. In this scenario, we investigate how phenolics impact lipase activity and introduce starch to observe whether starch captures the phenolics, subsequently affecting the inhibitory capabilities of phenolics on lipase,

conducted under physiologically relevant conditions. Thus, also addressing a notable gap in the existing literature. Utilising molecular docking analysis, it establishes a strong correlation between binding energy and solubility of phenolic acids, shedding light on the inhibition data obtained at pH 6.9. The study also unravels the complexation of phenolics with starch, revealing its potential to influence the capacity of phenolic acids to modulate pancreatic lipase. This dimension of the study further suggests a plausible mechanism by which the food matrix can modulate the bioavailability and efficacy of phenolic acids in inhibiting digestive enzymes.

By employing representative phenolic molecules, such as gallic acid, this thesis demonstrates how varying starch concentrations can alter phenolic-starch and phenolic-enzyme interactions. These alterations manifest through binding mechanisms involving hydrogen bonds and hydrophobic interactions, entrapment phenomena, and modifications in enzyme and phenolic diffusion within the food medium. Furthermore, by examining different sources of starch, the research explores the influence of starch chain length distribution on phenolic-starch interactions. The study further elucidates the influence of the timing of phenolic compound introduction on starch digestion kinetics (by means of promoting starch-phenolic interactions), carrying implications for food processing and preparation.

Lastly, this thesis uncovers the potential for a four-way interaction involving dietary fibre, starches, phenolics, and enzymes using a real simulated food model. It investigates how the presence of phenolic compounds affects the viscosity and water binding capacity of oat bran in both uncooked and cooked forms, properties crucial for the functionality and health benefits of dietary fibres in the digestive system. The conclusion drawn is that the health benefits associated with oats, particularly related to the digestive viscosity of β-glucans, may undergo alterations when co-formulated or co-ingested with phenolic compounds. This underscores the

dynamic interplay between phenolic compounds, starches, dietary fibres, and digestive enzymes, ultimately shaping the physiological effects of these compounds in the human body. Consequently, this research emphasises the significance of comprehending the intricate three-way interactions between different food components.

1.10. Thesis layout

The chapters of this thesis addressed the various research objectives comprehensively as follows:

In **Chapter two** of this doctoral thesis, the first objective was tackled by delving into the intricate triadic interactions involving starch, α-amylase and phenolics from tea. Additionally, this chapter fills a notable void in the existing body of knowledge by elucidating the significance of incubation duration and its ensuing effects on the binding kinetics between phenolics and digestive enzymes.

Chapter three addressed the second objective by studying the three-way relationship between phenolics, lipase and starch in a lipophilic food model. Furthermore, this chapter highlights the significance of comprehending these interactions under physiologically relevant conditions, along with the utilisation of phenolic types and concentrations that are pertinent to each specific food model.

In **Chapter four**, the third objective was addressed by introducing variations in the starch sources, including wheat, rice, maize, and potato, along with changes in starch concentration. This investigation aimed to shed light on how alterations in the micro and macro structure of starch and its physicochemical properties could impact the intricate triadic interplay involving

phenolic compounds, starches, and enzymes. Additionally, the chapter delved into the comprehension of phenolic-polysaccharide and phenolic-enzyme interactions by promoting either phenolic-starch or phenolic-enzyme interactions through the modification of starch cooking profiles.

In **Chapter five**, the fourth objective was tackled by gaining insight into the intricate three-way and four-way interactions involving various food components such as phenolics, starches, fibres, and enzymes within a real (whole grain) food system. This study provided further evidence of how diverse cooking profiles and the formulation of foods with varying concentrations of phenolic compounds can lead to significant modifications in the physicochemical and biological properties of food systems.

Chapter six of this doctoral thesis serves as the concluding chapter, providing a comprehensive summary of the major and minor findings derived from this research. Additionally, it offers valuable insights into potential avenues for further investigations in the field.

1.11. Acknowledgement

This work was funded by the Natural Science and Engineering Research Council of Canada – Discovery Grant #2018-04009. Author NB is a member of the Scientific Advisory Board of Agri fibre LLC (Mundelein, IL).

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Chapter 2: Investigating the interplay of Phenolics, Starches, and Enzymes: Kinetics of α-amylase binding and inhibition by green tea phenolics.

Chapter 2 : Investigating the interplay of Phenolics, Starches and Enzymes: Kinetics of α-amylase binding and inhibition by green tea phenolics.

2.1. Published contributions

D'Costa AS, Bordenave N. Inhibition of starch digestion by flavonoids: Role of flavonoid-amylase binding kinetics. *Food Chemistry*. 2021; 341:128256.

doi:10.1016/j.foodchem.2020.128256

- A.S. D'Costa, with the supervision from N. Bordenave, conceptualised the study, collected data, literature, conducted calculations and statistical analysis, and completed the writing up, with review and editing of subsequent versions. Author NB obtained funding for this study.

2.2. Abstract

In this study, kinetics of binding between α-amylase and green tea flavonoids were investigated by fluorescence quenching (FQ). Their effect on α-amylase inhibition was evaluated. Whereas epicatechin (EC) and epigallocatechin (EGC) exhibited slow binding kinetics (in the order of minutes), epicatechin gallate (ECG) and epigallocatechin gallate (ECGC) exhibited very rapid binding (in the order of seconds) with Human Salivary α-amylase (HSA) and Porcine Pancreatic α-amylase (PPA). EGCG reached maximum inhibition of HSA and PPA with short incubation time whereas maximum inhibition of HSA and PPA by EC was reached only after 45 to 60 min of incubation. Similar results with ECG and EGC, but not in line with FQ kinetics,

highlighted possible interferences of starch-flavonoid interaction in the binding and inhibition process. These results suggest that incubation times of enzymes and flavonoids shall be evaluated prior to enzyme inhibition testing in order to ensure consistent and reliable results.

2.3. Keywords

α-amylase, Tea polyphenols, Fluorescence quenching, α-amylase inhibition assay

2.4. Introduction

This paper reports the impact of flavonoid/α-amylase binding kinetics on the ability of flavonoids to inhibit the activity of the starch-digestive enzyme α-amylase.

The anti-hypercholesterolemic effect of flavonoids and in particular green tea flavonoids, have been demonstrated multiple times clinically and epidemiologically [1]. At the centre of the mechanisms involved in this effect is the flavonoid's ability to inhibit enzymes and brush-border transporters responsible for digestion and absorption of carbohydrates [2,3]. These enzymes include salivary and pancreatic α-amylases, as well as α-glucosidases (composed of sucrase-isomaltase and maltase-glucoamylase). Starch being the main source of energy in typical Western carbohydrate-rich diets [4], the salivary and pancreatic α-amylases play a key role in this process as they are responsible for the hydrolysis of starch into α-limit dextrins from the mouth to the small intestine. Therefore, the ability of flavonoids to inhibit α-amylases has been the subject of extensive research. This ability has been linked to the capacity of flavonoids to bind with α-amylases onto their active site *via* H-bonds between the peripheral hydroxyl groups of the flavonoids and the catalytic residues of the enzyme and collaborative conjugated π-stacking [5,6]. These insights were supported by demonstrations of the inhibitory capacity of

flavonoids *in vitro*, involving a comparison between flavonoids through their IC_{50}, the half maximal inhibitory concentration of flavonoids towards α-amylases. Multiple methods have been developed to perform such assays and they all essentially measure α-amylase activity through the determination of maltose concentration after incubation of the α-amylase, with or without inhibitor, with gelatinized amylopectin for 10 min at 37 °C. The most common method to determine maltose concentration is a colorimetric method using 3,5-dinitrosalicylic acid (DNS) [7]. Although this method has been found to be imperfect [7], the DNS assays have been optimized and produced numerous results on the relationship between structure of the flavonoids and their amylase inhibition capacity. Nevertheless, results of flavonoids IC_{50} have shown discrepancies with variations by factors up to 800 between studies [8]. A deep analysis of the relevant literature showed us that the α-amylase inhibition assays were relatively consistent apart from one aspect: a vast majority of these tests were carried out after a pre-incubation period of α-amylase with the flavonoid inhibitors, but this incubation period could vary from 5 to 30 min [6,9–20] or was not specified in the protocols [21–29].

Therefore, we hypothesised that binding kinetics could play a major role in the determination of the inhibitory capacity of flavonoids. This hypothesis was supported by seminal work on peptidic inhibitors of wheat α-amylase that determined a minimum incubation time necessary to achieve full inhibition at a defined inhibitor concentration [30]. Another observation supported this hypothesis, although more remotely related, is work characterizing the binding between apple flavonoids and apple cell wall materials, le Bourvellec *et al*. determined that 10 to 20 min were necessary to achieve full binding of the two components [31]. This suggested further that binding between macromolecules and flavonoids is not instantaneous and that in the case of flavonoid-α-amylase binding and inhibition, the time of incubation of flavonoid and α-amylase may affect the measurement of α-amylase activity.

To tests this hypothesis, we used four green tea flavonoids (epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG)) and two α-amylases (human salivary α-amylase (HSA) and porcine pancreatic α-amylase (PPA)). HSA and PPA were chosen for their relevance in human starch digestion and their common use in *in vitro* digestion models. EC, ECG, EGC, and EGCG were used at concentrations ranging from 15 to 90 μM as these concentrations were in the range of most IC_{50} values reported by the current literature and consistent with common dietary intakes on flavonoids [32,33]. Binding kinetics were measured by fluorescence quenching and the impact of binding kinetics was then assessed by measuring the activity of α-amylase activity by the DNS assay after flavonoid-α-amylase incubation ranging from 0 to 90min.

2.5. Materials and methods

2.5.1. Chemicals and reagents

α-amylase from human saliva (HSA, type XIII-A, lyophilized powder, 87.5 units/mg solid), α-amylase from porcine pancreas (PPA, type VI-B, 15 units/mg solid), amylopectin from maize (purity > 99 %), maltose monohydrate, reagents for PBS buffer (Na_2HPO_4, NaH_2PO_4, NaCl), colour reagents for α-amylase activity test ($KNaC_4H_4O_6$, 3,5-dinitrosalicylic acid, NaOH) were all purchased from Millipore-Sigma (Oakville, ON).

(-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG) were purchased from Adooq Bioscience (Irvine, CA) and had a purity > 98 % according to the vendor specifications.

All solutions were prepared with MilliQ water.

2.5.2. Fluorescence quenching

Fluorescence quenching of HSA and PPA by EC, EGC, ECG and EGCG was measured via fluorescence signal of tryptophan residue Trp[59] of HSA and PPA. In a 96-well plate, 100 µL of a 1.25U/mL enzyme solution (HSA or PPA) in PBS buffer (0.1M, pH 6.9) was mixed with 125µL of PBS buffer (blank) and either 25µL of PBS buffer for the blank or 25µL of flavonoid in PBS buffer in order to obtain a final flavonoid concentration of 15, 30, 60 or 90µM. Time t=0 min was set at mixing time. Samples were then placed in Tecan Spark multimode micro-plate reader (Baldwin Park, CA) at 25°C and shaken for 10s. Measurements of fluorescence emission at 350nm after 280nm excitation started 3min after initial mixing, and were taken at 5min intervals for the first 20 min and 10 min intervals up to 90 min. Relative attenuation of emission intensity relatively to the blank or relative fluorescence quenching (*FQ* in %) was calculated as,

$$100 - \frac{I_{sample} \times 100}{I_{blank}} \qquad \textit{Eq. 2-1}$$

where I_{sample} was the emission intensity of the sample and I_{blank} was the emission intensity of the blank. Relative fluorescence quenching (*FQ* in %) was then plotted against time (*t*) for each enzyme-flavonoid combination and each flavonoid concentration. Again, for each enzyme-flavonoid combination and each flavonoid concentration, the plot was fitted with a Michaelis-Menten model,

$$FQ = \frac{FQ_{max} \times t}{t_{1/2} + t} \qquad \textit{Eq. 2-2}$$

where FQ_{max} was the maximum fluorescence quenching and $t_{1/2}$ was the time needed to reach half of maximum signal attenuation.

Additionally, time of 95%-signal attenuation $t_{95\%}$ was calculated from this model as,

$$t_{95\%} = 19 \times t_{1/2}. \qquad \textit{Eq. 2-3}$$

2.5.3. α-Amylase inhibition assay

The inhibition assay was conducted in the absence and presence of polyphenols according to a previously reported method [34] with minor modifications. The method consists in measuring by a colorimetric method the amount of maltose generated by the hydrolysis of amylopectin by PPA or HSA. In detail, 200µL of the 1.25U/mL enzyme (HSA or PPA) was added to 50µL of PBS buffer (0.1M, pH 6.9) in 2mL tubes. To this, 50µL of PBS buffer (blank) or 50µL of flavonoid in PBS buffer solutions was added in order to obtain a final flavonoid concentration of 15, 30, 60 or 90µM. These solutions containing the enzyme and flavonoids were thoroughly mixed and incubated at 37°C for 0, 5, 10, 20, 30, 40, 50, 60, 70, 80 or 90min. After the incubation period, 200µL of a solution of 2.5mg/mL gelatinized amylopectin in MilliQ water was added and the tubes were incubated for 10min at 37°C. 1mL of a DNS colour reagent solution prepared according to the method described by Nyambe-Silavwe *et al.* [34] was added to the reaction mix and placed in a 100°C water bath for 10min to terminate the reaction and develop colour, and then on ice for 10min to cool the solution down to room temperature. 250µL of each sample solution was transferred into a 96 well micro-plate and absorbance was read at 540nm with a Tecan Spark multimode micro-plate reader (Baldwin Park, CA) at 25°C. Absorbance reading was then converted into a maltose concentration with a maltose standard

curve: maltose concentration ranged from 0 to 2mg/mL and the correlation between absorbance at 540nm and maltose concentration exhibited a linear relationship with a correlation coefficient R^2=0.9985. Each experiment was conducted in triplicate, for each concentration of each polyphenol and the enzyme. For each enzyme-flavonoid combination, each enzyme-flavonoid incubation time and each flavonoid concentration, maltose concentration values were expressed as percentage of maltose concentration obtained with the same enzyme without flavonoid, resulting in a value of percent inhibition of the enzyme by the flavonoids.

2.5.4. Numerical and statistical analysis

Data in this study are expressed as mean ± standard deviation of three replicates. Non-linear regression with the Michaelis-Menten model, one-way ANOVA to evaluate statistical significance of differences and Tukey post-hoc tests were performed with Minitab 19 (Minitab LLC, State College, PA).

2.6. Results and discussion

2.6.1. Fluorescence quenching

Previous *in silico* experiments has shown that flavonoids bind mammalian α-amylases mainly on their active site. For example, the biphenolic core structure of flavonoids, chalcone, has been proposed to bind specifically to the Trp^{59} and Tyr^{62} residues of the active site [35]. Docking analysis has also shown that one molecule of hesperetin, luteolin, quercetin, catechin or rutin binds to each amylase molecule at its active site [36]. Similar results were found through docking analysis with proanthocyanidins B1 and B2 [37]. Therefore, it has been shown that fluorescence quenching observed upon binding of polyphenols onto α-amylase is specific to the Trp^{59} residue of the active site and directly linked to the polyphenols' inhibitory activity [16]. Consequently, we used tryptophan fluorescence quenching to evaluate binding kinetics

between amylases and catechins in relation with their inhibitory activity. Fluorescence emissions of the tryptophan residues of the digestive enzymes were measured at 350nm, after initial excitation at 280nm in presence of catechins as a function of time of incubation. The intensity of fluorescence signal for an amylase-catechin complex was expressed relative to the intensity of the fluorescence signal of the enzyme alone. Therefore, data are presented as a percentage of fluorescence quenching (FQ) relatively to the enzyme alone by each catechin along time, (at time t=0 min). ***Figure 2-1*** shows a plot of FQ along time for all combinations of HSA or PPA with EC, ECG, EGC and EGCG. Additionally, these plots were fitted with a Michaelis-Menten kinetics model in order to extract meaningful data, namely maximum fluorescence quenching (FQ_{max}) and time of half-maximum FQ ($t_{1/2}$). These two parameters, reported in ***Table 2-1***, enabled the evaluation of magnitude and rate of binding for all combinations of HSA or PPA with EC, ECG, EGC and EGCG.

Figure 2-1 A and ***Figure 2-1 B*** show FQ by EC, on HSA and PPA, respectively, and the model parameters are reported in ***Table 2-1***. FQ exhibited similar patterns in magnitude and in rate for HSA and PPA in presence of EC. For HSA, FQ_{max} ranged between 36.8±0.4% and 31.5±0.7% at 15 and 60µM of EC, respectively. For PPA, FQ_{max} ranged between 43.2±0.7% and 36.9±1.2% at 15 and 90µM of EC, respectively. The spread of FQ_{max} values across all concentrations appeared to be narrow: relative standard deviation of FQ_{max} was 7.3% for HSA and 7.1% for PPA. Therefore, although these differences in FQ_{max} were statistically significant, all FQ were of the same order of magnitude across all concentrations for each enzyme and the apparent differences did not seem to be meaningful. Additionally, the ranges of signal attenuation observed with EC on PPA and HSA were the lowest of all combination tested in this work. Therefore, we could hypothesise that both HSA and PPA were saturated with EC even at concentration as low as 15 µM, leading to similar FQ_{max}. Despite these observations

about FQ_{max}, $t_{1/2}$ increased significantly for HSA from 1.50±0.23min to 40.13±4.45 min from 15 to 90µM EC. For PPA, $t_{1/2}$ increased from 3.05±0.39min to 25.74±2.56 min from 15 to 90µM EC. Contrary to magnitude of signal attenuation, time of half-signal attenuation exhibited a significant increase as concentration of EC was increased from 15 to 90µM, by a factor of 8.3 and 26.8 for HSA and PPA respectively. Therefore, both HSA and PPA seemed to be saturated with EC at all concentrations ranging 15-90µM, but the time required to reach this state of saturation increased significantly as the concentration of the EC increased. Molecular crowding and competition for binding sites may have played a role in affecting the binding kinetics of EC with the amylases.

Figure 2-1 C and ***Figure 2-1 D*** show FQ by ECG, on HSA and PPA, respectively, and the model parameters are reported in ***Table 2-1***. For HSA, FQ_{max} increased from 60.6±0.2% to 88.3±0.0% as concentration of ECG increased from 15 to 90µM, whereas for PPA an increase was seen from 57.9±0.2% to 88.2±0.0% as concentration of ECG increased from 15 to 90µM. FQ_{max} increased by a factor of 1.5 in both cases as concentrations were increased from 15 to 90µM, which seemed to be both significant and meaningful. For HSA, $t_{1/2}$ significantly decreased from 0.22±0.03min to 0.01±0.01min between 15 and 90µM ECG. For PPA, $t_{1/2}$ significantly decreased from 0.31±0.03min to 0.05±0.01min between 15 and 90µM ECG. From 15 to 90µM ECG, $t_{1/2}$ decreased by factors of 37 and 6 for HSA and PPA, respectively. Contrary to EC, HSA and PPA did not seem to be saturated with 15-90µM ECG and increasing ECG concentration normally led to faster binding.

Figure 2-1 E and ***Figure 2-1 F*** show FQ by EGC, on HSA and PPA respectively, and the model parameters are reported in ***Table 2-1***. Signal attenuation for both HSA and PPA demonstrated similar patterns in magnitude and rates in the presence of the catechin EGC. For

HSA, FQ_{max} ranged between 43.8±0.6% and 47.0±0.4% between 15 and 60μM EGC and increased to 60.2±0.5% for 90μM EGC. For PPA on the other hand, FQ_{max} ranged between 42.6±0.6% and 47.1±0.6% between 15 and 60μM EGC and increased to 58.4±0.6% for 90μM EGC. For both HSA and PPA, FQ_{max} were significantly but not meaningfully different between 15 and 60μM and seemed to increase meaningfully by a factor of 1.3 when EGC concentration reached 90μM. For HSA, $t_{1/2}$ ranged between 0.62±0.12min and 1.34±0.24min between 15 and 90μM EGC. For PPA, $t_{1/2}$ demonstrated a similar pattern and ranged between 0.90±0.15min and 1.73±0.29min between 15 and 90μM EGC. For both HSA and PPA, $t_{1/2}$ were statistically significantly different but did not seem to be meaningfully different as they all ranged closely around 1 min of time of half-maximum signal attenuation.

Figure 2-1 G and ***Figure 2-1 H*** show the relative fluorescence quenching by EGCG, on HSA and PPA, respectively and the model parameters are reported in ***Table 2-1.*** For HSA, FQ_{max} demonstrated a significant increase from 54.19±0.65% to 85.28±0.07% as concentration of EGCG increased from 15 to 90μM. A similar trend was seen in PPA with the FQ_{max} demonstrating a significant increase from 54.1±0.7% to 85.3±0.1% as concentration of EGCG increased from 15 to 90μM. The FQ_{max} increased by a factor of 1.56 in both cases which seemed both significant and meaningful. For HSA, $t_{1/2}$ decreased significantly by a factor of 23 from 1.36±0.19min to 0.06±0.02min from 15 to 90μM EGCG. For PPA, $t_{1/2}$ decreased significantly by a factor of 12 from 1.31±0.21min to 0.11±0.02min from 15 to 90μM EGCG.

From the fluorescence quenching experiments, we could observe that catechins displayed different binding kinetics with amylases, and that HSA and PPA had very similar behaviours. EC and EGC exhibited relatively slow binding rate ($t_{1/2}$ in the order of minutes) and moderate maximum signal attenuation (FQ_{max} generally below 50%), whereas ECG and EGCG exhibited

fast binding ($t_{1/2}$ in the order of magnitude of seconds) and higher maximum signal attenuation (FQ_{max} up to 90%). This is consistent with previous reports on amylase binding with green tea flavonoids [15]. Also, whereas increasing concentration of ligand increased binding kinetics with ECG and EGCG, the same was not observed with EGC and was the opposite with EC. The main structural difference between these two groups is the presence of peripheral, terminal galloyl groups for ECG and EGCG and their absence in EC and EGC. If steric effects related to the overall size of the ligand were involved in our observations, bigger molecules such as ECG and EGCG should exhibit the consequences of these effects, but it is not the case here. The difference in binding kinetics and extent of binding between these two groups may therefore be due to the position of the binding groups. It has been proposed that binding of flavonoids with amylase occurs more extensively with an increasing number of peripheral -OH groups [6], which is consistent with our data. Nevertheless, in the context of our study, we may take this proposition further by hypothesizing that binding occurs preferentially *via* the terminal galloyl groups for ECG and EGCG and *via* the B rings for EGC and EC. However, the proximity of the B ring with the bulky A and B rings may promote steric effects in the case of EC and EGC, whereas the relative greater distance between the terminal galloyl group and the bulky A and B rings may limit these steric effects. Molecular dynamics simulation may be used to test this hypothesis and understand better binding kinetics of flavonoids with amylases.

Beyond these structural considerations, the data presented in this section suggest that binding can be considered almost complete within minutes with ECG and EGCG whereas it could take up to hours to reach complete binding with EC and EGC. As amylase inhibition by flavonoids is based on binding of these two entities, the binding kinetics could be critical to the investigation of these inhibition phenomena.

A last note of caution should be expressed regarding binding kinetics measured by FQ of Trp[59] at 350nm. Variations in FQ upon addition of flavonoids may be attributed to redshift of Trp[59] emission as it was shown previously that this redshift can go up to 9 nm in the case of EGCG and concentrations of up to 0.8mg/mL. However, that work also showed that redshift was below 1nm for concentrations of green tea flavonoids below 0.05mg/mL. The maximum concentration used in the present study being 0.041mg/mL, it is safe to consider redshift as negligible in our report of the results. [16]

Table 2-1: Final signal attenuation (FQmax), time of half-signal attenuation (t1/2) and time of 95%-signal attenuation t95% for both HSA and PPA in the presence of EC, ECG, EGC and EGCG at different concentrations (15, 30, 60 and 90µM). Results are reported as average ± SEM of three replicates. Letters indicate results of ANOVA for each combination of flavonoid and enzyme: values sharing letters are not significantly different ($p < 0.05$).

Phenolic compound	Concentration (µM)	HSA			PPA		
		FQ_{max} (%)	$t_{1/2}$ (min)	$t_{95\%}$ (min)	FQ_{max} (%)	$t_{1/2}$ (min)	$t_{95\%}$ (min)
EC	15	36.8±0.4^{a}	1.50±0.23^{a,b}	28.5	43.2±0.7^{a}	3.06±0.39^{a}	58.1
	30	36.6±0.6^{b}	1.73±0.31^{a,b}	32.9	40.7±0.7^{b}	3.27±0.43^{a}	62.1
	60	31.5±0.7^{c}	5.73±0.80^{a}	108.9	38.1±0.54^{c}	11.41±0.73^{b}	216.8
	90	33.6±1.5^{c}	40.13±4.45^{b}	762.5	36.9±1.2^{c}	25.74±2.56^{c}	489.1
ECG	15	60.6±0.2^{a}	0.22±0.03^{a}	4.2	57.9±0.2^{a}	0.31±0.03^{a}	5.9
	30	70.8±0.1^{a}	0.07±0.01^{b}	1.3	68.6±0.1^{a}	0.09±0.01^{b}	1.7
	60	82.8±0.1^{a}	0.02±0.01^{b,c}	0.4	81.8±0.0^{a}	0.05±0.01^{b}	1
	90	88.3±0.0^{a}	0.01±0.01^{c}	0.2	88.2±0.0^{a}	0.05±0.01^{b}	1
EGC	15	47.0±0.4^{a}	0.89±0.14^{a,b}	16.9	45.3±0.5^{a}	1.34±0.19^{a,b}	25.5
	30	43.8±0.6^{b}	1.34±0.24^{a}	25.5	42.6±0.6^{b}	1.73±0.29^{a}	32.9
	60	45.2±0.6^{c}	1.01±0.20^{a,b}	19.2	47.1±0.6^{c}	1.34±0.23^{ab}	25.5
	90	60.2±0.5^{d}	0.62±0.12^{b}	11.8	58.4±0.6^{d}	0.90±0.15^{b}	17.1
EGCG	15	54.2±0.7^{a}	1.37±0.19^{a,b}	26	54.1±0.7^{a}	1.31±0.21^{a}	24.9
	30	64.2±0.2^{a}	0.13±0.04^{a}	2.5	65.2±0.2^{a}	0.15±0.03^{b}	2.9
	60	77.2±0.1^{a}	0.09±0.01^{a,b}	1.7	77.7±0.1^{a}	0.20±0.02^{b}	3.8
	90	85.3±0.1^{a}	0.06±0.02^{b}	1.1	85.3±0.1^{a}	0.11±0.02^{b}	2.1

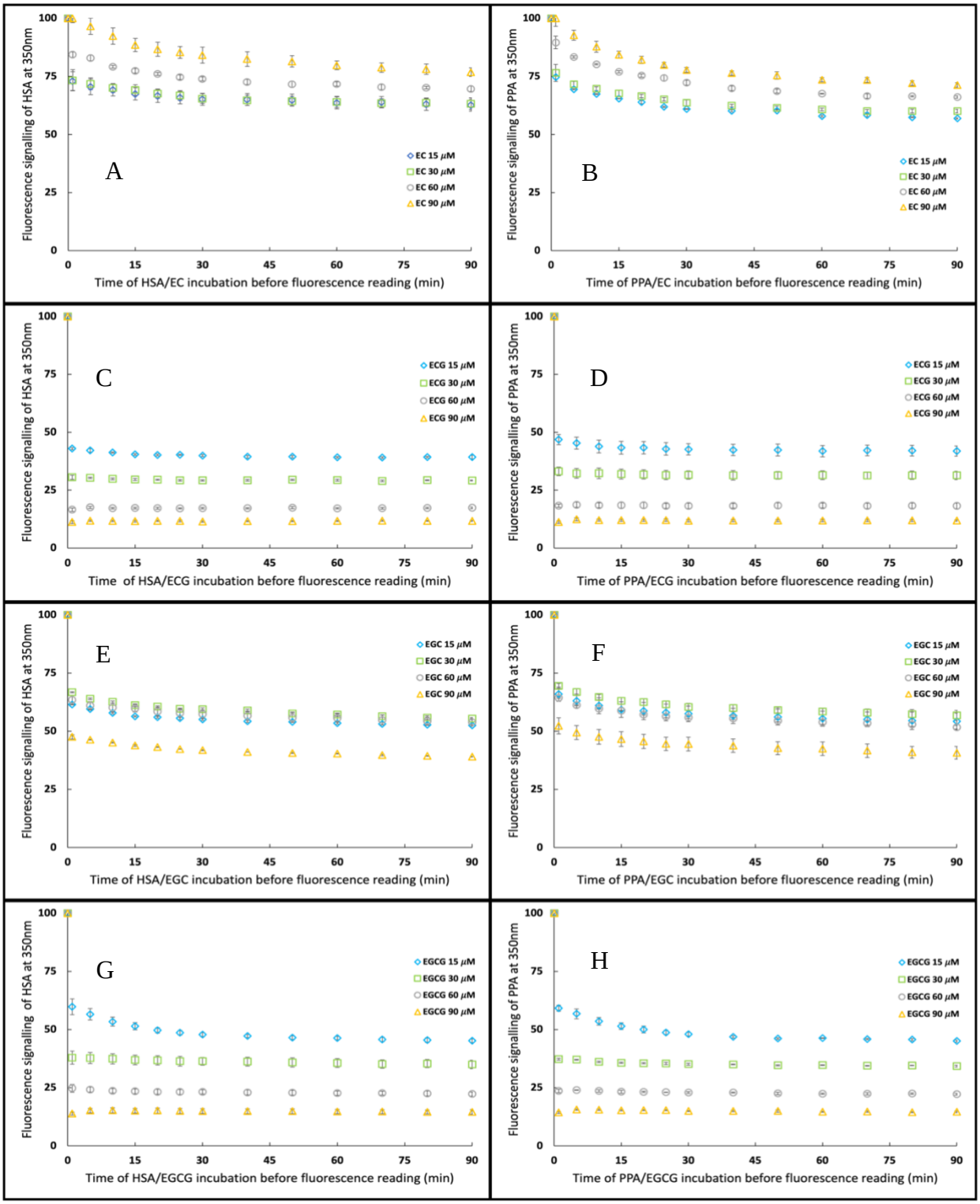

Figure 2-1: Attenuation of fluorescence signalling for HSA (left) and PPA (right) with different four flavonoids. From top to bottom, EC (A and B), ECG (C and D), EGC (E and F) and EGCG (G and H) at concentrations of 15μM (diamond), 30μM (square), 60μM (triangle) and 90μM (circle) over 90 minutes. Results are reported as mean ± standard deviation of three replicates.

2.6.2. α-Amylase assay

We measured the effect of enzyme-flavonoid incubation time on the inhibition of amylase enzymes by flavonoids. To this effect, HSA and PPA were incubated between 5 and 90min with EC, ECG, EGC or EGCG at concentrations ranging 15-90µM prior to measuring their activity on amylopectin. Results were reported as percent of amylase activity, relatively to the activity of then same enzyme alone, against time of incubation of the enzyme with polyphenols.

Results of amylase inhibition after 0, 30, and 90min flavonoid-amylase incubation are reported in ***Table 2-2*** for HSA and in ***Table 2-3*** for PPA. Complete dependence of enzyme inhibition on incubation time is displayed in ***Figure 2-2***.

Generally, all percentages of amylase inhibition increased with increasing incubation time prior to amylase activity test, for both HSA and PPA. Out of the 32 combinations reported in ***Table 2-2*** and ***Table 2-3*** (2 enzymes × 4 flavonoids × 4 flavonoid concentrations), 97% exhibited a significant difference in percent inhibition of amylase between 0 and 90min incubation (100% for HSA and 94 % for PPA), and 38% exhibited a significant difference in percent inhibition of amylase between 30 and 90min incubation (44% for HSA and 31% for PPA).

In more details, EGCG showed only one case of significant difference in percentage of inhibition between 30 and 90min incubation (60µM concentration with HSA) and all levels of inhibition were significantly different between 0 and 90min incubation. Additionally, ***Figure 2-2*** shows that full inhibition of HSA and PPA is reached within minutes of incubation. This is consistent with FQ data reported in ***Table 2-1*** and ***Figure 2-1***, if we approximate that enzyme inhibition is complete when 95% of enzyme-flavonoid binding is complete. Indeed, ***Figure 2-1*** and $t_{95\%}$ data in ***Table 2-1*** suggest that EGCG would reach full inhibition well before 30min

incubation at all concentrations. Similarly, EGC showed five cases of significant difference of percent inhibition between 30 and 90min incubation (30, 60 and 90µM concentration with HSA, 15 and 30µM concentration with PPA), which is in line with the relatively slow binding kinetics of EGC with HSA and PPA shown in ***Table 2-1*** and ***Figure 2-1***. However, there is a slight discrepancy between the $t_{95\%}$ values calculated for EGC (ranging from 12 to 33min) and continuously increasing level of enzyme inhibition between 0 and 90min incubation as shown in ***Figure 2-2***. To explain this discrepancy, we could hypothesize that there is competition between the enzymes and amylopectin for binding with EGC. Indeed, it has been show that starch can bind non-covalently to flavan-3-ols [38,39]. Nevertheless, as starch is digested by the non-complexed enzymes, this competition may decrease, and enzyme-phenolic binding and enzyme inhibition may increase, but with a delay.

This hypothesis may also support enzyme inhibition characteristics observed with EC and ECG. Whereas EC showed slow binding kinetics in ***Figure 2-1*** and ***Table 2-1***, inhibition level of HSA and PPA seemed to plateau relatively rapidly (around 60min with HSA and around 30min with PPA). This resulted in only two cases of significant difference of percent inhibition between 30 and 90min incubation (30µM concentration with HSA and PPA) whereas slow binding kinetics may have translated in more significant differences between 30 and 90min incubation. Contrarily, ECG exhibited rapid binding kinetics similar to EGCG in ***Figure 2-1*** and ***Table 2-1*** but seemed to reach an inhibition plateau only around 60min with HSA and no plateau with PPA. This resulted in four cases of significant difference of percent inhibition between 30 and 90min incubation (15 and 60 µM concentration with HSA, 30 and 90µM concentration with PPA) whereas rapid binding kinetics may have translated in less significant differences between 30 and 90min incubation. Once again, competition for binding between enzymes and starch may explain these observations. Indeed, we have shown previously that

size of the flavan-3-ols is a crucial factor for their binding with starch, with bigger flavan-3-ols binding less with starch possibly due to steric constraints and the size of the helical cavity of α-(1,4)-glucans [38]. Therefore, ECG may bind more with starch than EGCG making EGCG inhibition more aligned with binding kinetics with enzymes and introducing discrepancy for ECG. Similarly, EC may bind more with starch than EGC making EGC inhibition more aligned with binding kinetic with enzymes and introducing discrepancy for EC.

One major point of caution should be brought in the interpretation of these results. It has recently been shown that flavonoids can interfere with DNS colorimetric method of detection of maltose. This would be the case in our study. It has been shown by Lim *et al.* [7] that a method using HPAEC detection of maltose was more accurate than the DNS method and avoided under- or over-estimations of amylase inhibition by flavonoids [7]. Our results may then be discussed in light of Lim *et al*'s [7] work and criticized for their absolute accuracy. The following paragraph therefore aims to evaluate the error induced by the presence of flavonoids in the use of the DNS assay. By using the absorbances measured on test solutions and the absorbances measured on blanks containing the flavonoids and by correcting for the absorbance of the blank, we were able to measure the maximum contribution of flavonoids to the absorbance of the test solutions and therefore the maximum relative error induced by colour detection of the flavonoids in the determination of maltose concentration. Across all test samples, EC induced a relative error ranging from 7.3 to 14.5 % on maltose concentration; ECG induced a relative error ranging from 8.2 to 20.3 % on maltose concentration; EGC induced a relative error ranging from 5.4 to 11.6 % on maltose concentration; EGCG induced a relative error ranging from 4.7 to 11.8 % on maltose concentration. Although most of these errors were below 10 %, some exceeded 15 % which may not be acceptable for an analytical method. Nevertheless, one must keep in mind that these errors are maximum estimated errors and may actually be smaller,

although hard to quantify more precisely. Indeed, the blanks containing flavonoids and used to quantify colour detection due to the flavonoids themselves contain free flavonoids and therefore develop maximum colour DNS and maximum absorbance. By contrast, in test samples, the flavonoids are in large part bound either to α-amylase or to starch. Therefore, the flavonoids may not react to the same extent as the free flavonoids with DNS and their contribution to absorbance may be lower than estimated. Therefore, we feel confident these results are in large part accurate and reliable, despite the limitations exposed by Lim *et al.* [7] previously discussed.

In any case, the present study focuses on a dimension of enzyme inhibition by flavonoids that has not been explored before, namely binding kinetics, which is not affected by the interference of flavonoids with DNS. In other words, our results remain internally consistent from the perspective of the role of binding kinetics on amylase activity assays. It must also be noted that the DNS method is still one of the most widely used methods to assess amylase inhibition. Therefore, our study also reaches a practical goal by shedding light on limitations of a common method that has not been unaccounted for, to date.

Another point of caution is that the assays of this study were carried out at pH 6.9, a pH at which green tea flavonoids are notably unstable and can be degraded and lead to the formation of flavonoid oligomers which interfere with biological mechanisms in the small intestine [40]. Therefore, the variations in binding and enzyme inhibition may be influenced by these degradation reactions and we may find ourselves in a methodological conundrum: sufficient incubation is required to achieve complete binding but such incubation time my lead to binding of the enzyme with flavonoid derivatives different from the ones targeted initially for the study. Nevertheless, such adverse conditions are required to study enzyme-flavonoid interactions in

physiologically relevant conditions such as those found in the small intestine and we may consider that the flavonoid degradation product are actually physiologically relevant, but further exploration shall be carried out to study the role of these degradation products in enzyme inhibition.

Our observations about binding kinetics of enzymes with flavonoids vs. incubation time-dependent inhibition of the enzymes and competition between starch and enzyme for binding with flavonoids highlight crucial considerations for the design of enzyme inhibition assays with flavonoids. Indeed, whereas these assays have been the subject of important optimization works [34,41], the effect of incubation time of enzyme and flavonoids has never been tested, to the best of our knowledge. As a result, various protocols can be found in the literature with incubation varying from 5min [10,12,18], 10min [11,19], 15min [9], 20min [15,20] and 30min [6,13,14,16,17]. Additionally, many studies do not define or standardise incubation time [21–29]. Therefore, in addition to maltose detection method as already demonstrated by Lim *et al* [7], this discrepancy in protocols regarding enzyme-incubation time might be another explanation for the wide range of amylase inhibition levels observed in the literature as reported before [8].

Nevertheless, this potential need for standardisation of enzyme-flavonoid incubation time may only stand for investigations aimed at uncovering mechanisms of biding and inhibition of enzymes by flavonoids. Indeed, many protocols aiming at simulated ingestion of real food do not perform enzyme-flavonoid incubation, or do perform starch-flavonoid pre-incubation [42,43]. In these cases, such absence of incubation is necessary and required by the goal of the experiment which is to approach actual ingestion of foods containing both starch and flavonoids.

Therefore, whereas the present work does not suggest that there is an ideal protocol to evaluate enzyme inhibition by flavonoids, we suggest that if enzyme-flavonoid incubation is required by an experimental design, it should be consistent with the rational of the study in order to improve reliability of the data.

Additionally, whereas our work has been limited to α-amylases, it is likely that similar time-dependent effects could be observed with other enzymes involved in the glycaemic response to foods, glucosidases in particular [7,44], and this shall be tested as well.

Finally, this work may find clinical relevance is exploring the timing of flavonoid consumption to achieve maximum effect of post-prandial glycaemic response. Whereas the anti-hyperglycaemic effect of flavonoids has been well and widely documented clinically [1], one may hypothesize that the timing of a controlled pre-treatment with ingestion of flavonoids may affect the glycaemic response to a subsequent test meal.

Table 2-2: Percent inhibition of HSA by EC, ECG, EGC and EGCG (15-90μM) as a function of HSA-flavonoid incubation time. Results are reported as average ± SEM of three replicates. Letters indicate results of ANOVA for each combination of flavonoid and enzyme: values sharing letters are not significantly different ($p<0.05$).

Time of incubation of HSA with flavonoid	% inhibition of HSA			
	EC			
	15μM	30μM	60μM	90μM
0min	8.3±2.1%b	-1.6±4.9%c	-0.1±5.5%b	9.9±3.9%b
30min	22.8±0.6%a	16.1±0.4%b	20.4±1.9%a	17.2±4.8%a,b
90min	27.3±2.6%a	34.0±2.2%a	30.8±3.1%a	29.7±2.8%a
	ECG			
	15μM	30μM	60μM	90μM
0min	-0.7±0.5%c	-3.4±8.4%b	7.5±6.4%c	10.4±11.1%b
30min	24.2±1.9%b	31.5±5.2%a	34.5±3.0%b	39.6±1.8%a
90min	39.0±2.5%a	38.4±1.5%a	52.6±1.8%a	49.0±3.8%a
	EGC			
	15μM	30μM	60μM	90μM
0min	-3.1±5.3%b	0.6±1.7%c	4.1±2.2%c	1.5±2.1%c
30min	8.2±6.8%a,b	16.6±4.3%b	19.2±1.5%b	12.5±1.7%b
90min	29.6±0.6%a	35.5±3.8%a	39.6±1.6%a	38.5±1.5%a
	EGCG			
	15μM	30μM	60μM	90μM
0min	4.1±0.6%b	12.8±3.9%b	-4.7±1.1%c	2.6±4.1%b
30min	41.7±0.5%a	46.6±1.9%a	44.1±0.8%b	45.1±1.4%a
90min	42.5±0.1%a	46.5±1.5%a	47.4±0.8%a	47.5±0.7%a

Table 2-3: Percent inhibition of PPA by EC, ECG, EGC and EGCG (15-90μM) as a function of PPA-flavonoid incubation time. Results are reported as average ± SEM of three replicate. Letters indicate results of ANOVA for each combination of flavonoid and enzyme: values sharing letters are not significantly different ($p<0.05$).

Time of incubation of PPA with flavonoid	% inhibition of PPA			
	EC			
	15μM	30μM	60μM	90μM
0min	12.1±1.8%a	8.5±1.5%c	3.7±2.4%b	-0.9±8.0%b
30min	25.1±5.1%a	16.2±1.6%b	15.2±5.6%a,b	10.3±6.9%a,b
90min	19.9±6.1%a	25.2±1.3%a	25.6±3.1%a	23.8±4.5%a
	ECG			
	15μM	30μM	60μM	90μM
0min	-1.3±2.7%b	20.4±3.1%b	19.8±2.1%b	4.1±3.2%c
30min	25.9±11.7%a	22.9±1.2%b	29.2±2.5%a	16.4±2.6%b
90min	42.2±4.4%a	30.3±0.2%a	33.7±1.9%a	27.1±0.7%a
	EGC			
	15μM	30μM	60μM	90μM
0min	-0.9±2.9%c	2.9±2.6%c	2.6±2.4%b	1.1±4.4%b
30min	14.1±3.7%b	20.5±1.1%b	18.7±2.8%a,b	11.9±5.5%a,b
90min	31.2±0.8%a	37.6±2.0%a	30.4±7.4%a	25.5±2.8%a
	EGCG			
	15μM	30μM	60μM	90μM
0min	-10.8±2.3%b	1.6±2.7%b	-5.1±1.2%b	-4.1±3.2%b
30min	41.2±1.1%a	42.2±1.6%a	52.5±0.3%a	31.4±1.9%a
90min	43.6±0.9%a	43.9±0.6%a	55.1±1.2%a	34.9±1.8%a

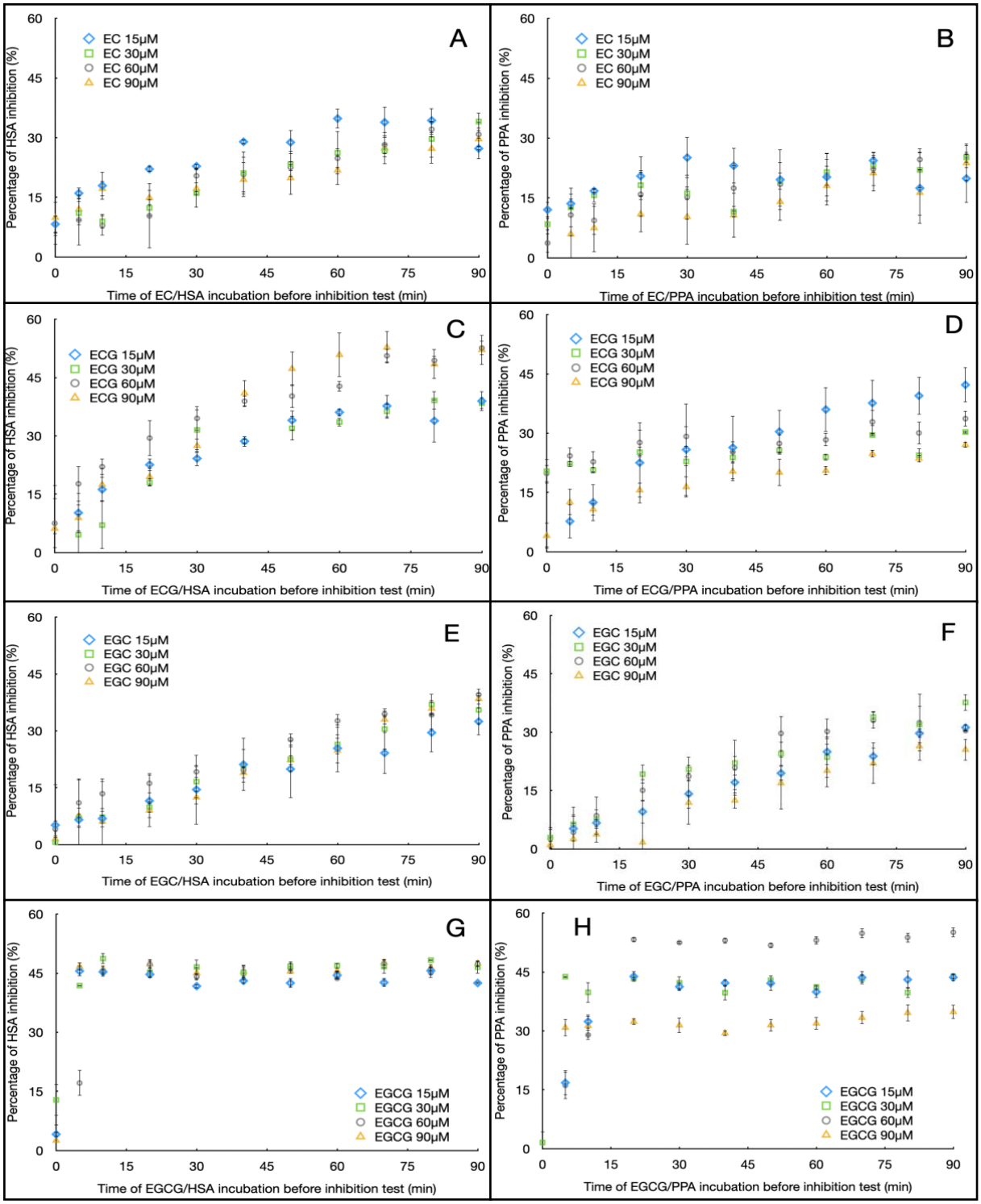

Figure 2-2: Percent inhibition of HSA (left) and PPA (right) with different four flavonoids. From top to bottom, EC (A and B), ECG (C and D), EGC (E and F) and EGCG (G and H) at concentrations of 15μM (diamond), 30μM (square), 60μM (triangle) and 90μM (circle) after enzyme flavonoid incubation time of 0 to 90 minutes. Results are reported as mean ± standard deviation of three replicates.

2.7. Conclusions

In this study, we found that the four main flavonoids of green tea have different binding kinetics with α-amylases and that this kinetics affected the flavonoids' ability to inhibit amylase activity depending on time of pre-incubation of flavonoids and amylase. Nevertheless, the correlation between binding kinetics observed by fluorescence quenching and amylase inhibition remained limited, possibly due to the formation of starch-flavonoid complexes. These findings add a critical perspective to the investigations on the anti-hyperglycaemic effects of flavonoids and suggests that the mechanisms at work in these effects may be more complex than previously thought.

2.8. Acknowledgements

This work was funded by Canada National Science and Engineering Research Council – Grant #2018-04009. The authors thank Pr. Mario G. Ferruzzi (NCSU) for the constructive discussions about interpretation of the results of this study. The authors have no conflict of interest to declare.

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Chapter 3: Unveiling the Complex Interplay of Potato phenolics, Starch, and Lipase: Inhibition and Binding Studies at Physiological pH.

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3.1. Published contributions

D'Costa AS, Chen AA, Hamann E, El Iraki R, Venugopal K, Bordenave N. Impact of potato starch on the inhibition of pancreatic lipase by potato phenolic acids. *Food Bioscience*. Published online November 30, **2023**:103414.

doi:10.1016/j.fbio.2023.103414

- A.S. D'Costa, with the supervision from N. Bordenave, conceptualised the study, collected data, literature, conducted calculations and statistical analysis, and completed the writing up, with review and editing of subsequent versions.
- A.A. Chen, with the supervision from A.S. D'Costa and N. Bordenave, conceptualised the study, collected data, literature, conducted calculations and statistical analysis.
- E. Hamann, R.El. Iraki and K. Venugopal, under the supervision of A.S. D'Costa assisted with data collection.

3.2. Abstract

Phenolic acids are known to bind with and inhibit digestive enzymes *in vitro*. In particular, their action on pancreatic lipase at physiological pH is not well understood. Therefore, this study evaluated the inhibitory potential of phenolic acids from potato (caffeic acid (CA), chlorogenic acid (ChA), p-coumaric acid (pCA), ferulic acid (FA)) towards lipase. All the

phenolic acids were weak inhibitors of lipase, and that ChA was the weakest of all. Binding energy between phenolic acids and lipase obtained by molecular docking and solubility of the phenolic acids seemed to correlate well with inhibition data obtained at pH 6.9. Additionally, phenolic acids can form complexes with polysaccharides. In food such as potato, the presence of significant amounts of starch could therefore prevent phenolic acids to modulate pancreatic lipase. Consequently, we evaluated the effect of potato starch (up to 1 % w/w) on the ability to inhibit the activity of pancreatic lipase. The results showed that the presence of starch had essentially no effect on the inhibition of lipase by FA and pCA, that it led to decreased ChA's inhibitory activity and to increased CA's inhibitory activity. These contradictory observations could be explained by starch-phenolic acid complexation although they may be modulated by physical effects due to starch (microphase separation, decreased diffusion), and potentially amplified by the limited solubility of the phenolic acids.

3.3. Keywords

Lipase inhibition; Starch; phenolic acid; potato.

3.4. Introduction

Epidemiological studies have shown that phenolic compounds are associated with health benefits, particularly metabolic health [1,2], although their mode of action hasn't been clearly uncovered yet. One of the leading hypotheses is that phenolic compounds could modulate food digestion and nutrient absorption, due to their ability to bind and inhibit digestive enzyme and intestinal transporters, as it has been shown in numerous *in vitro* studies [3,4]. However, phenolic compounds can form complexes with polysaccharides present in the food matrix or the food bolus [5–7], thereby affecting phenolics' availability and capacity to bind digestive enzyme and transporters and inhibit their activity. This phenomenon has been studied and observed

primarily on drivers of glycaemic response, namely on the activity of α-amylase, glucosidases and glucose brush border transporters [8–10].

Nonetheless, phenolic compounds can modulate the activity of the other digestive enzymes, such as lipase, which is critical in the hydrolysis of dietary lipids and consequently have an indirect impact on their absorption. Understanding the impact of phenolic compounds on lipase activity is therefore critical to understand their biological activity, including their potential health benefits, and has been studied extensively [11,12]. Nevertheless, considering the aforementioned influence of polysaccharides on the capacity of phenolic compounds to bind with and inhibit α-amylase, glucosidases, and intestinal glucose transporters, it is likely that polysaccharide-phenolic interactions could influence phenolics' ability to modulate the activity of lipase. The insights emerging from this approach would enable a deeper understanding of the effect of food matrix and composition on digestive processes related to lipids. To the best of our knowledge, this has been addressed in only one study so far [13]. In that study, the authors showed that a set of common gums (pectin, dextran, and gum arabic) formed complexes with banana condensed tannins, thereby decreasing tannin-lipase binding, and decreasing the inhibitory activity of the tannins against lipase (from porcine pancreas). Despite the value of this study, some questions remain open. Firstly, condensed tannins are known to be stronger inhibitors of digestive enzymes than lower molecular phenolic compounds such as phenolic acids [14–16]. There is also an inverse correlation between phenolic compounds' molecular weight and their ability to form complexes with polysaccharides [5,6]. Therefore, would the observations of Pu *et al*. (2023) [13] stand with common dietary phenolic acids? Secondly, these authors have studied pectin, dextran, and gum arabic as model polysaccharides. These are generally found at low concentrations in formulated foods (< 1 % w/w) [17]. In contrast, starch is by far the most commonly found polysaccharide in food systems, in concentrations in the order of tens of

percent in many foods. As starch has also been found to bind phenolic compounds [8,9,15], one could wonder whether starch could play the same role as gums towards lipase inhibition by phenolic compounds. To answer these questions, we have chosen potato as a model food. Indeed, potato is a common source of starch in western diets and is often associated with lipid rich meals (fried chips, baked potatoes, mashed potatoes, etc.), making the question of lipase activity practically relevant. Moreover, potato's main phenolic compounds are phenolic acids, namely, caffeic acid (CA), chlorogenic acid (ChA), p-coumaric acid (pCA), and ferulic acid (FA), making them adequately representative molecules for other low molecular weight phenolic compounds [18–20].

Therefore, we hypothesised in this study that the presence of starch would decrease the concentration of free CA, ChA, pCA and FA available to bind and inhibit porcine pancreatic lipase. To test this hypothesis, we chose to measure the half-maximal effective inhibitory concentration (EC_{50}) of the phenolic acids with and without the presence of starch, expecting that the presence of starch would lead to an increase of EC_{50} due to phenolic-starch binding. It is worth noting that it has been shown *in vivo* that starch itself does not have inhibitory activity against lipase [21]. Therefore, while testing our hypothesis, it is likely that any modulation of lipase activity would be due to phenolic-starch interactions modifying phenolic's ability to bind with lipase, and not to starch's lipase-inhibiting activity.

Beyond this central objective, a secondary (although important) objective of this study is to address methodological questions regarding lipase activity assays. Indeed, the vast majority of published work addressing the effect of phenolic compounds on lipase activity conducted the assays at pH 8.0-8.5 (often in Tris-HCl buffer) [22–28]. This is problematic in studies related to food functionality for two reasons. The first reason is that a pH of 8.0-8.5 is not physiologically

relevant. Reference *in vitro* digestion models use pH levels of 7.0-7.5 for the intestinal phase of digestion [29]. The second reason is that phenolic compounds are notably unstable at elevated pH and most would degrade rapidly [30]. Therefore, conducting lipase activity assays at pH 8.0-8.5 seems inadequate and most results published from such experimental conditions could be questioned. Therefore, in this study, we chose to address our central goal with an adapted lipase activity assay conducted at pH 6.9, in order to make our study more relevant to actual food systems and digestive processes.

3.5. Materials and methods

3.5.1. Chemicals and reagents

Type II lipase from porcine pancreas, p-nitrophenyl laurate (pNPL), reagents for PBS buffer (NaH_2PO_4, Na_2HPO_4, NaCl). Caffeic acid (CA), Chlorogenic Acid (ChA), Ferulic Acid (FA) and p-Coumaric Acid (pCA) were purchased from Sigma-Aldrich (St Louis, MO). Dimethyl sulfoxide (DMSO) and ethanol were purchased from Fischer Bioreagents. All solutions were prepared with MilliQ water (resistivity > 18.2 MΩ.cm at 25 °C).

Potato starch was supplied by Sigma-Aldrich (St Louis, MO). This starch was characterised in a previous study by high-performance size exclusion chromatography (HPSEC) coupled with multi-angle laser light scattering, viscometry, and differential refractive index detection (Chapter 4)[8]. Its structural characteristics are as follows:

- Total starch, values calculated over the whole starch peaks (amylose and amylopectin fractions):
    - Weight-average intrinsic viscosity: 129.1 $mL.g^{-1}$

  - Chain length distribution determined by HPSEC-DRI after debranching, expressed as % w/w of total starch, by Degree of Polymerization (DP): DP < 37 (amylopectin short branches) = 71 %, DP 37-100 (amylopectin long branches) = 9 %, DP>100 (amylose) = 20 %.

- Amylopectin fraction, values calculated over the whole amylopectin peak:
  - Weight-average molar mass, $\overline{M_w}$ = 129.1 ± 2.1 mL.g^{-1}
  - Dispersity, $\overline{M_w}/\overline{M_n}$ = 129.8 ± 5.2 g.mol^{-1}
  - Z-average radius of gyration, $\overline{R_G}$ = 180.3 ± 2.5 nm
  - Apparent density, $d_{G_{app}}$ = 6.0 g.mol^{-1}.nm^{-3}
  - Average branching degree, BD_{Hm} = 3.1 %

3.5.2. Estimation of phenolic-lipase binding by molecular docking

The crystal structure of pancreatic lipase was obtained from the Protein Data Bank (reference PDB: 1ETH) and was used to generate lipase-phenolic complex models with CA, ChA, FA and pCA. Docking analysis was performed with LePro software, following methods published previously [31,32]. Molecular docking results were a list plausible conformation of the predicted complex ranked by predicted binding energy (ΔE). To account for these results, predicted binding energy ΔE was expressed as mean ± standard deviation of the top 90 % most probable predicted conformations (with the highest binding energy).

3.5.3. Lipase activity assay

The lipase inhibition assay was conducted in the absence and presence of potato starch by method adapted from Marelli *et al*. (2012) and Martinez-Gonzalez *et al*. (2017), with

modifications [25,33], measuring absorption at 400 nm of *p*-nitrophenol released by lipase hydrolysis of pNPL.

3.5.3.1. Lipase stock solution

Lipase was added to 11 mL of 20 mM PBS buffer (pH 6.9) with 10 mM NaCl in a centrifuge tube placed in a 37 °C water bath for 10 min in order to reach a lipase concentration of 625 $U.mL^{-1}$. Following incubation, the solution was centrifuged (20 min, 1500 rcf, 21 °C). The supernatant was decanted, passed through a PDVF 0.22 μm filter and placed on ice.

3.5.3.2. Phenolic acid stock solution

Phenolic acids were dissolved in ethanol and serially diluted with 20 mM PBS buffer (pH 6.9) containing 10 mM NaCl, so that 50 μL of these dispersions would achieve final phenolic concentrations of 10, 7.5, 5, 2.5 and 1 mM in the assay solution. The dispersions were stored on ice and protected from light until used in the assay to prevent degradation.

3.5.3.3. Potato starch solutions

Weighed out starch (to achieve final starch concentrations of 0.1, 0.5 or 1 % w/w in the test solution) was wetted with 100 μL of ethanol, followed by the addition of 25 mL of MilliQ water and immediately placing it on a hot plate with a stirrer for 15 min. Once all the starch was dissolved and gelatinised, the sample was let to cool to room temperature.

3.5.3.4. Lipase activity assay

In a 2 mL microcentrifuge tube, 75 μL of the lipase solution were added either to 115 μL of PBS buffer without starch, or to 15 μL of PBS buffer and 100 μL of gelatinised potato starch

solution. To this, 50 µL of PBS buffer (for the lipase activity blank) or 50 µL of phenolic acid stock solution (CA, ChA, FA or pCA) were added. The resulting solution was thoroughly mixed and incubated at 37°C for 15 min. Following incubation, 10 µL of 10mM pNPL in DMSO also preincubated at 37 °C was introduced in the reaction mix. The microcentrifuge tube was then vortexed and 250 µL of its content was transferred into a 96 well micro-plate that was incubated at 37°C for 25 min, with shaking every 10 min. Following this final incubation, the absorbance of *p*-nitrophenol was read at 400 nm with a Tecan Spark multimode micro-plate reader (Baldwin Park, CA) at 37 °C.

3.5.4. Experimental design and processing of lipase data

At each starch concentration (0, 0.1, 0.5 and 1 %), the absorbance values (*A*) with CA, ChA, FA or pCA at concentrations of 10, 7.5, 5, 2.5 and 1 mM on the one hand, and without phenolic compound but at the same starch concentration on the other hand ($A_{control}$), were used to calculate the relative inhibition (*RI%*) of lipase with each phenolic compound and at each concentration as follows:

$$RI\% = 100 - 100 \times \frac{A}{A_{control}} \qquad \textit{Eq. 3-1}$$

For each phenolic compound and at each starch concentration, *RI%* was plotted against the concentration of phenolic (*c*) and the resulting curve was fitted with a log-logistic model as follows:

$$RI\% = RI\%_{max} - \frac{RI\%_{max}}{1 + e^{\alpha \times (\ln {}^{c}/_{EC_{50}})}} \qquad \textit{Eq. 3-2}$$

where α, $RI\%_{max}$ and EC_{50} are empirical parameters determined by the best fit. In this model and for each phenolic compound, EC_{50} is the concentration of phenolic compound achieving 50 % of the maximum achievable relative inhibition $RI\%_{max}$.

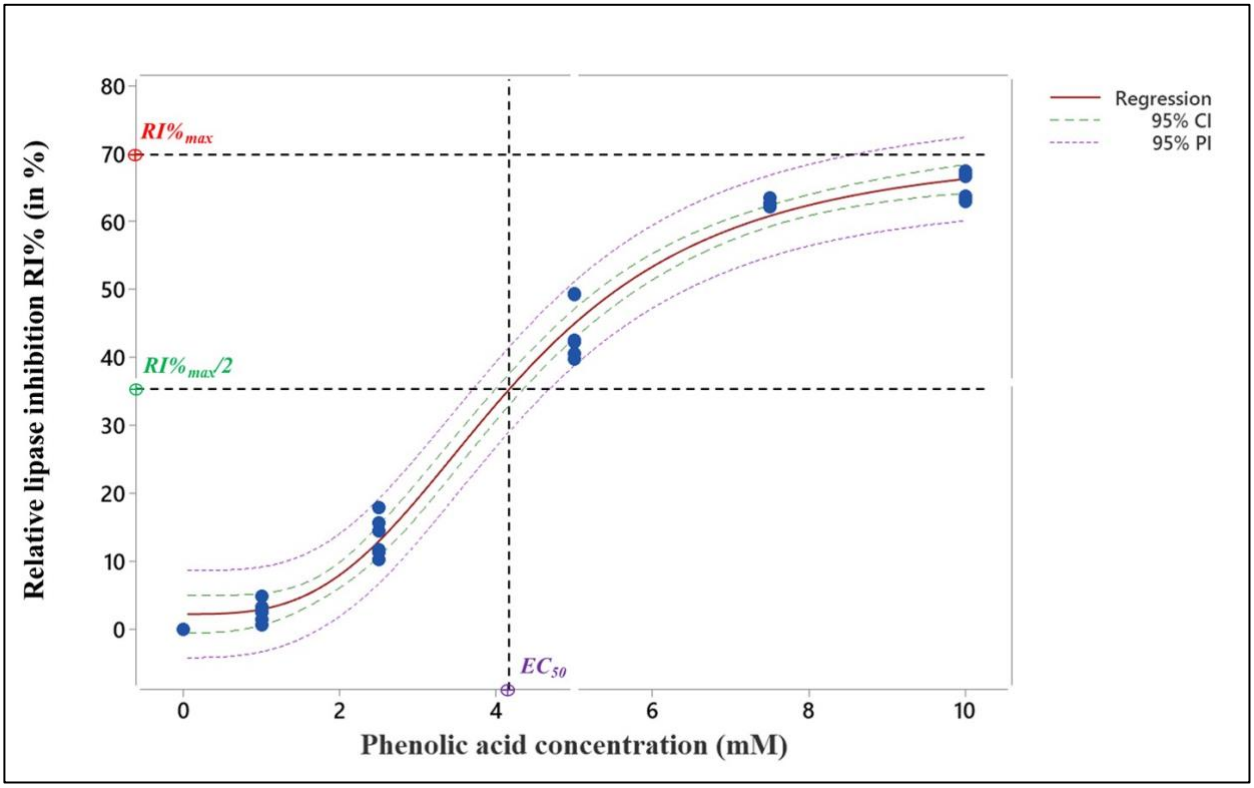

Figure 3-1: Representative example of a log-logistic fit of the plot of relative inhibition of lipase RI% by a phenolic compound against the concentration of that phenolic compound. $RI\%_{max}$ and EC_{50} are empirical parameters determined by the best fit. The green dotted line and the purple dotted line represent the 95 % confidence interval of data and the 95 % confidence prediction interval, respectively (95 % CI and 95 % PI).

3.5.5. Numerical and statistical analysis

Data in this study are expressed as mean ± standard deviation of 6 replicates. Analysis of percentage inhibition and activity of lipase were performed on Microsoft Excel 2016. Non-

linear log-logistic regression to determine $RI\%_{max}$ and EC_{50}, one-way ANOVA coupled with Tukey post-hoc tests to evaluate statistical significance of differences ($p < 0.05$), and two-way ANOVA were performed with Minitab 21 for Windows (Minitab LLC, State College, PA).

3.6. Results and discussion

3.6.1. Lipase activity assay methodological considerations

Through a literature review focusing on the study of pancreatic lipase and its inhibitors, we have found that almost all published work operated their lipase activity assays at pH 8.0-8.5 [22–28]. To the best of our knowledge, only a handful of studies reported a lipase activity assay conducted at pH 7.4 [34–36], although Guo *et al*. (2015) [34] operated in EtOH/H_2O systems, and two operated at pH that remained undefined and involved mixing equal volumes of pH 8.2 Tris buffer and pH 5.0 acetate buffer [37,38]. pH 8.0-8.5 is not physiologically and nutritionally-relevant to pancreatic lipase and its interaction with dietary compounds (pancreatic lipase is present in the small intestine, where pH is approximately 6.5-7.0) [29]. Moreover, elevated pH is unfavourable to the stability of the inhibitors, often phenolic compounds which can degrade rapidly in such conditions [30]. It seems that operating at pH 8.0-8.5 may pose a high risk of measuring the inhibition of lipase by the inhibitor's degradation product rather than the inhibitor itself, in the case of phenolic inhibitors. Therefore, one could wonder why the vast majority of studies on pancreatic lipase and its inhibitors operates at elevated pH levels. From a historical literature review, it seems that the use of pH 8.0-8.5 originates from the development of the earliest lipase activity assays aiming to prove the existence of lipase in human or cow milk, and characterize it [39,40]. These methods used (1) fresh raw milk or cream as a substrate for lipase, and (2) elevated pH to "*increase lipase activity and thus shorten the incubation time to* [limit bacterial growth] *and eliminate the need for a preservative*" [40]. In these conditions, automated addition of NaOH or KOH (pH-stat method) enabled to

simultaneously compensate the drop of pH due to the release of fatty acids associated with lipase activity and follow the advancement of the reaction. It seems that since then, lipase activity has been measured routinely at the pH of optimal lipase activity (8.0-8.5), without further consideration for physiological or nutritional relevance, particularly in the case of pancreatic lipase and its dietary inhibitors, and despite the aforementioned issues the conditions pose. Interestingly, other lipase activity assays based on colorimetric methods (using substrates absorbing in UV-visible domain upon hydrolysis) followed the same experimental conditions, although alkaline pH does not seem to be a requirement for their validity.

In view of these constraints and in order for our study to be nutritionally relevant, we have decided to adapt a colorimetric method (lipase-mediated hydrolysis of pNPL) at pH 6.9 with a PBS buffer. Additionally, we have decided to adapt a method using DMSO and ethanol to aid with dispersion of pNPL and starch respectively, which would be difficult in PBS buffer alone. In order to remain physiologically relevant, we have minimized the amount of DMSO in the system to approximately 4 % of the total experimental volume. Finally, the concentrations of starch added to the system (to measure the impact of starch on the capacity of phenolic acids to inhibit lipase) were chosen so as to remain consistent with starch concentrations that can be found in prepared foods (0.1 to 1 %).

In these conditions, the results of this study may be difficult to compare with previous studies as pH plays a critical role in the stability of all compounds present in the test system.

3.6.2. Molecular docking and inhibitory capacity of phenolic acids towards lipase

Results of molecular docking between type 2 porcine pancreatic lipase and CA, ChA, FA and pCA are reported in ***Table 3-1***, along with the oil-water partition coefficient of these phenolic acids.

Table 3-1: Prediction binding energy with type 2 porcine pancreatic lipase (in KJ.mol-1) and oil/water partition coefficient (Log P, obtained from pubchem.ncbi.nlm.nih.gov) of CA, ChA, FA and pCA.

Phenolic compound	Binding energy with lipase ($kJ.mol^{-1}$)	Log P
CA	-17.5 ± 0.4, b	1.2 [41]
ChA	-23.8 ± 5.5, c	-0.4 [42]
FA	-14.6 ± 0.2, a	1.5 [43]
pCA	-16.9 ± 0.3, b	1.5 [44]

These results show that binding energies of phenolic acids with type 2 porcine pancreatic lipase range approximately between -24 and -14 $kJ.mol^{-1}$. These values are lower than those reported previously on other phenolic compounds, which ranged from -40 to -30 $kJ.mol^{-1}$ for flavonoids [45] and for caffeoylquinic acids [24]. Although there is no reference regarding the binding of phenolic acids with lipase, these results are consistent with the binding of phenolic compounds with other digestive enzymes such as α-amylase, with which phenolic acids form weaker complexes than flavonoids [16].

Results of lipase activity assay with CA, ChA, FA and pCA in absence of starch are reported in ***Figure 3-2*** (EC_{50}) and ***Figure 3-3*** ($RI\%_{max}$) (data points for 0 % w/w starch concentration).

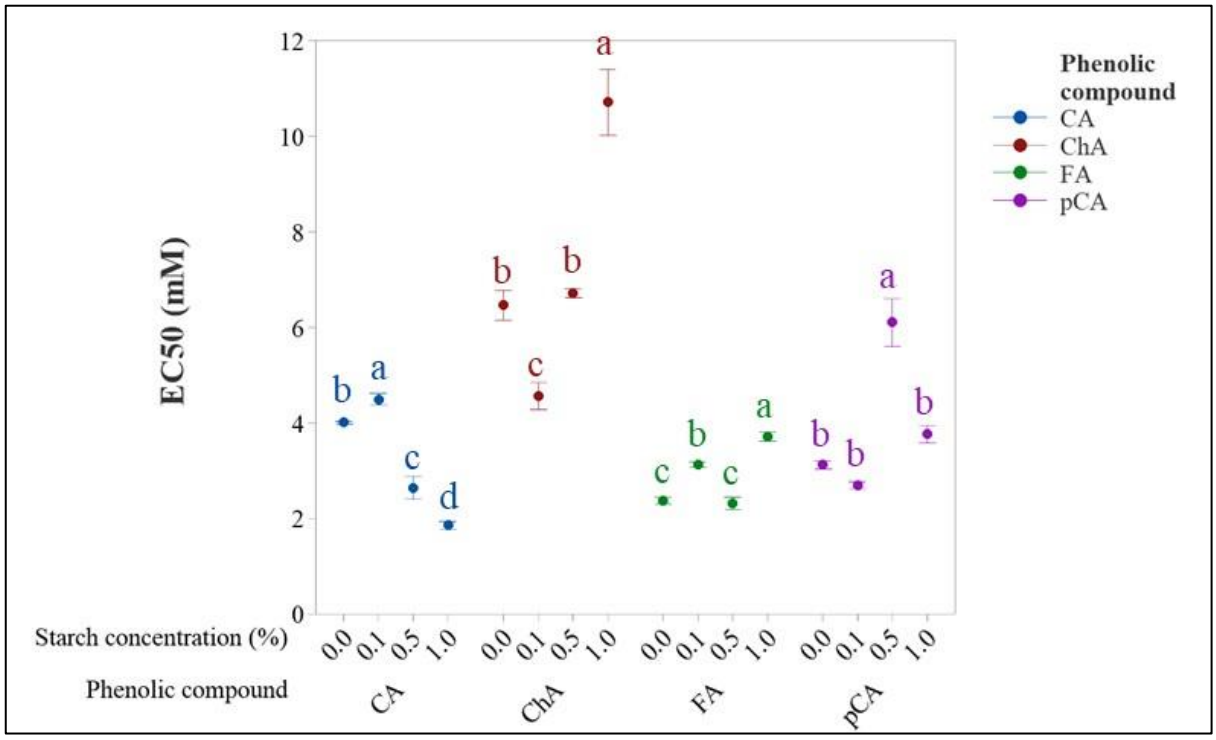

Figure 3-2: Half-maximal effective inhibitory concentration (EC50) towards pancreatic lipase of CA (blue), ChA (red), FA (green) and pCA (purple) in presence of starch (0 to 1 %). Results are reported as mean ± standard deviation of six replicates.

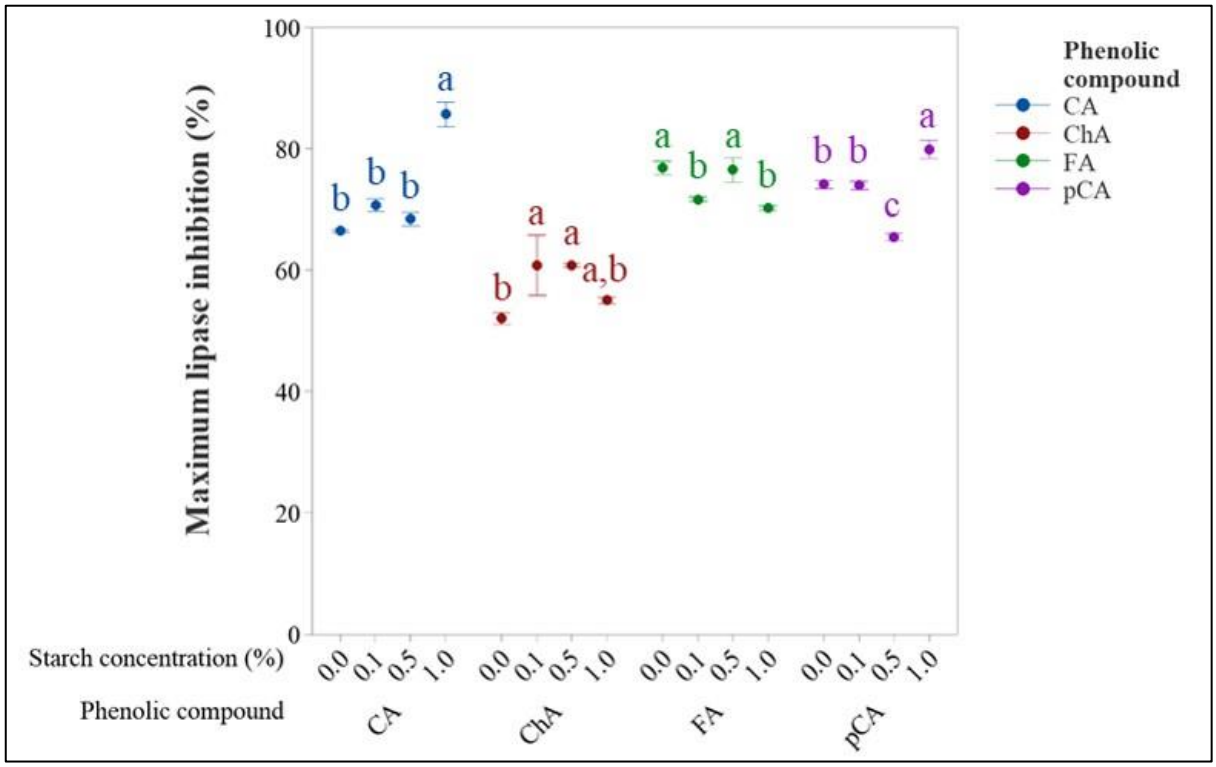

Figure 3-3: Maximum lipase inhibition capacity RI%max towards pancreatic lipase of CA (blue), ChA (red), FA (green) and pCA (purple) in presence of starch (0 to 1 %). Results are reported as mean ± standard deviation of six replicates.

The EC_{50} values of CA, ChA, FA and pCA without starch were respectively 4.0 ± 0.1, 6.5 ± 0.8, 3.1 ± 0.2 and 2.4 ± 0.2 mM, and all these values were significantly different from one another. The $RI\%_{max}$ values of CA, ChA, FA and pCA were respectively 66.4 ± 0.5, 52.0 ± 2.4, 74.0 ± 1.7 and 76.8 ± 2.7 mM, and all these values were significantly different from one another except FA and pCA. These results show that pCA was the strongest inhibitor of lipase (lowest EC_{50} and highest $RI\%_{max}$), closely followed by FA, while ChA was the weakest inhibitor (highest EC_{50} and lowest $RI\%_{max}$). Because previous studies used different concentrations of lipase in their assays, it is necessary to normalise these values according to the unit concentration of lipase to compare them with previous values found in the literature. The EC_{50} values of the present study (0.01 to 0.03 µmol of phenolic acid per unit of lipase)

were of the same order of magnitude as those found by Karamać and Amarowicz with CA, ChA, FA and pCA at pH 7.4 (0.008 to 0.012 µmol of phenolic acid per unit of lipase) [35], although our results differed as far as $RI\%_{max}$ was concerned (10-30 % inhibition in their case). However, they differed significantly from those found by Hu *et al.* with caffeoylquinic acids at pH 8.0 (0.2 to 0.9 µmol of phenolic acid per unit of lipase) [24]. This comparison may confirm the importance of pH in the study of lipase inhibition by phenolic compounds: higher pH required more phenolic acids to reach EC_{50}, probably because at pH 8.0 and above, significant amount of the phenolic acids can be degraded and therefore can't bind with and inhibit lipase.

These results also enabled us to correlate predicted binding data from molecular docking with experimental inhibition data. To approach this correlation, we considered that phenolics' ability to inhibit lipase *in vivo* depend essentially on their ability to form a complex with the enzyme in a biphasic system where lipids are emulsified in an aqueous phase. In such systems, different phenolic compounds would be partitioned between the aqueous and the lipid phase according to their hydrophilicity/lipophilicity, which would in turn affect their ability to form a complex with the lipase. Therefore, not only does phenolics' ability to inhibit lipase depend on the binding energy of the potential complex, but also on the phenolics' oil/water partition coefficient (Log P). Therefore, we analysed possible correlations between EC_{50} and $RI\%_{max}$ on the one hand, and binding energy with lipase and Log P on the other hand. It must be noted that our experimental systems were not biphasic as they consisted in ~ 4 % DMSO in PBS buffer. Nonetheless, the phenolic acids were likely to surround themselves with various ratios of water to DMSO locally and depending on their hydrophilicity/lipophilicity to ensure their stability in solution. As oil/water partitioning, this would affect their ability to form complexes with lipase. Therefore, Log P was a relevant modelling parameter in our systems as well.

Although they were based on four phenolic acids only, excellent linear correlations were obtained as follows:

$$EC_{50} = 10.6 + 0.9 \times \Delta E - 2.8 \times Log\ P,\ R^2 = 0.975 \qquad \textit{Eq. 3-3}$$

$$RI\%_{max} = 47.2 - 1.7 \times \Delta E + 13.5 \times Log\ P,\ R^2 = 0.967 \qquad \textit{Eq. 3-4}$$

EC_{50} was positively correlated with ΔE and negatively correlated with Log P, which is consistent with EC_{50} increasing (weaker inhibition) with weaker complexes and more hydrophilic phenolic acids. Similarly, $RI\%_{max}$ was negatively correlated with ΔE and positively correlated with Log P, which is consistent with $RI\%_{max}$ decreasing (weaker inhibition) with weaker complexes and more hydrophilic phenolic acids.

Overall, these results seem to be self-consistent and represent adequately the inhibitory capacity of phenolic acids towards porcine pancreatic lipase, despite the novelty added by modifying the pH of the assay.

3.6.3. Effects of starch on the inhibitory capacity of phenolic acids towards lipase

The underlying hypothesis tested here is that starch could form complexes with phenolic acids that would decrease these latter's availability and ability to bind with and inhibit lipase. This would materialise as an increase of EC_{50} in presence of starch compared to in absence of starch.

This approach tests indirectly a corollary hypothesis, namely that the extent to which EC_{50} is affected by the presence of starch is a measure of the extent to which starch and the phenolic acid form complexes.

To test these hypotheses, lipase from porcine pancreas was pre-incubated at 37°C for 15 min along with starch from potato (0.1 %, 0.5 % or 1%) and phenolics (CA, CGA, pCA or FA). This was followed by measuring the activity of lipase by the hydrolysis of pNPL. The pre-incubation time of 15 min was implemented in order to give enough time for the lipase-phenolic-starch system to reach equilibrium. Indeed, we have shown in previous work that such equilibrium can take up to several minutes to be reached and that control of this time was necessary to ensure control of the experimental conditions and reproducibility of the results [9].

The results of lipase inhibition by CA, ChA, FA and pCA in presence of starch are presented in ***Figure 3-2*** (EC_{50}) and ***Figure 3-3*** ($RI\%_{max}$). These results undergo significant statistical variation, although these variations didn't necessarily have experimental meaning. For example, the EC_{50} values of ChA between 0 and 0.5 % starch showed significant statistical differences but remained within 30 % of relative difference (between 4.6 and 6.7 mM for 0 and 0.5 % starch respectively), whereas EC_{50} increased very significantly at 1 % starch with a value of 10.7 mM. Similarly, for FA, EC_{50} and $RI\%_{max}$ values exhibited numerous statistically significant differences but remained within a relatively narrow range: between 2.3 and 3.7 mM for EC_{50} and between 70.2 and 76.7 % for $RI\%_{max}$, suggesting that these differences may not be experimentally meaningful.

Taking these observations into account, the effect of starch on EC_{50} and $RI\%_{max}$ can be analysed through the results of two-way ANOVA reported in ***Figure 3-4*** and ***Figure 3-5***.

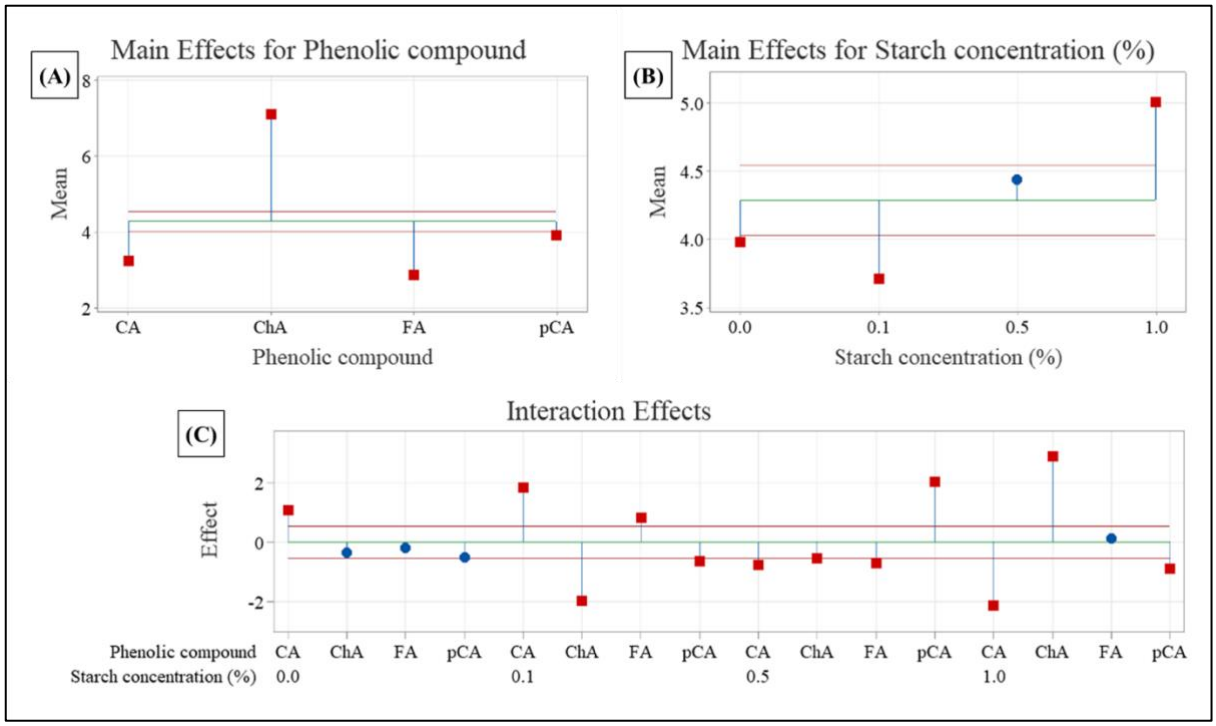

Figure 3-4: Results of two-way ANOVA on EC 50 according to type of phenolic compound (A), starch concentration (B) and starch x phenolic interactions (C). Horizontal lines represent the mean (green) and 95 % confidence interval (red). Data points are either individual point (interaction effects) or mean points by variable (individual effects of starch concentration and type of phenolic compound) and are coded as blue circles if they are not significantly different from the mean ($p < 0.05$), or as red squares if they are significantly different from the mean ($p > 0.05$).

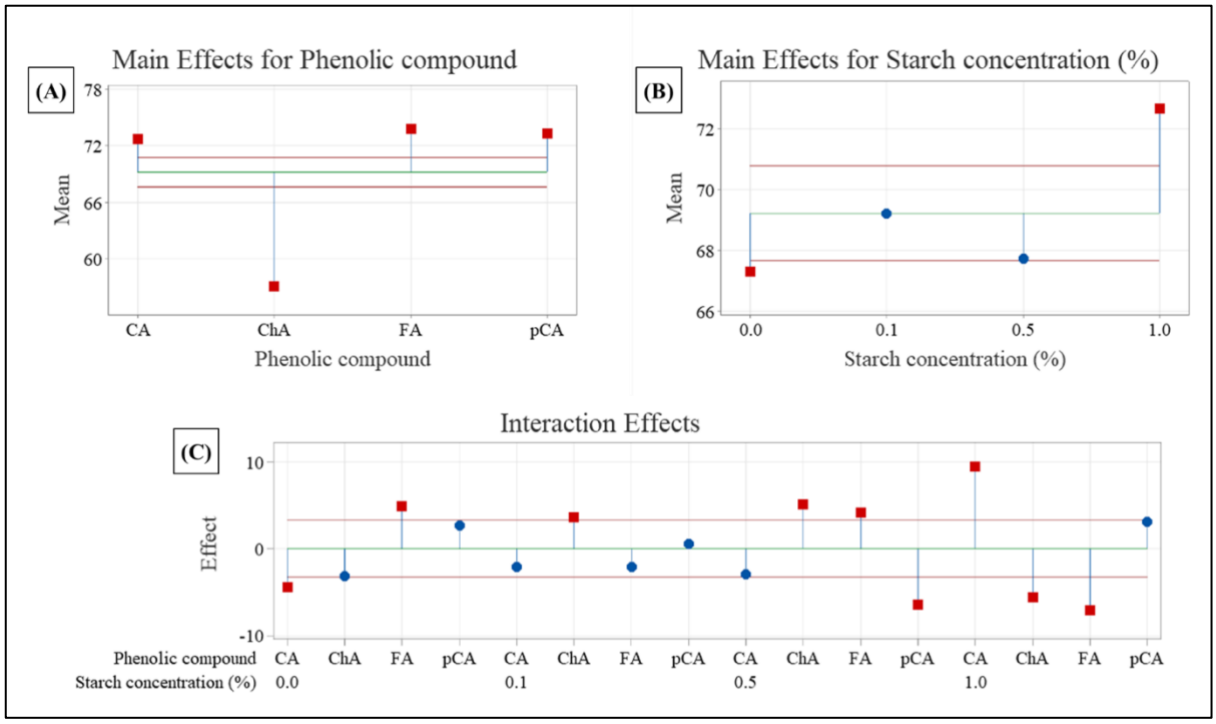

Figure 3-5: Results of two-way ANOVA on RI%max according to type of phenolic compound (A), starch concentration (B) and starch x phenolic interactions (C). Horizontal lines represent the mean (green) and 95 % confidence interval (red). Data points are either individual point (interaction effects) or mean points by variable (individual effects of starch concentration and type of phenolic compound) and are coded as blue circles if they are not significantly different from the mean ($p < 0.05$), or as red squares if they are significantly different from the mean ($p > 0.05$).

The results of two-way ANOVA by phenolic compound and across all starch concentrations (***Figure 3-4*** A and ***Figure 3-5*** A) confirmed the observations in section ***3.6.2*** without starch, that ChA was the weakest inhibitor of lipase among the four phenolic acids of this study (highest EC_{50} and lowest $RI\%_{max}$ on average).

The results of two-way ANOVA by starch concentration and across all phenolic compounds (***Figure 3-4*** B and ***Figure 3-5*** B) were contrasted regarding the effect of starch concentration

on the inhibitory effect of phenolic acids. Indeed, mean EC_{50} across all phenolic compounds (***Figure 3-4*** B) increased significantly from 0-0.1 % starch (both significantly lower than the overall mean), to 0.5 % starch (not significantly different from the overall mean), and to 1 % starch (significantly higher than the overall mean). This suggests that increasing starch concentration limited the inhibitory capacity of phenolic acids towards lipase. In contrast, mean $RI\%_{max}$ across all phenolic compounds (***Figure 3-5*** B) increased significantly from 0 % starch (significantly lower than the overall mean), to 0.1-0.5 % starch (both not significantly different from the overall mean), and to 1 % starch (significantly higher than the overall mean). This suggests that increasing starch concentration enhanced the inhibitory capacity of phenolic acids towards lipase. These seemingly contradicting results may be taken with caution, considering the magnitude of the effects observed and considering that statistical differences may not reflect experimentally meaningful differences. Indeed, the difference between the lowest and the highest mean EC_{50} values (for 0.1 and 1 % starch, respectively) was of 1.1 mM, i.e. 26 % of the overall mean EC_{50} value (4.2 mM), whereas the difference between the lowest and the highest mean $RI\%_{max}$ values (for 0 and 1 % starch, respectively) was of 5.4 %, i.e. 7 % of the overall mean $RI\%_{max}$ value (69.4 %). Therefore, it is unlikely that the effect of starch concentration on $RI\%_{max}$ was experimentally meaningful, contrary to its effect on EC_{50}. It is therefore likely that overall, increasing starch concentration limited the inhibitory capacity of phenolic acids towards lipase.

Nonetheless, these overall observations do not reflect the diversity of results when phenolic compounds were considered individually (***Figure 3-2*** and ***Figure 3-3***). With increasing starch concentration, EC_{50} of CA decreased from 4.0 ± 0.1 to 1.9 ± 0.2 mM, whereas its $RI\%_{max}$ increased from 66.4 ± 0.5 to 85.6 ± 4.9 %, from 0 to 1 % starch. ChA seemed to follow the opposite trend, with EC_{50} increasing from 4.6 ± 0.6 to 10.7 ± 1.4 mM and $RI\%_{max}$ decreasing

from 60.7 ± 9.9 to 55.0 ± 1.3 % (from 0.1 to 1 % starch and excluding 0 % starch). On the other hand, the EC_{50} and $RI\%_{max}$ of FA and pCA seemed to be generally constant from 0 to 1 % starch. For all the phenolic acids, the variations of EC_{50} and $RI\%_{max}$ with increasing starch concentration were self-consistent: when EC_{50} did not vary significantly, $RI\%_{max}$ did not vary significantly (case of FA and pCA); when EC_{50} increased, $RI\%_{max}$ decreased (decreasing inhibition, case of ChA); when EC_{50} decreased, $RI\%_{max}$ increased (increasing inhibition, case of CA). The cases of FA and pCA were consistent with either an absence of interaction of the phenolic acids with starch or an interaction that significantly weaker with starch than it was with lipase. The case of ChA suggests that significant starch-ChA interactions occurred that would affect lipase-ChA interactions and in turn decrease ChA's ability to inhibit lipase, which was consistent with the underlying hypothesis of this work. Only the case of CA was surprising, as its ability to inhibit lipase seemed to increase with increasing starch concentration. To explain these paradoxical observations, we may consider the relative affinity of the phenolic acids with starch as well as their partitioning coefficient. Indeed, it could be thought that a phenolic acid with limited water solubility and interacting weakly with starch would be concentrated in the liquid phase between starch colloidal particles in suspension, thereby increasing its apparent concentration and its ability to bind with and inhibit lipase. This could be in agreement with a former study by Han *et al*. (2020) [51]: their study concluded that CA may be too hydrophilic to form complexes with starch, particularly through inclusion complexes, as increasing concentrations of amylose led to decreased complexation between CA and maize starch [51]. These observations and conclusions could resonate well with the present study that used potato starch containing 30 % w/w of glucan chains with DP > 37 and therefore prone to engaging in inclusion complexes. The interactions of starch with the other phenolic acids of this study were scarcely studied (particularly pCA), but some results offer insights into the results of the present study. For example, it has been found that FA does not form complexes

with starch [46]. Taking into account that FA is poorly water soluble and unlikely to interact with lipase, this could explain why FA did not have any effect on EC_{50} and $RI\%_{max}$ here. Finally, starch-ChA complexes have been largely documented (particularly with lotus starch), which is consistent with our observation that the presence of starch decreased ChA's ability to inhibit lipase [47–50]. Finally, it is also possible that viscosity of the test solutions played a role in the inhibition as they were obtained. Indeed, from 0.1 to 1 % starch, viscosity of the test media increased drastically. This could have influenced diffusion of the reagents and their ability to bind. Whereas this factor not controllable in such experiment, it is possible that to some extent, inhibition of lipase at 0.5-1 % starch is composed of actual inhibition (due to phenolic-lipase binding) and apparent inhibition (due to starch-related physical effects). Overall, these considerations suggest that beyond the ability of phenolic acid to form complexes with starch, microphase separation or diffusion phenomena may become relevant to lipase inhibition when macromolecules such as starch are introduced in the system.

3.7. Conclusions

In conclusion, the present study aimed to investigate the impact of potential phenolic-starch interactions on the inhibitory capacity of phenolics on pancreatic lipase, by using starch from potato and phenolics such as CA, ChA, pCA and FA. We have observed that CA, pCA and FA were equivalently weak inhibitors of pancreatic lipase, and that ChA was even weaker than them. The inhibitory capacity of these phenolic acids towards pancreatic lipase seemed to correlate very well with simulated binding energy between lipase and the phenolic compounds on the one hand, and the phenolic acids' solubility on the other hand, which seems logical as lipase displays activity in lipid-aqueous biphasic systems. Additionally, starch exhibited contrasted effects on the phenolics' ability to modulate lipase activity: whereas ChA inhibitory activity seems disfavoured by the presence of starch, the opposite was true for CA, whereas

pCA and FA were unaffected by the presence of starch. These results provide information about the ability of potato phenolics to complex with and inhibit pancreatic lipase in presence of potato starch: generally, potato starch had marginal influence on the inhibition of pancreatic lipase activity by CA, ChA, FA and pCA, which was very limited anyways. We may extrapolate these results by considering that potato phenolics may be of very little use to modulate lipid digestion in fatty potato-base products, due to their limited effect on lipase itself and due to the further limiting effect brought by complexation with starch in some cases. This is important information as many phenolic compounds are evaluated *in vitro* for their enzyme inhibiting properties with little consideration for the food matrix they come with and the food components that are present along with the phenolic-lipase system. Therefore, this study provides more complete information about the biochemical properties of phenolic compounds in food systems during digestion.

Finally, this study has been the opportunity to revisit the *in vitro* digestion protocols used to evaluate the lipase inhibiting properties of phenolic compounds and assert the need to conduct such tests at physiological pH (6.5-7.0 in the case of digestion's intestinal phase).

3.8. Acknowledgements

This work was funded by the Natural Science and Engineering Research Council of Canada – Discovery Grant #2018-04009. Authors REI was financially supported by University of Ottawa's UROP program. The authors confirm that they have no other conflict of interest with respect to the work described in this manuscript.

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Chapter 4: Impact of Phenolic Timing and Starch Characteristics on α-Amylase Inhibition: Unravelling Mechanistic Insights and Nutritional Implications.

Chapter 4 : Impact of Phenolic Timing and Starch Characteristics on α-Amylase Inhibition: Unravelling Mechanistic Insights and Nutritional Implications.

4.1. Published contributions

D'Costa AS, Golding BA, Raval MK, Rolland-Sabaté A, Bordenave N. Probing gallic acid–starch interactions through Rapid ViscoAnalyzer in vitro digestion. *Food Research International*. 2023; 173:113409.

doi:10.1016/j.foodres.2023.113409

- A.S. D'Costa, with the supervision from N. Bordenave, conceptualised the study, collected data, literature, conducted calculations and statistical analysis, and completed the writing up, with review and editing of subsequent versions. Author N. Bordenave obtained funding for this study.
- B.A. Golding & M.K. Raval, with the supervision of A.S. D'Costa, assisted with data collection.
- Author A. Rolland-Sabaté, assisted with work completed at INRAE, and review and editing of accompanying sections.

4.2. Abstract

Phenolic compounds are known inhibitors of starch digestion through binding with α-amylase. However, a growing body of research shows that phenolic-starch interactions at the molecular level may interfere with this inhibition potential. In this study, we evaluated the effect of Gallic

Acid (GA) as a model phenolic compound on starch digestion kinetics carried out *in vitro* in a Rapid ViscoAnalyzer (RVA). The results showed that when GA was added before cooking of starch in order to promote starch-GA complexation, the rate of digestion of starch was similar to that of starch alone, and faster than when GA was added after cooking of starch. The results demonstrated that when GA was introduced after cooking of starch, GA inhibited α-amylase strongly and that inhibition increased with starch paste viscosity only for potato and wheat starches. No correlation was found between starch molecular characteristics and the inhibiting capacity of GA at different starch concentrations. However, the apparent influence of starch chain length distribution suggested that physical effects (such as the absorption of GA at the surface of the starch paste) may play a role in the capacity of GA to inhibit α-amylase.

4.3. Keywords

Starch; *in vitro* digestion; RVA; Gallic Acid; Inhibition; Complexation; Modelling.

4.4. Introduction

This paper describes the effect of interactions between starch on the one hand, and gallic acid (GA) on the other hand, on digestion kinetics of cooked starch pastes according to their concentration and viscosity.

Phenolic compounds and polysaccharides are often found co-existing in the same food sources (e.g., whole grain cereals), in formulated foods (e.g., granola bars with berries or cocoa), or co-ingested during meals (e.g., oatmeal with coffee or tea). *In vitro*, phenolic compounds have been shown to alter starch digestion kinetics by binding with and inhibiting digestive enzymes, salivary and pancreatic α-amylases, in particular [1–4], thereby holding the potential to decrease the glycaemic response to these types of foods *in vivo* [5–7]. However, phenolic compounds have

also been shown to bind non-covalently with polysaccharides [8,9], which can then make the phenolic compounds less available to bind with and inhibit glucose transporters [10], digestive enzymes [11] and α-amylases in particular [12]. Starch being a polysaccharide, starch-phenolic binding can occur, either by adsorption onto and absorption into granular starch [13] or by complexation with gelatinised starch [14–16]. Therefore, binding between starch and phenolic compounds could make the phenolic compounds less available to bind with and inhibit digestive α-amylases and could potentially and paradoxically limit the effects of the phenolic compounds on starch digestion kinetics. Indeed, it has been shown that through complexation with phenolics, starch itself paradoxically inhibited the inhibition of its own digestion by the phenolic compounds [17–19].

Despite the valuable insights brought by these studies and given the ever-growing interest about using phenolic compounds as modulators of glycaemia, numerous questions remain to understand further the effects of starch-phenolic interactions on starch digestion kinetics. Firstly, it has been shown that starch's macromolecular characteristics (e.g., molar mass, amylose/amylopectin ratio, chain length distribution) influence its ability to complex with phenolic compounds [20–25]. Therefore, studying these effects with starches containing a wide range of structural features is warranted. Secondly, in the aforementioned studies, the formation of starch-phenolic complexes was intentional. However, the timing of addition of phenolics to starch relatively to its cooking and digestion may affect the formation of these complexes, and therefore affect starch digestion kinetics. Furthermore, different timings of addition of phenolics to starch can simulate different dietary scenarios (e.g., co-ingestion of sources of starch and phenolics, co-formulation of starch and phenolics; sequential ingestion of sources of starch and phenolics, etc.). Therefore, studying the effect of timing of addition of phenolics to starch is warranted as well. Thirdly, starchy foods can be ingested in various forms that

affect their properties and the digestion kinetics of starch. This is particularly the case when starch is cooked, for example in porridges, which can vary dramatically in viscosity [26]. Beyond the effect of starch viscosity on its digestion kinetics, viscosity of starch is likely to affect its binding capacity with phenolic compounds, by modulating the diffusion of phenolic compounds through the starch paste, or through the competition between starch-starch and starch-phenolic interactions, for example. Therefore, studying the effect of starch paste viscosity is also warranted.

From these unanswered questions, we hypothesised that in paste form, starch itself could limit the ability of phenolic compounds to inhibit digestive α-amylases, and that this ability could be affected by paste viscosity, timing of addition of phenolics to the starch paste, and macromolecular characteristics of starch.

To test this hypothesis, we have used four different starches representative of common diets (maize, wheat, rice, and potato) and a model phenolic compound (GA, a known inhibitor of α-amylase). Phenolic compounds were introduced prior to or after cooking of starch in order to favour or disfavour starch-phenolic interactions, and starch was subjected to an *in vitro* digestion procedure with a Rapid ViscoAnalyzer (RVA), according to a method developed previously [27]. Starch digestion kinetics were then characterised by the decrease of RVA viscosity of starch in order to understand the effect of the presence and the time of addition of the phenolic compound.

4.5. Materials and methods

4.5.1. Chemicals and reagents

Gallic acid (GA, 98 % purity) was purchased from Alfa Aesar (Ward Hill, MA). Ethylgallate (EGA, synthesis grade, 98 % purity), maize, potato, rice, and wheat starches were purchased from Sigma-Aldrich (St Louis, MO). Porcine pancreatic α-amylase, reagents for PBS buffer (Na_2HPO_4, NaH_2PO_4, NaCl), dimethyl sulfoxide (DMSO) as well as salts, HCl and NaOH used for *in vitro* digestion were purchased from Sigma-Aldrich (Saint-Louis, MO).

For starch characterisation, isoamylase (isoamylase E-ISAMY from *Pseudomonas* sp., 200U.mL^{-1} in 3.2 M ammonium sulphate) and pullulanase (pullulanase M1 from *Klebsiella planticola*, 650U/mL in 3.2 M ammonium sulphate) were purchased from Megazyme (Bray, Ireland). Pullulan standards (from P5 to P800) were purchased from Showa Denko K.K. (Tokyo, Japan). Oligoglucan standards of DP1, DP2, DP3, DP6 and DP7 were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Oligoglucan standards of DP4 and DP5 were purchased from Elicityl (Crolles, France).

4.5.2. Starch characterisation by Size-Exclusion Chromatography

Starches were characterised for their molar mass, radius of gyration and chain length distributions according to the following methods.

4.5.2.1. Preparation of samples and solubilisation

For each type of starch, 0.5 g of starch was first dissolved in 20 mL 95% dimethyl sulfoxide (DMSO) with magnetic stirring for 5 days at room temperature. The sample was precipitated with 200 mL ethanol and stored overnight at 4 °C. The precipitate was then purified by three

successive washings in ethanol and recovered by centrifugation. The precipitate was finally washed with acetone and recovered by centrifugation. The pellet was then dried for seven days with a desiccant agent. 10 mg of purified starch was suspended in 20 mL of filtered ultrapure water (0.1 μm pore diameter), microwaved for 40 s at 900 W [28–30] and filtered (5 μm pore diameter) before injection for analysis in a high-performance size exclusion chromatography (HPSEC) coupled with multi-angle laser light scattering (MALLS), viscometry (VS), and differential refractive index (DRI) detection.

For chain length distribution analysis, starch samples were debranched according to the following procedure. 40 mg of purified starch was boiled for 30 min under stirring in 4 mL filtered ultrapure water. 1 mL of this starch solution was incubated for 40 h at 40 °C with 505 μL of a 50 mM acetate buffer at pH 3.6 with 0.02% sodium azide, 34 μL of a commercial isoamylase suspension and 13 μL of a commercial pullulanase suspension (commercial suspensions described in section ***4.5.1***). The resulting solution of debranched starch was filtered (0.45 μm pore diameter) before injection for analysis in a HPSEC-DRI system.

4.5.2.2. HPSEC analysis

Starch and debranched starch samples were analysed with a HPSEC system consisting of an Ultra-Fast Liquid Chromatography Prominence system including a LC-2OAD pump, a DGU-20A5 degasser, a SIL-20ACHT autosampler, and a CTO-20 AC column oven, all from Shimadzu K. K. (Kyoto, Japan).

Separation of 100 μL of each starch sample was achieved at 30 °C using one HPSEC column (Shodex KW802.5, 300 × 8 mm) and a guard column KW-G, both from Showa Denko K.K.

(Tokyo, Japan), with a MilliQ water mobile phase containing 0.2 $g.L^{-1}$ sodium azide, filtered (0.1 µm pore diameter) and degassed, eluting at 0.6 $mL.min^{-1}$.

Separation of 400 µL of each debranched starch sample was achieved at 30 °C using a set of PolySep-GFC-P3000, PolySep-GFC-P5000 and PolySep-GFC-P6000 (300 × 7.8 mm) HPSEC columns all from Phenomenex (Le Pecq, France), with a 0.01 M KOH in MilliQ water mobile phase, filtered (0.1 µm pore diameter) and degassed, eluting at 0.5 $mL.min^{-1}$.

Detection was achieved with a Viscostar viscosimeter and a DAWN Heleos 8+ MALLS detector fitted with a K5 flow cell and a GaAs laser at λ = 660 nm (Wyatt Technology, Santa Barbara, CA), and a RID-10A differential refractometer from Shimadzu K. K. (Kyoto, Japan).

Total analysis recovery was calculated from the mass of sample weighed and the mass of sample recovered after elution. For all samples analysed, total recovery was ranging between 66 and 100 %.

For starch samples, weight-average intrinsic viscosity mass ($\overline{[\eta]_w}$), number-average molar mass ($\overline{M_n}$), weight-average molar mass ($\overline{M_w}$), dispersity ($\overline{M_w}/\overline{M_n}$), z-average radius of gyration $\overline{R_G}$ and average branching degree BD_{Hm} (using corrected, modified ABC model) were all established for the amylopectin fraction using ASTRA v7.1.4 software from Wyatt Technology (Santa Barbara, CA) as previously described [29,30]. A refractive index increment value of 0.146 $mL.g^{-1}$ was used and the normalization of photodiodes was achieved using a monomeric BSA from Sigma-Aldrich (Saint-Louis, MO). For debranched starch samples, chain length distribution was characterised from the elution profiles of standard oligoglucans (DP1 to DP7) and pullulans (P5 to P800).

4.5.3. Sample preparation and *in vitro* digestion procedure

Starches with or without GA/EGA were subjected to an *in vitro* digestion procedure developed by Gamel *et al.* [31] with minor modifications as described by Northrop *et al.* [27,32] and without pancreatin and pepsin, as only the activity of α-amylase was targeted in this study with starch being the only digestible component of the test samples. Starches were subjected to *in vitro* digestion as pastes after a cooking procedure described thereafter. Starch pastes ranged 2-10 % w/w on dry weight basis.

Briefly, solutions of 1.5 M GA or EGA in DMSO were prepared fresh every day, stored at 4 °C and protected from light when not in use. Starch was cooked in an RVA canister with the sodium phosphate/NaCl buffer. Depending on the type of sample prepared, 83.3 µL of either sodium phosphate/NaCl buffer, GA solution or EGA solution were added to starch in the sodium phosphate/NaCl buffer prior to cooking starch or after cooking starch so that the total and final water content of the test samples amounted to 25 mL and the final concentration of GA/EGA was 5 mM (accounting for added buffered solutions, water/moisture in starch measured with an infra-red heated balance, and subsequent addition of α-amylase solution). Starch with or without GA/EGA was cooked according to the following temperature profile in the RVA: temperature was set at 37 °C, and when the target temperature was reached, the mixing paddle spun at 400 rpm for 10 s and was slowed down to 160 rpm throughout the rest of the experiment; then, the temperature was brought up to 90 °C at 15 °C.min^{-1} and held at 90 °C for 6 min; then, the temperature was brought down to 37 °C at 15 °C.min^{-1} and held at 37 °C. The *in vitro* digestion procedure was then started by the addition of 100 µL of 125 U.mL^{-1} α-amylase in 2.5 mM $CaCl_2$, resulting in final concentration of α-amylase of 0.5 U.mL^{-1}. Starting 20 s after α-amylase was introduced due to instrument limitations, viscosity of the

starch paste was measured every 4 seconds for 30 min. Digestograms of the samples were generated as plots of apparent RVA viscosity at 160 rpm against time.

4.5.4. Digestogram modelling

Digestograms obtained from the *in vitro* digestion procedure (plot of RVA viscosity against time) were first modelled according to the method described by Northrop *et al.* [27]: experimental data were fitted according to a first-order model viscosity decrease:

$$\eta_{model_1}(t) = (\eta_{(t=0)} - \eta_{(t=\infty)})e^{-kt} + \eta_{(t=\infty)} \qquad \textit{Eq. 4-1}$$

η_{model_1} is the modelled viscosity of starch being hydrolysed.

$\eta_{(t=\infty)}$ is the modelled viscosity of starch after an infinite time of digestion.

$\eta_{(t=0)}$ is the modelled viscosity of starch at the beginning of digestion when amylase is introduced.

k is the characteristic first-order rate of digestion.

t is time of digestion.

$\eta_{t=0}$, $\eta_{(t=\infty)}$ and k are model parameters that were determined empirically as described in section ***4.5.5***.

The same digestograms were also modelled according to a general sigmoidal model as follows:

$$\eta_{model\,2}(t) = \eta_{(t=\infty)} + \frac{\eta_{(t=0)} - \eta_{(t=\infty)}}{1 + e^{k \times \ln({}^{t}/_{t_{half}})}} \qquad \textit{Eq. 4-2}$$

$\eta_{model\,2}$ is the modelled viscosity of starch being hydrolysed.

$\eta_{(t=\infty)}$ is the modelled viscosity of starch after an infinite time of digestion.

$\eta_{(t=0)}$ is the modelled viscosity of starch at the beginning of digestion when amylase is introduced.

k is a characteristic measure of the rate of digestion. Increasing values of k model increasingly long initial and final plateaus of viscosity as well as increasingly steep decrease of viscosity between the plateaus.

t is time of digestion.

t_{half} is the time by which starch has decreased by 50 % of its initial viscosity.

$\eta_{t=0}$, $\eta_{(t=\infty)}$, t_{half} and k are model parameters that were determined empirically as described in section ***4.5.5***.

To evaluate and compare the goodness of fit of these two models, the relative difference between each model value and the experimental value was calculated for each digestogram and at each time point as follows:

$$\delta_1 = \left| \frac{\eta_{experimental} - \eta_{model\ 1}}{\eta_{experimental}} \right| \times 100 \qquad \textit{Eq. 4-3}$$

$$\delta_2 = \left| \frac{\eta_{experimental} - \eta_{model\ 2}}{\eta_{experimental}} \right| \times 100 \qquad \textit{Eq. 4-4}$$

For each digestogram, an average difference between experimental and model values (Δ_1 and Δ_2) was calculated over the entire duration of the digestion experiment (Δ_i is the average of δ_i over a single entire digestogram). Then, all Δ_1 and Δ_2 values obtained over all the digestions

performed in this work were tested for statistically significant difference with a Student's t-test and their average values $\overline{\Delta_1}$ and $\overline{\Delta_2}$ were calculated.

4.5.5. Numerical and statistical analysis

All samples were analysed in triplicate. All results reported here are therefore the average ± Standard Deviation of three independent measurements. Regression modelling of digestograms with $\eta_{model\ 1}(t)$ and $\eta_{model\ 2}(t)$ was performed on each digestogram with the Solver function of Microsoft Excel 2016 following a Least Squares Sum method, providing modelling parameters and fit of the regression model as Standard Error of the Regression (SER, in cP). One-way ANOVA tests and pairwise comparisons of digestion model parameters were performed with Fisher post-hoc tests ($p < 0.05$) with Minitab 19 for Windows (Minitab LLC, State College, PA).

4.6. Results and discussion

4.6.1. Characterisation of starches

This study was designed to investigate the binding of GA with starch as evidenced by changes of the effect of GA on the activity of α-amylase. As starch was the only structurally variable compound of this experimental design (sourced from potato, wheat, maize, or rice), possible binding events observed in this study could be influenced by the fine structure of the different starches used. Therefore, the macromolecular characterisation of these starches was warranted, and achieved by HPSEC-MALLS-VS-DRI. The macromolecular characteristics of starches as well as amylopectin and amylose making them up are reported in ***Table 4-1*** (intact starches) and ***Table 4-2*** (debranched starches).

As shown by the data in ***Table 4-1*** and ***Table 4-2***, these four starches, beyond being the most common sources of starch in human diet, covered a wide range of characteristics: their intrinsic viscosity mass $\overline{[\eta]_w}$ ranged from 88.4 to 130 mL.g^{-1}, their amylopectin's molar mass $\overline{M_w}$ ranged from 130 to 317×10^6 g.mol^{-1}, their radius of gyration $\overline{R_G}$ ranged from 180 to 226 nm, their apparent density $d_{G_{app}}$ ranged from 6.0 to 10.0 g.mol^{-1}.nm^{-3} and their average branching degree BD_{Hm} ranged from 3.1 to 7.0 %. These four starches were therefore adequate to study possible variations in binding with GA, according to their molecular characteristics.

Table 4-1: Macromolecular characteristics of wheat, maize, potato, and rice amylopectins and starches determined by HPSEC-MALLS-VS-DRI

Sample	Total starch	Amylopectin fraction				
	$\overline{[\eta]_w}$ (mL.g^{-1})[a]	$\overline{M_w} \times 10^6$ (g.mol^{-1}) [a]	$\overline{M_w}/\overline{M_n}$	$\overline{R_G}$ (nm)[a]	$d_{G_{app}}$ (g.mol^{-1}.nm^{-3})	BD_{Hm} (%)
Wheat starch	130.0	316.6	1.2	226.5	7.9	5.5
Maize starch	88.4	216.1	1.2	184.2	10.0	7.0
Potato starch	129.1	129.8	1.1	180.3	6.0	3.1
Rice starch	87.1	264.7	1.3	206.7	9.3	6.7

$\overline{[\eta]_w}$: weight-average intrinsic viscosity, calculated over the whole starch peaks (amylose and amylopectin fractions).

$\overline{M_w}$: weight-average molar mass, calculated over the whole amylopectin peak.

$\overline{M_w}/\overline{M_n}$: dispersity, calculated over the whole amylopectin peak.

$\overline{R_G}$: z-average radius of gyration, calculated over the whole amylopectin peak.

BD_{Hm}: average branching degree obtained from $\overline{M_w}$ and R_G data using the modified ABC model corrected according to the Hizukuri model [30] and calculated over the whole amylopectin peak.

[a] Pooled standard deviations were 1.6% for $\overline{[\eta]_w}$, 4% for $\overline{M_n}$ and $\overline{M_w}$, and 1.4% for $\overline{R_G}$.

Table 4-2: Chain length distribution of wheat, maize, potato, and rice starches determined by HPSEC-DRI after debranching.

Sample	Chain length fractions of starch [a]		
	DP < 37 (amylopectin short branches)	**DP 37-100 (amylopectin long branches)**	**DP > 100 (amylose)**
Wheat starch	72	3	25
Maize starch	75	3	22
Potato starch	71	9	20
Rice starch	82	3	15

DP: Degree of Polymerization

[a] composition given as % w/w on total starch basis.

4.6.2. Starch digestion kinetics

4.6.2.1. Preliminary experiments

GA was chosen as a model phenolic acid to study starch-phenolic acid interactions and their effect on inhibition of α-amylase. Several experiments had to be carried out to ensure the validity of the main experimental set and interpret our results through the lens of starch-GA interactions. In particular, we had to ensure that GA was stable through heat treatments and did not degrade, so potential degradation products did not interfere with the interpretation of experimental results. As *in vitro* digestion kinetics were measured through RVA viscosity, we had to ensure that GA did not affect the viscosity of starch pastes themselves, so that in the main experimental set, variations of viscosity and of starch digestion kinetics could be

attributed only the to the effect of GA on α-amylase activity. To achieve these control experiments, we used EGA, chosen for its structural proximity with GA and its lack of capacity to inhibit α-amylase, and we compared pasting profiles and digestion kinetics of starch alone, starch with GA, and starch with EGA.

Stability of GA through cooking: In a first series of samples, 6% starch (potato, maize, rice, or wheat) underwent the cooking procedure in the RVA canister without GA, while a solution of GA underwent the same heat treatment and for the same duration as starch, but separately. GA solution was then added to the starch paste so as to match the concentrations of starch and GA used in the main experimental set of this study. After thorough mixing in the RVA, paste viscosity was measured and *in vitro* digestion was carried out. A second series sample was prepared as the second sample, except that the solution of GA was freshly prepared and not heat treated. For both series samples and for each type of starch, there was no change of colour (degradation products of GA at pH 7 are blue to yellow coloured) as well as no significant difference in RVA paste viscosity and in starch digestion kinetics, indicating that GA retained its functionality and did not undergo significant degradation through the heat treatment applied to starches in the RVA. Consequently, we assumed that no hydrothermal degradation of GA interfered with the other experiments of this study.

Effect of GA and EGA on viscosity of starch pastes and on *in vitro* digestion: First, buffer or buffered solution containing GA or EGA was added to starches in the RVA canister before proceeding with the cooking procedure. No significant difference was detected between the viscosities of the resulting starch pastes. Secondly, starches were subjected to the RVA cooking procedure after which buffer or buffered solution containing GA or EGA was added to the starch paste in the RVA. For each starch, no significant difference was detected between the

viscosities of the resulting starch pastes (starch only, or starch with GA, or starch with EGA). Additionally, starches with and without EGA underwent the RVA cooking procedure followed by *in vitro* digestion. No significant difference was found in parameters of *in vitro* digestion kinetics obtained with or without EGA. These results indicated that GA did not affect starch paste viscosity. Therefore, it is unlikely that GA induced significant supramolecular arrangements of starch (such as the formation of extra junction zones, or aggregation, or changes in starch conformation), which would be evidenced by physical effect such as changes in paste viscosity. Therefore, variations in starch digestion kinetics observed in the rest of this study were unlikely to be due to such indirect effects of GA on starch supramolecular arrangements.

The results of these preliminary experiments are available in ***Supplementary Information (A1).*** Together, they indicate that samples containing starch only and starch with GA could be compared directly to understand starch-GA interactions and their effect on RVA viscosity and starch digestion kinetics.

4.6.2.2. Digestogram modelling

Kinetics of amylase hydrolysis of starch are usually fitted with a first-order kinetic model represented in ***Eq. 4-1*** [27,33,34]. While this was adequate for all starches alone in our experimental set, numerous digestograms of starches in presence of GA exhibited an initial plateau or lag phase of hydrolysis that could not be fitted with a first-order kinetic model, even though the later phases of hydrolysis could. Therefore, we devised a new model and evaluated its goodness of fit. We chose a general sigmoidal model represented in ***Eq. 4-2*** (see section ***4.6.2.2***. in Material and Methods) and illustrated in ***Figure 4-1***.

For each digestogram and at each time point, the relative difference between each model value and the experimental value was averaged over each complete digestogram (Δ_1 and Δ_2 for model 1 and model 2, respectively). These values were tested for statistically significant difference over all the digestions performed in this work with a Student's t-test, and their average values $\overline{\Delta_1}$ and $\overline{\Delta_2}$ were calculated. Over all the digestions performed in this work (n = 112), $\overline{\Delta_1}$ and $\overline{\Delta_2}$ were significantly different ($p < 0.01$) with $\overline{\Delta_1}$= 9.2 ± 9.7 % and $\overline{\Delta_2}$= 3.0 ± 2.2 %.

Across all digestograms, with or without GA, the sigmoidal model was therefore within 3.0 ± 2.2 % of corresponding experimental data on average, and it provided a significantly better fit than the simple first-order kinetic model at 95 % confidence. Modelling data are available in ***Supplementary Information (A1)***.

We chose to use this sigmoidal model for further analysis and interpretation of the experimental set-up on the effect of GA and viscosity on starch digestion kinetics. It must be noted that to the best of our knowledge, this fitting model has never been used in published work before and is distinct from fitting models usually used for starch digestion kinetics [27,33,34]. However, the development of this model was a necessity due to the inadequate fitting of usual first-order kinetic models when α-amylase inhibition was significant. This particular model was chosen so as to provide parameters (such as k and t_{half}) that would directly and practically help describe and understand starch digestion kinetics. It must be noted that while this sigmoidal model was adapted to cases of strong inhibition of digestion, it provided an excellent fit for control digestograms without inhibition, making it relevant and potentially usable for modelling a wide range of starch digestion conditions, although the introduction of a $1/e^{k\times\ln t}$ term containing logarithm of time doesn't allow a natural interpretation of digestion kinetics as opposed to first-order kinetic models which contain a simple $1/e^{kt}$ term.

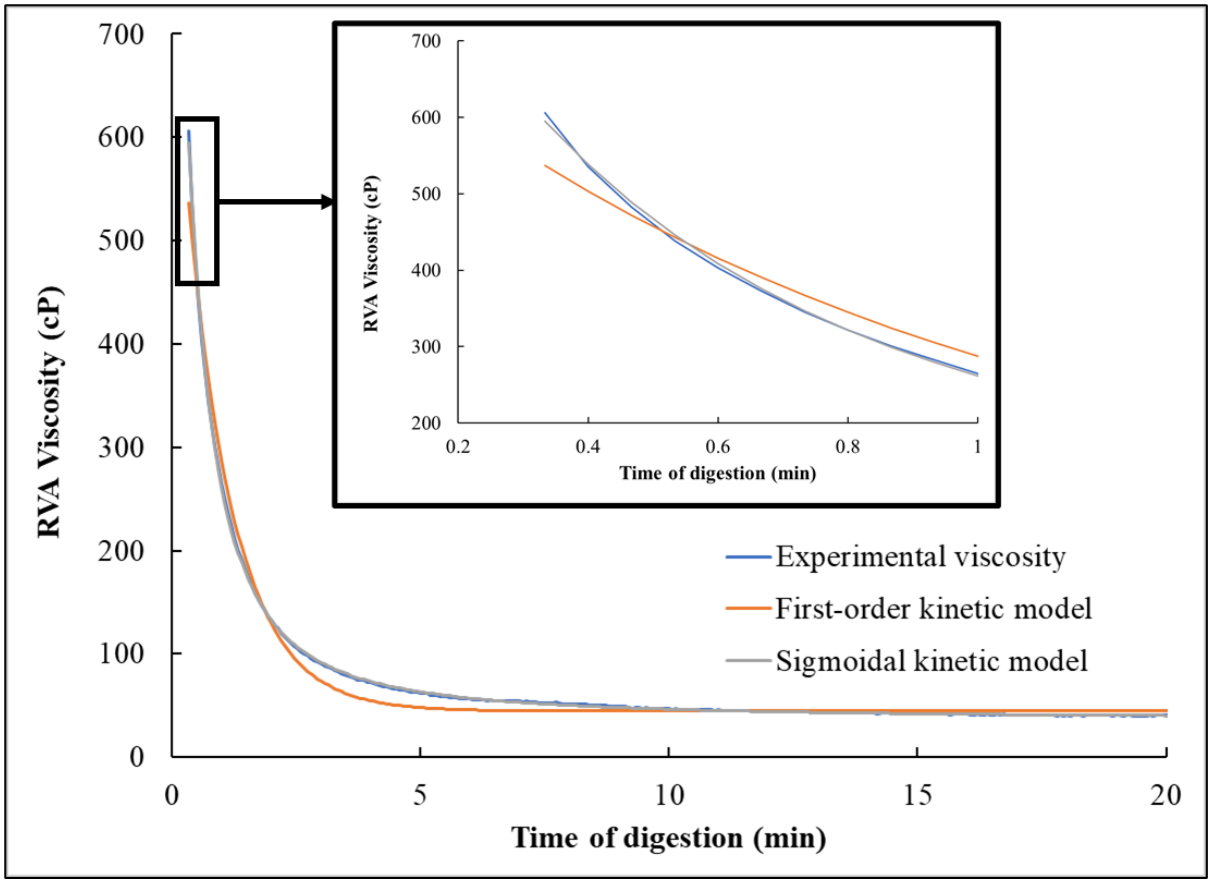

Figure 4-1: Illustration of fitting of experimental RVA viscosity data (blue line) with a first-order kinetic model (orange line) and a generalised sigmoidal model (grey line). The insert represents the same data over the first minute of digestion. These data were taken from the digestion of 6 % w/w potato starch without GA.

4.6.2.3. Effect of time of addition of GA on inhibition of α-amylase by GA

To test the possible occurrence of starch-GA binding, 6 % w/w starch pastes (potato, rice, wheat, and maize) were digested in presence of GA that was added into the RVA canister either before cooking of starch (in order to promote starch-GA interactions), or after cooking of starch, at the same time as α-amylase. These samples were compared with control starch pastes without GA. Although the pastes exhibited drastically different viscosities, it was chosen to

operate at constant starch and GA concentrations (6 % w/w and 5 mM, respectively) so possible starch-GA interactions could be comparable.

The digestograms that were obtained were modelled according to the sigmoidal model detailed in section ***4.6.2.2***, and modelling parameters $\eta_{(t=0)}$, t_{half} and *k* were reported for each sample type. The results are shown in ***Figure 4-2*** ($\eta_{(t=0)}$), ***Figure 4-3*** (*k*) and ***Figure 4-4*** (t_{half}).

Across all starches, parameters $\eta_{t=0}$ and *k* exhibited some statistical differences but none of them were deemed experimentally meaningful: within each type of starch all $\eta_{t=0}$ and *k* values never differed by more than 20 %. One exception was $\eta_{(t=0)}$ for potato starch where $\eta_{t=0}$ in the case of GA added after cooking was 42 % higher than when GA was added before cooking, and 35 % higher than the control. As the three samples had the same potato starch concentration and therefore the same starting viscosity before α-amylase was added, this difference could not be due to differences in α-amylase activity related to its diffusion within the viscous starch paste. Rather, this seems to indicate differences in α-amylase activity related to the availability of GA to bind and inhibit α-amylase, with α-amylase being effectively inhibited by GA only when GA was added after the cooking of starch. This is consistent with previous studies showing that promoting starch-phenolic interactions decreased the phenolics' ability to inhibit α-amylase [35].

This is consistent with the observations made on t_{half} parameter. Indeed, going from the least viscous to the most viscous starch pastes (rice, wheat, maize, potato, from left to right on ***Figure 4-3***), t_{half} is first significantly higher for the digestion with GA compared to the control, showing some inhibition of α-amylase by GA (in the case of rice starch). Then, the difference between the control and the samples with GA added before cooking disappeared (all

within 30 % of each other for each type of starch), whereas t_{half} of the samples with GA added after cooking became significantly higher than the other, with the difference increasing progressively. From the least viscous starch to the most viscous, t_{half} of the samples with GA added after cooking was:

- 1.7, 1.6, 5.6 and 13.2 times higher than the control (ratio of t_{half} for starch pastes with GA added after cooking over t_{half} of the control)
- 0.9, 1.5, 4.4 and 10.2 times higher than when GA was added before cooking (ratios of t_{half} for starch pastes with GA added after cooking over t_{half} of starch pastes with GA added before cooking)

These ratios were highly correlated with starch paste's initial viscosity (R^2 = 0.98 and 0.97 for the respective linear correlations). There was no such correlation between with the ratio of t_{half} values of the control and starch pastes with GA added before cooking, which were generally not significantly different. Therefore, there may be a significant association between the initial paste's viscosity and the GA's ability to inhibit α-amylase when GA was added after cooking of starch.

To explain these observations, we hypothesise that: (1) when GA was added prior to starch cooking, GA-starch interactions were promoted and reduced GA's availability to bind with and inhibit α-amylase, potentially through non-covalent binding as observed before; (2) when GA was added after starch cooking, the paste's viscosity made it difficult for GA to interact with starch and made it more available for binding with and inhibition of α-amylase. To explain this latter point, it is possible that simple viscosity effects were at play, limiting diffusion of GA through the paste, as previously shown by Zhang *et al.* [36]. It is also possible that starch-GA

interactions were disfavoured by starch-starch interactions. Indeed, it has been shown before that GA tends to bind primarily with amylose and amylopectin through hydrogen bonds involving the hydroxyl groups of the macromolecules. The formation of starch pastes involves the formation of intermolecular junction zones driven by hydrogen bonds and the hydroxyl groups of the starch macromolecules. The formation of starch paste may then reduce the number of available binding sites for GA. However, GA's ability to inhibit α-amylase when it was added after cooking was correlated with the paste's initial viscosity and not with starch's amylose content (or any other starch's structural features). Therefore, it is likely that our observations were only due to viscosity/diffusion effects. In this case, the interpretation of the results may differ according to the paste's initial viscosity. Indeed, starch-GA binding could occur and limit GA's ability to inhibit α-amylase at lower viscosities, when GA can diffuse more easily through starch paste than at high viscosities. However, in the sample set studied in this section, only rice and wheat starches exhibited relatively low viscosities and this hypothesis could not be tested. Therefore, a study of the effect of starch paste's viscosity on GA's ability to inhibit α-amylase when GA was added after cooking was warranted.

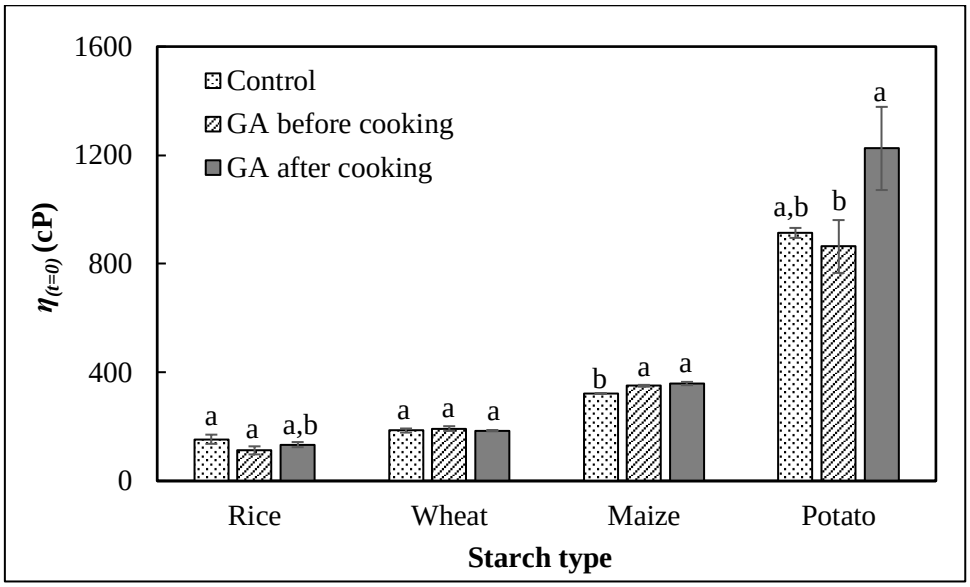

Figure 4-2: Modelling parameter η_((t=0)) of digestion of 6 % w/w starch pastes (from left to right: rice, wheat, maize, potato). Within each type of starch, bars from left to right represent the control, GA added to starch before cooking, GA added to starch after cooking. Data are reported as mean ± standard deviation. Within each type of starch, letters indicate the results of one-way ANOVA: bars sharing letters are not significantly different ($p < 0.05$).

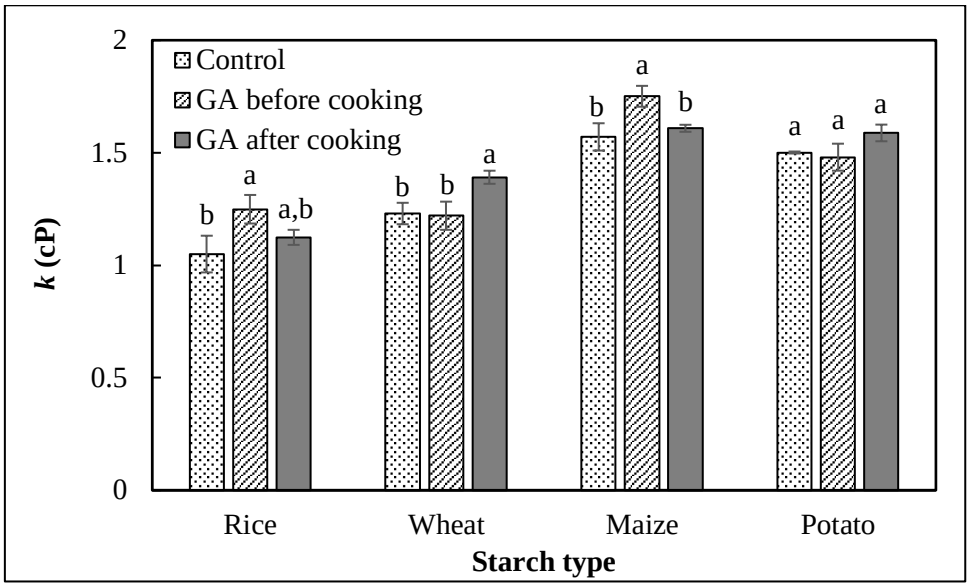

Figure 4-3: Modelling parameter k of digestion of 6 % w/w starch pastes (from left to right: rice, wheat, maize, potato). For each type of starch, bars from left to right represent the control, GA added to starch before cooking, GA added to starch after cooking. Data are reported as mean ± standard deviation. Within each type of starch, letters indicate the results of one-way ANOVA: bars sharing letters are not significantly different ($p < 0.05$).

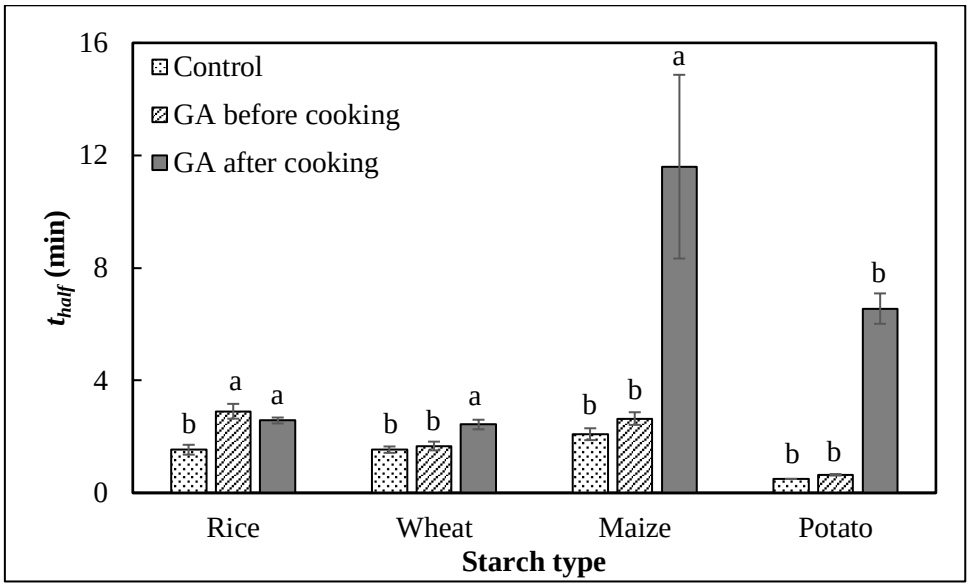

Figure 4-4: Modelling parameter t_half of digestion of 6 % w/w starch pastes (from left to right: rice, wheat, maize, potato). For each type of starch, bars from left to right represent the control, GA added to starch before cooking, GA added to starch after cooking. Data are reported as mean ± standard deviation. Within each type of starch, letters indicate the results of one-way ANOVA: bars sharing letters are not significantly different ($p < 0.05$).

4.6.2.4. Effect of starch concentration on inhibition of α-amylase by GA added to cooked starch after starch cooking

To test the potential existence of GA-starch binding events limiting GA's ability to inhibit α-amylase, we measured starch digestion kinetics in presence of GA added after cooking, over a large range of starch pastes' viscosities for each type of starch.

To produce starch pastes with viscosities ranging approximately 10^2 to 10^3 cP, different concentrations of starch were used for each starch: 6-10 % w/w for rice and wheat, 2-6 % w/w

for potato and 4-9 % w/w for maize. The pastes (with no GA as a control, or with GA added after starch cooking) were subjected to *in vitro* digestion in the RVA canister and the digestograms were modelled as in section ***4.6.2.3***. The results are shown in ***Table 4-3***.

As in section ***4.6.2.3***, $\eta_{(t=0)}$ and *k* exhibited some statistically significant differences between samples with GA and control. However, most of these differences were not experimentally relevant, such as $\eta_{(t=0)}$ values of 49 vs 57 cP for 2 % w/w potato starch, or 510 vs 554 cP for 7 % w/w maize starch, or k values 1.50 vs 1.59 cP.min-1 for 6 % w/w potato starch. Nonetheless, $\eta_{(t=0)}$ values for potato and rice starch at their highest concentrations showed statistically and experimentally significant differences, with $\eta_{(t=0)}$ increasing significantly with the addition of GA. This increase suggest that digestion was significantly slowed down in presence of GA. Indeed, the experimental protocol imposed a 20 second delay between the introduction of α-amylase in the canister and the beginning of viscosity measurement throughout digestion. During these 20 seconds, digestion could start for the control and materialise as a lower viscosity than the same sample with GA where α-amylase inhibition could happen. This interpretation of the impact of GA on $\eta_{(t=0)}$ value is supported by the statistically and experimentally significant increase of t_{half} in presence of GA compared to the controls (for all samples but one), which is evidence of slower digestion and α-amylase inhibition.

t_{half} increased by factors ranging from 1.3 to 16.5 in presence of GA compared to the controls, with each type of sample with GA compared with the same sample without GA. There was no consistent effect of GA across the four types of starch. Rather, the behaviour of the ratio $t_{half-with\ GA}/t_{half-control}$ with starch paste viscosity seemed to be specific to each type of

starch. For wheat starch, $t_{half-with\ GA}/t_{half-control}$ increased regularly from 1.6 to 2.0 over 6 to 9 % w/w starch and increased sharply to 4.1 for 10 % w/w wheat starch. For potato starch, $t_{half-with\ GA}/t_{half-control}$ increased sharply from 1.8 to 16.5 from 2 to 5 % w/w potato starch and plateaued over 5-6 % w/w potato starch. In contrast, $t_{half-with\ GA}/t_{half-control}$ remained constant around 1.3-2.2 for rice and maize starches, with the exception of a sharp increase up to 5.5 for 6 % w/w maize starch, which seems to be an artefact, although the measurement was replicated and the standard deviation of the t_{half} values was satisfactory.

Table 4-3: Modelling parameters of starch digestograms for starch alone (control) and starch with GA added after the cooking procedure (with GA), for rice, potato, wheat, and maize starches. $\eta_{t=0}$, $\eta_{(t=\infty)}$, t_{half} and k are model parameters determined from the fitting of digestograms according to *Eq. 4-2*, and SER is the Standard Error of Regression (fit). All results are reported as mean ± standard deviation of three replicates. Asterisk next to results indicate significant differences ($p < 0.05$) between model parameters of the control and the same sample

Starch concentration (% w/w)	Modelling parameters									
	$\eta_{(t=0)}$ (cP)		k (cP.min^{-1})		t_{half} (min)		$\eta_{(t=\infty)}$ (cP)		SER (cP)	
	Control	With GA	Control	With GA	Control	With GA	Control	With GA	Control	With GA
Rice starch										
6 %	154 ± 14	134 ± 8	1.05 ± 0.07	1.12 ± 0.03	1.54 ± 0.14	2.57 ± 0.09 *	6 ± 0	6 ± 1	0.58 ± 0.08	0.55 ± 0.06
7 %	260 ± 19	266 ± 8	1.21 ± 0.05	1.32 ± 0.05 *	1.90 ± 0.05	2.41 ± 0.03 *	10 ± 1	13 ± 3	0.66 ± 0.05	0.68 ± 0.02
8 %	398 ± 38	442 ± 22	1.32 ± 0.00	1.47 ± 0.02 *	1.96 ± 0.03	4.33 ± 0.18 *	12 ± 1	7 ± 2	1.28 ± 0.08	2.48 ± 0.11
9 %	721 ± 3	832 ± 16 *	1.45 ± 0.00	1.50 ± 0.05	2.26 ± 0.01	4.79 ± 1.81	11 ± 0	4 ± 0 *	1.28 ± 0.02	2.48 ± 0.13
10 %	902 ± 26	1158 ± 5 *	1.44 ± 0.01	1.53 ± 0.00	1.89 ± 0.04	4.07 ± 0.14 *	15 ± 1	0 ± 0 *	3.66 ± 0.27	8.86 ± 0.48
Potato starch										
2 %	49 ± 0	57 ± 0 *	2.15 ± 0.05	2.33 ± 0.05	0.83 ± 0.00	1.53 ± 0.01 *	2 ± 1	12 ± 0 *	0.37 ± 0.02	0.45 ± 0.01
3 %	100 ± 1	194 ± 14 *	1.89 ± 0.01	1.32 ± 0.03 *	0.84 ± 0.02	3.19 ± 0.20 *	4 ± 1	9 ± 1 *	0.66 ± 0.01	1.18 ± 0.06
4 %	367 ± 56	314 ± 5	1.43 ± 0.02	1.39 ± 0.03	0.45 ± 0.05	1.88 ± 0.04 *	13 ± 1	18 ± 1 *	1.51 ± 0.05	2.16 ± 0.04
5 %	591 ± 21	556 ± 21	1.52 ± 0.04	1.66 ± 0.09	0.52 ± 0.05	8.59 ± 0.76 *	24 ± 1	0 ± 0 *	1.51 ± 0.26	8.94 ± 0.11
6 %	914 ± 13	1225 ± 108	1.50 ± 0.00	1.59 ± 0.03 *	0.50 ± 0.00	6.55 ± 0.38 *	37 ± 0	0 ± 0 *	1.20 ± 0.04	9.51 ± 3.12
Wheat starch										
6 %	186 ± 6	185 ± 2	1.23 ± 0.04	1.39 ± 0.02 *	1.54 ± 0.09	2.43 ± 0.14 *	9 ± 1	11 ± 1	0.76 ± 0.06	0.82 ± 0.02
7 %	364 ± 11	364 ± 9	1.41 ± 0.00	1.57 ± 0.11	1.09 ± 0.00	1.82 ± 0.51 *	20 ± 2	22 ± 0	1.00 ± 0.10	1.06 ± 0.02
8 %	611 ± 5	652 ± 1 *	1.78 ± 0.01	1.78 ± 0.03	1.31 ± 0.02	2.29 ± 0.02 *	38 ± 0	42 ± 0 *	0.95 ± 0.02	3.07 ± 0.14
9 %	1068 ± 12	1163 ± 19 *	1.78 ± 0.02	1.67 ± 0.03	1.34 ± 0.01	2.63 ± 0.10 *	69 ± 1	95 ± 1 *	0.95 ± 0.30	3.07 ± 0.36
10 %	1927 ± 26	2003 ± 16	1.32 ± 0.04	1.37 ± 0.17	1.22 ± 0.03	5.04 ± 0.21 *	104 ± 1	63 ± 15 *	15.41 ± 0.68	18.66 ± 0.43
Maize starch										
4 %	70 ± 1	75 ± 0 *	2.35 ± 0.01	2.12 ± 0.04 *	4.32 ± 0.01	8.12 ± 0.09 *	10 ± 0	9 ± 1	0.73 ± 0.03	0.77 ± 0.02
5 %	170 ± 1	177 ± 7	1.58 ± 0.01	1.69 ± 0.00 *	2.97 ± 0.05	4.81 ± 0.20 *	9 ± 0	13 ± 1	0.84 ± 0.03	0.75 ± 0.06
6 %	322 ± 1	359 ± 4 *	1.57 ± 0.05	1.61 ± 0.01	2.09 ± 0.18	11.60 ± 2.31 *	13 ± 1	2 ± 0 *	0.88 ± 0.06	1.28 ± 0.01
7 %	510 ± 3	554 ± 2 *	1.58 ± 0.01	1.62 ± 0.01	2.62 ± 0.08	5.58 ± 0.36 *	20 ± 1	9 ± 2 *	0.88 ± 0.15	1.28 ± 0.09
9 %	1106 ± 2	1133 ± 23	1.42 ± 0.01	1.42 ± 0.01	2.89 ± 0.01	3.93 ± 0.07 *	22 ± 0	19 ± 2	5.16 ± 0.01	3.75 ± 0.19

Therefore, the inhibition of α-amylase by GA effectively increased with increasing initial viscosity of the starch paste in the cases of potato and wheat, whereas it varied minimally in the cases of rice and maize. One could hypothesise that increasing viscosity decreased the diffusion of GA through the starch paste, making it more available to inhibit α-amylase. Although this would be consistent with the observations of the previous section (addition of GA pre- and post-cooking at constant starch concentration) and previous studies [36], there is no evidence that viscosity itself played a direct role in the results of this section. Indeed, whereas the maximum viscosity of wheat starch paste was higher than the maximum viscosity of the other pastes (≈ 2,000 cP vs 900-1,100 cP), the maximum viscosities of rice and maize starch pastes were comparable to that of potato starch pastes. The effect of paste viscosity on inhibition of α-amylase by GA may then be altered by other effects. Due to the design of the experiments reported in this paper and the preliminary experiments reported in sections ***4.6.2.1***, it seems that only binding of GA by starch could explain the results obtained here and a possible interference with simple and straightforward viscosity-diffusion effects.

It is therefore possible that starch and α-amylase competed for binding with GA, which led to moderate α-amylase inhibition at low starch paste viscosity regardless of the type of starch. Differential effects appeared at higher starch concentration/viscosity where wheat and potato starches on the one hand, and rice and maize starches on the other hand behaved differently as far as inhibition of α-amylase by GA was concerned. It may be plausible that rice and maize starches have a greater ability to bind GA than potato and wheat starches. Under this hypothesis, rice and maize starches may capture GA more efficiently than potato and wheat starches, despite poor diffusion of GA within the gel. This hypothesis could be consistent with apparent inconsistencies in the magnitude of the effects of GA at high starch concentration. Indeed, starch digestion seemed to be slowed very significantly (t_{half} increased by a factor of

up to 16.5), which is inconsistent with the generally accepted property of GA as a weak inhibitor of α-amylase [37]. This discrepancy may then be explained by other effects of GA that were previously reported and that are consistent with the experimental conditions of the current study. Indeed, it has been shown that at high concentrations (not physiologically relevant), GA may not only inhibit α-amylase but denature it by interfering with its tertiary structure [13]. Here, GA was introduced as 83.3 µL of a 1.5 M solution along with 100 µL of the α-amylase solution. If GA was not absorbed by the starch pastes, it created a significant overconcentration of GA relatively to α-amylase in the added liquid phase which could led to the aforementioned denaturation and subsequent abnormally high inhibition. Therefore, our results are consistent with a possible difference in the ability of different starches or starch pastes to bind GA, possibly driven by starches' fine structure. Indeed, wheat and potato starches on the one hand, and rice and maize starches on the other hand, could be grouped according to their intrinsic viscosity (129-130 and 88-97 $mL.g^{-1}$ respectively) and their ratio of short glucan chains (DP < 37, from amylopectin) to long glucan chains (DP > 37, amylose and long amylopectin chains), with wheat and potato starches containing respectively 28 and 29 % w/w of long chains, and maize and rice containing respectively 25 and 18 % w/w of long chains. With a higher proportion of long chains and a more elongated conformation of starch macromolecules, wheat and potato starches used in this study were potentially more prone to intermolecular interactions and formation of junction zones than rice and maize starches [38]. Through increased formation of junction zones, an increased concentration of long chains could then decrease the availability of glucosidic chains to bind GA (as starch long chains are already engaged in intermolecular interactions) or decrease the absorption and diffusion of small molecules within the paste (due to a finer mesh of the polymeric network). This latter hypothesis may be preferred as it would be consistent with previous work that showed that the competition of starch and α-amylase for phenolic compounds may be mainly driven by physical effects than

by binding affinity, as the affinity of GA for α-amylase is very significantly higher than that of starch for GA [35,36]. This would be consistent with previous studies that modified the apparent capacity of GA to inhibit α-amylase through physical treatments of starch [17–19].

To test these hypotheses, statistical relationship between all experimental variables available were explored. Multivariate analysis of variance (MANOVA) was used to establish a relationship between several dependent variables (rate of starch digestion, time of half-digestion, extent of digestion) and several independent variables (molecular characteristics of starch, concentration of starch or paste viscosity). The results of MANOVA were inconclusive. Multivariate Regression Analysis (up to second order) was also attempted but only generated models with non-significant parameters ($p>0.05$) and poor goodness of fit (less than 30 % of the variance of the dependent variables explained by the variance of independent variables). Therefore, direct relationship between the characteristics of our systems (molecular characteristics of starch, concentration of starch) and their digestion patterns could be confidently ruled out, in particular between starch-GA binding on the one hand and starch paste viscosity on the other hand. However, this does not mean there is no valid relationship at all and our observations could be due to other factors having a significant contribution to paste viscosity, in particular macromolecular structures remaining after cooking of starch, such as ghost granules [39]. A deeper mechanistic understanding of the effect of starch breakdown on its binding with GA is therefore warranted.

4.7. Conclusions

Through *in vitro* digestion procedures carried out in an RVA instrument, we have demonstrated that the timing of addition of GA to cooked starch impacts its ability to inhibit α-amylase, as a result of starch-GA interactions. When GA was added after cooking of starch, GA's ability to inhibit α-amylase was then strongly dependent on the viscosity of the starch paste, although inconsistencies of the results across different types of starches suggest that paste viscosity is not the only driver of GA's availability to bind and inhibit α-amylase. These results are consistent with recent studies suggesting that the inhibition of starch digestion by phenolic compounds depends on competitive binding of the phenolic compounds with starch and α-amylase. The current study also demonstrated that physical effects may also be at play, favoured by starch macromolecular structure and conformation, and may even hinder the effects of binding at the molecular level.

Beyond the understanding of the three-way relationship between starch, phenolics, and α-amylase, these results could also have consequences from the point of view of nutrition. Indeed, phenolic compounds have been intensely studied for their potential as anti-hyperglycaemic compounds. However, the literature shows many inconsistencies in the results and this potential nutritional property. The present study showed that these effects may indeed be significantly affected by the concentration and viscosity of starchy foods ingested as well as the timing of ingestion of the source of phenolic compounds. Indeed, the experimental design used in this study suggests that GA may have drastically different effect on the rate of digestion of starch whether GA is included in the starchy food or GA is ingested (for example in a beverage) separately from the starchy food. In this latter case, the effect may also vary according to the viscosity of the starchy food and the ability of the beverage to mix in a homogeneous bolus with starch in the stomach. Although the current study was performed *in*

vitro, the results are sufficiently contrasted to warrant further studies to understand the nature of starch-GA-amylase interactions (and starch-phenolics-amylase more generally) at the molecular or supramolecular level, and their consequences *in vivo*. Such mechanistic studies could shed a new light on the potential hypoglycaemic effect of phenolic compounds and its realistic applicability in functional foods as it remains uncertain, and how it may be affected by strong matrix effects.

Finally, the specific experimental design of this study called for the development of a new fitting model for starch digestion kinetics, when α-amylase inhibition was significant enough to prevent the use of classic first-order kinetics models. This model involves empirical parameters that are directly related to the interpretation of starch digestion kinetics. This model could then be used as a generalised kinetic model for a wide range of starch digestion conditions.

4.8. Acknowledgements

This work was funded by the Natural Science and Engineering Research Council of Canada – Discovery Grant #2018-04009. Author NB is a member of the Scientific Advisory Board of Agrifiber LLC (Mundelein, IL). Authors BAG and MR were financially supported by the MITACS Globalink program. HPSEC-MALLS-VS-DRI measurements were made in TERSYS Federative Research Structure (Platform 3A project, CPER 2014-2020), supported by the French government, the department of Vaucluse, Avignon university, PACA region, European funds for regional development and CNRS.

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Chapter 5: Impact of Phenolic Compounds on β - Glucan Viscosity in Oat Bran: Insights into *In-Vitro* Digestion and Implication for Nutritional Properties.

Chapter 5 : Impact of Phenolic Compounds on β-Glucan Viscosity in Oat Bran: Insights into *In-Vitro* Digestion and Implications for Nutritional Properties.

5.1. Published contributions

Northrop G, D'Costa AS, Tosh SM, Bordenave N. Viscosity development from oat bran β-glucans through in vitro digestion is lowered in the presence of phenolic compounds. *Food Funct*. 2022;13(7):3894-3904.

doi:10.1039/D2FO00162D

- A.S. D'Costa, with the supervision from N. Bordenave, collected data, literature, conducted calculations and statistical analysis, and completed the writing up, with review and editing of subsequent versions.
- G. Northrop, with the supervision from S.M. Tosh and N. Bordenave, collected data, literature, conducted calculations and statistical analysis and completed writing up with review and editing of subsequent versions.
- N. Bordenave and S.M. Tosh conceptualised the study, N. Bordenave obtained funding for the study, completed writing up and review and editing of subsequent version.

5.2. Abstract

Dietary fibres have been shown to aggregate, lose viscosity, and water binding capacity in solution in presence of phenolic compounds. This study aimed to verify this observation in a complex grain system containing β-glucans. The viscosity of uncooked and cooked oat bran

digested *in vitro* was measured in presence of 1-30 mM phenolic acids or flavonoids and the digestograms were modelled to understand the effects of phenolic compounds on the drivers of viscosity. The final viscosity of the digesta, driven by β-glucans underwent a significant decrease of up to 31 % upon addition of the phenolic compounds. To account for the inhibitory activity of phenolic compounds on digestive enzyme, modelling of the digestogram was adjusted from previous work. The models suggest that phenolic compounds can simultaneously: (1) slow down the release of β-glucans by slowing down digestion through enzyme inhibition, and (2) decrease the viscosity of solubilised β-glucans, likely through colloidal aggregation as observed in solution before. These *in vitro* results suggest that the health benefits of oats linked to digestive viscosity of β-glucans may be altered by co-formulation with or co-ingestion of phenolic compounds.

5.3. Keywords

β-glucans; viscosity; dietary fibres; cholesterol; glycaemic response; phenolic compounds

5.4. Introduction

This paper describes the development of digestive viscosity from oat bran β-glucans upon the addition of individual phenolic compounds in a validated, simulated *in vitro* digestion system.

Viscosity development of mixed-linkage β-glucans from oats and barley have been shown to drive many of the physiological effects and health benefits of β-glucan-rich cereals [1-3]. However, we have shown in previous work that β-glucans lose viscosity and water binding capacity in solution upon the addition of individual phenolic compounds at concentrations consistent with common food composition, through the formation of aggregated colloidal complexes [4,5]. Upon complexation with 1-30 mM of vanillin (Van), caffeic acid (CA), ferulic

acid (FA), gallic acid (GA), ethyl gallate (EG), epicatechin (EC), epicatechin gallate (ECG), or epigallocatechin gallate (EGCG), solutions of 0.75 % w/w β-glucans underwent a reduction of up to 95 % in viscosity. If this phenomenon was observed in regular food matrices rather than in solution, it may have important physiological and nutritional consequences. Indeed, if that was the case, the ability of β-glucans to develop intestinal viscosity may be reduced in situations of co-consumption of phenolic compounds and β-glucans (for example a breakfast consisting of oatmeal and tea or coffee), leading to potentially decreased health benefits.

However, the simple physical aggregation observed in solution between phenolics, and β-glucans may be hindered by other effects in a complex β-glucan-rich food matrix, such as oatmeal. For example, food components such as starch and proteins may compete with β-glucans for binding with phenolic compounds, thereby decreasing the effects of aggregation on viscosity. Digestion kinetics may also be altered by the phenolic compounds, thereby altering starch, protein, and matrix breakdown, and ultimately altering solubilisation of β-glucans, although the direction of this effect is difficult to predict. Indeed, phenolic compounds may bind with enzymes, thereby contributing to decreased enzyme activity. They may also bind with starch or β-glucans reducing viscosity of the digesta and in turn contributing to higher enzyme activity. Therefore, in order to better understand phenolic-β-glucan interactions and their potential impact on β-glucans' health benefits, we propose a study similar to the aforementioned approach (that was carried out in solution)[4,5], consisting in measuring the digestive viscosity of oatmeal in presence of the same phenolic compounds at equivalent concentrations (1-30 mM of vanillin (Van), caffeic acid (CA), ferulic acid (FA), gallic acid (GA), ethyl gallate (EG), epicatechin (EC), epicatechin gallate (ECG), or epigallocatechin gallate (EGCG)).

To this effect, several methods have been investigated to evaluate the development of viscosity from β-glucans through *in vitro* digestion[6-13]. One of these methods has been accepted as a standard method by Cereals & Grains Association and monitors viscosity continuously during simulated digestion in a Rapid Visco-Analyser (RVA)[14-16]. Digestive viscosity measured after 120 min by this method has been correlated with physiological effects of β-glucans (alteration of glycaemic response and LDL-cholesterol level, in particular)[17-20]. This method generates digestograms of the test food (digestive viscosity vs. time of digestion) that can be modelled and analysed, as is demonstrated in our previous work[21]. The two simultaneous processes occurring during digestion (namely a decrease of viscosity caused by digestion of starch and proteins and an increase of viscosity caused by the release and solubilisation of β-glucans in foods such as oat bran) can be described by first-order kinetic models and their parameters. We have also demonstrated that oat bran serves as a relevant model to study β-glucan viscosity development during *in vitro* digestion, owing to its high β-glucan content, moderate particle size, good repeatability, low signal-to-noise ratio throughout *in vitro* digestion[21] and practical relevance as a common breakfast food. Consequently, this method seems appropriate to evaluate the effect of added phenolic compounds on the viscosity development of oat bran's β-glucans through digestion.

Therefore, we characterised the *in vitro* digestograms of oat bran test samples in the presence of: (1) 1-10 mM of Van, CA, FA, EG, EC, ECG and EGCG to evaluate the effect of various phenolic compounds, (2) 1-30 mM GA, to assess the possible effect of phenolic concentration on viscosity development during simulated digestion of oat bran. For this latter part, GA, a phenolic acid bearing four groups able to engage in hydrogen bonds with soluble dietary fibres, was chosen as a model compound representative of a large number of low molecular weight phenolic compounds.

5.5. Materials and methods

5.5.1. Chemicals and reagents

Oat bran was purchased from a local grocery store in Ottawa, ON and stored in -20°C freezer until sampling. Oat bran was determined to contain 7.9 ± 0.2 % w/w β-glucan on dry weight basis following AACC Method 32-23.01 [22]. Gallic acid was purchased from Alfa Aesar (Ward Hill, MA). Vanillin (Van), ferulic acid (FA) and ethyl gallate (EG) were purchased from Sigma-Aldrich (St Louis, MO). (-)-epicatechin gallate (ECG), (-)-epicatechin (EC), and (-)-epigallocatechin gallate (EGCG) were purchased from Adooq Bioscience (Irvine, CA). Porcine α-amylase, pepsin and pancreatin, as well as salts, HCl and NaOH used for *in vitro* digestion were purchased from Sigma-Aldrich (Saint-Louis, MO).

5.5.2. Experimental design and sample preparation

Oat bran samples were used uncooked or cooked. In the case of cooked oat bran, cooking was achieved according to the manufacturer's instructions (3 min at 100 °C, stirred at 160 rpm) in an RVA 4500 Rapid Visco-Analyser instrument (Perten Instruments, Winnipeg, MB).

The following step of the experimental design was the simulated digestion of these samples alone (control) or in the presence of phenolic compounds. *In vitro* digestive fluids (described in section ***5.5.3***) were added to the samples to obtain a final β-glucan concentration of 7.5 g/L and phenolic compounds were added to the *in vitro* digestion fluids to obtain a final total concentration of 1-30 mM. In order to study the influence of different phenolic compounds on oat bran *in vitro* digestion, standard concentration of phenolic compounds was 10 mM for Van, EG, EC and EGCG, except for FA (3 mM) and ECG (1 mM) due to their limited solubility. To study the influence of phenolic compound concentration on oat bran *in vitro* digestion, GA

which was chosen as a model phenolic acid, which was introduced to obtain a final concentration of 1, 3, 10 and 30 mM.

5.5.3. *In vitro* digestion procedure

Oat bran products cooked or uncooked, alone or in presence of phenolic compounds (see section ***5.5.2***) were subjected to an *in vitro* digestion procedure developed by Gamel *et al.* [14] with minor modifications as described by Northrop *et al.*[21]

Briefly, the samples and *in vitro* digestive fluids were placed in RVA canisters. 20 mM sodium phosphate buffer (pH 6.9) with 10 mM NaCl (and phenolic compounds when required by the experimental design) were added so that the total water content (buffer and water/moisture in oat bran sample) amounted to 25 mL. Enzymes were then added: 63µL salivary amylase (200 $U.mL^{-1}$ in 2.5 mM $CaCl_2$), 150 µL pepsin (1150 $U.mL^{-1}$ in 0.9 % NaCl) and 300 µL pancreatin (0.5 $U.mL^{-1}$ in 20 mM sodium phosphate buffer). It must be noted that although pepsin is inactive at pH 6.9 in principle, it was shown during the design of the procedure that its incorporation produces results more consistent with the objectives of the *in vitro* digestion system.

The content of the RVA canister was then placed in the RVA, kept at 37^{o}C for 10 seconds with a mixing speed of 460 rpm and followed by 2 hours at 160 rpm. Viscosity was measured every 4 seconds for 2 hours. Digestograms of the samples were generated as plots of apparent RVA viscosity at 160 rpm vs. time.

5.5.4. Digestogram modelling

Digestograms obtained from the *in vitro* digestion procedure (plot of RVA viscosity vs. time) were modelled according to the method described by Northrop *et al.*[21] . Briefly, experimental data were fitted according to a viscosity model

$$\eta_{model}(t) = \eta_1(t) + \eta_2(t) \qquad \textit{Eq. 5-1}$$

where η_1 and η_2 were respectively viscosity decrease and viscosity increase components with

$$\eta_1(t) = \eta_{1(t=0)} e^{-k_1 t} \qquad \textit{Eq. 5-2}$$

$$\eta_2(t) = \eta_{2(t=\infty)} - A_{2\infty} e^{-k_2 t} \qquad \textit{Eq. 5-3}$$

$\eta_{1(t=0)}$, k_1, $\eta_{2(t=\infty)}$, $A_{2\infty}$ and k_2 are model parameters empirically determined as described in section ***5.5.5*** and shown in ***Figure 5-1***.

Time constants k_1 and k_2 were converted into times t_1 and t_2 were as

$$t_1 = \frac{ln2}{k_1} \qquad \textit{Eq. 5-4}$$

$$t_2 = \frac{ln2}{k_2} \qquad \textit{Eq. 5-5}$$

$\eta_{1(t=0)}$ is the contribution of starch and proteins to total viscosity at t = 0 min. k_1 is the rate of viscosity decrease associated with the η_1 component. t_1 is the characteristic time for which the η_1 component has undergone half of its decrease after infinite time of digestion.

$\eta_{2(t=\infty)}$ is the viscosity of the component η_2 after infinite time of digestion (named "infinite viscosity"). $A_{2\infty}$ is the amplitude of viscosity development for infinite time of digestion (named "amplitude of infinite viscosity development"). k_2 is the rate of viscosity decrease associated with the η_2 component. t_2 is the characteristic time for which the η_2 component has undergone half of its increase after infinite time of digestion. Consequently,

$$\eta_{2(t=0)} = \eta_{2(t=\infty)} - A_{\infty} \qquad \textit{Eq. 5-6}$$

is the contribution of β-glucans to total viscosity at t = 0 min.

Additionally, $\eta_{(t=0)}$ is the initial viscosity and is the sum of the two components at t = 0 min, i.e.,

$$\eta_{(t=0)} = \eta_{1(t=0)} + \eta_{2(t=0)}. \qquad \textit{Eq. 5-7}$$

Regardless of digestogram modelling, viscosity of the digesta after 120 min ($\eta_{t=120}$) is the actual viscosity of the digesta at the end of the experimental digestion procedure and is different from the parameter $\eta_{2(t=\infty)}$. This value of final viscosity is the value that has been correlated with physiological effects of β-glucans, such as reduction of cholesterol or glycaemic response.[14]

5.5.5. Numerical and statistical analysis

All samples were analysed in triplicate. All results reported here are therefore the average ± Standard Error of the Mean (SEM) of three independent measurements. Regression modelling

of digestograms was performed on each digestogram with the Solver function of Microsoft Excel 2016 following a Least Squares Sum method. Fit of the regression model is given as Standard Error of the Regression (SER, in cP). One-way ANOVA tests and pairwise comparisons were performed with Fisher post-hoc tests with Minitab 19 for Windows (Minitab LLC, State College, PA) in order to find significant differences between reported results ($p < 0.05$).

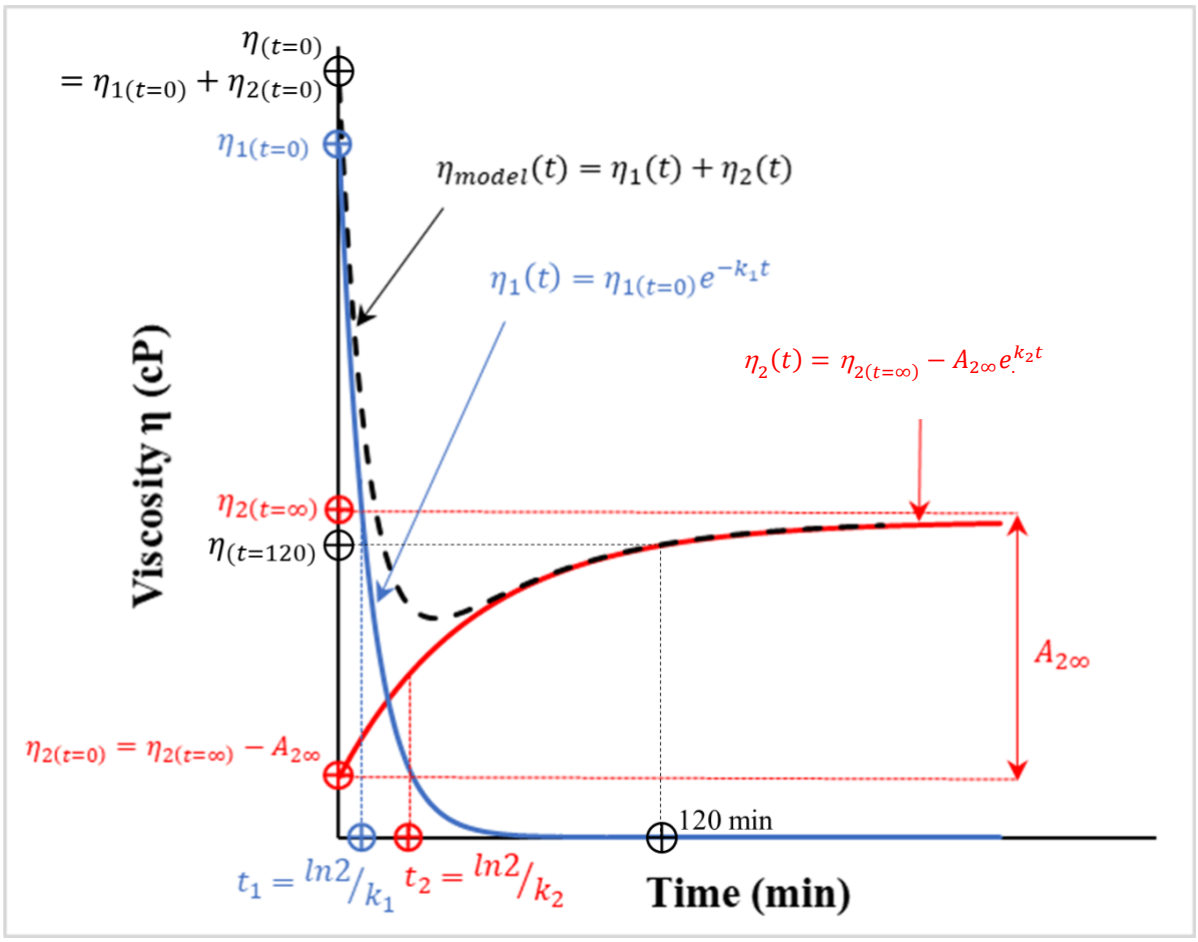

Figure 5-1: Representation of the proposed digestogram model: decreasing component of the model η_1 (blue line), increasing component of the model η_2 (red line) and total model (black dotted line). Model parameters $\eta_{1(t=0)}$, k_1, t_1, $\eta_{2(t=\infty)}$, $\eta_{2(t=0)}$, $A_{2\infty}$, k_2, t_2, $\eta_{(t=0)}$, as well as $\eta_{(t=120)}$ (digestive viscosity after 120 min of digestion) are represented on the axes.

5.6. Results and discussion

5.6.1. Effect of different phenolic compounds on the viscosity of oat bran through *in vitro* digestion

5.6.1.1. Uncooked oat bran

Uncooked and cooked oat bran were subjected to *in vitro* digestion in presence of Van, EG, EC, and EGCG (10 mM), as well as FA (3 mM) and ECG (1 mM) due to their lower solubility. Average digestograms of uncooked materials are shown in ***Figure 5-2***. All digestogram replicates were modelled and model parameters are reported in ***Table 5-1*** for uncooked material. GA (10 mM) was included in ***Figure 5-2*** and ***Table 5-1*** only for comparison purposes but is discussed in section ***5.6.2.1***.

All digestograms were adequately fitted with the viscosity increase component of the model (η_2) only, which is consistent with the uncooked state of oat bran. Indeed, in uncooked oat bran, bran particles contain starch and proteins in the form of insoluble crystalline granules and proteins bodies respectively, partially surrounded by indigestible cell walls. In this state and in absence of cooking, starch and protein do not contribute to the viscosity although they are partially and slowly digestible. Upon their digestion and the breakdown of the bran matrix, β-glucans are solubilised and contribute to the viscosity increase.

From the perspective of the β-glucans nutritional properties, $\eta_{t=120}$ is the critical experimental value of viscosity correlated with the functional properties of oat-based products. This value was significantly affected by the addition of phenolic compounds, showing a significant decrease ranging from 7 to 17 % from 81 ± 2 cP for the control. This observation suggests that phenolic compounds may limit β-glucans' capacity to develop digestive viscosity from

uncooked oatmeal and may limit oatmeal's well-known nutritional benefits (increased satiety, reduced cholesterol, etc.)[23,24].

This could simply be explained by a slower digestion induced by the phenolic compounds and their inhibitory capacity on digestive enzymes, a well-known effect of phenolic compounds[25-27]. Indeed, time of half infinite viscosity development t_2, a measure of digestion rate, was significantly increased by the addition of three phenolic compounds out of the seven tested, as shown in ***Table 5-1***. However, four other phenolic compounds decreased digestion rate (i.e., Van, FA, EG, EC). For these phenolics, the difference observed on $\eta_{t=120}$ with the control should be explained differently. To this effect, the other parameters of the digestogram models should be analysed.

The initial viscosity of the digesta $\eta_{(t=0)}$ (equal to $\eta_{2(t=0)}$ in this case) was not significantly affected by the addition of phenolic compounds (apart from FA and EGCG, but this statistical difference of 4 cP to the control seems experimentally irrelevant). On the contrary, infinite viscosity $\eta_{2(t=\infty)}$ and amplitude of infinite viscosity development $A_{2\infty}$ significantly decreased upon addition of the phenolics. Infinite viscosity decreased by 4 to 15 % from the control, matching the decrease observed with $\eta_{t=120}$. Additionally, amplitude of infinite viscosity development $A_{2\infty}$ decreased by 11 to 18 % from the control in presence of Van, EG and EC. Contrary to $\eta_{t=120}$, the parameters $\eta_{2(t=\infty)}$ and $A_{2\infty}$ are independent of the kinetics of digestion and β-glucans release, because they are characteristic of viscosity development until completion of digestion, regardless of how slow completion of digestion is achieved. Therefore, the decrease of $\eta_{2(t=\infty)}$ and $A_{2\infty}$ can't be attributed to limited release of β-glucans and can be attributed to the effect of phenolic compounds on β-glucans. This is consistent with our previous work which showed that soluble β-glucans aggregate and lose viscosity as well

as water holding capacity in solution in presence of phenolic compounds [4,5,28]. Although this inference would have been confirmed ideally by a direct observation of the aggregates, the use of light scattering techniques was made irrelevant here due to the heterogeneous nature of the digesta.

Another element supports the hypothesis of a digestive viscosity decrease due to phenolic-driven aggregation of β-glucans. In the present study, the magnitude of viscosity decrease tends to increase with higher molecular weight, more-prone-to-hydrogen-bonding phenolic compounds. This is very similar to the trend observed previously in solution and that supported the role of phenolic compounds in β-glucans aggregation [4,5].

Overall, these results on uncooked oatmeal suggest that at the concentrations used in this study, the phenolic compounds affected digestive viscosity after 120 min, mainly through aggregation of the β-glucans and marginally through slowing digestion. While this should be confirmed clinically, our observations suggest that in the upper gut, it is possible that phenolic compounds contribute to decreasing β-glucans' potential nutritional benefits while their nutritional benefits remain marginal.

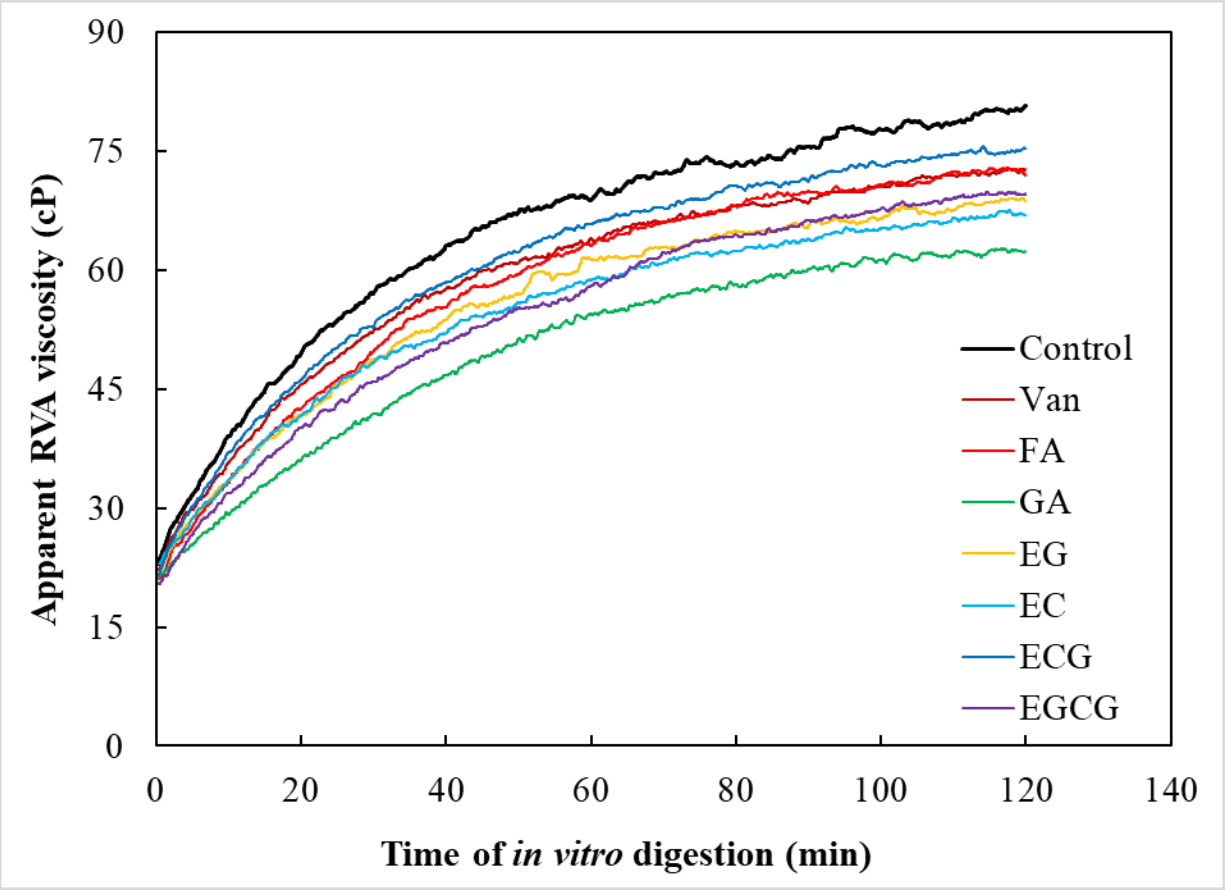

Figure 5-2: Digestograms of uncooked oat bran (control, black line) and uncooked oat bran in presence of 10 mM Van (dark red line), 3 mM FA (light red line), 10 mM EG (yellow line), 10 mM GA (green line), 10 mM EC (light blue line), 1 mM ECG (dark blue line) and 10 mM EGCG (purple line). Digestograms were obtained by *in vitro* digestion in an RVA instrument. Each digestogram is the average of three replicates. Concentrations of FA and ECG were limited to 3 and 1 mM respectively due to limitations of solubility. GA 10 mM is included only for illustration purposes.

Table 5-1: Model parameters of RVA viscosity η(t) during in vitro digestion of uncooked oat bran in presence of 10 mM Van, 3 mM FA, 10 mM EG, 10 mM GA, 10 mM EC, 1 mM ECG and 10 mM EGCG. SER is the Standard Error of Regression. Results are given as average ± SEM of three replicates. Letters within each column indicate results of one-way ANOVA: values sharing letters are not significantly different ($p < 0.05$).

Sample	Model parameters				$\eta_{2(t=0)}$ (cP)	t_2 (min)	$\eta_{(t=120)}$ (cP)
	$\eta_{2(t=\infty)}$ (cP)	$A_{2\infty}$ (cP)	k_2 (min^{-1})	SER (cP)			
Control	81 ± 2 a	55 ± 1 a	0.028 ± 0.002 a	1.0 ± 0.4	26 ± 2 a	25 ± 2 b	81 ± 2 a
Van 10 mM	74 ± 0 b,c	49 ± 1 b,c	0.028 ± 0.001 a	0.8 ± 0.1	24 ± 1 a,b	25 ± 1 b	73 ± 0 b,c
FA 3mM[a]	75 ± 1 b	53 ± 2 a,b	0.025 ± 0.002 a	0.6 ± 0.1	22 ± 0 b,c	28 ± 2 b	72 ± 1 c
GA 10 mM[b]	67 ± 1 d	46 ± 3 c,d	0.021 ± 0.001 b	0.7 ± 0.2	21 ± 2 c	33 ± 2 a,b	62 ± 0 e
EG 10 mM	71 ± 1 c,d	48 ± 1 c,d	0.026 ± 0.001 a	0.8 ± 0.1	23 ± 1 a,b,c	26 ± 1 b	69 ± 1 d
EC 10 mM	69 ± 2 d	45 ± 3 d	0.026 ± 0.002 a	0.6 ± 0.1	24 ± 0 a,b,c	27 ± 2 b	67 ± 2 d
ECG 1 mM[a]	77 ± 2 b	52 ± 2 a,b	0.027 ± 0.001 a	0.7 ± 0.2	24 ± 1 a,b	44 ± 12 a	75 ± 1 b
EGCG 10 mM	75 ± 2 b	53 ± 1 a,b	0.020 ± 0.001 b	0.8 ± 0.2	22 ± 1 b,c	35 ± 1 a,b	70 ± 0 c,d

[a] Concentrations of FA and ECG were limited to 3 and 1 mM respectively due to limitations of solubility.

[b] GA 10 mM is included only for illustration purposes.

5.6.1.2. Cooked oat bran

Average digestograms of cooked materials are shown in ***Figure 5-3***. All digestogram replicates were modelled and model parameters are reported in ***Table 5-2***. GA (1 mM) was included in ***Figure 5-3*** and ***Table 5-2*** only for comparison purposes but is discussed in section ***5.6.2.2***.

All digestograms of cooked materials were adequately modelled by the two model components η_1 and η_2. The initial viscosities were considerably higher than in uncooked materials, which was a result of cooking and starch gelatinisation as well as hydration of proteins. Therefore, hydrolysis of starch and protein during the digestion process had a considerable effect on the profile of the digestogram curve. Fitting of the digestogram with the complete model,

$$(\eta_1 + \eta_2) \qquad \textit{Eq. 5-8}$$

facilitated the quantification of the two separate processes that were occurring: decreasing viscosity due to digestive hydrolysis of starch and proteins; and increasing viscosity due to solubilisation of β-glucans.

Similar to uncooked oatmeal, viscosity after 120 min of digestion $\eta_{(t=120)}$ (which is the critical experimental value of viscosity correlated with the functional properties of oat-based products) was significantly lower with phenolic compounds than in the control, by 7 to 31 % from 153 ± 2 cP for the control. Once again, this observation suggests that phenolic compounds may limit β-glucans' capacity to develop digestive viscosity although potential mechanisms to explain it may be different than in cooked oatmeal, due to more complex digestion dynamics (two components η_1 and η_2 are involved instead of only one in uncooked oatmeal).

Both components η_1 and η_2 were significantly affected by the presence of phenolics, although in very different manners: whereas digestion of starch and proteins (evidenced by η_1 component) was slowed down in presence of phenolics, viscosity development (evidenced by η_2 component) was accelerated.

Indeed, on the one hand, the rate of digestion k_1 significantly decreased in presence of phenolic compounds by 8 to 41 %, which can be explained as in the case of uncooked oatmeal by possible inhibition of digestive enzymes by the phenolic compounds. On the other hand, the rate of viscosity build-up due to β-glucans solubilisation k_2 significantly increased by up to 79 %, which is counter-intuitive: one could think that slower digestion may lead to slower viscosity build up from β-glucans. To explain this apparent contradiction, we may look beyond digestion dynamics (k_1 and k_2) and observe that phenolic compounds not only affected digestion kinetics but also the viscosity of the digesta right from the start of the digestion procedure. Indeed, addition of EG, EC, and ECG led to a significant decrease of $\eta_{1(t=0)}$ by up to 34 %, although Van, FA, GA, and EGCG did not lead to any significant change. This trend towards a decrease of initial viscosity could be consistent with previously observed aggregation of proteins and starch with phenolic compounds [29,30] and in starch-soy protein composite porridges in presence of phenolic-rich grape pomace [31]. Additionally, in cooked oatmeal, β-glucans are already partially hydrated and solubilised due to the cooking procedure. Therefore, viscosity build-up due to β-glucans may be less dependent on β-glucans being released from the matrix (as in uncooked oatmeal) and more dependent on dynamics of hydration and competition for water with the other polymers of the system (starch and proteins). Because viscosity of starch and proteins was visibly decreased in presence of phenolics as shown above, it is likely that the competition for water and hydration was skewed in favour of β-glucans, thereby accelerating their viscosity build-up.

However, similarly to the case of cooked oatmeal, it is likely that interactions between β-glucans and phenolic compounds also affected viscosity development. Indeed, amplitude of infinite viscosity development $A_{2\infty}$ and infinite viscosity $\eta_{2(t=\infty)}$ were significantly affected by phenolic compounds. $A_{2\infty}$ decreased significantly by 25 to 47 % from the control in presence of phenolic compounds. Infinite viscosity $\eta_{2(t=\infty)}$ decreased significantly by 21 to 34 % from the control in presence of phenolic compounds. In other words, although the rate of viscosity development by β-glucans was faster in presence of phenolic compounds than in the control, these data suggest that upon completion of digestion (regardless of how slow it was and how long it took to complete), *i.e.*, when the matrix has been fully digested and all available β-glucans have been hydrated and solubilised, β-glucans were not able to develop viscosity to the same extent in presence of phenolics. Similar to uncooked oat bran, in this case, the decrease of $\eta_{2(t=\infty)}$ and $A_{2\infty}$ can't be attributed to limited release of β-glucans and may be attributed to the effect of phenolic compounds on β-glucans. Again, although colloidal particle size measurement could not be performed in cooked oat bran systems, it is likely that phenolic-driven aggregation of the β-glucans could explain this loss of viscosity as it had already been demonstrated previously in solution [4,5].

However, the role of individual phenolic compounds and the decrease of viscosity they induced in oat bran was different from solutions. In solutions, viscosity of β-glucans decreased by up to 95 % with phenolics at comparable concentrations, EC, ECG and EGCG leading to the most dramatic decreases, whereas the maximum viscosity decrease observed here was of 34 %. These differences could be attributed to interactions of the phenolic compounds with other components of oat bran. Indeed, phenolic acids and small catechins have been shown to from non-covalent complexes with dextrins, peptides, digestive enzymes as well as cell wall materials such as cellulose and arabinoxylans, which could all be present in the oat bran digesta

[29,30,32]. These components may therefore compete with β-glucans for the binding of phenolic compounds, thereby limiting the impact of phenolic compounds on viscosity development due to β-glucans.

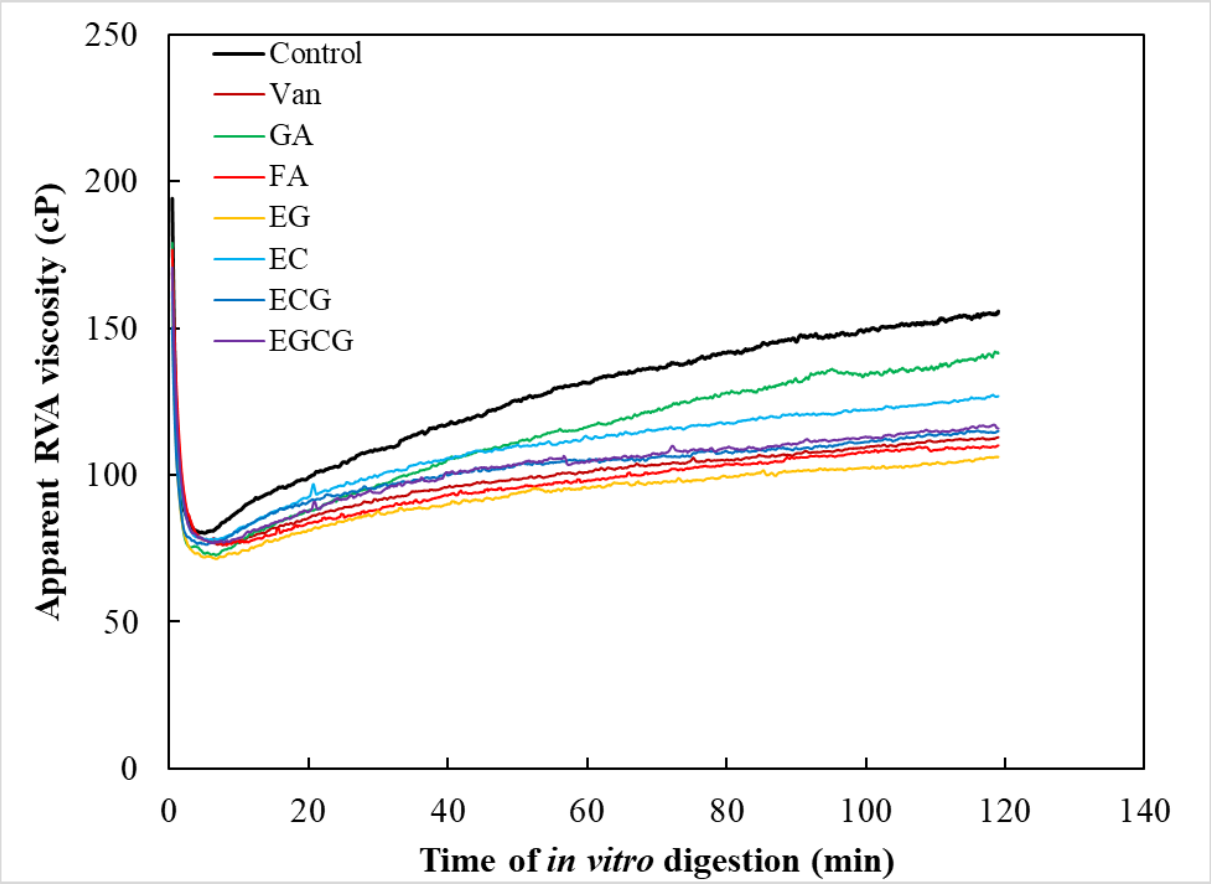

Figure 5-3: Digestograms of cooked oat bran (control, black line) and uncooked oat bran in presence of 10 mM Van (dark red line), 3 mM FA (light red line), 10 mM EG (yellow line), 1 mM GA (green line), 10 mM EC (light blue line), 1 mM ECG (dark blue line) and 10 mM EGCG (purple line). Digestograms were obtained by *in vitro* digestion in an RVA instrument. Each digestogram is the average of three replicates. Concentrations of FA and ECG were limited to 3 and 1 mM respectively due to limitations of solubility. GA 1 mM is included only for illustration purposes.

Table 5-2: Model parameters of RVA viscosity $\eta(t)$ during *in vitro* digestion of cooked oat bran in presence of 10 mM Van, 3 mM FA, 10 mM EG, 1 mM GA, 10 mM EC, 1 mM ECG and 10 mM EGCG. SER is the Standard Error of Regression. Results are given as average ± SEM of three replicates. Letters within each column indicate results of one-way ANOVA: values sharing letters are not significantly different ($p < 0.05$).

Sample	Model parameters						t_1 (min)	$\eta_{2(t=0)}$ (cP)	t_2 (min)	$\eta_{(t=120)}$ (cP)
	$\eta_{1(t=0)}$ (cP)	k_1 (min^{-1})	$\eta_{2(t=\infty)}$ (cP)	$A_{2\infty}$ (cP)	k_2 (min^{-1})	SER (cP)				
Control	115 ± 3 a,b	1.569 ± 0.050 a	173 ± 7 a	98 ± 1 a	0.014 ± 0.003 a	0.7 ± 0.1	0.4 ± 0.0 c	75 ± 6 a	52 ± 11 a,b	153 ± 2 a
Van 10 mM	93 ± 12 b,c	0.989 ± 0.238 c	118 ± 5 b,c	47 ± 5 c	0.019 ± 0.005 b	1.1 ± 0.2	0.8 ± 0.2 a	71 ± 2 a,b	39 ± 11 a,b,c	113 ± 2 d
FA 3mM[a]	134 ± 8 a	0.920 ± 0.022 c	120 ± 7 b,c	48 ± 7 b,c	0.015 ± 0.003 b	0.9 ± 0.3	0.8 ± 0.0 a	72 ± 0 a,b	51 ± 11 a,b	110 ± 2 d
GA 1 mM[b]	102 ± 4 b,c	1.357 ± 0.050 b	161 ± 1 a	94 ± 2 a	0.013 ± 0.001 a	0.9 ± 0.1	0.5 ± 0.0 b,c	67 ± 1 b	54 ± 4 a	142 ± 2 b
EG 10 mM	87 ± 5 c	1.265 ± 0.012 c	108 ± 0 c	41 ± 0 c	0.020 ± 0.002 b	0.8 ± 0.0	0.5 ± 0.0 a,b,c	68 ± 0 a,b	35 ± 2 a,b,c	106 ± 1 d
EC 10 mM	88 ± 15 c	1.111 ± 0.190 c	129 ± 9 b	58 ± 6 b	0.021 ± 0.002 b	1.3 ± 0.3	0.6 ± 0.1 a,b	71 ± 3 a,b	33 ± 3 b,c	127 ± 10 c
ECG 1 mM[a]	76 ± 16 c	1.448 ± 0.074 c	115 ± 1 b,c	41 ± 0 c	0.025 ± 0.001 b	1.3 ± 0.1	0.5 ± 0.0 b,c	80 ± 0 a,b	27 ± 1 c	115 ± 1 c,d
EGCG 10 mM	96 ± 6 b,c	1.008 ± 0.041 c	118 ± 3 b,c	48 ± 0 b,c	0.024 ± 0.006 b	1.5 ± 0.6	0.7 ± 0.0 a,b	70 ± 2 a,b	31 ± 8 b,c	116 ± 0 c,d

[a] Concentrations of FA and ECG were limited to 3 and 1 mM respectively due to limitations of solubility.

[b] GA 1 mM is included only for illustration purposes

5.6.2. Effect of gallic acid concentration on the viscosity of oat bran through *in vitro* digestion

In a previous study[5], we have shown that the effects of phenolic-driven aggregation of hydrocolloids on the physical properties of the hydrocolloids depended on the concentration of GA, a phenolic acid used as a representative model of other low molecular weight phenolic compounds. Aggregation increased and viscosity decreased with increasing concentration of GA, until hydrocolloids were saturated with GA and viscosity did not change any more despite higher concentrations of GA. In this section, the aim was to verify whether similar observations could be replicated in oatmeal, which would support the role of phenolic-driven aggregation of β-glucans in altering the development of digestive viscosity in oatmeal.

5.6.2.1. Uncooked oat bran

To investigate the effects of phenolic concentration on viscosity of oat bran through *in vitro* digestion, GA was used at concentrations ranging from 1 to 30 mM, corresponding to a maximum of 0.5 % w/w phenolic in food. GA was used as a model phenolic compounds in this study owing to its good solubility and its ability to affect the viscosity of β-glucans through complexation and aggregation[5]. Digestograms of uncooked oat bran in presence of GA (1 to 30 mM) are shown in ***Figure 5-4*** and modelling parameters are shown in ***Table 5-3***.

Similar to section ***5.6.1.1***, all digestograms of uncooked materials were adequately fitted only with the viscosity increase component of the model (η_2). Viscosity after 120 min of digestion $\eta_{(t=120)}$ was significantly affected by the addition of phenolic compounds, decreasing by 11 to 26 % from the control. As in section ***5.6.1.1***, this could be explained by a slower digestion induced by the phenolic compounds and inhibition of digestive enzymes. Indeed, time of half

infinite viscosity development t_2 significantly and meaningfully affected by the addition of GA, from 25 ± 2 min for the control up to 32 ± 1 min with GA 30 mM. Nonetheless, the modelling parameters of the digestion curves provided additional information on the effect of GA on viscosity from β-glucans.

Initial viscosity of the digesta $\eta_{2(t=0)}$ was statistically significantly affected by the addition of GA, but this decrease of up to 6 cP upon addition of GA may not be meaningful. On the other hand, infinite viscosity $\eta_{2(t=\infty)}$ and amplitude of infinite viscosity development $A_{2\infty}$ were significantly and meaningfully affected by the addition of GA. Infinite viscosity $\eta_{2(t=\infty)}$ decreased by up to 21 % from the control with increasing GA concentration. Amplitude of infinite viscosity development $A_{2\infty}$ decreased by up to 25 % from the control with increasing GA concentration.

These results are aligned with the results of section ***5.6.1.1*** with other phenolic compounds and show that beyond the effect of enzyme inhibition and slowing of digestion from GA, GA also affected the viscosity of β-glucans itself. These results also show that the magnitude of the effect of GA increased with increasing concentration of GA. This is also consistent with results we reported previously in homogeneous simple solutions [5] that showed that addition of GA drove loss of viscosity and water-binding capacity through aggregation of β-glucans in solution in direct relationship with concentration of GA. However, these previous data on β-glucans solutions (at a 0.75 % w/w concentration similar to this study based on oat bran β-glucan content) showed a saturation of the effect of GA between 3 and 10 mM [4], whereas a plateau/saturation effect seems to occur between 10 and 30 mM here, with uncooked oat bran. As hypothesised before, competition of starch, dextrins, proteins, and peptides with β-glucans to bind with GA might have driven saturation of β-glucans with GA to higher concentrations of GA.

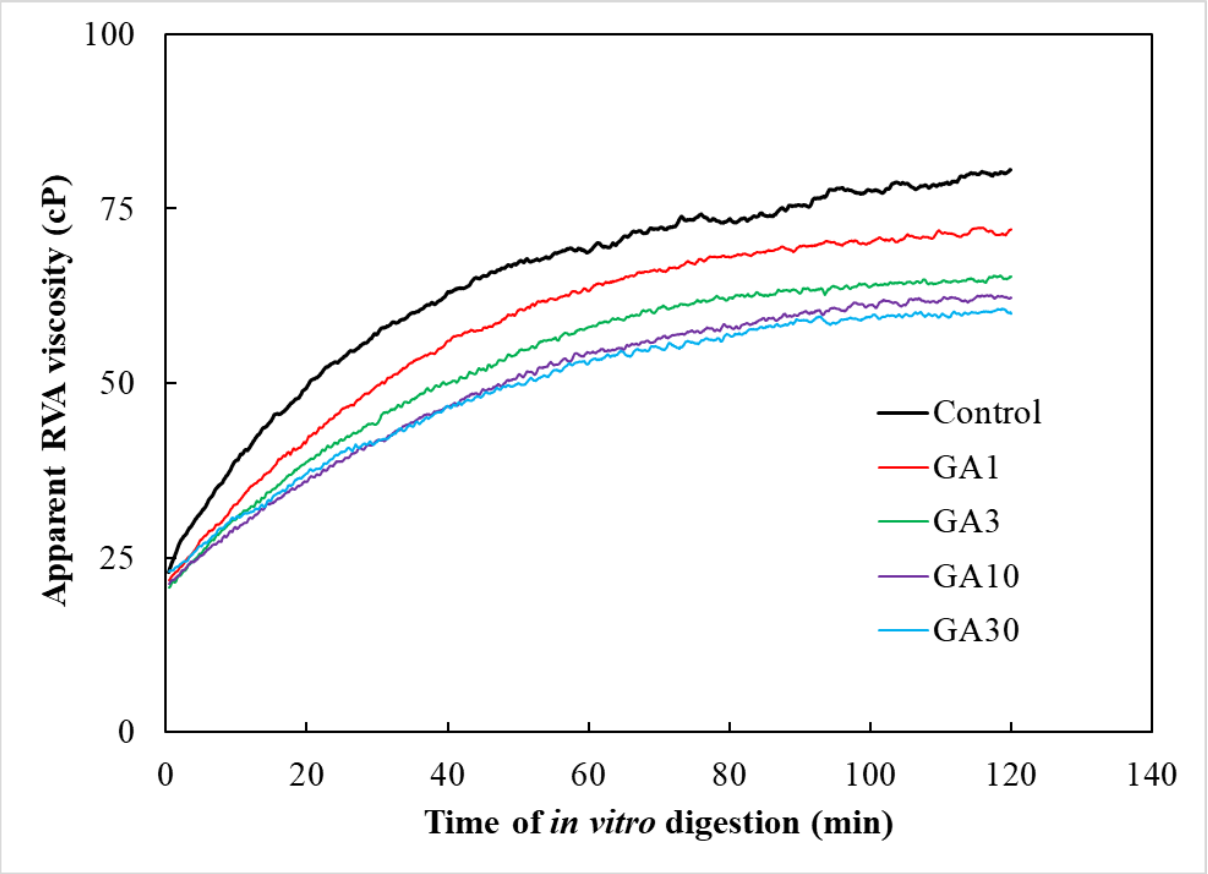

Figure 5-4: Digestograms of uncooked oat bran (control, black line) and uncooked oat bran in presence of 1 mM GA (red line), 3 mM GA (green line), 10 mM GA (purple line) and 30 mM GA (blue line). Digestograms were obtained by *in vitro* digestion in an RVA instrument. Each digestogram is the average of three replicates.

Table 5-3: Model parameters of RVA viscosity $\eta(t)$ during *in vitro* digestion of uncooked oat bran with GA 1 – 30 mM. SER is the Standard Error of Regression. Results are given as average ± SEM of three replicates. Letters within each column indicate results of one-way ANOVA: values sharing letters are not significantly different ($p < 0.05$).

Sample	Model parameters				$\eta_{2(t=0)}$ (cP)	t_2 (min)	$\eta_{(t=120)}$ (cP)
	$\eta_{2(t=\infty)}$ (cP)	$A_{2\infty}$ (cP)	k_2 (min^{-1})	SER (cP)			
Control	81 ± 2 a	55 ± 1 a	0.028 ± 0.002 a	1.0 ± 0.4	26 ± 2 a	25 ± 2 c	81 ± 2 a
GA 1mM	75 ± 2 b	54 ± 1 a	0.026 ± 0.002 a	0.6 ± 0.1	21 ± 2 b	26 ± 2 b,c	72 ± 3 b
GA 3mM	68 ± 3 c	48 ± 3 b	0.025 ± 0.002 a,b	0.6 ± 0.1	20 ± 1 b	28 ± 2 a,b,c	65 ± 4 c
GA 10mM	68 ± 1 c	47 ± 1 b	0.023 ± 0.001 b	0.5 ± 0.1	20 ± 1 b	30 ± 3 a,b	62 ± 0 c
GA 30mM	64 ± 1 c	42 ± 2 c	0.022 ± 0.001 b	0.6 ± 0.1	23 ± 2 a,b	32 ± 1 a	60 ± 1 c

5.6.2.2. Cooked oat bran

Digestograms of cooked oat bran in presence of GA (1 to 30 mM) are shown in ***Figure 5-5*** and modelling parameters are shown in ***Table 5-4***. All digestograms of cooked materials were modelled by the two components η_1 and η_2 of the model described in section ***5.5.4***.

Similar to uncooked oatmeal, viscosity after 120 min of digestion $\eta_{(t=120)}$ decreased by up to 31 % with GA, from 153 ± 2 cP for the control. Once again, this observation suggests that phenolic compounds may limit β-glucans' capacity to develop digestive viscosity, regardless of the mechanism of action.

Nevertheless, ***Figure 5-5*** shows that the digestograms with 10 and 30 mM GA exhibited unusual profiles compared to the other concentrations of GA, compared to the digestograms

presented in ***Figure 5-3*** and compared to observations in a previous study[21]. Both digestograms exhibited a marked inflexion point during the viscosity increase associated with solubilisation of β-glucans (η_2). Additionally, the digestogram of oat bran with 30 mM exhibited a significant delay of 4 min before viscosity started to decrease with starch and protein hydrolysis. Although the modelling of this digestogram was adjusted to this delay, these observations strongly suggest that 10 and 30 mM of GA led to a strong inhibition of the digestive enzymes and impacted the viscosity of the digesta. This was confirmed by the rate of viscosity decrease k_1 that decreased by a factor of 22 between the control and 30 mM GA. Consequently, the model led to nonsensical parameter values such as

$$\eta_{2(t=0)} = -276 \pm 46 \text{ cP} \qquad \textit{Eq. 5-9}$$

for GA 30 mM, or an increase of k_2 with increasing concentration of GA, meaning an acceleration of the release of β-glucans whereas the digestogram clearly showed a marked slowing of digestion. Finally, the Standard Error of Regression (SER) increased significantly to 3.4 and 5.5 at 10 and 30mM GA, compared to all other models of the current study for which SER was below 1.5, meaning that the fit of the model was sub-optimal.

Therefore, we devised a new model to fit these data and to account for lag times in viscosity decrease and increase components due to strong inhibition of the digestive enzymes. The introduction of a lag time during which variations of viscosity are slow before undergoing exponential increase or decrease (similar to the previous first-order mono-exponential model) was adequately taken into account by a logistic function (sigmoidal curve) with two contributions as follow:

- A decreasing component,

$$\eta_1(t) = \frac{\eta_{1max}}{1+e^{k_1(t-t_1)}} \qquad \textit{Eq. 5-10}$$

where k_1 is the rate of viscosity decrease due to starch and protein digestion, and t_1 is the time of half decrease of viscosity, assuming as before that the final viscosity of this component is 0 cP[21]. In this model η_{1max} represents a theoretical maximal viscosity of the food before digestion starts. Initial viscosity of the η_1 component at t = 0 min is therefore:

$$\eta_1(t=0) = \frac{\eta_{1max}}{1+e^{-k_1 \times t_1}} \qquad \textit{Eq. 5-11}$$

- An increasing component,

$$\eta_2(t) = \eta_{2min} + \frac{A_{2\infty}}{1+e^{-k_2(t-t_2)}} \qquad \textit{Eq. 5-12}$$

where $A_{2\infty}$ is the amplitude of infinite viscosity development due to release and solubilisation of β-glucans, k_2 is the rate of viscosity increase and t_2 is the time of half increase of infinite viscosity due to release and solubilisation of β-glucans. In this model η_{2min} represents a theoretical minimal viscosity of the food before digestion starts. Initial viscosity of the η_2 component at t = 0 min is therefore

$$\eta_2(t=0) = \eta_{2min} + \frac{A_{2\infty}}{1+e^{-k_2 \times t_2}} \qquad \textit{Eq. 5-13}$$

The digestograms of cooked oat bran with 0-30 mM GA were fitted with this model and the model parameters are shown in ***Table 5-5***.

Interestingly, SER for oat bran with 10 and 30 mM decreased from 3.4 and 5.5 with the previous model, to 2.5 and 2.4 with the new model, showing a significant improvement of the fit.

In this new model, rates of viscosity decrease (k_1) and viscosity increase (k_2) both decreased significantly by an order of magnitude, from the control to GA 30 mM. Similarly, t_1 increased from 0 min for the control to 9.6 min with 30 mM GA, whereas t_2 increased by a factor of 29 from the control to 30 mM. These data are consistent with a very significant slowdown of digestion associated with increasing concentration of GA.

The initial contribution of β-glucans to model viscosity $\eta_{2(t=0)}$ decreased by up to 72% with increasing concentration of GA. $\eta_{2(t=0)}$ is likely due to the β-glucans released and solubilised during the cooking process, when digestion has not impacted the matrix yet. Therefore, this decrease of $\eta_{2(t=0)}$ may not be attributed to effects of GA on the rate of digestion, but rather to the effect of GA on β-glucans solubilised at t = 0 min. Similarly, $A_{2\infty}$, the amplitude of infinite viscosity development due to β-glucans solubilisation (η_2, when no other digestible biopolymers are present in the digesta), decreased significantly by up to 50 % from the control with increasing concentration of GA. These two effects once again suggest that GA affected the viscosity of β-glucans themselves, very likely through the formation of colloidal aggregate leading to loss of viscosity, loss of pseudo-plasticity, and loss of water-binding capacity as observed before in simple solutions [4,5,28]. Once again, the present results suggest that despite a

complex digestive solution composition GA was still able to decrease viscosity development and final viscosity of β-glucans released from digestion of oats.

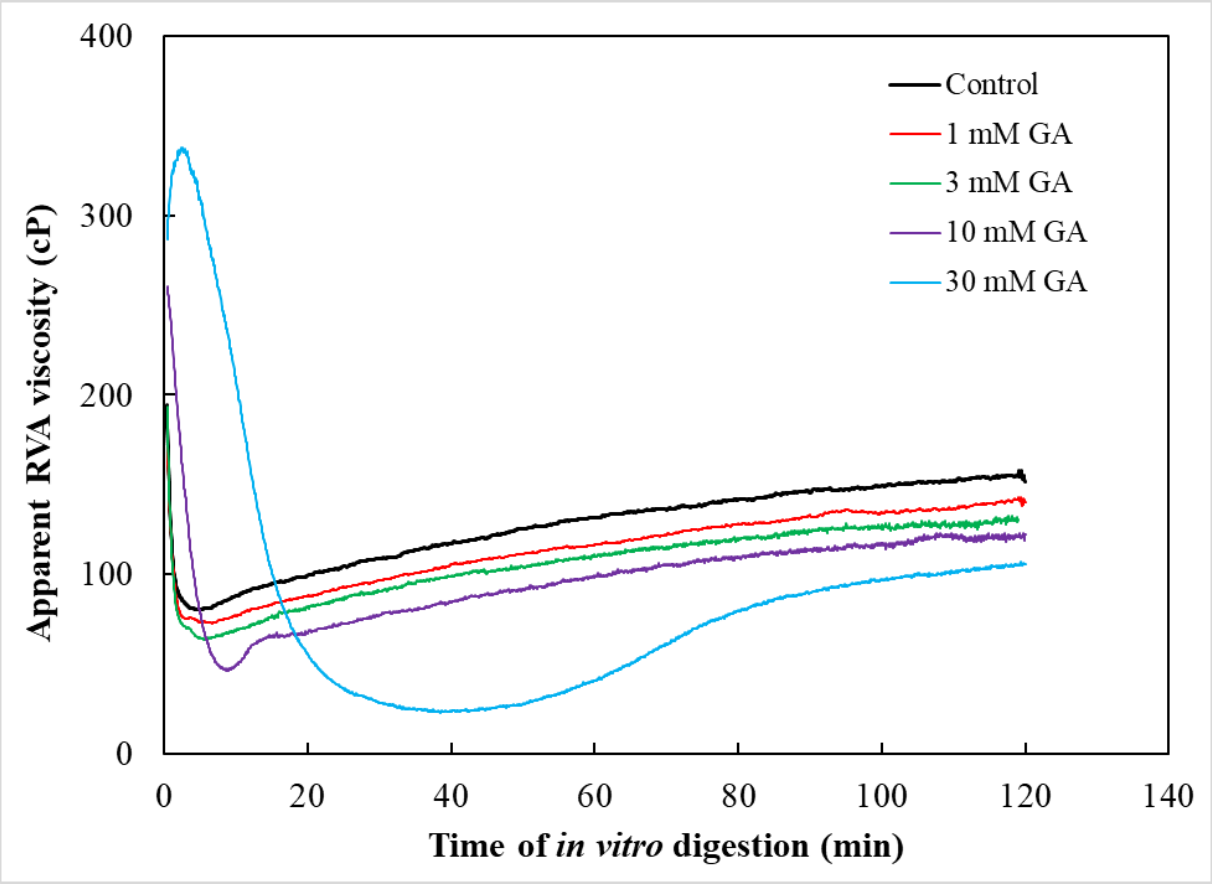

Figure 5-5: Digestograms of cooked oat bran (control, black line) and uncooked oat bran in presence of 1 mM GA (red line), 3 mM GA (green line), 10 mM GA (purple line) and 30 mM GA (blue line). Digestograms were obtained by *in vitro* digestion in an RVA instrument. Each digestogram is the average of three replicates.

Table 5-4: Model parameters (first order monoexponential model) of RVA viscosity $\eta(t)$ during in vitro digestion of cooked oat bran with GA 1 – 30 mM. SER is the Standard Error of Regression. Results are given as average ± SEM of three replicates. Letters within each column indicate results of one-way ANOVA: values sharing letters are not significantly different ($p < 0.05$). Data for GA 30 mM takes into account a 4 min delay to establish the model parameters that was added to t_1 and t_2.

Sample	Model parameters						t_1 (min)	$\eta_{2(t=0)}$ (cP)	t_2 (min)	$\eta_{(t=120)}$ (cP)
	$\eta_{1(t=0)}$ (cP)	k_1 (min^{-1})	$\eta_{2(t=\infty)}$ (cP)	$A_{2\infty}$ (cP)	k_2 (min^{-1})	SER (cP)				
Control	115 ± 3 c	1.57 ± 0.05 a	173 ± 7 a	97 ± 1 b	0.014 ± 0.003 b,c	0.7 ± 0.1	0.4 ± 0.0 c	51 ± 11 a,b	75 ± 6 a	153 ± 2 a
GA 1mM	111 ± 4 c	1.36 ± 0.05 b	161 ± 1 b	94 ± 2 b	0.013 ± 0.001 c	0.9 ± 0.1	0.5 ± 0.0 c	55 ± 4 a	67 ± 1 a	142 ± 2 b
GA 3mM	132 ± 16 c	1.34 ± 0.10 b	143 ± 3 c	85 ± 1 b	0.016 ± 0.002 b,c	1.1 ± 0.1	0.5 ± 0.0 c	43 ± 4 a,b,c	58 ± 3 a	130 ± 3 c
GA 10mM	256 ± 9 b	0.40 ± 0.02 c	132 ± 4 d	97 ± 2 b	0.018 ± 0.001 b	3.4 ± 0.3	1.7 ± 0.1 b	38 ± 2 b,c	36 ± 4 a	122 ± 1 d
GA 30mM	627 ± 47 a	0.07 ± 0.00 d	127 ± 3 d	403 ± 44 b	0.026 ± 0.001 a	5.5 ± 0.2	13.7 ± 0.5 a	30 ± 1 c	-276 ± 46 b	105 ± 4 e

Table 5-5: Alternate model parameters (logistic model) of RVA viscosity $\eta(t)$ during in vitro digestion of cooked oat bran with GA 1 – 30 mM. SER is the Standard Error of Regression. Results are given as average ± SEM of three replicates. Letters within each column indicate results of one-way ANOVA: values sharing letters are not significantly different ($p < 0.05$). Data for GA 30 mM takes into account a 4 min delay to establish the model parameters that was added to t_1 and t_2.

Sample	Model parameters								$\eta_{1(t=0)}$ (cP)	$\eta_{2(t=0)}$ (cP)	$\eta_{(t=120)}$ (cP)
	η_{1max} (cP)	k_1 (min^{-1})	t_1 (min)	η_{2min} (cP)	$A_{2\infty}$ (cP)	k_2 (min^{-1})	t_2 (min)	SER (cP)			
Control	361 ± 9 a,b	2.04 ± 0.06 a	0.0 ± 0.0 c	0 ± 0 b	161 ± 1 a	0.026 ± 0.005 b	2.5 ± 0.5 d	0.95 ± 0.08	180 ± 4 c	78 ± 7 c	153 ± 2 a
GA 1mM	323 ± 12 b	1.77 ± 0.07 b	0.0 ± 0.0 c	0 ± 0 b	148 ± 3 b	0.024 ± 0.002 b	5.0 ± 0.4 c	0.96 ± 0.16	162 ± 6 b	70 ± 1 c	142 ± 2 b
GA 3mM	386 ± 39 a	1.78 ± 0.10 b	0.0 ± 0.0 c	0 ± 2 b	134 ± 1 c	0.029 ± 0.002 b	5.8 ± 0.0 c	1.33 ± 0.05	159 ± 20 a,b,c	42 ± 5 b	130 ± 3 c
GA 10mM	257 ± 5 c	0.82 ± 0.03 c	2.2 ± 0.2 b	0 ± 0 b	126 ± 3 d	0.030 ± 0.001 b	16.9 ± 1.6 b	2.53 ± 0.15	221 ± 4 d	47 ± 4 b	122 ± 1 d
GA 30mM	396 ± 8 a	0.23 ± 0.00 d	9.6 ± 0.3 a	22 ± 4 a	80 ± 1 e	0.103 ± 0.002 a	71.5 ± 1.2 a	2.14 ± 0.07	357 ± 7 e	22 ± 4 a	105 ± 4 e

Ideally, our hypotheses related to phenolic-β-glucans complexation should be supported further by; 1- direct observation of the colloidal aggregates, and 2- direct measures of β-glucans concentration along the *in vitro* digestion process. However, the heterogeneity of the digestion medium prevents the former, and methodological limitations prevent the latter: it remains a challenge to quench digestion in order to measure solubilised β-glucans without affecting the oat matrix and the amounts of solubilised β-glucans. Nevertheless, through adequate mathematical modelling of the digestograms, we were able to extrapolate the digestive processes to infinite times, thereby overcoming these methodological limitations by considering virtually complete digestion and complete release of β-glucans, regardless of how long it would take experimentally. These extrapolations then seemed to support our conclusions.

Also, it has been established previously that the *in vitro* digestion method used in this work is a useful tool to predict the effects of oat and barley products on glycaemic response and cholesterol lowering: the higher the viscosity after 120 min, the lower the glycaemic response and the greater the cholesterol lowering effect [17-20]. Based on this established method and the observations reported in this work, one could wonder whether co-consumption of phenolic compounds with β-glucan-rich products, quite common in dietary recommendations [33,34], could alter the nutritional benefits associated with oats and β-glucans. This in turn could have profound consequences on our understanding of polyphenol-dietary fibre interactions and their effects on their respective nutritional properties. Indeed, current knowledge on combinations of dietary fibres and phenolic compounds shows contrasted effects: whereas it has been shown that phenolic-fibre complex formation could enhance transport of phenolics to the colon where they can be utilised by the gut microbiota, it has also been shown that these complexes can reduce the phenolics bioavailability, bioactivity, and absorption [35-39]. The current study

suggests further complexity as these complexes may reduce the benefits associated with soluble fibres and their viscosity development through digestion. These potential effects on glycaemic response, reduction of LDL-cholesterol levels or satiety would need to be tested and confirmed *in vivo*. Furthermore, these findings would need to be assessed when phenolics are present as real dietary combinations as opposed to individual models. This may contribute to our understanding of the mechanisms by which phenolic and fibre-rich foods such as whole grain cereal products, fruits, vegetables, and pulses contribute to positive health outcomes. Finally, these potential effects may also impact methodological considerations for the evaluation of the nutritional effects of whole grain cereals: even non-caloric but phenolic-rich beverages such as tea and coffee that are often co-consumed with whole grain cereals (such as oat or barley porridges) may affect the physiological outcomes of such clinical trials (glycaemic response, LDL-cholesterol levels, satiety, etc.). Therefore, supply of standard beverages not containing phenolic compounds (such as plain water) may be desirable to optimise the reliability of such trials.

5.7. Conclusions

We have demonstrated that addition of model phenolic compounds in concentrations relevant to actual food systems led to a significant reduction of β-glucan viscosity development in oat bran through *in vitro* digestion. These observations confirm previous observations in simple β-glucan solution. They suggest that dietary phenolic compounds may be able to interfere with β-glucan viscosity development in the small intestine and potentially with the physiological effects and nutritional properties of β-glucans. However, the magnitude of the effects of these model phenolic compounds was lower than in solution, possibly due to interactions with other food components. Therefore, further test with sources of phenolic compounds closer to real

foods and clinical trials will be needed to evaluate the actual impact of phenolic-fibre interactions on the physiological functions of fibres in the upper gut.

5.8. Acknowledgements

This work was funded by the Natural Science and Engineering Research Council of Canada – Discovery Grant #2018-04009. Author NB is a member of the Scientific Advisory Board of Agri fibre LLC (Mundelein, IL).

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Chapter 6: Conclusions

Chapter 6 : Conclusions

Throughout literature there have been enduring inconsistencies between *in-vitro* experimentation and clinical evidence concerning the consumption of nutritionally rich bioactive compounds, specifically, polysaccharides (comprising dietary fibres and starches) and phenolics, and in relation to their interactions with digestive enzymes. As shown in Chapter 1, previous research primarily focussed on some variation of a pairwise relationship between these three components. The main objective of this thesis was thus, to emphasise the significance of considering three-way interactions between phenolics, polysaccharides and digestive enzymes. The knowledge gained from this study is anticipated to not only help bridge the gap between observations made *in-vitro* and *in-vivo* observations, but also offer valuable insights into understanding/predicting potential interactions that would occur between our targeted bioactive components and the food matrix, throughout food preparation, consumption, and the digestive process.

The study was designed to explore multiple potential consequences of the multi-faceted interactions between phenolics, polysaccharides, and digestive enzymes, rather than exhaustively dissecting a singular aspect (on fibre viscosity, on starch viscosity, on enzyme inhibition/activity, on complexation, etc). Thereby, providing a stepping stone to understanding the overall impact on health and nutrition due to their interactions. This research is driven by four primary objectives, which have been formulated in response to seven key research questions outlined in Chapter 1.

The first two research questions played a pivotal role in shaping the first objective of this thesis, which has been thoroughly examined in Chapter 2. Using different tea phenolics (EG, EGC,

ECG, and EGCG) as a food model, this study delved into the intricate interactions between phenolics, digestive enzymes, specifically α-amylases and starch, while also shedding light on their binding kinetics and phenolic amylase inhibition mechanisms. Our findings revealed that phenolics, including EC and EGC, exhibited relatively slow binding kinetics with amylases, while ECG and EGCG displayed rapid and robust binding. The structural differences between the phenolics, namely the presence of terminal galloyl groups in ECG and EGCG appeared to contribute to their rapid binding, a phenomenon not observed in EC and EGC. Furthermore, through our investigations into the effect of incubation time between enzymes and phenolics revealed, that in general longer incubation times led to higher levels of enzyme inhibition, an observation vital to weaker inhibitors, while with stronger inhibitors specifically, EGCG containing abundant galloyl groups, displayed rapid and near complete rates of inhibition within minutes of incubation. Additionally, a significant discrepancy was observed between the amylase inhibition assay (containing starch) and the fluorescence quenching experiment (no starch). This observation demonstrated the existence of competition between digestive enzymes and starches for phenolic binding. These findings emphasise the importance of considering binding kinetics, matrix, and incubation times in the study of enzyme inhibition by phenolics. Such considerations may play a crucial role in the design of enzyme inhibition assays with phenolics, potentially impacting the reliability and reproducibility of results.

The second objective was inspired by the third, fourth, and fifth questions, and has been investigated in Chapter 3. The research steers into a different direction by looking into an alternative and simplified perspective to consider an altered system in which the digestive enzyme doesn't interact with starches, which is a substrate for α-amylase and a sequester for phenolic compounds. Lipase is a suitable example in this context, as it primarily works on lipids and not starches, thus starch would maintain its supramolecular structure throughout the

assay, thus acting as a dietary fibre. As such, this study utilised starch and phenolics from potatoes as a food model, to study the intricate interplay among starch, phenolic compounds, and pancreatic lipase. It unveiled the distinct inhibitory strengths of various phenolic compounds derived from potato starch. With CA, pCA, and FA being equivalently weak inhibitors of pancreatic lipase, while ChA exhibited comparatively weaker inhibitory characteristics. These findings parallel those observed in Chapter 2, where the structural distinctions among phenolic compounds played a significant role in their overall inhibitory potential.

Furthermore, the study indicated that the relative hydrophobicity/hydrophilicity of each phenolic compound may also contribute to its inhibitory capacity. Phenolics that readily dissolved displayed a higher potential for binding with lipase, while the converse was true for less soluble ones. Now, in contrast to Chapter 2, the structural conformation of starch remained unaltered throughout the assay, thus allowing for starch to maintain its viscosity, and phenolic sequestering capacity throughout. This circumstance provided an opportunity to elucidate how the interactions between phenolics and starch influenced the inhibitory capacity of phenolics on lipase activity over the study's duration.

The results uncovered intriguing nuances. Starch exerted varying effects on the ability of phenolic compounds to modulate lipase activity, contingent on the specific phenolic compound in question. Notably, ChA's inhibitory capacity decreased in the presence of starch, while the opposite trend was observed with CA. In contrast, the inhibitory capacity of FA and pCA remained unchanged, regardless of the presence or absence of starch. Generally, an increase in starch concentration limited the inhibitory potential of phenolic acids against lipase. This suggests that the presence of starch could impede the ability of phenolics to bind with and

inhibit lipase, potentially due to interactions between phenolics and starch, entrapment of phenolics by starch, or the heightened overall viscosity hindering the diffusion of phenolic compounds toward lipase.

Moreover, the study underscored the critical role of pH in the examination of lipase inhibition by phenolics. Previous research conducted at a higher pH level of 8 and above led to notable phenolic degradation. As the physiological pH typically ranges between 6.5-7.0 in the small intestine, this highlights the importance of conducting lipase inhibition studies at pH levels consistent with the physiological environment. In essence, this study enriches our comprehension of the intricate interplay among starch, phenolic compounds, and pancreatic lipase, offering comprehensive insights into the dynamics governing lipid digestion in the presence of dietary phenolic compounds. The research further emphasises the necessity for context-specific assessments of lipase inhibition and the significance of considering the complex interrelationships that influence nutritional outcomes in diverse dietary scenarios.

The third objective was influenced by the fourth, sixth and seventh questions, which has been explored in Chapter 4 of this thesis. In this study, interactions between gallic acid (a representative molecule for lower molecular weight phenolics) and various starches was thoroughly investigated with a specific focus on its impact on α-amylase activity, measured indirectly through the digestion of starch. The study utilised starches from various sources, namely, potato, rice, wheat, and maize, to explore the possible binding events influenced by the unique structural characteristics of each starch type.

By controlling time of GA introduction, we were able to promote either phenolic starch or phenolic enzyme interactions. The results showed that when GA was added before cooking of

starch (promoting starch-GA complexation), the rate of digestion of starch was similar to that of starch alone (absence of GA), and faster than when GA was added after cooking of starch (promoting competition between starch-enzymes for phenolics). The results further demonstrated that when GA was introduced after cooking of starch, GA inhibited α-amylase strongly and that inhibition appeared to increase with starch paste viscosity only for potato and wheat starches. No correlation was found between starch molecular characteristics and the inhibiting capacity of GA at different starch concentrations. Nonetheless, the inconsistencies in the outcomes across the four types of starches suggests that starch paste viscosity is not the sole contributor to GA's capacity to inhibit α-amylase. These findings are in agreement with recent research indicating that the inhibition of starch digestion by phenolics is dependent on the competitive binding between phenolics with starches and digestive enzymes. As with observations in Chapters 2 and 3, the interactions between the three components are highly dependent on the molecular structure of each individual component. However, the apparent influence of starch chain length distribution suggested that physical effects (due to diffusion kinetics and/or physical entrapment of phenolics, hinted at in Chapter 3), may play a role in the capacity of GA to inhibit α-amylase.

The final objective was influenced by the fourth, sixth and seventh questions, and has been thoroughly explored in Chapter 5 of this thesis. Using oat bran as a food model, the study investigated the interactions between phenolics, polysaccharides (starches and fibres), and digestive enzymes. In the context of uncooked oat bran, the study demonstrated that the introduction of phenolics results in a substantial decrease in viscosity after a 120-minute digestion period, profoundly impacting the intricate development of digestive viscosity (physicochemical property of dietary fibres). This observed reduction in digestive viscosity could be attributed to two potential mechanisms. Firstly, it is rationalised as a consequence of

the inhibition of digestive enzymes by phenolics, which leads to a slower digestion process. Secondly, it may be elucidated by the formation of aggregates between phenolics and β-glucan, leading to precipitation and a loss in viscosity. Moreover, this study found a significant correlation between the reduction in digestive viscosity development and the molecular weights of phenolic compounds, whereby phenolics with higher molecular weights, containing abundant -OH groups would have a greater propensity to form hydrogen bonds, eliciting a more pronounced decrease in viscosity, as seen in Chapters 2-4.

Similar to uncooked oat bran, the viscosity development after 120 minutes of digestion in cooked oat bran also saw a significant decrease following the addition of phenolics. While in uncooked oat bran increasing concentrations of GA saw a proportional drop in final digestive viscosity development, the same was not observed in the initial part of digestograms obtained with cooked oat bran. In cooked oat bran, there was a significant initial increase in digestive viscosity specifically at 10mM and 30mM respectively. This distinct behaviour can be ascribed to the combined contributions of gelatinised starch and β-glucans as addition of higher concentration of GA meant that a greater number of free phenolics are available to bind and inhibit α-amylase, thus leading to a temporary halt/slowdown of starch digestion.

The research in this thesis has effectively addressed the complex interactions between phenolics, polysaccharides (including dietary fibres and starches), and digestive enzymes through multiple new ways. The study has elucidated that these interactions are multifaceted, greatly dependent on factors such as the structural characteristics of phenolics and polysaccharides, incubation time, and pH. More importantly, this research emphasises the significance of three-way interactions rather than solely focusing on pairwise relationships, shedding light on the need for a more comprehensive understanding of the interplay between

these bioactive components. These findings have profound implications for the design of enzyme inhibition assays with phenolics, highlighting the importance of considering binding kinetics, matrix, and incubation times to enhance the reliability and reproducibility of results.

Furthermore, the study has shown that the interactions between these components can be significantly influenced by their immediate microenvironment, where the physical properties of polysaccharides, such as viscosity, play a critical role in the diffusion of phenolics and enzymes. This insight underscores the necessity of context-specific assessments in understanding the complex dynamics governing nutritional outcomes in diverse dietary scenarios.

6.1. Future directions

While the research has made substantial progress in unveiling the intricacies of these interactions, it also underscores the need for further investigation. Our present study has made use of pure phenolics and polysaccharides, as such there is a pressing need to study these multi-way interactions with more complex and multi-competitive interactions, for instance, with plant phenolic extracts rather than pure phenolic compounds, or with structured plant cell walls rather than solubilised dietary fibres, this approach would provide us with valuable insights into the potential synergistic or antagonistic effects of the different phenolics and polysaccharides when present in complex matrices. Future studies should also consider *in vivo* assessments and rigorous clinical trials to substantiate these multifaceted effects in real-world dietary contexts and further bridge the gap between *in-vitro* experimentations and *in-vivo* observations. Additionally, exploring the impact of other dietary combinations, especially when whole grain cereals are commonly ingested with phenolic-rich beverages, is essential to comprehensively understand the implications of these interactions on nutritional outcomes.

This research has opened the door to a deeper understanding of the relationships between bioactive compounds, polysaccharides, and digestive enzymes and their role in nutrition and health.

Appendices

Appendix A1

Supplementary information for Chapter 4

Supplementary table 1: Modelling parameters of starch digestograms for starch alone ("control"), and starch with either GA or EGA added either before or after the cooking procedure, for rice, potato, wheat, and maize starches. $\eta_{t=0}$, $\eta_{(t=\infty)}$, t_{half} and k are model parameters determined from the fitting of digestograms according to Eq. 4-2, and SER is the Standard Error of Regression (fit). All results are reported as mean ± standard deviation of three replicates. No significant differences ($p < 0.05$) between model parameters of the two samples were found one-way ANOVA test.

Sample type	Paste viscosity (cP)			
	Potato	Maize	Wheat	Rice
Control	1502 ± 4	392 ± 9	213 ± 10	120 ± 46
GA not heated, added after starch cooking	1503 ± 10	392 ± 7	206 ± 1	134 ± 41
GA not heated, added before starch cooking	1502 ± 7	398 ± 1	217 ± 4	142 ± 22
EGA not heated, added after starch cooking	1485 ± 4	389 ± 1	206 ± 2	147 ± 43
EGA not heated, added before starch cooking	1493 ± 11	388 ± 2	207 ± 1	166 ± 17

Supplementary table 2: Modelling parameters of starch digestograms for starch with GA added after the cooking procedure either fresh (not heated) or heated through the same cooking procedure as starch, for rice, potato, wheat, and maize starches. $\eta_{t=0}$, $\eta_{(t=\infty)}$, t_{half} and k are model parameters determined from the fitting of digestograms according to Eq. 4-2, and SER is the Standard Error of Regression (fit). All results are reported as mean ± standard deviation of three replicates. No significant differences ($p < 0.05$) between model parameters of the two samples were found through Student's t-test.

	Rice		**Maize**		**Potato**		**Wheat**	
	GA not heated, added after starch cooking	GA heated, added after starch cooking	GA not heated, added after starch cooking	GA heated, added after starch cooking	GA not heated, added after starch cooking	GA heated, added after starch cooking	GA not heated, added after starch cooking	GA heated, added after starch cooking
$\eta_{(t=0)}$ (cP)	134 ± 9	132 ± 42	359 ± 6	386 ± 20	1225 ± 153	1308 ± 91	185 ± 2	192 ± 6
k (cP.min^{-1})	1.12 ± 0.03	1.16 ± 0.05	1.61 ± 0.02	1.59 ± 0.06	1.59 ± 0.04	1.72 ± 0.36	1.39 ± 0.03	1.37 ± 0.13
t_{half} (min)	2.6 ± 0.1	2.0 ± 0.4	11.6 ± 3.3	16.8 ± 1.5	6.6 ± 0.5	7.2 ± 2.9	2.4 ± 0.2	6.4 ± 7.7
$\eta_{(t=\infty)}$ (cP)	6 ± 1	12 ± 6	1 ± 1	0 ± 0	0 ± 0	35 ± 46	11 ± 1	12 ± 2
SER (cP)	0.55 ± 0.07	0.50 ± 0.01	1.28 ± 0.01	0.81 ± 0.04	9.51 ± 4.42	8.20 ± 4.29	0.82 ± 0.03	0.74 ± 0.12

Supplementary table 3: Modelling parameters of starch digestograms for starch alone ("control") and starch with EGA added either before or after the cooking procedure ("with GA"), for rice, potato, wheat, and maize starches. $\eta_{t=0}$, $\eta_{(t=\infty)}$, t_{half} and k are model parameters determined from the fitting of digestograms according to Eq. 4-2, and SER is the Standard Error of Regression (fit). All results are reported as mean ± standard deviation of three replicates. Asterisk next to results indicate significant differences ($p < 0.05$) between model parameters of the control and the same samples with EGA from a one-way ANOVA test.

	Rice			**Maize**			**Potato**			**Wheat**		
	Control	EGA added before cooking	EGA added after cooking	Control	EGA added before cooking	EGA added after cooking	Control	EGA added before cooking	EGA added after cooking	Control	EGA added before cooking	EGA added after cooking
$\eta_{(t=0)}$ **(cP)**	154 ± 17	152 ± 16	161 ± 17	322 ± 1	323 ± 4	322 ± 4	914 ± 18	914 ± 35	875 ± 18	189 ± 7	194 ± 3	191 ± 3
k **(cP.min^{-1})**	1.05 ± 0.08	1.17 ± 0.10	1.07 ± 0.05	1.57 ± 0.06	1.53 ± 0.04	1.55 ± 0.02	1.50 ± 0.00	1.52 ± 0.03	1.54 ± 0.03	1.26 ± 0.02	1.25 ± 0.02	1.30 ± 0.03
t_{half} **(min)**	1.5 ± 0.2	1.3 ± 0.1	1.3 ± 0.0	2.1 ± 0.2	2.0 ± 0.1	2.0 ± 0.2	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	1.6 ± 0.1	1.2 ± 0.0	1.4 ± 0.0
$\eta_{(t=\infty)}$ **(cP)**	6 ± 1	11 ± 4	8 ± 0	13 ± 2	13 ± 0	13 ± 1	37 ± 0 *	0 ± 0 *	2 ± 2 *	10 ± 1	9 ± 0	9 ± 0
SER (cP)	0.58 ± 0.10	0.55 ± 0.05	0.55 ± 0.09	0.88 ± 0.07	0.82 ± 0.16	1.03 ± 0.21	1.20 ± 0.05	0.92 ± 0.11	1.04 ± 0.00	0.77 ± 0.10	0.74 ± 0.02	0.82 ± 0.09

Appendix A2

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Article Title	Impact of potato starch on the inhibition of pancreatic lipase by potato phenolic acids	Rightsholder	Elsevier Science & Technology Journals
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NEW WORK DETAILS

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Instructor Name	Nicolas Bordenave		

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		Start Page	113409
		Issue	Pt 2
Author/Editor	CANADIAN INSTITUTE OF FOOD SCIENCE AND TECHNOLOGY.	Volume	173
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