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# Strategies for reducing the allergenicity of hen egg by treatment with natural antioxidants

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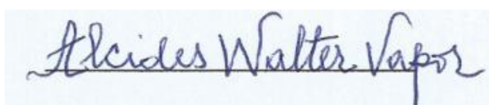

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# Dedicatory

To my beloved mother, my wife, daughters and son (**Isaly, Adjélva, Kelvin, Kélcia and Adjélcia**), who are the strength that moves me, for the affection and attention that they have given to me every day and supporting me.

"Science has bitter roots, but its fruits are sweet"

Aristóteles (384 a. C-322 a. C).

Strategies for reducing the allergenicity of hen egg by treatment with natural antioxidants

Alcides Vapor

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Strategies for reducing the allergenicity of hen egg by treatment with natural antioxidants

Alcides Vapor

# List of Publications

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I. Vapor, A., Mendonça, A., & Tomaz, C. T. (2022). Processes for reducing egg allergenicity: advances and different approaches. *Food Chemistry*, *367* (2022), 130568.

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### List of Scientific Communications

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II. Alcides W. Vapor, Cândida T Tomaz and António G. Mendonça, “Phenolic compounds: A Novel Approach to Reduce Egg Allergenicity? XIV annual CICS-UBI Symposium. 4th and 5th of July 2019, University of Beira Interior – Faculty of Health Science.

III. Alcides W. Vapor, “Phenolic Compounds: can be a trend against covid-19?” Respond to the pandemic: talk to professionals. 20th May 2020, 10:00. online broadcast – zoom ID: 915 038 1152.

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I. Sofia Soares, Alcides W. Vapor, Cândida T. Tomaz and António G. Mendonça, “Strategies for Reducing the Allergenic Capacity of Egg Proteins by Treating with Natural Antioxidants.” 5th Cycle of Conferences of Faculty of Sciences-

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II. Alcides W. Vapor, Cândida T. Tomaz and António G. Mendonça "Ovalbumin Structural Changes by Phenolic Compounds Interactions." XIII Annual CICS-UBI Symposium, 5th and 6th July 2018.

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# Resumo alargado

As alergias alimentares são causadas por respostas imunológicas anormais a componentes dos alimentos, nomeadamente proteínas (alergénios). Por esta razão, os ovos são um dos alimentos mais ricos da cadeia alimentar, sendo deste modo essenciais numa alimentação saudável. Contudo, a nível mundial cerca de 6-9 % da população infantil, e 4 % dos adultos, é alérgica aos ovos, o que impede o seu consumo ou de qualquer produto derivado. A alergia ao ovo de galinha tem aumentado em todo o mundo devido à omnipresença dos ovos na dieta, sendo uma das alergias alimentares mais comuns mediada por imunoglobulina E (IgE). A ovalbumina (OVA) é a proteína que existe em maior abundância na clara do ovo e a principal causa de alergia ao ovo em crianças e adolescentes. Até ao momento, os processos utilizados para a diminuição da alergenicidade das proteínas do ovo, como o tratamento térmico e digestão enzimática, entre outros, não têm sido totalmente eficazes, porque alguns alergénios apresentam elevada estabilidade e resistência. Assim, não existem ainda soluções industriais totalmente eficazes, nem medicamentos para a prevenção das reações alérgicas ao ovo, pelo que a sua evicção tem sido a única maneira de prevenir a alergia.

Os compostos fenólicos são reconhecidos como possuindo variadas atividades biológicas, como por exemplo: antioxidante; anti-inflamatória; antibacteriana; antiviral e antitumoral. Além disso, estes compostos, presentes na fruta e vegetais, ligam-se a péptidos e proteínas para formar complexos, podendo promover alteração da sua conformação nativa e, contribuir para a diminuição da alergenicidade. Assim, o objetivo deste trabalho foi desenvolver um processo para diminuição da alergenicidade das proteínas do ovo, nomeadamente da OVA, através da ação dos compostos fenólicos, em diferentes condições experimentais, de modo a promover alteração da sua estrutura nativa, e concomitantemente, da sua capacidade alergénica.

As técnicas espectroscópicas têm-se destacado pelo facto de necessitarem de pequenas quantidades de amostra, não serem destrutivas e poderem ser utilizadas sob diferentes condições experimentais. Deste modo, as principais alterações na estrutura proteica foram avaliadas através de dicroísmo circular (CD), fluorescência e espectroscopia de infravermelho com transformada de Fourier e reflexão total atenuada (ATR-FTIR). Como modelo, soluções de OVA em tampão fosfato 0.05 M, pH 7.40, foram incubadas a diferentes temperaturas, com vários compostos fenólicos (ácidos: gálico; cafeico; ferúlico; clorogénico e tânico; resveratrol e quercetina) preparados com a mesma solução tampão. Os resultados demonstraram que a estrutura da OVA foi afetada pela ligação dos compostos fenólicos. Os espectros de fluorescência mostraram que os

compostos fenólicos provocaram a extinção da fluorescência dos resíduos de triptofano da OVA, o que significa que as interações ocorreram diretamente ou próximo desses resíduos. A fluorescência das soluções de OVA diminuiu com o aumento da concentração dos compostos fenólicos. O mecanismo de *quenching* calculado a partir da constante de Stern-Volmer ($K_{SV}$) e da constante de *quenching* bimolecular, sugere um mecanismo de *quenching* estático. No entanto, ao considerar-se o efeito da variação da temperatura (298,15 a 318,15 K e 318,15 a 328,15 K) na extinção de fluorescência, verificou-se que $K_{SV}$ e Kq diminuíram quando a temperatura aumentou para alguns complexos OVA-compostos fenólicos, levando a um mecanismo de extinção dinâmico. Por outro lado, para outros complexos, $K_{SV}$ e Kq aumentaram com o aumento da temperatura, ou mantiveram-se constantes, sugerindo, respectivamente, um mecanismo de extinção estático e um mecanismo de extinção misto (estático e dinâmico). O mesmo efeito foi observado para a constante de ligação Kb, com mudança de temperatura. Além disso, a análise termodinâmica, de acordo com os valores encontrados de $\Delta H$ e $\Delta S$ para todos os complexos de OVA com compostos fenólicos testados, em ambas as temperaturas, sugeriu que as reações ocorreram espontaneamente ($\Delta G<0$) com a participação de interações fracas, principalmente hidrofóbica e pontes de hidrogénio. As experiências com CD e ATR-FTIR indicaram alterações na estrutura secundária da OVA, originadas principalmente pela conversão das hélices α em folhas β. O *docking* molecular mostrou que os compostos fenólicos testados podem interagir diretamente com os epítopos da OVA ou na sua proximidade, evitando presumivelmente a ligação de IgE. Em conclusão, todos os compostos fenólicos ligaram à OVA, alterando a sua estrutura terciária e secundária, sugerindo, deste modo, a aplicação destes antioxidantes naturais no tratamento das proteínas do ovo, como potencial alternativa para diminuir a sua alergenicidade.

Como perspetivas futuras, deverão ser realizados estudos com o ovo completo a que os compostos fenólicos selecionados serão adicionados, para avaliar a ligação da IgE-específica, pondo em prática a aplicação destes compostos como uma estratégia para redução da alergia aos ovos e produção de derivados de ovo hipoalergénicos. A confirmação da diminuição da alergenicidade, será realizada através de immunoblotting e ELISA com soros de doentes alérgicos ao ovo e comparando os níveis de IgE específica que se liga à OVA tratada e não tratada com compostos fenólicos. Posteriormente, a redução/eliminação da alergenicidade *in vivo* será avaliada por meio de testes de picada na pele em doentes alérgicos, utilizando extractos de OVA e de ovo inteiro, tratados com compostos fenólicos. Espera-se produzir um produto derivado do ovo que permite à população alérgica ao ovo, o seu consumo.

# Palavras-chave

Alergia ao ovo; alergia alimentar; alergenicidade; compostos fenólicos; ovalbumina; técnicas espectroscópicas.

# Abstract

Food allergies are caused by abnormal immune responses to food components (allergens), namely proteins. For this reason, eggs are one of the richest foods in the food chain and are therefore essential for a healthy diet. However, worldwide 6-9 % of the child population and 4 % of adults are allergic to eggs, which prevent their consumption or any derived products. Hen's egg allergy has increased worldwide due to the ubiquitous of eggs in the diet. Ovalbumin (OVA) is the most abundant protein in the egg white and causes allergy, especially in children and young children, being one of the most common food allergies, mediated by IgE. Until now, the processes used to decrease the allergenicity of egg proteins, such as cooking, thermal processing, and enzymatic digestion have not been totally effective, because the allergens have high stability and resistance. Thus, there are no drugs to prevent allergic reactions, and avoiding the egg has been the only way to prevent this allergy. Phenolic compounds (PC) are recognized as having a potent antioxidant, anti-inflammatory, antibacterial, antiviral and antitumor activity. In addition, these compounds, present in fruits and vegetables, can bind to peptides and proteins to form complexes, being able to promote alteration of its native conformation and to contribute to the reduction of allergenicity. Therefore, the goal of this work was the development of a process to reduce the allergenicity of hen egg proteins through the action of natural antioxidants, such as PC, under different experimental conditions, in order to promote alterations of proteins native structure, and concomitantly, of its allergenic capacity. Spectroscopic techniques have stood out among the various methods, as they use small amounts of sample, do not cause damage of the sample and can be used in different experimental conditions to analyse the conformational changes that can occur during the chemical, physical and enzymatic modifications of proteins. Thus, the OVA changes promoted by the different PC were evaluated using techniques such as circular dichroism (CD), fluorescence and total attenuated reflection - infrared Fourier transform (ATR-FTIR). The OVA solutions were incubated at different temperatures, with different PC (gallic, caffeic, ferulic, chlorogenic and tannic acids, resveratrol and quercetin) prepared with the same buffer solution. The results showed that the structure of OVA was affected by the binding of these compounds. The fluorescence spectra demonstrated that the PC quenched the fluorescent amino acid tryptophan, which means that the interactions occurred directly or close to this amino acid residue. It was also observed that the fluorescence of OVA solutions decreased with increasing PC concentration. The quenching mechanism

calculated from the Stern-Volmer constant ($K_{SV}$) and the bimolecular quenching constant (Kq) suggests a static quenching mechanism. However, when considering the effect of temperature variation (298.15 to 318.15 K and 318.15 to 328.15 K) on fluorescence quenching, it was found that $K_{SV}$ and Kq decreased with the increase of temperature in some OVA-PC complexes, leading to a dynamic extinction mechanism. On the other hand, for other complexes, $K_{SV}$ and Kq increased with increasing temperature, or remained constant, suggesting, respectively, a static extinction mechanism and a mixed extinction mechanism (static and dynamic). The same effect was observed for the binding constant Kb, with temperature change. Furthermore, the thermodynamic analysis, according to the values found for $\Delta H$ and $\Delta S$ for all OVA-PC complexes tested, at both temperatures, suggested that the reactions occurred spontaneously ($\Delta G<0$) with the participation of weak interactions, namely hydrophobic interactions and hydrogen. The experiments with CD and ATR-FTIR showed changes in the secondary structure of OVA, originated by the conversion of the α-helix into β-sheets. Molecular docking showed that the PC can interact directly with OVA epitopes or in its neighborhood, preventing IgE binding. Therefore, the application of natural antioxidants, such as PC, in the treatment of egg proteins appears to be a potential alternative to the current methods used in reducing the allergenicity of egg proteins. In fact, all PC bound to OVA, changing its secondary and tertiary structure.

As future perspectives, in-depth studies will be carried out with the whole egg treated with selected PC, to evaluate the binding of the specific IgE, as a strategy to reduce allergy to eggs and to produce hypoallergenic eggs. The confirmation of the decrease of allergenicity will be carried out by *immunoblotting* and ELISA tests with sera from patients allergic to egg and comparing the levels of specific IgE that binds to treated and untreated OVA with PC. Subsequently, the reduction/elimination of allergenicity *in vivo* will be evaluated by skin prick tests in allergic patients using OVA and whole egg extracts, treated with PC. It is expected to produce an egg-derived product that allows the egg-allergic population to consume it.

# Keywords

Allergenicity; Egg allergy; food allergy; phenolic compounds; ovalbumin; spectroscopic techniques.

Strategies for reducing the allergenicity of hen egg by treatment with natural antioxidants

Alcides Vapor

# Index

Dedicatory ........ v
Acknowledgements ........ vii
List of Publications ........ x
Resumo alargado ........ xiii
Abstract ........ xvii
Index ........ xx
List of Figures ........ xxiv
List of Tables ........ xxvi
List of Acronyms ........ xxviii
Thesis Overview ........ xxxi
**Chapter 1 ........ 1**
**1. Introduction ........ 1**
1.1. Food Allergy ........ 1
1.1.1. Epidemiology ........ 1
1.1.2. Risk Factors ........ 3
1.1.3. Pathogenesis ........ 4
1.2. Egg Allergy ........ 6
1.2.1. Egg protein allergens ........ 7
1.2.2. Egg protein allergy diagnosis ........ 8
1.2.3. Epidemiology ........ 9
1.2.4. Pathogenesis ........ 9
1.2. Processes for reducing egg allergenicity ........ 9
1.3.1. Heat treatment ........ 11
1.3.2. Microwave ........ 13
1.3.4. Maillard reaction ........ 15
1.3.5. Enzymatic treatment ........ 15
1.3.6. Food irradiation ........ 16
1.3.7. Ultrasound ........ 17
1.3.8. Microbial modification ........ 18
1.4. Phenolic Compounds ........ 20

1.4.1. Health benefits of phenolic compounds ... 21

1.4.2. Types of phenolic compounds (classification) ... 22

1.4.3. Interactions between proteins and phenolic compounds ... 24

1.4.3.1. Interactions between food proteins and phenolic compounds ... 26

1.4.3.2. Interactions of food proteins with phenolic compounds for reduction of allergenicity ... 27

1.4.3.3. Interactions of egg proteins with phenolic compounds for reduction of allergenicity ... 29

1.5. Spectroscopic techniques for analysis of protein-phenolic compounds interactions ... 31

1.5.1. Fluorescence spectroscopy ... 32

1.5.2. Circular Dichroism (CD) ... 33

1.5.3. Attenuated Total Reflection - Fourier Transform Infrared (ATR-FTIR) ... 35

**Chapter 2 ... 39**

**2. Objectives ... 39**

☐ General objective ... 39

☐ Specific objectives ... 39

**Chapter 3 ... 41**

**3. Materials and methods ... 41**

3.1. Materials ... 41

3.2. Methods ... 41

3.2.1. Fluorescence Spectra ... 41

3.2.3. Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) Spectra ... 43

**Chapter 4 ... 45**

**4. Results ... 45**

4.1. Interactions of ovalbumin with phenolic compounds ... 45

4.2. Fluorescence quenching of ovalbumin by phenolic compounds ... 45

4.2.1. Binding constants ... 54

4.2.2. Thermodynamic analysis of binding mode ... 55

4.3. Analysis of ovalbumin structural changes by CD ... 57

4.4. Analysis of ovalbumin structural changes by ATR-FTIR ... 62

4.5. Molecular docking ... 73

**Chapter 5 ... 80**

**5. Discussion, concluding remarks and future trends ........................... 80**

5.1. Methods for reducing egg allergenicity ........................................................ 80

5.2. Ovalbumin structural changes ........................................................................ 80

5.2.1. Fluorescence quenching ........................................................................ 80

5.2.2. Circular dichroism (CD) ........................................................................ 86

5.2.3. Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) ...87

5.2.4. Molecular docking ................................................................................ 90

5.3. Concluding remarks and future trends ........................................................ 91

**Chapter 6 ....................................................................................... 95**

**6. References ........................................................................... 95**

Strategies for reducing the allergenicity of hen egg by treatment with natural antioxidants

Alcides Vapor

# List of Figures

Figure 1. IgE mediated food allergy. Acute reactions (after exposure) and manifestations of food allergy....6
Figure 2. Selected methods for food processing ....11
Figure 3. Classification of phenolic compounds ....23
Figure 4. Structure of the phenolic compounds....24
Figure 5. CD spectrum of OVA, in 0.05 M phosphate buffer (pH 7.40) at 298.15 K......34
Figure 6. Fluoromax – 4 spectrofluorometer....42
Figure 7. JASCO J-815 CD spectrometer ....42
Figure 8. Thermo Nicolet-iS10 spectrometer. ....43
Figure 9. The fluorescence spectra emission of free OVA [4.44 μM] ($\lambda_{max}$ = 280nm), pH 7.40, and after 10 minutes of incubation in the presence of various concentrations of PC ....49
Figure 10. Stern-Volmer plots of OVA with PC after 10 minutes of incubation at 298.15 K, 318.15 K and 328.15 K.... 51
Figure 11. The Lineweaver-Burk equation (binding plots), plots of OVA [4.44 μM] with all PC tested, after incubation for 10 minutes, at 298.15, 318.15, and at 328.15 K.........55
Figure 12. CD spectra of free OVA and OVA with phenolic compounds in increasing concentrations after 10 minutes of incubation at 298.15 and 318.15 K....59
Figure 13. ATR-FTIR spectra (1700-1500 cm-1) of OVA [1.0 mM] and OVA with all PC [1.0 mM], concentration ratio 1:1....63
Figure 14. Curve-fitting of ATR-FTIR spectra of all OVA-PC using Voigt procedure, after 10 minutes of incubation .... 68
Figure 15. ATR-FTIR spectra second derivative using Savitsky-Golay, analyzing OVA-PC after 10 minutes of incubation, concentration [1.0 mM] and concentration ratio 1:1 ....72
Figure 16. Representation of crystal structure of OVA at 1.95 Å resolution, from protein data bank (PDB ID: 1OVA _1: shows chain A)....74
Figure 17. The molecular docking revealing the epitopes from OVA....76

Strategies for reducing the allergenicity of hen egg by treatment with natural antioxidants

Alcides Vapor

# List of Tables

Table 1. Risk factors of food allergy. ....................................................................4
Table 2. Egg allergenic proteins. ...........................................................................8
Table 3. Methods for reducing egg allergenicity. ........................................................... 19
Table 4. Application of phenolic compounds for reducing allergenicity of food proteins. ........................................................................................................ 28
Table 5. Application of phenolic compounds for egg reducing allergenicity. ................. 31
Table 6. Infrared bands of peptide bond..........................................................................36
Table 7. The quenching constants ($K_q$) and binding constants ($K_b$) between OVA and PC at three different temperatures (298.15, 318.15 and 328.15 K), after 10 minutes of incubation. .........................................................................................................53
Table 8. Thermodynamic parameters of OVA-PC interaction, at three different temperatures (298.15 K, 318.15 K and 328,15 K), after 10 minutes of incubation. ........56
Table 9. Van't Hoff equations (298.15, 318.15 and 328.15 K) ........................................57
Table 10. Phenolic compounds and ratio of OVA/PC tested in CD [OVA =4.44 μM] .... 57
Table 11. Secondary structure of OVA and OVA-PC solutions at 298.15K by Circular Dichroism ..........................................................................................................59
Table 12. Secondary structure of OVA and OVA-PC solutions at 318.15K by Circular Dichroism .......................................................................................................... 60
Table 13. Wavenumbers peaks in the region 1700-1600 cm-1 (amid I) from ATR-FTIR spectra at different temperatures (figure 13 A: 298.15 K; B: 318.15K and C: 328.15 K). ..........................................................................................................63
Table 14. Secondary Structure of OVA and OVA-PC (ATR-FTIR Spectra) at pH 7.40, OVA [1.0 mM] and PC [1.0 mM], concentration molar ratio OVA/ PC corresponding to 1:1; after 10 minutes incubation. ..........................................................................69
Table 15. ATR-FTIR peaks of second derivative for each solution of OVA, after incubation at 318.15K and 328.15 K. All spectra were obtained at room temperature after incubation. ..................................................................................................73
Table 16. Estimated ΔG of the interaction of the PC with OVA epitopes, given by Swissdock software. ..........................................................................................75

Strategies for reducing the allergenicity of hen egg by treatment with natural antioxidants

Alcides Vapor

# List of Acronyms

| | |
|---|---|
| AGEs | Glycation end-products |
| Ara h 3 (A3) | *Arachis hypogaea* |
| ATR-FTIR | Attenuated Total Reflection-Fourier Transform Infrared |
| BALB/c | Animal model |
| BATs | Basophil Activation tests |
| BLG | β-Lactoglobulin |
| BN rat | Animal model |
| BSA | Bovine serum albumin |
| CA | Caffeic Acid |
| CHA | Chlorogenic Acid |
| CD | Circular Dichroism |
| $CD_{23}$ | Low-affinity receptor for IgE |
| CDSSTR | Algorithm to estimate the secondary structure content |
| CN | Carbon and nitrogen |
| C=O | Carbonyl |
| CRD | Component Resolved Diagnostic |
| C3H/HeJ | Animal model |
| DBPCFC | Double blind placebo-controled food challenge |
| DSS | Dihedral space sampling |
| EAST | Enzyme allergoabsorbent test |
| e.g. | For example |
| EGCG | (-)-epigallocatechin 3-gallate |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FA | Ferulic Acid |
| FcεRI | High-affinity cell-surface receptor for IgE |
| FcεRII | Low-affinity receptor for IgE |
| GA | Gallic Acid |
| Gal d 1 | Ovomucoid |
| Gal d 2 | Ovalbumin |
| Gal d 3 | Ovotransferrin |
| Gal d 4 | Lysozyme |
| Gal d 5 | α-livitin |
| Gal d 6 | YGP42 protein |
| HDL | High density lipoprotein |
| HHP | High hydrostatic pressure |
| HSV-1 | Herpes simplex virus type 1 |
| HSV-2 | Herpes simplex virus type 2 |
| IgE | Immunoglobulin E |
| IgG | Immunoglobulin G |
| IL4 | Interleukin 4 |
| IL5 | Interleukin 5 |
| IL13 | Interleukin 13 |
| IR | Infrared |
| KDa | Kilo Dalton |

| | |
|---|---|
| kGy | Kilo gramma irradiation sterilization |
| LDL | Low density lipoprotein |
| Lr-0601 | Lactobacillus rhamnosus |
| MHC | Major Histocompatibility Complex |
| mAb | Monoclonal antibody |
| Mal d 3 | *Malus domestica* |
| MPa | Mile Pascal |
| MTGase | Microbial transglutaminase |
| MR | Maillard Reaction |
| NH | Amine |
| NRMSD | Normalized root means square deviation |
| OCN | Oxygen, carbon and nitrogen |
| OFC | Oral food challenge |
| OVA | Ovalbumin |
| PAMD@ | Precision allergy molecular diagnostic applications |
| PC | Phenolic Compounds |
| QCT | Quercetin |
| RES | Resveratrol |
| SARS | Severe acute respiratory syndrome |
| SPT | Skin Prick Test |
| TA | Tannic Acid |
| TCR | T-cell Receptor |
| TGase | Transglutaminase |
| Th1 | T-helper 1 |
| Th2 | T-helper 2 |
| Treg | T-regulatory cell |
| TP | Tea polyphenol |
| UV-vis | Ultraviolet-visible |
| UV-c | Ultraviolet-c (desinfectant for air) |
| VLDL | Very low density lipoprotein |
| WPI | Whey protein isolate |

# Thesis Overview

This Doctoral thesis comprises six chapters.

Chapter 1 consists of an introduction to the theme of this thesis, and it is divided into four sections.
The Introduction addresses the food allergy, its epidemiology, risk factors and pathogenesis, followed by a state of the art of the egg allergy, egg protein allergens, diagnosis, epidemiology, pathogenesis, and finally, a description of the most used processes for reducing egg allergenicity. In addition, it is provided an overview of the phenolic compounds, its health benefits, classification, interactions between proteins and phenolic compounds and interactions of food proteins with phenolic compounds for reduction of allergenicity.

Chapter 2 comprises the global objective of this thesis, and also detailed specific objectives in which are defined the tasks for the development of this work.

Chapter 3 reports the materials and methods used in experimental approach for the development of this thesis.

Chapter 4 presents the results of the application of spectroscopic techniques used for analysis of interactions of ovalbumin-phenolic compounds: Fluorescence spectra, Circular Dichroism (CD), Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) and Molecular Docking (*in silico* study).

Chapter 5 reports the main discussion of the results in the light of the state of the art, concluding remarks and future trends in this research topic.

Chapter 6 integrates the references used in the introduction and in discussion of this thesis.
Finally, is included in the introduction, the published review written within the scope of the topic of this thesis.

# Chapter 1

# 1. Introduction

The definition of "allergy" was firstly introduced in 1906 by Clemens von Pirquet (1874-1929) to describe a change in reactivity of the organism in time, quality and quantity, "different or strange reaction" to an antigenic stimulation that can lead to specific immunity or "hyper-" and "hyposensitivity reactions". Generally, allergy to human predisposition, Pirquet emembered that the alteration in reactivity it is not conditioned by exogenous substances (the allergens), depends also on endogenous factors of the organism itself (von Pirquet, 1906, cited by Huber, 2006; Niggemann and Beyer, 2015). Nowadays, the term allergy is used to designate IgE-mediated or type I hypersensitivity, not IgE-mediated, or an intermediate type that shows characteristics of both (Savage, Sicherer & Wood, 2016).

## 1.1. Food Allergy

Every adverse reaction to food consumption, mediated by immunological mechanisms, as well as reactions caused through dietary exposure to relevant allergens in food are designated as food allergy (Johansson et al., 2004; Verhoeckx et al., 2015; Savage et al., 2016). Such reactions can be derived of the dietary exposure to relevant allergens in food (Beyer & Niggemann, 2016). The main allergens in food are proteins (Savage et al., 2016). So far, there is no cure for offensive foods or allergies neither approved regulatory treatments for food allergies, whereby strict prevention is the best treatment to date (Carrard, Rizzuti, & Sokollik, 2015; Sathe, Liu, & Zaffran, 2016). Thus, clinical consultation and accurate diagnosis are essential in all suspected cases.

Therefore, the labeling of allergenic foods should be mandatory in all countries to ensure safety and prevent food allergies in the population (Taylor & Baumert, 2015; Mahdavinia, 2020). Moreover, it would be pertinent to raise public health awareness level of the importance of food allergies, the adoption and establishment of priority lists of allergenic foods.

### 1.1.1. Epidemiology

Up to 15 % of the world population already presented some adverse food reaction, having predominance in industrialized counties (Wróblewska, Jędrychowski, Hajós & Szabó, 2008; Sicherer & Sampson, 2014). Therefore, food allergy is a worldwide public health problem (Taylor & Baumert, 2015). Sicherer and Sampson (2018) reported that

the prevalence of adverse reactions to food is almost 8 % in children and 5 % in adults. On the other hand, it was demonstrated that the prevalence of food allergies is increasing every day, and as a public health problem is affecting millions of people, estimated at about 1-3 % in adults and 4-6 % in children, yet data on adults are limited (Loh & Tang, 2018; Leung, Wong, & Tang, 2018; Gupta et al., 2018; Sanchiz et al., 2018; Mahdavinia, 2020). So far, there is a weak understanding about the reasons why some food allergies are outgrown, and other are not. The understanding of the immune basis in the natural resolution of food allergies is critical for the development of new therapies for the persistent food allergies (Berin, 2018).

In Africa, there are few databases on food allergy. Only in South Africa this topic has been more researched and reported than in the other ten countries (Botswana, Democratic Republic of Congo, Ghana, Kenya, Marocco, Mozambique, Nigeria, Tanzania, Tunisia and Zimbabwe), which means that attention should be paid as an emerging disease (Kung, Steenhoff, & Gray, 2012; Gray & Kung, 2012; Gray et al., 2014; Levin et al., 2015; Basera et al., 2015; Gray, Levin & du Toit, 2016; Gray, 2017; El-Gamal, Hossny, El-Sayed & Reda, 2017; Botha et al., 2018). According to the National Institute of Public Health of Angola, there are no databases of food allergies in this country. However, many patients with food allergies often go to the hospital but clinicians do not have the means to diagnose it except through physical examinations. Thus, they only may suspect of food allergy without the tools to determine the food that originated the symptoms. It is important for the clinician to recognize the patterns of food allergy symptoms; otherwise, they will cause confusion and it will not be possible to help those who are suffering (Kazatsky & Wood, 2016).

On the other hand, it is assumed that the population is not well informed about food allergy. As well as the restaurants or foods where ingredients are not advertised properly are the source of many allergic reactions (Taylor & Baumert, 2015; Mahdavinia, 2020). Nonetheless, the exact prevalence of food allergy is difficult to determine because it is not reproducible due to variations in the definition of allergy, study population, geographical area, method, age, and food allergen studied (Loh & Tang, 2018; Sanchiz et al., 2018; Wai, Leung, Leung & Chu, 2019). Thus, it was reported the difficulty in understanding the epidemiology of food allergy, given the difficulty of identifying it on a large scale. Despite this lack of information, it was described that prevalence of food allergy is higher in younger age groups and decreases with age (Dunlop & Keet, 2018).

Therefore, the existing data on food allergy are not totally exact (Ohtani et al., 2016), and it will be necessary to disclose more information about the associated risks and how to deal with food allergy (De Silva et al., 2014).

### 1.1.2. Risk Factors

Food allergy is a condition that puts lives of everyone affected at risk, as it occurs not only in the gastrointestinal tract, but can also affect other organ systems (Valenta, Hochwallner, Linhart, & Pahr, 2015). Furthermore, children when grown up can feel bad because of stigmatization when they are among others (Antolín-Amérigo et al., 2016; Thijs et al., 2018).

The main risk factors that can predispose the onset of food allergy are: heredity (family history, sex and ethnicity), genetics (adaptative immunity) (Lack, 2008; 2012), change in diet, lack or excess of vitamin D, dietary fat, lack of natural antioxidants, obesity (Kaza, Knight & Bahna, 2007; Lack, 2008; 2012), impaired skin barrier function, microbial exposure - host microbiome (Atopic dermatitis at 3 months old), material factors (Anti-acid intake, cesarean section delivery, lack of multivitamin supplementation, maternal diet during pregnancy), environmental exposure (Sigurs, Bjarnason, Sigurbergsson, Kjellman & Björkstén, 1995; Kaza et al., 2007; Lack, 2008; 2012; Nutten, 2015) and intrinsic properties of the organism (Table 1) (Sigurs et al., 1995). Many studies reported about the delay in introducing the allergen in the baby food as the way to prevent allergies, but that hypothesis (Kaza et al., 2007) has been refuted (De Silva et al., 2014; Wei-Liang Tan et al., 2017) because of the factors presented above that affect food allergy.

Table 1. Risk factors of food allergy.

| Risk factors | Reference |
|---|---|
| Heredity (family history, sex and ethnicity) | Lack (2008; 2012) |
| Genetic (adaptative immunity) | |
| Change in diet | |
| Lack and excess of vitamin D | Kaza et al. (2007) |
| Dietary fat | |
| Lack of natural antioxidants | Lack (2008; 2012) |
| Obesity | |
| Impaired skin barrier function | Nutten (2015) |
| Microbial Exposure – host microbiome (Atopic dermatitis at 3 months old) | Kaza et al. (2007) |
| | Lack (2008; 2012) |
| Material factors (Anti-acid intake, cesarean section delivery, lack of multivitamin supplementation, maternal diet during pregnancy) | Sigurs et al. (1995) |
| Environmental exposure (Contact with furred animals, exposure to tobacco smoke, weight at second follow-up) | |
| Intrinsic properties of organism (Birth weight (g), gestational age (weeks), history of pertussis and infant diet) | Sigurs et al. (1995) |

### 1.1.3. Pathogenesis

The word "allergy" is widely accepted, but its use contrasts, as it was restricted to specific immune hypersensitivity reactions against harmless foreign antigens, as mentioned by Pirquet (Huber, 2006). Regarding the types of allergies, Coombs and Gell (1975), reported that there are 4 main types of allergic reactions based on the pathogenesis mechanisms, which trigger food allergy. Thus, the consumption of foods that are part of the allergen group can result in an IgE-mediated and non-IgE-mediated reaction (Sicherer & Sampson, 2018). However, immunopathogenesis of food allergy and food-induced disorders arise from the complex relationship between environmental and genetic influence (Sicherer & Sampson, 2014).

Most sources of allergens contain antigens that have little or no clinical relevance because they are weak inducers of allergic reactions, so food allergy is often confused with other adverse reactions to food (Valenta et al., 2015). Hence, it´s important learning about food allergy mechanism and attentive for discerning the difference between IgE-mediated and non-IgE-mediated reactions (van Ree, Poulsen, Wong, Ballmer-Weber, Gao & Jia, 2015).

When interaction with food antigens occur, IgE becomes cross-linked and binds to mast cells and basophils via the high-affinity receptor FcεRI (Bischoff & Crowe, 2005;

Valenta et al., 2015). This process activates these cells, leading to the release of granules that contain preformed inflammatory mediators (e.g. histamine), as well as de novo synthesis and/or release of inflammatory mediators (e.g. leukotrienes), proteases (e.g. tryptase), inflammatory cytokines (e.g. IL4). In a few minutes are activated mast cells and basophils, in the process called IgE-mediated reaction. Symptoms can occur shortly after allergen contact (Figure 1). While the symptoms develop directly in the places of contact with the allergen (for example, mouth, esophagus and/or intestine), or in other organs, through the entry of food allergens into the blood by gastrointestinal tract, systemic reactions occur, through the passage of cross-linked IgE bound to effector cells the mucosal barrier for circulation (Figure 1). Allergen can also reach nervous systems (Valenta et al., 2015; Dona & Suphioglu, 2020).

The denser load of allergen-specific IgE cells with IgE may account for the association between allergen-specific IgE levels against stable food allergens and the severity of allergic reactions that cause regulation of FcεRI in mast cells and basophils (MacGlashan et al., 1999; Yunginger, 2000; Valenta et al., 2015; Dona & Suphioglu, 2020). Interestingly, it has also been shown that enteric eosinophils not only contribute to inflammation, but also control dendritic cells to initiate primary immune responses mediated by Th2 cells, indicating a complex interaction between cells in food allergies (Chu et al., 2014). Delayed-type reactions can occur 24-48 hours after contact with the allergen, in the course of late-phase responses, and allergen-specific T cells can be activated via IgE-dependent and IgE-independent pathways (Figure 1). In fact, in patients with allergies, antigen presenting cells express FcεRI as well as the low-affinity receptor for IgE (also known as CD23). It was also related that the cells use FcεRII receptor for IgE-facilitated allergen presentation, the process is considered more effective for the activation of T-cells than the presentation of allergen without IgE (Novak, Bieber & Kraft, 2004; van Neerven, Knol, Ejrnaes & Würtzen, 2006; Valenta et al., 2015). Atopic dermatitis induced by food allergens has been shown to require Th2 cells (and Th2 cytokines such as IL4, IL13, and IL5 - a cytokine that activates eosinophils), also Th1 cells, which mediated delayed allergic inflammation (Reekers et al., 1996). Information on the time to onset of allergic reactions after eating food allergens (minutes-hours vs hours-days) and allergy phenotype (e.g. urticaria vs atopic dermatitis) can help to determine whether symptoms involve IgE-mediated mast cells immediate or activation of basophils or late or chronic allergic inflammation caused by T cells or activation of eosinophils (Valenta et al., 2015; Dona & Suphioglu, 2020).

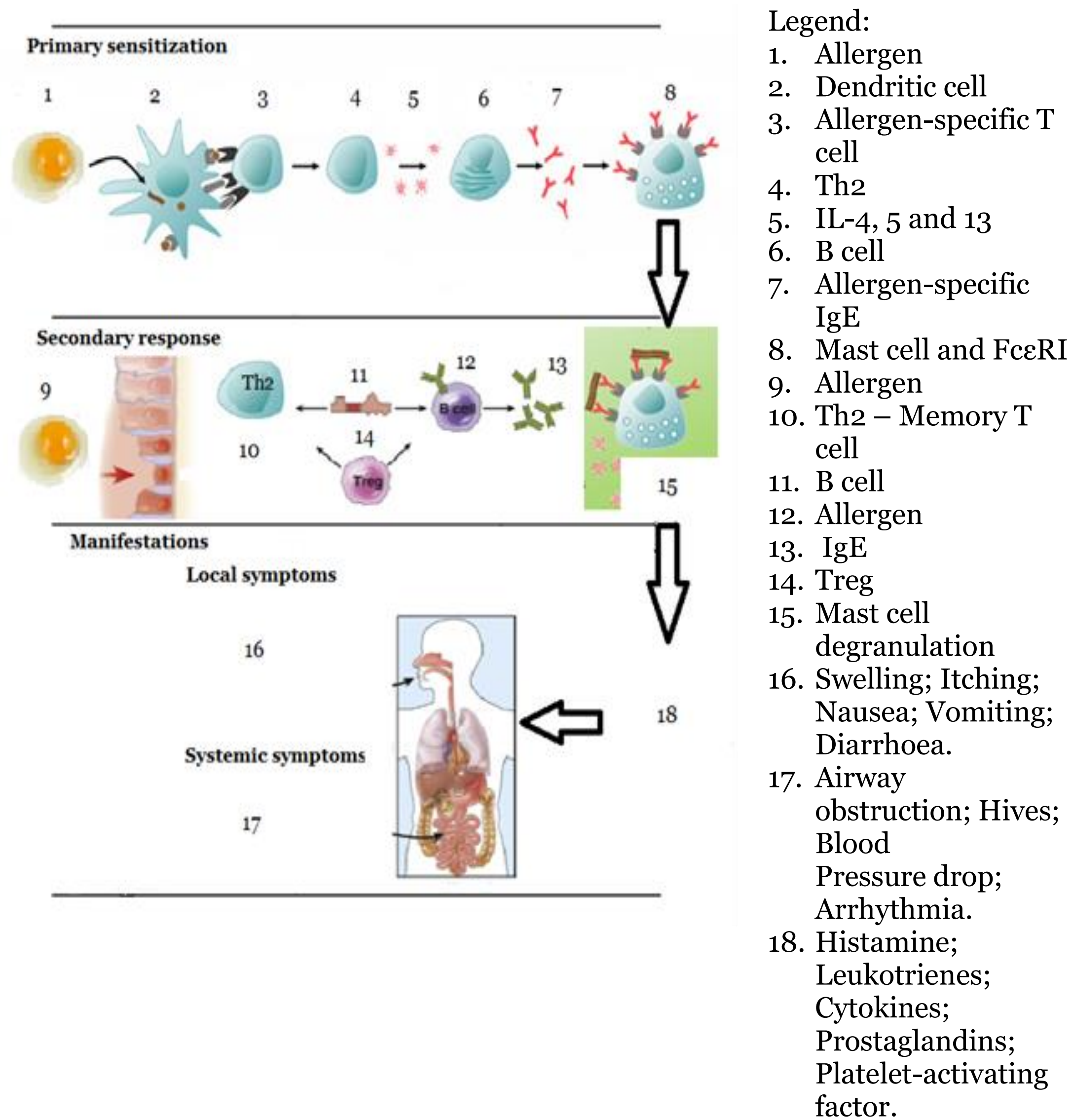



Figure 1. IgE mediated food allergy. Acute reactions (after exposure) and manifestations of food allergy. Allergen contact with dendritic cells induces T cells, Th2, IL (3, 4 and 13), B cells and IgE production (primary sensitization). After the binding of IgE to FcεRI, mast cells begin a degranulation with histamine release. Repeated allergen contact activates allergen-specific memory T cells (Th2) and B cells by regulatory T (Treg) cells, and induces IgE responses during the secondary response, whilst exposure could increase production of IgE, leading to manifestations. Allergy symptoms are caused by repeated contact with the oral allergen, via the immediate allergic reaction (allergen-induced crosslinking of mast cells bound IgE by allergen and then activation of allergen-specific T cells). After allergen ingestion, inflammation develops not only in the intestine, but in other organs, such as the skin, respiratory tract, and circulatory system. Adapted from Valenta et al. (2015).

## 1.2. Egg Allergy

After the allergy to cow's milk, egg allergy is the second most frequent food allergy among babies and children (Nwaru et al., 2014; Praticò et al., 2014). Most of the allergenic egg proteins are found in egg white, but eggs present allergens both in the egg white and yolk (Caubet and Wang, 2011; Ohtani et al., 2016), which can justify the

two-thirds of children more reactive to egg white (Kumar, Verma, Das, & Dwivedi, 2012).

Despite eggs are one of the most nutritious foods and therefore, essential for a healthy diet (Gupta et al., 2018), its direct or indirect consumption in prepared food, may be life-threatening for all with egg allergy.

Generally, egg allergy may have a good prognosis. A few of allergic outgrow their allergy in the fifth year of age (Kemp, 2007), however different studies suggest that egg allergy does not go away so soon for most people, but over time (Savage, Matsui, Skripak & Wood, 2007; Ohtani et al., 2016).

### 1.2.1. Egg protein allergens

The egg consists of approximately 8-11 % shell, 56-61 % white and 27-32 % yolk (Zhu, Vanga, Wang & Raghavan, 2018a). Egg white is considerably more allergenic than egg yolk, where in addition to allergenic proteins, it is also possible to find lipoproteins such as: HDL, LDL and VLDL (Dang et al. 2019). In fact, egg white contains numerous different proteins and glycoproteins which can trigger allergic reactions, and includes: ovomucoid (Gal d 1), about 11 % of egg white, with 28 KDa; ovalbumin (Gal d 2), about 54 % of egg white, with 42.7 KDa; Ovotransferrin (Gal d 3), about 12 % of egg white, with 76.6 KDa; and lysozyme (Gal d 4) about 3-4 % of egg white, with 14 KDa (Dhanapala et al., 2015; Lin, Wu, Huang, Cheng & Yeh, 2016; Park, Yoom, Kim, Han & Choi, 2017) (Table 2). Gal d 1 and Gal d 2 are the major allergens of egg white (Martorell et al., 2013). Initially, research on egg allergy had been more focused on Gal d 2 due to its high abundance in egg white. However, studies on Gal d 1 have shown that despite initially being considered a minor allergen, now it is postulated to be the dominant egg allergen due to its resistance to heat and high allergenic potential (Ognjenović et al., 2014; Wai, Leung, Leung & Chu, 2019). Although avidin is not considered an allergen, this egg white protein is widely studied since it forms a complex with biotin which prevents its absorption (Zhu et al., 2018a). The egg yolk has two minor allergens: Alpha-livetin or chicken serum albumin (Gal d 5), that can cause an egg syndrome to avian, and the YGP42 protein (Gal d 6) (Table 2) (Seweryn, Królewicz, Stach, & Kustrzeba-Wójcicka, 2018; Wai et al., 2019). It is important to highlight that there are minor food allergens that have not yet been thoroughly studied, which can limit the full understanding of its effect on protein allergenicity.

Another relevant issue is the cross-reactivity with different avian eggs allergens that must be taken in account among children with hen's egg allergy. In fact, and despite the low consume of eggs of other avian species compared to chicken eggs, the elimination of all type of avian eggs allergens in the diet of these children should to be performed (Moghtaderia, Nabavizadehb, & Teshnizic, 2020).

Table 2. Egg allergenic proteins.

| Egg proteins | Allergen name | Molecular weight | PI | Percentage content | Observations |
|---|---|---|---|---|---|
| **Ovomucoid (OVM)** | Gal d 1 | 28 KDa | 4.75 | 11 % | Dominant egg allergen Heat resistant |
| **Ovalbumin (OVA)** | Gal d 2 | 42,7 KDa | 5.19 | 54 % | Most abundant protein |
| **Ovotransferrin (OVT)** | Gal d 3 | 76,6 KDa | 6.85 | 12 % | - |
| **Lysozyme (LYS)** | Gal d 4 | 14 KDa | 9.36 | 3-4 % | - |
| **α-livitin** | Gal d 5 | 69 KDa | 5.51 | 40-60 % | The main egg yolk protein |
| **YGP42 protein** | Gal d 6 | 35 KDa | 9.16 | - | Egg yolk protein |

### 1.2.2. Egg protein allergy diagnosis

Different techniques have been used for egg allergy diagnosis (Sicherer & Sampson, 2018). The evaluation of patients' clinical history followed by a physical examination is the first approach to study egg allergy (Sicherer & Sampson, 2018). To make an accurate diagnosis it is important to consider all the symptoms and factors that cause allergy (Mahdavinia, 2020). The following steps usually involve in vivo Skin Prick Testing (SPT) and in vitro tests, such as ImmunoCAP specific IgE blood tests to evaluate the presence of egg allergen-specific antibodies (Muraro et al., 2014). The combination of egg specific serum IgE levels and SPT has been used to guide clinical decisions and to monitor the evolution of egg allergy in children (Graham, Bégin, Paradis, Lacombe-Barrios, Paradis & Des Roches, 2016). Moreover, combined ImmunoCAP test and oral food challenge (OFC) improve the diagnosis and the treatment approaches (Graham et al., 2016). Kim et al. (2019) concluded that the levels of specific IgE to egg white can be utilized as a predictor of tolerance in egg allergic children. Moreover, the specific IgE against to linear epitopes of Gal d 1 showed to be highly specific for identification of hen's egg allergic adults and can be an additional value in egg allergy diagnosis (Ehlers et al. 2020). Basophil activation tests (BATs) were also considered a crucial tool in the diagnosis of food allergy due to a superior specificity than SPT and specific IgE (Sato, Yanagida & Ebisawa, 2018). Component-resolved diagnostics (CRD) appears to provide additional information to determine if OFCs should be performed to diagnose allergies (Sato et al., 2018; Dona & Suphioglu, 2020). Recently, a new molecular based diagnostic, termed precision allergy molecular diagnostic applications (PAMD@), was described as an important tool in the

management of the allergic patients (Ansotegui et al., 2020). PAMD@ allows mapping the sensitization profile and assessing the food allergy risk and the cross-reactivities between different combinations of food and inhalant allergens. Thus, PAMD@ can contribute to understanding the molecular mechanisms underlying allergic sensitization and cross-reactivities of food allergens (Ansotegui et al., 2020).

### 1.2.3. Epidemiology

Currently, IgE-mediated egg allergy prevalence is climbing, reaching 6 to 9% of the child population and 4% of adults worldwide, which prevents their consumption or any derived products, this means that a quick intervention is needed to find out how to mitigate it (Savage et al., 2016; Gray et al., 2016; Dona & Suphioglu, 2020). Therefore, several studies about egg allergy, considering the head cause of IgE-mediated food allergy, and prevalence shows elevated values when compared to other allergies (Wei-Liang Tan et al., 2017; Peters et al., 2017; Dang et al., 2019; Dona & Suphioglu, 2020).

In addition, patients with egg allergy may be at increased risk of allergic reactions even to other types of eggs from other birds. Although systematic studies related to these themes have not been widely disseminated, based on clinical experiences (Kazatsky & Wood, 2016; Ohtani et al., 2016).

### 1.2.4. Pathogenesis

In the classification of food allergy, hen egg allergy belongs to type I, and is mainly mediated by IgE. The process occurs initially though sensitization mechanism where allergen is phagocytized by dendritic cells and these form specific T lymphocytes, that stimulate Th2 cells, once active, trigger the production of cytokines as IL4, IL5 and IL13, which stimulate the proliferation of B lymphocytes and will produce antigen specific IgE (Figure 1). The specific IgE bind to mast cells and basophils creating a state of sensitization mechanism in the patient. In a later contact with the allergen, the mast cells become degranulated with IgE bound to the surface and histamine, leukotrienes and prostaglandins are released and the allergic reaction with local symptoms or systemic symptoms is triggered (Valenta et al., 2015).

## 1.2. Processes for reducing egg allergenicity

The production of safe and innovative food products without losing nutrients has been the goal of food technicians during the last years. Food processing techniques can be applied to transform raw products into consumables and can provide many beneficial effects (Mattarozzi & Careri, 2019). Given that processing may affect the capacity of

proteins to promote allergic sensitisation, it is important to control the allergenicity of processed food (Verhoeckx et al., 2015). The protein changes depend on different factors like the type of food, allergen levels and processing requirements (Cabanillas & Novak, 2019). Among the allergenic foods, the egg is the one in which the processing or cooking most alters the allergenicity. In fact, the allergic potential of egg can be reduced or increased due to protein unfolding upon specific treatment which leads to structural alterations that can hide or destruct the epitopes of binding IgE (Jiménez-Saiz, Bénede, Molina & López-Expósito, 2015; Pablos-Tanarro, Ojalvo, Blanco, Fandiño & Molina, 2017; Cabanillas & Novak, 2019).

The evaluation of the effect of the food processing on the physicochemical properties of egg proteins and its impact on eggs allergenicity has been performed using different strategies, including assays for IgE-binding capacity and in vitro (mast cell, basophil granulocyte and basophil) and in vivo (animal models such as BALB/c mouse, C3H/HeJ mouse, and BN rat) experiments where an elicitation of an allergic response is induced (Huang et al. 2018). The SPT and food challenges, such as OFC and double-blind placebo-controlled food challenges (DBPCFC), despite being the best methods to assess the potential sensitising capacity of food proteins to the patients, are difficult to implement due to the economic and ethical limitations (Costa et al. 2021).

The determination of specific IgE only informs about sensitization, however it has been widely used to assess the allergenic potential of food proteins, although sensitization does not always culminate in the elicitation phase. The IgE-binding capacity of the allergens is usually determined by Immunoblotting, ELISA (enzyme-linked immunosorbent assay) and enzyme allergoabsorbent test (EAST)/ImmunoCAP using human sera or plasma of sensitised or allergic patients. These techniques are also applied to perform immunoreactivity assays using animal antibodies for measure of the immune reaction caused by an antigen. On the other hand, the allergenicity and the allergenic potential are assessed by a simulation of allergic reactions using *in vitro* or *in vivo* assays with cellular and animal models, respectively (Verhoeckx et al. 2015; Huang et al. 2018; Costa et al. 2021).

In the last years, there has been a great investment in the development of new processing techniques to produce hypoallergenic foods. Thermal and non-thermal techniques (Figure 2 and Table 3) have been shown to alter sensitization and allergenicity potential of egg proteins by modifying structural epitopes of the allergens, however such methods have not yet been able to completely eliminate egg allergenicity (Zhu et al., 2018a; Ma et al. 2020).

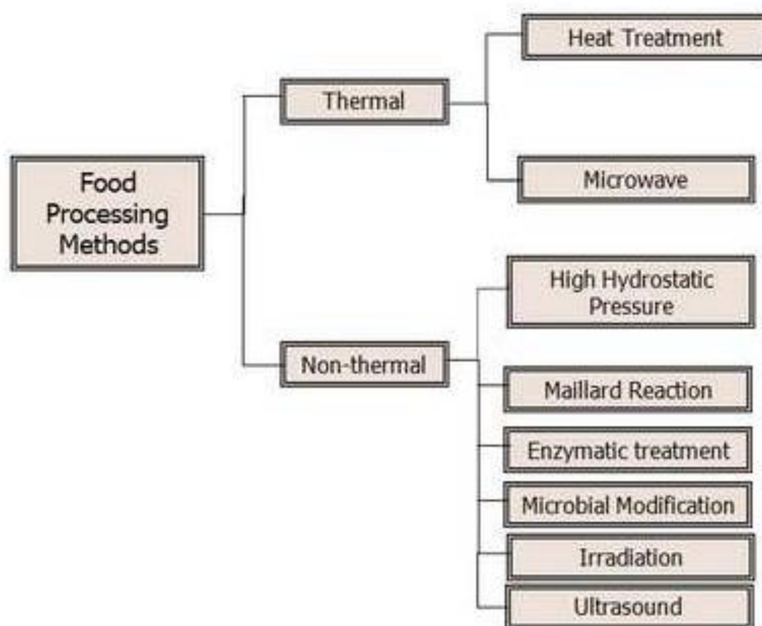



Figure 2. Selected methods for food processing.

### 1.3.1. Heat treatment

Thermal processing has been applied on foods to improve microbial safety, detoxification, texture and digestibility. In addition, the heat treatments cause modifications in the physical-chemical and functional properties of proteins. The effect of heating on allergic food components can decrease the allergenicity or increase it by promoting structure and conformational alterations in the native protein (Verhoeckx et al., 2015; Vanga, Singh & Raghavan, 2017). Egg products can be used after extensive heating, such as cakes, egg pasta and bread or with less heating such as fresh mayonnaise. Curiously, it was observed that eggs cooked with wheat or other substances are less IgE reactive than eggs heated on their own (Bloom et al., 2015). Thus, the patients allergic to eggs may present lower reactivity to foods like cakes, than to cooked eggs alone (Dhanapala et al., 2015). Heating by destroying or modifying conformational epitopes can decrease the allergenicity of diverse foods. Moreover, the heat induces protein denaturation, rearranging or forming novel intra and/or intermolecular bonds, which prevent epitopes from being recognized by IgE (Ekezie, Cheng & Sun, 2018).

Different studies have shown that heating Gal d 2 decreases the binding to IgE compared to unheated Gal d 2 (Leonard, 2016). Thus, it is important to find out how

heat treatments promote a change in the structure of food proteins and how it influences their digestibility, since both factors can affect their allergenicity (Ekezie et al. 2018). Pablos-Tanarro et al. (2017) applied moderate heat treatments to egg white and found a reduction of its allergenic potential in a BALB/c model of egg allergy. After heat treatment at specific pH, temperature, time, and concentration conditions, the egg white can aggregate and denature; however, the processing parameters could be monitored to control the protein aggregation (Gharbi & Labbafi, 2018). Claude et al. (2017) studied the effect of pH and ionic strength in Gal d 2 allergenicity during the aggregation process by thermal treatments. In fact, the aggregates formed at low ionic strength and near pH of stored egg, in which electrostatic repulsions compensate for hydrophobic attractions, exhibit a reduced allergenicity compared to the aggregates formed when electrostatic repulsions are minimal, at pH near the isoelectric point and high ionic strength. Moreover, the morphology and the structure of aggregates are relevant factors to take into account in the immune response during the sensitisation since they can induce differences in the development phase of allergy. Posteriorly, Claude et al. (2019) also studied the capacity of Gal d 2 and thermal aggregates, to promote degranulation of a humanized rat cell line that was sensitised with sera from children with egg allergy. They concluded that the aggregates had significantly lower ability to trigger degranulation than native Gal d 2.

Contrarily to Gal d 2, Gal d 1 is more resistant to heat denaturation and to proteolytic digestion due to the disulphide bonds that link the three subdomains. The principal responsible for the high allergenicity of Gal d 1 is the domain 3 which has a great stability and also the most dominant binding epitope (Verhoeckx et al., 2015). Nevertheless, recently it was described over 90% reduction of Gal d 1 antigenicity after a treatment at pH 9.5 and temperature of 100 ºC, due to the change in linear epitopes exposure and position of the conformational epitopes (Stănciuc, Cretu, Banu & Aprodu, 2018). Curiously, it was found that high levels of IgE towards Gal d 1 can suggest an incapability to tolerate extensively heated eggs (Chokshi & Sicherer, 2015).

The efficacy of a graduated exposure to extensively heated and baked egg to promote tolerance in egg allergic children under 2 years was evaluated and a significant decrease in time to resolve the allergy in these children was observed (Gotesdyner et al., 2019). The authors suggested an action mechanism involving a gradual decrease in specific IgE to egg and an increase in protective IgG and cellular regulatory pathways. Brossard et al. (2019) performed SPT in egg-allergic children and showed that all of them had reactive SPTs to raw egg white and 72 % to raw egg yolk. It was also observed that SPT-reactivity for some children decreased after heating, but hard-boiling was more

effective than pasteurization. Another study concluded that children with hyper-responsiveness to heated egg yolk in childhood can exhibit egg allergy for longer (Horino, Kitazawa, Satou & Miura, 2019). Recently, it was demonstrated that children allergic to eggs who eat regularly baked eggs are more likely to overcome allergy than egg allergic children who followed a strict diet without eggs. Additionally, they concluded that eat baked eggs daily seems to improve the tolerance to raw eggs (Pérez-Quintero, Martínez-Azcona, Balboa & Vila, 2020).

### 1.3.2. Microwave

The thermal treatments using new technologies, such as microwave, have increasing over the last years. The microwave generates heat instantly and significantly reduces the processing time and operational cost when compared to the conventional dry-heating methods (Vanga et al., 2017; Ekezie, Sun, Han & Cheng, 2017; Li, Sun, Ma, Jin & Sheng, 2018). The main advantages of microwaves are the less time to start and the fast and efficient heating, with reduced drying time, which can improve the product quality and food nutritional and sensory qualities. However, microwaves also show disadvantages, such as degradation of the products by dry heating and food dehydration. Since microwave heat induce alterations in the conformational structures and physical properties of food, different studies have been performed aiming the decrease of egg allergenicity (Cabanillas & Novak, 2019). Nevertheless, the microwave treatments can promote an increase as well as a decrease of the allergenicity, by changing the native structure of the proteins which can mask or unmask epitopes in the allergen, expose buried amino acids or generate neoallergens (Cabanillas & Novak, 2019).

Recently, a study analysed an allergen threshold data from OFC given to children allergic to cooked egg and cow's milk, where the egg was heated in a microwave 600 W, 2 x 30 s. The results showed that the dose-distribution curves for reaction thresholds to mildly baked eggs at microwave do not exhibited significant differences comparing to OFC using hard heated egg (Remington, Westerhout, Campbell & Turner, 2017). A FTIR-ATR and CD spectroscopy were used to evaluate the result of microwave treatment on the secondary structure of the egg white proteins and observed a reduction of α-helices levels and an increment of β-sheets (Zhu, Vanga, Wang & Raghavan, 2018b). Moreover, it was found that microwaves can remarkably inactivate the egg white avidin probably due to changes in the secondary structure. Modifications in the secondary and tertiary structures with aggregation of Gal d 2 were also found after glycation and microwave treatment (Hu et al., 2019). The changes affected the

potential allergenicity of egg white, however additional analyses should be performed. Findings from a recent study (Li, Jin & Sheng, 2020) showed that microwave heating affects negatively the rehydration of egg white powder, however its modification by phosphorylation was able to inhibit this effect to some extent. The described studies analysed the structural and/or the physicochemical properties of egg proteins after microwave treatment, nonetheless additional experiments must be performed to verify the impact of this processing method in allergenicity of egg proteins.

### 1.3.3. High hydrostatic pressure

High hydrostatic pressure (HHP) has become an alternative to thermal treatment since it can improve microbial safety, physical changes in food components, nutrition and functionality, as well as bioactive properties without affecting organoleptic properties (Naderi, House, Pouliot & Doyen, 2017). On the other hand, high pressure can modify the structure of allergenic proteins in foods and reduce their allergenicity (Huang, Hsu, Yang & Wang, 2014). Current high-pressure homogenizers permit pressures 10-15 times higher than conventional ones, with ranges between 300 and 400 MPa, without deterioration of color, flavour, texture, and nutritional value, since HHP does not break covalent bonds (Patrignani & Lanciotti, 2016). In fact, both high pressure processing and high-pressure homogenization can affect the digestibility and allergenicity of proteins by modification of the secondary and tertiary structures due to the breakage of the non-covalent interactions (Rahaman, Vasiljevic & Ramchandran, 2016). A 10 % decrease of immunoreactivity, analysed by an ELISA method, was observed after egg white high-pressure homogenization (Panozzo et al., 2014). The authors suggested that protein epitopes became bury during the unfolding and aggregation of the protein which promotes a reduction of immune response. Egg protein denaturation and aggregation can occur depending on the specific pressure range of the HHP and on factors, such as protein concentration, duration of treatment, temperature and pH. Contrarily to pressures above 150 MPa, pressures below 100 MPa did not cause egg white protein aggregation (Gharbi & Labbafi, 2018). On the other hand, the allergenic potential of egg white in a BALB/c model of egg allergy was analysed using heat and high-pressure treatments, and an improve in the allergenic potential of the egg white was observed with increased Th2-biased responses (Pablos-Tanarro et al., 2017). Despite some controversial results, the use of high-pressure processing technology as a pre-treatment procedure for food products seems to afford the production of hypoallergenic foods, (Huang et al., 2014), however more studies based on mice allergy models and also clinical studies have to be performed to validate this approach (Costa et al., 2021).

### 1.3.4. Maillard reaction

The non-enzymatic reaction of amino acids or proteins with reducing sugars during thermal processing is named Maillard reaction (MR) (Gupta et al., 2018; Toda, Hellwig, Henle & Vieths, 2019). The initial process involves the formation of a Schiff base, by the reaction between the carbonyl group of the reducing sugar and an amine group of the protein that is converted in a stable product, a ketomine termed the Amadori product. The protein glycation finally can result in an advanced glycation end-products (AGEs) that are present in thermal processed foods. The MR products are applied in the industrial food processing to confer functional characteristics to foods such as appearance, smell, taste and texture (Teodorowicz, van Neerven & Savelkoul, 2017). The AGE products have different sizes and chemical structures and can include compounds with adverse effects to human health, such as acrylamide and heterocyclic amines, known as carcinogens (Zhang, Wang & Fu, 2020a). Since glycation can change the structures of allergenic proteins, different studies have been performed regarding the effects of AGEs formation on food allergenicity (Gupta et al., 2018; Zhang et al., 2020a).

An increase of Gal d 2 IgE binding after glycation was observed which can indicate the exposure of the epitopes initially buried (Ma et al., 2013). Different studies have concluded that the effects of the MR are varied (Teodorowicz et al., 2017; Gupta et al., 2018; Toda et al., 2019; Wang et al., 2020a), and depend on different factors, such as type and concentration of reducing sugars, allergens thermal stability, the presence of basic amino acids residues in allergens, and processing conditions (e.g. temperature, pH, time). It is important to refer that glycation affects both linear and conformational structures of allergenic proteins and can hide the epitopes or form neoallergens which can result in a decrease, increase or even unchanged immunoreactivity (Rao, Jiang, Li, Samiwala, & Labuza, 2018). Thus, further research must be developed, in special in vivo studies, to confirm the impact of glycation in allergenicity of egg proteins.

Moreover, recently, it was suggested that MR products, like AGEs, when present in the diet, may act as inflammatory agents that affect the gut microbiome, and trigger the development of various pathologies, namely the allergic reactions (Toda et al. 2019; Zhang et al., 2020a).

### 1.3.5. Enzymatic treatment

Enzymatic hydrolysis is an efficient process for modification of food proteins allergenicity. Depending on the type of enzyme and the extent of hydrolysis, the

enzymatic hydrolysis may break polypeptide sequences producing bioactive peptides and modifying their allergenicity by cleavage of conformational and linear epitopes (Rahaman et al., 2016; Chang, Lahti, Tanaka & Nickerson, 2018). Hypoallergenic pepsin hydrolysates of Gal d 2 down control the Th2 responses promoted by egg allergens and increase Th1 responses, thus being a potential candidate for peptide-based immunotherapy of egg allergic patients (Lozano-Ojalvo, Molina & López-Fandiño, 2016). Findings of Park et al. (2017) revealed that the deglycosylation of Gal d 2 after N-acetylglucosaminidase treatment reduced the degree of Gal d 2 antigenicity and allergenicity, after the analysis using a competitive indirect ELISA performed with both egg-allergic sera of children and rabbit antiserum to Gal d 2, respectively. In another study by Tong et al. (2018), a polyphenol oxidase catalysed cross-linking of Gal d 2 with caffeic acid significantly reduced the allergenicity potential of this allergen in a mouse model of Balb/c, however it was not completely eliminated. Liu, Chen, Chen, Tong and Gao (2018) also found that enzymatic cross-linking of Gal d 2 with a polyphenol oxidase in the presence of caffeic acid, decreased the antigenicity, as a result of changes in the Gal d 2 conformation. In fact, it was observed a significant reduction in IgG and IgE binding to Gal d 2 epitopes after enzymatic treatment. These results are according to Tong et al. (2018) and suggest a new potential approach for producing hypoallergenic egg products. Nevertheless, since the reduction of allergenicity has been only partial, other strategies must be developed. Recently, Gazme, Rezaei & Udenigwe (2020) studied the hydrolysis of egg white proteins by immobilized and free enzymes Neutrase and Thermolysin and concluded that proteolysis promotes a significant reduction in the immunoreactivity of egg white proteins, compared to in vitro digestion. However, IgE-binding capacities were not completely eliminated due to the presence of intact proteins and also due to the several immunoreactive peptides from egg white proteins. Moreover, the epitopes more resistant to enzymatic hydrolysis were from Gal d 1.

### 1.3.6. Food irradiation

Radiation has been used for food conservation and to prevent changes in its nutritional and sensorial characteristics. Radiation can alter the immunoreactivity of certain food proteins by promoting structural and conformational modifications, such as cleavage of bonds, aggregation, crosslinking and amino acid alterations (Dong, Wang, & Raghavan, 2021). These effects are caused by free radicals produced due to water radiolysis during proteins irradiation (Rahaman et al., 2016).

Despite some interesting studies using pulsed UV have been performed to reduce allergens in foods, such as soy, peanut and milk, there are still some contradictory results for eggs (Dong et al., 2021). In fact, it was observed that UV-C exposure of egg proteins promoted denaturation with a decrease in IgG-binding capacity (Manzocco & Nicoli, 2012). On the other hand, in another study by Manzocco, Panozzo and Nicoli (2012), no difference was found in IgE-binding capacity between irradiated and untreated egg white.

The effect of γ-radiation on Gal d 2 also promotes contradictory effects on IgE-binding capacity, with an increase at low levels of radiation and a decrease at higher ones. In fact, when submitted to γ-radiation (≥ 10 kGy), the IgE-binding to Gal d 2 is greatly reduced, as well as the secretion of cytokines (ILs-4 and -5) suppressing Th2 dominant immune response in a Gal d 2-allergic mouse model (Seo et al. 2007).

In conclusion, although food irradiation seems to be a potential technique to reduce the allergenicity of eggs, further studies should be carried out for its application in the food industry (Dong et al., 2021).

### 1.3.7. Ultrasound

In the last years, ultrasound has been studied as potential alternative non-thermal process to decrease food allergenicity (Nayak, Li, Ahmed & Lin, 2017). Ultrasound can promote changes in both conformational and physical features of proteins, affecting emulsification and solubility properties which can alter its allergenicity (Zhu et al., 2018a). Nevertheless, different results have been found since the effect of ultrasound differs with distinct food proteins (Nayak et al., 2017). Stefanović et al. (2014) obtained hydrolysates of egg white with lower molecular weight and potentially with decreased allergenicity after combining ultrasound pre-hydrolysis treatment with proteases to improve functional properties of the proteins. On the other hand, Yang, Tu, Wang, Li and Tian (2017) showed a significant enhance in the IgG and IgE binding of Gal d 2 after high-intensity ultrasound. However, it was also considered a potential reduction in antigenicity since the protein unfolding and display of IgG and IgE epitopes can permit its alteration by the combination of other methods with ultrasound. In fact, glycation with mannose after ultrasound pretreatment at 0–600 W significantly reduced the IgG and IgE binding capacities of Gal d 2 due to the marked changes in its tertiary and secondary structures (Yang, Tu, Wang, Zhang & Song, 2018). Sheng et al. (2018) observed that high-intensity ultrasound has a significant effect on the egg white foaming properties by modification of the protein structure, however no immunoreactivity experiments were performed to confirm the reduction in

allergenicity. It has been suggested that ultrasound treatments seem to be more efficient when combined with other methods, such as heat or glycation, despite being considered as an alternative non-thermal processing in the reduction of food allergenicity (Dong et al. 2021).

### 1.3.8. Microbial modification

Microbial transglutaminase (MTGase) is a transferase that can modify the functional and structural properties of food proteins by crosslinking and also by covalent conjugation of polyamines, lipid esterification, or deamidation of glutamine. MTGase has a high potential in food processing since shows crosslinking efficiency as mammalian transglutaminases (TGases) in a great variety of food proteins, such as egg proteins (Reddy, Singh, Sofi & Reddy, 2013).

Despite the wide application of enzymatic crosslinking in the food industry, MTGase has not shown a great impact on the decrease of allergenicity of food proteins (Jimenéz-Saiz et al., 2015). Nevertheless, a reduction in allergenicity of the major allergen of peanuts (Wu et al. 2017) or tofu (Zhu et al., 2019) was observed to a certain extent.

Most of the studies that applied MTGase to egg proteins have described changes in protein structure and an improvement in its digestibility; however, there has been no noticeable reduction in allergenicity. Giosafatto, Rigby, Wellner, Ridout, Husband and Mackie (2012) proposed the use of TGase-modified Gal d 2 as a potential component to improve functional and mechanical properties, such as digestibility, and viscoelasticity, respectively. Despite the observation that MTGase crosslinking can modulate the gastric and duodenal digestibility of Gal d 2, its allergenic properties were not analysed in this study. A high pressure-assisted MTGase-induced Gal d 2 and egg white cross-linking was applied to improve gastroduodenal digestion, but no reducing in protein allergenicity was observed due to the formation of high amounts of monomeric proteins in addition to high-molecular weight polymers (Ma, Ojalvo, Chen, Fandiño & Molina, 2015). Nevertheless, it is important to refer the potential of enzyme crosslinking on gastroduodenal digestion of processed allergens, which in some cases can affect directly their allergenicity.

Besides the transglutaminases, the microbial fermentation has also a significant role in the nutritional and organoleptic properties of foods as well as in its preservation (Ross, Hill, Stanton, Sugrue, Hill & Arendt, 2017). Since is thought that food allergy results from a deficiency in mechanisms of immune tolerance, which are regulated by gut

microbiota, different researchers have associated gut microbiota dysbiosis with the emergence of food allergies. Thus, it has been suggested that certain probiotic strains can be used to prevent allergy, due to its action in immune responses. However, more studies should be carried out to confirm and to characterize the interrelation between probiotics ingestion, the microbiome and the immune response mechanisms in allergy (Canani et al., 2019; Fazlollahi et al., 2019).

The gut microbial community has also been recognised as bringing a health benefit. New terms are emerging, such as paraprobiotics or postbiotics, showing that microbial fractions, or cell lysates, can also allow health benefits, providing additional bioactivity (Huang et al., 2017). In a recent study, Song et al. (2020) found that a new strain of Lactobacillus rhamnosus (Lr-0601) modulates gut microbiota and show a potent immunoregulatory activity in mice sensitized to Gal d 2 which can be used as a potential approach against immune-related diseases.

Table 3. Methods for reducing egg allergenicity.

| Methods | Goal | Results | | References |
|---|---|---|---|---|
| | | Advantages | Disadvantages | |
| **Heat treatment** | Modification of physical-chemical and functional properties<br>Changing structural conformation<br>Reducing allergenicity through structured graduate exposure protocol | Partial decrease in allergenicity<br>Reduced intestinal absorption<br>Early eat introduction | Ovomucoid is heat resistant<br>Denaturation<br>Hiding of epitopes<br>Need of trained and experienced teams<br>Not completely effective<br>Mask or unmask epitopes in the allergen<br>Expose buried amino acids producing neoallergens, increasing the allergenicity | Bloom et al. (2015)<br>Brossard et al. (2019)<br>Claude et al. (2017)<br>Claude et al. (2019)<br>Gotesdyner et al. (2019)<br>Horino et al. (2019)<br>Leonard (2016)<br>Martorell et al. (2013)<br>Pablos-Tanaro et al. (2017)<br>Pérez-Quintero et al. (2020)<br>Shin et al. (2013)<br>Stănciuc et al. (2016)<br>Stănciuc et al. (2018) |
| **Microwave** | Changing structural conformation | Reduce allergenicity potential<br>Increase protein digestibility<br>Rapid heating<br>High energy efficiency<br>Reduce the drying time<br>Faster and efficient heating<br>Improve product quality<br>Better nutritional/sensory properties | Not completely effective<br>Mask or unmask epitopes in the allergen<br>Expose buried amino acids producing neoallergens, increasing the allergenicity<br>Degradation of the products by dry heating<br>Food dehydrating | Ekezie et al. (2017)<br>Hu et al. (2019)<br>Li et al. (2018)<br>Li et al. (2020)<br>Zhu et al. (2018b) |
| **High hydrostatic pressure** | Improve microbial safety<br>Change structure<br>Modify egg white foaming | Foods with no allergenic components<br>Reduce allergenicity<br>Increase profitability<br>Functionality and bioactive properties of food products, without affecting their organoleptic properties | Induction of denaturation and aggregation<br>Minimal deterioration of colour, flavour, texture, and nutritional value<br>Physical changes in food components<br>Changes in food nutrition and functionality | Huang et al. (2014)<br>López-Expósito et al. (2008)<br>Naderi et al. (2017)<br>Pablos-Tanarro et al. (2017)<br>Panozzo et al. (2014)<br>Patrignani & Lanciotti (2016)<br>Rahaman et al. (2016) |

| | | | | |
|---|---|---|---|---|
| | | Microbial food safety | | |
| **Maillard reaction** | Reducing sugars and compounds with free amino acids | Changes in immunoreactivity of linear and conformational epitopes | IgE-binding can decrease, remain unchanged or increase<br>Aggregation<br>Formation of new allergenic compounds<br>Decrease of nutritional value<br>Development of chronic inflammation | Ma et al. (2013)<br>Rahaman et al. (2016)<br>Teodorowicz et al. (2017)<br>Toda et al. (2019)<br>Wang et al. (2020) |
| **Enzimatic treatment** | Hydrolysis with enzymes<br>Breaking polypeptide sequences | Production of bioactive peptides<br>Cleavage of conformational and linear epitopes<br>Significant reduction in IgG and IgE binding capacity<br>Regulation Th2 | Partial IgE binding capacity<br>Formation of immunoreactive peptides | Chang et al. (2018)<br>Gazme et al. (2020)<br>Liu et al. (2018)<br>Lozano-Ojalvo et al. (2016)<br>Park et al. (2017)<br>Tong et al. (2018)<br>Yang et al. (2018) |
| **Food radiation** | Triggering oxygen radical generating radialysis | Reduction of IgE-binding<br>Products with longer shelf life | Changes in color, flavour, taste, and pH<br>Increase the potential allergenicity<br>Fragmentation, aggregation<br>No difference in IgE-binding capacity<br>Increase the risk of allergenic reactions | Manzocco et al. (2012)<br>Manzocco and Nicoli (2012)<br>Rahaman et al. (2016) |
| **Ultrasound** | Reduction of food allergenicity | Lead to changes in both conformational and physical properties of proteins which can affect the allergenicity<br>Reduced IgG and IgE binding capacity | Enhanced IgG and IgE binding capacity | Nayak et al. (2017)<br>Sheng et al. (2018)<br>Stefanović et al. (2014)<br>Yang et al. (2017)<br>Yang et al. (2018)<br>Zhu et al. (2018b) |
| **Microbial modification** | Modify properties in food products: the hydrophobicity, solubility, gelation, emulsification, foaming and viscosity | Provide fermented foods<br>Improved gastro-intestinal digestibility | Do not reduce potential allergenicity | Canani et al. (2019)<br>Fazlollahi et al. (2019)<br>Giosafatto et al. (2012)<br>Huang et al. (2017)<br>Jimenéz-Saiz et al. (2015)<br>Ma et al. (2015) |

## 1.4. Phenolic Compounds

Phenolic compounds (PC) are natural antioxidants and chemoprotective agents derived from secondary metabolites that are found in vegetables, fruits, grains, essential oils, tea, beverages and derived foods, widely used in food and medicine (Ozdal, Capanoglu & Altay, 2013). The interest in the study of PC has increased due to their widespread in the plants and the wide range of biological and physiological activities (Cuyckens & Claeys, 2004).

PC present at least a hydroxyl group attached to an aromatic ring in its structure (Figure 3). They are natural antioxidants and chemoprotective agents widely

distributed in plants secondary metabolism, which are not a source of energy for the body (Harnly, Bhagwat, & Lin, 2007; Ozdal, Capanoglu, & Altay, 2013).

### 1.4.1. Health benefits of phenolic compounds

Phenolic and polyphenolic compounds are found in vegetables, fruits, grains, essential oils, tea, and beverages, besides being widely used in food supplements and medicine (Xia, Wu, Shi, Yang, & Zhang, 2011; Zhang and Tsao, 2016; Chu, Bao & Wu, 2018). Nonetheless, it has been reported that the reduction in the rate of chronic diseases may be related to the high intake of plant-foods rich in PC. It is also assumed that the consumption of fruits, vegetables, teas, cereals-based foods are responsible for raising awareness about the effect of food on health (Gutiérrez-Grijalva, Ambriz-Pére, Leyva-López, Castillo-López & Heredia, 2016).

According to Tohma, Gülçin, Bursal, Gören, Alwasel and Köksal (2017), the use of natural antioxidants, or consumption of plant-food rich in them, could be promising as therapeutic candidates, providing prevention, and reducing or delaying the progression of diseases that oxidative stress provokes due to the lack of this supplementation.

In addition, to the antioxidant activity (Zhang & Tsao, 2016; Tohma et al., 2017; Lee, Chan & Mitchell, 2017; Guiomar, 2020), phenolic compounds attract attention from several areas of research, and exerts other health-benefits like anti-inflammatory (Zimmer, Leonardi, Miron, Schapoval, Oliveira & Gosmann, 2012; Silva, Rodrigues, Feas & Estevinho, 2012; Zhang and Tsao, 2016; Guiomar, 2020), antibacterial (Xia et al., 2011; Silva et al., 2012; Mphahlele, Fawole, Makunga & Opara, 2016; Guiomar, 2020), antifungal (Shalaby and Horwitz, 2015), antiulcer (Zakaria et al., 2011), cardiovascular diseases protective (Kuriyama et al., 2006; Mursu, Voutilainen, Nurmi, Tuomainen, Kurt & Salonen, 2008; Mulvihill and Huff, 2010), antiatherogenic (Liu, Zubik, Collins, Marko & Meydani, 2004; Mulvihill and Huff, 2010), antithrombotic (Han, Gu, Ye, Cao, Liu & Yin, 2012; Tao, Duan, Yang, Tang, Liu & Qian, 2012), immune modulating (Schütz, Saß, With, Graubaum & Grünwald, 2010), anticoagulant (Bijak, Bobrowski, Borowiecka, Podsędek, Golański & Nowak, 2011), vasodilatory (Mudnic et al., 2010), anticough and cold (Schütz et al., 2010; Mulaudzi et al., 2012), analgesic (Santoz et al., 2010), antipyretic (Santoz et al., 2010; Mulaudzi et al., 2012), anti-ultraviolet irradiated human skin (Park et al., 2014), cytotoxic (Mursu et al., 2008; Beara et al., 2012), antiviral (Hu et al., 2013) and antitumoral (Kuriyama et al., 2006; Chen et al., 2011; Weng and Yen, 2012; Anantharaju, Gowda, Vimalambike, & Madhunapantula, 2016) effects.

The antiviral activity of phenolic compounds, such as QCT, have been reported, namely inhibiting the replication of herpes simplex virus type 1 (HSV-1), and 2 (HSV-2), and SARS (Suárez et al., 2010; Anantharaju, Gowda, Vimalambike & Madhunapantula, 2016; Mphahlele et al., 2016).

### 1.4.2. Types of phenolic compounds (classification)

More than one phenol structural unit define a polyphenol. The phenolic hydroxyl group show its labile hydrogen, which makes phenols weak acids (Vermerris and Nicholson, 2006; Ozdal et al., 2013; Soares., 2015).

There are many known PC identified, which can be classified into two groups: basic PC and polyphenols (Vermerris & Nicholson, 2006; Ozdal et al., 2013), and the other classification was based on the number of phenolic rings they contain and the radicals that link these rings to each other. On the other hand, the same authors also suggested a classification of phenolic compounds that distinguishes between extractable and non-extractable phenols (Gutiérrez-Grijalva et al., 2016).

Currently, PC have been classified according to the pattern of their basic skeletons. However, as certain compounds discovered recently do not meet the structural criteria for inclusion in existing classes, classification system was modified. Then, they were divided into six classes; each class was divided into categories and families of compounds to fully hierarchize their structural differences. The first five classes were characterized by an increasing complexity of the basic skeletons of the members, and the sixth class included so-called hybrid phenolics as a separate group of compounds (Tsimogiannis & Oreopoulou, 2019).

The classification in five groups, was mainly based on the number of carbons in the molecule, like: the $C_6$ group, comprising simple phenols; the $C_6C_n$ group, which includes phenolic acid derivatives and hydroxycinnamic acid derivatives; the $C_6$-$C_n$-$C_6$ group, which includes stilbenes ($C_6$-$C_2$-$C_6$) and flavonoids ($C_6$-$C_3$-$C_6$); the $(C_6\text{-}C_3)_n$ group, consisting of lignans and lignins and; the tannins group (Vermerris and Nicholson, 2006; Ozdal et al., 2013; Soares., 2015). The following figure is the frame and highlight of PC used in the experiments, based on carbon number in the classification referred above (Figure 3).

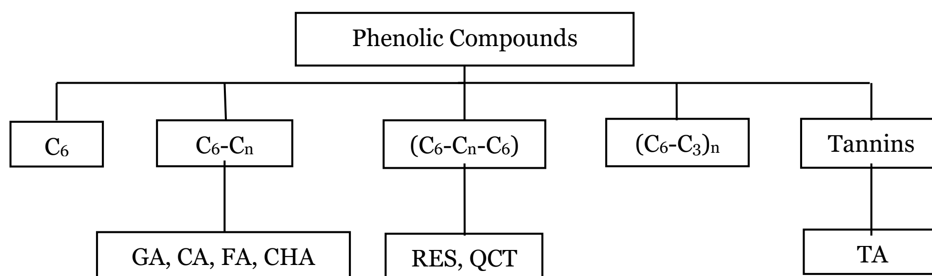



Figure 3. Classification of phenolic compounds. Highlighting and framing the main groups used (where: GA – Gallic Acid; CA – Caffeic Acid; FA – Ferulic Acid; RES – Resveratrol; QCT – Quercetin and TA – Tannic Acid).

Below are presented the structures of the most studied PC (Figure 4).

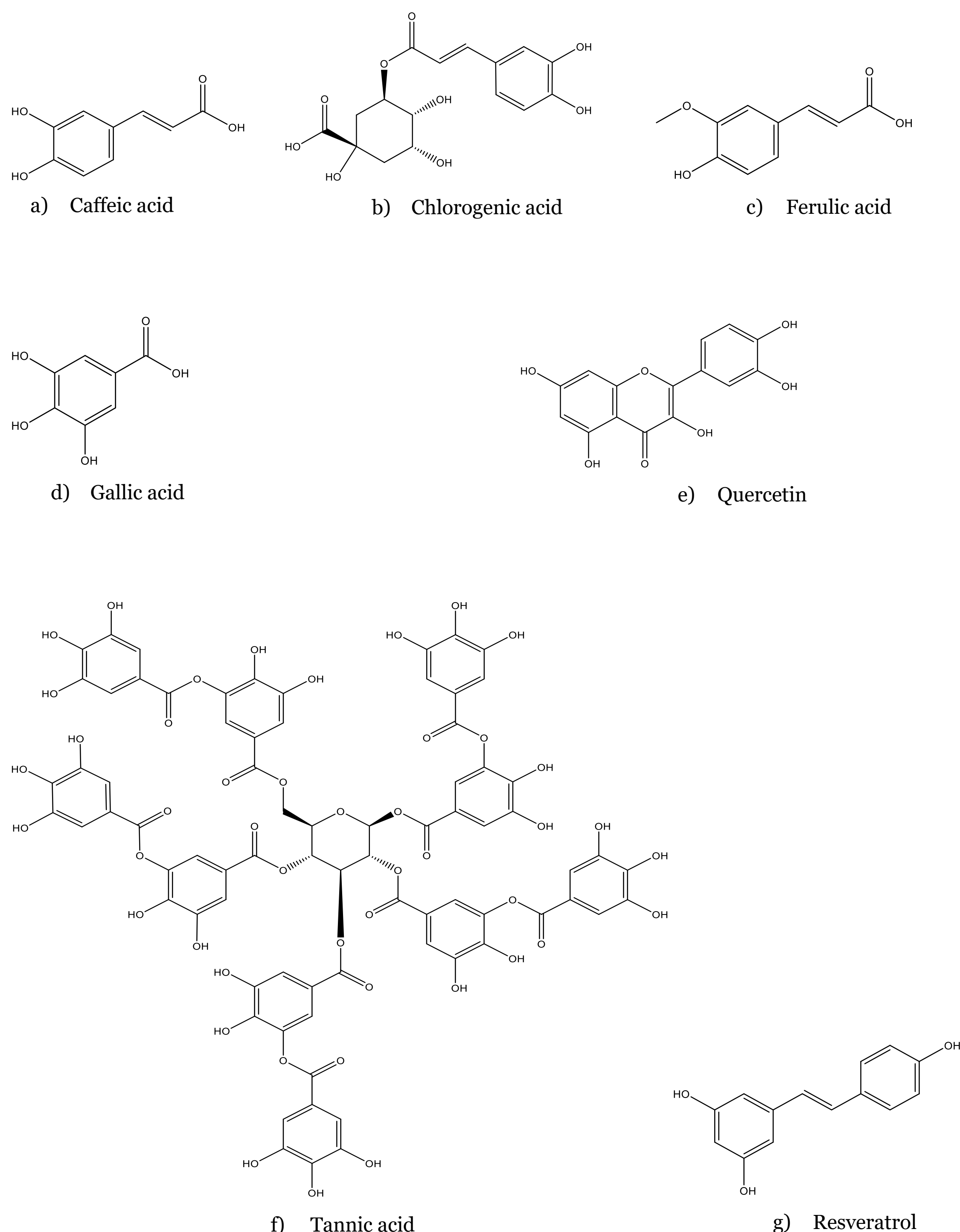



Figure 4. Structure of the phenolic compounds.

### 1.4.3. Interactions between proteins and phenolic compounds

For approaching the phenolic compounds-proteins interactions, non-covalent interactions formed by hydrogen bonds, hydrophobic bridges, van der Waals forces and ionic interactions (weak bonds), reversible interactions are highlighted. While in covalent bonds, irreversible interactions are involved. The covalent bond (strong

interactions) is promoted by the PC ability to produce the quinone radical. However, it is important to note that many studies focus on reversibility mainly due to the lack of adequate methodology to quantify the conjugates (Rohn, 2014; Buitimea-Cantúa, Gutiérrez-Uribe & Serna-Saldívar, 2018). These interactions depend on indispensable structural molecular characteristics such as: molecular weight, and methylation, hydroxylation, glycosylation, structural flexibility, abundance of hydroxyl groups and hydrogenation degrees. Thus, the molecular size of proteins is directly proportional to the increasing binding affinity of polyphenols (Buitimea-Cantúa et al., 2018). Two aspects can be perceived in interactions between PC and proteins: firstly, the interaction with PC changes the physico-chemical properties of proteins (Czubinski & Dwiecki, 2017).

Complexes formed may reduce the nutritional and technological value, enzymatic activity and other biological effects of proteins by changing their solubility, thermal stability and digestibility (Kroll, Rawel & Rohn, 2003; Tomás-Barberán & Andrés-Lacueva, 2012; Ozdal et al., 2013; Czubinski & Dwiecki, 2017). On the other hand, the formation of complexes with proteins could reduce the potential health-promoting features of PC by masking their antioxidant properties (Ozdal et al., 2013; Gonzales, Smagghe, Grootaert, Zotti, Raes & Van Camp, 2015; Czubinski & Dwiecki, 2017).

In addition, there is formation of hydrogen bonds by PC with the protein carboxyl groups since PC are excellent hydrogen donors. PC are small molecules, which allows them to have high affinity to protein and with the ability to penetrate the protein molecules, and at the same time cross-link peptide chains at more than one point (Mulaudzi et al., 2012; Ozdal et al., 2013).

It has been shown that interactions by phenolic compounds, such as GA, coumaric acid, CA, FA, CHA, TA, RES, and QCT , can influence protein structure, functionality, and characteristics (Jakobek, 2015).

Furthermore, the antioxidant activity of the protein increased in consequence of the complex (Heinonen, Rein, Satué-Gracia, Huang, German & Frankel, 1998; Hassan, 2013; Bandyopadhyay, Ghosh & Ghosh, 2012; Ozdal et al., 2013; Rohn, 2014; Mehanna, Hassan, El-Din, Ali, Amarowicz & El-Messery, 2014; Czubinski & Dwiecki, 2017).

However, the reaction between a PC and a protein can cause changes in the functional properties of the protein, such as emulsifying properties, gel properties, solubility, thermal stability, and foaming properties, implying changes at organoleptic in food

(Chu et al., 2018). Therefore, these interactions may affect astringency, protein digestibility, absorption, and bioavailability of antioxidants (Buitimea-Cantúa et al., 2018).

#### 1.4.3.1. Interactions between food proteins and phenolic compounds

According to Ozdal et al. (2013), the interactions between proteins and PC have been object of research, and resultant changes are observed in both proteins and PC. PC can bind to proteins by noncovalent interactions and also by stable covalent interactions (Chu et al., 2018, Perusko et al. 2017). Polyphenolic compounds can interact with food proteins forming complexes (Ozdal et al., 2013; He et al., 2019) that exhibit distinct physicochemical properties (Ulrih, 2015). These interactions promote modifications in the tertiary and secondary protein structures, and the formation of aggregates, depending on the type and size of the proteins (Ozdal et al., 2013).

It has been shown that interactions by phenolic compounds, such as GA, coumaric, CA, FA, CHA, TA, RES, and QCT, can influence protein structure, functionality, and characteristics. The reaction between phenolic compounds and proteins can alter the solubility, thermal stability, foaming, emulsifying and gel properties (Chu et al., 2018). There are important characteristics that affect phenolic-protein interactions, such as molecular weight, structural flexibility, and abundance of hydroxyl groups (Buitimea-Cantúa, Gutiérrez-Uribe & Serna-Saldívar, 2018).

Thereby, was reported that the fermentation/polymerization of catechin monomers, facilitate protein binding to larger polyphenols (Dubeau, Samson & Tajmir-Riahi, 2010; Buitimea-Cantúa et al., 2018).

In addition, the improvement of bioaccessibility, bioavailability and bioactivity of PC is influenced by the food matrix due to PC-food protein interactions (Jakobek, 2015). Therefore, the amount of any food constituent detected in the intestine, once it has been released from the food matrix to the point of crossing the intestinal barrier, is considered to be bioaccessibility. On the other hand, the fraction of the nutrient or ingested compound that reaches the systemic circulation and the specific places where it can exert its biological action, is called bioavailability, whilst bioactivity is conceptualized as the changes in health status exerted by the constituents of food or dietary supplements (Gutiérrez-Grijalva et al., 2016).

An *in vitro* study was carried out, and the predominance of hydrophobic binding was observed, after the binding of tea polyphenols with α- and β-casein, which is hydrophilic and hydrophobic at the same time (Hasni et al., 2011; Jakobek, 2015). Kanikis, Hasni, Bourassa, Tarantilis, Polissiou, Tajmir-Riahi (2011) found hydrophilic

and hydrophobic interactions in the complexation of polyphenols with β-lactoglobulin (BLG). In addition, they also suggested that the reactions in which polyphenol-protein complexes are formed occur due to various amino acid residues (Hasni et al., 2011; Jakobek, 2015). Von Staszewski et al. (2012) investigated BLG or caseino-macropeptide and green tea polyphenols associations, where the binding was hydrophobic. Shpigelman et al. (2010) found a combination of hydrophobic interactions and hydrogen bonds, when studied heated BLG binds to (-)-epigallocatechin-3-gallate from tea with high affinity than the native protein, in an *in vitro* experiment.

The interactions can also influence the functionality, quality and structure of the protein. In fact, the nutritional quality of proteins depends on the composition of amino acids, essential amino acids, digestion (Jakobek, 2015).

Many studies about interaction of food proteins (e.g. BLG) and egg (e.g. OVA) have been performed (Bandyopadhyay et al., 2012; Ozdal et al., 2013; You, Luo & Wu, 2014; Rohn, 2014; Wu et al., 2018; He et al., 2019), with PC mainly QCT, CHA, catechin, epigallocatechin 3-gallate (EGCG), and TA. Those works aid to clarify the nature of the interactions and the structural changes associated (Li & Yan, 2016; Lu, Li, Xu, Zhang, Lin & Wu, 2018; Wu et al., 2018; Tong et al., 2018; He et al., 2019).

#### 1.4.3.2. Interactions of food proteins with phenolic compounds for reduction of allergenicity

During the last years, different phenolic compounds have been tested to decrease or to eliminate the allergenic capacity of food proteins by changing their native conformation (He et al., 2019) (Table 4). Chung and Champagne (2009) conducted one of the first studies to verify the effect of PC on food proteins. Results showed that addition of the CA, CHA and FA to peanut extracts and liquid peanut butter promoted the irreversible precipitation of most of the major peanut allergens, Ara h 1 and Ara h 2 and, consequently, a 10- to 16-fold reduction of IgE binding in the soluble material. In a recent study, it was shown that complexation of polyphenols to peanut flour used in animal diet formulations can potentially lower plasma IgE of peanut-sensitized C3H/HeJ mice (Bansode, Randolph, Plundrich, Lila & Williams, 2019). The binding affinity between tannic acid and Gal d 2, bovine serum albumin and bovine BLG was evaluated by the analysis of intrinsic fluorescence of these proteins and it was observed that the binding affinities varied among the three proteins and depend on its structural differences (Xie, Wehling, Ciftici & Zhang, 2017). Moreover, according to CD spectra, all three proteins showed modifications in secondary and tertiary structures after binding with tannic acid, however no immunoreactivity studies were performed.

Curiously, Zhang, Liu, Su, Roux & Sathe (2018) observed a stabilizing effect of tannic acid, tannin, ellagic acid, tea tannin, and walnut tannin on Amandin against heat denaturation which prevents the reduction of its immunoreactivity as demonstrated by dot blot, Western blot and ELISA. On the other hand, a recent study (Wu et al., 2018) provided a novel strategy to produce hypoallergenic food with reduced BLG IgE-binding capacity due to structural change promoted by covalent conjugation of this protein with the phenolic compounds EGCG and CHA. Pessato et al. (2018) also suggested an approach to diminish the allergenicity of whey proteins by complexation with EGCG involving non-covalent interactions that induced secondary and tertiary structure modifications.

Table 4. Application of phenolic compounds for reducing allergenicity of food proteins.

| PC | Protein | Result | Changes | Reference |
|---|---|---|---|---|
| **CA** | Peanut<br>Whey proteins | Reduce allergenicity<br>- | Conformational changes and adjustment in epitopes | Chung and Champagne (2009)<br>Chung and Reed (2012)<br>Hasni et al. (2011)<br>Pessato et al. (2018)<br>Tong et al. (2018)<br>Zhang et al. (2018) |
| **CHA** | Ara h 3 (A3)<br>β-casein<br>BLG<br>WPI | Reduce allergenicity<br>Low capacity of IgE-binding | Conformational changes<br>Unfold proteins | Chung and Champagne (2009)<br>Chung and Reed (2012)<br>Hasni et al. (2011)<br>Kanakis et al. (2011)<br>Lu et al. (2018)<br>Wu et al. (2018)<br>Xu et al. (2019) |
| **EGCG** | BLG<br>Whey proteins<br>α-casein<br>β-casein<br>Peanut | Masking effect of polyphenol complexation on the antioxidative potential of the protein<br>Decreasing the IgE binding<br>Influence protein structure, functionality, and characteristics<br>- | OVA unfold<br>Promoted conformational changes | Chung and Champagne (2009)<br>Chung and Reed (2012)<br>Hasni et al. (2011)<br>Kanakis et al. (2011)<br>Pessato et al. (2018)<br>Wu et al. (2018) |
| **TA** | BSA<br>BLG<br>Amandin<br><br>Peanut | Form complexes used as nanoencapsulation systems for oral drug delivery<br>Reduce the mAb (monoclonal antibody) detectable amandin immunoreactivity | -<br>-<br>-<br>Promoted conformational changes | Chung and Champagne (2009)<br>Chung and Reed (2012)<br>Zhang et al. (2018) |
| **FA** | Peanut<br>Amandin | Reduce the allergenicity | Promoted conformational changes | Chung and Champagne (2009)<br>Chung and Reed (2012)<br>Zhang et al. (2018) |

**Abbreviations:** BLG – β-lactoglobulin; BSA – Bovin serum albumin; CA – Caffeic acid; CHA – Chlorogenic acid; EGCG – (-)-epigallocatechin 3-gallate; FA - Ferullic acid; TA – Tannic acid; WPI – Whey protein isolated.

Recent studies provided an innovative approach to produce hypoallergenic food by binding and reducing BLG IgE-binding capacity through PC EGCG and CHA (Wu et al., 2018).

Zhang, Liu, Su, Roux, and Sathe (2018) assessed amandin immunoreactivity after almond proteins were exposed to a variety of processing conditions in the presence of PC, sugars, or both. They concluded that amandin immunoreactivity was affected by the PC and the moist heat was the most effective treatment in reducing amandin immunoreactivity. However, TA, tannin, ellagic acid, tea tannin, and walnut tannin exhibited stabilizing effect on amandin against heat denaturation. In addition, Pessato et al. (2018), suggested that the reduction of whey proteins allergenicity might be promoted by complexation with caffeic acid and EGCG.

Recently the allergenicity, digestibility and functional properties of whey protein isolate (WPI) were investigated after covalent bond with CHA. The proteins (BLG and WPI) unfolded and showed a low capacity of IgE-Binding caused by CHA, which could provide a reduction of the allergenicity and an enhancement of proteins´ functional properties (Xu et al., 2019).

#### 1.4.3.3. Interactions of egg proteins with phenolic compounds for reduction of allergenicity

In addition to above mentioned food proteins, different PC, such as CA, CHA and TA, EGCG, QCT and tea polyphenol, also form complexes with egg proteins and have been tested for reduction of its allergenicity (Table 5).

Xie, Wehling, Ciftci and Zhang (2017), evaluated the TA interactions with OVA (Table 5), BSA and BLG. Results showed strong interactions and the complexes formed were used as nanoencapsulation systems for oral drug delivery. Ognjenović et al. (2014) showed that the catechin EGCG binds to the major allergen of egg white, Gal d 2, promoting modifications in conformation of the protein and overlaps with an IgE-binding epitope of OVA and concomitantly reduced allergenicity. Nevertheless, the study reveals that EGCG-binding site does not fully overlap with the dominant epitope to which IgE binds. In this way, it is possible the interaction between Gal d 2 and IgE which leads to basophils degranulation. Shen et al. (2014) concluded that the strong conformational changes of Gal d 2 and Gal d 4, in presence of tea polyphenol, can promote and inhibit digestion of these two egg white proteins. In fact, the structure of the polyphenol-protein complexes is affected by pH changes, resulting in different degrees of Gal d 2 and Gal d 4 unfolding and distinct digestibility. Li & Yan (2017)

demonstrated the binding of Gal d 2 to five dietary antioxidants with structural and thermodynamic changes that affect the allergen secondary structure and the folding. As previously mentioned, Tong et al. (2018) analyzed the Gal d 2 crosslinking by a polyphenol oxidase, in the presence of caffeic acid, and observed a partial reduction of allergenicity of Gal d 2 in a mouse model due to intermolecular aggregation and formation of polymers which affected the digestibility of this allergen. On the other hand, a promising method for hyposensitizing Gal d 2 using the CHA-Gal d 2 interaction, showed a reduction in the binding capacity of IgE by hiding the linear IgE epitope due to the binding of chlorogenic acid to Gal d 2 (Lu et al., 2018). Moreover, when Gal d 2 was crosslinked with CA as intermediary, marked structural changes in epitopes were observed, with a reduction of antigenicity and potential allergenicity. The decrease in binding to IgE and IgG result from the alteration of the linear epitope due to the crosslinking restriction site being positioned in the allergenic epitope. In addition, crosslinking promotes secondary structure disorder and exposure of hydrophobic regions in the surface of the protein, leading to conformational changes with destruction or alteration of conformational epitopes (Liu et al. 2018). The unfolding of Gal d 2 was analysed by spectroscopic methods after the covalent binding with EGCG. Besides the increase of digestibility and antioxidant activity, it was observed a lower IgE binding capacity compared with native Gal d 2, with a potential reducing of allergenicity (He et al., 2019). Recently, Zhang et al. (2020b) showed that the binding of QCT to Gal d 2 resulted to a reduction of its allergenicity *in vitro* and *in vivo* in mouse model. It was observed a change in the structure of Gal d 2 with destruction or mask of the epitopes, leading to a reduced capacity for binding to IgE and triggering cell degranulation. Also, the levels of specific IgE, IgG1, IgG and the expression of the high-affinity IgE receptor on sensitized mast cells surface were decreased. Moreover, a modulation of the Th1/Th2 immune response was observed, suggesting the suppressing of the allergic reaction promoted by Gal d 2 (Zhang et al., 2020b).

Table 5. Application of phenolic compounds for egg reducing allergenicity.

| PC | Protein | Results | Changes | References |
|---|---|---|---|---|
| **CA** | OVA | Partial reduced allergenicity in a mouse model | Conformational changes and adjustment in epitopes | Liu et al. (2018)<br>Tong et al. (2018) |
| **CHA** | OVA | Decreased IgE binding<br>Reduced allergenicity | Conformational changes | Lu et al. (2018) |
| **EGCG** | OVA<br>LYZ | Reduced allergenicity<br>Decreased IgE binding<br>Change in protein structure, functionality, and characteristics | OVA unfold<br>Conformational changes | He et al. (2019)<br>Ognjenović et al. (2014) |
| **QCT** | OVA | Reduced allergenicity *in vitro* and *in vivo*, in a mouse model.<br>Reduced ability for IgE-binding<br>Th1/Th2 modulation | Conformational changes and destruction or mask of the epitopes | Zhang et al. (2020) |
| **TA** | OVA | - | Formation of complexes to use in nanoencapsulation systems for oral drug delivery | Xie et al. (2017) |
| **TP** | OVA<br>LYZ | Reduced allergenicity<br>Improves pepsin digestion and prevents pancreatin digestion | Conformational changes<br>Structural change of substrate affects the proteins digestion | Shen et al. (2014) |

**Abbreviations**: CA – Caffeic acid; CHA – Chlorogenic acid; EGCG – (-)-epigallocatechin 3-gallate; IgE – Immunoglobulin E; LYZ – lysozyme; OVA – ovalbumin; QCT – quercetin; TA – Tannic acid; TP – Tea polyphenol.

Hen egg allergy has been extensively investigated in the past and in recent years, and the search for an effective and reliable methods to reduce hen egg allergenicity, is proportional to the increased need to create products that satisfies a specific market (Vapor et al., 2022). Therefore, a strategy to reduce hen's egg allergenicity using phenolic compounds seems to have a great potential to obtain hypoallergenic egg-derived products.

## 1.5. Spectroscopic techniques for analysis of protein-phenolic compounds interactions

Spectroscopic techniques are useful for studying proteins without destroying or modifying them and can detect conformational changes which occur in consequence of chemical, enzymatic, or physical modifications (Wang, Sun, Pu & Wei, 2017). Techniques such as Circular dichroism (CD), fluorescence, Fourier transform infrared (FTIR), Raman, and UV-vis absorption spectroscopy have been useful for studying interactions between proteins and between proteins and other compounds to determine the changes or how proteins unfold (He et al., 2019).

Diversified methods have been selected, based on spectroscopic principles to determine the interactions. However, other types of analysis as, molecular docking can also be used to evaluate the formation of complexes of phenolic compounds with proteins (Czubinski & Dwiecki, 2017). There are several techniques that have been used, to determine the interactions between proteins and between proteins with other compounds, based mainly on changes in secondary and tertiary structure of proteins (Czubinski & Dwiecki, 2017). The analysis more used to evaluate the formation of complexes and how proteins unfold, are Circular Dichroism (CD), ATR-FTIR, Fluorescence, Raman, and UV-vis absorption spectroscopic (He et al., 2019).

Many studies reported with accuracy the use of spectroscopic techniques and its efficacy to determine interactions between protein-protein and protein with several compounds (Wang et al., 2017; Pignataro, Herrera & Dodero, 2020; Chang et al., 2021).

Thus, in this work, the spectroscopic techniques CD, ATR-FTIR and Fluorescence were applied, to analyse the conformational changes that occur in proteins, affecting the secondary and tertiary structure due to phenolic compounds.

On the other hand, although these methods are important tools to study conformational changes in proteins, it is necessary to know that they do not accurately predict what the real values of secondary and tertiary structures are. Therefore, different spectroscopic techniques must be used to provide complementary information in studies involving protein interactions and conformational and structural changes (Feng et al., 2018).

### 1.5.1. Fluorescence spectroscopy

Fluorescence spectroscopy is a usual and suitable method for the study the interaction among molecules (Wu, Wu, Liu, Liu, Xu, & Lai, 2011; Li & Yan, 2017). Thus, supplies fundamental information on biomolecules structure, amino acids residues microenvironment and is often used in binding affinity studies (Czubinski et al., 2014; Stojadinovic et al., 2013; Shen et al., 2014; He, Chen, & Moser, 2015; Bose, 2016; Czubinski & Dwiecki, 2017).

Fluorescence results from a three-stage (excitation, excited-state lifetime, and fluorescence emission) process that occurs in chemical compounds or biomolecules, known as fluorophores, that can re-emit light after light excitation. Proteins have three amino acids [phenylalanine (Phe), Tyrosine (Tyr) and Tryptophan (Trp)] with intrinsic fluorescence properties that can be used in the studies involving the fluorescence quenching of proteins (Sheehan, 2009; Soares, 2015). Thus, the excitation of proteins

at the range 240-280 nm induces fluorescence emission in the range of 340-350 nm, as the result of the presence of aromatic amino acids, and a decrease in this fluorescence emission range or fluorescence intensity can be attributed to ligand binding (Rawel, Czajka, Rohn & Kroll, 2002a, 2002b; Möller & Denicola, 2006; Liu, Qi & Li, 2010; Czubinski et al., 2014; Ognjenović et al., 2014; Czubinski & Dwiecki, 2017).
Fluorescence measurements can provide binding information at the molecular level, such as binding mechanism, binding constants, thermodynamic analysis, etc., as well as information about its structure and dynamics. Different fluorescence studies were carried out to study the interactions between egg proteins and phenolic compounds. The fluorescence spectrum of OVA emission in the absence and presence of caffeine, theophylline and diprophylline, showed that the fluorescence of OVA regularly decreased, and caffeine demonstrated a strong extinction capacity (Wang et al., 2013). A comparative study of the interactions between OVA and five antioxidants was carried out by spectroscopic methods, reporting that Trp and Tyr residues are involved in fluorescence quenching. Afterwards, they demonstrated that only Trp is involved in fluorescence quenching and that Tyr residues do not participate in the molecular interaction between α-tocopherol and OVA (Li & Yan, 2017), in contrary, is necessary to know that, upon excitation at 280 nm, both Trp and Tyr are easily excited, but most of fluorescence come from Trp because of the efficient resonance energy transfer (RET) from Tyr to Trp. At 295 nm, only Trp emit fluorescence.
Nevertheless, Sheng et al. (2018) used fluorescence spectroscopy to study the impact of storage on the structure of hen's egg OVA, suggesting that there was a change in the protein structure caused by fluorescence decreasing.
On the other hand, Chang et al. (2021) reported that fluorescence quenching could weaken protein fluorescence intensity to study protein-polyphenol binding information and changes in protein tertiary structure. Furthermore, they added CA to the OVA and found that the fluorescence intensity of the OVA significantly decreased. Furthermore, the interaction between RES and OVA demonstrated conformational changes in the OVA microenvironment when evaluated using fluorescence spectroscopy (Cheng et al., 2021).

### 1.5.2. Circular Dichroism (CD)

Circular dichroism (CD) is an important and well-established spectroscopic tool and non-destructive technique for the determination of the structural characteristics of biomolecules, particularly the secondary structure of proteins (Spencer & Rodger, 2021). Moreover, the tertiary structure, the folding/unfolding properties, and protein interactions can be determined by CD (Wallace, Lees, Orry, Lobley & Janes, 2003;

Greenfield, 2006; Wang et al., 2017; Pignataro, Herrera & Dodero, 2020; Miles et al., 2021a).

The different types of the secondary structure of proteins show different bands in the CD spectra. Thus, the α-helices show a strong positive band at 191-193 nm and typical double negative bands at 208-210 and 222 nm, β-sheets produce an intense positive band at about 195-200 nm and a negative band at about 216-218 nm (Figure 5), whereas random coils have a strong negative band at 195-200 nm and a much weaker band (either positive or negative) between 215 and 230 nm (Pignataro, Herrera & Dodero, 2020).

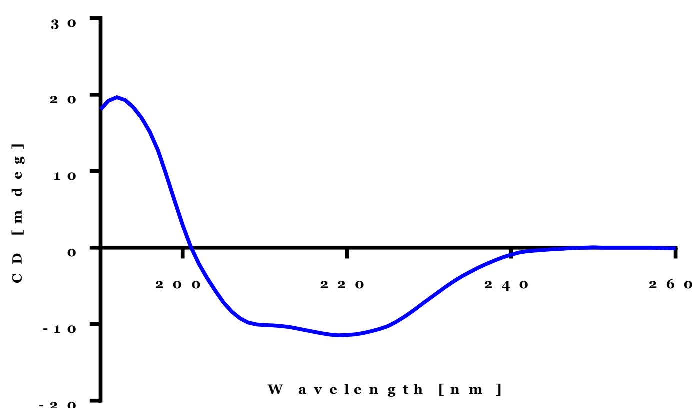



Figure 5. CD spectrum of OVA, in 0.05 M phosphate buffer (pH 7.40) at 298.15 K. α-helices show a strong positive band at 191-193 nm and a typical double negative band at 208-210 and 222 nm.

On the other hand, the CD spectrum in the near UV region (250-320 nm) can show useful information related to aromatic chromophores (Phe, Tyr, and Trp residues) of proteins in asymmetric environment, which has been widely used to assess the tertiary and occasionally quaternary structures of proteins during processing (Pignataro et al., 2020; Miles et al., 2021a). Generally, Phe residues have sharp fine structure between 255 and 270 nm with peaks near 262 and 268 nm, whereas the bands in near-UV arising from Tyr and Trp residues are located at 275-282 nm and 290-305 nm, respectively (Kelly et al. 2005; Martin & Schilstra, 2008; Wang et al., 2017).
However, CD does not give the residue-specific information that can be obtained by x-ray crystallography or NMR (Greenfield, 2006). Thus, an increase in the band magnitudes and intensities, indicate structural changes, which occur by the loss of the native structure and increasing interactions of the aromatic amino acid residues

(Pignataro et al., 2020; Miles et al., 2021a). Nonetheless, CD does not reveal the secondary structure of specific residues.

The different methods to analyze CD spectra are used assuming that the spectrum of a protein can be represented by a linear combination of the spectra of its secondary structural elements, plus a noise term, which includes the contribution of aromatic chromophores and prosthetic groups (Greenfield, 2006).

Ognjenović et al. (2014) in their research, for detecting subtle changes in the protein secondary structure utilized the CD spectroscopy. Their measurements show that the addition of EGCG caused an increase in β-sheet structure content in the OVA and then, suggested that the binding of EGCG to OVA near Trp160 could be able to explain why there were changes in the CD spectra. Moreover, a CD study by Pablos-Tanarro et al. (2017) showed changes in secondary structure of OVA after heat treatment.

CD spectroscopy was also applied to analyze the impact of EGCG conjugation on the secondary structure of OVA. In this study, the proportion of α-helix and unordered structure was decreased in OVA-EGCG complex comparing to free OVA, meaning that structural changes occurred (He et al., 2019).

### 1.5.3. Attenuated Total Reflection - Fourier Transform Infrared (ATR-FTIR)

Fourier Transform Infrared spectroscopy (FTIR) is one of the oldest experimental techniques recognized as a valuable tool for the analysis of secondary structure of proteins, structural dynamics, conformational changes (effects of ligand binding, temperature, pH, and pressure), structural stability and aggregation of polypeptides and proteins (Pannuru, Gupta, Horng & Lee, 2020). Kong and Yu (2007) recommended a combination of ATR-FTIR and CD for increasing prediction accuracy, when estimating the percentage contents of protein secondary structures.

Nowadays, Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR), as an evolution of classic IR is a non-destructive technique that requires less sample preparation that can be applied for structural characterization of proteins in different environments. Thus, ATR-FTIR has been used to obtain information on protein secondary structure and stability in both aqueous solution and non-aqueous or deuterated (D2O) forms and dried states (in single crystals, in aqueous solution, organic solvents, detergents micelles, lipid membranes) (Haris & Severcan, 1999). Another advantage of the IR technique comes from the development of reliable methods of data acquisition and digital subtraction, made by software on computers associated with the spectrometer, which allow the rapid processing and conversion of data (Carbonaro & Nucara, 2010).

The ATR-FTIR spectral data of high polymers are usually interpreted in terms of the vibrations of a structural repeat unit (Elliott & Ambrose, 1950; Krimm & Bandekar, 1986). The polypeptide chain gives rise to nine characteristic IR absorption bands, namely, amide A, B, and I-VII (Table 6). These nine amides I vibration modes were also presented by Banker (1992), with some standard conformations in detail in his review.

Table 6. Infrared bands of peptide bond.

| **Band** | **Frequency (cm$^{-1}$)** | **Description** |
|---|---|---|
| **Amide A** | 3300 | NH stretching |
| **Amide B** | 3100 | NH stretching |
| **Amide I** | 1600-1690 | C=O stretching |
| **Amide II** | 1480-1575 | CN stretching, NH bending |
| **Amide III** | 1229-1301 | CN stretching, NH bending |
| **Amide IV** | 625-767 | OCN bending |
| **Amide V** | 640-800 | Out-of-plane NH bending |
| **Amide VI** | 537-606 | Out-of-plane C=O bending |
| **Amide VII** | 200 | Skeletal torsion |

Data are from Elliott and Ambrose (1950), Krimm and Bandekar (1986), Banker (1992) and Miyazawa et al. (1956).

The most prominent vibrational bands of the protein backbone are amide I and II bands (Jia, Gao, Hao & tang, 2017). However, amide I band (1700-1600 cm$^{-1}$), is the most sensitive spectral region to the protein secondary structural components, which is attributed to C=O stretch vibrations of the peptide bond (approximately 80 %) with some contribution from in-plane N-H bending and C-H stretching modes. The frequencies of the amide I band components are found to be correlated closely to each secondary structural element of the proteins (Jia et al., 2017). Thus, the C=O stretching vibrations in proteins are very complex depending mainly on the details of the force field, the nature of side chains, secondary structures and inter- or intramolecular effects, including molecular geometry and hydrogen bonding pattern (Kong & Yu, 2007). The components that corresponding to specific secondary structures of the amide I band, are α-helices, β-sheets, β-turns, and random coils. However, as broad bands can hide components due to combination of very close bands that are instrumentally unresolvable (Carbonaro & Nucara, 2010). The amide II band arises mainly from in-plane NH bending (40-60 % of the potential energy) and from the CN stretching vibration (18-40 %), showing much less protein conformational sensitivity than its amide I counterpart (Krimm & Bandekar, 1986).

The second derivative using Savitsky-Golay procedure in the spectral region of 1700 – 1600 cm$^{-1}$, allowed the resolution of the major peaks for protein secondary structure. Moreover, this process can be used to achieve maximum band narrowing, reduce the

signal-to-noise ratio, to increase the signal and identify different types of secondary structures present in proteins, (Zhang & Yan, 2005; Zhou, Chen, Lin, Liu, Lyn & Ding, 2019).

In the analysis performed by Abrosimova et al. (2016) IR spectrum of egg white allowed to make an estimation of OVA secondary structure and to observe α-to-β structural transformation as a result of the heat denaturation. Moreover, it was observed a decrease of α-helix structures and the increase in number of intermolecular β-sheets. On the other hand, Milošević et al. (2020) report significant changes detected in the Amide I and Amide III region (decreased α-helix and increased β-sheet peaks) in the ATR-FTIR.

It must be considered that the interactions behind the conformations are revealed from the secondary structures observed in OVA. In addition, the strongest peak in the IR spectrum is due to C=O and its bending or elongation needs large interaction energy. The drastic reduction in the number of waves for the α-helix is due to the bending or coiling of the structure (Kavitha & Palaniappan, 2021).

# Chapter 2

# 2. Objectives

- **General objective**

➢ The main objective of this work is to develop processes for treatment eggs with natural antioxidants (PC), to be applied in the future to produce hypoallergenic eggs.

The prosecution of this project involves the treatment of egg proteins using PC in several temperature and incubation time, with the aim of modifying its native structure and consequently, its allergenic capacity.

- **Specific objectives**

➢ To evaluate the interaction of a various PC with OVA, under different experimental conditions, such as concentration and temperature, in order to find the best PC to be applied in further experiments.

➢ To promote changes in the native structure of the OVA by its interaction with selected PC and evaluate these changes and the type of interactions by using different analytical approaches.

- The changes in the secondary structure of proteins due to PC were monitored by CD and ATR-FTIR.
- Changes in the tertiary structure were detected by fluorescence spectroscopy. This technique determined whether the quenching effect due to binding and the major type of interaction occurred. Thermodynamic and binding analysis were also evaluated.

- Molecular docking using *in silico* analysis allowed to detect the specific binding zones/sites between PC-proteins, thus supporting data previously found by fluorescence, ATR-FTIR and CD. Additional information can be obtained about the binding to some epitopes of peptides or proteins.

Strategies for reducing the allergenicity of hen egg by treatment with natural antioxidants

Alcides Vapor

# Chapter 3

# 3. Materials and methods

## 3.1. Materials

OVA from hen egg white (OVA), caffeic acid (CA), ferulic acid (FA), gallic acid (GA), chlorogenic acid (CHA), tannic acid (TA), quercetin (QCT), and resveratrol (RES) were purchased from Sigma-Aldrich (Sintra, Portugal) and used without further purification. All the solutions of OVA and PC were prepared with 0.05 M phosphate buffer to keep the pH of all the solutions at 7.40, in Type 1 water (18.2 MΩ.cm at 298.15 K).

## 3.2. Methods

### 3.2.1. Fluorescence Spectra

A Fluoromax – 4 spectrofluorometer (Horiba Jobin Yvon-Edison, USA) (Figure 6) equipped with 1.0 cm pathlength quartz cuvette was used for fluorescence measurements of OVA and OVA + PC solutions. The excitation wavelength was set at 280 nm and the emission spectra were recorded between 285 and 420 nm at room temperature, after samples incubation (10 minutes) at 293.15, K 318.15 and 328.15 K. Both the excitation and emission slit widths were set at 2.5 nm. OVA concentration was fixed at 4.44 µM while PC concentration varied in the range of 0 to $9.76 \times 10^{-5}$ M, depending on the PC. The corresponding phosphate buffer and PC backgrounds were subtracted from all the raw spectra. Each solution (OVA or OVA+PC) was recorded three times and the mean was calculated. The software supplied with the spectrofluorometer and OriginPro 2016 64-bit (OriginLab, Northampton, USA) were used for data collection, spectra visualization and data analysis. The thermodynamic calculations were performed with Microsoft 365 Excel (Microsoft, Redmond, WA, USA), figures were prepared in Microsoft 365 Excel software. Data were expressed as mean of 3 measurements.

Figure 6. Fluoromax – 4 spectrofluorometer.

### 3.2.2. CD Spectra

A JASCO J-815 CD spectrometer (Jasco, Tokyo, Japan) (Figure 7) was used to register CD spectra of OVA [4,44 μM] and OVA + PC [0 to 3.99 mM] solutions (Table 9) in the range of 190-260 nm, 298.15 K and 318.15 K after 10 minutes of incubation, using a 1.0 mm path length water-jacketed quartz cuvette and a circulating water bath (LAUDA RE-104, Lauda, Germany). All CD spectra were corrected through subtraction from 0.05 M phosphate buffer pH 7.40 and PC spectra. The ellipticity was measured in mdeg with 100 nm/min speed step and resolution 0.2 nm. All collected data were uploaded to Dichroweb (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) and analyzed with CDSSTR algorithm to estimate the secondary structure content in OVA. The results were considered significant when the normalized root mean square deviation was lower than (NRMSD) 0.1 (Whitmore & Wallace, 2008). Figures were prepared in Microsoft 365 Excel and GraphPad Prism 7.01 software (trial version, USA), and OriginPro 2016 64-bit (OriginLab, Northampton, USA) were used for data analysis. Data were expressed as mean of 3 measurements.

Figure 7. JASCO J-815 CD spectrometer.

### 3.2.3. Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) Spectra

A Thermo Nicolet-iS10 spectrometer (Thermo Fisher Scientific, Massachusetts, USA) (Figure 8) was used to record FTIR-ATR spectra of 30 µL samples of OVA [1.0 mM] or OVA+PC [1:1] solutions in 0.05 M phosphate buffer pH 7.40, incubated previously at 298.15 K, 318.15 K and 328.15 K for 10 minutes. Spectra data within the range of 4000 to 600 $cm^{-1}$ were registered, and 128 scans were averaged for each spectrum of samples and background. Buffer and PC spectra were recorded and digitally subtracted to the OVA+PC spectrum. OMNIC software (Thermo Fisher Scientific, Massachusetts, USA) was used for treating peaks area (curve-fitting) through Voigt function, which is the combination of Gaussian and Lorentzian functions, and data was transferred to GraphPad Prisma 7.01 software (trial version, USA) for performing the graphics (Bradley, 2007). The OriginPro 2016 64-bit software (OriginLab, Northampton, USA) was used for data processing, and figures were prepared in Microsoft 365 Excel and GraphPad Prisma 7.01 software (trial version, USA). Data was expressed as mean of 3 measurements.

Figure 8. Thermo Nicolet-iS10 spectrometer.

### 3.2.4. *In silico* analysis

Molecular docking simulations were performed using a web-based interface (SwissDock, available at: www.swissdock.ch) that is based on the docking software EADock dihedral space sampling (DSS) through CHARMM force field and the FACTS solvation model (Grosdidier, Zoete & Michielin, 2011a, 2011b). It allows the generation of binding modes and simultaneously their CHARMM energies are estimated on a grid. Thus, the binding modes with the most favorable energies were evaluated with FACTS (Zoete et al., 2010), clustered and visualized at molecular graphic software, Chimera 1.11.2 (http://www.cgl.ucsf.edu/chimera/) (Pettersen et al., 2004). Simulations were performed between OVA (PDB ID: 1OVA) and each phenolic compound to find the

most favorable binding sites of PC to OVA as well as its proximity to fluorescent amino acids residues and epitopes. All the PC three-dimensional structures were obtained on ZINC database (http://zinc.docking.org) or drawn with MarvinView 19.9 (ChemAxon Ltd, USA) and Marvin Sketch 20.5 (ChemAxon, Cambridge, USA) and submitted in the SwissDock platform.

# Chapter 4

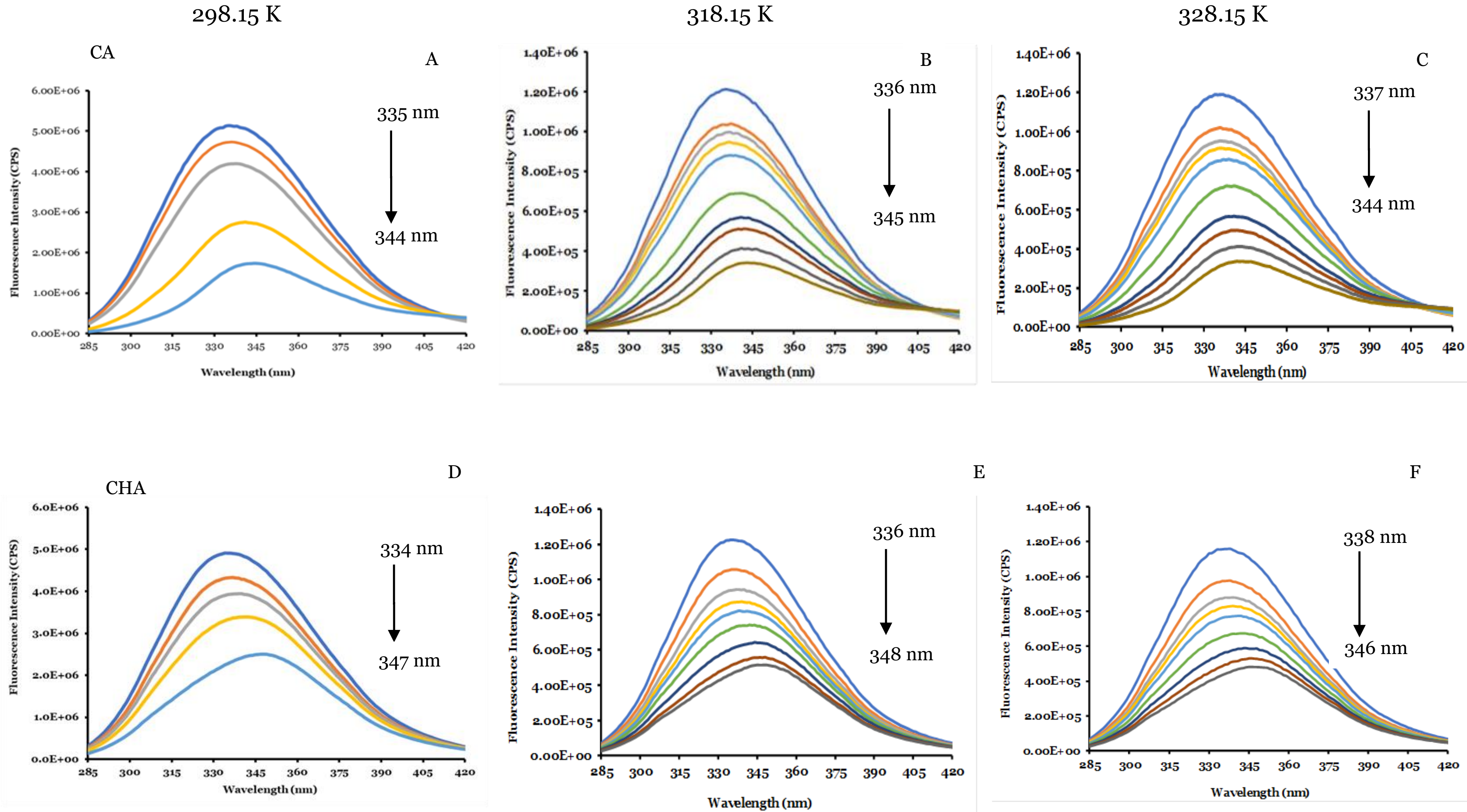
# 4. Results

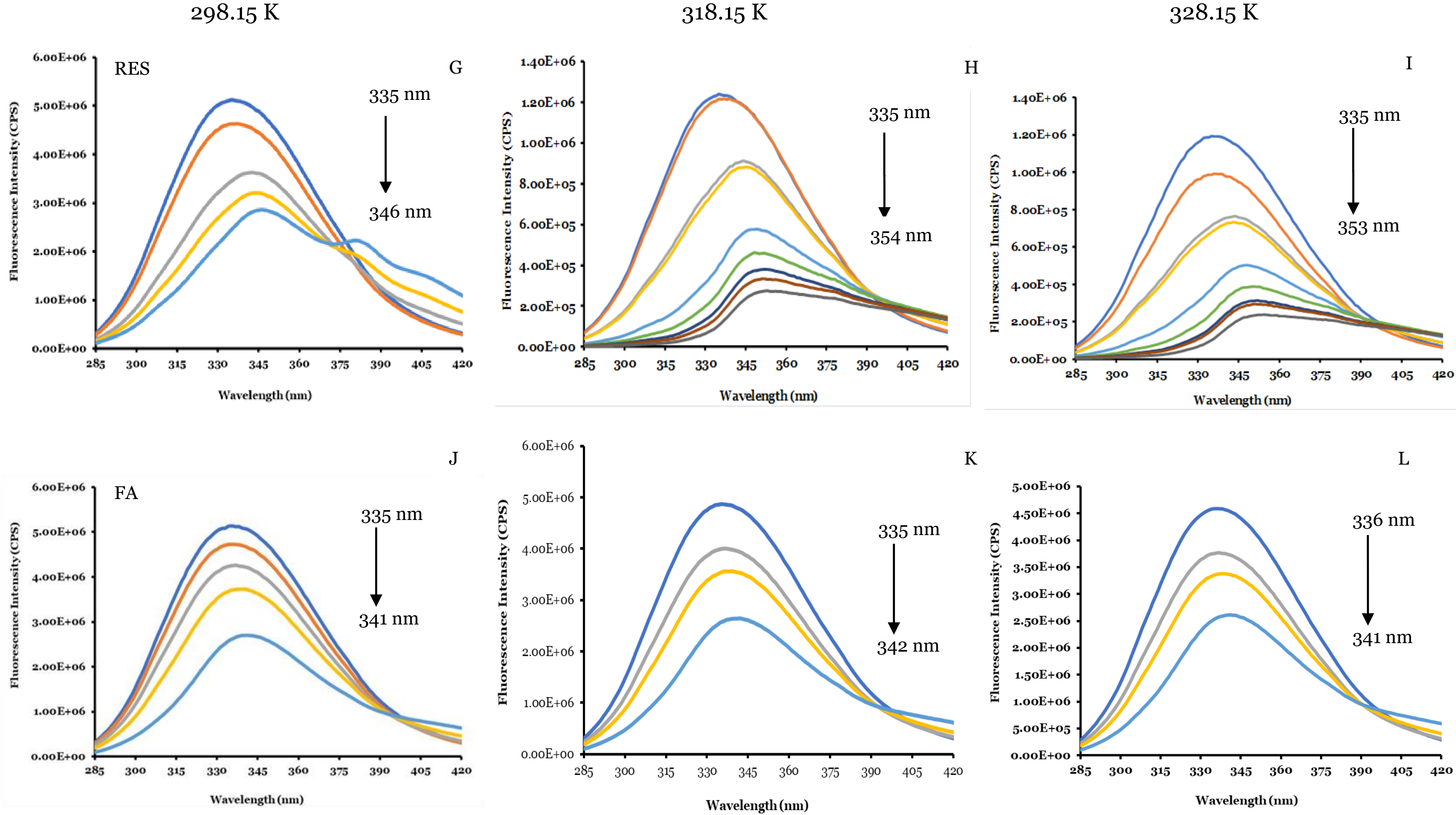
## 4.1. Interactions of ovalbumin with phenolic compounds

### 4.2. Fluorescence quenching of ovalbumin by phenolic compounds

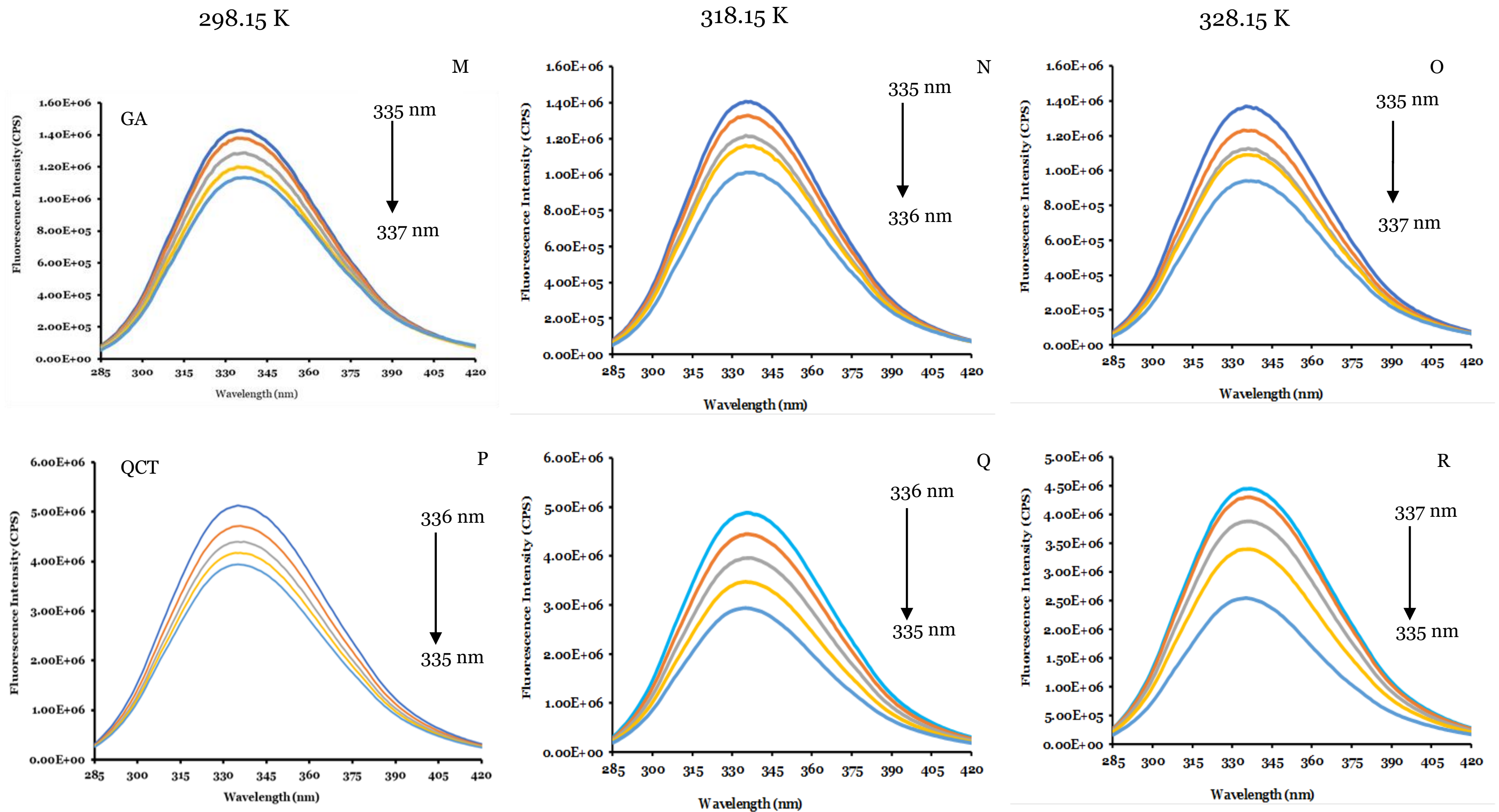
Despite the three aromatic amino acids (Phe, Trp, and Tyr) residues were excited at 280 nm, the registered emission spectra are considered due to the Trp residues since these are the dominant intrinsic fluorophores. Thus, the spectra at physiological pH 7.40 of the intrinsic fluorescence intensity of solutions of OVA with PC allow the evaluation of the effects of these compounds on OVA tertiary structure.

The results showed that for all PC tested, the fluorescence of OVA decreased gradually with increasing PC concentrations. Thus, Figure 9 (A-U) shows the emission fluorescence spectra of OVA in the absence and presence of the PC (CA, CHA, RES, FA, GA, QCT, TA), at three different temperatures (298.15, 318.15 and 328.15 K), after 10 minutes of incubation time. In addition, the λmax of OVA fluorescence is found between 335-336 nm in a solution without PC and in the range 335-354 nm for OVA solutions with increasing PC concentrations (Figure 9 A-U). Furthermore, a red shift is observed for almost all OVA-PC solutions (Figure 9 A-O and S-U), except for OVA-QCT which exhibit a blue shift (Figure 9 P-R). GA presented a small red shift (Figure 9 M-O), indicating that the Trp is not exposed to any change in polarity or there is a small change in the environment. RES and CHA promoted the major shifts at different temperatures with a difference of 11, 18 nm, and 19nm (Figure 9 G-I) and 13 nm, 12nm and 8nm (Figure D-F), respectively at 298.15, 318.15K and 328.15K. Moreover, the RES spectrum (Figure G-I) indicates a very marked effect with increase of the temperature, not only on fluorescence decreasing, but also on the magnitudes of the observed shifts.


298.15 K
318.15 K
328.15 K
CA
A
335 nm
344 nm
B
336 nm
345 nm
C
337 nm
344 nm
CHA
D
334 nm
347 nm
E
336 nm
348 nm
F
338 nm
346 nm
Fluorescence Intensity (CPS)
Wavelength (nm)



298.15 K
318.15 K
328.15 K
RES
G
H
I
335 nm
346 nm
335 nm
354 nm
335 nm
353 nm
FA
J
K
L
335 nm
341 nm
335 nm
342 nm
336 nm
341 nm
Fluorescence Intensity (CPS)
Wavelength (nm)


298.15 K
318.15 K
328.15 K
M
GA
335 nm
337 nm
N
335 nm
336 nm
O
335 nm
337 nm
P
QCT
336 nm
335 nm
Q
336 nm
335 nm
R
337 nm
335 nm
Fluorescence Intensity (CPS)
Wavelength (nm)

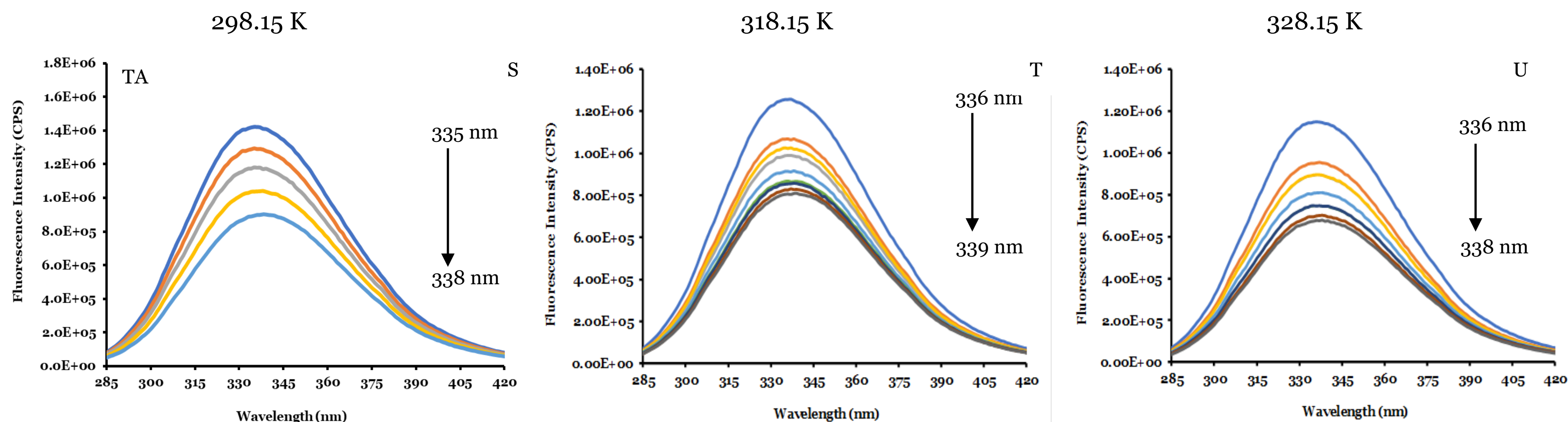



Figure 9. The fluorescence spectra emission of free OVA [4.44 µM] ($\lambda_{max}$ = 280nm), pH 7.40, and after 10 minutes of incubation in the presence of various concentrations of PC. The arrow shows the quenching effect of PC on OVA fluorescence intensity as well as the shift of $\lambda_{max}$ (from top to bottom): **(A)** CA [0 µM, 1.15 µM, 5.69 µM, 40.8 µM, and 80.1 µM] at 298.15 K. **(B)** CA [0 µM, 1.15 µM, 3.43 µM, 5.69 µM, 11.2 µM, 31.5 µM, 40.8 µM, 49.6 µM, 66.4 µM, and 80.1 µM] at 318.15 K. **(C)** CA [0 µM, 1.15 µM, 3.43 µM, 5.69 µM, 11.2 µM, 31.5 µM, 40.8 µM, 49.6 µM, 66.4 µM, and 80.1 µM] at 328.15 K. **(D)** CHA [0 µM; 2.79 µM, 8.22 µM, 18.5 µM, and 42.4 µM] at 298.15 K. **(E)** CHA [0 µM, 2.79 µM, 6.44 µM, 9.98 µM, 15.1 µM, 21.7 µM, 29.5 µM, 35.4 µM and 42.4 µM] at 318.15 K. **(F)** CHA [0 µM, 2.79 µM, 6.44 µM, 9.98 µM, 15.1 µM, 21.7 µM, 29.5 µM, 35.4 µM and 42.4 µM], at 328.15 K. **(G)** RES [0 µM; 3.68 µM, 24.7 µM, 53.5 µM and 97.6 µM] at 298.15 K. **(H)** RES [0 µM, 3.68 µM, 17.9 µM, 31.4 µM, 44.3 µM, 62.4 µM, 73.8 µM, 84.7 µM and 97.6 µM] at 318.15 K. **(I)** RES [0 µM, 3.68 µM, 17.9 µM, 31.4 µM, 44.3 µM, 62.4 µM, 73.8 µM, 84.7 µM and 97.6 µM], at 328.15 K. **(J)** FA [0 µM, 0.86 µM, 5.87 µM, 15.3 µM and 47.6 µM], at 298.15 K. **(K)** FA [0 µM, 5.87 µM, 15.3 µM and 47.6 µM], at 318.15 K. **(L)** FA [0 µM, 5.87 µM, 15.3 µM and 47.6 µM], at 328.15 K. **(M)** GA, [0 µM, 3.50 µM, 8.57 µM, 29.9 µM and 43.1 µM] at 298.15K. **(N)** GA [0 µM, 3.50 µM, 8.57 µM, 19.7 µM and 43.1 µM], at 318.15 K. **(O)** GA [0 µM, 3.50 µM, 8.57 µM, 19.7 µM and 43.1 µM], at 328.15 K. **(P)** QCT, [0 µM, 1.10 µM, 7.54 µM, 23.6 µM and 58.9 µM], at 298.15 K. **(Q)** QCT [0 µM, 1.10 µM, 7.54 µM, 23.6 µM and 58.9 µM] at 318.15 K. And **(R)** QCT [0 µM, 1.10 µM, 7.54 µM, 23.6 µM and 58.9 µM], at 328.15 K. **(S)** TA [0 µM, 0.389 µM, 1.15 µM, 2.44 µM and 3.50 µM] at 298.15 K. **(T)** TA [0 µM, 0.389 µM, 0.773 µM, 1.15 µM, 1.71 µM, 2.44 µM, 2.80 µM, 3.15 µM and 3.50 µM], at 318.15 K. **(U)** TA [0 µM, 0.389 µM, 0.773 µM, 1.71 µM, 2.80 µM, 3.15 µM and 3.50 µM], at 328.15 K. Each spectrum is the average of 3 scans.

The Stern-Volmer equation (1) was used to evaluate the type of fluorescence quenching mechanism present in the interaction between OVA and the PC:

$$F_o/F = 1 + K_{SV} \times [Q] \qquad (1)$$

where $F_o$ and F are the steady-state fluorescence intensities in the absence and in presence of the quencher, respectively; $K_{SV}$ is the Stern-Volmer quenching constant, and [Q] is the concentration of the quencher. $K_{SV}$ was obtained from equation (1) through the plot of $F_o/F$ versus [Q] (Figure 10 A-G). Since a linear plot is not sufficient to distinguish between the static or dynamic mechanisms of quenching, the value of bimolecular quenching rate constant, $K_q$, was calculated by equation (2) to confirm the mechanism (Tables 7 and 8):

$$K_q = K_{SV}/\tau_o \qquad (2)$$

where $\tau_o$ is the average lifetime of the fluorophore without the quencher, normally considered as $10^{-8}$ s for a biomolecule.

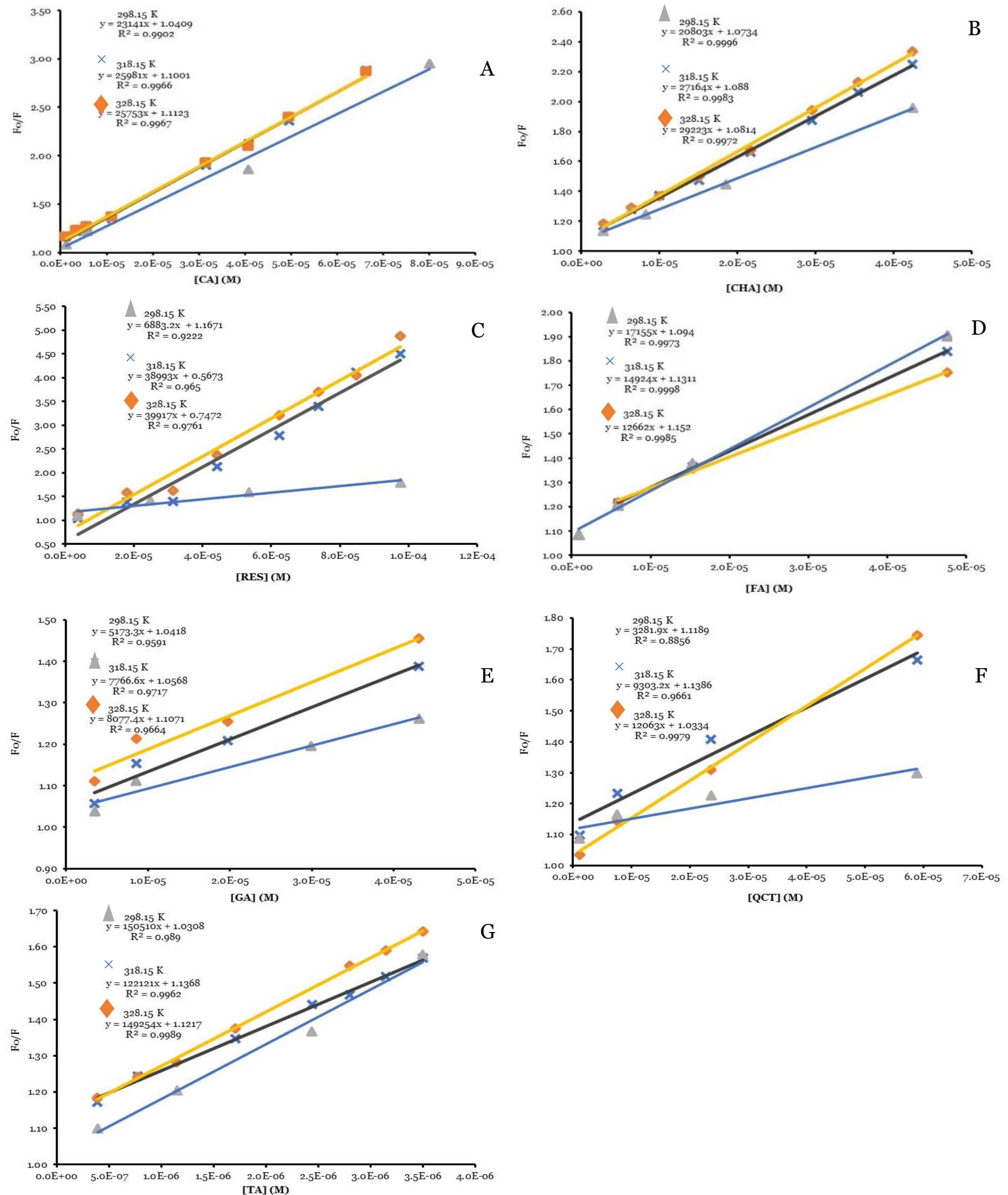



Figure 10. Stern-Volmer plots of OVA with PC after 10 minutes of incubation at 298.15 K, 318.15 K and 328.15 K**.** (**A**) CA, (**B**) CHA, (**C**) RES. (**D**) FA, (**E**) GA, (**F**) QCT, (**G**) TA. Each experimental point represents the average of 3 scans.

The Stern-Volmer plots (Figure 10) are linear for all OVA-PC solutions after 10 minutes of incubation at 298.15 K, 318.15 K, and 328.15 K. The linearity was the base for the distinction of the main quenching mechanism type in the OVA-PC interaction. From equation (2) the values of $K_q$ (Table 7) for all PC, at three temperatures, are greater than the maximum scattering collision $K_q$ of the biomolecule ($K_q$ = 2.0 × $10^{10}$ $M^{-1}$ $s^{-1}$), which suggested that the quenching mechanism of PC interaction with OVA was originated from static quenching rather than a dynamic collisional mechanism. The obtained Ksv and $K_q$ values are presented in Table 7, where can be observed that when temperature increases from 298.15 K to 318.15 K, $K_{SV}$ and $K_q$ increase for CA, CHA, RES, GA and QCT, indicating a dynamic quenching mechanism. On the other hand, for FA and TA a decrease in $K_{SV}$ and $K_q$ was observed meaning that static quenching mechanism is present in and OVA-FA and OVA-TA interaction. After the increase of temperature from 318.15 K to 328.15 K, $K_{SV}$ and $K_q$ increase for CHA, RES, TA and QCT. On the other hand, for CA and GA, $K_{SV}$ and $K_q$ kept constant, whilst for FA a decrease was observed.

Table 7. The quenching constants ($K_q$) and binding constants ($K_b$) between OVA and PC at three different temperatures (298.15, 318.15 and 328.15 K), after 10 minutes of incubation.

| T (K) | PC | $K_{SV}$ ($\times10^4$ $M^{-1}$) ± SE | $K_q$ ($\times10^{12}$ $M^{-1}$ $s^{-1}$) | $K_b$ ($\times10^4$ $M^{-1}$) | T (K) | Ksv ($\times10^4$ $M^{-1}$) ± SE | $K_q$ ($\times10^{12}$ $M^{-1}$ $s^{-1}$) | $K_b$ ($\times10^4$ $M^{-1}$) | T (K) | Ksv ($\times10^4$ $M^{-1}$) ± SE | $K_q$ ($\times10^{12}$ $M^{-1}$ $s^{-1}$) | $K_b$ ($\times10^4$ $M^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 298.15 | CA | 2.31 ± 0.16 | 2.31 | 7.76 | 318.15 | 2.60 ± 0.06 | 2.60 | 3.37 | 328.15 | 2.58 ± 0.06 | 2.58 | 2.32 |
| | CHA | 2.08 ± 0.03 | 2.08 | 5.67 | | 2.72 ± 0.04 | 2.72 | 3.77 | | 2.92 ± 0.06 | 2.92 | 3.62 |
| | RES | 0.69 ± 0.14 | 0.69 | 3.19 | | 3.90 ± 0.30 | 3.90 | 1.11 | | 3.99 ± 0.26 | 3.99 | 1.67 |
| | TA | 15.10 ± 1.12 | 15.10 | 28.70 | | 12.21 ± 0.34 | 12.21 | 22.40 | | 14.93 ± 0.22 | 14.93 | 20.50 |
| | FA | 1.72 ± 0.06 | 1.72 | 12.00 | | 1.50 ± 0.02 | 1.50 | 4.49 | | 1.30 ± 0.05 | 1.30 | 4.74 |
| | GA | 0.52 ± 0.08 | 0.52 | 1.16 | | 0.78 ± 0.09 | 0.78 | 1.81 | | 0.81 ± 0.11 | 0.81 | 4.07 |
| | QCT | 0.33 ± 0.08 | 0.33 | 11.90 | | 0.93 ± 0.12 | 0.93 | 11.00 | | 1.21 ± 0.04 | 1.21 | 3.32 |

### 4.2.1. Binding constants

Lineweaver-Burk, equation (3) gives the equilibrium among free and bound molecules to the equivalent sites of protein that PC can bind:

$$1/(F_o\text{-}F) = 1/F_o + 1/(K_b \times F_o \times [Q]) \qquad (3)$$

where $K_b$ is the binding constant between protein and ligand and is obtained from the plot of $(1/(F_o\text{-}F))$ versus $1/[Q])$, Figure 11 (A-G). Thus, the application of equation (3) to all PC-OVA complexes allowed $K_b$ determination for each temperature (Table 7).
The highest $K_b$ at 298.15 K, was found for TA, followed by FA and QCT. With the increase of temperature to 318.15 K and also to 328.15 there was a diminution of $K_b$ for almost all OVA-PC solutions, except for GA. Besides, the increment to 328.15 K also promoted a slightly increase in $K_b$ of RES and FA Interestingly, the lowest $K_b$ value at 298.15 K was for GA, and at 318.15 K were RES and GA (Table 7).

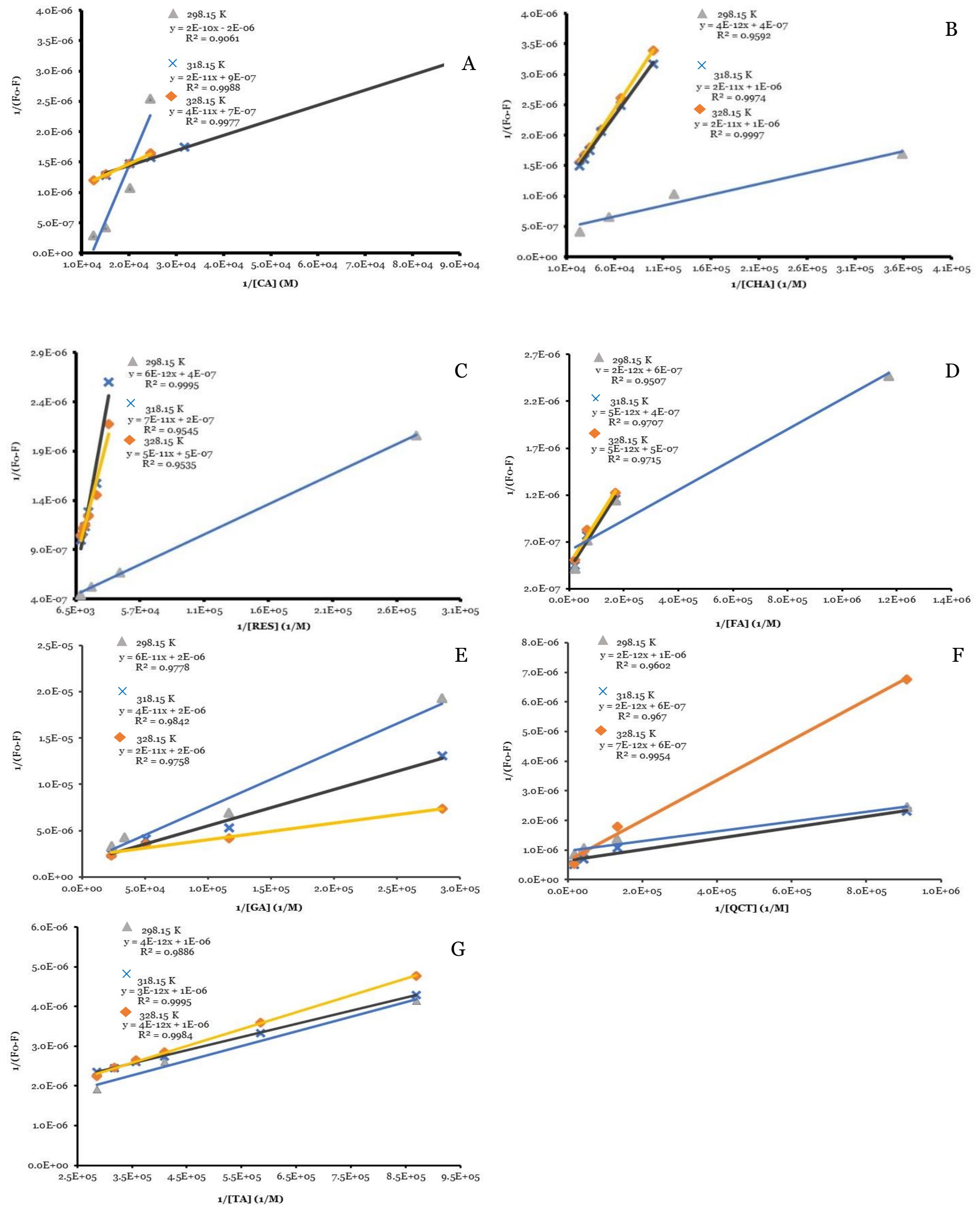



Figure 11. The Lineweaver-Burk equation (binding plots), plots of OVA [4.44 µM] with all PC tested, after incubation for 10 minutes, at 298.15 K, 318.15 K, and at 328.15 K. **(A)** CA **(B)** CHA **(C)** RES. **(D)** FA. **(E)** GA. **(F)** QCT], and **(G)** TA. The experimental points represent an average of 3 scans.

## 4.2.2. Thermodynamic analysis of binding mode

In terms of equilibrium, $K_b$ for OVA and PC solutions can be defined by equation (4):

$$K_b = [OVA – PC]/[OVA][PC] \quad (4)$$

where [OVA-PC] is the protein-PC complex concentration, [OVA] is the free OVA concentration and [PC] the PC concentration.

Thermodynamic parameters ($\Delta H$ and $\Delta S$) associated to the system shown in Table 8, were calculated according to the linearization of the van't Hoff equation (5). The value and sign of the thermodynamic parameters $\Delta S$ and $\Delta H$ can be considered an important piece of information for the determination of the main forces involved in the protein-ligand interactions, and consequently to protein-ligand stability. Under conditions of constant pressure in equilibrium, the binding constant ($K_b$) is related to the Gibbs free-energy change ($\Delta G$) of the reaction through, equations (5) and (6):

$$\ln K_b = - \Delta H/RT + \Delta S/R \quad (5)$$

$$\Delta G = \Delta H – T \times \Delta S = - RT \times \ln K_b \quad (6)$$

where R is the gas constant (8.3145 J $mol^{-1}$ $K^{-1}$) and T is the temperature (K).

Table 8. Thermodynamic parameters of OVA-PC interaction, at three different temperatures (298.15 K, 318.15 K and 328,15 K), after 10 minutes of incubation.

| PC | T (K) | ΔG (×10³ J mol⁻¹) | ΔH (×10³ J mol⁻¹) | ΔS (J mol⁻¹ K⁻¹) |
|---|---|---|---|---|
| CA | 298.15 | -27.91 | -32.78 | -16.33 |
| | 318.15 | -27.58 | | |
| | 328.15 | -27.42 | | |
| CHA | 298.15 | -27.09 | -12.83 | 47.83 |
| | 318.15 | -28.04 | | |
| | 328.15 | -28.52 | | |
| RES | 298.15 | -25.41 | -21.54 | 12.99 |
| | 318.15 | -25.67 | | |
| | 328.15 | -25.80 | | |
| TA | 298.15 | -31.15 | -9.24 | 73.48 |
| | 318.15 | -32.62 | | |
| | 328.15 | -33.35 | | |
| FA | 298.15 | -28.82 | -27.44 | 4.63 |
| | 318.15 | -28.92 | | |
| | 328.15 | -28.96 | | |
| GA | 298.15 | -23.00 | 31.21 | 181.82 |
| | 318.15 | -26.64 | | |
| | 328.15 | -28.46 | | |
| QCT | 298.15 | -29.36 | -29.36 | 0.00 |
| | 318.15 | -29.36 | | |
| | 328.15 | -29.36 | | |

All PC presented negative values for $\Delta G$, with the lowest $\Delta G$ values obtained were for OVA-TA and, also for OVA-FA which indicate that the formation of the complex

occurred spontaneously and is thermodynamically favorable. In addition, ΔH values were found negative for CA, CHA, FA, QCT, RES and TA and positive only for GA. ΔS was found negative for CA, zero for QCT, while for CHA, FA, GA, RES and TA was positive (Table 8). These results could inform about exothermic or endothermic reactions that occurred and the type of interactions that support them. Up to all PC tested, GA is the one who presented positive ΔH and a large positive value for ΔS.

Table 9. Van't Hoff equations (298.15, 318.15 and 328.15 K)

| PC | Van't Hoff equation |
|---|---|
| CA | Y=3942.5x-1.9642 |
| QCT | Y=3531.5x-0.0014 |
| FA | Y=3300.6x+0.5574 |
| CHA | Y=1542.4x+5.7529 |
| GA | Y=-3753.5x+21.869 |
| RES | Y=2590.3x+1.562 |
| TA | Y=1111.1x+8.8377 |

Table 9 present the Van't Hoff equations for each PC at three temperatures (298.15, 318.15 and 328.15K). The equations represented the behavior of the slope of each PC, expressing exothermic and endothermic reactions.

## 4.3. Analysis of ovalbumin structural changes by CD

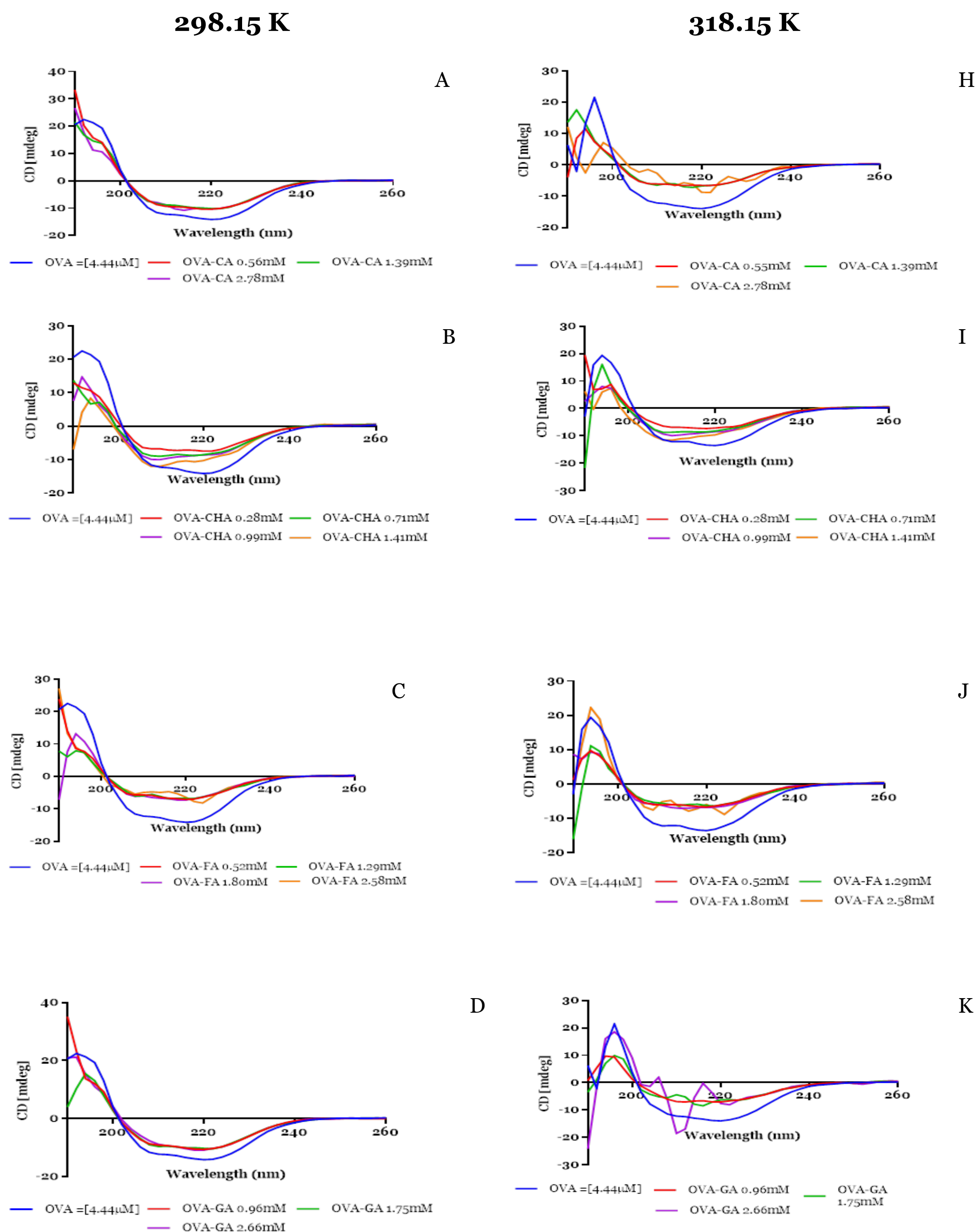
The CD spectra were recorded using OVA and OVA in the presence of increasing amounts of PC in different concentrations and ratios (OVA/PC), (Table 10).

Table 10. Phenolic compounds and ratio of OVA/PC tested in CD [OVA =4.44 μM]

| **PC** | **Concentration (mM)** | | | | **ratio (OVA /PC)** | | | |
|---|---|---|---|---|---|---|---|---|
| CA | 0.56 | 1.39 | 2.78 | | 1:125 | 1:313 | 1:626 | |
| CHA | 0.28 | 0.71 | 0.99 | 1.41 | 1:64 | 1:159 | 1:223 | 1:318 |
| FA | 0.52 | 1.29 | 1.8 | 2.58 | 1:116 | 1:291 | 1:405 | 1:581 |
| GA | 0.96 | 1.75 | 2.66 | | 1:216 | 1:394 | 1:599 | |
| QCT | 0.46 | 0.86 | 1.85 | | 1:104 | 1:194 | 1:417 | |
| RES | 0.44 | 1.1 | 1.53 | 2.19 | 1:99 | 1:248 | 1:345 | 1:493 |
| TA | 0.03 | 0.06 | 0.15 | 0.21 | 1:7 | 1:13 | 1:33 | 1:46 |

The CD spectra in Figure 12 showed that the negative band at 208 nm, characteristic of α-helical structures of a protein, was lower in OVA-PC complexes than in OVA, which can indicate a decrease in the content of this type of secondary structure after the

addition of PC to OVA. In addition, almost of β-sheet shows a negative band at 2016-218 nm, which is also attributed to secondary structures characteristics of proteins (Tables 11 and 12; Figure 12 A-G). This phenomenon was verified for almost all tested PC, and OVA-PC molar ratios incubated 10 minutes at 298.15 K and 318.15 K.



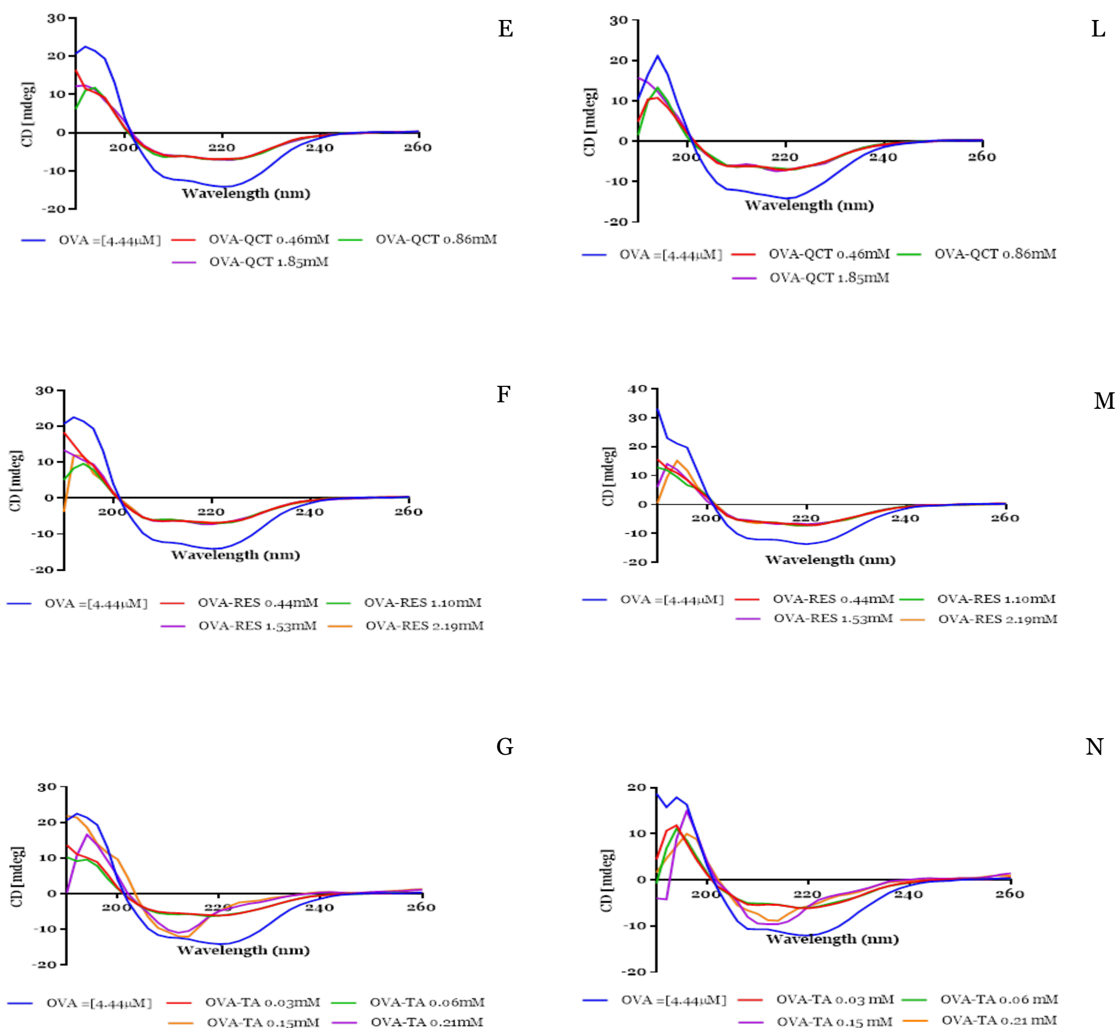



Figure 12. CD spectra of free OVA and OVA with phenolic compounds in increasing concentrations after 10 minutes of incubation at 298.15 and 318.15 K. OVA [4.44 µM] in all experiments. (**A**) CA, (**B**) CHA, (**C**) FA, (**D**) GA, (**E**) QCT, (**F**) RES, (**G**) TA, (**H**) CA, (**I**) CHA, (**J**) FA, (**K**) GA, (**L**) QCT, (**M**) RES and (**N**) TA. The spectra were obtained as the average of 3 scans.

All data from CD were uploaded to Dichroweb software (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) to analyze and quantify the components of the secondary structure. Results are presented in Tables 11 and 12.

Table 11. Secondary structure of OVA and OVA-PC solutions at 298.15K by Circular Dichroism

| Compound | Concentration of Ligand (mM) | α-helix (%) | β-sheets (%) | β-turns (%) | Random coil (%) | NRMSD |
|---|---|---|---|---|---|---|
| OVA | 0.00 | 79 | 8 | 6 | 7 | 0.001 |
| CA | 0.56 | 56 | 12 | 15 | 17 | 0.015 |
| | 1.39 | 59 | 14 | 14 | 13 | 0.011 |
| | 2.78 | 66 | 5 | 1 | 28 | 0.002 |
| CHA | 0.28 | 44 | 29 | 7 | 20 | 0.001 |
| | 0.71 | 43 | 30 | 6 | 21 | 0.002 |
| | 0.99 | 41 | 33 | 5 | 21 | 0.000 |
| | 1.41 | 52 | 22 | 5 | 21 | 0.001 |
| FA | 0.52 | 44 | 28 | 7 | 21 | 0.001 |
| | 1.29 | 43 | 29 | 6 | 22 | 0.000 |
| | 1.80 | 47 | 26 | 6 | 21 | 0.001 |
| | 2.58 | 39 | 27 | 3 | 31 | 0.001 |
| GA | 0.96 | 44 | 24 | 9 | 23 | 0.000 |
| | 1.75 | 48 | 25 | 8 | 19 | 0.001 |
| | 2.66 | 49 | 24 | 5 | 22 | 0.001 |
| RES | 0.44 | 46 | 22 | 8 | 25 | 0.001 |
| | 1.10 | 61 | 5 | 14 | 20 | 0.002 |
| | 1.53 | 46 | 28 | 11 | 15 | 0.001 |
| | 2.19 | 44 | 29 | 6 | 21 | 0.000 |
| QCT | 0.46 | 45 | 27 | 4 | 24 | 0.001 |
| | 0.86 | 52 | 18 | 6 | 24 | 0.001 |
| | 1.85 | 41 | 31 | 7 | 21 | 0.001 |
| TA | 0.03 | 51 | 18 | 14 | 17 | 0.011 |
| | 0.06 | 48 | 14 | 15 | 23 | 0.014 |
| | 0.15 | 46 | 26 | 7 | 21 | 0.000 |
| | 0.21 | 54 | 20 | 13 | 13 | 0.001 |

Table 12. Secondary structure of OVA and OVA-PC solutions at 318.15K by Circular Dichroism

| Compound | Concentration of Ligand (mM) | α-helix (%) | β-sheets (%) | β-turns (%) | Random coil (%) | NRMSD |
|---|---|---|---|---|---|---|
| OVA | 0.00 | 57 | 16 | 6 | 21 | 0.001 |
| CA | 0.56 | 53 | 22 | 6 | 19 | 0.001 |
| | 1.39 | 50 | 24 | 5 | 21 | 0.002 |
| | 2.78 | 21 | 48 | 6 | 25 | 0.008 |
| CHA | 0.28 | 42 | 32 | 7 | 19 | 0.000 |
| | 0.71 | 44 | 29 | 6 | 23 | 0.000 |
| | 0.99 | 44 | 28 | 8 | 20 | 0.000 |
| | 1.41 | 46 | 25 | 7 | 22 | 0.001 |
| FA | 0.52 | 53 | 23 | 5 | 19 | 0.001 |
| | 1.29 | 48 | 23 | 6 | 23 | 0.001 |
| | 1.80 | 48 | 27 | 4 | 21 | 0.001 |
| | 2.58 | 45 | 23 | 3 | 29 | 0.001 |
| GA | 0.96 | 55 | 18 | 7 | 20 | 0.002 |
| | 1.75 | 54 | 21 | 8 | 17 | 0.002 |
| | 2.66 | 53 | 23 | 7 | 17 | 0.001 |
| RES | 0.44 | 45 | 10 | 17 | 28 | 0.001 |
| | 1.10 | 45 | 25 | 8 | 22 | 0.001 |
| | 1.53 | 45 | 30 | 6 | 19 | 0.001 |
| | 2.19 | 48 | 19 | 12 | 21 | 0.001 |
| QCT | 0.46 | 44 | 28 | 7 | 21 | 0.001 |
| | 0.86 | 43 | 29 | 6 | 22 | 0.001 |
| | 1.85 | 44 | 31 | 5 | 20 | 0.000 |
| TA | 0.03 | 50 | 38 | 7 | 5 | 0.002 |
| | 0.06 | 51 | 23 | 7 | 19 | 0.002 |
| | 0.15 | 46 | 26 | 7 | 21 | 0.000 |
| | 0.21 | 48 | 23 | 10 | 19 | 0.003 |

The NRMSD (normalized root mean square deviation) in equation 7, allows the comparison between the data set available in Dichroweb and experimental data. The results were considered statistically significant since the obtained NRMSD was lower than 0.1 (Whitmore & Wallace, 2004, 2008).

$$\text{NRMSD} = [\sum\theta_{exp} - \theta_{cal})^2/\sum(\theta_{exp})^2]^{1/2} \qquad (7)$$

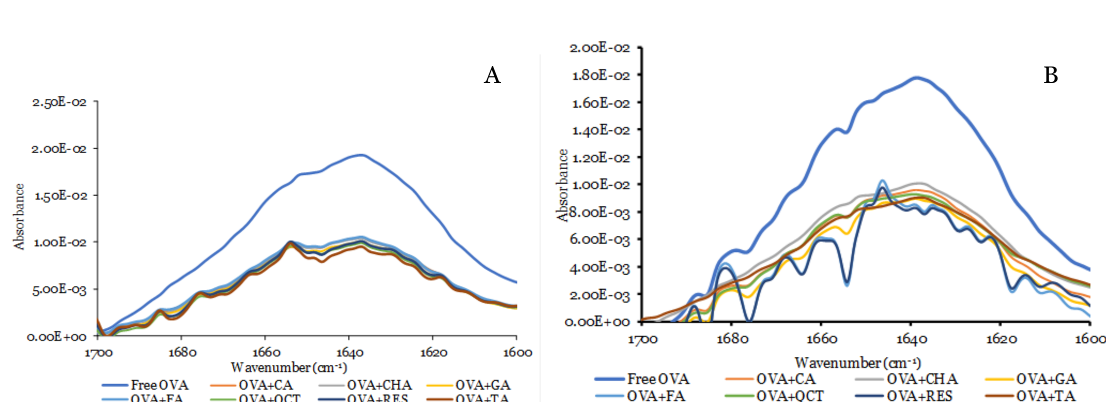
where $\theta_{exp}$ and $\theta_{cal}$ are the experimental ellipticities and the ellipticities of the back-calculated spectra for the derived structure, respectively. Thus, the NRMSD is not used for matching or ranking identified results. However, NRMSD is a measure of the correspondence between the experimental and calculated spectra and should be <0.1 for a good result.

Furthermore, for all PC at 298.15 and 318.15 K, even for the lowest concentrations there was an effect of decreasing the percentage of α-helices in relation to free OVA. On the other hand, there was an increase in the percentage of β-sheets in almost all cases (Tables 11 and 12). However, at 298.15 K there was also an increase in β-turns for CA, RES and TA, and in random coil for all PC (Table 11). At 318.15 K, an increase in β-turns was observed at the lowest concentration of RES and at the highest concentration of TA, but no significant changes in random coil were observed (Table 12). So, this means that significant changes occurred in the secondary structure of OVA caused by interactions with PC.

The CD broad negative band of α-helix and β-sheet are well defined as referred above, showing a significant decrease and increase respectively, when OVA interact with the PC (Figure 12). Therefore, in general an increase of β-sheet structures and a decrease α-helix was observed for all PC and temperatures tested (298.15 and 318.15 K) (Tables 11 and 12; Figure 12 A-N).

## 4.4. Analysis of ovalbumin structural changes by ATR-FTIR

The conformational changes of OVA after incubation with PC were evaluated by ATR-FTIR spectroscopy. The spectra were obtained in the range 1700-1600 cm-1 that is informative about the changes in the secondary structure of proteins. Indeed, there is evidence of structural changes in amide I band of OVA following the interaction with each PC (Figure 13 A-C) with a decrease of absorbance in result of the correspondent spectra peaks found in Table 13 A-C. Thus, for each peak in amide I band, its area was analyzed, and results as percentage of secondary structures were compared. FTIR spectra of OVA-PC showed shifts in the wavenumber values of amide I band, suggesting that changes occurred due to the interactions of PC with OVA (Figure 13 A-C, and Table 13).



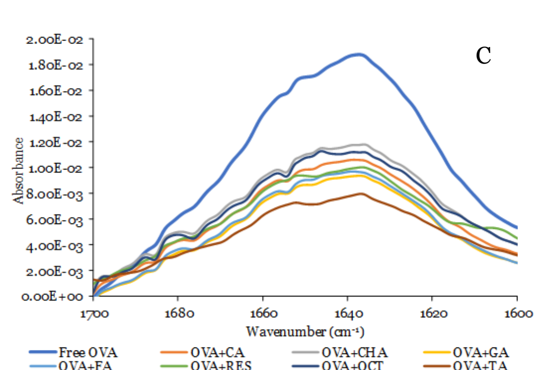



Figure 13. ATR-FTIR spectra (1700-1500 cm-1) of OVA [1.0 mM] and OVA with all PC [1.0 mM], concentration ratio 1:1. (A) 298.15; (B) 318.15 K ;(C) 328.15; K. The spectra were obtained as an average of 3 scans.

The results show that all the PC influenced the FTIR profile of OVA, as shown in Figure 13 A-C. Furthermore, there are deviation of OVA wavenumbers in consequence of the interaction with PC (Figure 13, and Table 13), after incubation at three different temperatures.

At 298.15 K, it was possible to observe an increase in the amide I band maximum wavenumber of OVA with CA, CHA, GA, QCT, RES and TA, comparing to free OVA. At 318.15 K, it was observed that the amide I band maximum wavenumber increases for CA, CHA, GA and QCT, decreases for TA and FA and remains constant for RES. On the other hand, at 328.15 K it was observed only a slight increase for FA. It is important to refer that the maximum value of the amide I band of OVA do not change when temperature increases from 298.15 to 318.15 K but decrease to 1636 $cm^{-1}$ with the increment to 328.15 K (Figure 13 A-C, and Table 13).

Table 13. Wavenumbers peaks in the region 1700-1600 cm-1 (amid I) from ATR-FTIR spectra at different temperatures (figure 13 A: 298.15 K; B: 318.15K and C: 328.15 K).

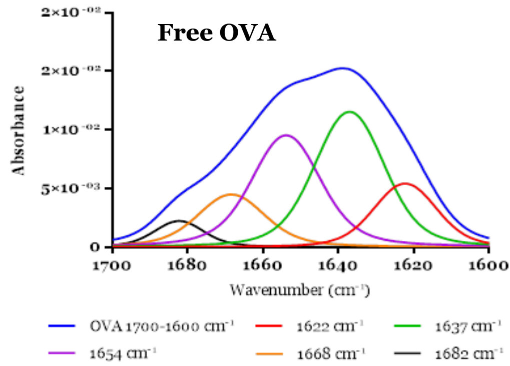
| PC [1.0 mM] | T (K) | Wavenumber ($cm^{-1}$) peaks | T (K) | Wavenumber ($cm^{-1}$) peaks | T (K) | Wavenumber ($cm^{-1}$) peaks |
|---|---|---|---|---|---|---|
| | | Amide I | | Amide I | | Amide I |
| **OVA** | | 1638 | | 1638 | | 1636 |
| **OVA-CA** | | 1639 | | 1639 | | 1636 |
| **OVA-CHA** | | 1639 | | 1639 | | 1636 |
| **OVA-FA** | | 1638 | | 1638 | | 1637 |
| **OVA-GA** | **298.15** | 1642 | **318.15** | 1641 | **328.15** | 1636 |
| **OVA-QCT** | | 1641 | | 1640 | | 1636 |
| **OVA-RES** | | 1643 | | 1638 | | 1636 |
| **OVA-TA** | | 1645 | | 1636 | | 1636 |

For the amide I band (1700 – 1600 $cm^{-1}$), after curve-fitting, bands with peaks at the following wavenumbers were obtained: 1624 $cm^{-1}$, 1639 $cm^{-1}$, 1653 $cm^{-1}$, 1668 $cm^{-1}$ and 1685 $cm^{-1}$, corresponding to α-helix, β-sheets, turns, and random coil, respectively (Figure 14 A-X). Thereafter, the bands integration was performed by GraphPad Prisma

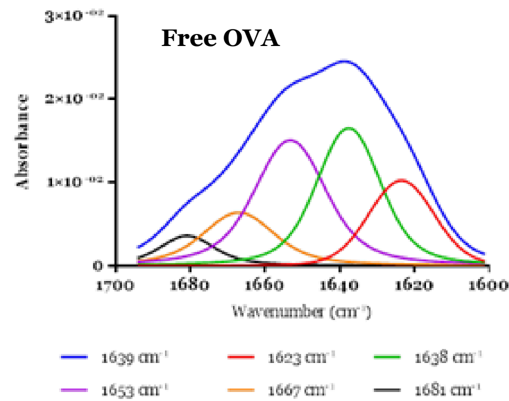
7.01 (trial version, USA), and the values of each area were analyzed and calculated as a percentage (Figure 14 A-X, and Table 14).

**298.15 K** | **318.15 K** | **328.15 K**

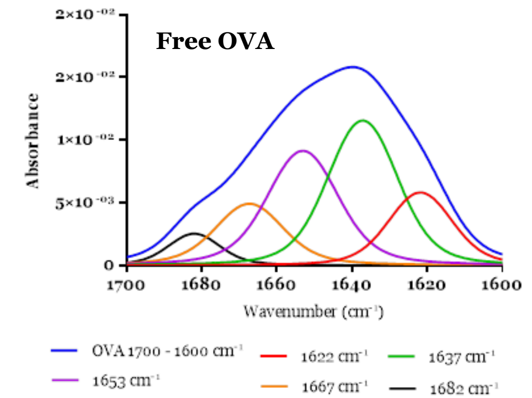
A



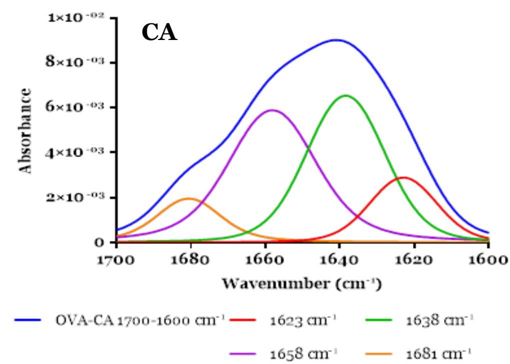

I



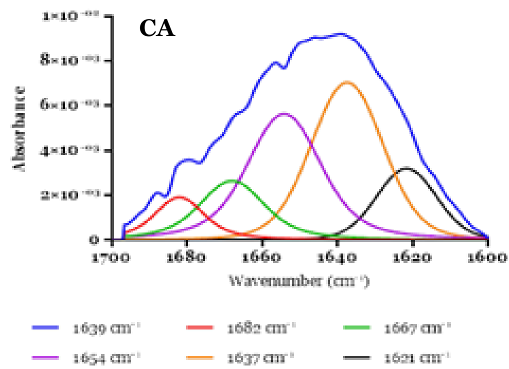

Q



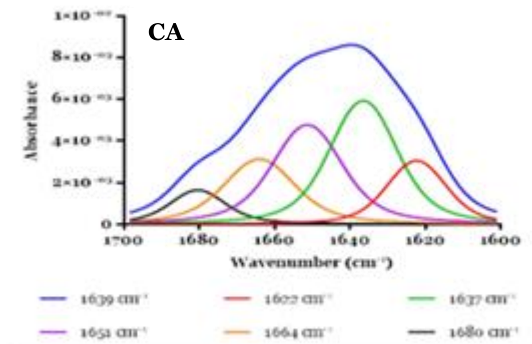

B




J

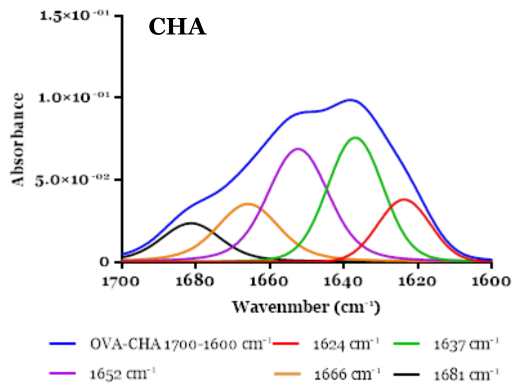



R

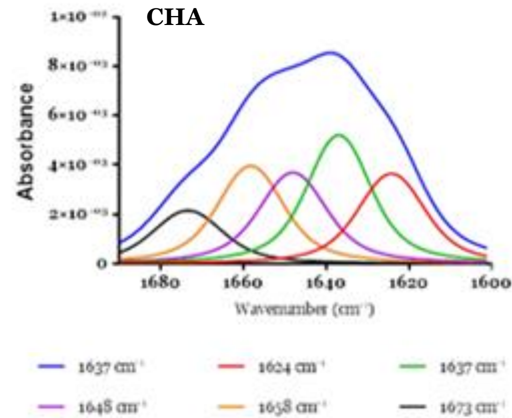


**298.15 K** **318.15 K** **328.15 K**

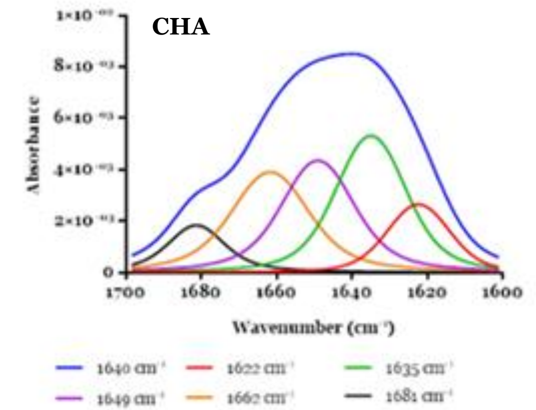
C



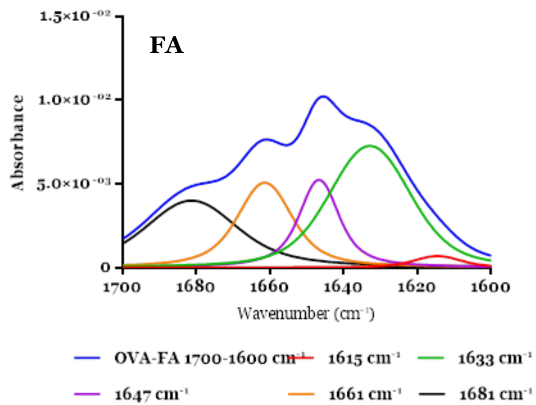

K



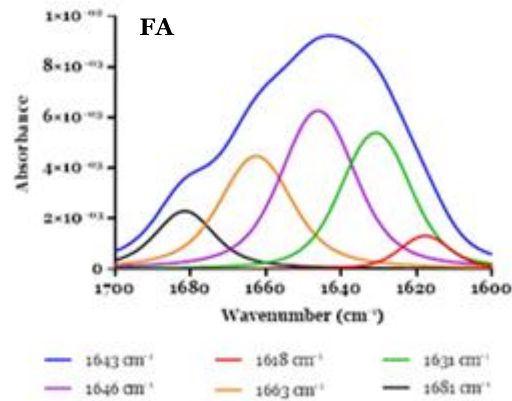

S



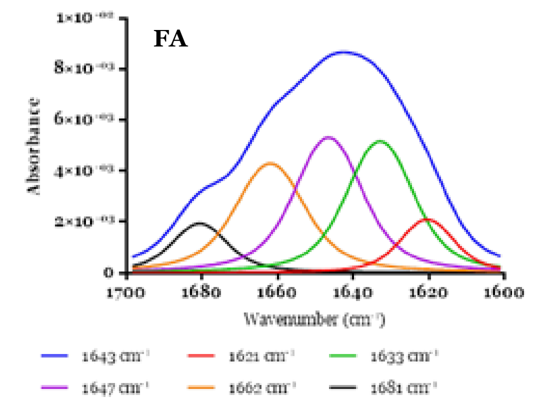

D



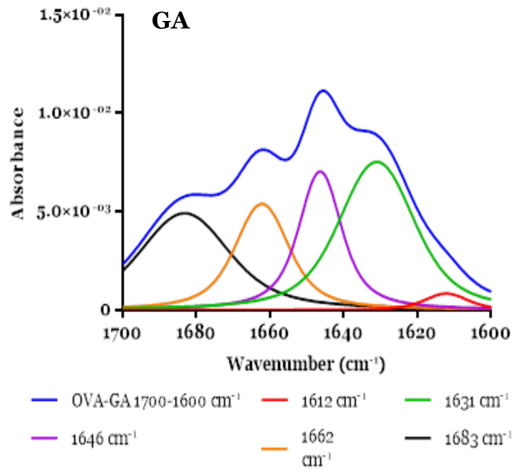

L



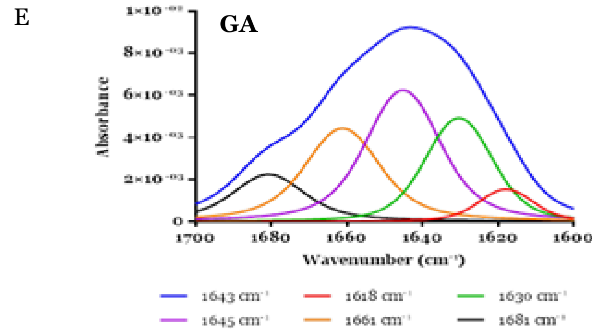

T



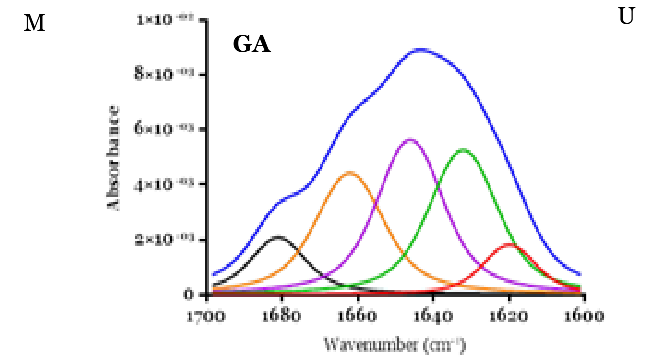
**298.15 K** **318.15 K** **328.15 K**



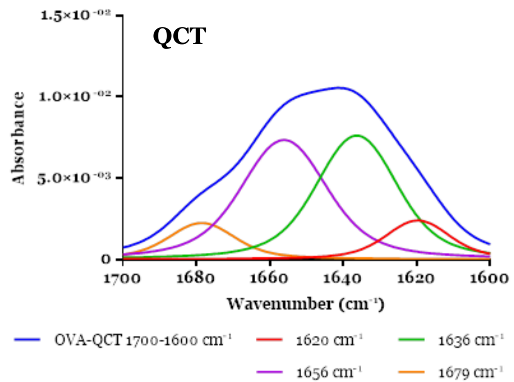

E



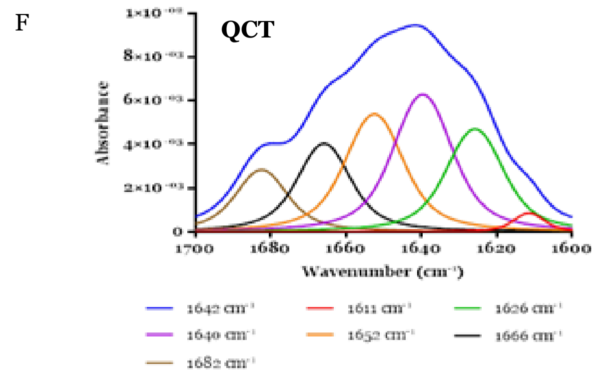

M



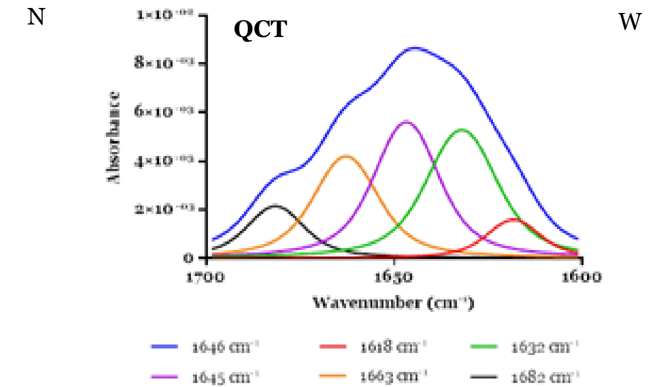

U




F




N




W

**298.15 K** **318.15 K** **328.15 K**

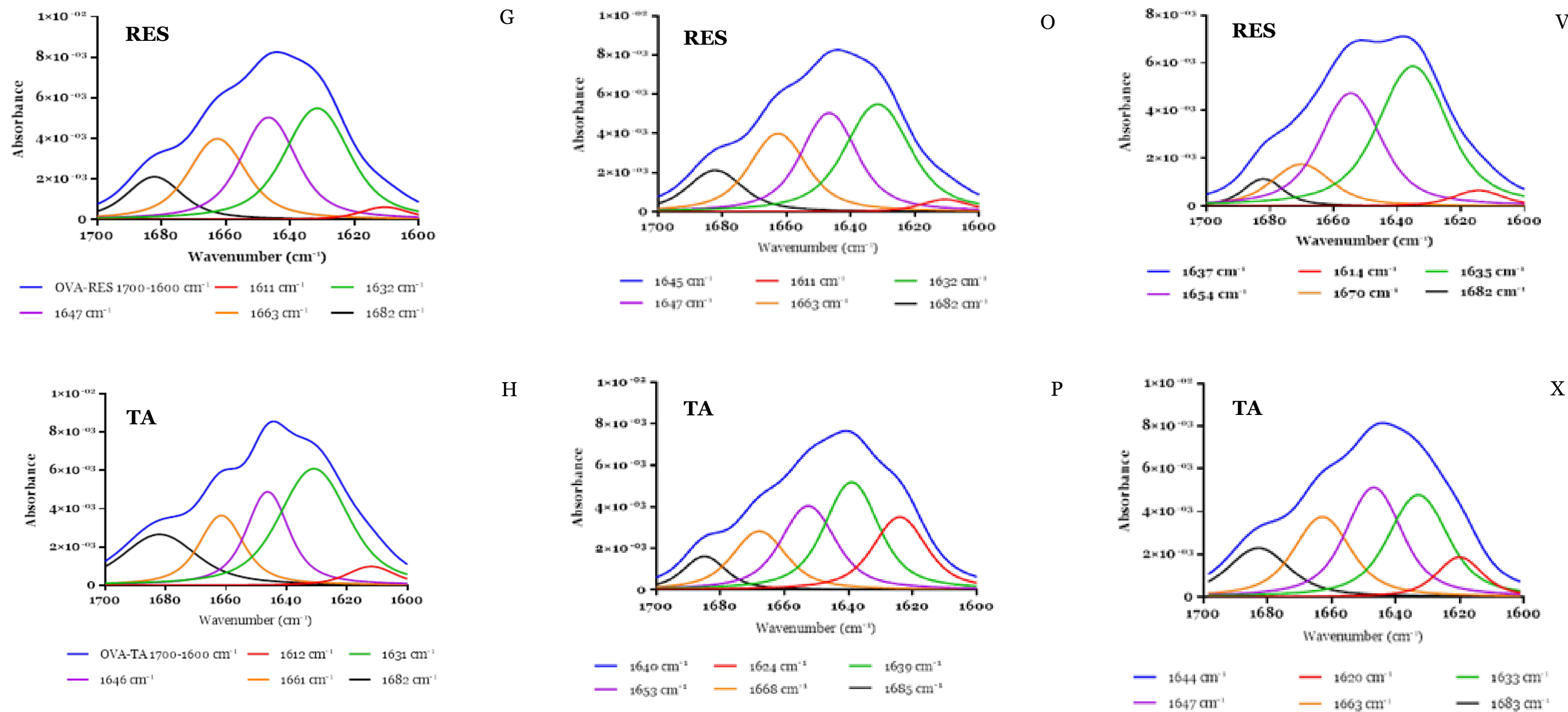


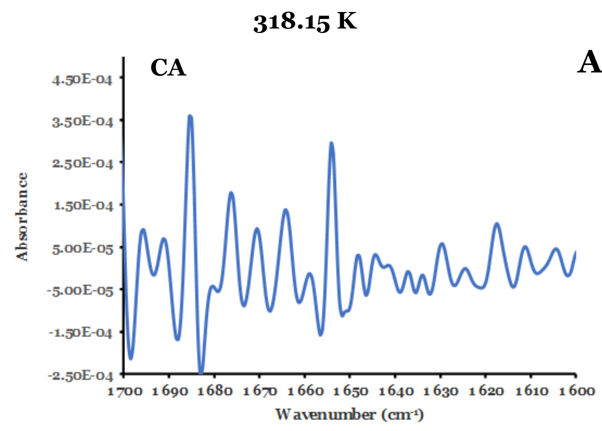

Figure 14. Curve-fitting of ATR-FTIR spectra of all OVA-PC using Voigt procedure, after 10 minutes of incubation. Wavenumbers below the spectra refer to the maximum wavenumber of each curve (**A**) free OVA, (**B**) CA, (**C**) CHA, (**D**) FA, (**E**) GA, (**F**) QCT, (**G**) RES, (**H**) TA at 298.15 K; (**I**) free OVA, (**J**) CA, (**K**) CHA, (**L**) FA, (**M**) GA, (**N**) QCT, (**O**) RES, (**P**) TA at 318.15 K; (**Q**) free OVA, (**R**) CA, (**S**) CHA, (**T**) FA, (**U**) GA, (**V**) QCT, (**W**) RES and (**X**) TA at 328.15 K. The experimental points represent an average of 3 scans.

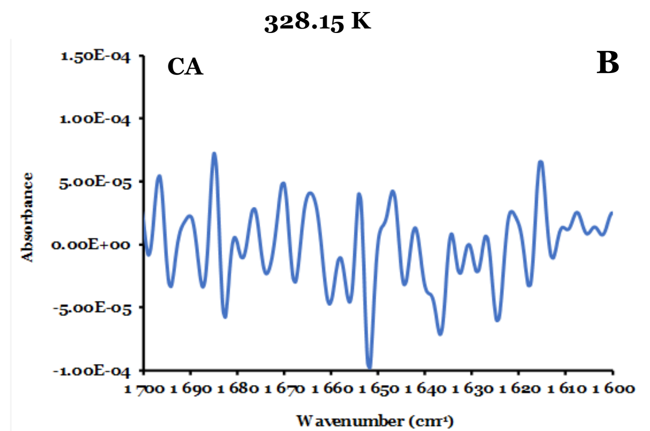
Table 14. Secondary Structure of OVA and OVA-PC (ATR-FTIR Spectra) at pH 7.40, OVA [1.0 mM] and PC [1.0 mM], concentration molar ratio OVA/ PC corresponding to 1:1; after 10 minutes incubation.

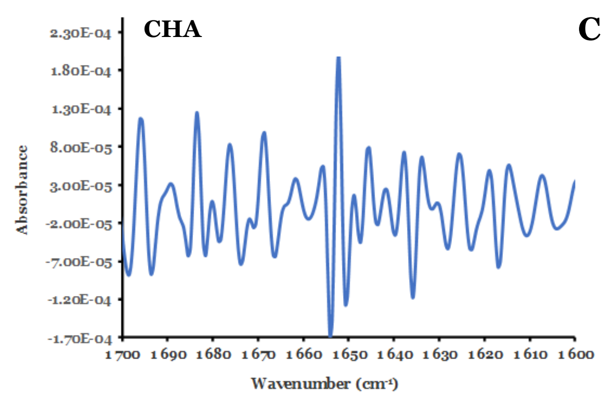
| Complex OVA/ PC [1:1] | T (K) | α-helix (%) | β-sheet (%) | β-turn (%) | random coil (%) | T (K) | α-helix (%) | β-sheet (%) | β-turn (%) | random coil (%) | T (K) | α-helix (%) | β-sheet (%) | β-turn (%) | random coil (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OVA | | 30 | 51 | 19 | 0 | | 32 | 19 | 17 | 32 | | 28 | 52 | 20 | 0 |
| OVA-CA | | 0 | 30 | 38 | 32 | | 34 | 48 | 8 | 10 | | 25 | 45 | 30 | 0 |
| OVA-CHA | | 31 | 43 | 27 | 0 | | 21 | 46 | 12 | 50 | | 26 | 42 | 33 | 0 |
| OVA-FA | | 0 | 41 | 43 | 16 | | 22 | 30 | 19 | 29 | | 25 | 21 | 25 | 29 |
| OVA-GA | 298.15 | 0 | 37 | 43 | 20 | 318.15 | 0 | 29 | 37 | 34 | 328.15 | 22 | 27 | 17 | 34 |
| OVA-QCT | | 41 | 49 | 10 | 0 | | 24 | 22 | 26 | 28 | | 25 | 25 | 20 | 30 |
| OVA-RES | | 0 | 38 | 34 | 28 | | 34 | 47 | 19 | 0 | | 37 | 26 | 10 | 21 |
| OVA-TA | | 0 | 44 | 34 | 22 | | 0 | 31 | 22 | 47 | | 27 | 47 | 27 | 0 |

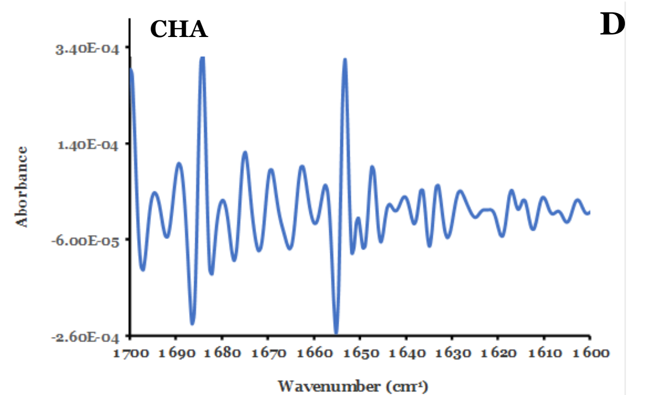
According to the Table 14, in general for all temperatures the content of α-helix for some OVA-PC decreased in comparison with free OVA values, while β-sheet increased only at 318.15 K. At 298.15, the α-helix structures increased for QCT, remained constant for CHA, whilst decreased for CA, FA, GA, RES and TA. On the other hand, β-sheet decreased for CA, GA and RES, whereas β-turns and random coil structures increased for almost all PC, with some exceptions, like QCT. At 318.15 K, it was found that β-sheet increased for all PC, in consequence of the reduction of α-helix in some cases (CHA, FA, GA, QCT and TA). However, α-helix increased for CA and RES. β-turns and random coils also increased for FA, GA, QCT, RES and TA. At 328.15 K, α-helix increase only for RES, while decrease for almost all PC. β-sheet decrease for all PC, in contrary, β-turns increase for CA, CHA, FA, QCT and TA, have decreased for GA and RES. Thus, random coil increase for FA, GA, QCT and RES and for other PC, decreased. Random coils increase for CHA, GA, QCT and TA. In contrary for CA, FA and RES, decreased. These all results compared from the free OVA.

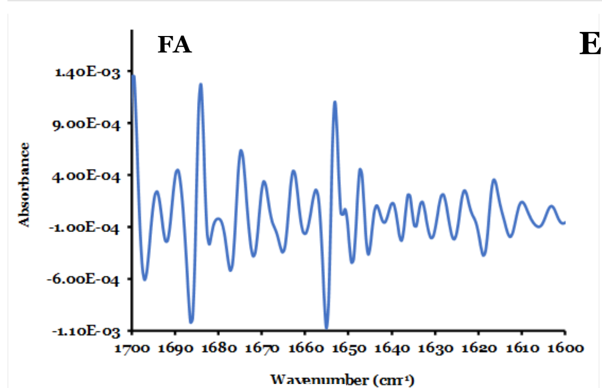
The results obtained for ATR-FTIR and CD are not completely in agreement, since these techniques have different sensitivity, and the assays were also performed at different experimental conditions. Effectively, while in CD the spectrum was recorded at the incubation temperature, in IR the spectrum was obtained at room temperature after incubation at 298.15, 318.15 and 328.15 K.

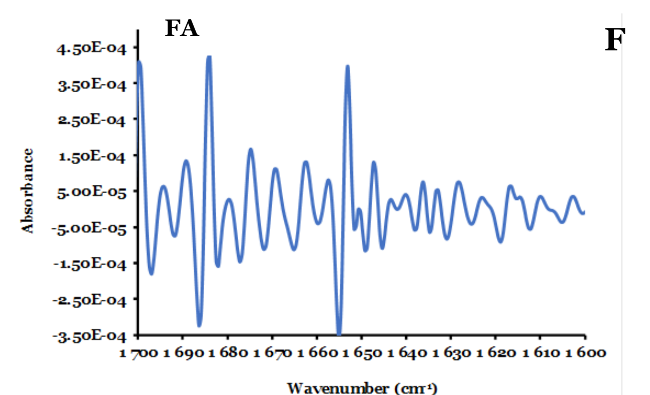

318.15 K
CA
A
Absorbance
4.50E-04
3.50E-04
2.50E-04
1.50E-04
5.00E-05
-5.00E-05
-1.50E-04
-2.50E-04
1700 1690 1680 1670 1660 1650 1640 1630 1620 1610 1600
Wavenumber (cm-1)

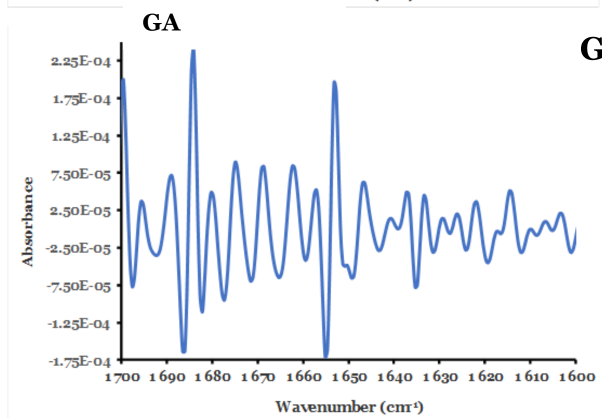

328.15 K
CA
B
Absorbance
1.50E-04
1.00E-04
5.00E-05
0.00E+00
-5.00E-05
-1.00E-04
1700 1690 1680 1670 1660 1650 1640 1630 1620 1610 1600
Wavenumber (cm-1)

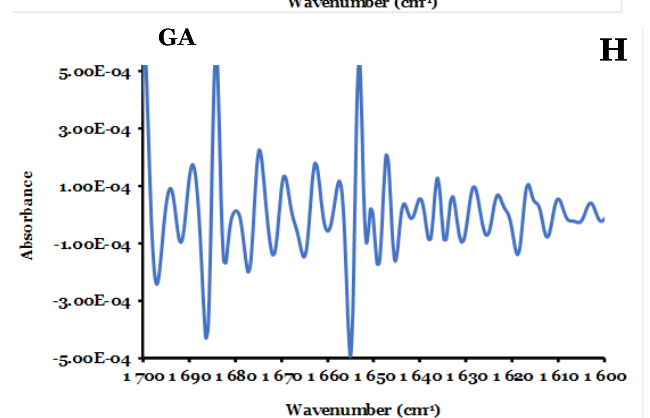

CHA
C
Absorbance
2.30E-04
1.80E-04
1.30E-04
8.00E-05
3.00E-05
-2.00E-05
-7.00E-05
-1.20E-04
-1.70E-04
1700 1690 1680 1670 1660 1650 1640 1630 1620 1610 1600
Wavenumber (cm-1)


CHA
D
Absorbance
3.40E-04
1.40E-04
-6.00E-05
-2.60E-04
1700 1690 1680 1670 1660 1650 1640 1630 1620 1610 1600
Wavenumber (cm-1)


FA
E
Absorbance
1.40E-03
9.00E-04
4.00E-04
-1.00E-04
-6.00E-04
-1.10E-03
1700 1690 1680 1670 1660 1650 1640 1630 1620 1610 1600
Wavenumber (cm-1)


FA
F
Absorbance
4.50E-04
3.50E-04
2.50E-04
1.50E-04
5.00E-05
-5.00E-05
-1.50E-04
-2.50E-04
-3.50E-04
1700 1690 1680 1670 1660 1650 1640 1630 1620 1610 1600
Wavenumber (cm-1)


GA
G
Absorbance
2.25E-04
1.75E-04
1.25E-04
7.50E-05
2.50E-05
-2.50E-05
-7.50E-05
-1.25E-04
-1.75E-04
1700 1690 1680 1670 1660 1650 1640 1630 1620 1610 1600
Wavenumber (cm-1)


GA
H
Absorbance
5.00E-04
3.00E-04
1.00E-04
-1.00E-04
-3.00E-04
-5.00E-04
1700 1690 1680 1670 1660 1650 1640 1630 1620 1610 1600
Wavenumber (cm-1)

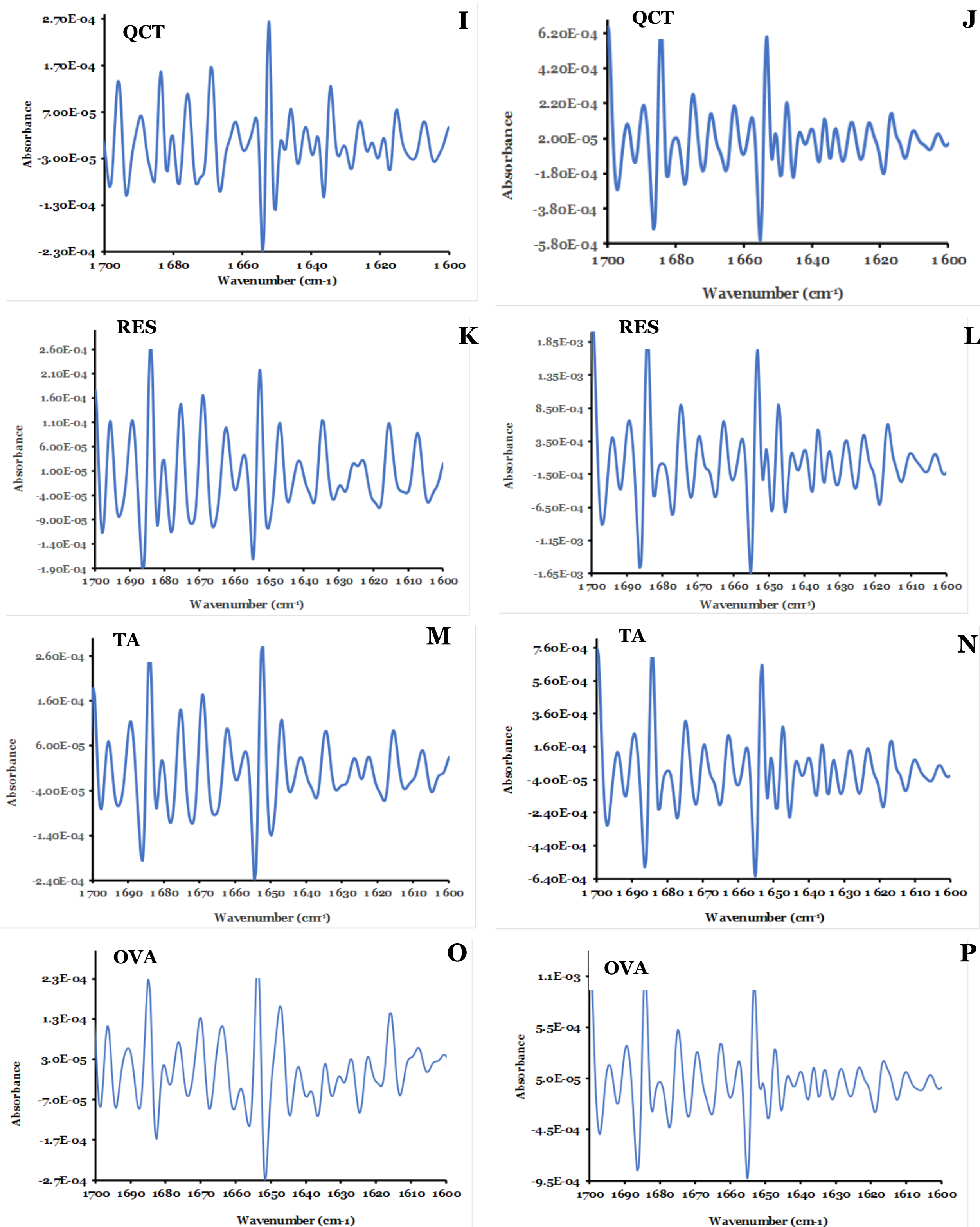



Figure 15. ATR-FTIR spectra second derivative using Savitsky-Golay, analyzing OVA-PC after 10 minutes of incubation, concentration [1.0 mM] and concentration ratio 1:1. At 318.15 K and 328.15 K peaks are, respectively at **(A)** 1683 $cm^{-1}$ and **(B)** 1652 $cm^{-1}$ for OVA-CA; **(C)** 1654 $cm^{-1}$ and **(D)** 1655, 1686 $cm^{-1}$ for OVA-CHA; **(E)** 1655, 1686 $cm^{-1}$ and **(F)** 1655, 1686 $cm^{-1}$ for OVA-FA; **(G)** 1655, 1686 $cm^{-1}$ and **(H)** 1655, 1686 $cm^{-1}$ for OVA-GA; **(I)** 1654 $cm^{-1}$ and **(J)** 1655, 1686 $cm^{-1}$ for OVA-QCT; **(K)** 1655, 1686 $cm^{-1}$ and **(L)** 1655, 1686 $cm^{-1}$ for OVA-RES; **(M)** 1655, 1686 $cm^{-1}$ and **(N)** 1655, 1686 $cm^{-1}$ for OVA-TA; **(O)** 1652, 1683 $cm^{-1}$ and **(P)** 1655, 1686 $cm^{-1}$ for Free OVA.

Furthermore, the analysis of second derivative (Figures 15 A-P), could determine with accuracy, the peaks that represent the changes of secondary structure of OVA as consequence of the interactions with PC. These peaks are visible and correspond to the wavenumbers in Figure 15 A-P, and Table 15. However, observing the values of (amide I band), at both temperatures (318.15 and 328.15 K) in the Figure 15 there are differences between the values of free OVA and OVA-PC complexes (Table 15). In the Table 15, the values of wavenumber of second derivative presented, correspond to α-helix (1649-1656 $cm^{-1}$), β-sheets (1612-1639 $cm^{-1}$), β-turns (1660-1698 $cm^{-1}$) and random coil (1645 $cm^{-1}$). The prominent peaks correspond to α-helix and to β-turns.

Table 15. ATR-FTIR peaks of second derivative for each solution of OVA, after incubation at 318.15K and 328.15 K. All spectra were obtained at room temperature after incubation.

| Compounds | T (K) | Peaks ($cm^{-1}$) | T (K) | Peaks ($cm^{-1}$) |
|---|---|---|---|---|
| OVA | 318.15 | 1698 – 1693 – 1683 – 1679 – 1667 – 1660 – 1656 – 1652 – 1645 – 1637 – 1633 – 1624 – 1618 – 1612 | 328.15 | 1696 – 1692 – 1686 – 1683 – 1678 – 1672 – 1665 – 1660 – 1655 – 1649 – 1645 – 1639 – 1637 – 1630 – 1625 – 1619 – 1611 |
| OVA-CA | | 1698 – 1688 – 1683 – 1656 – 1652 | | 1683 – 1652 – 1637 – 1624 |
| OVA-CHA | | 1654 – 1651 – 1617 | | 1697 – 1686 – 1682 – 1655 |
| OVA-FA | | 1697 – 1686 – 1677 – 1655 | | 1697 – 1686 – 1682 – 1655 |
| OVA-GA | | 1698 – 1686 – 1682 – 1677 – 1659 – 1655 – 1649 – 1635 | | 1697 – 1692 – 1686 – 1682 – 1677 – 1665 – 1655 – 1649 – 1635 |
| OVA-QCT | | 1698 – 1694 – 1685 – 1678 – 1673 -1666 – 1654 – 1650 – 1636 – 1654 | | 1697 – 1686 – 1682 – 1677 – 1672 – 1665 – 1655 |
| OVA-RES | | 1698 – 1693 – 1686 – 1682 – 1678 – 1672 – 1666 – 1660 – 1655 -1651 – 1645 – 1637 – 1631 – 1618 | | 1697 – 1692 – 1686 – 1682 – 1677 – 1672 – 1665 – 1655 – 1649 – 1645 – 1638 – 1634 – 1631 – 1626 – 1619 |
| OVA-TA | | 1698 – 1683 – 1686 – 1682 – 1678 – 1672 – 1665 – 1655 – 1650 – 1645 – 1637 – 1631 – 1618 | | 1697 – 1692 – 1686 – 1683 – 1677 – 1672 – 1665 – 1660 - 1655 – 1652 – 1649 – 1645 – 1638 – 1634 – 1631 – 1626 – 1619 |

## 4.5. Molecular docking

The molecular docking technique was used to predict the binding parameters of the PC-OVA (ligand-receptor) complex and its stability. It is important to highlight that in SwissDock analysis the protein is considered to have a rigid structure, which implies that there is a conformational adaptation of the PC to establish binding to the protein. The chain A of the crystal structure of OVA (PDB ID: 1OVA, Figure 16) that presents 15 α-helices and 16 β-sheets was selected through Protein Data Bank

(http://www.rcsb.org/pdb/home/home.do), for the simulations in SwissDock. As referred above, this software uses the whole protein as a rigid structure, and the ligand-receptor complexes can be scored considering for steric fit, and chemical complementarity. The molecular docking (*in silico*) with SwissDock (www.swissdock.ch) included thermodynamics-based simulations to locate the most favorable localizations for the PC-protein (OVA) binding. Moreover, some details as the hydrogen bonds length between PC and OVA could be seen when the output were visualized by using UCSF Chimera 1.11.2.

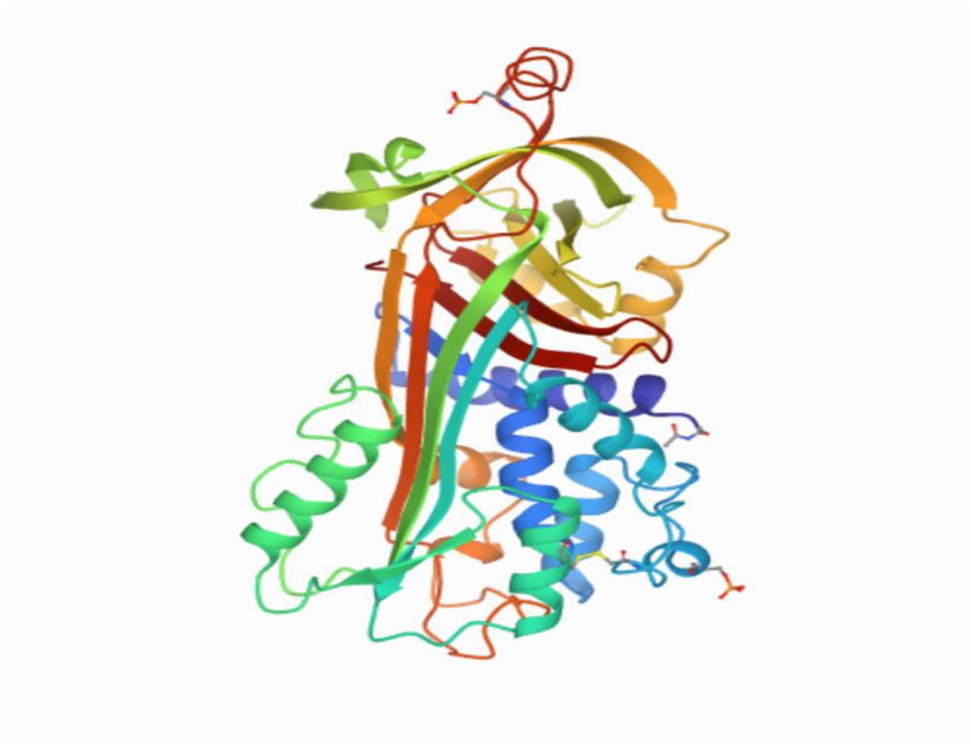


Figure 16. Representation of crystal structure of OVA at 1.95 Å resolution, from protein data bank (PDB ID: 1OVA _1: shows chain A) deposited by Stein, Leslie, Finch & Carrell (1991) Doi: 10.2210/pdb1OVA/pdb.

The docking results support the data previously obtained by fluorescence, and CD, concerning to the existence of interactions between OVA and each PC.

Figure 17 A-P presents the docking simulation in which was possible to bind theoretically as a presumption each PC to OVA.

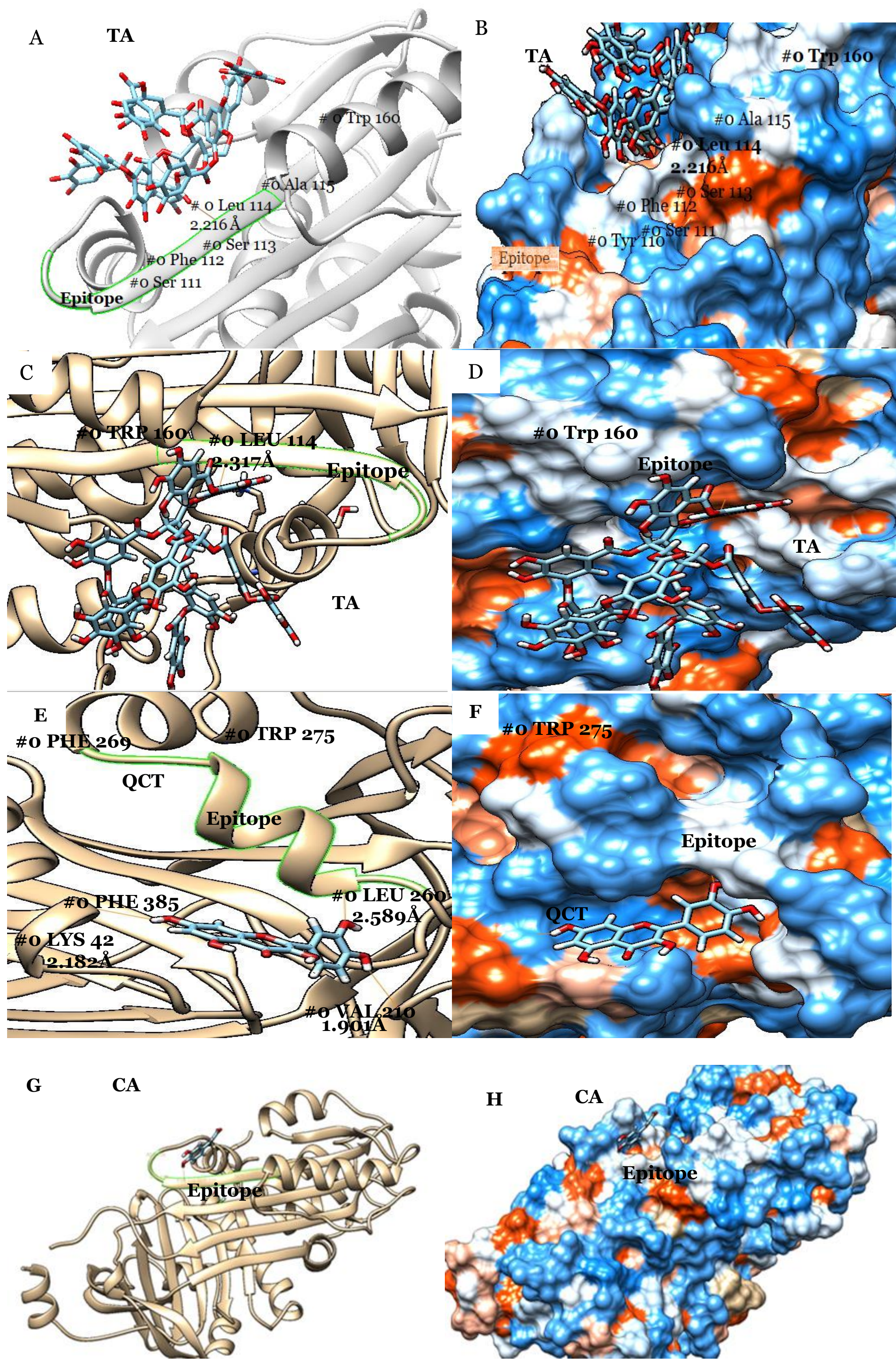
Thus, seven ligands were used in docking simulations (CA, CHA, FA, GA, TA, QCT and RES) and all of them bound to OVA with negative binding energies, with emphasis on GA, CA, TA, and CHA, that present the most negative values, from -9.46 KJ $mol^{-1}$ up to -18.09 KJ $mol^{-1}$ (Table 16). Thus, a *in silico* interaction between each PC and OVA was performed and analyzed in more detail to verify if binding sites involved directly the OVA epitopes or areas close to epitopes (Figures 17 A-P).

Thereby, it was found that CA (D95-A102), CHA (E191-V200), QCT (D95-A102; G251-N260) and TA (D95-A102) bind directly to the OVA epitope while FA (D95-A102) and

RES (V243-E248), bind closely to the epitope with hydrophobic interactions. GA (E191-V200) bounds closely to the epitope with hydrogen bonds (Figure 17).

Table 16. Estimated ΔG of the interaction of the PC with OVA epitopes, given by Swissdock software.

| PC | OVA amino acid sequence | Positions | Estimated ΔG (KJ mol$^{-1}$) |
|---|---|---|---|
| QCT | LAMVYLGAKDST | 38-49 | -7.68 |
| | DVYSFSLA | 95-102 | -6.77 |
| | EDTQAMPFRV | 191-200 | -6.73 |
| | VLLPDE | 243-248 | -6.37 |
| | GLEQLESIIN | 251-260 | -6.63 |
| | GITDVF | 301-306 | -6.97 |
| | ISQAVHAAHA | 323-332 | -6.71 |
| | AVLFFGRCVS | 375-384 | -6.32 |
| CHA | LAMVYLGAKDST | 38-49 | -10.50 |
| | DVYSFSLA | 95-102 | -6.77 |
| | EDTQAMPFRV | 191-200 | -18.09 |
| | VLLPDE | 243-248 | -6.73 |
| | GLEQLESIIN | 251-260 | -6.63 |
| | GITDVF | 301-306 | -7.65 |
| | ISQAVHAAHA | 323-332 | -7.65 |
| | AVLFFGRCVS | 375-384 | -7.96 |
| GA | LAMVYLGAKDST | 38-49 | -6.77 |
| | DVYSFSLA | 95-102 | -6.78 |
| | EDTQAMPFRV | 191-200 | -9.46 |
| | VLLPDE | 243-248 | -6.24 |
| | GLEQLESIIN | 251-260 | -6.19 |
| | GITDVF | 301-306 | -6.04 |
| | ISQAVHAAHA | 323-332 | -6.40 |
| | AVLFFGRCVS | 375-384 | -6.15 |
| TA | LAMVYLGAKDST | 38-49 | -9.73 |
| | DVYSFSLA | 95-102 | -9.73 |
| | EDTQAMPFRV | 191-200 | -11.51 |
| | VLLPDE | 243-248 | -11.51 |
| | GLEQLESIIN | 251-260 | -11.51 |
| | GITDVF | 301-306 | -11.19 |
| | ISQAVHAAHA | 323-332 | -9.31 |
| | AVLFFGRCVS | 375-384 | -10.17 |
| CA | LAMVYLGAKDST | 38-49 | -7.34 |
| | DVYSFSLA | 95-102 | -7.34 |
| | EDTQAMPFRV | 191-200 | -9.61 |
| | VLLPDE | 243-248 | -6.94 |
| | GLEQLESIIN | 251-260 | -6.10 |
| | GITDVF | 301-306 | -6.75 |
| | ISQAVHAAHA | 323-332 | -7.12 |
| | AVLFFGRCVS | 375-384 | -7.17 |
| FA | LAMVYLGAKDST | 38-49 | -6.64 |
| | DVYSFSLA | 95-102 | -6.61 |
| | GITDVF | 301-306 | -6.73 |
| | ISQAVHAAHA | 323-332 | -6.73 |
| | AVLFFGRCVS | 375-384 | -6.88 |
| RES | LAMVYLGAKDST | 38-49 | -6.49 |
| | DVYSFSLA | 95-102 | -6.43 |
| | EDTQAMPFRV | 191-200 | -6.74 |
| | VLLPDE | 243-248 | -6.74 |
| | GLEQLESIIN | 251-260 | -6.74 |
| | GITDVF | 301-306 | -6.81 |
| | ISQAVHAAHA | 323-332 | -6.85 |
| | AVLFFGRCVS | 375-384 | -6.81 |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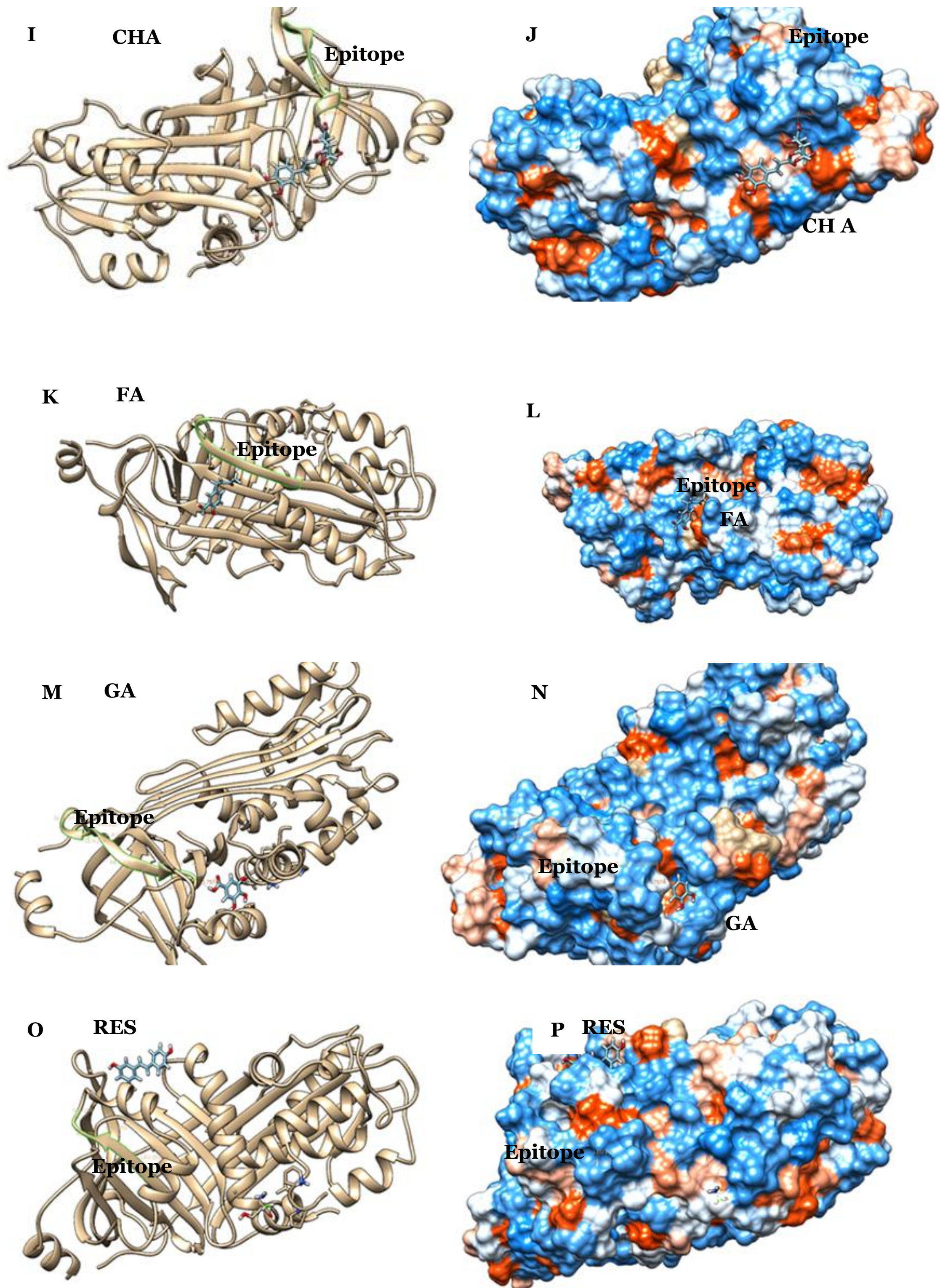


Figure 17. The molecular docking revealing the epitopes from OVA. **(A)** Tannic Acid (TA)-binding to OVA and Trp160 solvent exposed. Epitope (green color border) with the sequence of its amino acid residues; hydrogen bond (orange color) stabilized by Ser111 H-B 2.216 Å, ΔG = -11.5 KJ mol$^{-1}$ and the Trp160 is well visible; **(B)** Hydrophobic surface of OVA IgE-binding site and TA showing amino-acids sequence and overlapped sequence **(C)** TA bind directly to OVA epitope (**D95A102**) through hydrogen bond between its OH group, and OH of Ser111, and the carbonyl oxygens of Phe112, Ser113, Leu114 and Ala115, close to fluorescent residue Phe112, from the epitope nearby Ala115, Tyr110 and exposed Trp160. **(D)** A hydrophobic surface representation diagram of the OVA-TA complex with IgE epitope highlighted. **(E)** The strong QCT-binding with three H-B Lys42, 2.182 Å; Leu260, 2.589 (bound directly to the IgE epitope (**L38T49**); Val210, 1.901 Å; ΔG = -6.63 KJ mol$^{-1}$ exposed Trp275. **(F)** A hydrophobic surface representation diagram of the OVA-QCT complex with IgE epitope highlighted. **(G)** CA binding to OVA in

the epitope (**D95A102**) with hydrogen bond at Ser111 residue, 1.945 Å. **(H)** Hydrophobic surface representation diagram of OVA-CA. **(I)** CHA binding directly to the OVA epitope (**E191V200**) with hydrogen bond at Glu201 residue, 2.114 Å. **(J)** CHA Hydrophobic surface representation of OVA-CHA. **(K)** FA binding to OVA close to the epitope (**D95A102**), representing clearly the hydrophobic interaction. **(L)** OVA-FA hydrophobic surface representation**. (M)** GA binding nearby OVA epitope (**E191V200**), with two hydrogen bonds at Thr211 residue, 1.757 Å and 1.994 Å, in the same residue. **(N)** Hydrophobic surface representation of OVA-GA. **(O)** RES binding close to the epitope (**V243E248**), by hydrophobic interaction. **(P)** OVA-RES hydrophobic surface representation. Blue color is hydrophilic part of the molecule and red color the hydrophobic. H-B hydrogen bond.

# Chapter 5

# 5. Discussion, concluding remarks and future trends

## 5.1. Methods for reducing egg allergenicity

Egg allergy is a public health concern affecting mostly children and some adults. Until now, there is no treatment for this disorder, and the eviction of food is the best way to prevent allergy. There are many methods already used to reduce egg allergenicity (Table 3), but so far this task did not yet be successful, due to the high stability and resistance of the allergens. Nevertheless, natural antioxidants such as PC, can be a good trend in the fight against food allergy, specifically in the case of hen's egg proteins (Vapor et al., 2022). In fact, it has been described that PC interacts specifically with food allergens and may modulate the immune response in humans, which could be useful in preventing and treating allergic diseases (Hassan, 2013; Li & Yan, 2017). Therefore, in this study, spectroscopic techniques and molecular docking were used to analyze OVA-PC interactions and OVA conformational changes that can contribute to the reduction of egg allergy.

## 5.2. Ovalbumin structural changes

### 5.2.1. Fluorescence quenching

Fluorescence spectroscopy can be used to obtain information on changes in the tertiary structure of the proteins. The fluorescence spectrum of OVA is mainly owing to Trp residues (Trp148, Trp184 and Trp267), since Phe residues have the lowest quantum yield and a weaker fluorescence (Sheehan, 2009;), and Tyr residues fluorescence is almost totally quenched when an amino group and carboxyl group are ionized (Lu, Wang, Gao, Wang, Yan, & Chen, 2009). Trp residues show the highest fluorescence and are the main responsible for the intrinsic fluorescence of the protein when excited at 280 nm (Xie et al., 2017). Thus, three Trp residues in OVA are positioned differently in the structure: Trp160 is solvent exposed, Trp194 is buried in the hydrophobic pocket, and Trp275 is partially exposed (Ognjenović et al., 2014).

The conformational changes of the proteins in the vicinity of aromatic amino acid residues, in particular Trp, induced by ligand interactions and/or protein unfolding, can alter the intensity of fluorescence and the wavelength of the emission spectrum

(Möller & Denicola, 2002). The results showed that for all PC tested, the fluorescence of OVA decreased gradually with increasing PC concentrations (Figure 9 A-U) which can be attributed to ligand binding (Liu et al., 2010; Czubinski et al., 2014; Fu et al., 2016). The addition of PC to OVA solutions caused a decrease in fluorescence intensity as result of quenching (Figure 9 A-U) and also a shift in λmax of OVA-PC fluorescence with increasing PC concentrations (Figure 9 A-U). The red shift suggests that the OVA fluorophores became more accessible to the solvent, which increases the propensity of the Trp side chain to form hydrogen bonds (Ognjenović et al., 2014). Furthermore, red shift may also result from a change in the polarity of the environment of the OVA Trp residues or the unfolding of the OVA and change in its conformation, thus disturbing the protein tertiary structure (He et al., 2019; Liang, et al., 2008; Soares, 2015). A CA-induced red shift was already seen when CA binds to OVA (Chang et al., 2021). Cheng et al. (2021) observed that RES and OVA formed a more stable complex because of various forces, and through their molecular docking simulation, demonstrated that complex could bind by hydrogen bonds and hydrophobic interactions. However, RES has got an issue of lower solubility in aqueous solution, that can lead to the aggregability or denaturation of OVA. In fact, the OVA fluorophores become more accessible to the solvent since its side chains form hydrogen bonds both with water and with OH of PC molecules, in addition to other type of interactions (e.g. Van der Waals forces). Moreover, the shift of the emission peak to higher wavelengths, suggest an increase in polarity around Trp residues (Wang et al., 2017) due to a conformational change in OVA because of the interactions with PC.

The blue shift in the fluorescence spectrum suggested that the Trp residues of OVA moved into a more hydrophobic environment, as proposed for BSA (Möller & Denicola, 2002; Madaeni, & Rostami, 2008).

Thus, the results demonstrated that the binding of PC to OVA promotes conformational changes that can affect the allergenic epitopes and so may reduce its allergenic capacity (Wu et al., 2011), as well as in the case of covalent conjugation of BLG with polyphenols (Lu et al., 2018). As well as, happened with peanut and whey proteins, in which study with CA, conformational changes and adjustment of epitopes occurred, and the allergenicity was reduced (Chang & Champagne, 2009, Tong et al., 2018). In addition, CHA was studied with Ara h3 (A3), β-casein, BLG, WPI, where protein unfolded and conformational changes happened (Chung & Champagne, 2009; Wu et al., 2018). Also, in another study, TA was examinated with BSA, BLG, Amadin and peanut, where were found conformational changes and complexes formation used as nanoencapsulation systems for oral drug delivery and reduce the mAb (Chung & Champagne, 2009; Chang & Reed, 2012; Zhang et al., 2018).

PC were also applied largely for reducing hen´s egg allergenicity, by Liu et al. (2018) and Tong et al. (2018), when studied OVA with CA. In that research, allergenicity were reduced partially in a mouse model and occurred conformational changes and adjustment of epitopes. CHA with OVA was evaluated by Lu et al. (2018), finding a decreased of IgE-binding, reduced allergenicity and conformational changes. Ognjenović et al. (2014) and He et al. (2019), applied EGCG to OVA and LYZ, where OVA allergenicity reduced, IgE-binding decreased, and LYZ structure, functionality and characteristics changed and occurred conformational changes. But OVA unfolded. QCT when applied to OVA, the allegenicity in vitro and in vivo, in a mouse model, IgE-binding was reduced, Th1 and Th2 were modulated, and OVA presented conformational changes and destrution or mask of the epitopes (Zhang et al., 2020). TA with OVA was analysed by Xie et al. (2017) in vitro and in vivo, in a mouse model, where allergenicity were reduced, the ability for IgE-binding was reduced and occurred complexes formation to use in nanoencapsulation systems for oral drug delivery.

The Stern-Volmer plots (Figure 10), of all OVA-PC solutions were linear. However, linearity cannot distinguish if the type of quenching mechanism is static or dynamic (Lu et al., 2009; Chang et al., 2021). Nevertheless, the values greater than $2.0 \times 10^{10}$ $M^{-1}$ $s^{-1}$ of the quenching rate constant ($K_q$) in the table 7, for all PC-OVA complexes suggest a static quenching mechanism (Annad et al., 2011). There are three types of mechanisms for fluorescence quenching: dynamic quenching, static quenching (Jahanban-Esfahlan & Panahi-Azar, 2016), and quenching including both dynamic and static (Chang et al., 2021), being predominantly dynamic quenching (Valeur & Berberan-Santos, 2012). This means that the linear Stern-Volmer plots are characteristics of static or dynamic quenching mechanisms and are not sufficient to define the quenching type (Lu et al., 2009). Otherwise, other factors, mainly temperature, and $K_{SV}$ values, disrupt the scattering quenching rate constant established as static, suggesting a dynamic quenching mechanism (Annad & Mkherjee, 2013; Wang et al., 2013; Wang et al., 2013; Sinisi et al., 2015; Müllertz, Perrie & Rades, 2016; Wang et al., 2017). From the results, is proposed a mixed quenching mechanism, because both static and dynamic mechanism are present. Moreover, the main reason for the quenching of tryptophan fluorescence, was assigned to static in nature however, dynamic quenching may also be found (Anand et al., 2011). In addition, dynamic and static quenching mechanism can be distinguished by the temperature dependence of the quenching. Furthermore, for the static quenching $K_{SV}$ and $K_q$ values decrease with an increase in temperature, and for dynamic quenching $K_{SV}$ and $K_q$ values increase with the temperature (Anand & Mukherjee, 2013). The obtained results showed that when temperature increased from 298.15 K to 318.15 K $K_{SV}$ and $K_q$ values increased for some

OVA-PC (CA, CHA, GA, QCT and RES) suggesting a dynamic quenching mechanism. In contrary, for FA and TA, $K_{SV}$ and $K_q$ decrease with the increment of temperature implying a static quenching mechanism. However, the increase of temperature from 318.15 K to 328.15 K, only promote the increase of KSV and Kq for CHA, QCT, RES and TA indicating a dynamic quenching mechanism (Jahanban-Esfahlan & Panahi-Azar, 2016) whereas, keep constant for CA and GA, and decrease for FA, which can be attributed to static mechanism (Table 7), that contribute to a complex formation (Chaves at al., 2018; Fu et al., 2020). Furthermore, the dynamic behavior may be owing to the destruction of the complex promoted by the kinetic increase in particle velocity or due to the action of temperature (Li & Yan, 2017). However, the $K_b$ results (Table 7) suggested that all PC bind to OVA and form stable complexes in 0.05 M phosphate buffer pH 7.40. Thus, we may be facing the case of a quenching including both dynamic and static processes for all OVA-PC interaction.

Kb values (Table 7) are in the range of $10^4$ $M^{-1}$, which correspond to the magnitude of the binding constant of PC to proteins (Wang et al., 2013). Thus, PC in low concentrations, at physiological pH, bind to OVA by non-covalent interactions of high affinity (Ognjenović et al., 2014). However, it was found that $K_b$ values for GA, increased with the increase of temperature from 298.15 to 318.15 and also for FA, GA and RES from 318.15 K to 328.15 K, (Table 7), which could be attributed to a great interaction and stability of the complex. On the other hand, $K_b$ values for CA, CHA, FA, RES and TA, decreased from 298.15 to 318.15 K. and from 318.15 to 328.15 K, decreased for CA, CHA, QCT and TA, what presumably was due to the instability of the complex (Table 7). The opposite causes the complex to stabilize (Lu et al., 2009).

In addition, the highest $K_b$ was found for OVA-TA at all temperatures, followed by OVA-FA and OVA-QCT. The smallest values of $K_b$ are observed for OVA-RES, almost at the three different temperatures, and also for GA at 328.15 K (Table 7). This could be also attributed to the lack of solubility of RES, as mentioned above. However, Chang et al. (2021) reported that one RES molecule could bind to only one OVA molecule, changing the conformation and microenvironment of OVA. Furthermore, this can be the reason of the aspect of the fluorescence spectra of OVA-RES (Figure 9 H-I).

Thus, in all cases there were interactions between OVA and PC. $K_b$ depends on interactions that include those considered to have a broad enthalpy contribution, such as van der Waals interactions, hydrogen bonds and electrostatic interactions, as well as entropy-dominated interactions (Chang et al., 2007; Olsson et al., 2008; Zhang et al., 2012; Jahanban-Esfahlan & Panahi-Azar, 2016).

Moreover, each type of PC affects the OVA microenvironment in a different way, depending on its structural characteristics and capacity to bind to the protein (Lu et al.,

2009; Wang et al., 2013). This fact may be related to the exposure of the OVA Trp residues after the PC binding and the establishment of hydrogen bonds in the complex with the consequent fluorescence quenching. It is important to refer that as described by other researchers (Wang et al., 2013; Fu et al., 2016) and also found in this work, some hydrogen bonds occurred exactly in or near the allergenic epitopes according to the docking results (Figure 17 A, B, O and P).

In consequence of all the interactions, complexes between all PC and OVA were formed, with $K_b$ in the order of magnitudes of $10^4$ $M^{-1}$. However, Li and Yan (2017) in their research, reported that the values of $K_b$ between ovalbumin and EGCG and QCT were in the range of $10^4$-$10^5$ $M^{-1}$. The same order of magnitude. were also obtained for the interaction of a polyphenol (theaflavin) with OVA through noncovalent bonds, such as hydrophobic interactions and hydrogen bonds (Wu et al. 2020).

The data resulting from the thermodynamic analysis allowed the understanding of the nature of the interaction forces. Thus, the thermodynamic parameters calculated for three different temperatures between all complexes OVA-PC tested, showed a negative $\Delta G$, meaning that are most favored thermodynamically (Table 8). $\Delta H$ were found negative for all OVA-PC except for OVA-GA. On the other hand, a positive $\Delta S$ was observed for almost all OVA-PC while for QCT and CA zero and negative, respectively (Table 8).

Therefore, exothermic and endothermic reactions occurred, as shown in the Van't Hoff equations of Table 9. In fact, for OVA-CA $\Delta H<0$ and $\Delta S<0$ showed that the formation of the respective complex will be only spontaneous at lower temperatures. Moreover, these results indicate that the binding processes are spontaneous ($\Delta G<0$) in all the cases, and enthalpy driven ($\Delta H<0$) to some complexes OVA-PC tested (Chang et al., 2007; Wang et al., 2013). Thereby, the main relationships amongst the forces involved in the interaction process, namely the enthalpy and entropy, and the type of interaction, are as follows (Wu et al., 2011): $\Delta H>0$ and $\Delta S>0$ (hydrophobic forces); $\Delta H<0$ and $\Delta S>0$ (electrostatic forces) and $\Delta H<0$ and $\Delta S<0$ (hydrogen bonding and van der Waals forces) (Wang et al., 2013). Thus, results suggested the involvement of electrostatic interactions, hydrophobic forces, hydrogen bonding and van der Waals forces, between OVA and PC (Yuan, Liu, Kang, Lv & Zou, 2008; Williams, 2013; Fu et al., 2016; Stănciuc, et al., 2016; Jahanban-Esfahlan & Panahi-Azar, 2016). It is important to highlight that GA presented positive $\Delta H$ and $\Delta S$ which corresponds to hydrophobic interactions. Many researchers showed strong interactions due to high stability of the complex OVA-PC (Kang, Liu, Xie, Li, Jiang, & Wang, 2004; Frazier et al., 2010; Wang et al., 2013) which is also confirmed in this work. As referred above, the binding analysis showed that OVA-PC complex formation was driven by enthalpy

(exothermic or endothermic reactions) and entropy variation (Wang et al., 2013). Huang et al. (2013), studied the interaction of RES with S-OVA, and observed that van der Waals forces and hydrogen bonds stabilized the binding, due to the negative values for ΔH, ΔS, and ΔG. The conformational changes of S-ovalbumin were mainly caused by the microenvironmental changes of Tyr and Trp residues (Huang et al., 2013).

Thermodynamic analysis showed that the formation of the OVA-PC complex was driven by ΔH (Lima et al., 2019). However, temperatures of 298.15, 318.15 and 328.15 K were considered for CA and TA, which presented significant values. In contrary, for other PC (CHA, FA, GA, QCT and RES) results for two temperatures (318.15 and 328.15 K) were significant.

The reason of complex formation of OVA-PC may be due to the various hydroxyl groups in these PC that could form hydrogen bonding, in addition to electrostatic and hydrophobic interactions (Jahanban-Esfahlan & Panahi-Azar, 2016). Furthermore, the non-covalent bonds formed are potentially reversible and have high energy (Zhang et al., 2012; Quan, Benjakul, Sae-leaw, Balange & Maqsood, 2019), promoting the stabilization of the complex.

It was suggested that hydrogen bonds formed between polar groups on the surface of the protein and hydroxyl groups of PC were owing by the negative values of variation of enthalpy and Gibbs energy, as observed in Table 8 (Wang et al., 2013; Jahanban-Esfahlan & Panahi-Azar, 2016; Fu et al., 2016). The lowest ΔG values obtained in the fluorescence were for OVA-TA (Table 8) which suggest that TA form stable complexes with the protein and can be tested in future studies for potential application in reducing of allergenic potential of OVA. The change in free energy (ΔG) itself is composed of enthalpy and entropy (equation 6), related with the heat given out or taken up upon making and breaking bonds (ΔH) and the energetic consequences of changes to the degree of order within the system (ΔS) (Olsson et al., 2008; Chang et al., 2007; Zhang et al., 2012). Thus, for those PC that ΔH values were negative, means that exothermic reactions occurred. The opposite is related with endothermic reactions. Positive entropy values suggested that the solvent surrounding molecules arranged themselves in a less orderly way when the interaction occurred (Stănciuc, et al., 2016).

From a thermodynamic point of view, the interactions in OVA-CA involved van der Waals forces or hydrogen bonds formation, since $\Delta S<0$ and $\Delta H<0$ (Stănciuc, et al., 2016). In turn, CHA FA, RES and TA, interact with OVA through electrostatic forces because $\Delta S>0$ and $\Delta H<0$. Finally, for GA is proposed hydrophobic interaction with OVA (Jahanban-Esfahlan & Panahi-Azar, 2016), since $\Delta S>0$ and $\Delta H>0$. However, in a previous study by a member of our group (Soares, 2015), at 310.15 K TA interact mainly by hydrophobic interactions, which can be a good prognosis to be changes in epitopes

of OVA, but only GA and TA promote changes (decrease) in β-sheet and β-turn, respectively.
In conclusion, OVA fluorescence decreased by increasing PC concentration, showing that significant changes in tertiary structure of OVA occurred. From the results, a mixed quenching mechanism was proposed, since both static and dynamic mechanism were present. Thermodynamic analysis suggested electrostatic, hydrophobic, hydrogen bonds and van der Waals interactions involved in the binding process.

### 5.2.2. Circular dichroism (CD)

The low value for NRMSD is a necessary but not sufficient condition for concluding that an analysis produced a good result. That is, if the NRMSD is high (>0.1) the correspondence of the calculated secondary structure with the actual one is unlikely to be 'correct' (Whitmore & Wallace, 2004).
Experimental data were analyzed by different algorithms, namely: SELCON3, CONTINLL, and CDSSTR that gave lower NRMSD values. High NRMSD values can arise from different/unusual features in the experimental spectrum compared with the standard spectra in the dataset. Thus, they can also indicate an error in concentration or cell pathlength measurements, resulting in an incorrect spectral magnitude and it may be useful to scale (with caution) the spectrum to compensate thereby reducing the NRMSD. Only the VARSLC algorithm does not provide a calculated spectrum or an NRMSD value (Miles, Ramalli & Wallace, 2021b).
The structure of OVA-PC complexes was analyzed and quantified by CD spectroscopy, since this technique can detect changes in secondary structure of the proteins (Pignataro et al., 2020; Miles et al., 2021). In brief, the results showed a decrease in α-helices and an increase in β-sheet and random coil content after the binding of PC to OVA (Tables 11 and 12; Figure 12 A-N), suggesting that OVA-PC interaction promote a conformational change in protein, which agrees with the results of fluorescence spectroscopy obtained by Zhou et al. (2019). The reduction of α-helix content and the increase in β-sheets lead to changes in the structure of OVA (Tables 11 and 12; Figure 12 A-N) (Jia et al., 2017). Moreover, it was reported that the interconversion of α-helix to β-sheet, depend on PC concentration, molecular weight, temperature and incubation time (Hu & Du, 2000; Hassan, 2013; Ognjenović et al., 2014; Jia et al., 2017; Pablos-Tanarro et al., 2017). The quantitative determination of secondary structure (Whitmore & Wallace, 2008) confirmed this observation since an increase in β-sheets and random coil content was observed for most of the OVA/PC molar ratios tested, as well as a decrease in α-helix structures (Kanakis et al., 2011; He et al., 2019). The increase in β-

sheets for TA is explained by the configuration of the TA molecule that has a high molecular weight, but in low concentrations it can bind effectively when interact with proteins (Tables 11 and 12) (Xie, Wehling, Ciftci, & Zhang, 2017).

The α-helix to β-sheets transition of OVA was induced by PC action which can promote denaturation of the protein, resulting in molecular aggregation and precipitation (Hu & Du, 2000; Jia et al., 2017). Nevertheless, in the case of CA, CHA, FA, GA, QCT and TA (Tables 11 and 12; Figure 12 A-N) for β-sheet there was an increase whilst α-helix structure decrease, which means a lack of stability for OVA (He et al., 2019).

In the CD analysis, at 298.15 K, there was an increase in the percentage of β-sheets in almost all cases, except for CA and RES. There was also an increase in random coils for all PC, as a consequence of the decrease in the α-helix (Table 11).

At 318.15 K, there was an increase in the percentage of β-sheets for almost all PC, except for the first lowest concentration of RES. In addition, an increase in the percentage of random coils was also observed in some cases, except for the lowest concentration of TA and the highest concentration of GA. So, this implies that significant changes occurred in the secondary structure of the OVA caused by interactions with PC.

It should be noted that there was not difference in the profile with changes in PC concentrations.

In addition, PC have different and high molecular weight. Other PC have low solubility in aqueous solutions. For those with solubility, the concentration was increased to see if more changes might occur to OVA.

Furthermore, there were conformational changes in each OVA-PC with a reduction in α-helix content according to the Tables 11-12 and a partial unfolding of OVA. This agrees with the conclusions of Jia et al. (2017) for other proteins, where CD data revealed that the increasing amount of PC concentration changed OVA secondary structure inducing α-helix to β-structures transition. Therefore, changes in the secondary structure of the OVA were caused by all PC and evidenced by CD analysis.

### 5.2.3. Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR)

The main information about the structural changes in proteins obtained by Infrared spectroscopy is contained in Amide I band, 1600-1700 $cm^{-1}$ (Wilcox, 2014; Jia et al., 2017). The amide I band is the result of C=O stretch vibrations from peptide bond and is more sensitive to the protein secondary structural components (Abrosimova et al., 2016), than to amide II band (Li & Hao, 2015; Zhang et al., 2012). Thus, the changes in

spectra were investigated mainly in the Amide I band. Absorption bands associated with C=O bond stretching are usually very strong because a large change in the dipole takes place in that mode (Baxter et al., 2008; Amenabar et al., 2013).

The results showed that all the PC influenced the ATR-FTIR profile of amide I band of OVA, because changes were observed in the maximum wavenumber of OVA-PC complex at several temperatures, some complexes increase the wavenumber, other decreased, and another kept constant (Figure 13 A, B and C). However, the amide I band curve fitting, allowed to obtain peaks correspondent to following secondary structure components: α-helix, β-sheet, turn and random coil structures (Figure 14) (Abrosimova et al., 2016; Milošević et al., 2020). Furthermore, the outcomes (Figure 14 A-X, and Table 14) support the results obtained by Zhang et al. (2012) and Milošević et al. (2020), attributed the following values to amide I band components: 1615–1637 $cm^{-1}$ to β-sheet, 1638–1648 $cm^{-1}$ to random coil, 1649–1660 $cm^{-1}$ to α-helix, 1660–1680 $cm^{-1}$, and 1680–1692 $cm^{-1}$ to β-turn structures and, usually, major values from 1680-1692 $cm^{-1}$ are assigned also to β-antiparallel and β-sheets, respectively (Table 14). The characteristic band for random coil conformation is located at 1648 ± 2 $cm^{-1}$ (Yang et al., 2015; Milošević et al., 2020).

Regarding table 14, there are changes in the number of peaks of OVA-PC ATR-FTIR spectra resulting from the action of all tested PC. However, in FTIR, irregular structures are not often resolved at the boundaries of the broad amide I band of the experimentally obtained spectra. There are empirically determined guidelines for the secondary structure of proteins based on their characteristic amide I frequencies (Abrosimova et al., 2016; Milošević et al., 2020). It was observed that the α-helix and irregular structures have very close bands and peaks (1649-1660 $cm^{-1}$), which complicates the analysis. On the other hand, amide groups in β-sheet structures give rise to major diagnostic bands between 1611 and 1638 cm-1 and weaker bands around 1672-1698 $cm^{-1}$. For example, for TA, the percentage of the α-helix, β-sheets, 3β-turns and random coil in FTIR which assigned to respective peaks, when applied curve-fitting and area analysis, presented the following wavenumbers: 1624 $cm^{-1}$, 1639 $cm^{-1}$, 1653 $cm^{-1}$, 1668 $cm^{-1}$ and 1685 $cm^{-1}$, from those areas derived the information about secondary structure of OVA (Figure 14 A-X; Table 14). However, these percentages were not like those obtained by CD not only due to the different sensitivity and specificity of these techniques o but also because of the different experimental conditions used to obtain the spectra (Fellows et al., 2020).

In a recent study OVA–QCT conjugates were compared with native OVA, and it was observed that the peak positions of the amide I band moved from 1653.08 $cm^{-1}$ to 1657.05 $cm^{-1}$ and 1651.90 $cm^{-1}$, respectively (Zhang et al., 2020), depending on the

experimental conditions. In the present study it was also observed that peaks position of complex formed OVA-PC moved when compared the native OVA.

The secondary structure dependence on the amide I band arises from couplings between neighboring and intramolecular amide groups. Moreover, β-sheets in the OVA treated with PC are more evidenced than observed in native protein (free OVA). The wavenumbers presented in Table 13, reflect differences in transition of α-helices to β-sheets, and to random coil as well as to β-turns. These facts can be attributed to changes that occurred in the environment of the complex and guaranteed the stability of the molecule. It means that hydrogen bond, can offer larger strength to α-helix due the existence of C=O group in the same strand of the molecule. According to the Figure 14 A-X, and Table 14, at 298.15 K there was an increase in the β-turns of almost all PC, except the QCT. The random coil also increased except for CHA and QCT. This increase occurred at the expense of the decrease in the α-helix. Therefore, at 318.15 K, PC largely affected the secondary structure, thus inter and intramolecular hydrogen bonds lead to β-sheet increases and α-helix decreases, respectively. As observed for the three temperatures, β-turns increase corresponding to the small secondary pattern (Figure 14 A-X, and Table 14) in proteins and cannot be possible to ignore its contributions for the stability of protein. Thus, this suggested that OVA binds strongly to PC and unfold. As reported by Kavitha & Palaniappan (2021) the content in β-turns and random coil gives additional support to the protein stability.

The peaks from second derivative suggest that most of the OVA-PC complexes presented α-helix and mainly β-turn structures (Figure 15, and Table 15), according to the correspondence of the peaks to secondary structure contents, as found in recent studies (Abrosimova et al., 2016; Milošević et al., 2020). Moreover, it was reported that second derivative spectra can be used as fingerprints of changes in OVA conformation (Sęczyk et al., 2019).

Although CD and FTIR-ATR have the objective of analyzing the alterations in the secondary structure of the proteins, the comparison of the results must be done with care, because since they have different sensitivity and were carried out under different conditions. However, in both, there was a significative increase in beta sheets, due to the decrease in alpha helices, mainly at temperatures of 298.15 and 318.15 K. This also translates into a significant change in the secondary structure of the protein.

### 5.2.4. Molecular docking

Molecular docking is a powerful tool to explore the interactions among small molecules and macromolecules (Wu et al., 2017, Meng, Zhang, Mezei & Cui, 2011). The docking methods can estimate interactions based on energy, namely Gibbs free energy. Thus, the docking technique is used to predict the provisional binding parameters of the ligand-receptor complex and the complex stability (Dar & Mir, 2017).

Docking can be achieved through two steps linked itself: first using sample conformations of the ligand in the active site of the protein; then ranking these conformations via a scoring function. However, the goal of the scoring function is to define correct poses from incorrect poses, or inactive compound binders in a feasible computation time. Therefore, scoring functions involve estimating rather than calculating the binding affinity between the protein and the ligand. Different scoring functions can be considered, namely force-field-based, empirical, and knowledge-based scoring functions (Meng et al., 2011; Dar & Mir, 2017).

In the present study, the most favorable PC-OVA binding mode was chosen based on the lowest binding energy. Thus, in silico calculations demonstrated that all PC bind to OVA surface in different locations with low binding energies. Moreover, some of these locations are in epitopes or its neighborhood. Furthermore, one predicted affinity binding site are located near Trp160 (Figure 17 A-P) that is solvent exposed and located on the most dynamic secondary structure element in the serpins, from which OVA belongs and is often used to monitor conformational and structural changes (Mine & Rupa, 2003). These estimated negatives $\Delta G$, suggested that were mainly originated by the hydrogen bonds, and van der Waals and electrostatic contributions as reported by Wang, Dadmohammadi, Jaiswal, & Abbaspourrad (2020). Mine and Rupa (2003) found five distinct regions with dominant allergenic IgE epitopes, such as: L38T49, D95A102, E191V200, V243E248 and G251N260. However, TA binding near this Trp160 (Figure 17 A-P) could explain why fluorescence quenching effects and changes in CD and FTIR spectra were observed when PC were added to the OVA. Observing the findings of Mine and Rupa (2003) and Benedé et al. (2014), the existent epitopes studied by them, are presumably close to the sequences suggested by the in silico analysis, in the four PC highlighted with the sequences of some PC, positioned close to Trp160. QCT interact with the epitope with Leu260 residue and interact with other residues out of the epitope such as Val240 and Phe385. The interactions with these residues are stabilized through hydrogen bonds length 2.589 Å, 1.90 Å and 2.182 Å respectively, whilst in this situation Trp275 is solution exposed (Figure 17 E and F). Moreover, TA interacts through the hydrogen bonds formed between its OH groups and OH of Ser111, and carbonyl oxygens of peptide bonds of Phe112, Ser113, Leu114 and

Ala115. The interaction with Ser111 is stabilized by hydrogen bonds length 2.317 Å nearby Ala115 and Tyr110 besides the solution exposed Trp160 (Figure 17 C). This is important for predicting and understanding the changes in OVA-PC fluorescence, CD and ATR-FTIR spectra.

From the conclusions of the in silico simulations, it has been found that PC (CA, CHA, QCT and TA) bind directly to OVA epitopes (Table 16) at least by hydrogen bonds that are presumed to be high affinity. However, FA and RES bind closely to the amino acid sequence that corresponds to the epitope, and GA also, bind closely, linked hydrogen bonds with Thr211 residues. Hence, it was possible to conclude that estimated ΔG contributed to the interaction of OVA-PC. Together with the thermodynamic results, hydrophobic, electrostatic and van der Waals interactions should be also considered, such as verified by Wang et al. (2020), which confirm the interactions between PC and OVA reported in this work. Therefore, docking studies showed that the tested PC can interact directly with OVA epitopes, or with its neighbors, thus avoiding the IgE binding.

## 5.3. Concluding remarks and future trends

Many efforts have been made to reduce food allergy at all levels, using thermal and non-thermal methods, however these treatments have not been fully effective due to the high resistance of the allergens. Egg allergy has been causing a series of health problems in children, adolescents and, in some cases in adults, reaching worldwide and being considered a public health problem.

It is known that there is a limitation regarding treatment, which leads health professionals to advise avoiding the consumption of allergenic foods.

Currently, PC are considered possible tools to achieve the reduction of egg allergenicity due to their ability to bind to proteins, besides the antioxidant activity. Many studies report the interaction between PC and proteins, both covalently and non-covalently. Such interactions are strong or weak, and reversible or irreversible and may lead to the formation of complexes that in turn alter the native conformation of the protein, and at the same time decrease or even eliminate, the allergenic activity of IgE.

Therefore, the present doctoral work aimed to develop a strategy to reduce allergenicity to hen's eggs by using natural antioxidants such as PC. Although there are already previous studies in which the interactions between a single PC and OVA were analyzed, in this work seven different PC were studied in order to analyze and compare their effect on the alteration of the OVA structure. Thus, CA, CHA, FA, GA, TA, QCT and RES

were tested for binding to OVA, the most common and abundant protein in hen's eggs, with the aim of using it in the future to reduce egg allergenicity.

OVA was incubated with each PC and the solutions were analyzed applying spectroscopic techniques, such as Fluorescence, CD, and ATR-FTIR to analyses the changes in its tertiary and secondary structure. Thus, with CD, showing an increase of β-sheets and a decrease of α-helix. This phenomenon occurred because OVA unfold and lose its stability. ATR-FTIR also confirmed the changes at the secondary structure of OVA in result of interactions with PC. These results lead to assumption that changes occurred in the secondary structure of the protein and PC bound to the epitopes or overlap with it, being able to inhibit the IgE-binding and concomitantly reducing the allergenicity.

The Fluorescence of OVA solutions with PC decreased when the concentration of PC increased. Moreover, the results confirm that tertiary structural changes occurred in result of OVA-PC interactions, and the nature of the favored-thermodynamically, interactions were hydrogen bonds, and electrostatic interactions. The quenching mechanism calculated from $K_{SV}$ and $K_q$ was higher than the rate value established, which suggested static quenching mechanism, according to other studies. However, when considering the effect of temperature (298.15 to 318.15 K and 318.15 to 328.15 K) on fluorescence quenching, it was found that $K_{SV}$ and $K_q$ decreased when temperature increased for some OVA-PC, leading to a dynamic quenching mechanism. On the other hand, for some OVA-PC complexes, $K_{SV}$ and $K_q$ increased with increasing temperature, and other kept constant, suggesting respectively, a static quenching mechanism and a mixed quenching mechanism (static and dynamic).

The same effect was observed for $K_b$, with temperature change. Furthermore, the thermodynamic analysis according to the values found for ΔH and ΔS for all tested OVA-PC complexes, at both temperatures, suggested that the reactions occurred spontaneously with the participation of weak interactions. Therefore, the use of different spectroscopic methods for estimate protein secondary and tertiary structure supports the goal of the present research, in which the non-covalent interaction of OVA with PC occurs. In addition, the diversity of ways in which interactions between PC and OVA occur at the different temperatures is highlighted. Changes in the secondary structure of the OVA were caused by all PC and evidenced by CD and FTIR-ATR analysis. On the other hand, docking studies showed that the tested PC could interact directly with OVA epitopes, or with its neighbors, thus avoiding the IgE binding.

All PC can bind to OVA, but TA was the one that binds strongly, according to the results. So, TA was the most promising PC and can be used in egg foods potentially to decrease egg allergenicity. Information obtained from OVA-PC interactions by

fluorescence spectrometry, CD, ATR-FTIR and molecular docking are essential to study and minimize food allergy.

As future work, it is necessary to carried out immunoblotting and ELISA assays with sera from allergic patients to confirm the decrease in allergenicity of OVA treated with PC. Thus, the levels of IgE-specific that binds to OVA treated and non-treated with PC will be compared. Subsequently, *in vivo* reduction or elimination of allergenicity will be evaluated by skin prick tests in allergic patients using OVA and whole egg, treated with PC.

As future perspectives the great trend is to provide in large scale the production of hen egg treated by PC, to guarantee the production of hypoallergenic whole eggs and egg white, to help who suffer with hen egg allergy, since egg is a ubiquitous food. In addition, PC studies with OVA should be the starting point for mitigating allergy to several other allergenic foods.

The application of PC in the treatment of egg proteins, thus appears as a potential alternative to the current methods used to reduce the allergenicity of egg proteins.

Additional research is necessary to obtain hypoallergenic egg products. The study of whole egg powder with PC covalent bonds, in the same conditions of temperature and incubation time, must be performed to design novel strategies for promoting reduction of egg allergy.

# Chapter 6

# 6. References

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