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Atmospheric Cold Plasma Interaction with Allergens in Food Processing

By

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Table of Contents

Table of Contents I

Abstract VIII

Statement of Original Authorship XI

Acknowledgement XII

List of Tables XIII

List of Figures XVII

List of Appendices XXVIII

Abbreviations XXXI

Chapter 1 Introduction 1

1.1 Scope of the project 1

1.1.1 Research questions and objectives 2

1.2 Atmospheric cold plasma (ACP) 5

1.2.1 Types of plasma sources 6

1.2.2 Chemical reactions of ACP 6

1.2.3 Plasma activated liquids 12

1.2.3.1 pH 13

1.2.3.2 Redox potential 14

1.2.3.3 Conductivity 15

1.2.4 Measurement of ACP effectors 16

1.2.5 Application of ACP for microbial decontamination 19

1.2.6 Application of ACP for protein inactivation 21

1.2.7 Effect of ACP on food allergens 23

1.3 Food Allergen Safety, Advisory and Industry Environment 27

1.4 Food Allergy Review 30

1.4.1 Food Allergy and Intolerance 33

1.4.2 Food Allergens 37

1.4.2.1 Milk and Milk Allergens 41

1.4.2.2 Wheat and Wheat Allergens 43

1.5 Food Allergen Management in Food Industry 45

1.5.1 Allergens in Food Supply Chain 45

1.5.2 Centralised Food Processing Units for Allergen Management... 49

1.5.3 Food Industry Regulation for Allergen Management 50

1.5.4 Allergen Control Systems 52

1.5.5 Allergen Labelling 56

1.5.6 Allergen Cleaning and Removal 56

1.5.7 Food Allergen Analytical Methods 60

1.6 Current Technology Interactions with Allergens 62

1.6.1 Thermal Processing 62

1.6.2 Enzymatic Hydrolysis 64

1.6.3 High hydrostatic pressure 66

1.6.4 High intensity ultrasound 68

1.6.5 Gamma irradiation 70

1.6.6 Ultraviolet light 71

Chapter 2 Methods and Materials 73

2.1 Plasma setup and diagnostics 73

2.1.1 RSS set-up 73

2.1.1.1 ACP treatment of samples using RSS system 74

2.1.2 In-package DBD set-up... 75

2.1.2.1 ACP treatment of samples using DBD system 76

2.1.3 PB set-up 76

2.1.3.1 ACP treatment of samples using PB system 77

2.1.4 Plasma Diagnostics 77

2.1.4.1 Measurement of Voltage and Current 77

2.1.4.2 Measurement of Temperature 77

2.1.4.3 Measurement of pH 78

2.1.4.4 Measurement of Conductivity 78

2.1.4.5 Measurement of Redox Potential 78

2.1.4.6 Measurement of reactive oxygen species concentrations ... 78

2.1.4.7 Measurement of reactive nitrogen species concentrations.. 79

2.1.4.8 Fast Imaging Techniques 80

2.2 Characterising protein properties 80

2.2.1 Preparation of milk and wheat proteins 80

2.2.2 Preparation of protein-fouled ss coupon 81

2.2.3 Protein extraction after plasma treatment 81

2.2.3.1 Protein extraction of milk and wheat proteins 81

2.2.4 SDS-PAGE analysis 82

2.2.4.1 Measurement of protein concentrations 82

2.2.4.2 Preparation of SDS-PAGE samples 82

2.2.4.3 Preparation of gel 83

2.2.4.4 Preparation of electrophoresis 83

2.2.5 Secondary structure determination 84

2.2.6 Amino acid profile analysis 85

2.2.7 Enzyme-linked immunosorbent assay (ELISA) 86

2.2.7.1 Sandwich ELISA of Rbiopharm ®IDASCREEN 87

2.2.7.2 Sandwich ELISA of ELISAGenie 87

2.3 Sample preparation for PAW treatment study 88

2.4 Statistical analysis 89

Chapter 3 Physicochemical properties of PAW generated by spark and glow (RSS discharges in air) 90

3.1 Experimental Design 90

3.2 Results and Discussion 92

3.2.1 Characteristics of electrical discharges above the ultrapure water 92

3.2.2 Thermal, chemical and electrical characteristics of SDPAW and GDPAW 95

3.2.3 Selective generation of hydrogen peroxide, nitrate and nitrite . 101

3.3 Conclusion 107

Chapter 4 Plasma diagnostics of spark and glow (RSS discharges in air)108

4.1 Experimental Design 108

4.2 Results and Discussion 110

4.2.1 Formation of RONS in the liquid phase 110

4.2.2 Time resolved discharge imaging 113

4.2.3 Wavelength resolved discharge imaging 115

4.3 Conclusion 120

Chapter 5 Effect of direct RSS plasma treatment on the antigenicity of milk and wheat proteins 122

5.1 Experimental Design 122

5.2 Results and Discussion 125

5.2.1 Characteristics of SD and GD plasma treatment 125

5.2.2 SDS-PAGE analysis 129

5.2.3 Secondary structural analysis 136

5.2.4 Amino acid profile analysis 141

5.2.5 Antigenicity analysis 150

5.3 Conclusion 156

Chapter 6 Effect of indirect treatment using PAW on bovine casein antigenicity 158

6.1 Experimental Design 158

6.2 Results and Discussion 160

6.2.1 Chemical composition in SDPAW and GDPAW after 10 min treatment time 160

6.2.2 Effect of PAW treatment on casein antigenicity in combination with mild heat treatment with respect to casein concentrations 161

6.2.3 Effect of PAW and PAW-mimicking treatment on casein antigenicity 164

6.3 Conclusion 168

Chapter 7 Effect of DBD in-package plasma treatment on the antigenicity of milk and wheat proteins 169

7.1 Experimental Design 169

7.2 Results and Discussion 171

7.2.1 SDS-PAGE analysis 171

7.2.2 Secondary structural analysis 177

7.2.3 Amino acid profile analysis 181

7.2.4 Antigenicity analysis 188

7.3 Conclusion 194

Chapter 8 Effect of direct surface treatment using plasma brush on the antigenicity of milk and wheat proteins 196

8.1 Experimental Design 196

8.2 Results and Discussion 198

8.2.1 SDS-PAGE analysis 198

8.2.2 Secondary structural analysis 203

8.2.3 Amino acid profile analysis 206

8.2.4 Antigenicity analysis 214

8.3 Conclusion 220

Chapter 9 Overall discussion, conclusions, and future recommendations 222

9.1 Summary and major findings 222

9.2 Conclusions 240

9.3 Future recommendations 242

Bibliography 245

List of Publications 286

Conferences 288

Appendices 290

Abstract

Approximately 5-15% of allergen recalls are associated with consumer reactions, which are largely attributed to cross contamination with allergens. Therefore, it is essential to mitigate against allergen cross-contamination during food processing, distribution and storage.

Atmospheric Cold plasma (ACP) was found to effect functionality and modify proteins such as enzymes, owing to the chemical and bioactive radicals generated known collectively as reactive oxygen (ROS) and nitrogen species (RONS), together with physical stress such as electric field and UV irradiation. Allergens are comprised of proteins; thus, ACP was investigated as a promising tool to mitigate allergen cross-contamination in food processing sectors.

The aim of this work is to understand and develop ACP based approaches for control and prevention of allergen cross-contamination within food processing. This program of research focused on developing a mechanistic understanding of how ACP interacts with food allergens and affects the antigenicity of food allergens. RSS systems, which comprised two modes of tunable plasma discharge, spark discharge (SD) and glow discharge (GD), were used as plasma source for the liquid application environment. To optimise the efficacy of application, diagnostics studies of the plasma devices and the chemical composition of plasma activated water (PAW) generated by RSS system were investigated. SDPAW predominantly contains H_2O_2 and NO_3^-. GDPAW predominantly contains peroxide, NO_2^- and NO_3^-. The concentration of ROS and RNS was dependent on applied voltage and frequency of the plasma.

To understand the mechanism of plasma in gas-phase, the ignition and propagation of air discharges of SD and GD were examined using fast imaging

diagnostic techniques in collaboration with the University of Liverpool. SD drives both anode-directed and cathode-directed streamers, while GD drives only anode directed streamers. SD had higher OH emission than GD at both positive and negative half-periods.

The effect of SD and GD on the antigenicity reduction of milk-derived and wheat-derived allergens were then studied. Casein, α-lactalbumin and β-lactoglobulin are the major allergens in bovine milk while gliadin is the major allergen in wheat gluten. These allergen solutions were treated directly by SD and GD. The antigenicity of caseins, α-lactalbumin and gliadin decreased while β-lactoglobulin increased. The results showed the modification of chemical structure of plasma treated allergens. A concentration dependence also emerged across the different studies. For example, indirect PAW treatment reduced casein antigenicity but only at low concentrations of 0.01 mg/ml, when applied in combination with a mild heat treatment at 60 °C. The ROS generated in SDPAW is attributed to antigenicity reduction of casein, while RNS generated in either SDPAW or GDPAW was not involved.

The mode of application was probed, where liquid mediated direct SD and GD RSS were compared with indirect PAW treatment, and further compared to direct gaseous dry plasma approaches of dielectric barrier discharge (DBD) in-package system and plasma brush (PB) system. The antigenicity reduction of milk-derived and wheat-derived allergens were studied in terms of sample concentrations and plasma process duration. The results showed the conformational and linear epitopes of plasma treated allergens were altered. The efficacy for antigenicity reduction is correlated with protein composition, sample concentration and plasma process duration.

Overall, dry or liquid mediated plasma processes can be tailored to mitigate allergen residues in food processing. The food allergens from two important foods were investigated to reveal the universality of ACP. The effectiveness of antigenicity reduction was well explained by the modification of conformational and linear structures of allergens treated by ACP.

Statement of Original Authorship

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the Title Page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

Sing Wei Ng **Date:** 2 August 2022

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List of Tables

Table 1.1 Principles of different cold plasma generating devices. Adapted from Ng, Tsoukou, Chaple, Boehm, and Bourke (2021). 7

Table 1.2 Chemical reactions during plasma-water interactions resulting in the formation of RONS. Adapted from Ng et al. (2021). 9

Table 1.3 Detection methods for liquid phase reactive species. Adapted from Ng et al. (2021). 18

Table 1.4 Summary of the previously conducted research on allergen mitigation by atmospheric cold plasma. 23

Table 1.5 Lifetime and point prevalence of food allergy for all ages (self-reported). Adapted from Nwaru et al. (2014). 30

Table 1.6 Prevalence estimates for probable food allergy by age group in the United States and Canada (self-reported). Adapted from Soller et al. (2015). 31

Table 1.7 Eliciting doses (ED) for common allergens causing allergies or intolerances. Adapted from Commission (2011); Kupińska-Adamczyk et al. (2018). 32

Table 1.8 Molecular weights of milk allergens. Adapted from Verhoeckx et al. (2015). 42

Table 1.9 Wheat allergens. Adapted from EFSA Panel on Dietetic Products and Allergies (2014). 44

Table 5.1 Temperature, volume and pH value of milk protein solutions collected from untreated, and SD and GD treated samples. 128

Table 5.2 Secondary structures of SD and GD treated caseins and whey proteins at different treatment times (10, 20, 30 min). 138

Table 5.3 Amino acid profile in untreated and treated caseins by SD and GD at different treatment times (10, 20, 30 min) (g amino acid/100 g protein) 143

Table 5.4 Amino acid profile in untreated and treated whey proteins by SD and GD at different treatment times (10, 20, 30 min) (g amino acid/100 g protein) 144

Table 5.5 Amino acid profile in untreated and treated glutens by SD with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein). 147

Table 5.6 Amino acid profile in untreated and treated glutens by GD with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein). 149

Table 7.1 Amino acid profile in untreated and treated casein proteins by DBD with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (g amino acid/100 g protein) 182

Table 7.2 Amino acid profiles in untreated and treated whey proteins by DBD with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration

(0.2 mg/ml); LC: low concentration (0.02 mg/ml) (g amino acid/100 g protein) 185

Table 7.3 Amino acid profile in untreated and treated glutens by DBD with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein) 187

Table 8.1 Amino acid profile in untreated and treated casein proteins by PB with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (g amino acid/100 g protein)... 208

Table 8.2 Amino acid profile in untreated and treated whey proteins by PB with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (g amino acid/100 g protein)... 211

Table 8.3 Amino acid profile in untreated and treated glutens by PB with different protein concentrations (10, 1, 0.1 mg/ml) at 10 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein)..... 213

Table 9.1 Relative susceptibility of amino acids of casein resulting from different plasma treatments in descending order... 232

Table 9.2 Relative susceptibility of amino acids of whey resulting from different plasma treatments in descending order 233

Table 9.3 Relative susceptibility of amino acids of gluten resulting from different plasma treatments in descending order 234

Table 9.4 Relative susceptibility of amino acids of different proteins (casein, whey and gluten) resulting from spark discharge treatment in descending order 235

Table 9.5 Relative susceptibility of amino acids of different proteins (casein, whey and gluten) resulting from glow discharge treatment in descending order 236

Table 9.6 Relative susceptibility of amino acids of different proteins (casein, whey and gluten) resulting from dielectric barrier discharge plasma treatment in descending order 237

Table 9.7 Relative susceptibility of amino acids of different proteins (casein, whey and gluten) resulting from plasma brush treatment in descending order 238

List of Figures

Figure 1.1 Chemical pathway of RONS generation from gas phase to liquid phase. Adapted from Renwu Zhou et al. (2018). 11

Figure 1.2 Classification of adverse reactions to food. 35

Figure 1.3 Food supply chain model. Adapted from Wallace et la. (2018). ... 45

Figure 2.1 Schematic of RSS system: spark discharge and glow discharge. 74

Figure 2.2 Schematic of DBD system. 75

Figure 2.3 PB system adapted from https://www.relyon-plasma.com/relyon-plasma-produkte/plasmabrush-pb3/?lang=en. 76

Figure 3.1 Liquid-phase plasma diagnostics of SD and GD – experimental design. 91

Figure 3.2 Applied voltage waveforms of spark discharges (SDs) with different applied voltages and glow discharges (GDs) with different applied frequencies. (A) SD applied with 6 kV, (B) SD applied with 7.5 kV, (C) SD applied with 9 kV, (D) SD from Lu et al. (2017), (E) GD applied with 30 kHz, (F) GD applied with 27.5 kHz (G) GD applied with 25 kHz, (H) GD from previous literature. 94

Figure 3.3 Properties of SDPAWs generated by SD with different voltages of 6, 7.5, 9 kV and GDPAWs generated by GD with different frequencies of 25, 27.5, 30 kHz. Temperatures of (A) SDPAW and (B) GDPAW. pH of (C) SDPAW and (D) GDPAW. ORPs of (E) SDPAW and (F) GDPAW. Conductivities of (G) SDPAW and (H) GDPAW. *slope is presented in unit of µSmin^{-1} in legend of (G) and (H). (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 97

Figure 3.4 Thermal images of SD operated above ultrapure water applied with 9 kV and 25 kHz for (A) 0 min, (B) 1 min, (C) 3 min, (D) 5 min, (E) 7 min, (F) 10 min, (G) 15 min, (H) 20 min, (I) 25 min, (J) 30 min. *Bx1: temperature of SDPAW, Sp1: environmental temperature. (K) Average temperatures of SDPAW. 99

Figure 3.5 Thermal images of GD operated above ultrapure water applied with 5 kV and 25 kHz for (A) 0 min, (B) 1 min, (C) 3 min, (D) 5 min, (E) 7 min, (F) 10 min, (G) 15 min, (H) 20 min, (I) 25 min, (J) 30 min. *Bx1: temperature of GDPAW, Sp1: environmental temperature. (K) Average temperatures of GDPAW.... 100

Figure 3.6 Chemical compositions of SDPAW with different voltages of 6, 7.5, 9 kV and GDPAW with different frequencies of 25, 27.5, 30 kHz. Hydrogen peroxide concentrations in (A) SDPAW and (B) GDPAW measured by $TiOSO_4$, peroxides concentrations in (C) SDPAW and (D) GDPAW measured by buffered KI, nitrate concentrations in (E) SDPAW and (F) GDPAW, nitrite concentrations in (G) SDPAW and (H) GDPAW. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 104

Figure 3.7 the ratio of H_2O_2 to peroxides concentrations in SDPAW with different voltages of 6, 7.5, 9 kV for up to 30 min treatment time. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 105

Figure 4.1 Gas-phase plasma diagnostics of spark discharge (SD) and glow discharge (GD) – experimental design. 109

Figure 4.2 Concentration plots of H_2O_2, NO_2^-, and NO_3^- in plasma activated water (PAW) produced by (a) GD and (b) SD. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 111

Figure 4.3 UV absorbance spectrum of GDPAW, SDPAW, 1 mM H_2O_2, 1 mM HNO_2, and 5 mM HNO_3. 113

Figure 4.4 Time resolved fast iCCD imaging to resolve the entire plasma period using 500ns steps when the system is in GD (a) and SD (b). The distance was measured from the tip of the needle to the liquid surface. 115

Figure 4.5 Species-resolved images of GD and SD at positive and negative half- periods through narrow bandpass filters of 310 nm, 340nm, 390 nm, and 780 nm for emissions from OH, N_2 (SPS), N_2^+ (FNS) and atomic O, respectively. The upper red line indicates the location of the tip of HV needle while the lower red line indicates the location of the water surface. 119

Figure 5.1 RSS treatment of casein and whey proteins – experimental design. 122

Figure 5.2 RSS treatment of wheat gluten proteins – experimental design. 124

Figure 5.3 Applied voltage and current waveforms of (a) spark discharge (SD) and (b) glow discharge (GD).. 126

Figure 5.4 SDS-PAGE profiles of untreated; (a) SD and (b) GD treated caseins; (c) SD and (d) GD treated whey proteins subjected to different exposure times. M: the standard protein marker. Lane 1-4: control and samples treated for 10, 20, 30 min, respectively, under non-reducing condition. Lane 5-8: control and samples treated for 10, 20, 30 min, respectively, under reducing condition. 133

Figure 5.5 SDS-PAGE profiles of untreated; (a) SD and (b) GD treated glutens subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Gluten control. Lane 3-4: high (10 mg/ml) and medium (1 mg/ml) protein concentrations, respectively, treated for 30 min, respectively. Lane 5-7: low (0.1 mg/ml) protein concentrations treated for 30, 20, 10 min, respectively........ 135

Figure 5.6 Secondary structures of SD treated gluten proteins (a) with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time (b) with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 139

Figure 5.7 Secondary structures of GD treated gluten proteins (a) with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time (b) with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 140

Figure 5.8 ELISA responses of casein subjected to different (a) SD and (b) GD treatment times against IgE binding activity (%). ELISA responses of α-lactalbumin (ALA) subjected to different (c) SD and (d) GD treatment times against IgG binding activity (%). ELISA responses of β-lactoglobulin (BLG) subjected to different (e) SD and (f) GD treatment times against IgE binding activity (%). 152

Figure 5.9 ELISA responses of gliadin subjected to SD treatment against IgE binding activity (%). SD treated gluten proteins with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time and with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml). 154

Figure 5.10 ELISA responses of gliadin subjected to GD treatment against IgE binding activity (%). SD treated gluten proteins with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time and with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) 155

Figure 6.1 PAW treatment of milk casein – experimental design................. 159

Figure 6.2 Chemical composition of SDPAW and GDPAW treated for 10 min. 161

Figure 6.3 Bovine casein antigenic responses to SDPAW and GDPAW with different casein concentrations of 1, 0.1 and 0.01 mg/ml under conditions of 20 °C. 163

Figure 6.4 Bovine casein antigenic responses to SDPAW and GDPAW with different casein concentrations of 1, 0.1 and 0.01 mg/ml under conditions of 60 °C. 164

Figure 6.5 Bovine casein antigenic responses to SDPAW and GDPAW under conditions of (A) 20 °C and (B) 60 °C for contact treatment times from day 0 to day 5. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))..................... 165

Figure 6.6 Bovine casein antigenic responses to PAW and RONS mimicking solutions at 60 °C. (A) effect of SDPAW and its mimicking solution on bovine casein antigenicity, (B) effect of GDPAW and its mimicking solutions on bovine casein antigenicity. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 167

Figure 6.7 RONS concentrations of (A) SDPAW and (B) GDPAW during casein-PAW reaction at 60 °C. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 167

Figure 7.1 DBD treatment of casein, whey and gluten proteins – experimental design. 170

Figure 7.2 SDS-PAGE profiles of untreated and DBD treated caseins subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Casein control. Lane 3-4: high (2 mg/ml) and medium (0.2 mg/ml) protein concentrations, respectively, treated for 30 min. Lane 5-7: low (0.02 mg/ml) protein concentrations treated for 30, 20, 10 min, respectively. 173

Figure 7.3 SDS-PAGE profiles of untreated and DBD treated whey proteins subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Whey control. Lane 3-4: high (2 mg/ml) and medium (0.2 mg/ml) protein concentrations, respectively, treated for 30 min. Lane 5-7: low (0.02 mg/ml) protein concentrations treated for 30, 20, 10 min, respectively. 175

Figure 7.4 SDS-PAGE profiles of untreated and DBD treated glutens subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Gluten control. Lane 3-4: high (10 mg/ml) and medium (1 mg/ml) protein concentrations, respectively, treated for 30 min. Lane 5-7: low (0.1 mg/ml) protein concentrations treated for 30, 20, 10 min, respectively. 176

Figure 7.5 Secondary structures of DBD treated casein proteins (a) with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time (b) with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 178

Figure 7.6 Secondary structures of DBD treated whey proteins (a) with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time (b) with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 179

Figure 7.7 Secondary structures of DBD treated gluten proteins (a) with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time (b) with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 180

Figure 7.8 ELISA responses of casein subjected to DBD treatment against IgE binding activity (%). DBD treated casein proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml)... 189

Figure 7.9 ELISA responses of α-lactalbumin (ALA) subjected to DBD treatment against IgG binding activity (%). DBD treated whey proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) 190

Figure 7.10 ELISA responses of β-lactoglobulin (BLG) subjected to DBD treatment against IgE binding activity (%). DBD treated whey proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) 192

Figure 7.11 ELISA responses of gliadin subjected to DBD treatment against IgE binding activity (%). DBD treated gluten proteins with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time and with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) 193

Figure 8.1 PB treatment of casein, whey, and gluten proteins – experimental design. 197

Figure 8.2 SDS-PAGE profiles of untreated and PB treated caseins subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Casein control. Lane 3-4: high (2 mg/ml) and medium (0.2 mg/ml) protein concentrations treated for 10 min, respectively. Lane 5-9: low (0.02 mg/ml) protein concentrations treated for 10, 5, 2, 1, 0.5 min, respectively. 199

Figure 8.3 SDS-PAGE profiles of untreated and PB treated whey proteins subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Whey control. Lane 3-4: high (2 mg/ml) and medium (0.2 mg/ml) protein concentrations treated for 10 min, respectively. Lane 5-9: low (0.02 mg/ml) protein concentrations treated for 10, 5, 2, 1, 0.5 min, respectively. 200

Figure 8.4 SDS-PAGE profiles of untreated and PB treated glutens subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Gluten control. Lane 3-4: high (10 mg/ml) and medium (1 mg/ml) protein concentrations treated for 10 min, respectively. Lane 5-9: low protein concentrations (0.1 mg/ml) treated for 10, 5, 2, 1, 0.5 min, respectively. 201

Figure 8.5 Secondary structures of PB treated casein proteins (a) with different protein concentration (2, 0.2, 0.02 mg/ml) at 10 min treatment time (b) with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 204

Figure 8.6 Secondary structures of PB treated whey proteins (a) with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time (b) with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 204

Figure 8.7 Secondary structures of PB treated gluten proteins (a) with different protein concentrations (10, 1, 0.1 mg/ml) at 10 min treatment time (b) with low protein concentration of 0.1 mg/ml at different treatment times (10, 5, 2, 1, 0.5

min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6)) 205

Figure 8.8 ELISA responses of casein subjected to PB treatment against IgE binding activity (%). PB treated casein proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml). 215

Figure 8.9 ELISA responses of α-lactalbumin (ALA) subjected to PB treatment against IgG binding activity (%). PB treated whey proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml). 216

Figure 8.10 ELISA responses of β-lactoglobulin (BLG) subjected to PB treatment against IgE binding activity (%). PB treated whey proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) 218

Figure 8.11 ELISA responses of gliadin subjected to PB treatment against IgE binding activity (%). PB treated gluten proteins with different protein concentrations (10, 1, 0.1 mg/ml) at 10 min treatment time and with low protein concentration of 0.1 mg/ml at different treatment times (10, 5, 2, 1, 0.5min). HC:

high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) 219

List of Appendices

Appendix 1 Percentage reduction of amino acid contents of casein treated by spark discharge (SD) and glow discharge (GD) treatments at different treatment times (10, 20, 30 min) (%) 291

Appendix 2 Percentage reduction of amino acid contents of whey treated by spark discharge (SD) and glow discharge (GD) treatments at different treatment times (10, 20, 30 min) (%) 292

Appendix 3 Percentage reduction of amino acid contents of gluten treated by spark discharge treatment with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (%) 293

Appendix 4 Percentage reduction of amino acid contents of gluten treated by glow discharge treatment with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (%) ... 294

Appendix 5 Percentage reduction of amino acid contents of casein treated by dielectric barrier discharge treatment with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (%) 295

Appendix 6 Percentage reduction of amino acid contents of whey treated by dielectric barrier discharge treatment with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (%) 296

Appendix 7 Percentage reduction of amino acid contents of gluten treated by dielectric barrier discharge treatment with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (%) 297

Appendix 8 Percentage reduction of amino acid contents of casein treated by plasma brush treatment with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (%) 298

Appendix 9 Percentage reduction of amino acid contents of whey treated by dielectric barrier discharge treatment with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (%) 299

Appendix 10 Percentage reduction of amino acid contents of gluten treated by dielectric barrier discharge treatment with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (%) 300

Appendix 11 Standard deviation of amino acid profile, shown in Table 5.3, in untreated and treated caseins by SD and GD at different treatment times (10, 20, 30 min) (g amino acid/100 g protein) 301

Appendix 12 Standard deviation of amino acid profile, shown in Table 5.4, in untreated and treated whey proteins by SD and GD at different treatment times (10, 20, 30 min) (g amino acid/100 g protein) 302

Appendix 13 Standard deviation of amino acid profile, shown in Table 5.5, in untreated and treated glutens by SD with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein). 303

Appendix 14 Standard deviation of amino acid profile, shown in Table 5.6, in untreated and treated glutens by GD with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein). 304

Abbreviations

·NO	nitric oxide
·OH	Hydroxyl radical
AC	Alternating current
ACP	Atmospheric cold plasma
AD	Acid detergent
ANOVA	one-way analysis of variance
ATR-FTIR	Attenuated Total Reflection Fourier Transform Infrared
BCA	Bicinchoninic acid
BMPO	5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide
BSA	Bovine serum albumin
CAC	Codex Alimentarius Commission
CAD	Chlorinated alkaline detergent
CD	Corona discharge
CPU	Central production unit
CrI	Credible Interval
DBD	Dielectric barrier discharge
DCF	dichlorofluorescein
$DCFH_2$	2,7-dichlorodihydrofluorescein
DMP	2, 6-dimethyl phenol
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
DTT	Dithiothreitol
EAACI	European Academy of Allergy and Clinical Immunology
EAST	Elevated arm stress test
EC	European Commission
ED	Eliciting doses
EFSA	European Food Safety Authority
EHOs	Environmental Health Officers
ELISA	Enzyme-linked immunosorbent assay
EPR	Electron paramagnetic resonance
ESI	Electrospray Ionisation
ESR	Electron spin resonance

EU	European Union
FAO	Food and Agricultural Organisation
FARRP	Food Allergy Research and Resource Program
FDA	Food and Drug Administration
FSA	Food Standards Agency
FSAI	Food Safety Authority of Ireland
FTIR	Fourier Transform Infrared
GAP	Good Agricultural Practices
GD	Glow discharge
GDPAW	Glow discharge plasma activated water
GDTC	GD treated casein
GDTW	GD treated whey
GHP	Good Hygiene Practices
GMP	Good Manufacturing Practices
H_2O_2	Hydrogen peroxide
HACCP	Hazard Analysis & Critical Control Point
HC	High concentration
HHP	High hydrostatic pressure
HILIC	Hydrophilic Interaction Chromatography
HMW	High molecular weight
HV	High voltage
IFT	Institute of Food Technologists
Ig	Immunoglobulin
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISO	International Organisation for Standardisation
LC	Low concentration
LFD	Lateral flow device
LIF	Laser induced fluorescence
LMW	Low molecular weight
MC	Medium concentration

MD	Microwave discharge
MRM	multiple reaction monitoring
NIR	near-infrared
NMP	N-metheyl-2-pyrrolidone
NO_2^-	Nitrite
NO_3^-	Nitrate
O_2NOO^-	peroxynitrate
O_3	ozone
OECD	Organisation for Economic Co-operation and Development
OES	Optical emission spectroscopy
$ONOO^-$	peroxynitrite
ORP	Redox potential
PALs	Plasma activated liquids
PAW	Plasma activated water
PB	Plasma brush
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PID	Proportional Integral Derivative
PIV	Particle image velocimetry
PJ	Plasma jet
PPLs	Plasma processed liquids
PPO	Polyphenol oxidase
PRPs	Prerequisite programmes
PSLs	Plasma stimulated liquids
PTLs	Plasma treated liquids
PUV	Pulsed ultraviolet
QMS	Quality management system
RBL	Rat basophil leukaemia
RF	Radio frequency
RNS	Reactive nitrogen species
RONS	Reactive oxygen and nitrogen species

ROS	Reactive oxygen species
RSS	Reactive species specificity
SD	Spark discharge
SDPAW	Spark discharge plasma activated water
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDTC	SD treated casein
SDTW	SD treated whey
SPR	Surface plasmon resonance
SRM	Selected reaction monitoring
SSOPs	Sanitation standard operating procedures
TB	Tris buffer
TFA	terephthalic acid
$TiOSO_4$	Titanium oxysulphate
UHT	Ultra-high temperature
UPLC-MS/MS	Ultra-high-performance liquid chromatography-tandem mass spectrometry
UPLC-Q-TOF-MS	Ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
US	United States
UV	ultraviolet
WHO	World Health Organisation
α-LA	α-lactalbumin
β-LG	β-lactoglobulin

Chapter 1 Introduction

1.1 Scope of the project

Food contaminants, such as allergens, are challenging the food industry due to the inefficiency of conventional decontamination techniques. In food supply chains, allergens are ubiquitous in food formulations (Boye & Godefroy, 2010). It is critical to ensure the processing lines are thoroughly and effectively cleaned between runs. The increased awareness of food allergies and the relative populations effected, drives the demand for methods to control, reduce or prevent allergen cross-contamination of products during food processing, distribution, and storage. Good hygiene, awareness of allergens, the risk of allergen cross-contamination and effective cleaning methods are essential. Considerable improvement has been made in the past decade by the food industry in controlling allergen contamination in food processing chains (Kupińska-Adamczyk, Sady, & Pachołek, 2018). However, unlabelled allergens can still be unintentionally present in food products via cross-contamination during manufacturing and inadequate sanitation of shared equipment or processing. The wet-based and dry-based cleaning protocols were designed and studied adequately to remove different allergenic residues from a variety of food contact surfaces (such as stainless steel, plastics, cloth, and rubber). However, good cleaning procedures take time and money to perform (Gupta et al., 2017; Roeder et al., 2010). Consequently, it is important to provide the food industry with variable and novel options for allergen cross-contamination mitigation and control to meet its specific requirements by exploring possible in-built technologies.

The effectiveness of ACP is related to the composition of plasma, such as reactive species, energised ions and electrons, and ultraviolet radiation. Food allergy is caused by an adverse immunoglobulin E (IgE)-mediated response to food allergen-derived proteins. The cross-reactivity between the allergen epitope and the IgE antibody is determined by the conformational structure of the allergen protein and the sequence of the amino acids. This composition of plasma can induce certain interactions with food allergens and change their conformational structure (Tolouie, Mohammadifar, Ghomi, & Hashemi, 2018). Thus, ACP holds promise to reduce the allergen response through modification or degradation of the chemical structure of food allergens.

Few studies have reported the influence of ACP on food allergens to date; device, critical process factors, process control and the lack of reports on important food groups remain under-investigated. Significant questions remain as to how ACP can be developed to mitigate allergen cross contamination in food processing environments, which may prove beneficial to fundamental understanding of how ACP may be developed for allergen control to foods.

1.1.1 Research questions and objectives

This research aims to build an understanding of the influence of ACP system and process parameters on important food allergens, with the key research aim being how ACP may be harnessed as a technology for mitigating against allergen cross contamination. Within this, a number of key research questions were explored.

- What are the systems and process parameters that can govern ACP mitigation of food allergens?

- What are the different modes of ACP exposure suitable for allergen mitigation?
    - Direct treatment: Target exposed directly to ACP
    - Indirect treatment: Target exposed to ACP-treated medium
    - Liquid-based plasma: generated RONS in a liquid medium
    - Gaseous-based plasma: generated gaseous RONS
- How do ACP effectors interact with known allergens?
- Is there a universality of ACP effect applied to allergen target in terms of protein structures and composition?
- Is there a potential risk of antigenicity enhancement by ACP?

Based on the previously reported research findings and the questions above, the specific objectives defined for this work were:

- To determine the physicochemical properties (pH, ORP, conductivity, temperature, H_2O_2, NO_2^- and NO_3^- concentration) of plasma activated water (PAW) generated by RSS air spark and glow discharges with various parameters (such as applied voltage, frequency) – reported in Chapter 3.
- To advance understanding of the RSS setup by evaluating the physical behaviours of air spark and glow discharges in gas phase – reported in Chapter 4.
- To determine the influence of RSS spark discharge (SD) and glow discharge (GD) on the proteomic profiles and antigenicity of milk and wheat proteins – reported in Chapter 5.

- To determine the influence of indirect PAW treatment on the antigenicity of bovine casein with respect to protein concentrations and temperature – reported in Chapter 6.
- To determine the influence of dielectric barrier discharge in-package (DBD) on the proteomic profiles and antigenicity of milk and wheat proteins – reported in Chapter 6.
- To determine the influence of surface treatment with plasma brush (PB) on the proteomic profiles and antigenicity of milk and wheat proteins – reported in Chapter 8.

1.2 Atmospheric cold plasma (ACP)

In recent years, ACP has been utilised in food processing as an innovative method to control and mitigate the ingress of key risks factors to the food chain. Plasma is defined as highly energised matter in a gaseous state. The ACP equipment consists mainly of two electrodes with dielectric discharge material, a power outlet, and a treatment chamber. A gas, such as air, argon, or nitrogen, is supplied between the electric fields. During plasma discharge, the energised matter reacts with gas or food molecules to generate charged particles in the form of positive ions, negative ions, free radicals, electrons, and quanta of electromagnetic radiation (photons). High-energy electrons cause ionisation of the energised matter as well as the excitation of the particles present. The electrons cause ionisation and dissociation of molecules leading to alterations in the orientation of ions and molecules. Therefore, there are physical and chemical reactions taking place on the targets.

Cold plasma is generated by supplying sufficient energy to ionise the neutral gas, leading to the generation of a variety of active electrically charged particles, such as charged ions and particles, electric fields, ultraviolet photons, and reactive oxygen and nitrogen species (RONS) (Fridman, Chirokov, & Gutsol, 2005). RONS, such as hydroxyl radical ($\cdot OH$), nitric oxide ($\cdot NO$), hydrogen peroxide (H_2O_2), nitrite (NO_2^-), nitrate (NO_3^-), peroxynitrite ($ONOO^-$), peroxynitrate (O_2NOO^-) ions, are generated in the gaseous phase and dissolve into irradiated liquids in certain amounts. These features of ACP enable the exploitation of a novel field of chemical reactions both in aqueous and dry conditions (Furusho, Kitano, Hamaguchi, & Nagasaki, 2009; Garcia-Reyes et al., 2009).

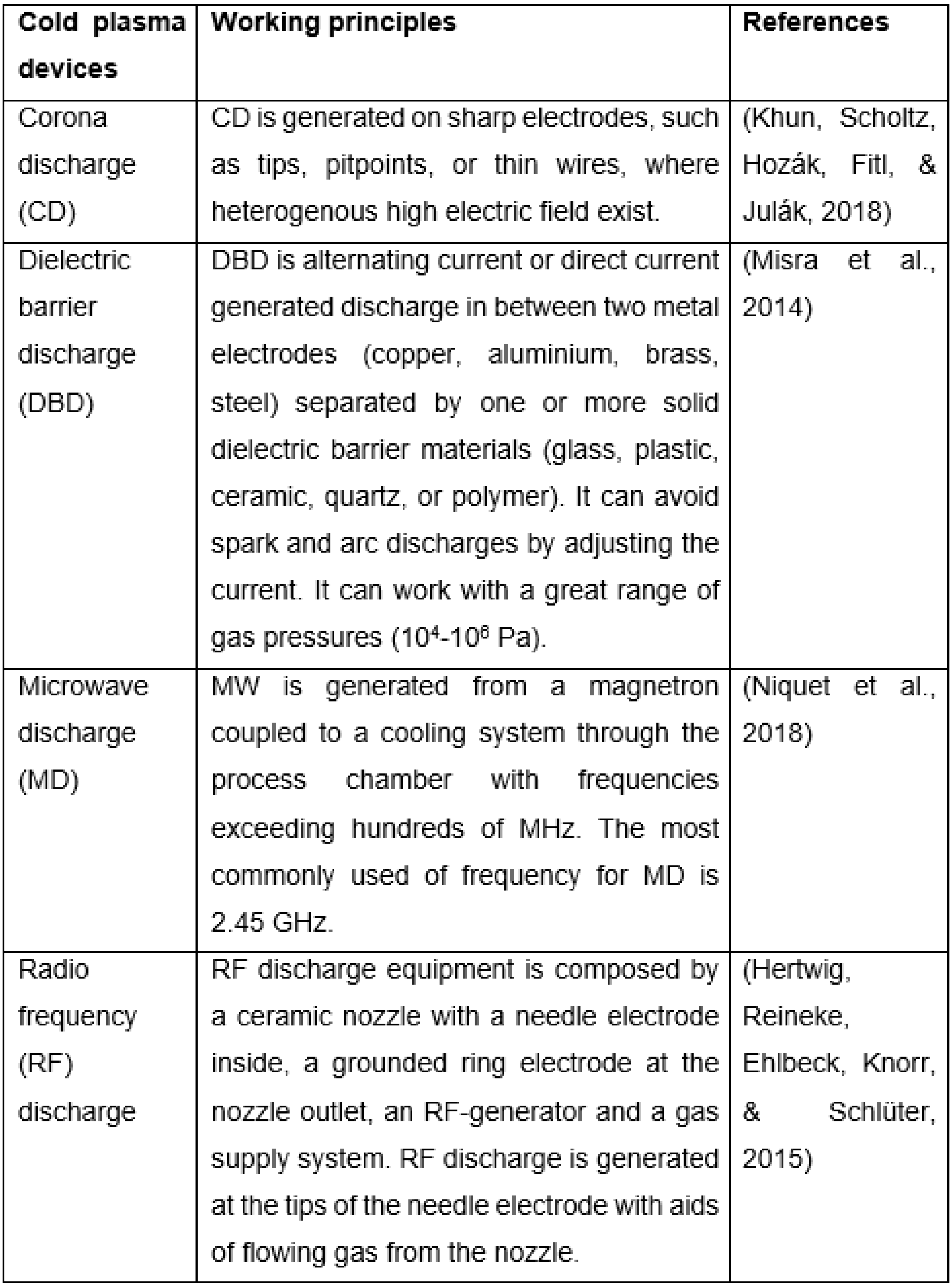
1.2.1 Types of plasma sources

There are several cold plasma generating devices that can treat the liquids in existence. The principles of cold plasma generating devices depend on the equipment configuration, shown in Table 1.1.

1.2.2 Chemical reactions of ACP

Cold plasma technologies are intricately involved in many electrical discharge processes, which possess their specific physicochemical properties. To better utilise cold plasma technology in liquid products efficiently, the physicochemical properties of liquids treated by cold plasma need to be studied. Many studies are conducted on plasma activated water (PAW) because water is a simple and the most commonly used liquid. The type of discharge generated, excited voltage, electrode geometry, electrode used, conductivity, discharge phase distribution, variety of liquid and chemical environment affect the generation of different types and concentrations of reactive oxygen and nitrogen species (RONS) (Brisset & Pawlat, 2016; Lukes, Dolezalova, Sisrova, & Clupek, 2014). There are two types of reactive species. In the case of PAW production, electrons and photons impact the parent molecules (O_2, N_2, and H_2O) and further form a number of primary species (atomic and singlet oxygens, superoxide, hydroxyl radicals, hydrogen radicals, and atomic and excited nitrogen). These primary species further react with other primary species or with the surrounding gas or liquid molecules to produce oxygen-and nitrogen-based secondary species (hydrogen peroxide, nitric oxide, superoxide, nitrite, nitrate, peroxynitrite and peroxynitrate) (Brisset & Pawlat, 2016; Lukes, Locke, & Brisset, 2012). The possible chemical reactions occurring during plasma-water interaction are shown in Table 1.2.

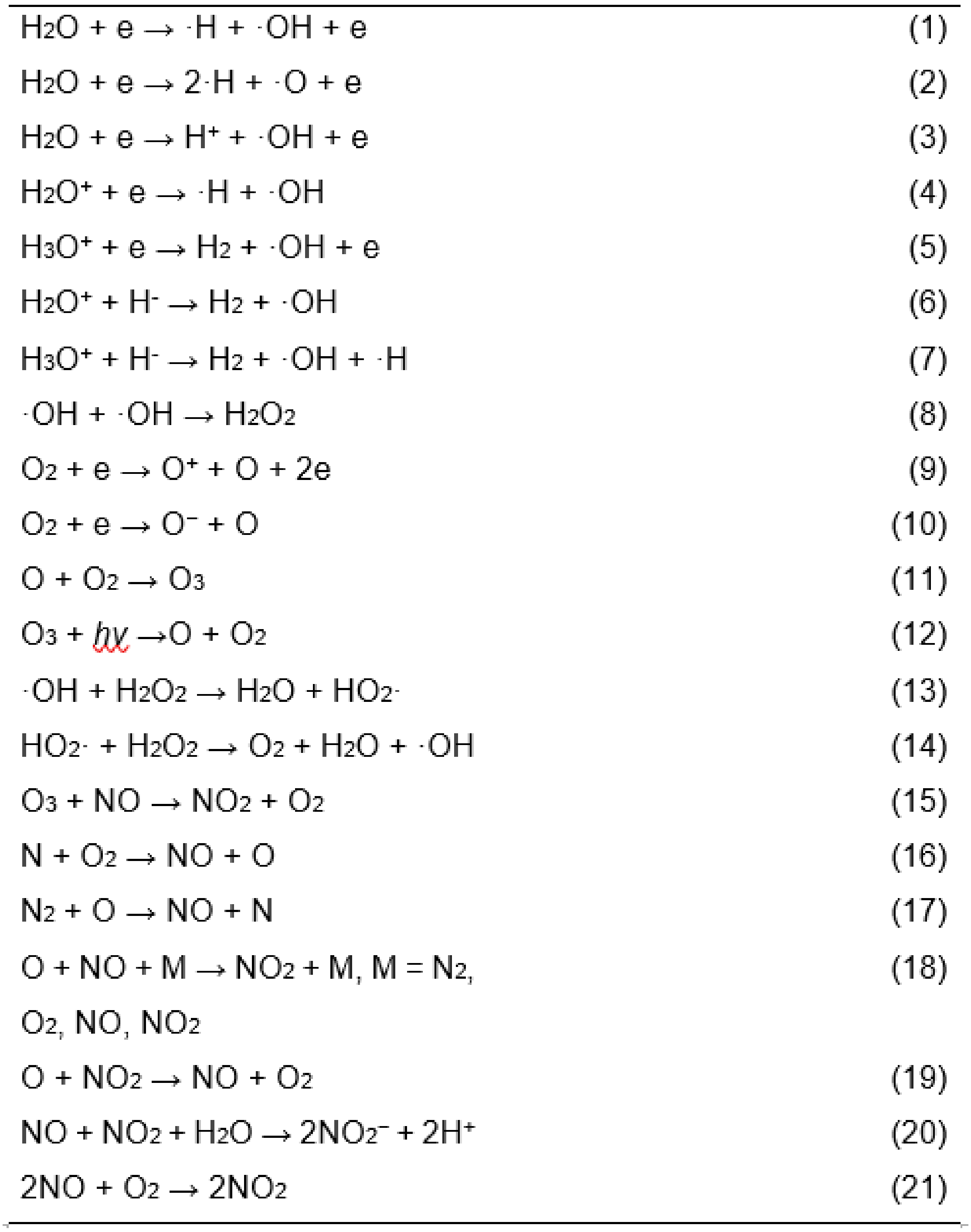
Table 1.1 Principles of different cold plasma generating devices. Adapted from Ng, Tsoukou, Chaple, Boehm, and Bourke (2021).



Cold plasma devices	**Working principles**	**References**
Corona discharge (CD)	CD is generated on sharp electrodes, such as tips, pitpoints, or thin wires, where heterogenous high electric field exist.	(Khun, Scholtz, Hozák, Fitl, & Julák, 2018)
Dielectric barrier discharge (DBD)	DBD is alternating current or direct current generated discharge in between two metal electrodes (copper, aluminium, brass, steel) separated by one or more solid dielectric barrier materials (glass, plastic, ceramic, quartz, or polymer). It can avoid spark and arc discharges by adjusting the current. It can work with a great range of gas pressures (10^4-10^6 Pa).	(Misra et al., 2014)
Microwave discharge (MD)	MW is generated from a magnetron coupled to a cooling system through the process chamber with frequencies exceeding hundreds of MHz. The most commonly used of frequency for MD is 2.45 GHz.	(Niquet et al., 2018)
Radio frequency (RF) discharge	RF discharge equipment is composed by a ceramic nozzle with a needle electrode inside, a grounded ring electrode at the nozzle outlet, an RF-generator and a gas supply system. RF discharge is generated at the tips of the needle electrode with aids of flowing gas from the nozzle.	(Hertwig, Reineke, Ehlbeck, Knorr, & Schlüter, 2015)

Gliding arc discharge	Gliding arc discharger is generated between the tip of high voltage electrode and either at wall of reactor body or at the edge of the reactor with the aids of gas flow from the gas input.	(Ananthanarasimhan, Rao, Shivapuji, & Dasappa, 2019)
Plasma jet (PJ) or plasma brush (PB)	The plasma is generated by electrode area by flowing auxiliary gas to another surface. There are four types of plasma jets, such as DBD jets, dielectric-free electrode jets, DBD-like jets, and single electrode jets.	(Laroussi & Akan, 2007)
Spark discharge (SD)	SD is generated between the high voltage needle tip and the water surface. The water sample was placed on the ground electrode plate.	(Lu, Boehm, Bourke, & Cullen, 2017)
Glow discharge (GD)	GD is generated between the high voltage needle tip and the water surface. The ground electrode was submerged into water sample.	(Lu et al., 2017)

Table 1.2 Chemical reactions during plasma-water interactions resulting in the formation of RONS. Adapted from Ng et al. (2021).



$H_2O + e \rightarrow \cdot H + \cdot OH + e$ (1)

$H_2O + e \rightarrow 2 \cdot H + \cdot O + e$ (2)

$H_2O + e \rightarrow H^+ + \cdot OH + e$ (3)

$H_2O^+ + e \rightarrow \cdot H + \cdot OH$ (4)

$H_3O^+ + e \rightarrow H_2 + \cdot OH + e$ (5)

$H_2O^+ + H^- \rightarrow H_2 + \cdot OH$ (6)

$H_3O^+ + H^- \rightarrow H_2 + \cdot OH + \cdot H$ (7)

$\cdot OH + \cdot OH \rightarrow H_2O_2$ (8)

$O_2 + e \rightarrow O^+ + O + 2e$ (9)

$O_2 + e \rightarrow O^- + O$ (10)

$O + O_2 \rightarrow O_3$ (11)

$O_3 + h\nu \rightarrow O + O_2$ (12)

$\cdot OH + H_2O_2 \rightarrow H_2O + HO_2\cdot$ (13)

$HO_2\cdot + H_2O_2 \rightarrow O_2 + H_2O + \cdot OH$ (14)

$O_3 + NO \rightarrow NO_2 + O_2$ (15)

$N + O_2 \rightarrow NO + O$ (16)

$N_2 + O \rightarrow NO + N$ (17)

$O + NO + M \rightarrow NO_2 + M, M = N_2,$ (18)
O_2, NO, NO_2

$O + NO_2 \rightarrow NO + O_2$ (19)

$NO + NO_2 + H_2O \rightarrow 2NO_2^- + 2H^+$ (20)

$2NO + O_2 \rightarrow 2NO_2$ (21)

$$NO_2 + \cdot OH \rightarrow HNO_3 \tag{22}$$

$$H_2O_2 + hv \rightarrow 2\cdot OH \tag{23}$$

$$3NO_2 + H_2O \rightarrow 2HNO_3 + NO \tag{24}$$

$$H_2O_2 + H^+ + NO_2^- \rightarrow ONOOH + H_2O \tag{25}$$

$$NO + NO \rightarrow N_2 + O_2 \tag{26}$$

$$NO + \cdot OH \rightarrow HNO_2 \tag{27}$$

$$HNO_2 + \cdot OH \rightarrow NO_2 + H_2O \tag{28}$$

$$NO_2^- + hv \rightarrow NO\cdot + \cdot O^- \tag{29}$$

$$NO_3^- + hv \rightarrow NO_2\cdot + \cdot O^- \tag{30}$$

$$NO_3^- + hv \rightarrow NO_2^- + O \tag{31}$$

$$NO_2 + NO_3 \rightarrow N_2O_5 \tag{32}$$

$$N_2O_5 + H_2O \rightarrow 2HNO_3 \tag{33}$$

$$2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \tag{34}$$

$$3NO_2^- + 3H^+ \rightarrow 2NO + NO_3^- + H_3O^+ \tag{35}$$

$$OH + NO_2 \rightarrow [O{=}N{-}OOH] \rightarrow O{=}N{-}OO^- + H^+ \tag{36}$$

$$O{=}N{-}OO^- \rightarrow NO_3^- \tag{37}$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \tag{38}$$

$$4OH^- \rightarrow O_2 + 2H_2O + 4e \tag{39}$$

$$O_3 + H_2O \rightarrow \cdot OH + O_2 + HO_2\cdot \text{ (basic condition)} \tag{40}$$

The types of RONS generated by plasma from the gas phase to the liquid phase are different due to the different chemical reactions which occur. These plasma chemical reactions can be divided into four categories (Lukes et al., 2012).

1. Acid-base reactions. The formation of chemical products (hydrogen peroxide, nitrite, and nitrate) by plasma cause acidification in water.
2. Oxidation reactions. The formation of RONS (·OH, superoxide, O_3, H_2O_2, peroxynitrite) produced by plasma cause oxidation, as they are oxidants.
3. Reduction reactions. Reductive species (hydrogen radicals, hydroperoxyl radicals) generated by plasma cause reduction.
4. Photochemical reactions initiated by ultraviolet (UV) radiation from the plasma (photolysis of H_2O_2, O_3, NO_2, NO_3, photocatalysis).

The chemical pathways of RONS generation from the plasma to the water are summarised and illustrated in Figure 1.1. These chemical reactions generate short-lived and long-lived RONS in PAW that possess antimicrobial activity and can react with target molecules in liquids.

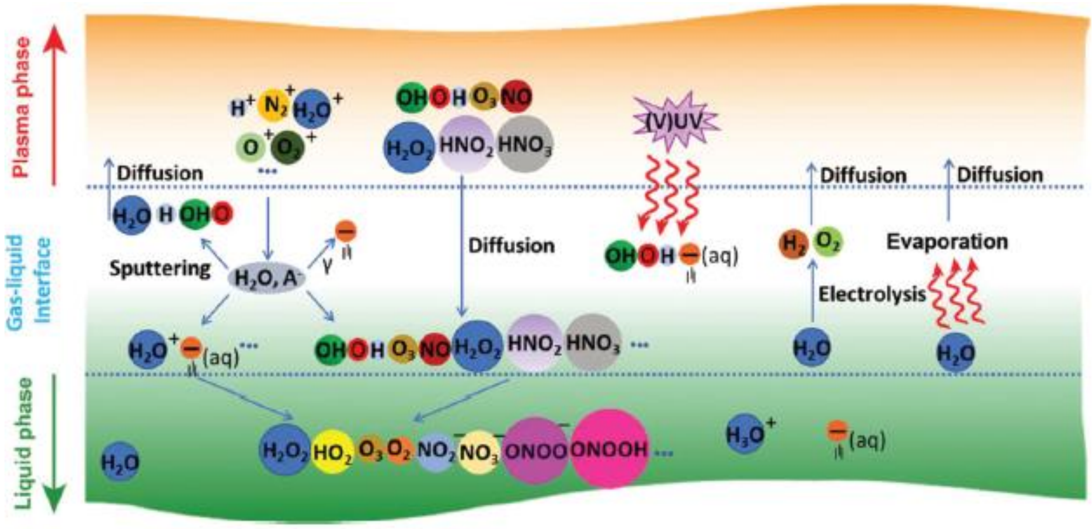


Figure 1.1 Chemical pathway of RONS generation from gas phase to liquid phase. Adapted from Renwu Zhou et al. (2018).

1.2.3 Plasma activated liquids

Plasma activated liquids (PAL) are generated from the exposure of liquids to a plasma discharge. The plasma discharges in and in contact with liquids have demonstrated efficacy as an indirect mode of application (Bruggeman & Leys, 2009). These liquids exposed to plasma can be termed as plasma activated liquids (PALs) (Thirumdas et al., 2018), plasma treated liquids (PTLs) (Shaw et al., 2018), plasma stimulated liquids (PSLs) (Chen, Cheng, Lin, & Keidar, 2016) and plasma processed liquids (PPLs) (Schnabel et al., 2017). Plasma activated water (PAW), as an example of PALs, creates an acidic environment which results in changes of redox potential, conductivity and in the generation of reactive oxygen and nitrogen species (RONS). The RONS are mainly generated near the gas-liquid interface and transported into the liquid phase. In the gas-liquid interface, the primary RONS are composed of charged particles (electrons and photons), ions and reactive species such as O_3, $^1O_2$, O_2^-, OH, NO·, and NO_2·. The dissolution of gas phase electrons and reactive species into liquid phase stimulates dynamic chemical reactions (such as acid-base reactions, oxidation, reduction and photochemical reaction by ultraviolet (UV) radiation) and further generates a series of aqueous reactive species, which are termed as secondary RONS, such as O_3, OH, HOONO, HNO_2, HNO_3 and H_2O_2 (Gorbanev, Privat-Maldonado, & Bogaerts, 2018). Some of these secondary RONS are short lived and difficult to measure reliably as they interact and affect each other in complex ways (Graves, 2012). The formation of hydrogen peroxide (H_2O_2), nitrite (NO_2^-) and nitrate (NO_3^-) has been well studied as the stable long-lived reactive species in PAWs.

1.2.3.1 pH

pH is a measure of the acidity or alkalinity of a solution. pH is one of the key factors that affect the chemistry and biological effects of PAW. A great number of publications have reported that the pH value of plasma activated liquids is decreased with an increase in plasma treatment time (Ercan et al., 2013; Machala et al., 2013; Oehmigen et al., 2010; Thirumdas et al., 2018; Tsoukou, Bourke, & Boehm, 2018). The acidification of PAL (plasma activated liquids) occurred when reactions took place between the chemical species formed in the plasma and water. The amount of ionisation and the accumulation of reactive species corresponds to the acidity of the PAW (Fajar, Ramadhanty, Nicholas, & Abuzairi, 2019). Oehmigen et al. (2010) reported that the acidification of PAL occurred as a result of the generation of nitrite and nitrate induced by RNS like NO from the plasma phase. The general mechanism to describe the decrease of pH and the formation of nitrite and nitrate is illustrated in the reactions (20, 21, 34) in Table 1.2. The NO from gas phase plasma rapidly reacts with oxygen (reaction (21)) and produces NO_2. NO_2 can subsequently dissolve in water and further dissociate into nitrite and nitrate via electron capture by NO_2 (reaction 34) or oxidation by NO (reaction 20) (Lukes et al., 2012). The hydrogen ions formation in reactions (20, 34) is the evidence for the reduction of pH of PAW. However, some authors investigated that the decrease in pH in PAW treated by plasma in a nitrogen-free atmospheric (Radu Burlica & Locke, 2008; Porter, Poplin, Holzer, Finney, & Locke, 2009; Shainsky et al., 2011). The results of oxygen, argon, helium, and carbon dioxide gas, used as working gas in discharge with water, were showing a decrease in pH in PAW. This means that these pH changes not only due to the generation of nitrite and nitrate since NO was not found in these cases. The formation of H^+ and H_3O^+ ions in water due to electronic and ionic bombardment could be a possibility to explain the reduction of pH because the conductivities of these cases were increased (Lukes et al., 2012; Shainsky et al., 2011). H_2O_2 is known as a weak acid and its pH, which is concentration-dependent, is at least 4.5. The final concentration of H_2O_2 in water generated by plasma was reported at 5.5 mM in one study (Tsoukou et al., 2018). This concentration is unlikely to cause an obvious reduction in pH. The reduction in pH in PAW is attributed to the well-known nitrite and nitrate formation and the hydrogen ions which are not fully understood. H_2O_2 contributes only partially or not at all to the decrease in pH in PAW. Lu, Boehm, Bourke, and Cullen (2017) and Tsoukou et al. (2018) observed a sharp drop in pH during the initial phase of plasma exposing to non-buffered solution and were stable thereafter. Jie Shen et al. (2019) observed the pH of PAW different temperature (25 °C, -4 °C, -20 °C, -80 °C) for post-storage time of 30 days. The pH did not change significantly after 30 days post storage time. pH is not the main factor to inactivate the microorganisms (Oehmigen et al., 2010; Satoh, MacGregor, Anderson, Woolsey, & Fouracre, 2007), however, the synergy of acidic condition and RONS can increase antibacterial efficacy (Naitali, Kamgang-Youbi, Herry, Bellon-Fontaine, & Brisset, 2010; Traylor et al., 2011).

1.2.3.2 Redox potential

Oxidation reduction potential (ORP) is a measurement of the amounts of oxidants and reductants in solution. It is the voltage measurement between the oxidation reaction occurring at the (positive) anode and the cathode (negative) reduction reaction in the electrochemical cell in unit of millivolts (mV). At higher

ORP levels in the solution, the solution contains more oxidants to oxidize substances. Abuzairi et al. (2018) reported that the value of ORP of PAW is increasing as a function of plasma treatment time. Q. Zhang et al. (2013) observed that PAW is effective in inactivating bacteria due to the high ORP in the PAW. They observed the ORP of PAW at 4 °C for post storage time of 24 hours. The ORP dropped from 490 mV to 450 mV after storage of 24 hours. The ORP is an indicator to measure the amount of ROS (·OH, O, O_3, H_2O_2) and RNS (NO·, nitrite, nitrate and peroxynitrite) (Qian Zhang et al., 2016). Hence, ORP is regarded as a key factor to investigate the oxidation capability of PAW.

1.2.3.3 Conductivity

Electrical conductivity is a measure of the ability of a solution to conduct electricity in the unit of micro-Siemens per cm (µS/cm). It is also used to measure the ionic content in a solution. Higher conductivity measurement of a solution indicates the higher concentration of ions present in the solution. The reactive species and ions generated by the plasma are readily dissolved in water, which evidently changes the conductivity. Xiang et al. (2018) reported that the electrical conductivity of PAW increased dramatically as a function of plasma treatment time. Ma et al. (2015) found that PAW generated by a plasma jet with a working gas mixture of argon and oxygen for 20 min obtained the conductivity of 450 µS/cm whilst Tian et al. (2015) obtained the conductivity of 18.8 µS/cm. Both authors worked with the same working gas but different applied voltage. Ma applied 18 kV to generate plasma, whereas Tian applied 0.4-0.42 kV to generate plasma. The applied voltage to generate plasma for Tian is too low, compared to Ma, this could be the reason for low conductivity of PAW obtained. Tian further studied the discharge in contact with and in liquids. The conductivity of discharge in water was higher than discharge above water.

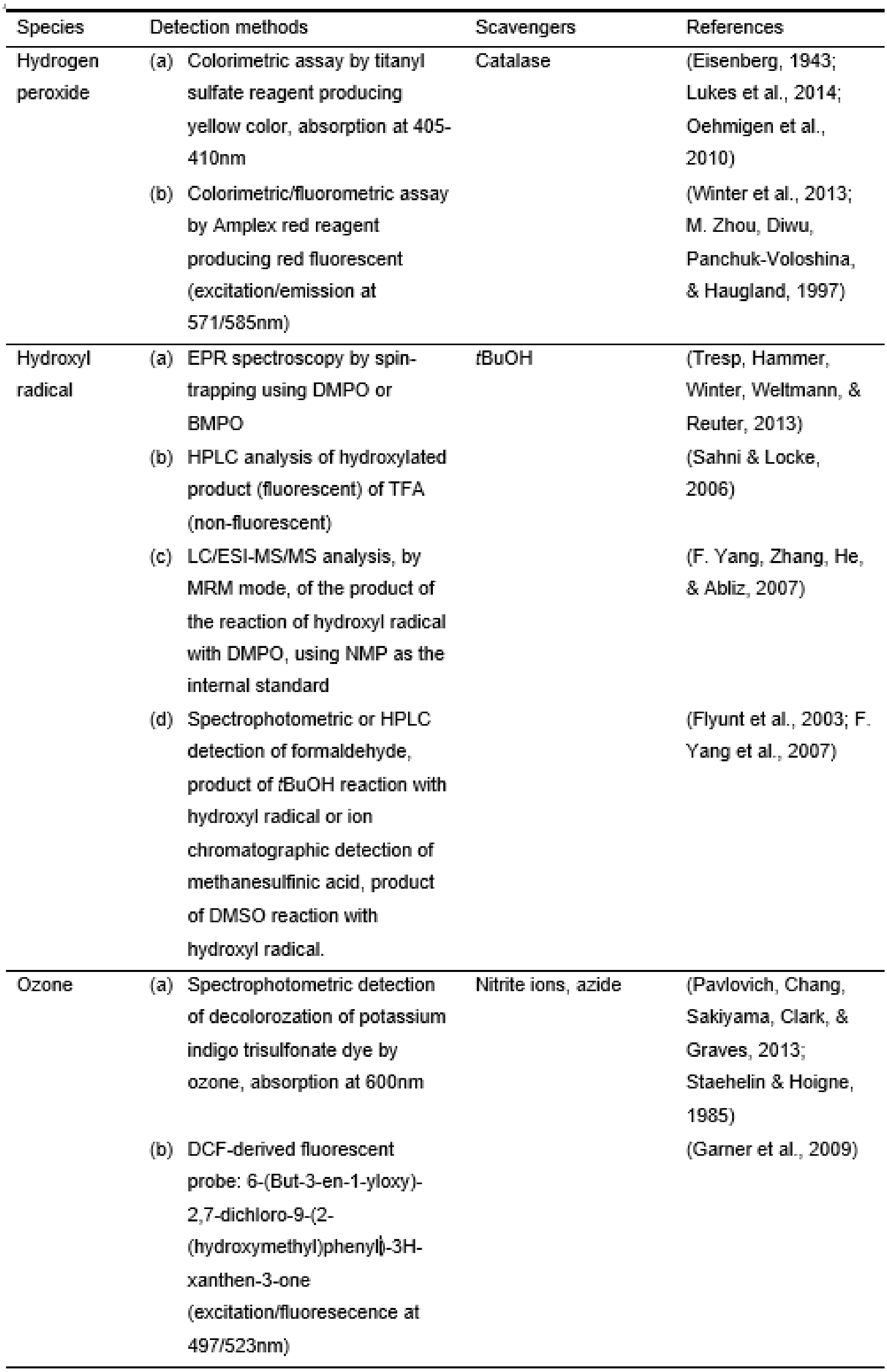
1.2.4 Measurement of ACP effectors

As discussed initially, liquid phase and gas phase RONS are generated by plasma. In this section, we focus only on liquid phase RONS generated by plasma. Some of the liquid phase RONS have high capability of oxidation that can inactivate microorganisms (Jin Shen et al., 2016; Jie Shen et al., 2019; Zheng, 2017). The RONS production may be controlled in a wide range and depends on type of plasma, applied voltage, pH, conductivity, type of working gas, flow rate, humidity, and chemical environment (Brisset & Pawlat, 2016; Lu et al., 2017; Lukes et al., 2014). RONS can be classified into two categories namely reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS includes hydroxyl radical, hydrogen peroxide, ozone, and singlet oxygen. Meanwhile, RNS includes nitrites, nitrates and peroxynitrites. The production of these RONS is varied, and the lifetime can be either short or long. Hence, it is very important to identify the type, concentration, and survival rate of RONS in order to establish a PAW of high potential.

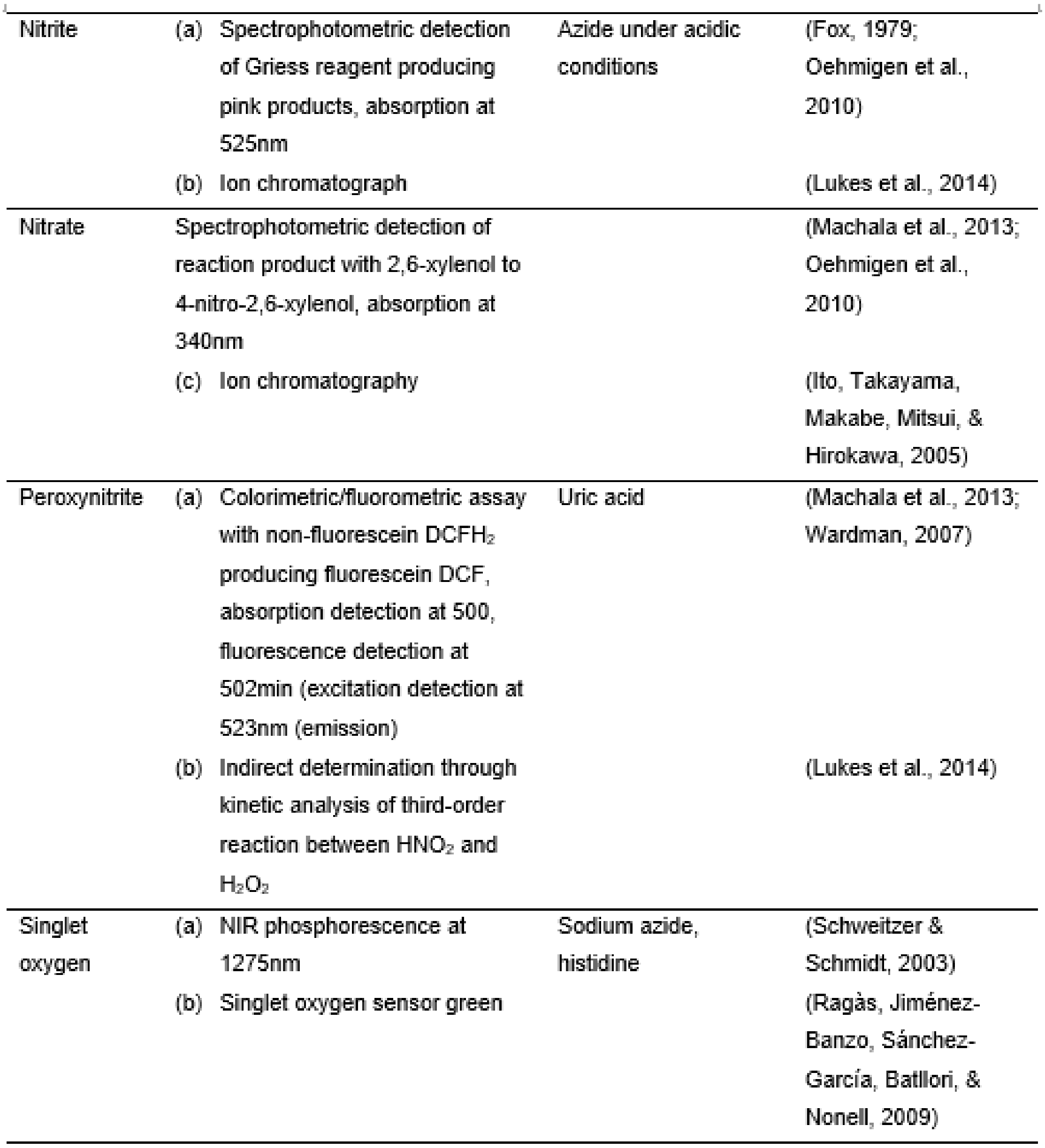
Hydroxyl radical is the major ROS found in PAW. It has high oxidizing potential, can serve as a strong oxidant in aqueous environment but it is a short-lived species. Hydrogen peroxide is a long-lived ROS formed in PAW which plays a significant role in the microbial inactivation. One mechanism of hydrogen peroxide formation is the recombination of hydroxyl radicals, shown in reaction (8) (Lukes, Clupek, Babicky, & Sunka, 2008). In the acidic environment, H_2O_2 accompanied with superoxide contributes to the most important part of the oxidation effects of PAW (Shainsky et al., 2011). Ozone is a powerful oxidant, and it has the highest standard oxidation potential among other oxidants, such as chlorine, chlorine dioxide, permanganate, and hydrogen peroxide. However, it is less soluble in aqueous solution. It decays slowly in water and decomposes into hydroxyl radicals and hydroperoxyl radicals (reaction (40)) (Wende, von Woedtke, Weltmann, & Bekeschus, 2018). Nitrites and nitrates are RNS which are formed in PAW through the dissolution of nitrogen oxide generated by plasma. The productions of nitrite and nitrate in PAW are described in reaction (20, 34, 35). Peroxynitrite is a RNS that can serve as nitrating agent and nitrosating agent (Naïtali, Herry, Hnatiuc, Kamgang, & Brisset, 2012). It is generated by the reaction of nitrite ion with hydrogen peroxide (reaction (20)), or the reaction of nitrogen dioxide and hydroxyl radical (reaction (36)). Peroxynitrite is usually stable in basic condition. In acidic condition, the protonated form of peroxynitrite will convert into nitrate ion (reaction (37)) (Renwu Zhou et al., 2018).

To better understand the RONS, there are various methods that can be used to detect these reactive species in PAW namely colorimetric assays, reactive species scavengers, spin trapping reagents, UV-VIS spectrometry, laser-induced fluorescence techniques, light emission spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, fluorescence spectrometry and ion chromatography. More details are provided in Table 1.3.

Table 1.3 Detection methods for liquid phase reactive species. Adapted from Ng et al. (2021).



Species	Detection methods	Scavengers	References
Hydrogen peroxide	(a) Colorimetric assay by titanyl sulfate reagent producing yellow color, absorption at 405-410nm	Catalase	(Eisenberg, 1943; Lukes et al., 2014; Oehmigen et al., 2010)
	(b) Colorimetric/fluorometric assay by Amplex red reagent producing red fluorescent (excitation/emission at 571/585nm)		(Winter et al., 2013; M. Zhou, Diwu, Panchuk-Voloshina, & Haugland, 1997)
Hydroxyl radical	(a) EPR spectroscopy by spin-trapping using DMPO or BMPO	*t*BuOH	(Tresp, Hammer, Winter, Weltmann, & Reuter, 2013)
	(b) HPLC analysis of hydroxylated product (fluorescent) of TFA (non-fluorescent)		(Sahni & Locke, 2006)
	(c) LC/ESI-MS/MS analysis, by MRM mode, of the product of the reaction of hydroxyl radical with DMPO, using NMP as the internal standard		(F. Yang, Zhang, He, & Abliz, 2007)
	(d) Spectrophotometric or HPLC detection of formaldehyde, product of *t*BuOH reaction with hydroxyl radical or ion chromatographic detection of methanesulfinic acid, product of DMSO reaction with hydroxyl radical.		(Flyunt et al., 2003; F. Yang et al., 2007)
Ozone	(a) Spectrophotometric detection of decolorozation of potassium indigo trisulfonate dye by ozone, absorption at 600nm	Nitrite ions, azide	(Pavlovich, Chang, Sakiyama, Clark, & Graves, 2013; Staehelin & Hoigne, 1985)
	(b) DCF-derived fluorescent probe: 6-(But-3-en-1-yloxy)-2,7-dichloro-9-(2-(hydroxymethyl)phenyl)-3H-xanthen-3-one (excitation/fluoresecence at 497/523nm)		(Garner et al., 2009)



Nitrite	(a) Spectrophotometric detection of Griess reagent producing pink products, absorption at 525nm	Azide under acidic conditions	(Fox, 1979; Oehmigen et al., 2010)
	(b) Ion chromatograph		(Lukes et al., 2014)
Nitrate	Spectrophotometric detection of reaction product with 2,6-xylenol to 4-nitro-2,6-xylenol, absorption at 340nm		(Machala et al., 2013; Oehmigen et al., 2010)
	(c) Ion chromatography		(Ito, Takayama, Makabe, Mitsui, & Hirokawa, 2005)
Peroxynitrite	(a) Colorimetric/fluorometric assay with non-fluorescein $DCFH_2$ producing fluorescein DCF, absorption detection at 500, fluorescence detection at 502min (excitation detection at 523nm (emission)	Uric acid	(Machala et al., 2013; Wardman, 2007)
	(b) Indirect determination through kinetic analysis of third-order reaction between HNO_2 and H_2O_2		(Lukes et al., 2014)
Singlet oxygen	(a) NIR phosphorescence at 1275nm	Sodium azide, histidine	(Schweitzer & Schmidt, 2003)
	(b) Singlet oxygen sensor green		(Ragàs, Jiménez-Banzo, Sánchez-García, Batllori, & Nonell, 2009)

Note: DMPO = 5,5-dimethyl-1-pyrroline-N-oxide, BMPO = 5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide, TFA = terephthalic acid, MRM = multiple reaction monitoring, NMP = N-metheyl-2-pyrrolidone, $DCFH_2$ = 2,7-dichlorodihydrofluorescein, DCF = dichlorofluorescein, NIR = near-infrared.

1.2.5 Application of ACP for microbial decontamination

A range of studies have reported that direct application of cold atmospheric plasma technology is a promising tool for microbial decontamination in food industry (Han et al., 2016; Los et al., 2018; Pankaj, Wan, & Keener, 2018; Patange, Boehm, Bueno-Ferrer, Cullen, & Bourke, 2017; Patange, Boehm, et al., 2019; Patange, Lu, Boehm, Cullen, & Bourke, 2019; Schnabel, Handorf, et al., 2019; Scholtz, Pazlarova, Souskova, Khun, & Julak, 2015). The mechanism of microbial inactivation by plasma is complex, with the role of various charged particles and generated reactive species under current investigation. A number of antimicrobial agents including ROS, RNS, UV, ionized gas, and charged particles can be generated in a plasma discharge. The antimicrobial efficacy of plasma depends on the bacterial type, substrate on which the bacteria are present, plasma designs and plasma process parameters (voltage, plasma treatment time, gas, post treatment storage time, storage duration, temperature). In general, higher applied voltage is correlated with higher concentration of ROS, which leads to the inactivation of microorganisms. More recently it is reported that higher post treatment storage time during in package treatment, can enhance antimicrobial activity because of the increased diffusion and interaction time with microbial targets (Niquet et al., 2018). These parameters can also have an impact on the chemical changes which take place in liquids exposed to plasma discharge and leading to the generation of so-called plasma activated liquids (PAL) which possess antibacterial and other biological activity.

ACP sources generate a plethora of reactive chemical species. As a result, PAL such as plasma activated water (PAW) has a different chemical composition than the untreated liquid, such as water, and can serve as an alternative method for decontamination of surfaces or processing environments on a scale compatible with industrial food processing. It is therefore a promising strategy in poultry industries and stock farming. An important question is if PAL can be as effective as the existing microbial decontamination processes. The sanitizers and disinfectants usually employed in the food industry include chlorine compounds, organic acids, quaternary ammonium compounds, iodine compounds and trisodium phosphate (A. Phillips, 2016). These products possess high antimicrobial activity against most microorganisms. Novel techniques for microbial decontamination are urgently needed and plasma treated liquids represent a promising new approach, if the sensory attributes of the plasma treated food products can be maintained and the safety of the products ensured (Y. Xu, Tian, Ma, Liu, & Zhang, 2015).

1.2.6 Application of ACP for protein inactivation

Protein plays a vital role in food processing (Fernandes, 2010). Some enzymes are critical for improvements of the food processing such as amylase, which are used to break down complex carbohydrate into simple sugars. The amylase are widely used in bakery, brewing process, confectionary and starch industry (Souza & Magalhães, 2010). On contrast, some enzymes naturally present in food can possess adverse effect during food processing or post-processing making food unfit or undesirable for consumption. For example, polyphenol oxidase presents in apple caused browning (Illera et al., 2019).

ACP has been reported to inactivate a range of proteins, such as lysozyme (Takai, Kitano, Kuwabara, & Shiraki, 2012), polyphenol oxidase (Illera et al., 2019) and peroxidase (Surowsky, Fischer, Schlueter, & Knorr, 2013). Takai et al. (2012) observed the inactivation of lysozyme by ACP. They suggested that the reactive species generated by the plasma affect lysozyme. The plasma treatment decreased the enzymatic activity and changed the secondary structure that results from the increased molecular weight of lysozyme with chemical modification. The inactivation of polyphenol oxidase in cloudy apple juice by ACP has been studied by (Illera et al., 2019). They applied spark discharges plasma to the cloudy apple juice up to 5 min and studied the quality and inactivation activity during controlled storage. They found the reduction of the polyphenol oxidase residual activity by increasing the treatment time. Surowsky et al. (2013) reported the inactivation of peroxidase in a model system sing a plasma jet with various working gases. They observed a significant decrease in the α-helix contents, which led to the loss of enzymatic activity.

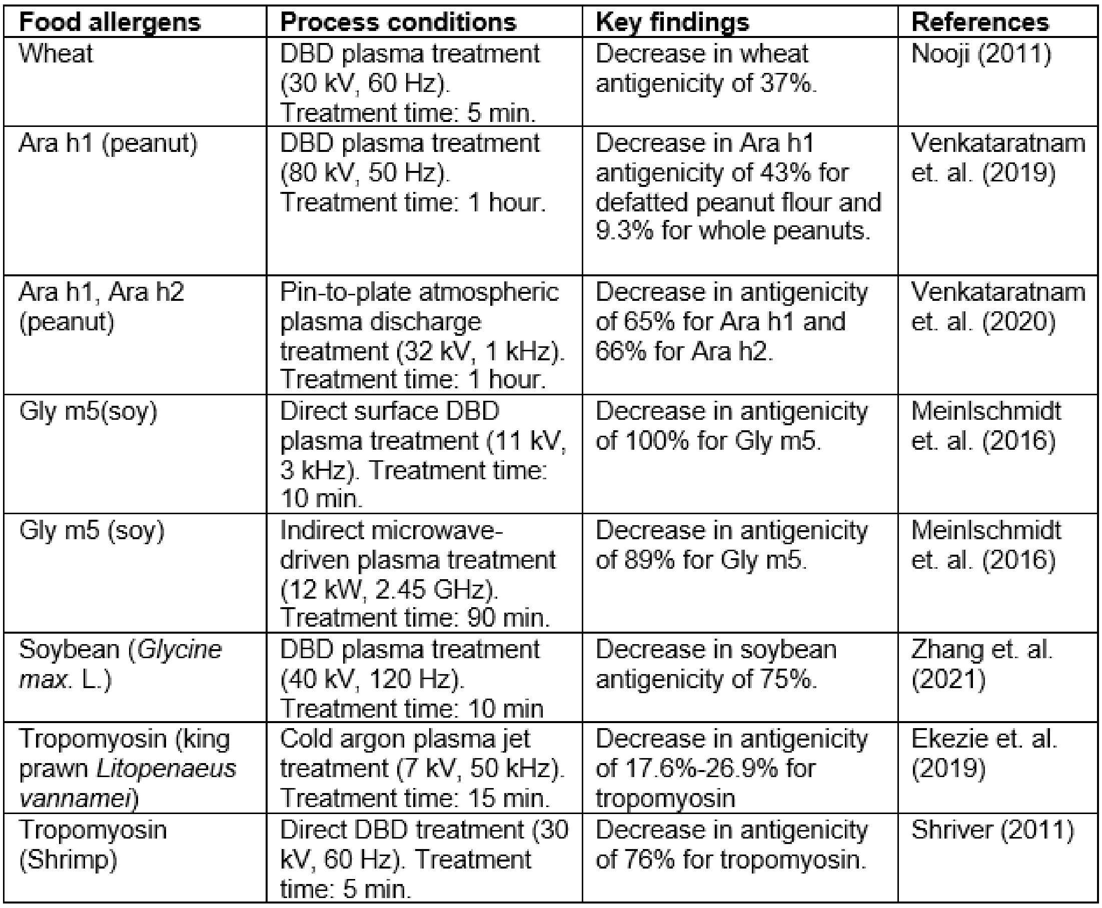
Proteins are comprised of a series of amino acids organized into complex three-dimensional structures (classified into primary, secondary, tertiary and quaternary), which is associated with their functionality. Many reports revealed that the loss of secondary structures after the proteins are exposed to cold plasma (NN Misra, Pankaj, Segat, & Ishikawa, 2016; Segat, Misra, Cullen, & Innocente, 2016; Surowsky et al., 2013; Takai et al., 2012).

As reported by Amos, Chan, Easton, and Radom (2015), the amino acids of proteins are subject to attack from free radicals and non-free radicals. However, each amino acid is not equality susceptible to the reactive species generated from plasma. The reactive species attack the amino acid side chains and the peptide backbone. The amino acids with reactive side chains, such as sulfoxide, methionine, amino group, imidazole ring and indole ring, are particularly susceptible to the plasma-induced oxidation (Takai et al., 2014). Aromatic amino acid are easily nitrated by the RNS in plasma, as reported by Lin, Chen, Liu, Chu, and Lin-Shiau (2000); van der Vliet, O'Neill, Halliwell, Cross, and Kaur (1994).

1.2.7 Effect of ACP on food allergens

Recently, atmospheric cold plasma technology has gained considerable attention from researchers as a potent technology suitable for effective elimination of food allergens (Bourke, Ziuzina, Boehm, Cullen, & Keener, 2018). Cold plasma can interact with the compounds present in the food, further changing their properties (Ramazzina et al., 2016). From a microscopic view, Takai et al. (2014) and R. Zhou et al. (2016) suggested that cold plasma can modify the structure of amino acids' side chains. From a macroscopic view, several authors have reported that cold plasma induced the structural modification of proteins (Attri et al., 2015; Chauvin, Judee, Yousfi, Vicendo, & Merbahi, 2017; Segat, Misra, Cullen, & Innocente, 2015; Takai et al., 2012). Researchers have recently explored the feasibility of elimination of food allergens by applying cold plasma (Table 1.4).

Table 1.4 Summary of the previously conducted research on allergen mitigation by atmospheric cold plasma.



Food allergens	**Process conditions**	**Key findings**	**References**
Wheat	DBD plasma treatment (30 kV, 60 Hz). Treatment time: 5 min.	Decrease in wheat antigenicity of 37%.	Nooji (2011)
Ara h1 (peanut)	DBD plasma treatment (80 kV, 50 Hz). Treatment time: 1 hour.	Decrease in Ara h1 antigenicity of 43% for defatted peanut flour and 9.3% for whole peanuts.	Venkataratnam et. al. (2019)
Ara h1, Ara h2 (peanut)	Pin-to-plate atmospheric plasma discharge treatment (32 kV, 1 kHz). Treatment time: 1 hour.	Decrease in antigenicity of 65% for Ara h1 and 66% for Ara h2.	Venkataratnam et. al. (2020)
Gly m5(soy)	Direct surface DBD plasma treatment (11 kV, 3 kHz). Treatment time: 10 min.	Decrease in antigenicity of 100% for Gly m5.	Meinlschmidt et. al. (2016)
Gly m5 (soy)	Indirect microwave-driven plasma treatment (12 kW, 2.45 GHz). Treatment time: 90 min.	Decrease in antigenicity of 89% for Gly m5.	Meinlschmidt et. al. (2016)
Soybean (*Glycine max*. L.)	DBD plasma treatment (40 kV, 120 Hz). Treatment time: 10 min	Decrease in soybean antigenicity of 75%.	Zhang et. al. (2021)
Tropomyosin (king prawn *Litopenaeus vannamei*)	Cold argon plasma jet treatment (7 kV, 50 kHz). Treatment time: 15 min.	Decrease in antigenicity of 17.6%-26.9% for tropomyosin	Ekezie et. al. (2019)
Tropomyosin (Shrimp)	Direct DBD treatment (30 kV, 60 Hz). Treatment time: 5 min.	Decrease in antigenicity of 76% for tropomyosin.	Shriver (2011)

Nooji (2011) reported that dielectric barrier discharge (DBD) plasma treatment decreased the wheat antigenicity by 37%. Two recent studies on the effect of different cold plasma treatments (DBD and pin-to-plate plasma discharge) on peanuts demonstrated a significant reduction of antigenicity of peanut allergen (Venkataratnam, Cahill, Sarangapani, Cullen, & Barry-Ryan, 2020; Venkataratnam, Sarangapani, Cahill, & Ryan, 2019). The authors found that the plasma-induced modifications in secondary structure could lead to the modification of the antigen epitopes and their binding capacity, thereby affecting its antigenicity. Moreover, Meinlschmidt et al. (2016) investigated the effects of different cold plasma treatments (direct surface DBD and indirect microwave-driven plasma) on soy allergens (Gly m5). The authors observed that new protein bands at 50 kDa and insoluble aggregates were formed after plasma treatment shown in the results of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). According to the study, 10 min of direct surface DBD treatment decreased the antigenicity of Gly m5 in soy by 100%. 90 min of indirect microwave-driven plasma treatment decreased the antigenicity of Gly m5 soy by 89%. Similarly, Zhang et al. (2021) demonstrated that 10 min of DBD plasma treatment with a voltage of 40 kV and a frequency of 120 Hz decreased the antigenicity of soybean by 75%. A recent attempt on treating king prawn using cold argon plasma jet for 15 min showed up to 17.6%-26.9% reduction of IgE recognition to tropomyosin (Ekezie, Sun, & Cheng, 2019). Likewise, Shriver (2011) demonstrated that 5 min of DBD plasma treatment with a voltage of 30 kV and a frequency of 60 Hz decreased the antigenicity of tropomyosin in shrimp by 76%.

Contrary to the above research, Alves Filho et al. (2019) recently reported that cold plasma treatment did not affect cashew nut allergenicity. Similarly, Tammineedi et al. (2013) reported the effects of cold plasma treatment on milk proteins (α-casein, β-lactoglobulin and α-lactalbumin). According to the paper, the results showed no noticeable change in the protein profiles of plasma-treated milk proteins in SDS-PAGE results and no significant reduction in antigenicity. The reason for the absence of attenuation in milk antigenicity could be poor plasma performance associated with this particular set-up and process design. These authors used cold plasma equipment with a power of 60W and the sample were exposed to the plasma afterglow (indirect plasma). The inefficiency of attenuation in milk antigenicity could be the low power used and the indirect plasma exposure treatment. Besides, there are few studies found antigenicity reduction with a long plasma treatment (Meinlschmidt et al., 2016; Venkataratnam et al., 2020; Venkataratnam et al., 2019). Advancing cold plasma devices is critical to enhance the cleaning efficiency in food processing while lower the cost, labour and potential worker injuries at a minimum. More research is still required to validate the effectiveness of cold plasma treatment against food allergens.

1.3 Food Allergen Safety, Advisory and Industry Environment

Food safety is unquestionably one of the most serious worldwide challenges in the food supply chain (Gendel, 2013). The World Health Organisation (WHO) clearly defined food safety as the level of confidence that food is innoxious and innocuous to the consumer when it is prepared, served and eaten according to its intended use. Food safety is a multi-disciplinary subject involving the food processing chain, preparation and storage in ways that reduce foodborne illness and food recalls. Food incidents were mainly caused by food contamination that causes adverse effects in humans if consumed (FSA, 2009). Millions of people in the Organisation for Economic Co-operation and Development (OECD) countries are affected by food contamination every year (Rocourt, Moy, Vierk, & Schlundt, 2003). Food allergy cases explosively increased as measured by hospitalisations and self-report over the past 3 decades (Keet, 2018). Therefore, cross-contamination of food allergens in the food production chain should increase awareness towards prevention.

To lower the incidence of foodborne illness, the global food sector operates in a food industry where policies, standards, regulations, guidelines, education and advice relating to food, including those related to food allergen safety, are continuously being developed and updated (Kupińska-Adamczyk et al., 2018). In Ireland, the Food Safety Authority of Ireland (FSAI) was established as a statutory body under the Food Safety Authority of Ireland Act 1998 (FSAI, 2019). Its main role as stated in its founding regulations is to ensure that food produced, distributed, or marketed in Ireland meets the highest standards of food safety and hygiene reasonably available and to ensure that food complies with legal requirements and recognised codes of good practice. FSAI is also regularly linking up with the European Commission, the European Parliament and the European Food Safety Authority (EFSA). EFSA is an independent body of the European Union (EU). Its main function is to provide independent advice and perform risk assessment and communication on food and feed safety topics, on the basis of science, to support risk managers at EU level, including the European Commission (EC), European Parliament and EU Member States (EC, 2002). There are several food safety standards extensively adopted in the worldwide food industry, such as Hazard Analysis & Critical Control Point (HACCP).

HACCP is a systematic preventive approach to identifying, evaluating and controlling hazards, whether biological, chemical, or physical, in food processing chains that could pose a threat to the finished product and developing measures to reduce these risks to a safe level. Currently, the CAC, EU and United States (US) food legislation recommends HACCP as the most effective system to maintain the assurance of a food safety system (Beulens, Broens, Folstar, & Hofstede, 2005; Trienekens & Zuurbier, 2008). HACCP attempts to prevent hazards rather than end-of-pipe inspection. It addresses in all realms of the food industry, going to poultry, seafood, dairy, agricultural; ranging from growing, harvesting, processing, distribution and retail for consumption. It involves seven principles:

- conduct a hazard analysis (biological, chemical or physical, including allergen);
- identify critical control points (The points of a food processing chain at which the potential hazard can be prevented, eliminated, or reduced to an accepted level.);

- establish preventive measures with critical limits for each critical control point;
- establish procedures to monitor the critical points to ensure the process is under control at each critical control point;
- establish corrective actions to be taken when monitoring indicates a deviation from an established critical limit;
- establish procedures for verifying the HACCP system is working appropriately;
- establish record keeping procedures to document the HACCP.

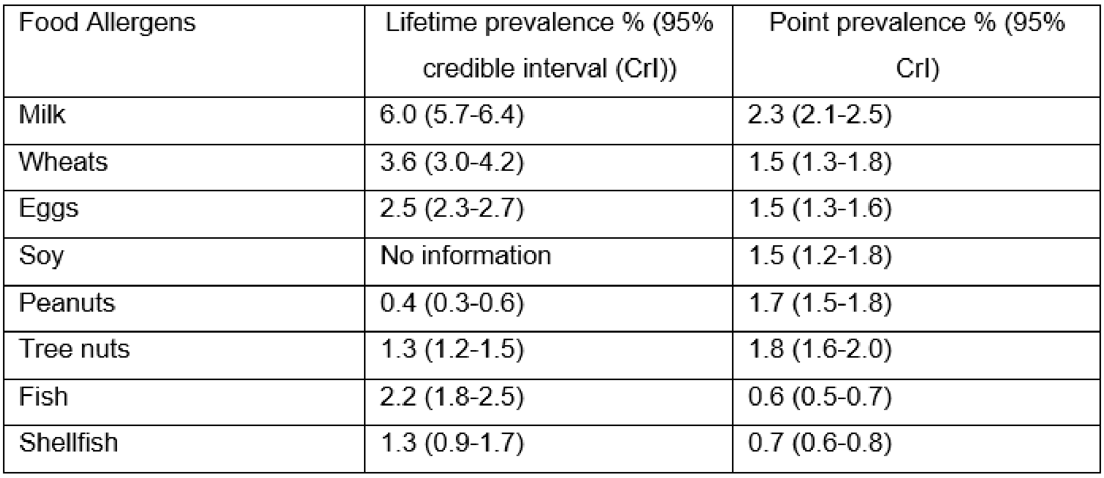
Good Agricultural Practices (GAP), Good Hygiene Practices (GHP) and Good Manufacturing Practices (GMP) are accepted as prerequisites or foundations for HACCP in the overall food safety management (Huss, Ababouch, & Gram, 2004).

Apart from the food safety standards, new food safety intervention strategies are required across the increasingly complex global supply chain (Nrusimhanath Misra, Schlüter, & Cullen, 2016). Sanitation methods are the conventional methods to maintain good industrial hygiene standards. However, more efficient strategies are still needed to reduce the food allergen safety risks of food products, while maintaining product quality characteristics with the combined goal of shelf-life extension. Consequently, the industry needs to be provided with viable options to meet its specific needs.

1.4 Food Allergy Review

Food allergens have become increasingly important to the food industry because they represent chemical hazards. The rise in allergies in recent decades has been particularly noticeable in Western countries. In 2013, it was reported that more than 17 million people in Europe suffer from food allergies, and 25% of school-age children in Europe suffer from allergic diseases (EAACI, 2013). A systematic review of self-reported lifetime prevalence of food allergy for all age groups was studied and is shown in Table 1.5 (Nwaru et al., 2014). In Europe, allergies to cow’s milk and egg were more common among children, while allergies to peanuts, tree nuts, fish and shellfish were more common among adults.

Table 1.5 Lifetime and point prevalence of food allergy for all ages (self-reported). Adapted from Nwaru et al. (2014).

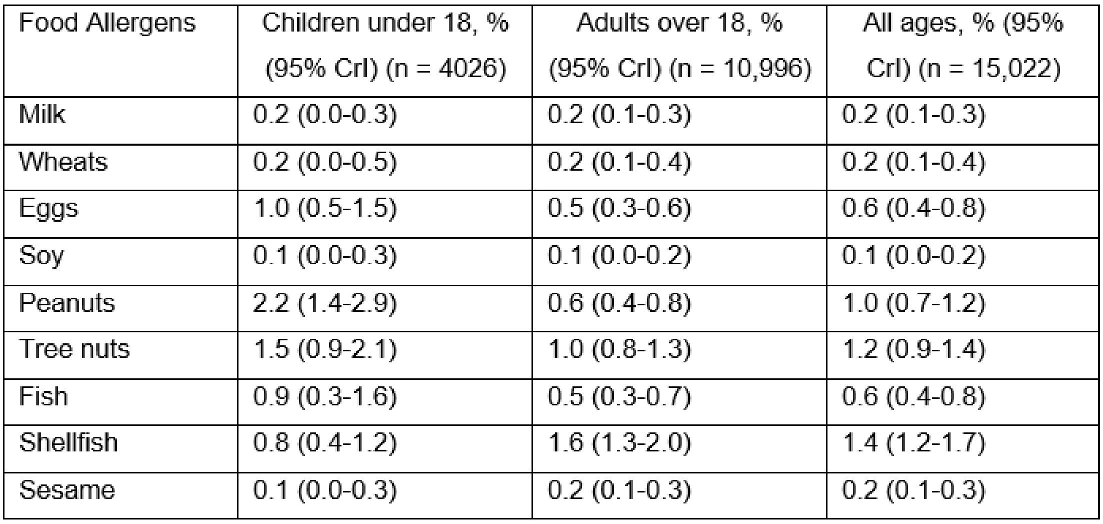


Food Allergens	Lifetime prevalence % (95% credible interval (CrI))	Point prevalence % (95% CrI)
Milk	6.0 (5.7-6.4)	2.3 (2.1-2.5)
Wheats	3.6 (3.0-4.2)	1.5 (1.3-1.8)
Eggs	2.5 (2.3-2.7)	1.5 (1.3-1.6)
Soy	No information	1.5 (1.2-1.8)
Peanuts	0.4 (0.3-0.6)	1.7 (1.5-1.8)
Tree nuts	1.3 (1.2-1.5)	1.8 (1.6-2.0)
Fish	2.2 (1.8-2.5)	0.6 (0.5-0.7)
Shellfish	1.3 (0.9-1.7)	0.7 (0.6-0.8)

In 2015, a telephone survey of over 5700 households (15,022 individuals) estimated the self-reported prevalence of food allergy in the United States and

Canada ranges between 8.1% and 9.1%, shown in Table 1.6 (Soller et al., 2015).

Table 1.6 Prevalence estimates for probable food allergy by age group in the United States and Canada (self-reported). Adapted from Soller et al. (2015).

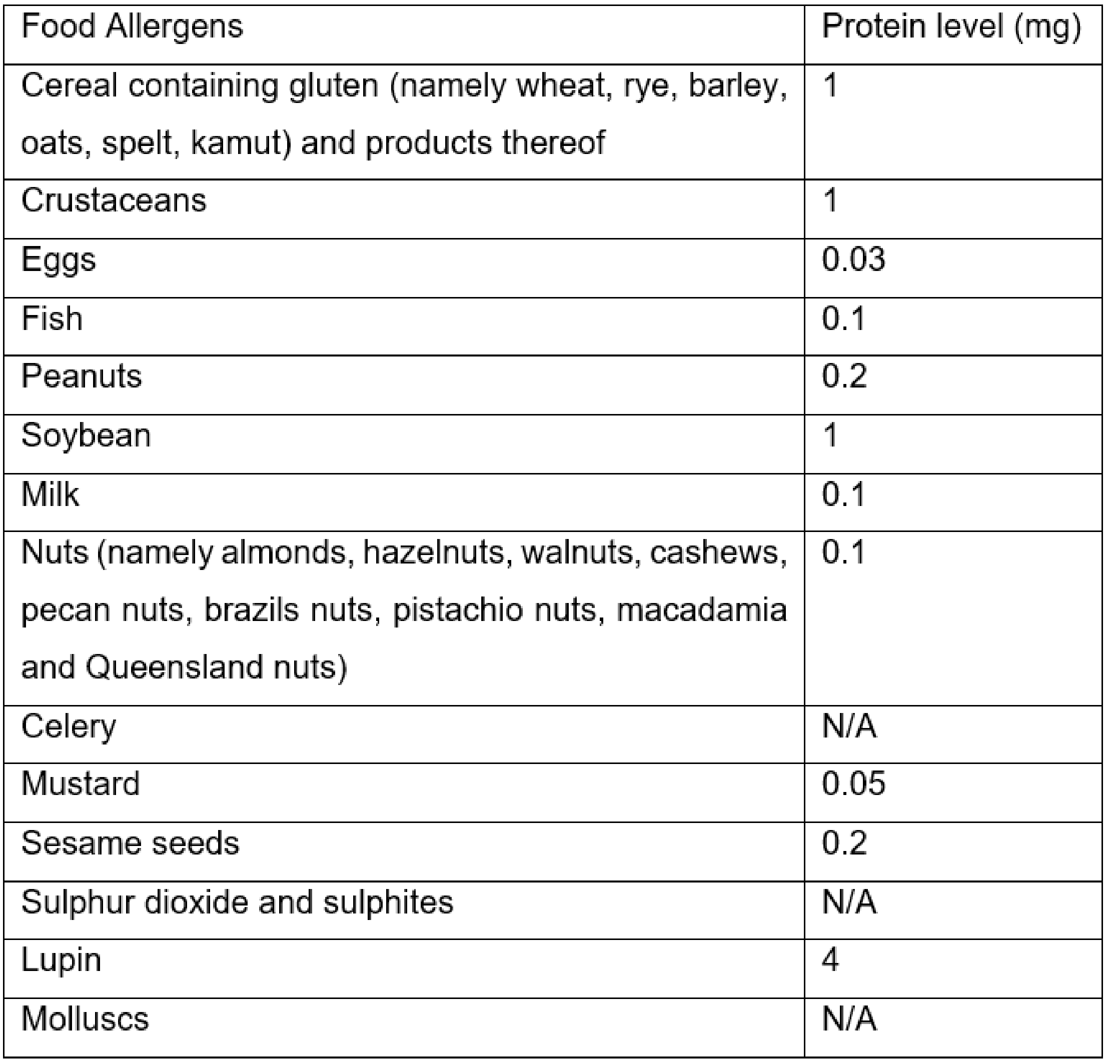


Food Allergens	Children under 18, % (95% CrI) (n = 4026)	Adults over 18, % (95% CrI) (n = 10,996)	All ages, % (95% CrI) (n = 15,022)
Milk	0.2 (0.0-0.3)	0.2 (0.1-0.3)	0.2 (0.1-0.3)
Wheats	0.2 (0.0-0.5)	0.2 (0.1-0.4)	0.2 (0.1-0.4)
Eggs	1.0 (0.5-1.5)	0.5 (0.3-0.6)	0.6 (0.4-0.8)
Soy	0.1 (0.0-0.3)	0.1 (0.0-0.2)	0.1 (0.0-0.2)
Peanuts	2.2 (1.4-2.9)	0.6 (0.4-0.8)	1.0 (0.7-1.2)
Tree nuts	1.5 (0.9-2.1)	1.0 (0.8-1.3)	1.2 (0.9-1.4)
Fish	0.9 (0.3-1.6)	0.5 (0.3-0.7)	0.6 (0.4-0.8)
Shellfish	0.8 (0.4-1.2)	1.6 (1.3-2.0)	1.4 (1.2-1.7)
Sesame	0.1 (0.0-0.3)	0.2 (0.1-0.3)	0.2 (0.1-0.3)

Allergies to peanuts, tree nuts, fish and eggs were more common among children, while allergies to shellfish were more common among adults, according to the study. Each year, many people are hospitalised due to food-related allergic reactions and individuals suffering anaphylaxis account for 30% of fatalities (Yue, Ciccolini, Avilla, & Waserman, 2018).

There are more than 160 foods known globally to produce allergic reactions. However, only 14 accounts for more than 90% of the reactions in Europe. The 14 most common food products that are required to be declared are listed in Annex II in regulation (EU) No 1169/2011 of the European Parliament and of the Council: cereal containing gluten (namely wheat, rye, barley, oats, spelt, kamut), crustaceans, eggs, fish, peanuts, soybeans, milk, tree nuts (namely almonds, hazelnuts, walnuts, cashews, pecan nuts, brazil nuts, pistachio nuts, macadamia and Queensland nuts), celery, mustard, sesame seeds, sulphur dioxide and sulphites, lupin and molluscs, shown in Table 1.7 (Commission, 2011; Kupińska-Adamczyk et al., 2018). A study was conducted to determine the thresholds of food allergens' reactivity shown in Table 1.7.

Table 1.7 Eliciting doses (ED) for common allergens causing allergies or intolerances. Adapted from Commission (2011); Kupińska-Adamczyk et al. (2018).



Food Allergens	Protein level (mg)
Cereal containing gluten (namely wheat, rye, barley, oats, spelt, kamut) and products thereof	1
Crustaceans	1
Eggs	0.03
Fish	0.1
Peanuts	0.2
Soybean	1
Milk	0.1
Nuts (namely almonds, hazelnuts, walnuts, cashews, pecan nuts, brazils nuts, pistachio nuts, macadamia and Queensland nuts)	0.1
Celery	N/A
Mustard	0.05
Sesame seeds	0.2
Sulphur dioxide and sulphites	N/A
Lupin	4
Molluscs	N/A

1.4.1 Food Allergy and Intolerance

Consumption of food allergens can cause various disorders in allergic sufferers. The definitions of food allergy and food intolerance are distinctive.

Food intolerance occurs through non-immunological reactions to food, such as those involving enzyme defects (such as lactose intolerance) or pharmacological reactions to certain substances (such as vasoactive amines) (Hayder, Mueller, & Bartholomaeus, 2011). Symptoms of food intolerance are reported to affect the skin, gastrointestinal tract, respiratory tract and central nervous system (Hodge, Swain, & Faulkner-Hogg, 2009). There is a wide range of symptoms reportedly associated with food intolerance including headache, urticaria, fatigue, behavioural problems, irritable bowel, asthma and migraines. Food intolerance is far more common to occur.

True food allergy, known as hypersensitivity, is a reproducible adverse immune reaction to a specific food that affects numerous organs in the body (Valenta, Hochwallner, Linhart, & Pahr, 2015). This reaction comprises basically all types of immune-mediated reactions, including those caused by the adaptive and innate immune system (Panel, 2010). Food allergic reactions are classified into four forms: type I, II, III and IV reactions. Moreover, the immune-mediated food allergic reaction can be further categorised into immunoglobulin E (IgE)-mediated and non-IgE-mediated allergic reactions (Asero et al., 2007). The type I reactions categorise into IgE-mediated allergic reactions, while type II, III and IV reactions categorise into non-IgE-mediated allergic reactions.

The most common forms of immune-mediated adverse reactions to foods (type I reactions) are characterised by the production of IgE against food allergens (Longo, Berti, Burks, Krauss, & Barbi, 2013). During the initial exposure to the food allergen, the allergen, as an antigen, binds to T cells which subsequently activate B-cells. The B-cells produce and release IgE which is cross-linked to mast cells or basophils (Tanabe, 2007). This process is known as sensitisation. Upon re-exposure to the allergen, IgE bound to mast cells or basophils cross links with the allergens which cause degranulation of the mast cells or basophils. Vasoactive mediators such as histamine and prostaglandin are released that ultimately cause inflammation in the individual.

Type II reactions involve food allergen-specific immunoglobulin G (IgG)- and immunoglobulin M (IgM)-mediated cytotoxic response against cell surface and extracellular matrix proteins. The immunoglobulins involved in type II reactions destroy cells via complement activation or phagocytosis. Type III reactions are also mediated by IgG and IgM that bind with soluble food allergenic fractions forming antigen-antibody complexes. The complexes in various tissues induce complement activation and an ensuing inflammatory response mediated by massive infiltration of neutrophils via the release of chemotactic agents. Although food antigen-specific IgG can cause adverse reactions via type II or type III in theory, there is no solid experimental evidence to support the relevance of these reactions to food allergies that develop in patients. Moreover, several papers recommends not using food antigen-specific IgG as a food allergy diagnostic tool (Ballmer-Weber & Knol, 2010; Bock, 2010).

Type IV reactions are allergen-specific T cell-mediated hypersensitivity that can damage the gut mucosa, which is associated with disorders such as coeliac disease and food protein-induced enterocolitis. Sensitised T helper type 1 cells release cytokines that activate macrophages or cytotoxic T cells that mediate direct cellular damage. T helper type 2 cells and cytotoxic T cells mediate similar responses. Coeliac disease is characterised by a hypersensitivity reaction against the wheat gluten peptide composed of alcohol-soluble gliadin and acid- and alkali-soluble glutenin subunits, accompanied by an autoimmune component (Schuppan, Junker, & Barisani, 2009).

Food protein-induced enterocolitis is characterised by a food allergen-induced inflammation via direct activation of the innate immune system (Ruiter & Shreffler, 2012). Figure 1.2 illustrates the broad classification of adverse reactions to food.

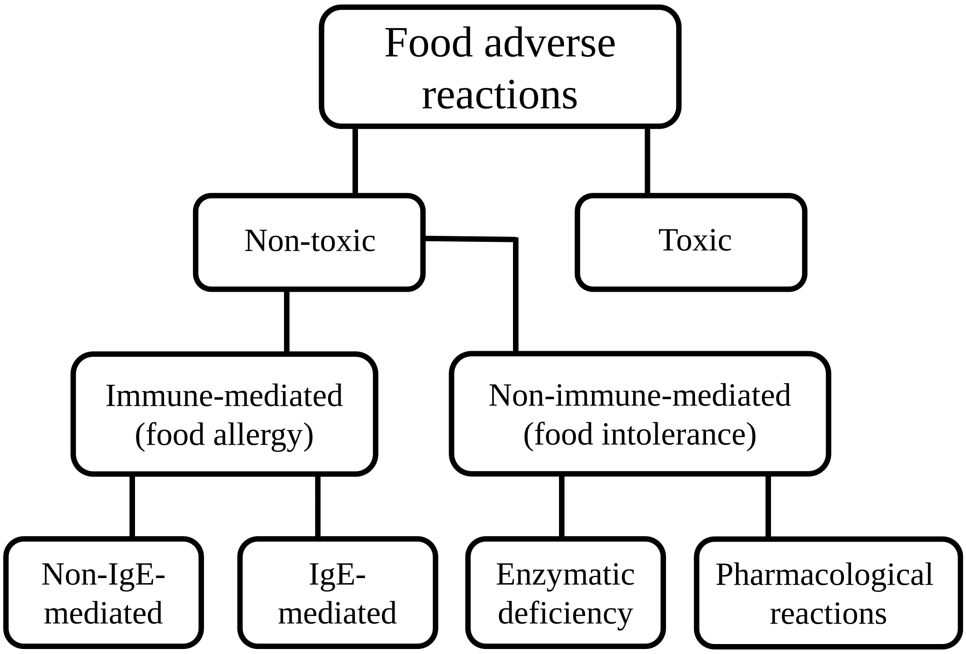


Figure 1.2 Classification of adverse reactions to food.

Food allergies are the leading cause of anaphylaxis, a severe allergic reaction that is potentially fatal. Symptoms of anaphylaxis can affect either localised or entire bodies, and they may impact each body system differently. Although not every allergic reaction will provoke anaphylaxis, each allergic case should be taken very seriously because any episode can result in severe anaphylaxis. An indication of a mild allergic reaction affecting the skin can include visible irritation of the skin, including swelling of the lips, tongue or face and hives on the skin. More severe symptoms involve the respiratory system, including asthma via swelling of the throat, tightness in the chest or coughing. Gastrointestinal tract symptoms with mild reactions include vomiting, diarrhoea, nausea, abdominal pain; and severe reactions include coeliac disease and enterocolitis. A severe case of food allergies can cause anaphylaxis, a condition involving multiple organs in the body that requires immediate medical attention. Every allergic sufferer responds differently to his or her offending food depending on the individual's overall health status. For example, people with asthma tend to have a more serious reaction after consuming the offending food allergens (Caffarelli, Garrubba, Greco, Mastrorilli, & Povesi Dascola, 2016). An allergic reaction can occur within minutes of ingesting the food or up to one hour after ingestion. Factors, such as food intake and digestion time, can determine the onset and location of symptoms. The onset of these symptoms may begin right away or take up to 2 hours after exposure to the food allergen, but life-threatening reactions may worsen after several hours upon ingestion.

Once an individual is diagnosed with one or more food allergies, the only prevention-based treatment available to date is the safest and most economical method for eliminating the foods that trigger the immune reaction. Sometimes, allergy-producing ingredients are used in foods that people would not normally associate with them. For instance, almonds or hazelnuts could be used as protein sources or dressings containing milk. There have been cases where food allergy reactions have occurred due to undeclared ingredients on labels. For this reason, regulation (EU) No 1169/2011 of the European Parliament and of the Council was established to declare food allergens on the labels. Under this law, food manufacturers are required to clearly label foods that are or contain one or more of the top 14 food allergens. This law is extremely important because food allergic sufferers do not have any preventive approaches to avoid food allergies other than avoiding the food entirely. However, some precautionary labelling is not sufficiently regulated at the EU level (EAACI, 2013). For example, some foods have the label “may contain milk” or “may contain peanuts”. Labelling is an essential aspect of managing food allergies, especially since such precautionary labelling can also imply the possibility of high levels of contamination and high levels of risk. In order to do so, the EU should set clear guidelines for labelling food products for allergens. This includes products with vague labelling (for example, they may contain allergen derivatives). This should be clearly linked to the name of the allergen to avoid confusion. Manufacturers should have efficient and effective GMP in place to remove all detectable allergenic residues from food processing surfaces in order to prevent cross-contamination (S. L. Taylor & Baumert, 2010).

1.4.2 Food Allergens

Food allergies are caused by adverse immunological responses to proteins, or antigens, which are components of food matrices. In the case of foods, these antigens are known as food allergens (Aalberse, 2000). The majority of food allergens are water-soluble glycoproteins in the range of 10 kDa to 70 kDa of molecular weights (Ebo & Stevens, 2001) and are often characterised by having the following characteristics: the ability to sensitise a genetically predisposed individual by triggering the production of IgE antibodies, the ability to bind those particular IgE antibodies, and the ability to cause an allergic reaction following

IgE binding (Aalberse, 2000). However, some allergens, such as gluten, associated with Coeliac disease, can be non-IgE-mediated, where the reaction is arbitrated by the lymphatic system (Sabra et al., 2003).

Extensive proteomic studies have been conducted over recent decades to identify the actual cause of food allergies. According to these studies and state of the art of food allergy, it is now commonly understood that IgE binds to the specific site on the allergen or the surface of the allergenic protein known as an epitope (Tanabe, 2007). The epitope is categorised into linear or conformational epitopes depending on the epitope structure (Bannon, 2004; Davis & Williams, 1998). A linear epitope is an epitope that has a portion of continuous amino acids along a protein, while a conformational epitope is an epitope that has 3-dimensional folding of a protein (Tanabe, 2007). The cross reactivity between the allergen epitope and the antibody is determined by the conformational structure of the allergen protein and the sequence of the amino acids.

In order to fully understand allergen epitopes, a short review from macroscopic to microscopic levels of allergen epitopes is essential. The first macroscopic level of proteomics is the quaternary structure. Quaternary structures are characterised by more than one polypeptide chain linked with each other via non-covalent interactions (Damodaran, 2008). The tertiary structure is a 3-dimensional organisation whereby the linear protein chain with secondary structure segments folds into a 3-dimensional arrangement via disulphide bonds. The secondary structure refers to the locally folded 3-dimensional segments that form within polypeptides. The secondary structural elements are α-helices, β-sheets, β-turns and random coils. The secondary structural elements are formally stabilised and defined by the pattern of hydrogen bonds between the amino hydrogen and carboxyl oxygen atoms within a single protein chain. The α-helix resembles a curled ribbon. The β-sheet is in the form of zigzag. The β-turn is in the form of peptide plane flipping (180° rotation of carbonyl oxygen and amino hydrogen peptide plane, with little positional alteration to side chains and surrounding peptides). The random coil is a non-regular pattern along the polypeptide chain. This structure is formed when amino acid side chains prevent the formation of α-helices or β-sheets. The final microscopic level is the primary structural component of proteins. The primary structure refers to the linear sequence of amino acids connected to each other via peptide bonds. A linear epitope in allergens is composed of multiple short amino acid sequences. A conformational epitope in allergens is composed of various amino acids on the polypeptide chain that are brought together by folding or identified by secondary, tertiary, or quaternary structures of the protein. Therefore, studies of IgE binding to the linear or conformational epitope, such as secondary or tertiary structure, of a protein are necessary (Bannon, 2004). During heating, hydrolysis and other physical or chemical treatments, there can be a significant effect on protein structure. Some of the proteins' secondary and tertiary structures are degraded, exposing smaller amino acid sequences to the exterior of the protein (Sathe & Sharma, 2009). However, there are hydrophobic sites on proteins, which hide the linear epitopes from surface water, other solvents or binding to the IgE (Sampson, 1999; Tanabe, 2007). The linear epitopes are exposed upon denaturation. Conversely, this denaturation can cause conformational changes and further destruction of conformational epitopes (Rahaman, Vasiljevic, & Ramchandran, 2016). It is possible that denaturation deriving from an applied process can increase or decrease antigenicity.

A study involving 36 milk-allergic children reported that patients had a significantly higher ratio of specific IgE reactivity to linearised epitopes than to the allergen’s native state, which is known as conformational epitope (Vila et al., 2001). The linear epitopes of allergens were exposed by denaturing the secondary and tertiary structures of the native milk proteins (α- and β-caseins) via boiling with dithiothreitol (DTT). A similar study was done to observe the IgE binding to linear and conformational epitopes of egg proteins (ovomucoid and ovalbumin) (K. M. Järvinen et al., 2007). The egg-allergic patients had higher IgE reactivity to both linear and conformational epitopes of ovomucoid and ovalbumin. The authors also speculated that crosslinking between IgE and linear epitopes must be considered in order to identify the actual allergenic nature of food proteins. Since the allergen protein undergoes digestion upon ingestion, which includes enzymatic hydrolysis, the linear epitopes are exposed in the gut via the denaturation of secondary and tertiary structures of proteins (De Zorzi, Curioni, Simonato, Giannattasio, & Pasini, 2007; Pasini, Simonato, Giannattasio, Peruffo, & Curioni, 2001).

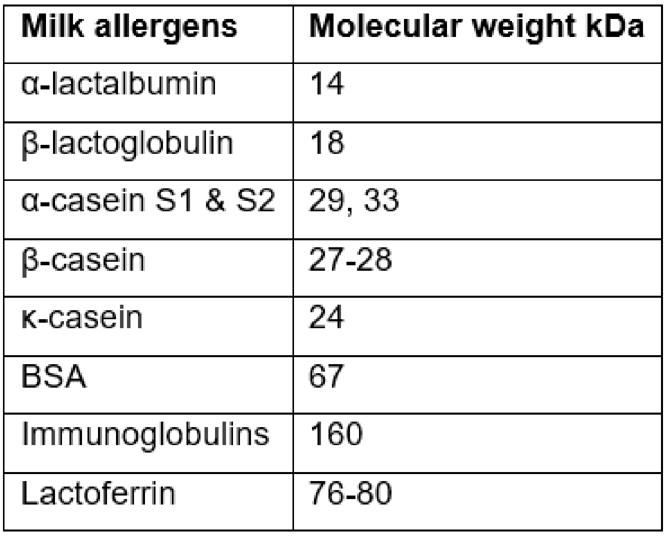
Many food allergens are resistant to denaturation, which allows them to retain the ability to trigger immune reactions under conditions such as heating and enzymatic degradation (Ebo & Stevens, 2001). Thus, proteins that are resistant to digestive conditions or high heat can enter the gastrointestinal tract with unchanged linear or conformational epitopes and cause an allergic response. For example, tropomyosin, a major allergenic fraction in shellfish (Shanti, Martin, Nagpal, Metcalfe, & Rao, 1993), and ovomucoid, a major egg allergen

(Bernhisel-Broadbent, Dintzis, Dintzis, & Sampson, 1994), are both heat-stable formations.

1.4.2.1 Milk and Milk Allergens

Milk allergens account for approximately 2% of food allergies in children ages 2 and under, but most of them become tolerant past 6 years of age (Poms, Klein, & Anklam, 2004). After the age of three, 85% of children have the tendency to outgrow cow’s milk allergy (Vila et al., 2001). Bovine milk protein is a prevalent source of antigens encountered in large quantities in infancy (Isolauri & Turjanmaa, 1996). The major allergens present in bovine milk are α-, β-, κ-caseins, while other proteins, such as bovine serum albumin (BSA), bovine immunoglobulins and lactoferrin (present in trace amounts) are also potential allergens (Fritsché, 2003; Poms et al., 2004; Sharma, Kumar, Betzel, & Singh, 2001). Most proteins in milk, including allergens, are glycoproteins (O'Riordan, Kane, Joshi, & Hickey, 2014). Approximately 80% of milk protein is casein and the remaining 20% comprises whey and other proteins. Table 1.8 lists the molecular weights of all milk allergens (Verhoeckx et al., 2015).

Table 1.8 Molecular weights of milk allergens. Adapted from Verhoeckx et al. (2015).



Milk allergens	Molecular weight kDa
α-lactalbumin	14
β-lactoglobulin	18
α-casein S1 & S2	29, 33
β-casein	27-28
κ-casein	24
BSA	67
Immunoglobulins	160
Lactoferrin	76-80

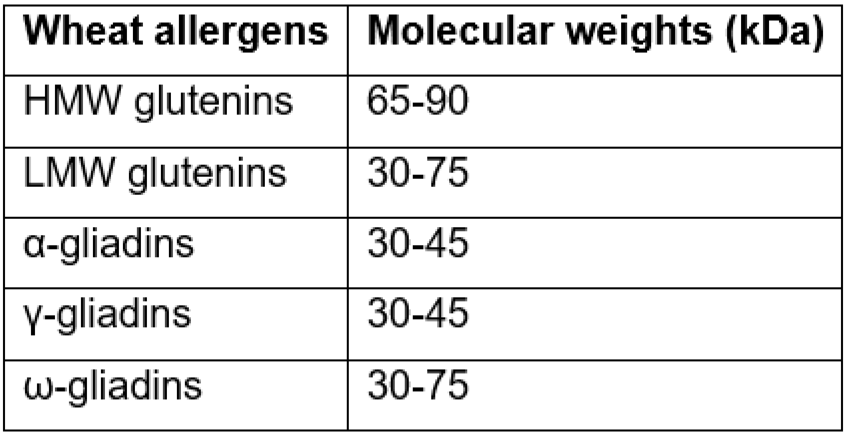
Generally, bovine milk is extensively processed prior to consumption in developed countries. Liquid raw milk consumption is rare, although raw milks are often included in regional lacto-fermented food products. Hypoallergenic milk products are vastly researched and have the potential to keep infants safe. Several attempts have been made to reduce the allergenicity of milk proteins using different technologies, including heat treatment. Most commonly used heat treatment conditions are: pasteurisation (heating milk to 60-70 °C for 30 minutes, 70-80 °C for 15-20 seconds), sterilisation (110-120 °C for 10-20 minutes) and ultra-high temperature (UHT) (135-145 °C for 0.5-4 seconds) (Claeys et al., 2013). UHT processing is extensively utilised in Europe because it inactivates bacteria and spores without the occurrence of Maillard reactions. This minimises the impact on taste and colour of the milk (Tamime, 2009). To produce condensed milk, milk is condensed by vacuum evaporation (Kasinos, Le, & Van Der Meeren, 2014). To produce milk powders, the milk concentrate is spray dried with a controlled flow of hot air (200 °C) (Schuck, le Floch-Fouere, & Jeantet, 2013).

Researchers found that each allergen or protein fraction had different resistance to heat treatments. Casein is a heat-stable allergen, while whey proteins are heat-labile allergens (El-Agamy, 2007).

1.4.2.2 Wheat and Wheat Allergens

Food allergies to wheat are prevalent in both children and adults. Allergic reactions to wheat have been categorised as immediate (within 2 hours) or delayed (within 1 week) (Majamaa, Moisio, Majamaa, Turjanmaa, & Holm, 1999). Some allergic reactions reported in patients allergic to wheat include atopic dermatitis, nausea, abdominal pain, anaphylaxis and gastrointestinal symptoms. Wheat proteins are mainly divided into four groups based on their solubility: water-soluble albumin (15% of the total); salt-soluble globulin (5%); and 70% ethanol-soluble prolamins (seed storage proteins rich in proline and glutamine), which include gliadin (40%) and acid- or alkali-soluble glutenin (40%). Gliadin subunits comprised of α-, γ- and ω-gliadin and glutenin subunits comprised of high molecular weight (HMW) and low molecular weight (LMW) glutenin subunits are the constituents of gluten, shown in Table 1.9.

Table 1.9 Wheat allergens. Adapted from EFSA Panel on Dietetic Products and Allergies (2014).



Wheat allergens	Molecular weights (kDa)
HMW glutenins	65-90
LMW glutenins	30-75
α-gliadins	30-45
γ-gliadins	30-45
ω-gliadins	30-75

The gluten protein fragments have different resistance to heat treatment. Gliadin is a heat-labile allergen (Rumbo, Chirdo, Fossati, & Añón, 2001); while glutenin is a heat-stable allergen (Branlard et al., 2020). Allergic reactions to wheat have been identified in various wheat protein fractions. The α-amylase/trypsin inhibitor (12-18 kDa) was determined as one of the major allergens present in water/salt soluble albumin and globulin fractions (Simonato et al., 2001). ω-5-gliadin has been reported as the major allergen in gliadin subunits responsible for allergic reactions in children (Palosuo et al., 2001).

Some wheat allergen reactivity appears to be stronger in baked wheat bread compared to unbaked dough (Pasini et al., 2001; Simonato et al., 2001). In one case, the researchers speculated that an increase in allergen reactivity was attributed to browning reactions that caused modification of the allergens and enhanced IgE-binding epitopes (Simonato et al., 2001). Another study found that the digestibility of the allergens decreased after baking, possibly caused by aggregation and crosslinking, which made the proteins insoluble for digestion (Pasini et al., 2001).

1.5 Food Allergen Management in Food Industry

1.5.1 Allergens in Food Supply Chain

Figure 1.3 illustrates the pathway of raw materials, from land, sea or farm, transforming into end food products that the end consumer can finally consume (Wallace, Sperber, & Mortimore, 2018).

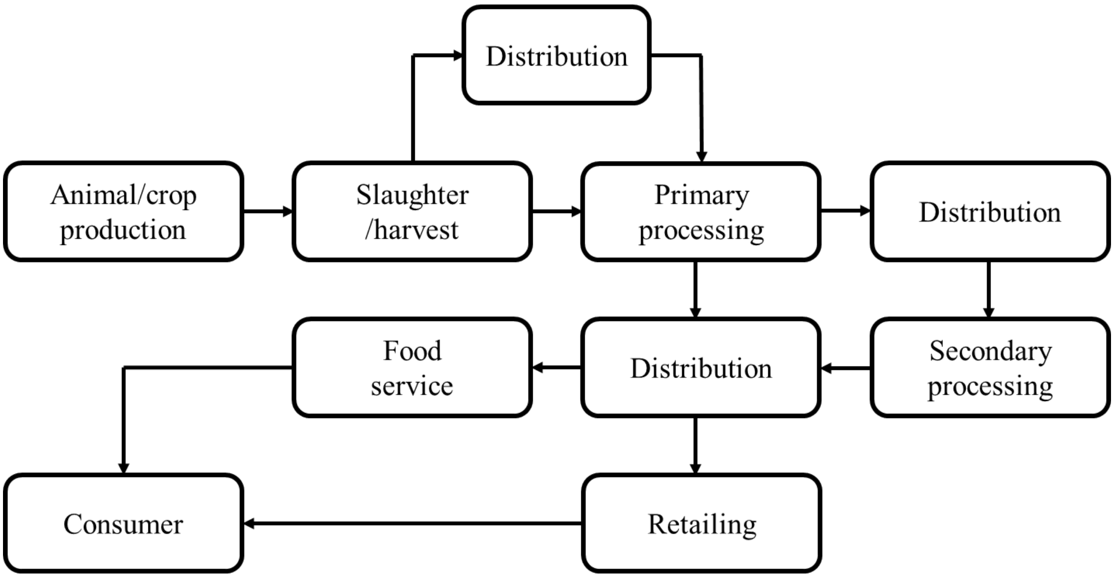


Figure 1.3 Food supply chain model. Adapted from Wallace et la. (2018).

Allergen hazards to consumers from the food supply can be categorised as arising from the following:

1. Mispack, for instance putting a product in the wrong packaging;
2. Inadequate labelling of allergenic ingredients;
3. Cross-contamination or cross-contact.

These categories are the root causes for recalls (Gendel & Zhu, 2013). Allergen cross-contamination can arise at any point in the food chain (S. L. Taylor & Baumert, 2010):

1. Shared farm fields, harvesting equipment and on-farm storage facilities;
2. Shared off-farm storage facilities, where relevant, such as grain elevators;
3. Shared transportation vehicles for transporting agricultural crops to processing sites;
4. Shared processing facilities and shared processing equipment within those facilities;
5. Shared food preparation facilities, equipment and cookware.

Allergen cross contamination is very difficult to control, however, it can be reversed by product-sorting practices at food processing facilities, but sorting is impossible with small materials of relatively similar size (such as oat and wheat grains). The use of shared storage facilities and transportation vehicles for bulk commodities also contributes to the likelihood of cross-contamination. Food manufacturers often use shared equipment and facilities for the production of processed foods with a variety of different formulations. In most cases, one or several formulations contain a particular allergen. Therefore, proper management of the use of such shared equipment and facilities is necessary to prevent cross-contamination that could allow potentially hazardous residues of a food allergen into another non-allergenic product (Hefle & Taylor, 2004).

Food products can also be semi-finished or finished complex processed recipes made with a variety of ingredients. Each step of these processes would modify or leave a trace on the product which, in the case of allergen presence, should be listed either on a label, if the product is prepacked, or documented if the product is not packed; thus, modification or any other process should be noted. It is recognised that in some manufacturing operations, due to a variety of factors, total avoidance of cross contact is not always practicable; therefore, it might well be that specific allergens which are not part of the ingredients by design end up as part of the formulation (Madsen et al., 2010). While large food manufacturers are usually aware of the allergen situation and have in place appropriate measures of control, it is not necessarily the same for small scale companies. Lack of knowledge and very limited budgets are factors which do not always allow for the assessment of the situation of the production site (Boye & Godefroy, 2010).

By European regulation, food suppliers are required to have production methods in place to guarantee food safety at each and every step of the process and like any business operator in the supply chain, are responsible for ensuring that foods satisfy the requirements of food law (Popping & Diaz-Amigo, 2018). With a strict management system under Regulation (EC) 178/2002, safe products should be the result of adequate control at each step of the supply chain rather than complicated corrective actions further down the process (EC, 2002). This will ensure that the previous supplier has in place a food safety management system that will guarantee the delivery of safe food with accurate information which will assist further processors to produce food that will have no adverse effect on the consumers, even on food allergic sufferers. These systems are intended to reduce risks to an acceptable level. The HACCP plan, which is developed to address the identified risks, has strategic value in improving relations in the supply chain. Effective communication between the supplier and the customer is critical to demonstrate that the supplier understands the intended and possible requirements of the product (Surak, Cawley, & Gavoor, 2007). Within the EU, HACCP has been a favoured food safety management system and it is a legal requirement for all businesses to manage food safety according to its principles (Commission, 2004). Different food legislation across the globe adds to the complexity of food allergy management.

Throughout the whole supply chain suppliers are important guardians of food safety management and are required to ensure that their supplies will not cause any harm to consumers due to their inadvertent lack of transfer of the accurate information to the third party (EC, 2002). This is communicated through labelling in prepacked foods, as required by law (Ward et al., 2010). The potential risks of cross contamination from mishandling activities at the raw material suppliers' sites and during transportation also needs to be taken into consideration when approving reputable suppliers (FSA, 2008). Small companies might find that ensuring the products bought from suppliers are safe could create a burden on the practice employed to run their operations (E. Taylor, 2001). Food manufacturers are closely regulated by the EFSA who want assurance that the food purchased is safe. To achieve this level of confidence, the agencies could request an onsite and operational audit of the food manufacturer’s facility (Kotsanopoulos & Arvanitoyannis, 2017). This could work well for large food businesses. However, for small companies, it is difficult for suppliers to carry out an on-site food safety audit of the facility due to technical problems (E. Taylor, 2001). In theory, a certificate of compliance to food safety management by the effective application of a HACCP system (Losito, Visciano, Genualdo, & Cardone, 2011) endorsed by a reputable accreditor, such as a certification body, can provide assurance that the products meet the required standards including allergen-free products (Boye & Godefroy, 2010), although this might not be true in practice, as many intermediate factors could change the state of the product. When it comes to auditing, small companies may find it difficult to obtain such certificates of compliance, particularly if they are also buying from small suppliers.

1.5.2 Centralised Food Processing Units for Allergen Management

As costs of industrial cooking equipment rise and the physical space is limited by factors such as finance, food business operators with such constraints might opt to engage the services of a central production unit (CPU) to prepare finished or semi-finished meals that contain allergens before these are served to the end consumer. Food production is a complex process that involves many situations where the equipment used for production could be shared between various food products. From transportation of raw materials through manufacturing and final processing, small amounts of food allergenic ingredients could inadvertently be introduced into the food which could possibly pose a risk to allergic individuals (Madsen et al., 2010). If properly managed, remote food production could ensure improved consistency and greater control of food safety. This depends greatly on the commitment of the management to verify that the supplies and their operations are being controlled and monitored and that any deviations are immediately corrected. This principle is well established amid food safety systems with the intention of proactively ensuring that the food is produced with quality assurance rather than quality control (Coutts & Fielder, 2009). In other words, the food is assured to be safe rather than tested and determined as safe. This principle is also relevant to food allergens. Although most of the preparation is done offsite, the obligation to provide safe food still lies with the end food manufacturers. This practice puts small companies dependent on the food safety management of the CPU and thus at a greater risk due to a lack of direct control over food production.

To this effect, food manufacturers should have in place a food allergy management policy robust enough to reflect the intricacy of the operation (Madsen et al., 2010). Prepared meals need to have documentation that would declare their ingredients and other information that is mandatory and legally required by EU regulation 1169/2011. Further preparation should ensure that the information delivered by the CPU is transmitted accurately throughout the receiving operation and that the information is accurately recorded. The food processing industry needs to consider the impact that the food provided by a CPU has on its own operations to avoid allergen cross-contamination (Madsen et al., 2010).

1.5.3 Food Industry Regulation for Allergen Management

The European Union (EU) Regulation 1169/2011 addresses the provision of food information to the consumer with the scope of providing a basis for the assurance of a high level of protection of the consumer’s information needs, including non-pre-packed foods, also known as loose food. Article 44 of the same regulation states that the provision entrenched in Article 9(1) refers to, “any ingredient or processing aid listed in Annex II or derived from a substance or product listed in Annex II causing allergies or intolerances used in the manufacture or preparation of a food and still present in the finished product, even if in an altered form;” needs to be clearly declared to the consumer in order to provide an assurance of a high level of protection in relation to the food information (Commission, 2011). Food manufacturers are legally bound to devise systems to ensure that the food they supply is safe (EC, 2002). The same regulation also goes on to state that it aims to protect the interests of the consumer and that it would provide a basis for the consumer to make informed choices about the food to be consumed. Article 14(4)c states that food should not be placed for sale if it could be injurious to particular health sensitivities of a specific category of consumer, where the food prepared is intended for that category of consumer. This suggests that when food is prepared for allergic or intolerant consumers, it should not in any way put their health at any risk of injury. The information provided should help consumers determine if the consumption of a particular food or a category of foods could have an adverse health effect (EC, 2002) This clearly puts a moral responsibility on the food producer to offer only food that is safe to the consumer, including sensitive individuals.

HACCP systems identify food safety hazards and through control and monitoring, help ensure food safety. It is therefore logical that allergens should be controlled through the HACCP plan, which is under the control of chemical hazards and thus control of allergens in food service should not be a new concept. Regulation (EC) 854/2004, Article 3 requires that onsite visits by competent authorities be held to approve the operation, however it has been noted that Environmental Health Officers (EHOs) have limited knowledge of allergen control (I. S. Leitch, Walker, & Davey, 2005). This could indicate that when designs are approved, little if any consideration is given to the control of these foods within a food business. This might be due to oversight by inspectors who may look at HACCP plans or food safety systems and not necessarily spot the absence of allergen control. To rectify this recognised deficit, specialised food allergy training was initiated in 2006 with the aim of delivering knowledge of allergen control to EHOs. The scope was to equip EHOs with new knowledge and skills that could flow down to the food service industry (I. Leitch & McIntosh, 2014). So far, no evaluation of this training has been published.

Food safety systems need to be just as complex as the food industry itself and should only be installed by knowledgeable individuals. As small scale companies might tend to obtain such information or systems via consultants, it is important that such information is accurate and written by a reputable source (Wallace et al., 2018). Skilled trainers who can interpret the application of HACCP to small companies are seen as an important factor in ensuring a successful implementation of a food safety management system based on the principles of HACCP which will include the identification, control and management of food safety hazards including food allergens (Mayes, Mayes, & Mortimore, 2001).

1.5.4 Allergen Control Systems

Even trace amounts of allergen residues in products may pose an adverse reaction to consumer health. In response, food manufacturers are increasingly focusing on allergen control systems that minimise or eliminate the use of allergenic substances. Food manufacturers are solely responsible for making statements on the absence or presence of allergens in food products and for assuring that no cross-contamination with allergens occurs to non-allergenic food products during the production process. Food products without allergens can be labelled with words (for example gluten-free products or lactose-free) to indicate that the consumer may enjoy allergen-free food. Food products with allergens must also be labelled and listed in the ingredient list.

The allergen risks in the food industry comprise product ingredients and accidental cross-contamination from production processes and shared facilities, reusable equipment, untrained employees, technological and packaging processes, cleaning and disinfection activities. One of the key factors in ensuring food safety is to effectively assess the risk of food allergen cross-contamination at every stage of food production, considering all ingredients, additives, personnel, workwear, machinery and equipment, packaging, transportation, environmental hygiene and the risk of cross-contamination.

There are two fundamental approaches to control allergen contamination in production processing: dedication-harmonisation and allergen control management (Jackson et al., 2008). Dedication-harmonisation refers to the design and operation of production processes so that all products being produced either are allergen free or contain the same allergen(s). However, this approach forces many food companies to produce similar food products with dissimilar allergen content in the same manufacturing facilities and on the same production processes due to insufficient product volume and high cost of equipment and personnel (Deibel et al., 1997). Therefore, those companies that manage the variety of products produced in their production process must implement allergen control systems to ensure that the dissimilar allergens do not contaminate those products where such allergens are not desired.

Allergen control systems must be implemented by food manufacturers in order to ensure food safety including Good Manufacturing Practice (GMP), Good Hygiene Practice (GHP), Hazard Analysis and Critical Control Points (HACCP) and Allergen Control Plan. The allergen risk analysis (which involves biological, physical and chemical factors and food production conditions that may pose a risk to consumer health) should be assessed for all areas of the facility and all stages of the production process where the allergens may be present.

Food safety management system (FSMS) is one of the larger management systems used to evaluate food production. It provides programmes, plans, policies, procedures, practices, goals, objectives, methods, controls, roles, responsibilities, relationships, documents, records and resources. FSMS relies on trained employees to implement HACCP to ensure that allergen handling is done correctly according to the food safety plan addressing the appertaining issues. HACCP is one of the most widely used tools in the food industry for the prevention of food safety hazards through FSMS based on product design, hazards analysis and process control. FSMS implementing HACCP are intended to control food safety hazards which are mainly divided into three categories (physical, biological and chemical). Allergens are considered chemical hazards. The EU list of allergens, as entered in Annex II of the most recent Regulation EU 1169/2011, are common food products in many food businesses that cause allergic reactions to consumers in Europe. All food industries, including restaurants, are now obliged to provide information on allergens to all customers as part of their HACCP plan. HACCP principles have been used for many years as a tool to assess the risks associated with food production. It is very common that food industries get a sense of having adopted HACCP when in fact these would have in place prerequisite programmes (PRPs). Although PRPs provide the hygienic foundation for many food industries, HACCP is not a mere sanitation programme (Wallace et al., 2018). Used correctly, HACCP principles together with the application of good hygiene practices should eliminate or reduce risks to acceptable levels (Commission, 2004). Supported by documentation, where necessary, to provide evidence of due diligence, HACCP provides the key allergen control of the food throughout each stage of food production from primary production up to the final consumer. A study of food industry professionals surveyed from the United States, Asia, Australia, Canada, Central America, Europe, Mexico, South America, United Kingdom and other countries reported that their companies manufactured products containing at least one allergen, and nearly all had an allergen control plan in place (Gupta et al., 2017). The allergen control plan is meant to protect the health and confidence of consumers. It is also about protecting the financial health and reputation of the company (FARRP, 2019). The allergen control plan should categorise six elements as below (Jackson et al., 2008).

- Allergen control team and training
- Segregation of allergenic foods or ingredients during storage, handling and processing
- Supplier control programs for ingredients and labels
- Prevention of cross-contamination during processing
- Product label review; label and packaging usage and control
- Validation allergen cleaning program

1.5.5 Allergen Labelling

In some cases, food manufacturers do not follow good manufacturing practices (GMPs) in the production plant, such as improper sanitation methods and shared equipment. This could result in the unintended presence of allergens in the final product. The manufacturers expect the regulations to clearly set a threshold amount of allergens allowing the food products to be considered allergen-free. However, no statement was made to define the threshold amount of allergen by the regulation. In order to address the needs of the consumers and protect the financial health of the companies, food manufacturers may consider including some warning statements, such as “may contain…” or “possible presence of…”, to indicate that the food product may contain an allergen as a result of possible cross-contamination.

1.5.6 Allergen Cleaning and Removal

Cleaning effectiveness is crucial in combating adverse reactions. Adequate sanitation for shared equipment is pivotal. Rarely do food manufacturers have two sets of equipment for allergenic and non-allergenic food products. A study conducted by the FDA from 2002 to 2004 shows that around 80% of the facilities visited that shared equipment had one or more specific control measures to avoid cross-contamination. 76% of the facilities that attempted to control cross-contamination used shared equipment with cleaning procedures for allergen-containing and allergen-free products (Jackson et al., 2008).

Food soils are defined as unwanted matter on food-contact surfaces (Schmidt, 2015). Soils are usually classified based on the methods of removal from the surface cleaned. The two main categories are water soluble and non-water soluble. Water soluble soils are those that can dissolve in water that contains no cleaning agent, and these are inorganic salts, sugars, starches and minerals (Marriott, Schilling, & Gravani, 2018). Among the water insoluble soils are those that can dissolve in alkaline detergents, solutions with a pH above 7, such as fatty acids and proteins, and those that dissolve in acid media (pH below 7) like mineral deposits (Marriott et al., 2018). There are four types of food soils commonly found on equipment and these are carbohydrates, proteins, fats and minerals. Proteins (including allergenic proteins) are the most difficult to remove, and it is even harder if they have been denatured and adhered to surfaces (Schmidt, 2015).

Cleaning is defined as the complete removal of food soils using detergents under manufacturer's specifications. The appropriate sequence should be 1) Rinse, 2) Clean, 3) Rinse and 4) Sanitise. The last step is to reduce the levels of microorganisms to safe levels. Detergents are commonly used to aid in cleaning of food soils by lowering the surface tension of the water and loosening the soils (Marriott et al., 2018). They can be classified according to the extent of the effects they produce upon contact with the soil. The two main types of detergents are alkaline and acid cleaners. Alkaline detergents are detergents with a pH higher than 7 and they are sub-divided as the alkalinity (pH) increases. In general, fats, oils and proteins require a pH of 11 or higher for effective removal. Conversely, acid detergents are detergents with a pH lower than 7. They are useful for the removal of mineral deposits (Schmidt, 2015). Other factors that affect detergent performance are time, temperature and quality of the water (Marriott et al., 2018).

Alkaline detergents are commonly used to remove proteins and oxidizing agents can be added to solutions to further solubilise the soil (Jackson et al., 2008). Removal of protein films requires alkaline detergents in conjunction with chlorine (usually hypochlorite) (Schmidt, 2015). The condition of the soil also affects their ability to be removed. In addition, soils that fall into cracks or crevices or on uneven surfaces are harder to remove, and thus soil removal also depends on surface qualities like smoothness, porosity and wettability (Marriott et al., 2018).

Allergen removal protocols can involve wet and dry cleaning. Wet cleaning is performed in facilities where high-water-activity foods are processed; therefore, the accommodation is to have water available everywhere as well as equipment that is resistant to constant moisture. The existing four types of wet-cleaning methods are

1) clean in place, where equipment is not disassembled;
2) clean out of place, where equipment is partially disassembled;
3) foam or gel cleaning;
4) manual cleaning, where fully dismantled equipment is cleaned by hand.

Many facilities choose clean in place systems because they can be automated and constantly applied. Recent strategies in the food chain emphasise reduction in water and energy use, so effective alternatives that use minimal water and reduce energy consumption are desirable.

Conversely, dry allergen cleaning is much more difficult to successfully achieve since it is applied in facilities where dry products are produced, and no water contact is desired. Normally, these are plants designed so that water is not readily available, if at all, to avoid contact with the product. Some of the most commonly used methods of cleaning are vacuuming, sweeping, scraping, wiping and using compressed air. According to an Institute of Food

Technologists (IFT) report of allergen control practices in the food industry, more than 50% of companies use these dry cleaning techniques for allergen removal (Jackson et al., 2008).

Good material construction should be in place that is easy to clean and examine for food allergen residues. Proper process design and cleaning should minimise allergen cross-contamination. Visual verification should be easy to perform by providing clear access to all pieces of equipment. Additionally, deep cleaning between product runs is imperative to avoid contamination (Huggett & Hischenhuber, 1998).

Cleaning of milk handling equipment requires chemical, thermal and physical action (Reinemann, Wolters, Billon, Lind, & Rasmussen, 2003). A water rinse at 38-55 °C should be performed immediately. A chlorinated alkaline detergent should be used to remove organic soils, such as fat and protein, within a temperature range of 43-77 °C. These detergents contain alkali, phosphates and other agents that help dissolve fat, protein and carbohydrates (Jones, 2009). Moreover, Jones (2009) has proposed that it is necessary to know the water hardness for effective cleaning of milking equipment.

In 2004, a study was conducted to determine the removal of peanut allergens from several food-contact surfaces (Jackson et al., 2004). Various food-contact materials (stainless steel, Teflon, polyethylene, urethane and polycarbonate) were contaminated with peanut butter, and washed for 30 min with either water, chlorinated alkaline detergent (CAD), or acid detergent (AD), each at room temperature and at 62.8 °C. Results indicated that CAD and AD solutions removed residues from all food-contact surfaces at 62.8 °C, but room temperature CAD was not effective in some plates. Room temperature water did not remove residues from any of the materials, and hot water (62.8 °C) was not effective in urethane and Teflon plates.

Another study investigated milk-derived soil removal from heated stainless-steel surfaces using ozonated water as a pre-rinse (Guzel-Seydim, Wyffels, Greene, & Bodine, 2000). Stainless steel coupons were cleaned, passivated and soiled by autoclaving (121 °C at 15 psi for 15 min) with reconstituted non-fat dry milk (20% solids). Plates were subjected to a 15 min treatment of either warm water (40 °C) or ozonated cold water (10 °C) to compare the pre-rinse cleanability. Results showed that ozone treatment removed 84% of soil versus 51% by the warm water treatment. Using electron microscopy (at 200x and 2000x magnification), it was determined that the amount of soil present in the plates washed with ozonated water was significantly less than in those washed with warm water.

Combining the results from the two studies above, heat promotes the effectiveness in the removal of soils because it can penetrate them and can also allow for better chemical action in detergents. Heat also affects proteins because it denatures them, but some proteins retain their allergenic properties even after heat is applied. Therefore, the addition of chlorine to alkaline detergents is recommended to aid in the protein removal process since it helps in the breakage of proteins into smaller units for easier removal.

1.5.7 Food Allergen Analytical Methods

An effective method should have excellent specificity as well as sensitivity because a wide variety of food components can interfere with the assay, while to some people, even a low dose can cause severe reactions. At the current stage, several analytical methods exist for the quantitative and qualitative detection of allergenic residues in foods. Most detection methods involve immunoassays, which utilise antibodies raised against the target food or food protein extracts. A drawback of this method is that it may underestimate the risk of allergenic food proteins since the exact epitopes may be masked. Meanwhile, cross-reactivity between allergenic proteins can result in false positives in protein-based assays.

The most commonly employed detection methods in the industry include enzyme linked immunosorbent assays (ELISAs), polymerase chain reaction (PCR) and mass spectrometry; these methods are currently available commercially for detecting residues from allergenic sources (Van Hengel, 2007). Enzyme-linked immunosorbent assays (ELISAs) have been the most favoured analytical method used by the food industry and regulatory agencies to monitor the allergen residues for their relatively satisfactory sensitivity and specificity. With continuous development and improvement, lateral flow device (LFD) and dipstick tests are two of the most widely used ELISA platforms applied by the food industry today which allows rapid determination of allergen residue (Baumert & Tran, 2015), without interrupting the production process (Sajid, Kawde, & Daud, 2015). Strip assays allow the detection of allergens as a ration of food matrices, such as eggs (0.02 mg/kg), gluten (10 mg/kg), gliadin (2.5 mg/kg), milk (< 5 mg/kg), peanuts (1-5 mg/kg), almonds (1 mg/kg), hazelnuts (1-5 mg/kg), crustaceans (5 mg/kg) and molluscs (5 mg/kg) (Schubert-Ullrich et al., 2009). Surface plasmon resonance (SPR) is a promising technique in the food allergen detection field, using biosensor techniques. SPR is capable of detecting even trace amounts of food allergens without complex sample preparation (Van Hengel, 2007). SPR is also a labour-free detecting method in which the binding event can be monitored in real-time. Other new technologies like microfluidic devices (B. Zhang, Feldman, & Wang, 2013) and optical thin film biochips (Hamilton, 2015) have also been introduced as novel detection methods, but remain unproven in food processing. Novel food allergen detection methods should be accurate, robust, comparable and easy-to-interpret regardless of the variability in sampling, food matrix extraction and methods that can be considered routinely applied in allergen risk management processes when the aforementioned criteria are met.

1.6 Current Technology Interactions with Allergens

1.6.1 Thermal Processing

Thermal processing is often applied in food processing to obtain better flavour and texture or to pasteurise or sterilise food.

Thermal processing has been studied extensively and has been shown to both decrease and increase allergen reactivity of nuts. Boiling decreased the allergens in peanuts, because the allergens moved into the cooking water, thus reducing the overall antigenicity of the peanut. Roasting increased IgE-binding capacity of Ara h1 and Ara h2 compared to raw and boiled peanut. However, there was no change in IgE immunoreactivity between whole protein extracts from raw and roasted peanut (Mondoulet et al., 2005). Roasted peanut had a higher level of IgE binding compared to raw peanut (Maleki et al., 2000). Ara h1 and Ara h2 were more resistant to digestion after undergoing Maillard reaction. In contrast, roasted hazelnuts triggered fewer allergic reactions than raw hazelnuts, indicating that the hazelnut allergen is heat-labile (Hansen et al., 2003). Roasting reduced the antigenicity of hazelnuts. Native hazelnuts required less amount of allergen extract concentration than the roasted hazelnuts to induce the same weal reaction and basophil activation (Messens, Van Camp, & Huyghebaert, 1997; Worm et al., 2009).

Extrusion processing, which is heating in combination with pressure, was used to treat soy proteins. Extrusion did not alter the antigenicity of two allergens located in 38 and 50 kDa regions, while the IgE reactivity of soybean protein at 31 to 34 kDa was not observed. Thus, the reactivity of total major soybean allergens was decreased (Wilson, Blaschek, & de Mejia, 2005).

The fish major allergen, parvalbumin, has been reported unchanged in antigenicity after undergoing high temperature (90 °C) or proteolytic digestion in the gastro intestine (Arif & Hasnain, 2010). However, canned tuna and salmon have significantly less protein content than raw tuna and salmon, thus the antigenicity of canned tuna and salmon was drastically decreased, as demonstrated by ELISA and oral challenges (Bernhisel-Broadbent, Strause, & Sampson, 1992). After boiling treatment, the IgE binding to overall shrimp extract was decreased although the antigenicity of tropomyosin was enhanced, as indicated by in vitro analyses (G. M. Liu et al., 2010).

Taheri reported that β-lactoglobulin, a major milk allergen, lost most of its antigenicity under the heating temperature at 85-95 °C (Taheri-Kafrani et al., 2009). Heat treatment considerably reduced the antigenicity of whey proteins, but it did not reduce the antigenicity of casein (Y.-H. Lee, 1992). Skin prick tests showed that boiled milk induced weaker immunoreactions in children. Most children allergic to milk showed tolerance to extensively heated milk (Nowak-Wegrzyn et al., 2008). Boiling at 100 °C for 20 min could not eliminate the immunoactivity of ovalbumin and ovomucoid in eggs (Hoffman, 1983). However, children allergic to eggs have more tolerance to heated eggs than unheated forms (Nowak-Wegrzyn et al., 2008).

Baking increased the wheat allergens reactivity because the Maillard reaction increased the resistance of allergens to proteolytic digestion, which allowed them to reach the gastrointestinal system without changing of the epitopes and induced immune reactions (Simonato et al., 2001). Thermal processing does not significantly eliminate the allergen potency of many foods significantly to prevent a food allergy. Besides, it could cause other undesirable changes to flavours, qualities and functional properties of food.

1.6.2 Enzymatic Hydrolysis

Researchers have explored using enzymatic means to alter allergen reactivity. The addition of molecules to an allergen by enzymatic processes may alter the protein conformation or physically obstruct IgE antibodies from binding to conformational or linear epitopes. Enzymatic digestion can also alter linear epitopes via fragmentation. An example are fermented foods, which inherently undergo changes by endogenous and microbial enzymes, which can ultimately modify allergen reactivity (Frias, Song, Martínez-Villaluenga, De Mejia, & Vidal-Valverde, 2007; Song, Frías, Martinez-Villaluenga, Vidal-Valdeverde, & de Mejia, 2008; Yu, Ahmedna, Stowe, & Goktepe, 2005). Digestive enzymatic hydrolysis did not change IgE binding to the peanut allergen, Ara h2, despite the changes in secondary structure and reduction of disulphide bonds, because the linear epitopes of Ara h2 were unaltered even when subjected to digestive enzymatic hydrolysis (Sen et al., 2002). For the roasted peanuts and hazelnuts treated with pancreatic enzymes, the antigenicity of peanut proteins was persistent during gastric digestion, as shown in elevated arm stress test (EAST) and rat basophil leukaemia (RBL) cell mediator release assays, while the IgE reactivity of digested hazelnuts was reduced to less than 10% (Vieths, Reindl, Müller, Hoffmann, & Haustein, 1999).

Enzymatic hydrolysis was found to significantly reduce the potency of soy allergen, but other proteins were also decomposed during this process. Protein hydrolysates had significantly lower antigenicity compared to the original proteins, but protein fragments or native proteins remaining in the hydrolysed formulas could still trigger a severe allergenic reaction (Wilson et al., 2005). Fermentation hydrolysed the soy protein into peptides by protease from the microorganisms, which led to the reduction in allergen reactivity. However, the flavour was altered and became unacceptable after fermentation. The effect of fermentation on the reduction of allergen relies on the type of microorganism used and the level of hydrolysis (Wilson et al., 2005). The acceptability of the final product properties post fermentation is likely dependent on the food itself and would be suitable for foods where the flavour profiles deriving from fermentation processes are already palatable and known. Combining fermentation or enzymatic processes with another thermal or non-thermal process has potential to increase the range of allergens where antigenicity may be controlled.

In the mechanism of enzymatic polyphenol oxidation, polyphenol oxidase (PPO) helps to oxidize phenols to produce o-quinones derivative. O-quinones react with amino group, tryptophan residues or sulfhydryl groups in another protein, thus generating cross-linking of proteins. Researchers found PPO with phenolic compounds, such as caffeic acid and epicatechin reduced antigenicity of cherries major allergen Pru av 1 (Gruber, Vieths, Wangorsch, Nerkamp, &

Hofmann, 2004). PPO/caffeic acid effectively reduced IgE binding and allergen of peanut by crosslinking induced by PPO and/or caffeic acid (Si-Yin Chung, Kato, & Champagne, 2005). Overall, enzymatic digestion, whether through fermentation processes or applied enzymatic process, may fragment linear protein epitopes, making the antibodies unable to bind to the altered epitopes.

1.6.3 High hydrostatic pressure

High hydrostatic pressure (HHP) is a non-thermal technology which utilises water or a dilute aqueous solution (~100 to 1000 MPa) as a pressure transmitting medium. The liquid surrounding the food inside the pressure vessel is compressed; and the resulting high hydrostatic pressure is applied uniformly to the food product (Naderi, House, Pouliot, & Doyen, 2017). HHP has been employed in the food industry to inactivate microorganisms and enzymes and improve product texture by protein alteration and denaturation (Chawla, Patil, & Singh, 2011). The effects of high pressure have been attributed to changes in noncovalent bonds, such as electrostatic and hydrophobic interactions, which cause conformational modification. Pressures greater than 200 MPa have been used to alter the secondary and tertiary structure of major proteins, such as those found in meat and milk; however, greater pressures are needed to induce protein changes in egg and soy (400 and 300 MPa, respectively) (Messens et al., 1997).

As HHP has been shown to induce conformational changes in proteins, it has been theorised that HHP can alter allergen reactivity by altering the structure of food allergens. Kleber et al. (2007) discovered that HHP had a negative effect on the allergen reactivity of milk. Specifically, they found that the major milk allergen, β-lactoglobulin, was more reactive after treatment under HHP parameters ranging from 200 to 600 MPa. Combining the treatment with heat further enhanced allergen potency. It was speculated that β-lactoglobulin unfolds under high pressure and heat, which allows for protein aggregation. In this case, conformational changes may expose new linear epitopes, or IgE-binding sites, which would allow for greater allergen reactivity.

Conversely, Kato et al. (2000) found that increasing HHP actually reduced the presence of rice allergens. By treating rice grains with pressures of 300 MPa for up to 120 min, it was found that rice endosperm cells were damaged. The researchers revealed that, following structural damage of rice grains due to pressurisation, the surrounding buffer was able to permeate the rice grain, causing allergen solubilisation and subsequent extraction into the buffer. However, the amount of allergen extraction was dependent on the solvent used and the solubility of the rice allergens; thus, solvent compatibility may restrict the efficacy of this method. The researchers also performed a study using proteases, which further enhanced the decrease in allergen reactivity.

When applied to pasteurised liquid whole hen's egg, HHP caused the total egg allergen reactivity to decrease by 3.3-fold, as measured by EAST inhibition (Hildebrandt et al., 2010). When the samples were subjected to thermal treatment (70 °C), a 1.5-fold reduction in allergen reactivity was noted. However, when samples were heat treated prior to HHP treatment, a reduction of 8.9-fold was observed compared to the control. In this study, heat treatment was performed at 70 °C, and HHP was carried out at 600 MPa for 10 min at 20 °C.

In the case of soybeans, HHP (100-300 MPa for 15 min) reduced the immunoreactivity of an important soybean allergen, Gly m1 (Peñas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011). Based on this data, HHP-treated samples had less intense protein bands in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); thus, a reduction in allergen content was noted. Furthermore, sprouts that grew from HHP-treated soybean seeds had significantly reduced antigenicity compared with ones grown from untreated seeds, and it was suggested that HHP can be used to produce hypoallergenic soybean sprouts without notable nutrient loss.

1.6.4 High intensity ultrasound

High intensity ultrasound, which is also known as power ultrasound, is a technology that uses waves of ultrasound frequency from 20-100 kHz with high intensities of 10–1000 Wcm^{-2} (Tiwari, 2015). The use of these high intensity waves causes the waves to impact the food matrix and create cavitation bubbles in the food. The cavitation bubbles are the result of opposite compression and shearing waves. As these bubbles form and then eventually implode, it creates areas of high localised pressure and heat that go up to 1000 atmospheric pressure (atm) and 5000 Kelvins (K), along with the formation of free radicals in the food, which subsequently cause physical and chemical disruptions in the food structure.

High intensity ultrasound has been recently implemented for food processing such as peeling tomatoes (Rock, Yang, Shriver, Anugu, & Nooji, 2010), increasing shelf life and reducing the oil content of potato chips (Wambura & Yang, 2011), reducing the amount of time necessary to parboil rice (Wambura, Yang, & Wang, 2008), and many other processes. The high-energy waves promote formation of sonication bubbles in foods, which undergo compression and rarefaction intermittently, until they eventually collapse at critical bubble sizes. Following implosion, localised regions of high pressure and temperature (up to 1000 atm and 5000 K, respectively) surrounding these collapsed cavities can physically change the conformation of allergens and alter their reactivity. Furthermore, regions of high shear stress and high velocity gradients can create micro-streams that induce physical and chemical effects, and free radicals generated from water can contribute to protein modification (Soria & Villamiel, 2010).

To date, a modest amount of data is presented on the effects of high intensity ultrasound on food allergen reactivity. However, Li et al. (2006) found that treating shrimp with high intensity ultrasound reduced IgE binding to both isolated tropomyosin and crude shrimp protein extract from treated shrimp. The isolated proteins and whole shrimp were treated with 30 Hz for 130-180 min. Following treatment, there was a larger decrease in IgE binding to the isolated shrimp protein than to the extract prepared from the treated shrimp muscle. According to immunoblot analysis and ELISA, IgE binding to the treated isolated shrimp allergen decreased by approximately 81.3-88.5%, yet the protein extracted from the muscle of the treated shrimp had a reduction in IgE binding of approximately 68.9% (determined by ELISA only). It was further noted that during treatment of the allergen isolate, a new protein fraction with a low molecular weight increased in quantity as treatment time elapsed. Thus, fragmentation of the shrimp allergen may occur during high intensity ultrasound under the conditions stated (Z.-x. Li et al., 2006). No indication of elevated temperature or product quality was indicated following the relatively long ultrasound treatment of 130-180 min.

1.6.5 Gamma irradiation

Gamma (γ) irradiation refers to ionising radiation from the gamma portion of the electromagnetic spectrum. It can disrupt the chemical bonds in a food through free radicals. There are different uses for gamma irradiation in food applications, including food decontamination, optimisation of yield and other quality functions. In comparison with other processing methods, such as microwave, ultraviolet light, high hydrostatic pressure and hydrothermal treatments, irradiation treatment is rapid, convenient, but more expensive (Bao, Ao, & Jane, 2005). Among non-thermal processing methods, gamma irradiation has been used to monitor food borne pathogens, reduce the population of microbial load and insect infestation, inhibit the germination of root crops, and prolong the shelf life of perishable products. Furthermore, it has been employed to reduce food allergen reactivity. It has been speculated that gamma irradiation structurally alters the IgE-binding epitopes found on eggs (Seo et al., 2007), milk (J.-W. Lee et al., 2001), and shrimp allergens (Byun et al., 2000; Zhenxing, Hong, Limin, & Jamil, 2007) by creating free radicals, which can cause protein fragmentation and aggregation. Seo et al. (2007) described a reduction in the egg allergen ovalbumin when treated with gamma irradiation (cobalt-60) doses of 100 kGy, yet samples treated with 10 kGy were not affected. The disappearance of this allergen was attributed to a change in molecular weight. Gamma irradiation is thought to enhance protein crosslinking, including the formation of disulphide bonds, and cause hydrophobic interactions that could lead to protein aggregation (Davies & Delsignore, 1987). However, a dose of 100 kGy is rather high for food applications, and a maximum dose of approximately 10 kGy has been shown to be safe for human consumption (Byun et al., 2000).

1.6.6 Ultraviolet light

Ultraviolet (UV) light has been widely utilised in the food industry as a pasteurisation technology (Kathiravan Krishnamurthy, Irudayaraj, Demirci, & Yang, 2008). There are two types of UV modes: continuous and pulsed. The UV spectrum is further divided into short wave (UVC) from 200 nm to 280 nm, medium wave (UVB) from 280 nm to 320 nm and long wave (UVA) from 320 nm to 400 nm. The pulsed ultraviolet (PUV) radiation encompasses a wide range of spectra, from vacuum UV to far infrared radiation with a wavelength range between 100-1100 nm. Although both methods have been utilised for microbial inactivation, PUV is more effective and rapid for microbial inactivation than continuous UV light, because the energy is multiplied manifold (Oms-Oliu, Martín-Belloso, & Soliva-Fortuny, 2010).

In a PUV light system, high voltage electrical energy is stored in a capacitor and released in a single burst, which passes through a lamp filled with inert gas, such as xenon. The gas becomes ionised and emits radiation as ultraviolet (54%), visible (26%), and infrared (20%) light, which occurs within several nanoseconds (Oms-Oliu et al., 2010). It is speculated that PUV light has photothermal, photophysical, and photochemical effects on food systems, which could alter protein conformation or cause protein aggregation, resulting in the loss of conformational epitopes (S. Y. Chung, Yang, & Krishnamurthy, 2008; K Krishnamurthy, Demirci, & Irudayaraj, 2007). Specifically, PUV light can ionise molecules due to its high-energy contribution, and visible and infrared waves are responsible for vibration and rotation of molecules, respectively (Kathiravan Krishnamurthy et al., 2008).

Traditionally, UV light has been used for sterilisation practices, but more recently, PUV light has been shown to decrease allergen potency of peanut products (S. Y. Chung et al., 2008), soybean extract (Yang et al., 2010), shrimp extract (Shriver, Yang, Chung, & Percival, 2011), egg extract (Anugu, Yang, Shriver, Chung, & Percival, 2010), milk (Anugu, Yang, & Krishnamurthy, 2009), and wheat extract (Panozzo, Manzocco, Lippe, & Nicoli, 2016). When applied to peanut extracts and liquid peanut butter, PUV-treated samples had a decrease in IgE binding of 6- to 7-fold compared to the controls (S. Y. Chung et al., 2008). It was speculated that PUV light treatment caused protein aggregation of the major peanut allergen Ara h1, thus altering protein conformation and IgE-binding epitopes. These researchers discovered that the proteins, which corresponded to the molecular weight of Ara h1 (63 kDa), were insoluble in concentrated urea (2 M) or sodium chloride (1 M). The solubility of Ara h2 (18-20 kDa) was unaffected, as noted by SDS-PAGE.

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Chapter 2 Methods and Materials

All chemicals and reagents used were purchased from Sigma-Aldrich, Arklow, Ireland, unless otherwise stated.

2.1 Plasma setup and diagnostics

2.1.1 RSS set-up

The plasma treatment used in Chapter 3, Chapter 4, Chapter 5 and Chapter 6 was performed using a Reactive Species Specificity (RSS) system which is an in-house design and set up. The RSS system consisted of two modes: spark discharge (SD) and glow discharge (GD). The system has been previously described by Lu et al. (2017). Both setups were in different electrical discharge configurations. The schematic plans of both are represented in the Figure 2.1. Both setups were composed of a high voltage (HV) half bridge resonant inverter circuit, known as PVM500 plasma driver (Information Unlimited Inc., USA), with maximum output of 20 kV with a variable frequency of 20-70 kHz as a power supply and Simran THG5000UD 5000 W step up-step down transformer. The system was composed of a stainless-steel needle which served as the HV electrode and was fixed perpendicular to the liquid surface. The distance between the HV needle tip and the liquid surface was adjusted to 2.5-5 mm in all experiments. For each treatment of the liquid sample, a volume of 10 ml liquid sample was accurately placed into a polystyrene petri dish with inner diameter of 55 mm via a pipette, which corresponded to a liquid layer of about 4.2 mm depth. The ground electrode connections of both setups were different. In SD setup, the polystyrene petri dish was placed on a stainless-steel plate which was connected to the ground, shown in Figure 2.1. In GD setup, a thin stainless-steel ground electrode was submerged into the liquid sample contained in the petri dish, shown in Figure 2.1. The system was operated at atmospheric pressure using atmospheric air as working gas.

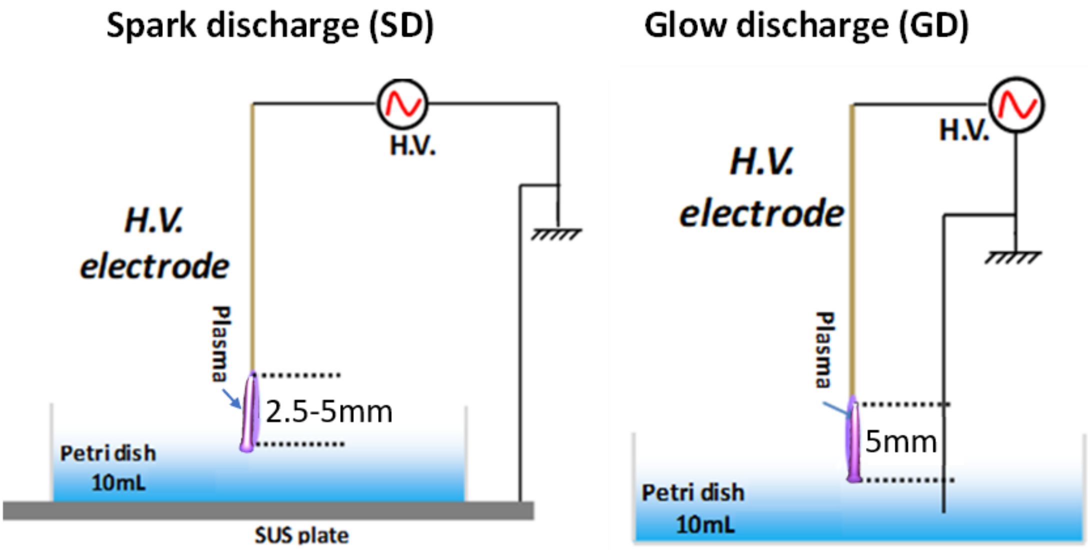


Figure 2.1 Schematic of RSS system: spark discharge and glow discharge.

For SD plasma treatment, the distance of HV between electrode tip and sample surface was varied from 2.5-5 mm due to the stability of plasma discharge. The distance was adjusted to 2.5 mm when the sample contained buffer and proteins. The distance was adjusted to 5 mm when the sample was only ultra-pure water.

2.1.1.1 ACP treatment of samples using RSS system

10 ml of liquid sample were placed in polystyrene petri dishes and treated using the RSS system. The samples used in these experiments were deionised water, casein solution, whey solution and gluten solution. The sample preparation is discussed in Chapter 2.2.1.

2.1.2 In-package DBD set-up

The plasma treatment used in Chapter 6 was performed using a Dielectric Barrier Discharge (DBD) system with a maximum high voltage output of 120 kV at a frequency of 50 Hz. The system has been previously described by Ziuzina et al. (2015). The schematic diagram is presented in Figure 2.2. The gap distance between the two aluminium electrodes was approximately equal to the height of the polypropylene container for a unit of 2.2 cm, used as a sample holder and a dielectric barrier. The plasma working gas was atmospheric air.

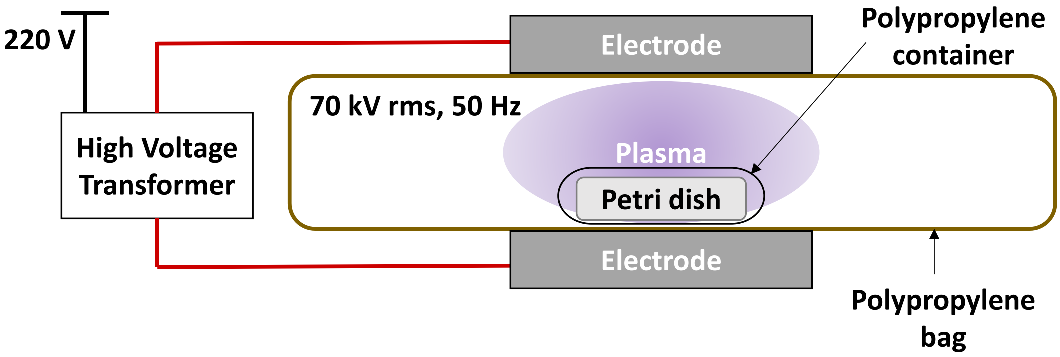


Figure 2.2 Schematic of DBD system.

The liquid samples held in a Petri dish without the lid in a polypropylene container were subjected to plasma treatment at an input voltage of 70 kV rms under atmospheric pressure. Each container was sealed in a polypropylene bag (B2630; Cryovac Sealed Air Ltd, Dunkan, SC, USA) and placed between the aluminium electrodes connected to the transformer. The temperature of the sample and the temperature inside the container was equal to room temperature. The temperature increase inside the container and at the surface of the samples due to plasma treatment was < 5 °C.

2.1.2.1 ACP treatment of samples using DBD system

10 ml of liquid sample were placed in polystyrene petri dishes and treated using the DBD system. The samples used in this experiment were casein solution, whey solution and gluten solution. The sample preparation is discussed in Chapter 2.2.1.

2.1.3 PB set-up

The plasma treatment used in Chapter 8 was performed using the Plasma Brush (PB) system from Relyon Plasma GmbH, Germany. This commercially available industrial scale system was kindly provided for these experiments by Advanced Plasma Technology and Mr Peter Dobbyn, who are the sole suppliers of Relyon equipment in Ireland. The schematic diagram of the system set up used is illustrated in the Figure 2.3. The system is a high-power system with pulsed atmospheric plasma technology composed of PS2000 high-power voltage supply and PB plasma generator (model 3) with A250 nozzle, connected through a flexible 10 m cable. The maximum open circuit voltage and maximum output power of PS2000 are 20 kV and 1000 Watt with a frequency range of 40 to 65 kHz.

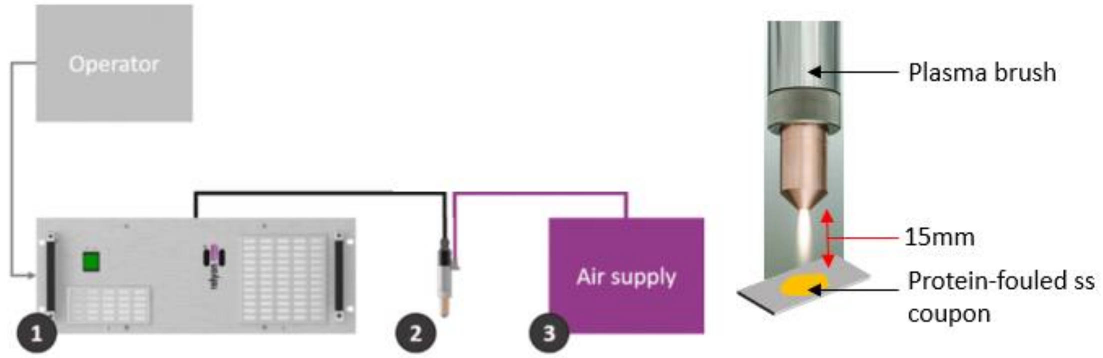


Figure 2.3 PB system adapted from https://www.relyon-plasma.com/relyon-plasma-produkte/plasmabrush-pb3/?lang=en.

(1) PS2000 high voltage supply, (2) PB plasma generator equipped A250 nozzle, (3) air compressor.

The samples were subjected to plasma treatment at approximately a voltage of 1 kV and a frequency of 55 kHz. The gas flow of compressed air was adjusted to approximately 35 SLM. The distance between nozzle tip and sample surface was 15 mm.

2.1.3.1 ACP treatment of samples using PB system

1 ml of sample dried on stainless steel (ss) coupon were treated using PB system. The samples used in this experiment were casein, whey, and gluten. The sample preparation is discussed in Chapter 2.2.1.

2.1.4 Plasma Diagnostics

The plasma diagnostics were carried out in Chapter 3 and Chapter 4.

2.1.4.1 Measurement of Voltage and Current

Applied voltage and discharge current were monitored by a Tektronix P6015A HV probe and a Pearson 4100 0.5-1.0 W wideband current monitor. The voltage probe and current probe were connected to the high-impedance inputs of a DSO-X 2014A digital oscilloscope, InfiniiVision 2000 X-Series with 100 MHz bandwidth and 2-G sample/s sampling rate (Agilent Technologies).

2.1.4.2 Measurement of Temperature

The temperature of plasma activated water (PAW) was measured by an infrared thermometer GM320 (Radionics Ltd, Dublin, Ireland).

The thermal imaging of PAW generation process was recorded by a FLIR One Pro thermal camera coupled with an Android phone (Radionics Ltd, Dublin, Ireland).

2.1.4.3 Measurement of pH

The pH analysis was measured by an Orion pH meter (model 420A, Life technologies Ltd, Park Paisley, UK).

2.1.4.4 Measurement of Conductivity

The conductivity was measured by a conductivity meter (model 150A+, Life technologies Ltd, Park Paisley, UK).

2.1.4.5 Measurement of Redox Potential

The redox potential was measured by an Orion ORP meter (model 420A, Thermo Electron Corporation, USA).

2.1.4.6 Measurement of reactive oxygen species concentrations

Hydrogen peroxide concentrations in plasma activated water (PAW) were quantified using titanium oxy-sulphate ($TiOSO_4$, Sigma-Aldrich, Arklow, Ireland) and spectrophotometric measurement. The calibration curve of known hydrogen peroxide concentrations (0, 82, 163, 326, 653, 979, and 1632 µM) was prepared by diluting 30% hydrogen peroxide standard solution (Perhydrol® for analysis EMSURE® ISO) and used to convert absorbance into hydrogen peroxide concentrations. Briefly, 10 µl of $TiOSO_4$ solution and 100 µl of PAW or calibration curve samples were added to a 96-well microtiter plate. After 10 min incubation at room temperature, absorbance was read on a spectrophotometric plate reader at 405 nm (Lu et al., 2017).

Total oxidizing species concentrations in PAW were quantified using oxidation of potassium iodide to iodine and spectrophotometric measurement. The calibration curve of known hydrogen peroxide concentrations was prepared as described above and used to convert absorbance into total oxidizing species concentrations. 50 µl of PAW or calibration curve samples, 50 µl of phosphate buffer (10 mM) and 100 µl of potassium iodide (1 M) were added to a 96-well microtiter plate. After 30 min incubation at room temperature, absorbance was read on a spectrophotometric plate reader at 390 nm (Boehm, Heslin, Cullen, & Bourke, 2016).

Trichloroacetic acid solution (100%) was added into the sample to a final concentration of 30% if the sample contained proteins before conducting ROS measurement.

2.1.4.7 Measurement of reactive nitrogen species concentrations

Nitrite ion (NO_2^-) concentrations were measured by Griess reagent (Sigma-Aldrich, Arklow, Ireland). A range of known concentrations of sodium nitrite solution (0, 20, 40, 60, 80, and 100 µM) was used to prepare a NO_2^- calibration curve and to convert absorbance into NO_2^- concentrations. 50 µl of Griess reagent and 50 µl of PAW or calibration curve samples were added to a 96-well microtiter plate. After 30 min incubation in the dark, absorbance was read at 548 nm.

Nitrate ion (NO_3^-) concentrations were determined by 2,6-dimethyl phenol (DMP) using the Spectroquant® nitrate assay kit (Merck Chemicals, Darmstadt, Germany) using the manufacturer's instructions with minor modification. Samples were pre-treated with sulfamic acid to eliminate nitrite interference. A set of standard concentrations of sodium nitrate solution (0, 0.1, 0.25, 0.5, 1, 2.5, and 5 mM) was used to prepare NO_3^- calibration curve and to convert absorbance into NO_3^- concentrations. Briefly, 200 µl of reagent A, 25 µl of PAW or calibration curve samples and 25 µl reagent B was added in order into a 1.5 ml microtube and then mixed vigorously. After 20 min incubation at room temperature, 100 µl of the total mixture was added to a 96-well microtiter plate and the absorbance was read at 340 nm.

Trichloroacetic acid solution (100%) was added into the sample to a final concentration of 30% if the sample contained proteins before conducting ROS measurement.

2.1.4.8 Fast Imaging Techniques

The plasma images were taken by intensified charge coupled device (iCCD) iStar DH734 camera (Andor, Belfast, UK). The plasma images were taken with or without bandpass filters of 250, 310, 340, 390 and 780 nm (Thorlabs, Exeter, UK). Without bandpass filter, the exposure time was 500 nsec. With bandpass filter, the exposure time was 20 µsec and the delay time was 15 µsec for the negative half-period while 35 µsec for the positive half-period. The plasma images were then analysed by Andor SOLIS.

2.2 Characterising protein properties

2.2.1 Preparation of milk and wheat proteins

Casein from bovine milk (powder, technical grade) and gluten from wheat (powder, technical grade) were purchased from Sigma Aldrich, Arklow, Ireland. Whey proteins were purchased from Fonterra in Auckland, New Zealand.

For solution preparation, casein protein was dissolved in phosphate buffered saline (PBS) with 0.01 M phosphate buffer, 2.7 mM potassium chloride and 0.137 M sodium chloride, pH 7.4, and adjusted to a final concentration of 0.02, 0.2, and 2 mg/ml. Whey protein was dissolved in PBS as well and adjusted to a final concentration of 0.02, 0.2, and 2 mg/ml. Gluten solution was dissolved in 0.5 M Tris buffer (TB), and adjusted to a final concentration of 0.1, 1, and 10 mg/ml.

2.2.2 Preparation of protein-fouled ss coupon

The coupon material used was polished 304 stainless steels (ss) (purchased from Brendan Buggy Steel Fabrication Ltd, Dublin, Ireland). The size of coupon was 25 * 25 mm^2 in square shape and 1 mm in thickness. The 304 stainless steel (ss) coupons were polished by 240 grit finish.

For protein-fouled ss coupon preparation, 0.002, 0.02, or 0.2 mg of casein/whey proteins were fouled on ss coupons, respectively: 1 ml of casein or whey solution prepared above was left on the ss coupons and dried at room temperature for 24 hours. Gluten solution was dissolved in 50% ethanol, and adjusted to a final concentration of 0.1, 1, and 10 mg/ml. For gluten-fouled ss coupon preparation, 0.01, 0.1, and 1 mg of gluten proteins were fouled on ss coupons: 1 ml of gluten/ethanol solution was left on the ss coupon and dried at room temperature for 24 hours. After plasma treatment, the fouled proteins were extracted from fouled ss coupons by re-dissolving the proteins in solvent for further analysis. The casein and whey proteins were extracted by re-dissolving the proteins in PBS, while the gluten protein was extracted by re-dissolving the proteins in 0.5 M Tris buffer.

2.2.3 Protein extraction after plasma treatment

2.2.3.1 Protein extraction of milk and wheat proteins

The protein sample after plasma treatment was extracted by filtering the protein solution through either dialysis or Amicon®Ultra-15 Centrifugal filter unit (UFC9003, Sigma-Aldrich, Arklow, Ireland). For dialysis, the protein solution was filtered by dialysis membrane with a 3 kDa molecular-weight cut-off

(MWCO) for 24 hours with deionised water. For centrifugal filtration, the protein solution was poured into the centrifugal filter unit with a 3 kDa MWCO and centrifuged at 5000 rpm for 1 hour. The extracted protein was dried in a vacuum for 24 hours.

2.2.4 SDS-PAGE analysis

A molecular weight marker was purchased from Abcam, Cambridge, UK (ab116029) to determine the molecular weights.

2.2.4.1 Measurement of protein concentrations

Protein concentrations were quantified using bicinchoninic acid (BCA) reagent (Pierce^{TM} rapid gold BCA protein assay kit) and spectrophotometric measurement. The calibration curve of known bovine serum albumin (BSA) concentrations (0, 125, 250, 500, 750, 1000, 1500, and 2000 µg/ml) was prepared and used to convert absorbance into protein concentrations. BCA working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. Briefly, 20 µl of standard or unknown samples and 200 µl of BCA working reagent were added to a 96-well microtiter plate. After 5 min incubation at room temperature, absorbance was read on a spectrophotometric plate reader at 480 nm.

2.2.4.2 Preparation of SDS-PAGE samples

The dried extract was re-dissolved in specific buffers as described above. The concentration of casein/whey protein samples was re-adjusted to 2 mg/ml using BCA reagents. The concentration of gluten samples was re-adjusted to 10 mg/ml.

Laemmli 2x concentrate was used as a sample buffer for denaturing protein samples in SDS-PAGE. Laemmli 2x concentrate was prepared by adding 4%

sodium dodecyl sulphate (SDS), glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl buffer at pH 6.8. Another sample buffer was prepared by adding 4% sodium dodecyl sulphate (SDS), 20% glycerol, 0.004% bromophenol blue and 0.125 M Tris-HCl buffer at pH 6.8 and used for sample loading without breaking the disulphide bonds. The protein sample was then mixed with sample buffer at the ratio of 1 to 1. The mixture was boiled at 100 °C for 5 min. The sample mixture was then cooled to room temperature for further SDS-PAGE.

2.2.4.3 Preparation of gel

The 12% separating gel was prepared by adding 2 ml of acrylamide/bis-acrylamide (30%/0.8%, w/v), 1.25 ml of 1.5 M Tris-HCl (pH 8.8), 50 µl of sodium dodecyl sulphate (SDS) (10%, w/v), 50 µl of ammonium persulphate (AP) (10%, w/v), 8 µl of N,N,NN-tetramethyl ethylenediamine (TEMED) and 1.65 ml of deionised water in the casting frame.

The 10% separating gel was prepared by adding 1.65 ml of acrylamide/bis-acrylamide (30%/0.8%, w/v), 1.25 ml of 1.5 M Tris-HCl (pH 8.8), 50 µl of SDS (10%, w/v), 50 µl of AP (10%, w/v), 8 µl of TEMED and 2.05 ml of deionised water in the casting frame.

The 5% stacking gel was prepared by adding 0.33 ml of acrylamide/bis-acrylamide (30%/0.8%, w/v), 0.25 ml of 0.5 M Tris-HCl (pH 6.8), 20 µl of SDS (10%, w/v), 20 µl of AP (10%, w/v), 8 µl of TEMED and 1.35 ml of deionised water in the casting frame.

2.2.4.4 Preparation of electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Bio-Rad mini-gel slab electrophoresis unit (Bio-Rad, Life technologies Ltd, Park Paisley, UK). Briefly, the casein-mixture/whey-mixture samples in fixed volumes (10 µl per lane) were loaded on 5% polyacrylamide stacking gel (pH 6.8) and separated on 12% polyacrylamide separating gel (pH 8.8). The gluten-mixture samples in fixed volumes (10 µl per lane) were loaded on 5% polyacrylamide stacking gel (pH 6.8) and separated on 10% polyacrylamide separating gel (pH 8.8). The gel was subjected to electrophoresis at 200 V for 1 hour using a Mini-Protein electrophoresis cell (Model 161-3301, Bio-Rad) connected to a power supply (Model 3000, Bio-Rad).

After electrophoresis, the gel was then stained with Coomassie Blue (R) in 25% (v/v) 2-propanol and 10% acetic acid solution for 2 hours or more. After staining, the gels were destained with two changes of a 10% acetic acid solution for a total period of 24 hours. Gel pictures were taken by Syngene G Box Chemi XRQ (Scientific Laboratory Supplies Limited, Nottingham, UK) for visible analysis.

2.2.5 Secondary structure determination

The Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy was performed on untreated and treated proteins to determine conformational changes in the secondary structure before and after plasma treatment. A Nicolet iS7 Fourier FTIR spectrometer coupled with iD7 ATR (Thermo Fisher Scientific Inc., USA) was employed under ambient conditions in this study as per the method described in G. Liu et al. (2009) with slight modification. In brief, the solid protein was prepared by dialysing untreated and treated protein solution against distilled water and freeze dried. The solid protein was pressed into a small pellet within two aluminium foils by hand to make good contact with ATR crystal. Each FTIR spectrum was recorded from 4000 to 400 cm^{-1} with 64 scans with 4 cm^{-1} resolutions. The secondary structural analysis was estimated by conducting the second derivative of the amide I peak in the wavenumbers ranging from 1580 to 1720 cm^{-1} for deconvolution analysis with the aid of OMNIC software. The deconvolved spectrum was curve-fitted with Gaussian band shapes. The resultant peaks were assigned to different secondary structures using guideline of Y. Zhang, Deng, and Zhao (2017). The measurements were repeated 6 times, and the values were averaged to reduce any baseline effects.

2.2.6 Amino acid profile analysis

The protein solution samples were readjusted into 100 µg/ml before acid hydrolysis. The protein solution samples were placed into sealed glass tubes and hydrolysed with 6 M HCl at 110 °C for 24 hours (Rafiq et al., 2016). The samples were transferred to a vial for further amino acid analysis. Amino acid analysis was performed following the method from Kennedy and Bivens (2017). Ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis was performed using an Agilent 6470 triple quadrupole mass spectrometer system, equipped with a G1332A degasser, a G7120A binary pump, a G7129B vial sampler, a G7116B multicolumn thermostat and an Agilent Jet Stream Electrospray ionisation source (Agilent Technologies, Dublin, Ireland). Poroshell 120 A Hydrophilic Interaction Chromatography (HILIC)-Z column (2.1 × 100 mm, 2.7 µm particles) was used. The UPLC-MS/MS system was operated at a flow rate of 0.8 ml/min using 20 mM ammonium formate in water (eluent A) and 20 mM ammonium formate in acetonitrile (eluent B). The following gradient of eluent B was applied: 0 min:

100% B; 0-10 min: ramped to 70% B; 10-11 min ramped to 100% B and held for 5 min. With column equilibrium, the run time was 16 min. The column temperature was kept to 30 °C. The injection volume was 0.1 µl. The triple quadrupole instrument was operated in selected reaction monitoring (SRM) mode with Electrospray Ionisation (ESI) positive. All characteristic SRM settings can be found in literature (Kennedy & Bivens, 2017). In the case of cystine, the precursor ion, product ion, fragmentor and collision energy used were 241 m/z, 151.9 m/z, 75 V and 12 V, respectively. The amino acid standard curve was made by known concentrations of amino acid standard (0, 31.25, 62.5, 125, 187.5, 250, 312.5, 625 and 1250 µmol/L) and used to convert response into amino acid concentrations. The amino acid content was calculated as amino acid concentration*amino acid molecular weight. The amino acid contents were expressed as g/100g protein.

2.2.7 Enzyme-linked immunosorbent assay (ELISA)

Rbiopharm ®IDASCREEN ELISA kits used were purchased from Fannin Ltd., Dublin, Ireland. ELISAGenie ELISA kit used was purchased from AssayGenie, Dublin, Ireland. The antigenicity of casein, β-lactoglobulin, and α-lactalbumin in milk, and gliadin in gluten were determined by using commercial ELISA kits (RBiopharm ®IDASCREEN Fast Casein, RBiopharm ®IDASCREEN β-lactoglobulin, ELISAGenie bovine α-lactalbumin ELISA kits, and RBiopharm ®IDASCREEN Fast gliadin, respectively). The detailed procedures were performed according to the manufacturer’s instructions and are briefly described as follows. The standard curve of known protein concentration was prepared by diluting the specific protein provided by the manufacturer. The standard curve was used to convert absorbance into antigenicity of protein concentrations. The percentage of antigenicity of the protein was calculated as (treated concentration/untreated concentration) × 100. The measurements were repeated 6 times.

2.2.7.1 Sandwich ELISA of Rbiopharm ®IDASCREEN

The antigenicity of casein, β-lactoglobulin and gliadin was determined using the sandwich ELISA assays. The wash buffer provided in the kit was used for sample dilution and well washing. Standards were provided in the kit. The sample for ELISA was prepared by diluting it to 200 ng/ml. A sufficient number of wells was inserted into the microwell holder for all standards and samples to be run in duplicate. The positions of standards and samples were recorded. 100 µl of each standard or sample was added to separate duplicate wells and incubated for 10 min at room temperature. The liquid was discarded, and the washing step was repeated 3 times. 100 µl of the conjugate was added to each well and incubated for 10 min at room temperature. The liquid was discarded, and the washing step was repeated 3 times. 100 µl of chromogen was added to each well and incubated for 10 min at room temperature in the dark. 100 µl of the stop solution was added to each well and mixed gently by shaking the plate manually. The absorbance was read at 450 nm.

2.2.7.2 Sandwich ELISA of ELISAGenie

The α-lactalbumin antigenicity was determined using the sandwich ELISA assay. The standard, sample diluent and wash buffer were provided in the kit. The sample for ELISA was prepared by diluting it to 200 ng/ml. A sufficient number of wells was inserted into the microwell holder for all standards and samples to be run in duplicate. The positions of standards and samples were recorded. 100 µl of each standard or sample was added to separate duplicate wells and mixed gently. The plate was incubated for 120 min at 37 °C. The liquid was discarded. 100 µl of detection antibody was added to each well and mixed gently. The plate was incubated for 60 min at 37 °C. The washing step was repeated 4 times. 100 µl of the conjugate was added to each well and mixed gently. The plate was incubated for 60 min at 37 °C. The liquid was discarded, and the washing step was repeated 6 times. 90 µl of chromogen was added to each well and incubated for 10 min at 37 °C. 50 µl of the stop solution was added to each well and mixed gently by shaking the plate manually. The absorbance was read at 450 nm.

2.3 Sample preparation for PAW treatment study

1 mM of H_2O_2, 1 mM of NO_2^-, 1 mM of NO_3^-, 5 mM of NO_3^-, mixture of 1 mM of H_2O_2 and 5 mM of NO_3^-, and mixture of 1 mM of NO_2^- and 1 mM of NO_3^- were made and served as PAW-mimicking solutions.

4 mg/ml of casein solution was mixed with PAW/mimicking solutions to a final concentration of 1 mg/ml (3 parts of PAW/mimicking solutions and 1 parts of 4 mg/ml of casein solutions). 2 mg/ml of casein solution was mixed with PAW/mimicking solutions to a final concentration of 0.1 mg/ml (19 parts of PAW/mimicking solutions and 1 parts of 2 mg/ml of casein solutions). 2 mg/ml of casein solution was mixed with PAW/mimicking solutions to a final concentration of 0.01 mg/ml (199 parts of PAW/mimicking solutions and 1 parts of 2 mg/ml of casein solutions).

The PAW-casein/mimicking-casein mixtures were incubated at 20 °C and 60 °C in a water bath. The antigenicity of the mixtures was examined with different contact times from day 0 to day 5 by ELISA.

2.4 Statistical analysis

Statistical analysis was performed using Prism 8.0 (GraphPad Software Inc., La Jolla, USA) and the results were statistically analysed by one-way analysis of variance (ANOVA). All values are mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6). The significant differences among the means of samples were determined by a significant difference test at $p < 0.05$.

Chapter 3 Physicochemical properties of PAW generated by spark and glow (RSS discharges in air)

3.1 Experimental Design

Water exposed to cold plasma, termed as plasma activated water (PAW), creates an acidic environment which results in changes of redox potential, conductivity and in the generation of reactive oxygen and nitrogen species (RONS). The long-lived reactive species (such as H_2O_2, NO_2^- and NO_3^-) act as mediators for reactions with biological or chemical targets. PAWs retain their activity post-plasma exposure and are of interest as novel decontamination agents in their own right (Schnabel et al., 2017; Shaw et al., 2018; Thirumdas et al., 2018). Therefore, plasma and liquid diagnostics are essential for evaluating plasma treatment systems to enrich the diagnostic information.

In this chapter, the physicochemical properties of plasma activated water (PAW) generated by the Reactive Species Specificity (RSS) system (consisting of spark discharge (SD) and glow discharge (GD)) were investigated. The electrical properties of both discharges were determined by observing the voltage waveforms. The H_2O_2, NO_2^-, NO_3^- and total oxidizing species concentration of PAW generated from both discharges were measured. Different levels of applied voltage (6, 7.5, 9 kV) and frequency (25, 27.5, 30 kHz) are included in this study and the results are compared with the previous literature published on this plasma system and set-up (Lu et al., 2017). The approach used is illustrated in Figure 3.1. 10 ml of deionised water was exposed to spark discharge (SD) or glow discharge (GD).

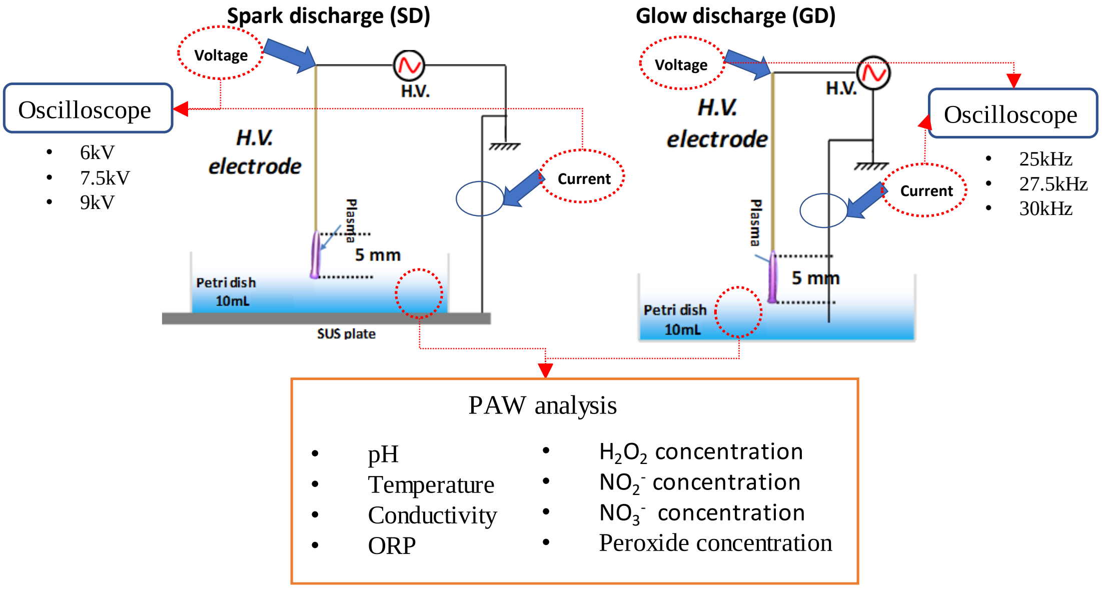


Figure 3.1 Liquid-phase plasma diagnostics of SD and GD – experimental design.

3.2 Results and Discussion

3.2.1 Characteristics of electrical discharges above the ultrapure water

Ultrapure water was exposed to SDs with different applied voltages and GDs with different applied frequencies. The voltage waveforms can be observed in Figure 3.2.

In previous literature, Lu et al. (2017) observed the SD with an applied voltage of 7 kV ignited only at the positive polarity half period of the applied voltage presented in Figure 3.2D. In Figure 3.2A to C, the SDs with different applied voltages of 6, 7.5 and 9 kV ignited twice at the positive and negative polarity half periods of the applied voltage in a cycle are presented. The high pulse repetition rate of SD was controlled to 25 kHz. As seen by observing the applied voltage waveform of all SDs, the discharge ignition at the positive polarity half period was further away from the crest point of the half period of the applied voltage waveform when higher voltage was applied to ignite SD, shown in Figure 3.2A to C. Another discharge ignition at the negative polarity half period was on the opposite side of the ignition at the positive polarity half period of the applied voltage. The GDs with different applied frequencies of 30, 27.5 and 25 kHz ignited at the negative half period of the applied voltage as shown in Figure 3.2E to G were similar as in previous literature (Lu et al., 2017) illustrated in Figure 3.2H. The applied voltage of GDs with different frequencies of 30, 27.5 and 25 kHz was adjusted to 5 kV, which was different to previous literature where GD was applied with approximately 7 kV. In fact, due to the differences in the power supply used, the maximum of applied voltage for GD treatment was up to 5 kV, approximately. By observing the applied voltage waveforms of GDs, their waveforms were analogous to the previous literature, except the period of waveform, as shown in Figure 3.2E to H. The period of waveform was directly dependent on the applied frequency as the frequency is the reciprocal of the time.

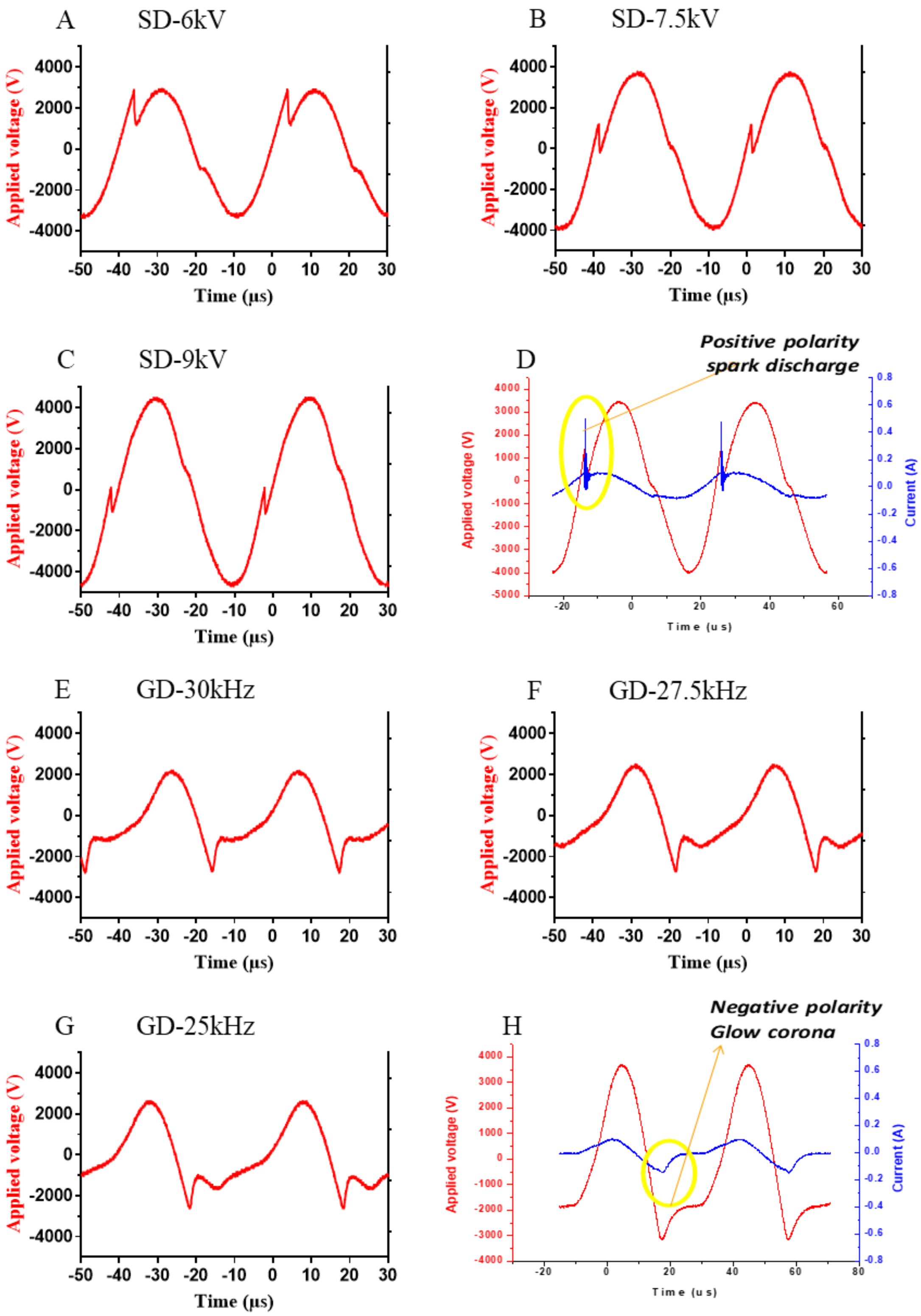


Figure 3.2 Applied voltage waveforms of spark discharges (SDs) with different applied voltages and glow discharges (GDs) with different applied frequencies. (A) SD applied with 6 kV, (B) SD applied with 7.5 kV, (C) SD applied with 9 kV,

(D) SD from Lu et al. (2017), (E) GD applied with 30 kHz, (F) GD applied with 27.5 kHz (G) GD applied with 25 kHz, (H) GD from previous literature.

3.2.2 Thermal, chemical and electrical characteristics of SDPAW and GDPAW

The spark discharge PAWs (SDPAWs) were generated by SD above ultrapure water with different voltages of 6, 7.5 and 9 kV; while the glow discharge PAWs (GDPAWs) were generated by the GD above ultrapure water with different frequencies of 25, 27.5 and 30 kHz. Either SD or GD treatments of ultrapure water were operated for up to 30 min. The properties of SDPAW with different voltages of 6, 7.5 and 9 kV and GDPAW with different frequencies of 25, 27.5 and 30 kHz are shown in Figure 3.3. The temperatures of SDPAWs and GDPAWs were recorded immediately after plasma treatment by infrared thermometer GM320, shown in Figure 3.3A and B, respectively. The temperature of SDPAW with 9 kV and GDPAW with 25 kHz increased sharply to 50-51 °C and gradually reached a plateau. The maximum temperature of SDPAW with 9 kV was 56.5 °C after a 30 min treatment time. The maximum temperature of GDPAW with 25 kHz was 55.9 °C after a 30 min treatment time. The overall temperatures of SDPAW and GDPAW were higher when higher applied voltage and lower applied frequency were operated, respectively. Ly et al. (2018) reported that the applied voltage was positively correlated to temperature. The pH plots for both types of PAW recorded by Orion pH meter are shown in Figure 3.3C and D. As observed the SDPAW and GDPAW showed a sharp drop in pH after 1 min exposure to plasma and decreased slightly afterwards. The curves on the pH graphs were unchanged for SDPAW with different voltages and GDPAW with different frequencies. Both types of

PAW showed an initial decline to about pH 2.7 in the first minute. These results were different, compared to the previous study. The pH of SDPAW subsequently decreased gradually to 2.2 after 10 min treatment, while GDPAW showed an initial decrease to about pH 3.7 in the first minute; the pH level remained constant thereafter. Both types of PAW after 30 min treatment gave a pH 2. The redox potentials (ORP) for both types of PAW recorded by Orion ORP meter are shown in Figure 3.3E and F. As observed the SDPAW and GDPAW showed a logarithmic growth in ORP level after exposure to plasma. The ORP value of SDPAW with different voltages of 6, 7.5 and 9 kV increased logarithmically from 322±12 mV to 530±21 mV, 506±23 mV and 570±29 mV, respectively, after 30 min of SD treatment. While the ORP value of GDPAW with different frequencies of 25, 27.5, 30 kHz increased logarithmically from 322±12 mV to 631±21 mV, 598±23 mV and 587±25 mV, respectively, after 30 min of GD treatment. Renwu Zhou et al. (2018) reported that the ORP values of PAW increased while the pH values decreased as a function of plasma generation time.

Figure 3.3G and H illustrates the conductivity of the SDPAW with different voltages and GDPAW with different frequencies, respectively. The conductivities of both types of PAW increased as a function of plasma generation time. The slope increased from 152 to 226 $\mu Smin^{-1}$ when increasing the applied voltages from 6 to 9 kV. The slope increased from 66 to 146 $\mu Smin^{-1}$ when decreasing the applied frequencies from 30 to 25 kHz. The rate of water conductivity increased as a function of plasma generation time. Lu et al. (2017) reported that the water conductivity of PAW increased with plasma treatment time.

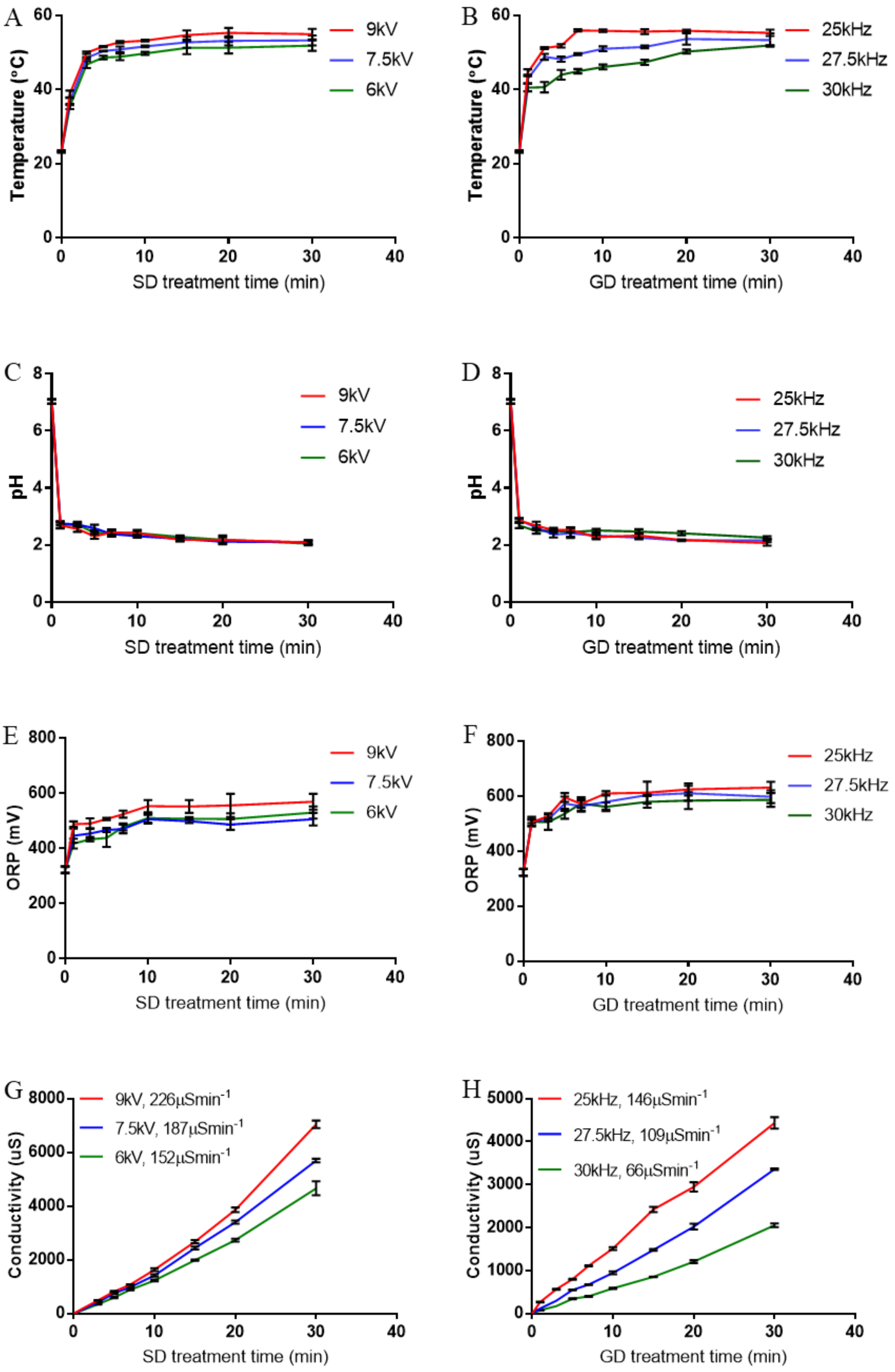


Figure 3.3 Properties of SDPAWs generated by SD with different voltages of 6, 7.5, 9 kV and GDPAWs generated by GD with different frequencies of 25, 27.5, 30 kHz. Temperatures of (A) SDPAW and (B) GDPAW. pH of (C) SDPAW and

(D) GDPAW. ORPs of (E) SDPAW and (F) GDPAW. Conductivities of (G) SDPAW and (H) GDPAW. *slope is presented in unit of µSmin^{-1} in legend of (G) and (H). (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

The real-time temperatures of SDPAW and GDPAW were recorded using FLIR One Pro thermal camera coupled with an Android phone, as illustrated in Figure 3.4 and Figure 3.5, respectively. Box 1 (Bx1) comprises the detected area of temperature of PAW. Spot 1 (Sp1) comprises the detected spot of environmental temperature, which served as a control. The environmental temperature was about 21.6 to 23.3 °C. The temperature of SDPAW and GDPAW increased logarithmically. The highest average temperature of SDPAW was 57.3 °C after 30 min treatment, while GDPAW was 60.9 °C after 25 min treatment. The temperature results taken using thermal imaging were in real-time whilst those taken by an infrared thermometer gun were done manually. Even though the temperature was recorded immediately by infrared thermometer gun, heat energy could be lost rapidly. The advantage of the temperature results taken by thermal imaging was the temperature was detected in a certain area. To ensure the accuracy of thermal data, the results were taken from the plasma spot to the margins. To ensure the accuracy of thermal data, the experiments were conducted in a low lightness and isolated from heat-producing substances (such as power supply). The emissivity of substances (such as water, petri dish, working bench) that were detected was above 0.5, which can yield accurate temperature measurements at this level as advised by the manufacturer.

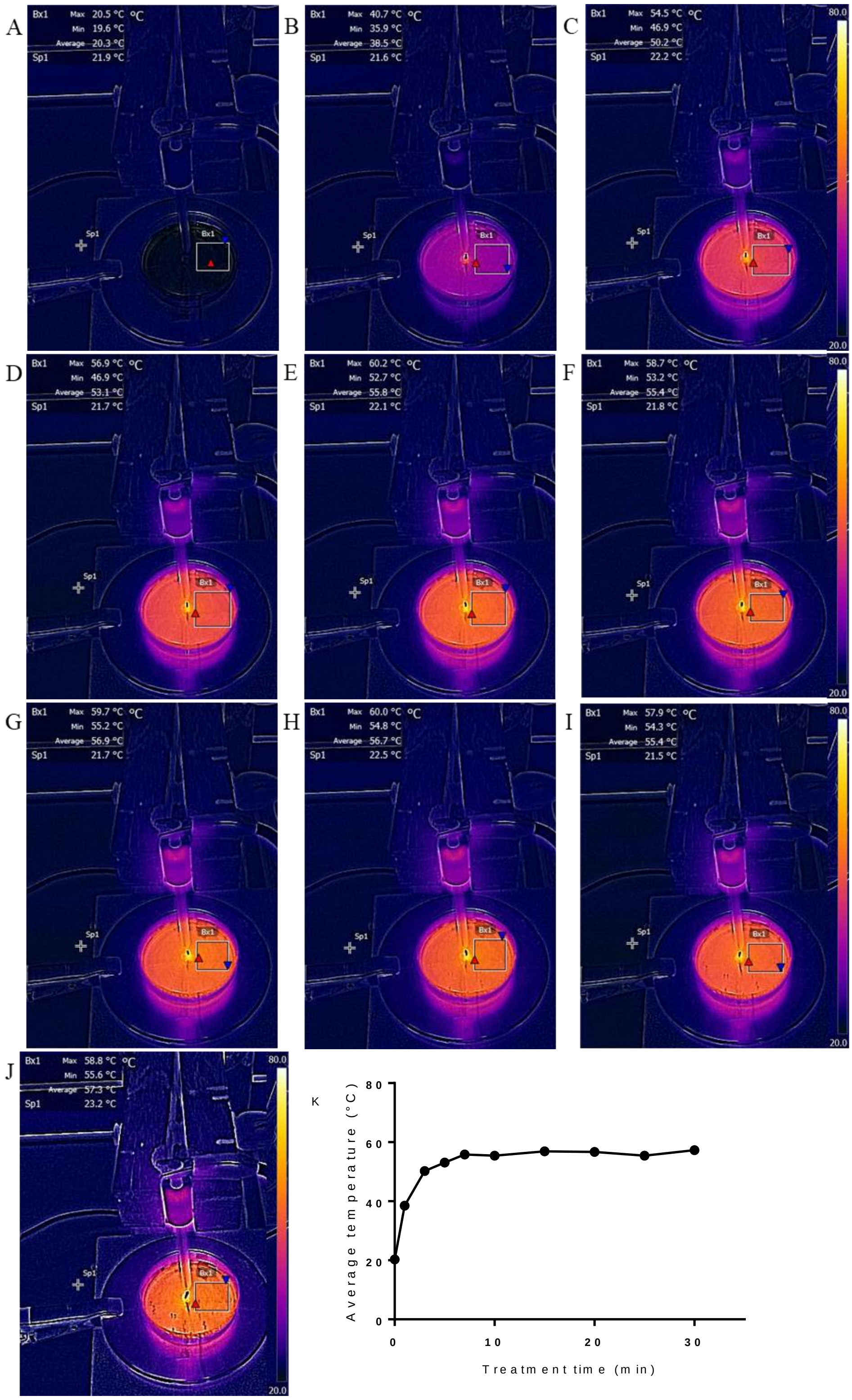


Figure 3.4 Thermal images of SD operated above ultrapure water applied with 9 kV and 25 kHz for (A) 0 min, (B) 1 min, (C) 3 min, (D) 5 min, (E) 7 min, (F) 10 min, (G) 15 min, (H) 20 min, (I) 25 min, (J) 30 min. *Bx1: temperature of SDPAW, Sp1: environmental temperature. (K) Average temperatures of SDPAW.

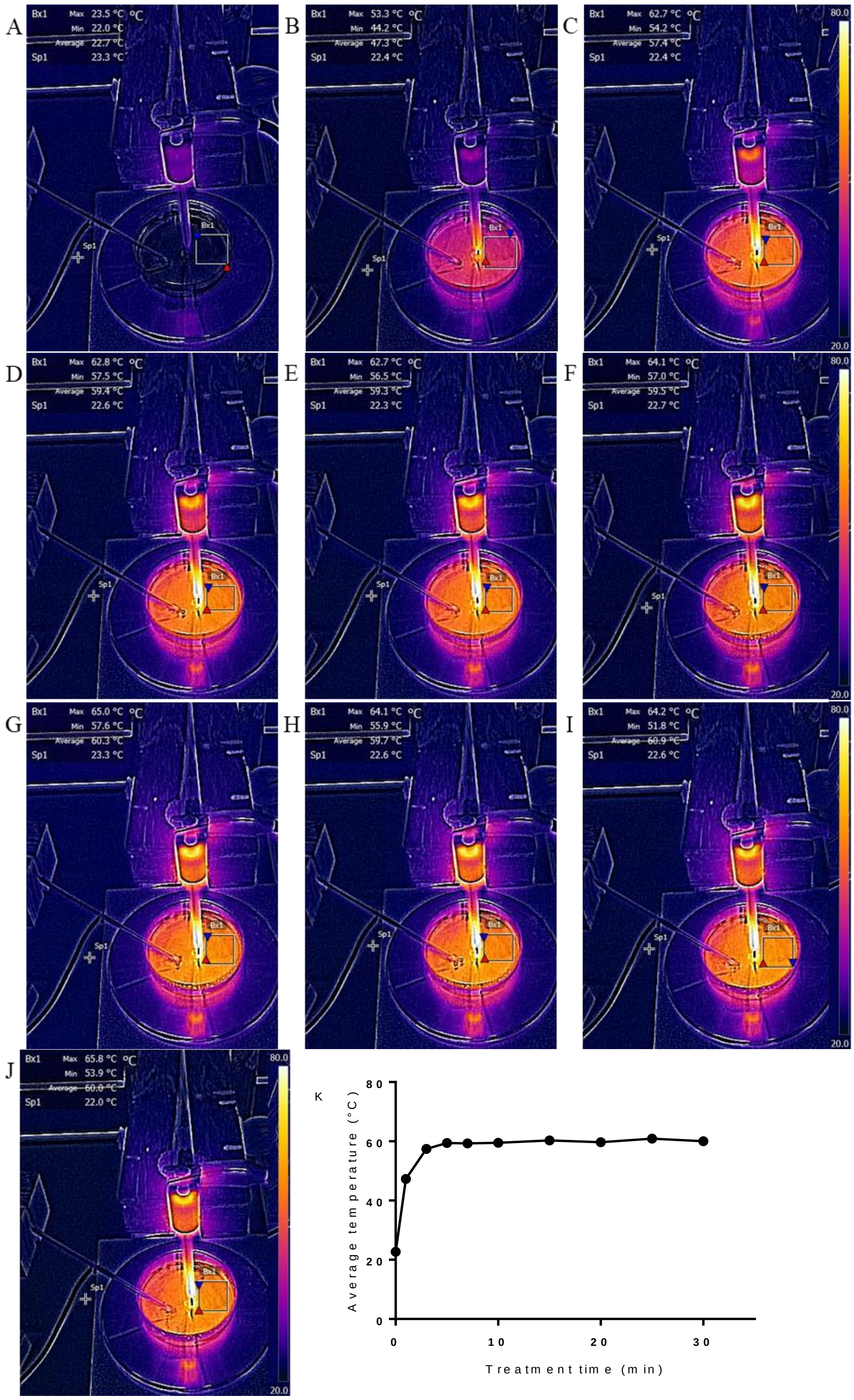


Figure 3.5 Thermal images of GD operated above ultrapure water applied with 5 kV and 25 kHz for (A) 0 min, (B) 1 min, (C) 3 min, (D) 5 min, (E) 7 min, (F) 10 min, (G) 15 min, (H) 20 min, (I) 25 min, (J) 30 min. *Bx1: temperature of GDPAW, Sp1: environmental temperature. (K) Average temperatures of GDPAW.

3.2.3 Selective generation of hydrogen peroxide, nitrate and nitrite

The concentrations of H_2O_2, nitrate , nitrite and total oxidizing species for both types of PAW were quantitatively measured, as presented in Figure 3.6. H_2O_2 was specifically measured by $TiOSO_4$. Total oxidizing species was measured by buffered KI as PAW may contain total oxidizing species other than H_2O_2, such as superoxide, peroxynitrite and peroxynitrate.

In Figure 3.6A and C, the H_2O_2 and total oxidizing species concentrations of SDPAW with 9 kV increased linearly to approximately 800 µM for both within the first 5 min and increased gradually afterward. The H_2O_2 and total oxidizing species concentrations of SDPAW with 9 kV were 1.3 mM and 2.1 mM after 30 min treatment, respectively. Similarly, the H_2O_2 and total oxidizing species concentrations of SDPAW with 7.5 kV increased linearly to approximately 600 µM and 720 µM, respectively, within 5 min and increased gradually afterward. The H_2O_2 and total oxidizing species concentrations of SDPAW with 7.5 kV were 1.2 mM and 1.8 mM after 30 min treatment, respectively. In SD treatment with 6 kV, H_2O_2 was hardly detectable within the first 3 min and started to be generated afterward, while the total oxidizing species were generated from the start. The H_2O_2 and total oxidizing species concentrations of SDPAW with 6 kV were 553 µM and 790 µM, respectively, after 30 min treatment. Figure 3.7A shows the ratio of H_2O_2 to total oxidizing species concentrations in SDPAW applied with different voltages of 6, 7.5 and 9 kV. Taken together, the ratios of H_2O_2 to total oxidizing species concentrations in SDPAW were mostly in the range from 50% to 100% and for treatment times above 5 min, total oxidizing species consisted predominantly of H_2O_2. Only SD with 6 kV showed that H_2O_2 composed only 15% of the total oxidizing species for up to 3 min treatment time.

NO_2^- concentrations in SDPAW increased up to 223 µM and 63 µM for 1 min SD treatment time with low applied voltages of 6 and 7.5 kV, respectively. NO_2^- was likely converted into NO_3^- by reacting with H_2O_2 after 3 min SD treatment time (Machala et al., 2013). NO_3^- concentrations of SDPAW with 6, 7.5 and 9 kV linearly increased to 13.5, 16.7 and 20 mM, respectively, within 30 min treatment (Figure 3.6E). Overall for SD treatment with 6, 7.5 and 9 kV, the concentration of RONS in PAW generated by SD was higher when applying higher voltage (Figure 3.6A, C, E and G). The SDPAW predominantly contained NO_3^- and peroxides with moderate to high ratios of H_2O_2.

The GDPAW up to 7 min GD treatment time predominantly contained NO_2^- and NO_3^-. The GDPAW with 25 kHz did not generate peroxides up to 7 min and further started generating peroxides after 10 min GD treatment (Figure 3.6D). The peroxides concentration of GDPAW with 25 kHz after 10 min increased linearly to 2.1 mM within 30 min treatment. In the GD treatment with high frequencies of 27.5 and 30 kHz, the peroxides concentration of GDPAW was not detected for 15 min and further increased linearly to 1.3 mM and 0.2 mM, respectively. The GDPAW contained no detectable H_2O_2. The peroxides generated could be superoxide, peroxynitrite and peroxynitrate. The GDPAW after 30 min treatment time predominantly contained NO_2^-, NO_3^- and peroxides. NO_2^- concentrations in GDPAW logarithmically increased to 594 µM within 15 min GD treatment time (Figure 3.6H). NO_2^- concentrations in GDPAW with 25 and 27.5 kHz reduced after 30 min GD treatment time. NO_3^- concentrations of GDPAW with 27.5 and 30 kHz increased linearly to 9.5 mM and 5.7 mM, respectively (Figure 3.6F). Notably, NO_3^- concentrations of GDPAW with 25 kHz increased linearly with a slope of 0.45 mM/min for 20 min and increased again with another slope of 0.99 mM/min. The absence of H_2O_2 and decrease in NO_2^- is closely related to the increase in slope of NO_3^-. It could be attributed to the reaction of NO_2^- with H_2O_2 and further conversion into NO_3^- (Silanikove, Shapiro, Silanikove, Merin, & Leitner, 2009). In summary, for GD treatment with 25, 27.5 and 30 kHz, the concentration of RONS in PAW generated by GD was higher when applying lower frequency. In comparison with the results in Figure 3.6 and in Lu et al. (2017), the trends of RONS generation in PAW were similar but the concentrations of RONS in PAW were different. The concentrations of NO_2^- and H_2O_2 in PAW in this study with similar parameters used were lower than Lu et al. (2017) while NO_3^- concentration in this study was higher than Lu et al. (2017). The higher NO_3^- concentration in both types of PAW compared to Lu et al. (2017) is possibly due to its higher plasma temperature, which favours the formation of NO_2 gas phase with higher densities (Malik, 2016). The difference in concentration of RONS may have been caused by differences in the power supply used.

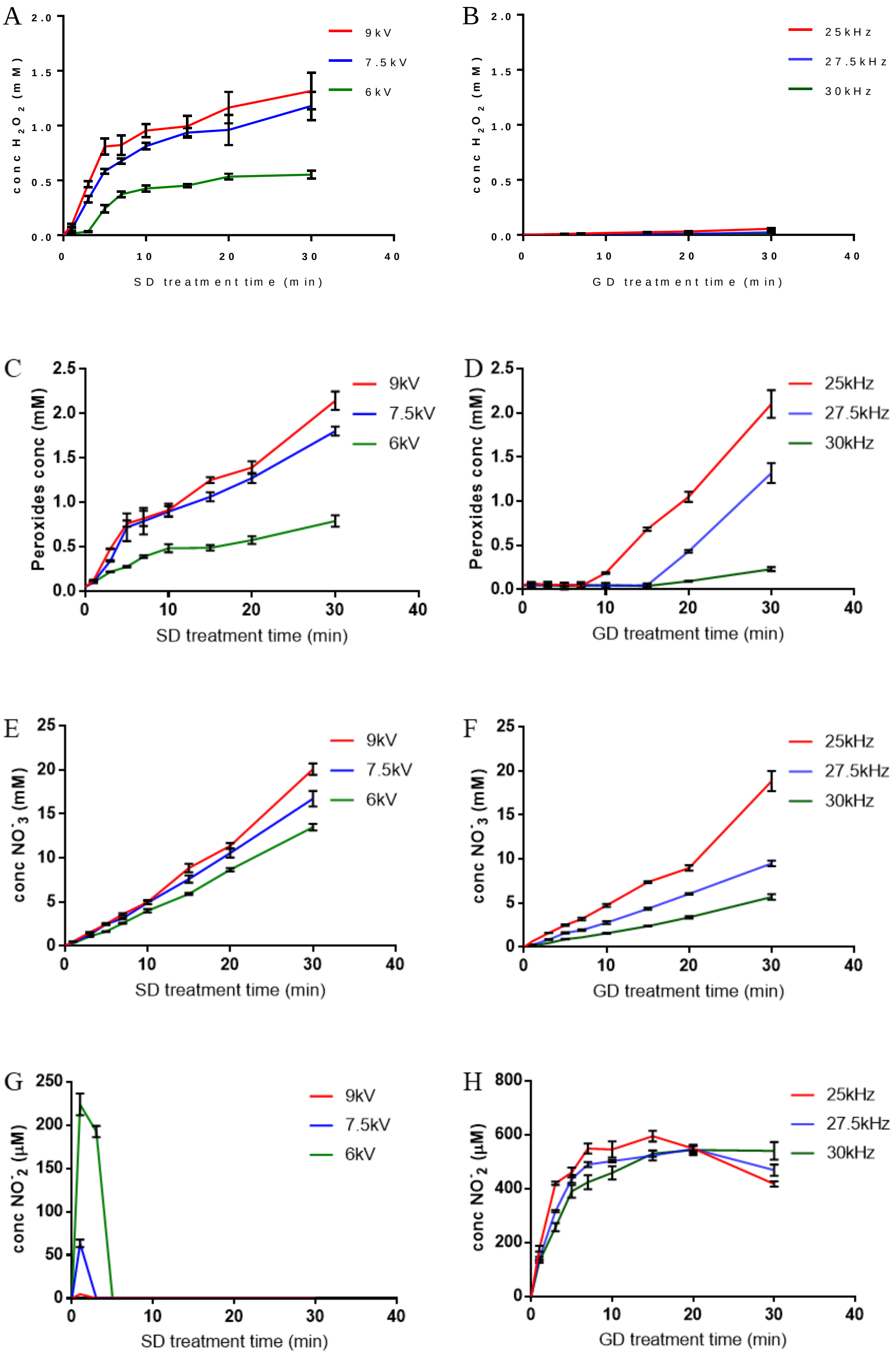


Figure 3.6 Chemical compositions of SDPAW with different voltages of 6, 7.5, 9 kV and GDPAW with different frequencies of 25, 27.5, 30 kHz. Hydrogen peroxide concentrations in (A) SDPAW and (B) GDPAW measured by $TiOSO_4$,

peroxides concentrations in (C) SDPAW and (D) GDPAW measured by buffered KI, nitrate concentrations in (E) SDPAW and (F) GDPAW, nitrite concentrations in (G) SDPAW and (H) GDPAW. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

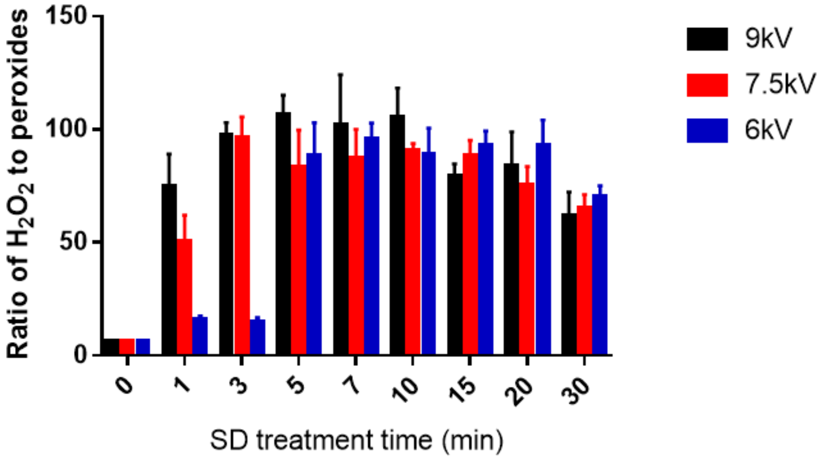


Figure 3.7 the ratio of H_2O_2 to peroxides concentrations in SDPAW with different voltages of 6, 7.5, 9 kV for up to 30 min treatment time. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

In summary, it can be concluded that the differences in the configurations of the plasma setup gave rise to significantly different liquid chemistry within the PAW. Moreover, the studies showed that PAW generated by either SD or GD was an acidic, strong oxidant with high water conductivity and this result is in close agreement with Thirumdas et al. (2018). The temperature of PAW during plasma generation was below 60 °C at a longer duration of treatment. Furthermore, the studies showed the generation of different long-lived reactive oxygen and nitrogen species (RONS) (such as peroxides, H_2O_2, NO_2^- and NO_3^-)

in the ultrapure water exposed to open air AC spark and glow discharges. Many studies have been published showing that these long-lived RONS were typically found in PAW. PAW generated by dielectric barrier discharge and microwave plasma contains NO_3^- and H_2O_2 (Schnabel et al., 2017; Tsoukou et al., 2018). Moreover, PAW generated by plasma jets or surface discharges contains H_2O_2, NO_2^- and NO_3 (Z. Liu et al., 2019; Renwu Zhou et al., 2018).

PAW has gained considerable attention for application in food and agriculture. PAW promotes a synergistic effect on the disinfection of food and while it can also promote growth of seeds (Los, Ziuzina, Boehm, Cullen, & Bourke, 2017, 2019). The RSS system provides opportunities for tailoring these reactive species in PAW for many aspects of the applications. Lu et al. (2018) demonstrated that the principal reactive species composition within PAW is closely related to the inhibition of cell growth in mammalian cells.

3.3 Conclusion

In this chapter, the characteristics of spark and glow discharges above the ultrapure water were studied. Spark discharge (SD) occurred in the pulse mode at the positive polarity half cycle and at the negative polarity half cycle, whereas the glow discharge (GD) was ignited at the negative polarity half period. SDPAW predominantly contains NO_3^- and peroxides with moderate to high ratios of H_2O_2 while GDPAW predominantly contains NO_2^- and NO_3^- at the initial stage and contains NO_2^-, NO_3^- and peroxides without H_2O_2 at the late stage. Compared to the previous literature, there were some differences between both results, particularly for GD, some of which may have resulted from minor difference in the power supply used. The applied voltage of GD maximally went up to 5 kV, whereas it previously reached 6.5 kV. The NO_2^- concentration increased to a maximum of approximately 600 µM, which was expected to increase linearly to 2 mM for 15 min GD treatment time with 25 kHz, reported by Lu et al. (2017). The liquid chemistry of PAW can be governed by the configurations of plasma setup and the plasma process parameters (such as applied voltage, frequency). In the next chapter, we study the relationship between the gas phase and liquid phase generated by SD and GD by looking at their characteristics in both phases.

Chapter 4 Plasma diagnostics of spark and glow (RSS discharges in air)

4.1 Experimental Design

A complete physical understanding of the mechanisms and nature of the plasma discharges was warranted. In this study, the Reactive Species Specificity (RSS) system was employed which consisted of two modes: spark discharge (SD) and glow discharge (GD). Notably, plasma activated water generated in spark discharge (SDPAW) predominantly contains H_2O_2 and NO_3^-, while plasma activated water generated in glow discharge (GDPAW) is rich in NO_2^- and NO_3^-.

In this chapter, we extend on the physical characterisation of the system to further understand the gas-phase discharge propagation mechanism, space and wavelength resolved imaging was performed. Using narrow bandpass filters at 310 nm, 340nm, 390 nm, and 780 nm the emission from excited OH, N_2 (SPS, Second Positive System), N_2^+ (FNS, First Negative System) and atomic O, respectively, were captured. The chemical composition of plasma activated water (PAW) generated by both discharges was examined by colorimetric measurement and UV-Vis absorption spectroscopy. The optical and electrical measurements were used to explain the result of the colorimetric tests and tentatively identify the link between the configuration of the plasma system and the generation of H_2O_2 in the bulk liquid.

The applied frequency was controlled to 25 kHz. The approach used is illustrated in Figure 4.1. 10 ml of deionised water was exposed to spark discharge (SD) with 7.5 kV or glow discharge (GD) with 5 kV.

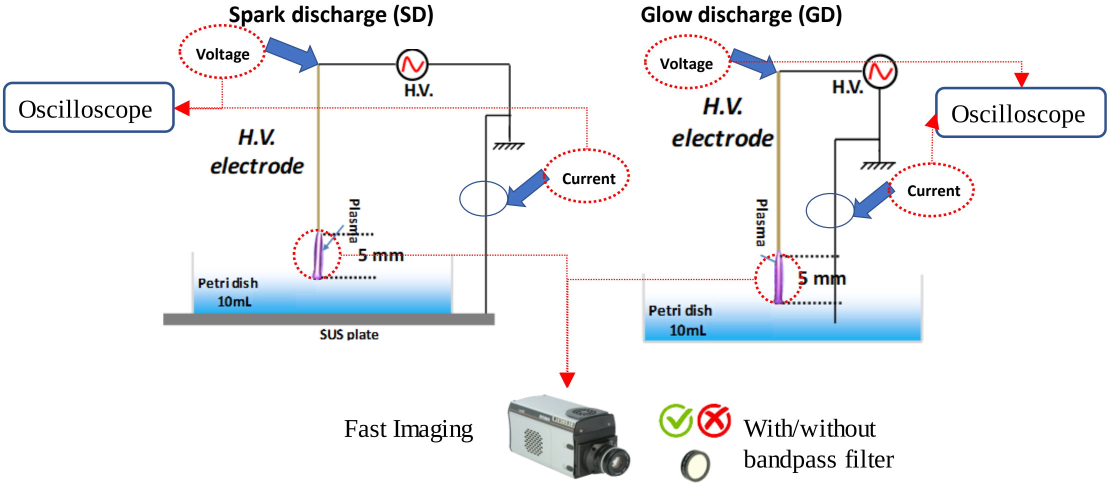


Figure 4.1 Gas-phase plasma diagnostics of spark discharge (SD) and glow discharge (GD) – experimental design.

4.2 Results and Discussion

4.2.1 Formation of RONS in the liquid phase

PAW generated by both spark and glow discharges was analysed by quantifying the concentration of H_2O_2, NO_2^- and NO_3^- using colorimetric analysis. It was determined that for GD, shown in Figure 4.2a, the NO_2^- concentration in µmol/L increased logarithmically in time *t* in min following:

$$[NO_2^-] = 67.784 \ln t + 152.23$$

The NO_3^- concentration of the GDPAW was found to increase linearly with a slope of 0.1135 mM/min while measurements revealed that no H_2O_2 was produced. In SD, shown in Figure 4.2b, it was determined that both the H_2O_2 and NO_3^- concentration increased linearly with a slope of 92.43 µM/min and 0.3737 mM/min, respectively. However, no NO_2^- was detected in SD. Consequently, it can be summarised that GDPAW was predominantly composed of NO_2^- and NO_3^-, while SDPAW was predominantly composed of H_2O_2 and NO_3^-. These results are in close agreement with our previous work (Lu et al., 2017).

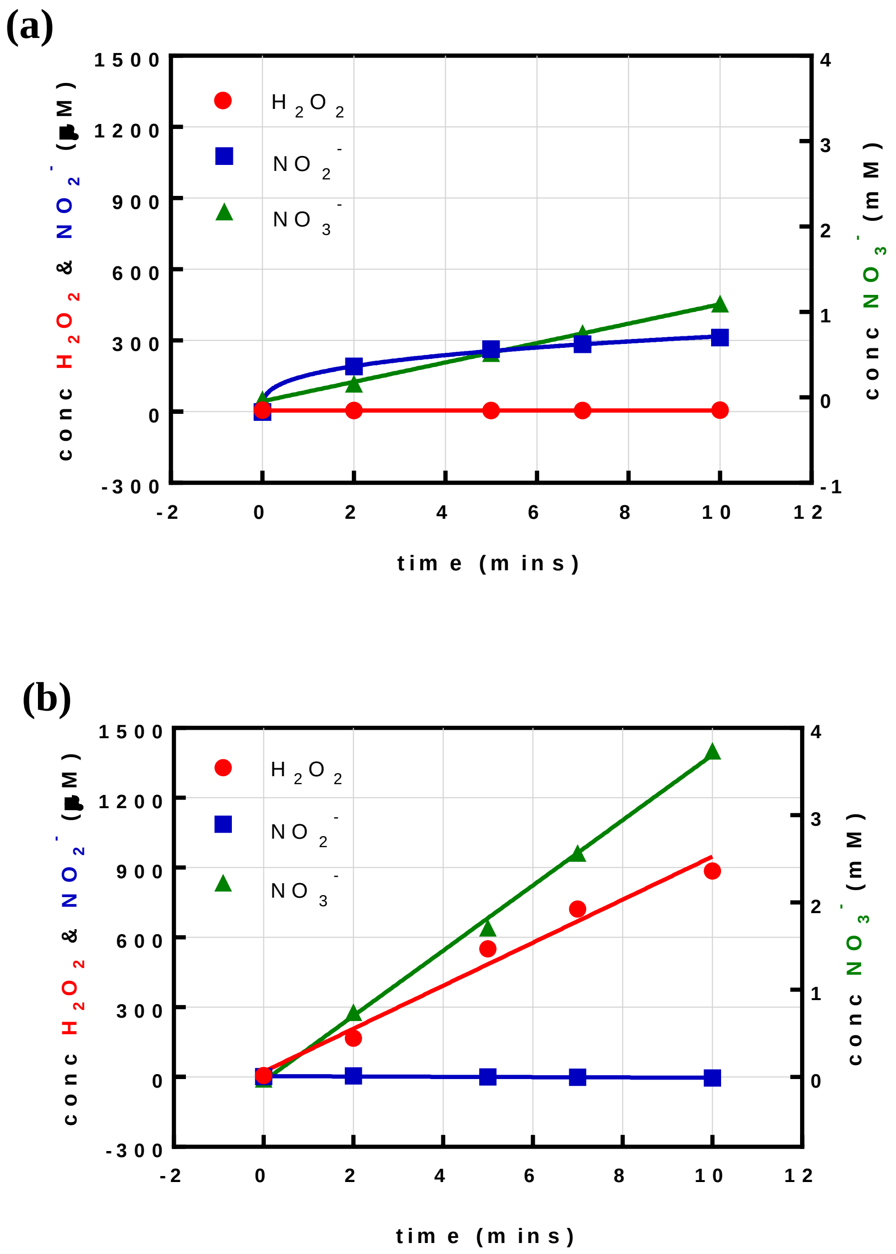


Figure 4.2 Concentration plots of H_2O_2, NO_2^-, and NO_3^- in plasma activated water (PAW) produced by (a) GD and (b) SD. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

To further assess the composition of PAW created in both discharges its UV absorption spectra were analysed and compared with those of standard solutions of 1 mM H_2O_2, 1 mM HNO_2 and 5 mM HNO_3. Figure 4.3 shows the absorbance spectra of PAW and the standard solutions. This figure highlights three main contributions corresponding to the absorbance of H_2O_2 around 260 nm, NO_3^- peaking at 300 nm and NO_2^- in the range of 330-400 nm as reported by Brubaker et al. (2017); Girard et al. (2016). The spectra of HNO_2 in the range of 330-400 nm observed as five finger-like peaks while the spectra of $NaNO_2$ or NO_2^- occurred around 350 nm (Brubaker et al., 2017). H_2O_2 in PAW from both discharges was difficult to detect from the UV spectra because no obvious peak at 260 nm was present in the UV spectra. PAW generated by both discharges was found to contain NO_3^-, evident by the peak observed at 300 nm. Notably, the distinctive five peaks associated with HNO_2 was only observed in the absorption spectra of GDPAW. The SDPAW had an absorption peak at 300 nm, aligning well with the absorption maximum of HNO_3. These results agree well with those obtained by the colorimetric measurements; GD was found to create substantial quantities of both NO_2^- and NO_3^-, which are both observed in the absorption spectra. In SD, no NO_2^- was detected in the PAW, while no HNO_2 is observed in the absorption spectra.

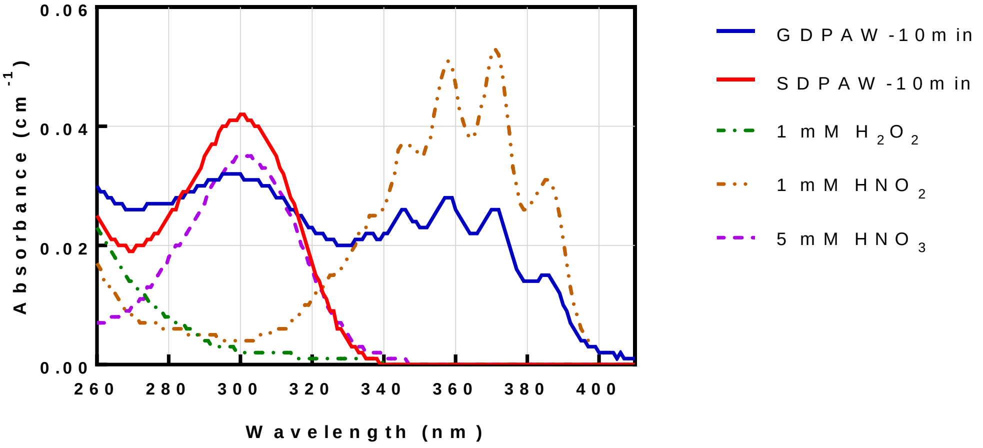


Figure 4.3 UV absorbance spectrum of GDPAW, SDPAW, 1 mM H_2O_2, 1 mM HNO_2, and 5 mM HNO_3.

Critically, RONS play an important role in the field of plasma medicine and decontamination applications, with H_2O_2 being reported to be involved in the antimicrobial action of PAW (R. Burlica, Grim, Shih, Balkwill, & Locke, 2010; Naitali et al., 2010). Furthermore, Afzali et al. (2020); R. Weller and Finnen (2006) reported that the acidified nitrite was able to accelerate wound healing in rats with type 2 diabetic. It was also reported that acidified nitrite was a novel antimicrobial agent for cutaneous pathogens, such as *Trichophyton mentagrophytes*, *T. rubrum*, *Candida albicans*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Propionibacterium acnes* (R Weller, Price, Ormerod, Benjamin, & Leifert, 2001). Naitali et al. (2010) proposed that nitrate had an antimicrobial effect in combination with nitrite in acidic condition. Hence, the ability to manipulate and tune the composition in PAW is of vital importance from an application perspective.

4.2.2 Time resolved discharge imaging

To understand how each electrode configuration yields a distinct PAW composition, time resolved short-exposure imaging was performed. The iCCD imaging for the discharges occurring when the system is in GD and SD is shown in Figure 4.4a and b, respectively. When the system was operated in GD, a single breakdown event in one complete applied voltage period was observed, this occurred in the negative half period at 22 µs (Figure 4.4a). It was observed that an intense streamer discharge was initiated at the powered pin electrode and propagated toward the liquid surface. As the discharge occurred only in the negative half-period of the applied voltage waveform, the pin electrode was acting as instantaneous cathode, thus indicating the streamer is anode directed. The light intensity was found to be the highest when in close proximity to the pin electrode. The intense streamer discharge was only visible for ~1.5 µs, after which an afterglow that was spatially confined to the pin electrode was observed to last for a further 20 µs.

Figure 4.4b shows that the discharge dynamics when the system is operated in SD differ significantly from those observed in GD. At approximately 3 µs into the positive-half cycle of the applied voltage waveform, when the pin electrode was the instantaneous anode, an intense cathode-directed streamer was observed. Following streamer propagation, a period of afterglow was observed to persist for approximately 10 µs at the water surface. During the negative half period a secondary breakdown event was observed at 21 µs. The dynamics of this streamer were very similar to those observed in GD, yet the intensity was approximately 5-fold lower. The afterglow for the negative streamer occurs only at the vicinity of the HV electrode, following a similar pattern to that observed in Figure 4.4a.

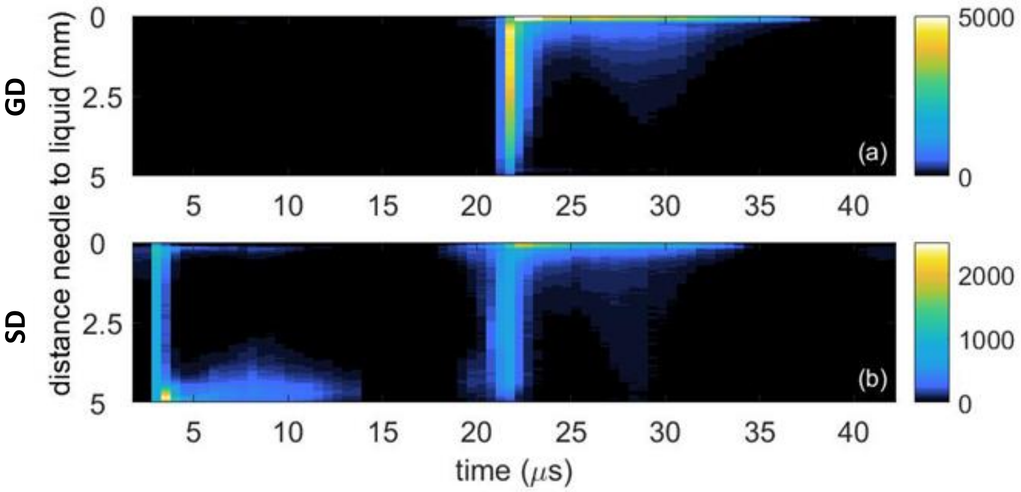


Figure 4.4 Time resolved fast iCCD imaging to resolve the entire plasma period using 500ns steps when the system is in GD (a) and SD (b). The distance was measured from the tip of the needle to the liquid surface.

4.2.3 Wavelength resolved discharge imaging

Wavelength resolved imaging was used to gain an appreciation for the generation of excited states within the discharge created by each discharge system. Figure 4.5 shows the accumulated light emission for the positive and negative half period with the system in GD and SD separately. The upper and lower red lines indicate the locations of HV electrode and water surface.

During the positive half-period there is no emission at any wavelength in GD, matching the results shown in Figure 4.4a. During the negative half-period, emission observed using the BPF of 310 nm (including OH emission and N_2 SPS (0,0) band) reached an intensity of $5x10^3$ counts at 1 mm from the liquid surface, and $1x10^4$ counts were detected closer to the HV electrode. Critically, the emission from excited OH does not appear to contact with the liquid surface, a finding that may explain the lack of H_2O_2 detected in the liquid phase when using GD, as OH in the gas phase is a precursor of H_2O_2 in the liquid phase (Norberg, Tian, Johnsen, & Kushner, 2014). Light emissions at 340 and 390 nm (representing N_2 and N_2^+ emissions) appear to show contact with the liquid, although the intensities are an order of magnitude higher at the electrode ($3x10^4$ counts), reducing by an order of magnitude away from the electrode. The light emission at 780 nm (atomic O) was very weak throughout the entire discharge. With the system in SD, the wavelength resolved emission clearly highlighted the presence of a streamer during the positive half-period of the applied voltage.

Emission at 310 nm reached a maximum of 2x10^4 counts close to the HV electrode, while still reaching approximately 1x10^4 counts close to the liquid surface. The presence of OH emission in close proximity to the liquid surface when using this discharge system may account for the presence of H_2O_2 in the bulk liquid due to interactions occurring at the liquid interface. The light emissions at 340 and 390 nm appeared similar to those observed at 310 nm.

The differences in spatio-temporal profile of the discharges in each discharge system clearly lead to a significant variation in the composition of the resultant PAW, most notably in the generation of H_2O_2, which is an important molecule for biological applications. In GD, H_2O_2 was completely absent, which is in stark contrast to that observed in SD, where µM concentrations were measured. We hypothesise that this major difference can be attributed to two key factors: (1) OH emission, which may be used as an indirect indicator of ground-state OH density, does not appear to reach the liquid surface in GD, indicating a reduced concentration of an important precursor for H_2O_2 formation. To support this hypothesis, computational studies of streamers in atmospheric pressure air have shown that the electric field in an anode directed streamer, i.e. the only discharge observed in GD, is considerably weaker than that in a corresponding cathode directed streamer (Luque, Ratushnaya, & Ebert, 2008). From this it may be inferred that a weaker electric field leads to a reduction in the generation of OH and consequently H_2O_2 in the liquid phase. Previous studies have also shown that OH formed in the plasma phase is the primary source of H_2O_2 in the bulk liquid (Norberg et al., 2014).

It is clear that some OH states have not been detected in optical emission imaging studies. Undetected OH can reach the liquid surface and consequently form H_2O_2 in the liquid phase. In GD, no H_2O_2 was detected in PAW because only small amounts of OH might be generated above the water surface these reacted with NO, forming NO_2^- and NO_3^- (Lu et al., 2017). Secondly, H_2O_2 in the liquid phase could be electrolysed and form oxygen bubbles around the grounded electrode (Rumbach, Witzke, Sankaran, & Go, 2013).

(2) an afterglow lasting ~10 µs was formed at the liquid surface during the positive half-cycle of the applied voltage waveform in SD, no such afterglow was observed in either half-period when GD was adopted. In GD, the plasma was observed to briefly interact with the liquid surface for a maximum of 2 µs during the negative half-cycle of the applied voltage cycle. Under such circumstances, it may be assumed that longer-lived chemical species, such as NO, created in the plasma phase diffuse to the liquid surface and significantly influence the liquid chemistry. Conversely, in SD, the plasma had a significantly longer contact time with the liquid, enabling short-lived species, such as O and OH to take part in reactions at the interface.

Overall, the results showed that the OH emitted from a cathode-directed streamer of SD was denser than the OH emitted from an anode-directed streamer of any discharge. These results are in good agreement with the results of Nikiforov et al. (2011); Xiong, Yang, and Bruggeman (2015). Nikiforov et al. (2011) found that the density of OH radicals emitted from an anode-directed streamer is almost 2-fold lower than from a cathode-directed streamer. Xiong et al. (2015) also discovered that higher OH density was observed during cathode-directed streamer discharge. The higher OH density presented in the cathode-directed streamer can be attributed to the fact that the positive column is constricted by bombarded ions and therefore the current density is higher than in the anode-directed streamer, according to those authors. Combining the studies between liquid chemistry and plasma diagnostics, the production of H_2O_2 in the SDPAW can be attributed to the higher OH density emitted from the cathode-directed streamer of SD. The OH radical is a strong oxidant (Ehhalt, Dorn, & Poppe, 2011). In other words, the degree of oxidation induced by SD is higher than GD, due to the presence of high OH density.

From an electrical point of view, the interplay between the impedance of the power source and the coupling of the plasma, through the liquid, to ground causes a shift in the dynamics of the discharge which ultimately impacts the chemistry of the generated PAW. To improve the tunability of chemistry in PAW, instead of using a container, using a grounded electrode coated with various dielectric materials with different thicknesses might give an additional insight. In addition, to improve the measurement of the dynamics of all states of OH, laser-induced fluorescence could be employed in further studies.

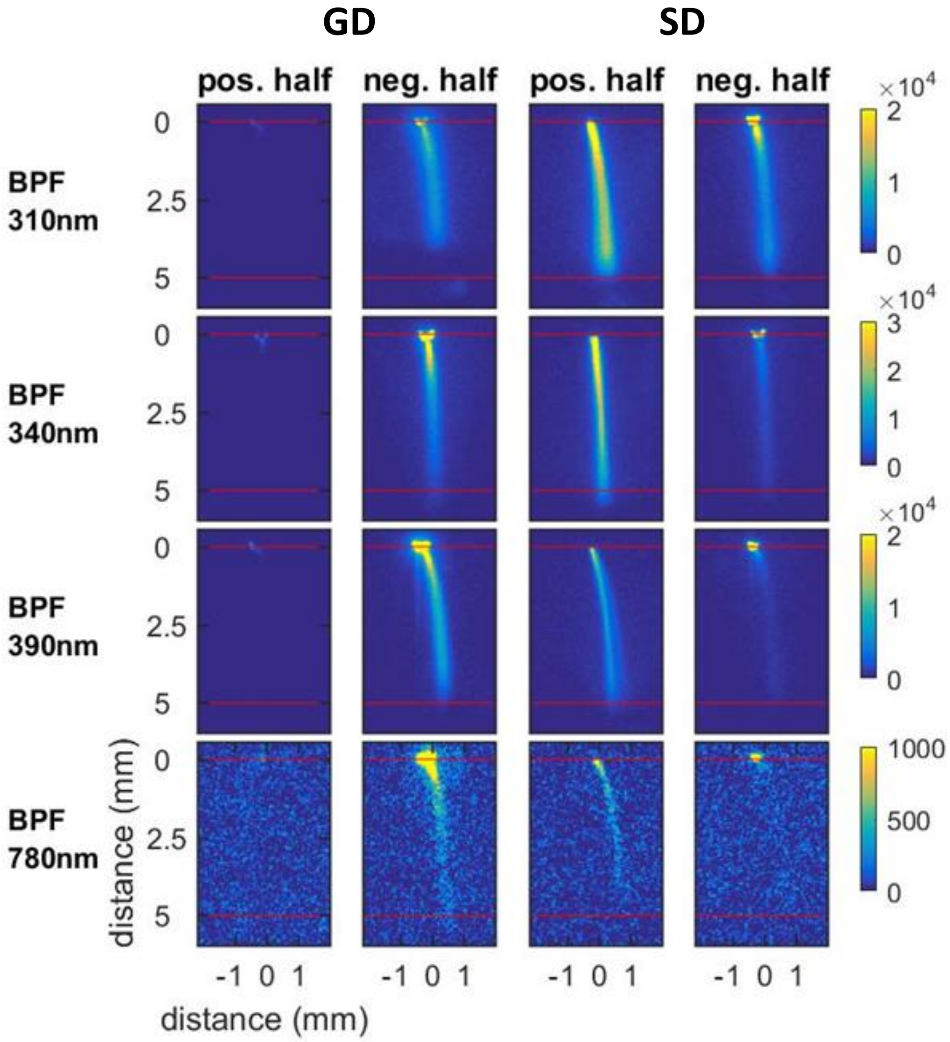


Figure 4.5 Species-resolved images of GD and SD at positive and negative half- periods through narrow bandpass filters of 310 nm, 340nm, 390 nm, and 780 nm for emissions from OH, N_2 (SPS), N_2^+ (FNS) and atomic O, respectively. The upper red line indicates the location of the tip of HV needle while the lower red line indicates the location of the water surface.

4.3 Conclusion

This study advanced the characterisation of a single pin-to-liquid plasma system operated in two different discharge modes. In GD the liquid was directly electrically grounded via a stainless-steel rod, while in SD, a large ground plate was placed beneath the petri dish reservoir holding the liquid. It was shown that in GD no H_2O_2 is created in the liquid, with the chemistry being dominated by NO_2^- and NO_3^-. When the system was operated in SD, high concentrations of H_2O_2 and NO_3^- were detected alongside with no measurable NO_2^-.

Ultimately, it can be concluded that the differences in the grounding of the plasma system gave rise to significantly different discharge dynamics, with SD producing both anode-directed and cathode-directed streamers, while GD produced only anode directed streamers. Critically, both discharges were capable of generating RONS within a liquid volume, yet the composition of the PAW created was different in each case. In GD it was observed that only a single, anode directed streamer was created during each period of the applied voltage, which was assumed to compromise OH and subsequently H_2O_2 production in the liquid. In SD, it was observed that the higher OH density emitted from the cathode-directed streamer of SD was converted into liquid form of H_2O_2. Hence, it could indicate that the degree of oxidation induced by SD is higher than GD, due to the presence of high OH density.

From an application perspective this is important as it confirms that the composition of plasma reactive species and PAW can be tailored simply by varying the electrode configuration. Many potential applications of this RSS system, such as microbial decontamination (Tsoukou, Delit, Treint, Bourke, & Boehm, 2021) or immune inhibitory effects (Ng et al., 2020), are enhanced by the presence of OH radicals and H_2O_2, thus this work demonstrates a way in which OH radicals and H_2O_2 production can be significantly enhanced. The fundamental understanding of the translation of plasma discharge into liquid chemistry provides the foundation for the following chapters, where the effect of plasma treatments on food allergens is investigated.

The following paper has been published from this study:

Ng, S. W., Slikboer, E., Dickenson, A., Walsh, J. L., Lu, P., Boehm, D., & Bourke, P. (2021). Characterization of an atmospheric pressure air plasma device under different modes of operation and their impact on the liquid chemistry. *Journal of Applied Physics, 129* (12). doi:10.1063/5.0039171

Chapter 5 Effect of direct RSS plasma treatment on the antigenicity of milk and wheat proteins

5.1 Experimental Design

Cross-contamination of allergens in food processing environments is a persistent concern for the food industry. Milk and milk-derived ingredients are one of the most common food allergens present in the food sector. Casein and whey proteins are the major allergens in bovine milk. This study investigated whether ACP interacts with milk proteins to evaluate if ACP can present an alternative for preventing allergen cross-contamination in the food industry. The approach used is illustrated in Figure 5.1.

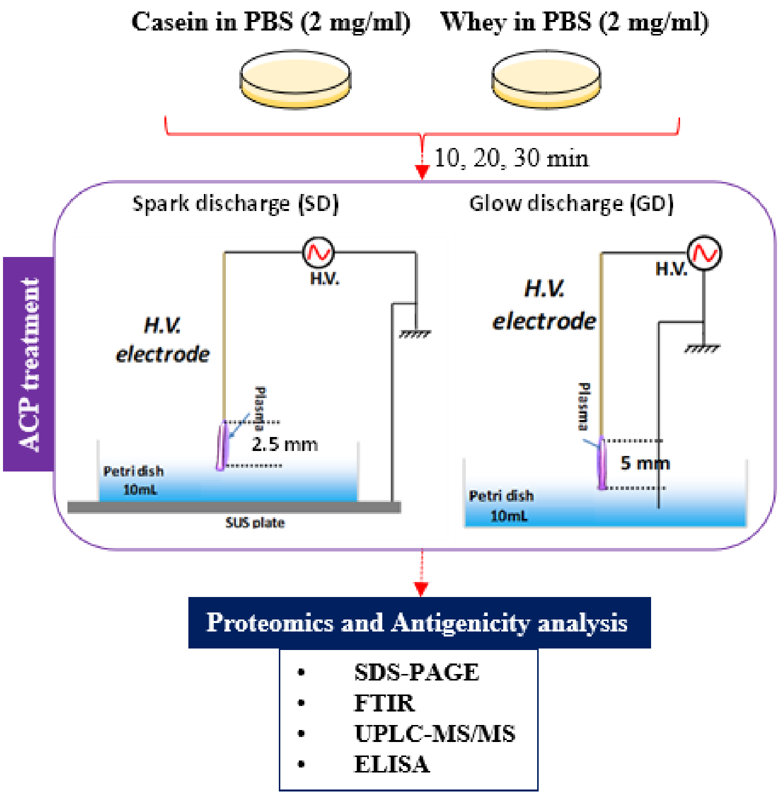


Figure 5.1 RSS treatment of casein and whey proteins – experimental design.

In this study, the ACP treatment was performed using the Reactive Species Specificity (RSS) system. The RSS system consisted of two modes: spark discharge (SD) and glow discharge (GD). The influence of ACP treatment using SD and GD on the antigenicity of milk proteins (casein, β-lactoglobulin and α-lactalbumin) was investigated. The influence of both discharges on the proteomic profile of casein and whey proteins was studied to provide a better understanding of the potential interaction of two characterised and different plasma discharges with casein and whey proteins.

In addition, wheat gluten is another common food allergen present in the food industry, especially in the baking and food processing industries, where they present thermal resistant challenges. Gliadin protein is the major allergen in wheat gluten. The proteins associated with milk- and wheat-derived allergens are different in terms of protein structure and composition, and this study was extended to include wheat allergens to assess the universality of the ACP treatments. The approach used is illustrated in Figure 5.2. To obtain a better knowledge of the possible interaction of these two plasma discharges with the antigenicity of wheat gluten protein, the impact of both discharges on the proteomic profile of wheat gluten proteins was investigated.

The effect of initial protein concentration was considered in the wheat allergen trials, to assess if protein concentration affected the potential for antigenicity reduction using ACP. The protein concentrations used were 10, 1, and 0.1 mg/ml. These amounts are 100 or 10 times higher or equal to the allergic-eliciting threshold level established by EU regulations 1169/2011 and were selected to provide a challenge range to quantify effects.

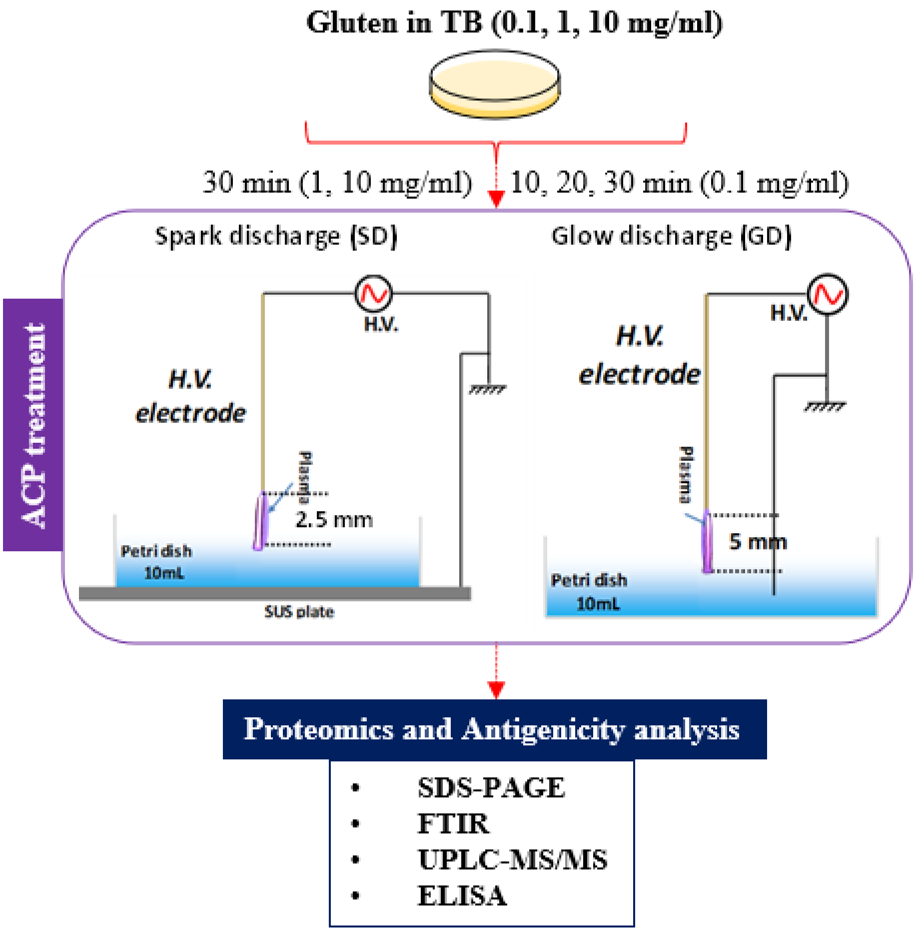


Figure 5.2 RSS treatment of wheat gluten proteins – experimental design.

5.2 Results and Discussion

5.2.1 Characteristics of SD and GD plasma treatment

Two types of discharges above the liquids were monitored through an oscilloscope coupled with a HV probe and current probe. The applied voltage and current waveforms differentiate between SD and GD, as are observed in Figure 5.3. SD was ignited at the positive polarity half period of the applied voltage shown in Figure 5.3a, while GD was ignited at the negative polarity half period of the applied voltage shown in Figure 5.3b. SD predominantly generates reactive oxygen species while GD predominantly generates reactive nitrogen species (Lu et al., 2017; Lu et al., 2018).

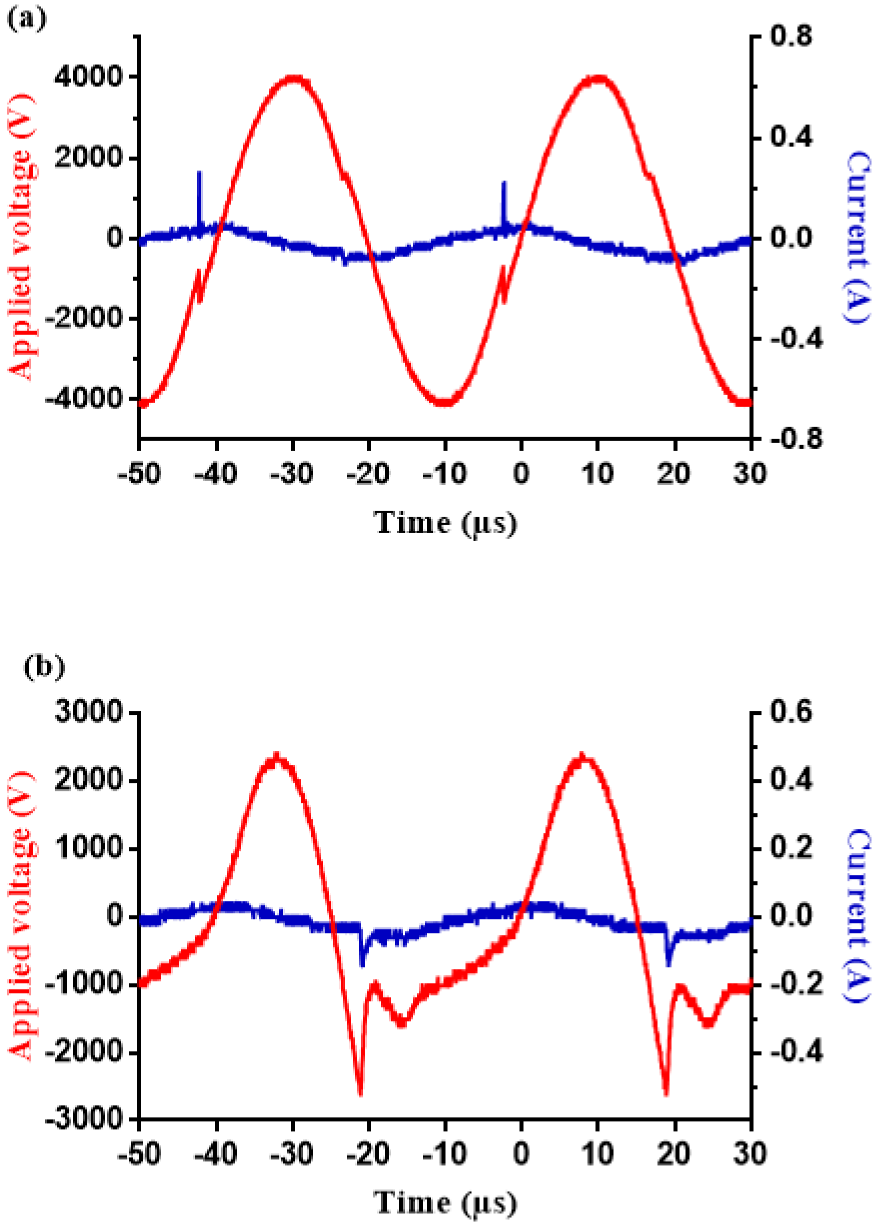
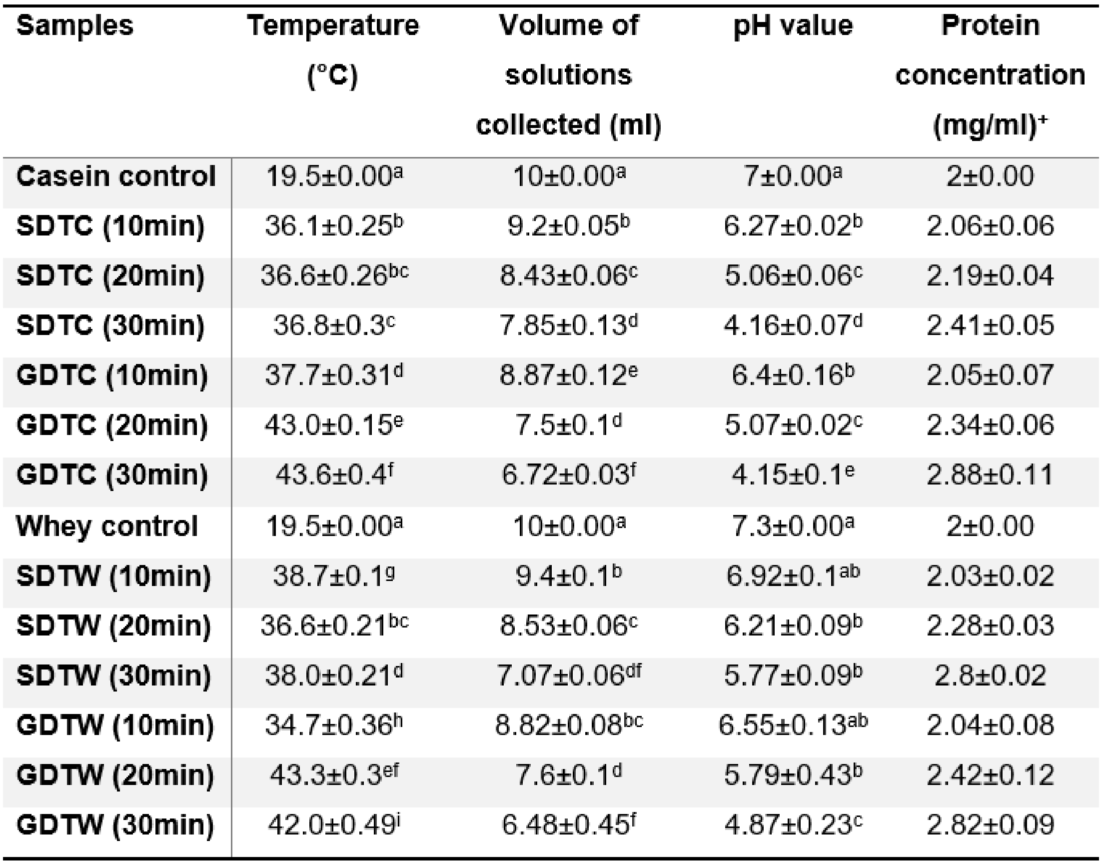


Figure 5.3 Applied voltage and current waveforms of (a) spark discharge (SD) and (b) glow discharge (GD).

Each plasma treatment was observed to reduce the liquid volume of casein and whey solutions (Table 5.1). The volume decreased with increasing treatment time. This moisture loss was due to increase in temperature (up to 43.3 °C recorded) which led to some evaporation of water molecules. Approximately 6.2 ml was collected after 30 min of plasma treatment from 10 ml of initial sample. This had the effect of concentrating the protein concentrations further (Table 5.1). Hence, it was necessary to recover the protein concentration of treated samples as per control (2 mg/ml) before further analysis. In addition, phosphate, present in the buffer, precipitated during GD treatment.

Applying either SD or GD treatments decreased the pH value of the protein solutions significantly within 10 min. The pH value was reduced from 7 and 7.3 (untreated casein and whey solutions, respectively) to a minimum of 4.15 and 4.87 after 30 min of SD or GD treatment time, respectively (Table 5.1). The decrease of pH value could be attributed to nitrogen oxides produced in the plasma interacting with liquids and producing nitric and nitrous acids (Tsoukou et al., 2018).

Table 5.1 Temperature, volume and pH value of milk protein solutions collected from untreated, and SD and GD treated samples.



Samples	Temperature (°C)	Volume of solutions collected (ml)	pH value	Protein concentration (mg/ml)$^{+}$
Casein control	19.5±0.00^{a}	10±0.00^{a}	7±0.00^{a}	2±0.00
SDTC (10min)	36.1±0.25^{b}	9.2±0.05^{b}	6.27±0.02^{b}	2.06±0.06
SDTC (20min)	36.6±0.26^{bc}	8.43±0.06^{c}	5.06±0.06^{c}	2.19±0.04
SDTC (30min)	36.8±0.3^{c}	7.85±0.13^{d}	4.16±0.07^{d}	2.41±0.05
GDTC (10min)	37.7±0.31^{d}	8.87±0.12^{e}	6.4±0.16^{b}	2.05±0.07
GDTC (20min)	43.0±0.15^{e}	7.5±0.1^{d}	5.07±0.02^{c}	2.34±0.06
GDTC (30min)	43.6±0.4^{f}	6.72±0.03^{f}	4.15±0.1^{e}	2.88±0.11
Whey control	19.5±0.00^{a}	10±0.00^{a}	7.3±0.00^{a}	2±0.00
SDTW (10min)	38.7±0.1^{g}	9.4±0.1^{b}	6.92±0.1^{ab}	2.03±0.02
SDTW (20min)	36.6±0.21^{bc}	8.53±0.06^{c}	6.21±0.09^{b}	2.28±0.03
SDTW (30min)	38.0±0.21^{d}	7.07±0.06^{df}	5.77±0.09^{b}	2.8±0.02
GDTW (10min)	34.7±0.36^{h}	8.82±0.08^{bc}	6.55±0.13^{ab}	2.04±0.08
GDTW (20min)	43.3±0.3^{ef}	7.6±0.1^{d}	5.79±0.43^{b}	2.42±0.12
GDTW (30min)	42.0±0.49^{i}	6.48±0.45^{f}	4.87±0.23^{c}	2.82±0.09

SDTC, GDTC, SDTW and GDTW stand for SD treated casein, GD treated casein, SD treated whey and GD treated whey, respectively.

$^{+}$This column shows the protein concentration before and after plasma treatment.

*Values are expressed as mean ± SD. Values with different letters in a column are significantly different ($p < 0.05$). (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

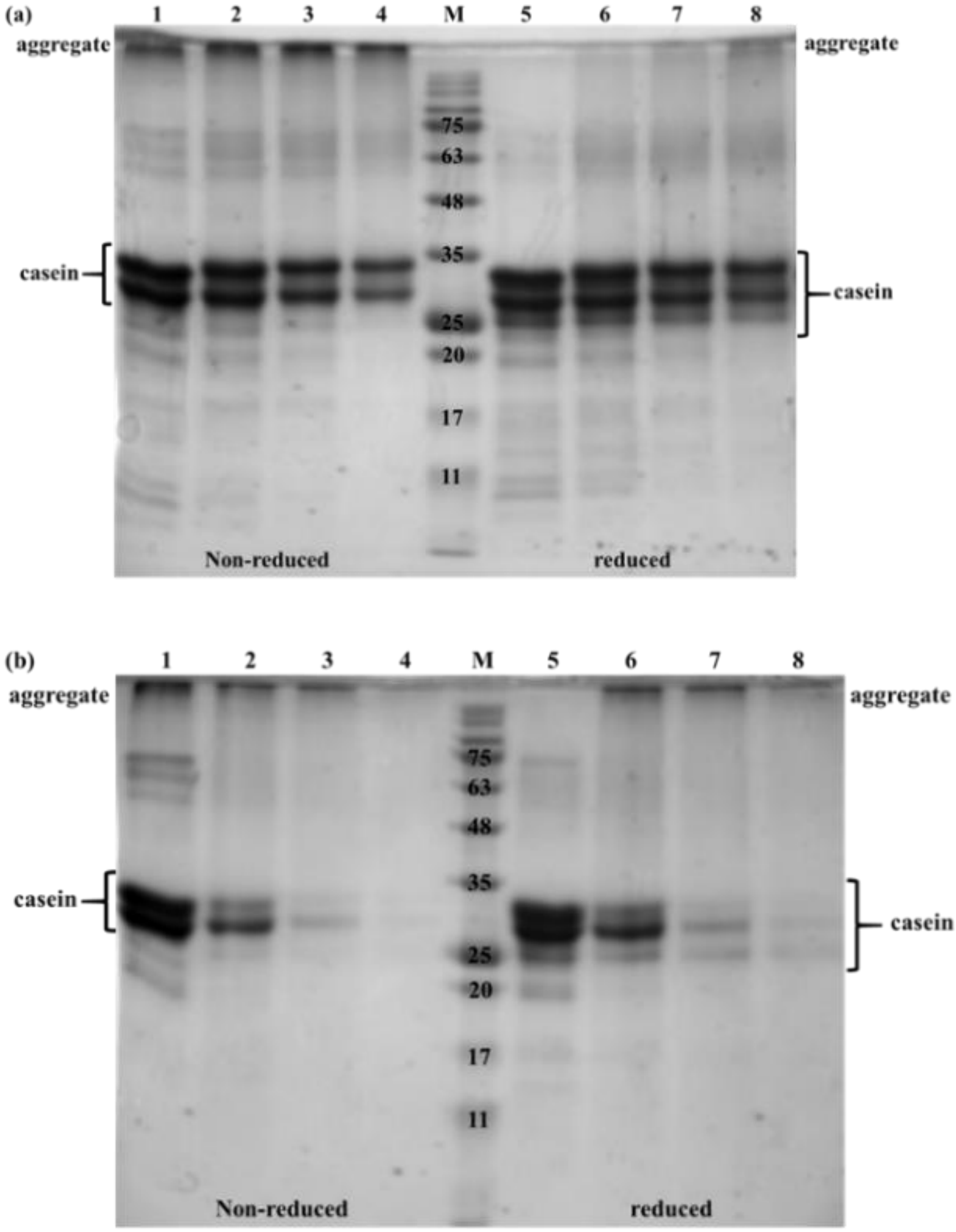
For wheat allergen studies, the gluten solution was subjected to SD and GD. The applied voltage and frequency were 8 kV and 25 kHz, to mimic the process parameters applied to the milk allergens. 10 mg/ml of gluten solution (considered as high concentration) and 1 mg/ml of gluten solution (considered as medium concentration) were subjected to ACP for a treatment time of 30 min. 0.1 mg/ml of gluten solution (considered as low concentration) was subjected to ACP treatment for 10, 20, and 30 min. The protein concentration of treated samples was re-adjusted to 10 mg/ml for further analysis.

5.2.2 SDS-PAGE analysis

In the bovine milk studies, SD and GD treated caseins were analysed using SDS-PAGE under non-reducing and reducing conditions for their electrophoretic profiles, shown in Figure 5.4a and b, respectively. Casein is known as the major allergen in milk. The band of native casein occurred around the protein marker of 25-35 kDa under non-reducing and reducing conditions. As shown in Figure 5.4a, the band intensity of casein slightly decreased with SD treatment time for both non-reducing and reducing conditions. Observing the control in lanes 1 and 5 at the top of the gel in Figure 5.4a, the buffer soluble aggregate disappeared after reducing the disulphide bond with β-mercaptoethanol as a reducing agent. However, in the presence of reducing agent, the soluble aggregates were found after SD treatment in the gel compared to the control. This could be explained by a change in solubility as a result of SD treatment, which is induced by the new formation of higher molecular weight aggregates via the formation of disulphide bonds, hydrophobic and electrostatic interactions and inter protein crosslinking (Tammineedi et al., 2013). As shown in Figure 5.4b, the band intensity of casein declined dramatically with increasing GD treatment time in both conditions. Observing the untreated and GD treated casein in non-reducing conditions, no protein band was found in lane 4 of the gel (Figure 5.4b). The lost protein band in lane 4 could be attributed to the formation of new, buffer insoluble aggregates via the generation of disulphide bonds, hydrophobic and electrostatic interactions, and intra/inter protein crosslinking. After 10 min and 20 min GD treatment, we can observe the soluble aggregate in reducing conditions, however, after 30 min GD treatment, we can see very little soluble aggregate in reducing condition (Figure 5.4b). This is attributed to insolubility of protein after extended GD treatment. In the presence of reducing conditions (Figure 5.4b, lane 8) the existence of soluble aggregate was attributed to the formation of disulphide bonds by comparison with the GD treated casein (30 min) in non-reducing and reducing conditions, lanes 4 and 8, respectively. This emphasises the overall greater changes in the protein profile of casein induced by GD than by SD treatment.

The SDS-PAGE profiles of SD and GD treated whey proteins under non-reducing and reducing conditions are shown in Figure 5.4c and d. Whey proteins, as major allergens in milk, are composed of β-lactoglobulin and α-lactalbumin. As shown in Figure 5.4, the band at 17 kDa corresponds to β-lactoglobulin; and the band at 11 kDa corresponds to α-lactalbumin. The band located approximately at 35 kDa is the dimer of β-lactoglobulin. Applying SD, the results in Figure 5.4c showed that the band intensity of native and dimer β-lactoglobulin was largely unchanged even at the maximum treatment duration of 30 min. However, the band of native and dimer β-lactoglobulin was more smeared and broader. This could indicate some SD induced structural modification of β-lactoglobulin, resulting in changes to the molecular weight of β-lactoglobulin. R. Zhou et al. (2016) reported the modification of amino acids by reactions induced by plasma, such as nitration, oxidation, hydroxylation, dehydrogenation, sulphonation, and dimerization. These reactions can modify the protein structure and cause changes in the molecular weight of proteins. The band intensity of bovine serum albumin (BSA) remained unchanged, except for a slight decrease in band intensity following 30 min of SD treatment. Similar trends were recorded for the band intensity of α-lactalbumin in the presence of reducing agent. However, the α-lactalbumin band density faded upon prolonged exposure to SD treatment in the absence of reducing agent. This could be explained by the new generation of disulphide bonds during SD exposure.

Employing GD, Figure 5.4d shows notable changes in the profile of both β-lactoglobulin and α-lactalbumin when compared to the control. The native and dimer of β-lactoglobulin bands and BSA bands gradually decreased with increased GD treatment time. Similar to GD treated α-lactalbumin, its band intensity diminished after a treatment time of 30 min in non-reducing condition. Moreover, the protein bands in lanes 6 and 7 in Figure 5.4d were smeared, indicating that the structure of whey proteins could be modified by GD treatment. As shown in Figure 5.4c and d, little or no soluble aggregate was found under reducing condition at the top of the gel. Presumably, the formation of insoluble aggregates, particularly in the case of Figure 5.4d, were induced by the plasma treatment which led to disappearance of protein bands in SDS-PAGE, as only soluble proteins can be detected in the electrophoretic profile. Analysis of whey proteins demonstrated a higher notable change in the protein profile of whey treated by GD than by SD.


(a)
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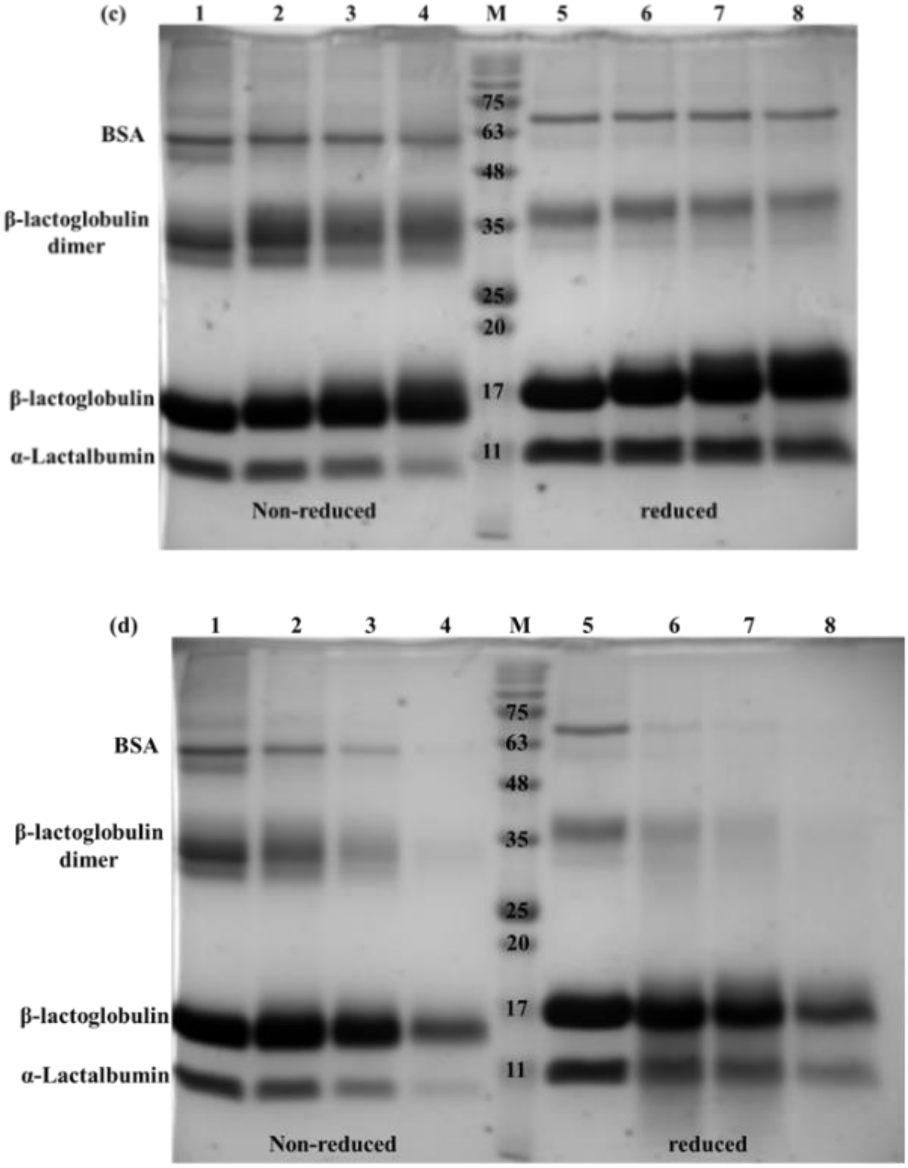


Figure 5.4 SDS-PAGE profiles of untreated; (a) SD and (b) GD treated caseins; (c) SD and (d) GD treated whey proteins subjected to different exposure times. M: the standard protein marker. Lane 1-4: control and samples treated for 10, 20, 30 min, respectively, under non-reducing condition. Lane 5-8: control and samples treated for 10, 20, 30 min, respectively, under reducing condition.

In the gluten studies, SD and GD treated glutens were analysed using SDS-PAGE under the reduced conditions for their electrophoretic profiles, shown in Figure 5.5a and b, respectively. Gluten is the major allergen in wheat. Gluten protein fragments were separated in the gel. The bands of high molecular weight (HMW)- and low molecular weight (LMW)-glutenin subunits occurred around the protein markers of 100-125 kDa and 45-55 kDa, respectively, under reducing condition. The band at 75 kDa corresponds to ω5-gliadin. The bands located in the range of 60-70 kDa are ω1,2-gliadins. The bands located in the range of 30-40 kDa are α-/β-gliadins. Figure 5.5 shows notable changes in the profile of gluten after plasma treatment when compared to the control in lane 2. The results of both treatments were similar. The band intensities of high gluten concentration (10 mg/ml) were distinctly decreased with both spark and glow discharge plasma treatments for 30 min treatment time compared to the controls in lane (Figure 5.5a and b). The band intensities of medium (1 mg/ml) and low (0.1 mg/ml) gluten concentrations disappeared with both discharge modes for 30 min treatment time. This is attributed to denaturation of proteins after plasma treatment. At a treatment time of 10 min, the band intensities of gluten with low protein concentration in lane 7 had disappeared. This could indicate the gluten structure can be completely denatured at a treatment time of 10 min or less when a gluten concentration of 0.1 mg/ml was subjected to plasma treatment; or at a treatment time of 30 min or less when a gluten concentration of 1 mg/ml was subjected to plasma treatment.

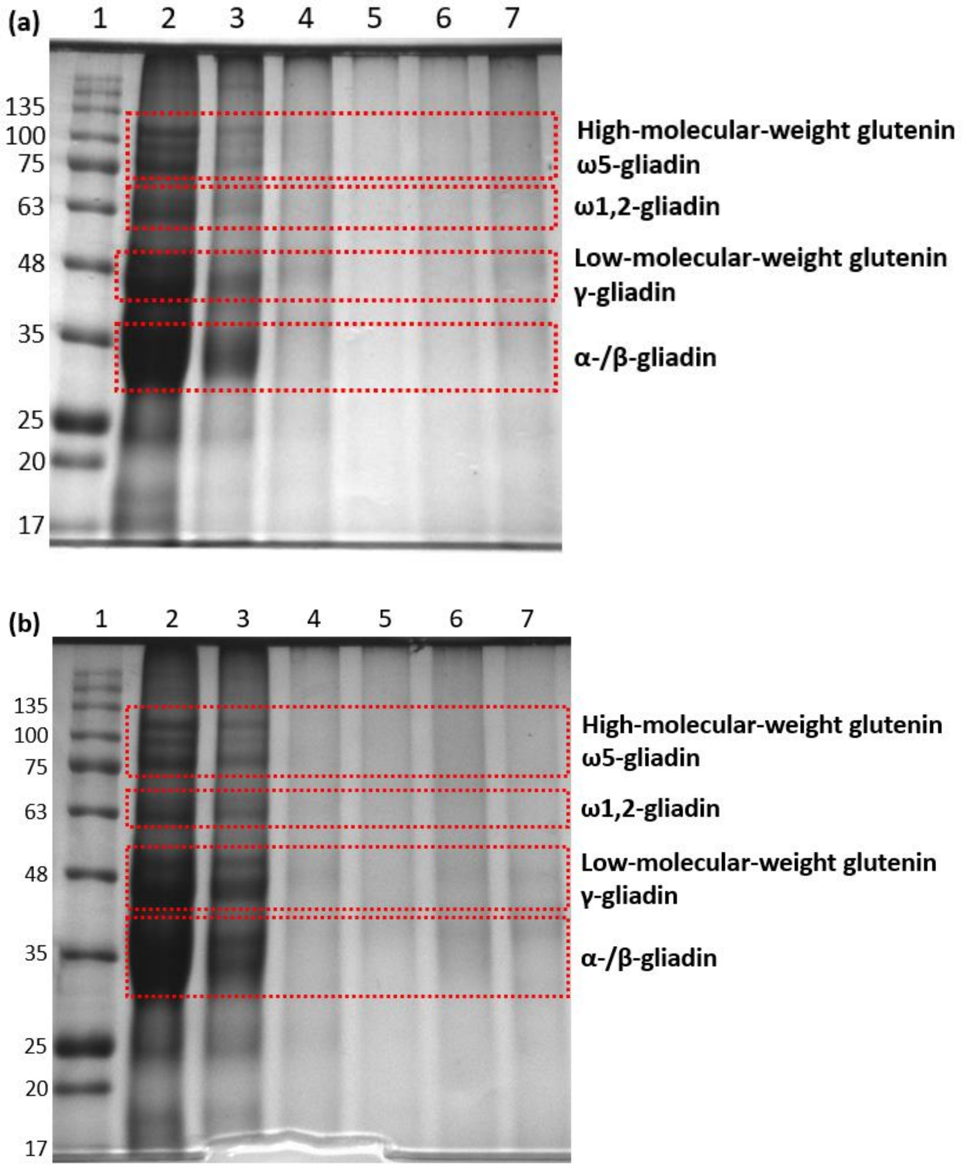


Figure 5.5 SDS-PAGE profiles of untreated; (a) SD and (b) GD treated glutens subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Gluten control. Lane 3-4: high (10 mg/ml) and medium (1 mg/ml) protein concentrations, respectively, treated for 30 min, respectively. Lane 5-7: low (0.1 mg/ml) protein concentrations treated for 30, 20, 10 min, respectively.

Atmospheric cold plasma offers a source of reactive oxygen and nitrogen species along with UV irradiation, which have the potential to modify protein structures (Ramazzina et al., 2016). A previous study on the effect of a plasma jet system using the noble gas argon as the working gas on major milk allergens α-casein, β-lactoglobulin and α-lactalbumin showed no significant change in SDS-PAGE band intensity compared to the control (Tammineedi et al., 2013). However, the samples were not directly exposed to the plasma discharge or afterglow, unlike the current work, where samples in solution were directly exposed to the tunable discharge. Moreover, there is no study to date on the effect of ACP on gluten by observing the SDS-PAGE of gluten profiles. In this study, the SDS-PAGE results showed significant changes in casein, whey, and gluten protein profiles post RSS treatment.

5.2.3 Secondary structural analysis

The FTIR spectra of casein and whey proteins were used to define the extent of secondary structural changes induced by cold plasma. The deconvoluted amide I (1600-1700 cm^{-1}) region is widely used in the analysis of protein secondary structure. The resultant peaks at 1620 ± 20 cm^{-1}, 1645 ± 5 cm^{-1}, 1654 ± 4 cm^{-1}, and 1680 ± 20 cm^{-1} corresponded to β-sheets, random coils, α-helices, and β-turns, respectively, in the fitting procedure (Hu, Zheng, Liu, Deng, & Zhao, 2016).

As shown in Table 5.2, the FTIR analysis of the amide I band showed that SD and GD treatment changed the secondary structural components of casein, particularly β-sheets and β-turns. The β-sheet content of both SD and GD treated casein decreased. The β-turn content of SD treated casein increased slightly ($p < 0.05$) after 30 min treatment, while the β-turn content of GD treated casein sharply increased. The α-helix content of both SD and GD-treated caseins increased slightly ($p < 0.05$) compared to the control. However, the random coil content of SD treated and GD treated caseins was largely unaffected. These data suggested that the proteins remained unfolded or were only folded partially following plasma exposure.

The FTIR analysis of the deconvoluted amide I band indicated that the secondary structural components of whey proteins, particularly β-sheets and β-turns, changed considerably after either SD or GD treatment (Table 5.2). The β-sheet content of SD treated whey sharply increased; while the β-sheet content of GD treated whey drastically decreased with increased treatment time. The β-turn content of SD treated whey decreased, while the β-turn content of GD treated whey increased. SD and GD treatment showed an opposite effect on the fractions of β-sheet and β-turn of whey. The α-helix content of SD-treated and GD-treated whey slightly decreased ($p < 0.05$) compared to the control. However, similarly to casein, the random coil content of SD treated and GD treated whey was largely unaffected.

Table 5.2 Secondary structures of SD and GD treated caseins and whey proteins at different treatment times (10, 20, 30 min).

Samples	Secondary structure (%)			
	α-helix	β-sheet	β-turn	random
Casein				
Control	18.80±0.26^{a}	45.44±0.21^{a}	23.17±0.25^{a}	12.6±0.21^{a}
SD10	19.24±0.29^{b}	44.6±0.24^{b}	23.56±0.37^{a}	12.6±0.17^{a}
SD20	19.46±0.1^{bc}	44.06±0.25^{c}	23.60±0.19^{a}	12.88±0.09^{ab}
SD30	19.68±0.15^{c}	43.59±0.25^{d}	23.65±0.17^{b}	13.08±0.14^{b}
GD10	19.06±0.21^{ad}	42.16±0.27^{ef}	26.35±0.28^{c}	12.42±0.14^{a}
GD20	19.36±0.15^{de}	41.74±0.32^{f}	26.59±0.33^{cd}	12.31±0.17^{ac}
GD30	19.71±0.24^{e}	37.99±0.34^{g}	30.33±0.53^{e}	11.97±0.24^{c}
Whey				
Control	18.89±0.16^{a}	43.04±0.13^{a}	24.77±0.24^{a}	13.3±0.26^{a}
SD10	18.52±0.29^{a}	47.03±0.27^{b}	21.74±0.15^{bc}	12.71±0.16^{ab}
SD20	17.41±0.26^{bc}	48.64±0.38^{cd}	21.5±0.3^{bc}	12.45±0.29^{bc}
SD30	17.27±0.18^{c}	48.91±0.39^{d}	21.48±0.34^{b}	12.35±0.13^{c}
GD10	18.75±0.26^{ad}	39.71±0.21^{e}	28.01±0.15^{d}	13.52±0.06^{a}
GD20	18.37±0.16^{d}	40.45±0.38^{f}	27.54±0.3^{e}	13.63±0.11^{a}
GD30	17.85±0.39^{e}	41.29±0.33^{g}	27.17±0.15^{f}	13.69±0.21^{a}

*Values are expressed as mean ± SD. Values with different letters in the same column are significantly different ($p < 0.05$). (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

For gluten sample, it was same as casein and whey samples. The gluten sample was sandwiched into a small pellet, placed on the ATR crystal and further performed the FTIR spectrum. As shown in Figure 5.6, the FTIR analysis of the amide I band showed that SD treatment changed the secondary structural components of gluten, particularly α-helix, β-sheet, and β-turn. Following 30 min treatment time, the β-sheet contents of SD treated gluten with various gluten concentrations (0.1, 1, 10 mg/ml) had increased from 33.5% to 45% approximately while the α-helix contents had decreased from 20% to 14% approximately as compared to the control (Figure 5.6a). The β-turn contents were decreased exponentially when the lower gluten concentration was subjected to SD treatment. At the low gluten concentration (0.1 mg/ml), the β-sheet contents of SD treated gluten increased logarithmically while β-turn content decreased linearly after extended SD treatment (Figure 5.6b). The α-helix contents of SD treated gluten decreased logarithmically. The random coil contents did not change significantly (p > 0.05).

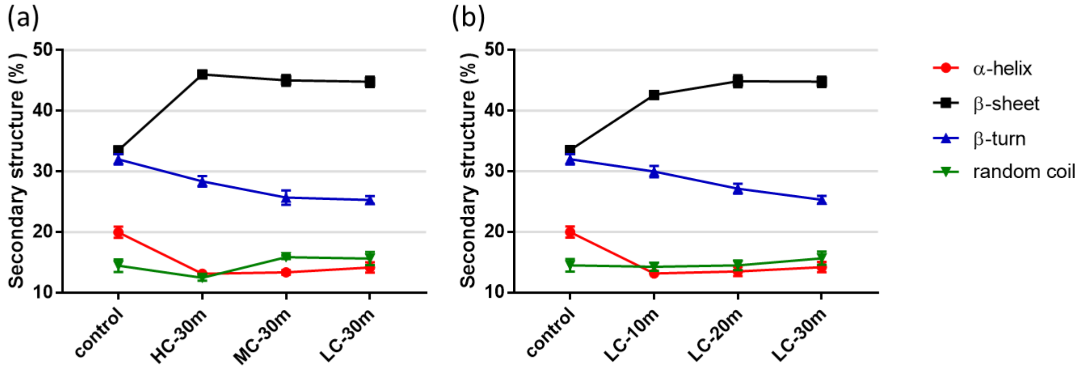


Figure 5.6 Secondary structures of SD treated gluten proteins (a) with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time (b) with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

The secondary structural components of gluten proteins, particularly α-helix, β-sheet, and random coil, were significantly altered after GD treatment, as revealed by FTIR analysis of the deconvoluted amide I band (Figure 5.7). GD treatment induced changes in the secondary structure contents of treated gluten at the low (0.1 mg/ml) and medium (1 mg/ml) concentrations, however, at the high gluten concentration (10 mg/ml) no changes were observed, compared to the control (Figure 5.7a). The β-sheet contents of GD treated gluten increased while the α-helix and random coil contents decreased when low (0.1 mg/ml) and medium (1 mg/ml) gluten concentrations were subjected to GD treatment. The changes in the secondary structure contents of GD treated gluten were more pronounced when a lower gluten concentration was subjected to GD treatment. At the low gluten concentration (0.1 mg/ml), the β-sheet contents of GD treated gluten increased logarithmically by 16.8% while α-helix, β-turn and random coil contents of GD treated gluten decreased by 6%, 3.2% and 7.7%, respectively after extended treatment (Figure 5.7b).

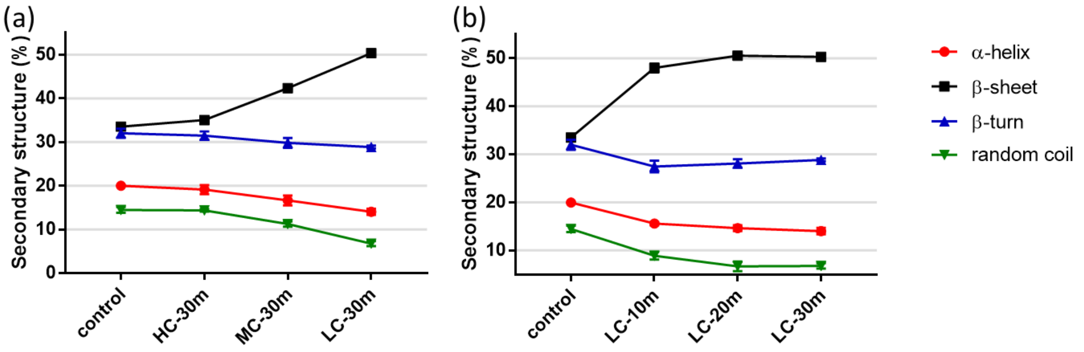


Figure 5.7 Secondary structures of GD treated gluten proteins (a) with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time (b) with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

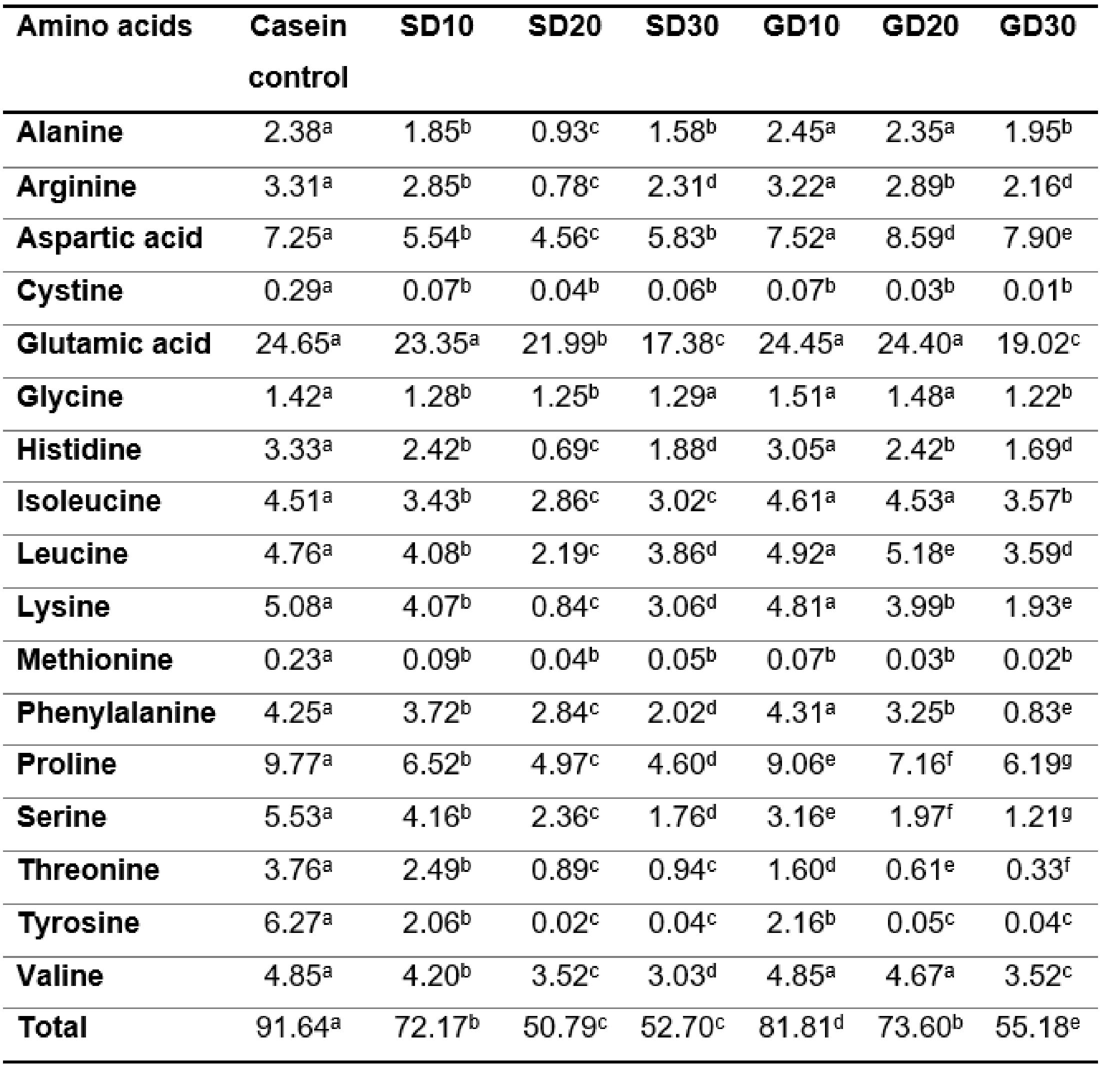
The SD and GD plasma processes significantly affected secondary structures of proteins, revealing that the protein structural modifications were dominated by partial unfolding. Venkataratnam et al. (2020) also observed changes in α-helix, β-strands and β-turns on exposure to pin-to-plate plasma discharge of peanuts. The β-turn structure is considered a product of protein unfolding of any higher order structures, whereas the anti-parallel β-sheet structure could be formed in aggregated protein molecules (Zhao et al., 2013). Cold plasma discharges of various configurations, can provide RONS in gaseous or liquid form; these can then modify the amino acid side chains, leading to the disruption of non-covalent bonds and alteration in secondary structures (Ekezie et al., 2019). Such rearrangement, destruction and disruption in protein secondary structures due to protein folding/unfolding can ultimately lead to modification of antigen epitopes and attenuation of their IgE-binding capacities (Zhu, Wang, Chen, & Zhou, 2018).

5.2.4 Amino acid profile analysis

Primary protein structure can be changed by modifying amino acid profiles, therefore the amino acid compositions of untreated and treated caseins by SD and GD treatments were assessed (Table 5.3). The total amino acid content of SD and GD treated casein declined with increasing treatment time. The concentrations of all of the amino acids decreased with increasing SD or GD treatment time. The total amino acid content of SD and GD treated whey also decreased with increased treatment time (Table 5.4). Alanine, arginine, cystine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine contents decreased significantly, while aspartic acid, glycine and leucine contents increased with SD and GD treatments of whey proteins. However, the glutamic acid content of whey proteins was unchanged after either SD or GD treatments.

Sulphur-containing amino acids, such as cystine and methionine, which have been described as particularly susceptible to reactive oxygen species (ROS) (Takai et al., 2014), showed a significant decrease ($p < 0.05$) after 10 min SD and GD treatment of casein and whey proteins. The tertiary structures of casein and whey proteins may have collapsed due to the reduction of cystine content after either SD or GD treatment. Aromatic amino acids, such as tyrosine and phenylalanine, quantitatively declined using either SD or GD treatments of casein and whey proteins. Five-membered ring amino acids, such as histidine and proline, also declined post-treatment. The structures of sulphur-containing, aromatic and five-membered ring amino acids were reported as modified by hydroxylation, dehydrogenation, nitration, and dimerization induced by atmospheric-pressure air micro-plasma (R. Zhou et al., 2016). Leucine, as a hydrophobic, aliphatic amino acid, showed a slight increase after either SD or GD treatment of whey proteins, in accordance with results from Zhou et al (2016). Glutamic acid and aspartic acid, as acidic, hydrophilic amino acids, were largely unaffected by plasma treatments; these amino acids were previously reported as less susceptible to ROS (Pal et al., 2016).

Table 5.3 Amino acid profile in untreated and treated caseins by SD and GD at different treatment times (10, 20, 30 min) (g amino acid/100 g protein)



Amino acids	**Casein control**	**SD10**	**SD20**	**SD30**	**GD10**	**GD20**	**GD30**
Alanine	2.38^{a}	1.85^{b}	0.93^{c}	1.58^{b}	2.45^{a}	2.35^{a}	1.95^{b}
Arginine	3.31^{a}	2.85^{b}	0.78^{c}	2.31^{d}	3.22^{a}	2.89^{b}	2.16^{d}
Aspartic acid	7.25^{a}	5.54^{b}	4.56^{c}	5.83^{b}	7.52^{a}	8.59^{d}	7.90^{e}
Cystine	0.29^{a}	0.07^{b}	0.04^{b}	0.06^{b}	0.07^{b}	0.03^{b}	0.01^{b}
Glutamic acid	24.65^{a}	23.35^{a}	21.99^{b}	17.38^{c}	24.45^{a}	24.40^{a}	19.02^{c}
Glycine	1.42^{a}	1.28^{b}	1.25^{b}	1.29^{a}	1.51^{a}	1.48^{a}	1.22^{b}
Histidine	3.33^{a}	2.42^{b}	0.69^{c}	1.88^{d}	3.05^{a}	2.42^{b}	1.69^{d}
Isoleucine	4.51^{a}	3.43^{b}	2.86^{c}	3.02^{c}	4.61^{a}	4.53^{a}	3.57^{b}
Leucine	4.76^{a}	4.08^{b}	2.19^{c}	3.86^{d}	4.92^{a}	5.18^{e}	3.59^{d}
Lysine	5.08^{a}	4.07^{b}	0.84^{c}	3.06^{d}	4.81^{a}	3.99^{b}	1.93^{e}
Methionine	0.23^{a}	0.09^{b}	0.04^{b}	0.05^{b}	0.07^{b}	0.03^{b}	0.02^{b}
Phenylalanine	4.25^{a}	3.72^{b}	2.84^{c}	2.02^{d}	4.31^{a}	3.25^{b}	0.83^{e}
Proline	9.77^{a}	6.52^{b}	4.97^{c}	4.60^{d}	9.06^{e}	7.16^{f}	6.19^{g}
Serine	5.53^{a}	4.16^{b}	2.36^{c}	1.76^{d}	3.16^{e}	1.97^{f}	1.21^{g}
Threonine	3.76^{a}	2.49^{b}	0.89^{c}	0.94^{c}	1.60^{d}	0.61^{e}	0.33^{f}
Tyrosine	6.27^{a}	2.06^{b}	0.02^{c}	0.04^{c}	2.16^{b}	0.05^{c}	0.04^{c}
Valine	4.85^{a}	4.20^{b}	3.52^{c}	3.03^{d}	4.85^{a}	4.67^{a}	3.52^{c}
Total	91.64^{a}	72.17^{b}	50.79^{c}	52.70^{c}	81.81^{d}	73.60^{b}	55.18^{e}

* Values with different letters in a row are significantly different ($p < 0.05$). (mean of triplicate measurements from two independent experiments (n=6))

Table 5.4 Amino acid profile in untreated and treated whey proteins by SD and GD at different treatment times (10, 20, 30 min) (g amino acid/100 g protein)

Amino acids	Whey control	SD10	SD20	SD30	GD10	GD20	GD30
Alanine	4.84^{a}	4.58^{b}	3.73^{c}	3.93^{c}	4.87^{a}	4.69^{b}	4.20^{e}
Arginine	2.27^{a}	2.14^{a}	2.00^{ab}	1.92^{b}	2.18^{a}	2.33^{a}	1.90^{b}
Aspartic acid	11.32^{a}	12.32^{b}	12.39^{b}	12.69^{c}	13.37^{d}	11.78^{ab}	12.22^{b}
Cystine	2.86^{a}	0.09^{b}	0.00^{c}	0.00^{c}	0.19^{d}	0.00^{c}	0.00^{c}
Glutamic acid	17.14^{a}	17.46^{b}	17.02^{a}	16.63^{c}	17.52^{b}	17.62^{b}	17.19^{a}
Glycine	1.38^{a}	1.42^{a}	1.40^{a}	1.76^{a}	1.69^{a}	1.53^{a}	1.75^{a}
Histidine	2.11^{a}	1.91^{a}	1.66^{b}	1.44^{b}	2.07^{a}	1.53^{b}	1.22^{c}
Isoleucine	6.43^{a}	6.26^{a}	5.14^{b}	4.89^{b}	6.25^{a}	5.34^{b}	4.62^{c}
Leucine	7.13^{a}	8.17^{b}	9.35^{c}	7.97^{b}	7.22^{a}	7.76^{ab}	7.94^{b}
Lysine	10.08^{a}	9.51^{b}	8.01^{c}	7.23^{d}	9.70^{b}	7.24^{d}	5.91^{e}
Methionine	2.25^{a}	0.13^{b}	0.06^{c}	0.05^{c}	0.21^{e}	0.26^{e}	0.15^{b}
Phenylalanine	2.92^{a}	2.80^{a}	2.41^{b}	2.25^{c}	2.93^{a}	2.87^{a}	2.33^{bc}
Proline	3.94^{a}	3.27^{b}	2.84^{c}	2.72^{c}	3.39^{b}	2.92^{c}	2.58^{d}
Serine	3.47^{a}	2.82^{b}	2.14^{c}	2.08^{c}	3.09^{b}	2.65^{b}	1.71^{d}
Threonine	4.73^{a}	3.85^{b}	2.56^{c}	2.21^{c}	4.48^{a}	3.03^{d}	1.34^{e}
Tyrosine	3.17^{a}	0.14^{b}	0.05^{c}	0.05^{c}	0.10^{c}	0.08^{c}	0.04^{c}
Valine	5.11^{a}	4.55^{b}	4.07^{c}	3.94^{c}	5.04^{a}	4.90^{a}	3.78^{c}
Total	91.15^{a}	81.38^{b}	74.81^{c}	71.75^{d}	84.29^{b}	76.54^{c}	68.86^{d}

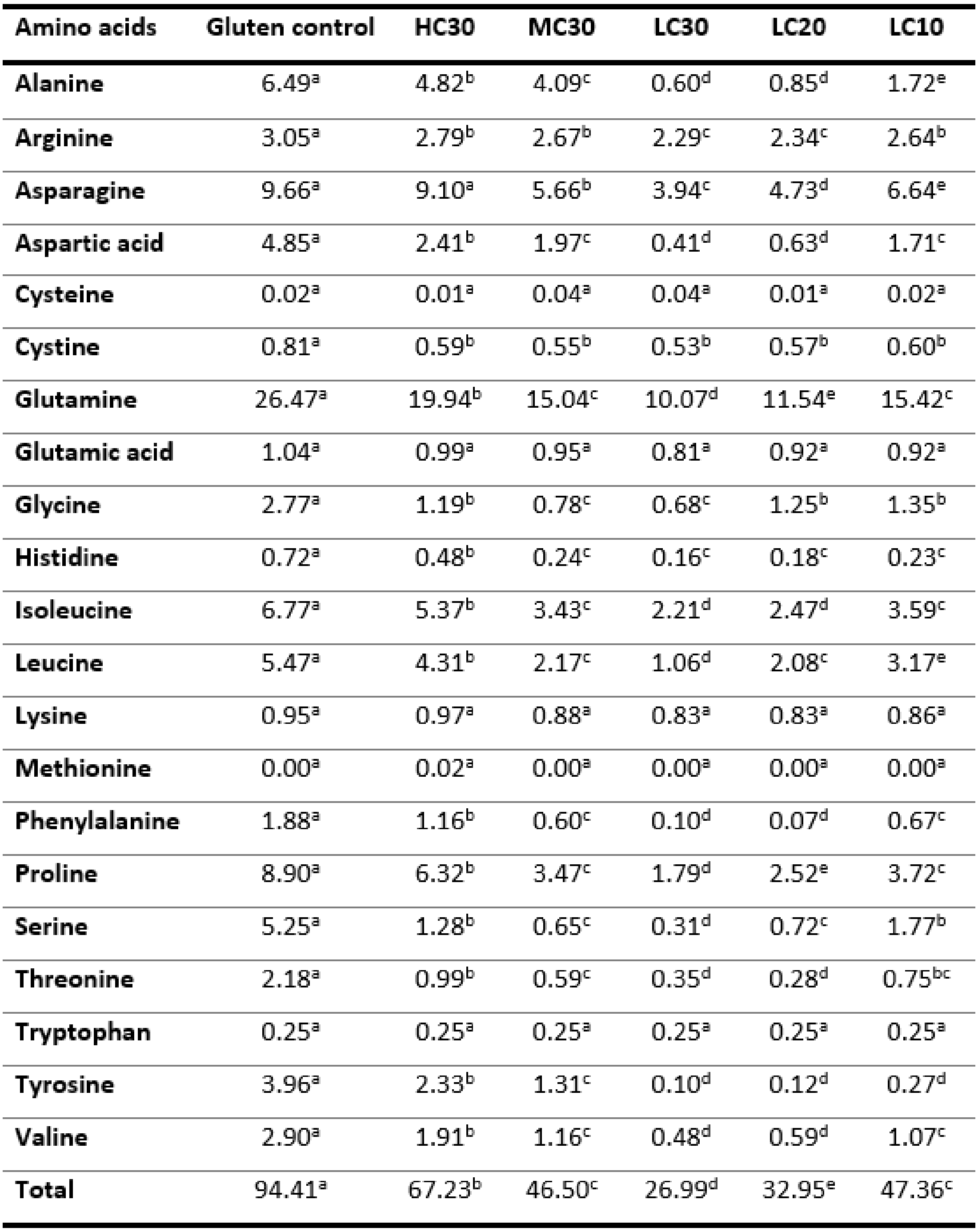
* Values with different letters in a row are significantly different ($p < 0.05$). (mean of triplicate measurements from two independent experiments (n=6))

The amino acid composition of glutens treated by plasma could influence the primary protein structures of glutens. When observing the results with low gluten concentrations (0.1 mg/ml), the total amino acid content of SD and GD treated gluten declined with increasing treatment time (Table 5.5 and Table 5.6). The results with medium (1 mg/ml) and high (10 mg/ml) gluten concentrations also showed that the total amino acid content of SD and GD treated gluten declined compared to gluten control. For the lower gluten concentrations subjected to plasma treatment, the decrease in the total amino acid content of plasma treated gluten was more pronounced. Glutamine, isoleucine, leucine, phenylalanine, and proline are the common amino acids presented in IgE-binding epitopes on wheat gluten (Battais et al., 2005; Denery-Papini et al., 2011). When looking at the results for low gluten concentrations, these particular amino acids contents decreased significantly with extended SD and GD treatment time. In addition, the particular amino acids contents of SD and GD treated gluten with medium and high gluten concentrations decreased compared to gluten control.

Sulphur-containing amino acids, such as cystine, cysteine and methionine, are susceptible to reactive oxygen species (Takai et al., 2014). In Table 5.5 and Table 5.6, cystine contents of SD and GD treated glutens showed a significant decrease ($p < 0.05$). The reduction of cystine content after SD and GD treatment may collapse the tertiary structures of gluten proteins. Cystine is the oxidized dimer form of cysteine. It was assumed that cysteine contents would increase after the decrease in cystine contents. However, in those tables, cysteine contents of SD and GD treated glutens were approximately zero after the decrease in cystine contents. Both cystine and cysteine were affected by the plasma processes applied, however cystine did not breakdown into cysteine, suggesting it was modified into other different chemical structures. There is a possibility that cystine was broken down into cysteine first and further degraded again.

Gluten is rich in glutamine and proline. These two amino acids are the high portion amino acids present in IgE-binding epitopes on wheat gluten proteins. After SD and GD treatment, glutamine and proline contents decreased, which held true both for low gluten concentration with extended treatment time and for different gluten concentrations with 30 min treatment time. It was reported that glutamine was oxidized when subjected to helium plasma jet treatment (Takai et al., 2014). Proline, as a five-membered ring amino acid, was ring-opened when subjected to air micro-plasma jet treatment (R. Zhou et al., 2016).

Table 5.5 Amino acid profile in untreated and treated glutens by SD with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein).



Amino acids	Gluten control	HC30	MC30	LC30	LC20	LC10
Alanine	6.49^{a}	4.82^{b}	4.09^{c}	0.60^{d}	0.85^{d}	1.72^{e}
Arginine	3.05^{a}	2.79^{b}	2.67^{b}	2.29^{c}	2.34^{c}	2.64^{b}
Asparagine	9.66^{a}	9.10^{a}	5.66^{b}	3.94^{c}	4.73^{d}	6.64^{e}
Aspartic acid	4.85^{a}	2.41^{b}	1.97^{c}	0.41^{d}	0.63^{d}	1.71^{c}
Cysteine	0.02^{a}	0.01^{a}	0.04^{a}	0.04^{a}	0.01^{a}	0.02^{a}
Cystine	0.81^{a}	0.59^{b}	0.55^{b}	0.53^{b}	0.57^{b}	0.60^{b}
Glutamine	26.47^{a}	19.94^{b}	15.04^{c}	10.07^{d}	11.54^{e}	15.42^{c}
Glutamic acid	1.04^{a}	0.99^{a}	0.95^{a}	0.81^{a}	0.92^{a}	0.92^{a}
Glycine	2.77^{a}	1.19^{b}	0.78^{c}	0.68^{c}	1.25^{b}	1.35^{b}
Histidine	0.72^{a}	0.48^{b}	0.24^{c}	0.16^{c}	0.18^{c}	0.23^{c}
Isoleucine	6.77^{a}	5.37^{b}	3.43^{c}	2.21^{d}	2.47^{d}	3.59^{c}
Leucine	5.47^{a}	4.31^{b}	2.17^{c}	1.06^{d}	2.08^{c}	3.17^{e}
Lysine	0.95^{a}	0.97^{a}	0.88^{a}	0.83^{a}	0.83^{a}	0.86^{a}
Methionine	0.00^{a}	0.02^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Phenylalanine	1.88^{a}	1.16^{b}	0.60^{c}	0.10^{d}	0.07^{d}	0.67^{c}
Proline	8.90^{a}	6.32^{b}	3.47^{c}	1.79^{d}	2.52^{e}	3.72^{c}
Serine	5.25^{a}	1.28^{b}	0.65^{c}	0.31^{d}	0.72^{c}	1.77^{b}
Threonine	2.18^{a}	0.99^{b}	0.59^{c}	0.35^{d}	0.28^{d}	0.75^{bc}
Tryptophan	0.25^{a}	0.25^{a}	0.25^{a}	0.25^{a}	0.25^{a}	0.25^{a}
Tyrosine	3.96^{a}	2.33^{b}	1.31^{c}	0.10^{d}	0.12^{d}	0.27^{d}
Valine	2.90^{a}	1.91^{b}	1.16^{c}	0.48^{d}	0.59^{d}	1.07^{c}
Total	94.41^{a}	67.23^{b}	46.50^{c}	26.99^{d}	32.95^{e}	47.36^{c}

* Values with different letters in a row are significantly different ($p < 0.05$). (mean of triplicate measurements from two independent experiments (n=6)

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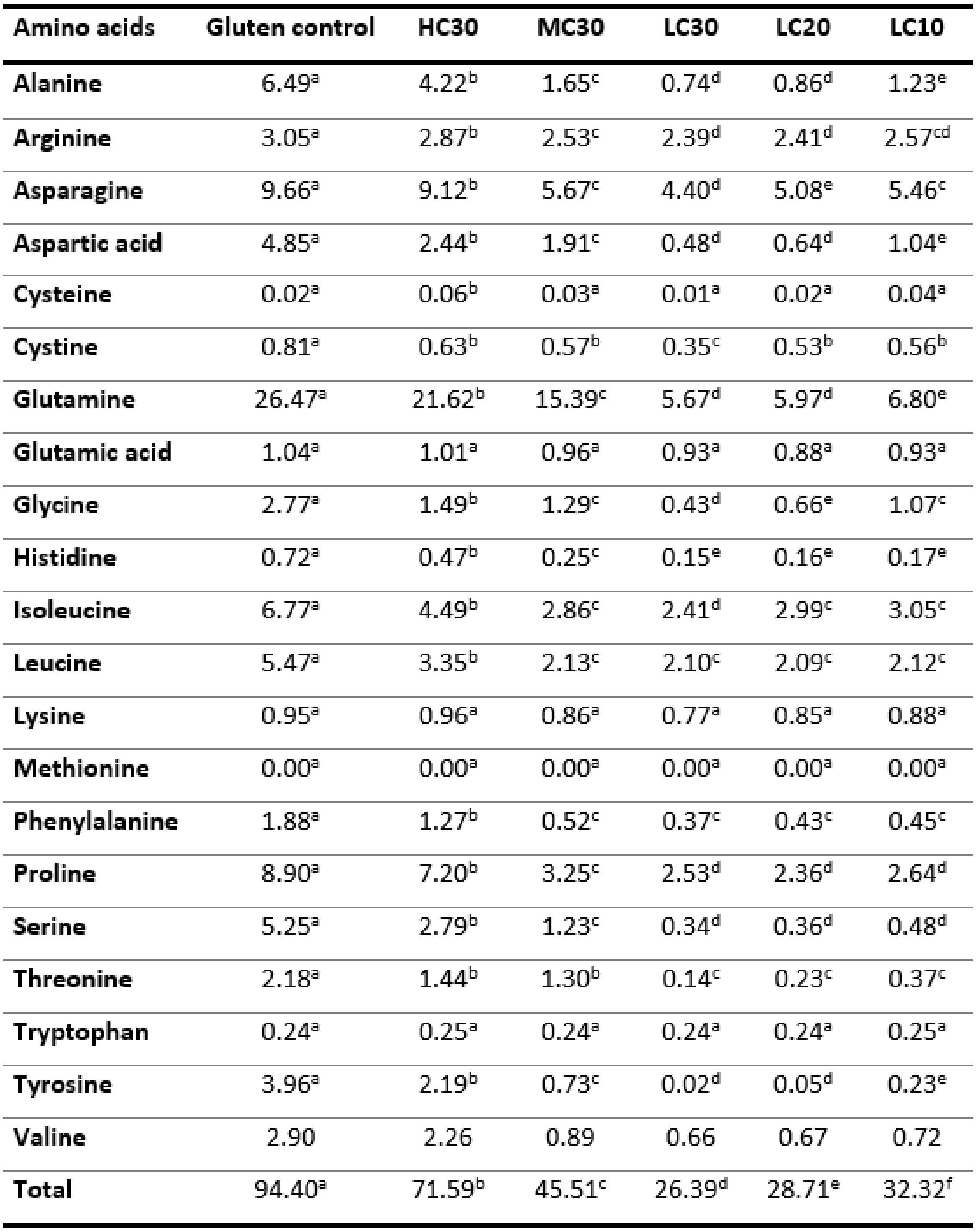
Table 5.6 Amino acid profile in untreated and treated glutens by GD with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein).



Amino acids	Gluten control	HC30	MC30	LC30	LC20	LC10
Alanine	6.49^{a}	4.22^{b}	1.65^{c}	0.74^{d}	0.86^{d}	1.23^{e}
Arginine	3.05^{a}	2.87^{b}	2.53^{c}	2.39^{d}	2.41^{d}	2.57^{cd}
Asparagine	9.66^{a}	9.12^{b}	5.67^{c}	4.40^{d}	5.08^{e}	5.46^{c}
Aspartic acid	4.85^{a}	2.44^{b}	1.91^{c}	0.48^{d}	0.64^{d}	1.04^{e}
Cysteine	0.02^{a}	0.06^{b}	0.03^{a}	0.01^{a}	0.02^{a}	0.04^{a}
Cystine	0.81^{a}	0.63^{b}	0.57^{b}	0.35^{c}	0.53^{b}	0.56^{b}
Glutamine	26.47^{a}	21.62^{b}	15.39^{c}	5.67^{d}	5.97^{d}	6.80^{e}
Glutamic acid	1.04^{a}	1.01^{a}	0.96^{a}	0.93^{a}	0.88^{a}	0.93^{a}
Glycine	2.77^{a}	1.49^{b}	1.29^{c}	0.43^{d}	0.66^{e}	1.07^{c}
Histidine	0.72^{a}	0.47^{b}	0.25^{c}	0.15^{e}	0.16^{e}	0.17^{e}
Isoleucine	6.77^{a}	4.49^{b}	2.86^{c}	2.41^{d}	2.99^{c}	3.05^{c}
Leucine	5.47^{a}	3.35^{b}	2.13^{c}	2.10^{c}	2.09^{c}	2.12^{c}
Lysine	0.95^{a}	0.96^{a}	0.86^{a}	0.77^{a}	0.85^{a}	0.88^{a}
Methionine	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Phenylalanine	1.88^{a}	1.27^{b}	0.52^{c}	0.37^{c}	0.43^{c}	0.45^{c}
Proline	8.90^{a}	7.20^{b}	3.25^{c}	2.53^{d}	2.36^{d}	2.64^{d}
Serine	5.25^{a}	2.79^{b}	1.23^{c}	0.34^{d}	0.36^{d}	0.48^{d}
Threonine	2.18^{a}	1.44^{b}	1.30^{b}	0.14^{c}	0.23^{c}	0.37^{c}
Tryptophan	0.24^{a}	0.25^{a}	0.24^{a}	0.24^{a}	0.24^{a}	0.25^{a}
Tyrosine	3.96^{a}	2.19^{b}	0.73^{c}	0.02^{d}	0.05^{d}	0.23^{e}
Valine	2.90	2.26	0.89	0.66	0.67	0.72
Total	94.40^{a}	71.59^{b}	45.51^{c}	26.39^{d}	28.71^{e}	32.32^{f}

* Values with different letters in a row are significantly different ($p < 0.05$). (mean of triplicate measurements from two independent experiments (n=6))

5.2.5 Antigenicity analysis

ELISA was performed to quantify the change in the IgE binding of plasma treated casein, β-lactoglobulin and gliadin; as well as in the IgG binding of α-lactalbumin treated with SD and GD plasma treatment. The antigenicity of casein after SD and GD treatment is presented in Figure 5.8a and b, respectively. The reduction in antigenicity of SD and GD treated caseins was aligned with increasing plasma treatment time. The IgE-binding capacity of caseins treated for 30 min with either SD or GD discharge was reduced by 49.9% and 91.1% respectively, by comparison with untreated control. GD treatment was more effective for antigenicity reduction of casein than SD treatment.

The antigenicity of α-lactalbumin after SD and GD treatment is presented in Figure 5.8c and d, respectively. Similarly, α-lactalbumin antigenicity was reduced by 49.5% by SD treatment and 45.5% by GD treatment for 30 min. Apart from the plasma treatment, the mild heat induced by the cold plasma process may decrease the α-lactalbumin antigenicity. Xu (2015) reported that the α-lactalbumin antigenicity was significantly decreased after heat treatment of 65 °C for 30 min. The antigenicity of β-lactoglobulin after SD and GD treatment is presented in Figure 5.8e and f, respectively. In contrast to other proteins, there was a notable increase in antigenicity of β-lactoglobulin observed after either SD or GD treatments. β-lactoglobulin antigenicity increased by approximately 2.5 to 3 times compared to the control after either SD or GD treatment for 10 min. An increase in β-lactoglobulin antigenicity by high hydrostatic pressure has been reported by Meng, Bai, Gao, Li, and Chen (2017). It is attributed to the exposure of hidden epitopes upon protein unfolding which increased the antigenicity of β-lactoglobulin. It was reported that the allergy eliciting doses for milk-derived and wheat-derived products were 0.1 mg and 1 mg, respectively (Commission, 2011; Kupińska-Adamczyk et al., 2018). This implies the importance of assessing the potential risk of allergenicity enhancement.

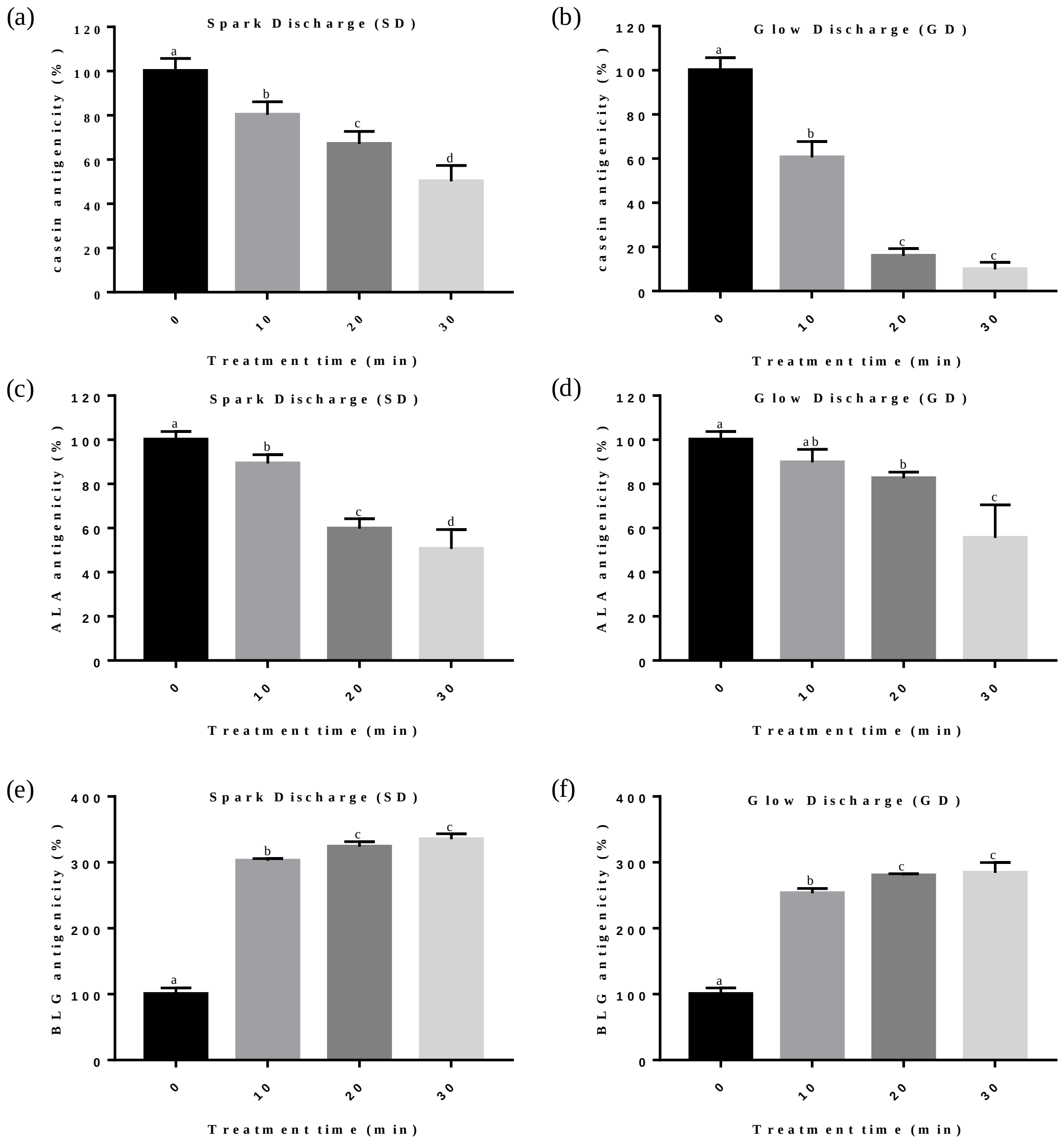


Figure 5.8 ELISA responses of casein subjected to different (a) SD and (b) GD treatment times against IgE binding activity (%). ELISA responses of α-lactalbumin (ALA) subjected to different (c) SD and (d) GD treatment times against IgG binding activity (%). ELISA responses of β-lactoglobulin (BLG) subjected to different (e) SD and (f) GD treatment times against IgE binding activity (%).

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

The reduction in antibody binding potential could be attributed to modification of conformational structure and release of hydrophobic or hydrophilic residues by the cold plasma treatments, which permit reorientation or destruction of antigenic epitopes. Conversely as observed with β-lactoglobulin, an increase in IgE binding potential could be due to the formation of new epitopes (Sathe, Liu, & Zaffran, 2016). The IgE-binding epitopes on casein were characterised by a high concentration of alanine, glutamic acid, leucine, lysine, proline, serine and valine (Busse, Järvinen, Vila, Beyer, & Sampson, 2002; Chatchatee, Jarvinen, Bardina, Beyer, & Sampson, 2001; Chatchatee, Järvinen, et al., 2001). Combining the results of amino acid profile analysis and antigenicity analysis, the decrease in these amino acid contents induced by both plasma treatment led to the decrease in casein antigenicity. Asparagine, isoleucine, lysine and valine were the major components of IgG binding epitopes on α-lactalbumin; while aspartic acid, glutamic acid and leucine were the major components of IgE-binding epitopes on β-lactoglobulin (K.-M. Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001). The decrease in these corresponding amino acid contents induced by plasma treatment led to the decrease in α-lactalbumin antigenicity; whilst the increase in these corresponding amino acid contents by plasma treatment led to the increase in β-lactoglobulin antigenicity.

The gliadin antigenicity of gluten after SD and GD treatment is illustrated in Figure 5.9 and Figure 5.10, respectively. Two parameters (gluten concentrations and treatment time) are shown. In both figures, the results showed that for the lower gluten concentration which was subjected to either SD or GD treatment, the decrease in the gliadin antigenicity was more pronounced. Additionally, the gliadin antigenicity of SD and GD treated gluten decreased as treatment time increased. Both figures formed a ‘V’ trend. The trough is low gluten concentration (0.1 mg/ml) in 30 min treatment time. The IgE-binding capacity of gliadins, when the high gluten concentrations were subjected to SD and GD treatment for 30 min treatment time, was reduced by 30.1% and 41.2%, respectively, by comparison with untreated gluten control. However, when the low gluten concentrations (0.1 mg/ml) were subjected to SD and GD treatment for 30 min, the IgE-binding capacity of gliadins was reduced by 82.2% and 73%, respectively, by comparison with untreated gluten control. For the high gluten concentration, GD treatment was more effective for allergenicity reduction of gliadin than SD treatment. Conversely, for low gluten concentration, SD treatment was more effective for allergenicity reduction of gliadin than GD treatment.

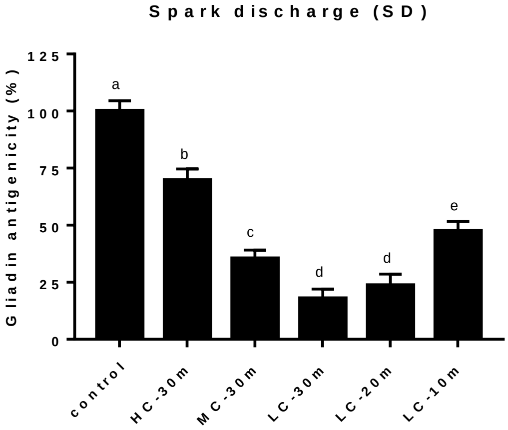


Figure 5.9 ELISA responses of gliadin subjected to SD treatment against IgE binding activity (%). SD treated gluten proteins with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time and with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml).

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

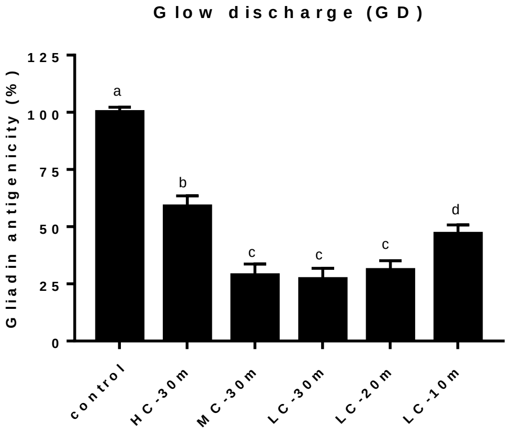


Figure 5.10 ELISA responses of gliadin subjected to GD treatment against IgE binding activity (%). SD treated gluten proteins with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time and with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml)

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

Although there are multiple mechanisms affecting food allergen reactivity, the underlying concept is the modification of the conformational as well as linear epitopes. For example, conformational epitopes can be changed by aggregation into insoluble aggregates or crosslinking of proteins as result of a loss of protein solubility, whereas sequence epitopes can be affected by fragmentation. Reactive species induced by cold plasma may modify amino acids, which are sensitive to oxidation, hydroxylation, and nitration, presumably resulting in reconciliation or disruption of binding sites for the antibodies investigated in this study.

5.3 Conclusion

The study investigated the effect of tunable atmospheric cold plasma treatment on the structures and antigenicity of milk protein (casein, β-lactoglobulin and α-lactalbumin) and wheat protein (gluten). The results clearly showed that direct cold plasma treatment was effective for attenuation of casein, α-lactalbumin and gliadin antigenicity as assessed by ELISA. However, β-lactoglobulin antigenicity was increased by direct cold plasma treatment, of either SD or GD. Moreover, allergen concentration plays an important role in allergen mitigation efficacy. Additionally, cold plasma treatments resulted in changes in the content of secondary structures and changes in the amino acid compositions of milk and wheat proteins. These modifications in secondary and primary structures may collectively affect the antibody binding capacity of milk proteins and wheat proteins. Thus, these results show the universality of the RSS system that poses a promising alternative approach to reduce milk-derived and wheat-derived allergenicity. There was a strong relationship between either Spark or Glow plasma process duration, allergen concentration and efficacy for allergenicity attenuation. This cold plasma technology is a bench-scale device. Hence, scaling-up of the technology is needed for application to food processing environments while maintaining the capability of allergen mitigation using direct or indirect treatments. The results achieved point to a potential application for reducing the allergenicity of food allergen residues in food processing environments.

The following paper has been published from this study:

Ng, S. W., Lu, P., Rulikowska, A., Boehm, D., O'Neill, G., & Bourke, P. (2020). The effect of atmospheric cold plasma treatment on the antigenic properties of bovine milk casein and whey proteins. *Food Chem, 342*, 128283. doi:10.1016/j.foodchem.2020.128283

Chapter 6 Effect of indirect treatment using PAW on bovine casein antigenicity

6.1 Experimental Design

Plasma activated water (PAW) is a medium with high redox potential and can serve as an oxidizing agent. H_2O_2 is a reactive oxygen species (ROS) generated in PAW and also serves as an oxidizing agent. Oxidizing agents are thought to react strongly with thiol groups in proteins thereby oxidizing protein side chains (Finnegan et al., 2010). This structural modification could help to reduce the antigenicity of some allergens.

Following on from results showing the modification of milk and wheat antigenicity using direct treatment with the RSS SD and GD in chapter 5, the potential of indirect treatment with PAW for allergen mitigation was further investigated. In this study, the influence on bovine casein antigenicity of application of PAW generated from air spark and glow discharges was investigated. The allergen concentration, temperature of PAW and contact time were considered as target and process variables, respectively. Heat energy aids in speeding up protein oxidation. The mechanism of casein antigenicity reduction of RONS in spark discharge PAW (SDPAW) and glow discharge PAW (GDPAW) were examined within contact times ranging from 0 to 5 days. The approach used is illustrated in Figure 6.1. SDPAW was generated by exposing 10 ml of deionised water to spark discharge (SD) with 7.5 kV and 25 kHz. GDPAW was generated by exposing 10 ml of deionised water to glow discharge (GD) with 5 kV and 25 kHz.

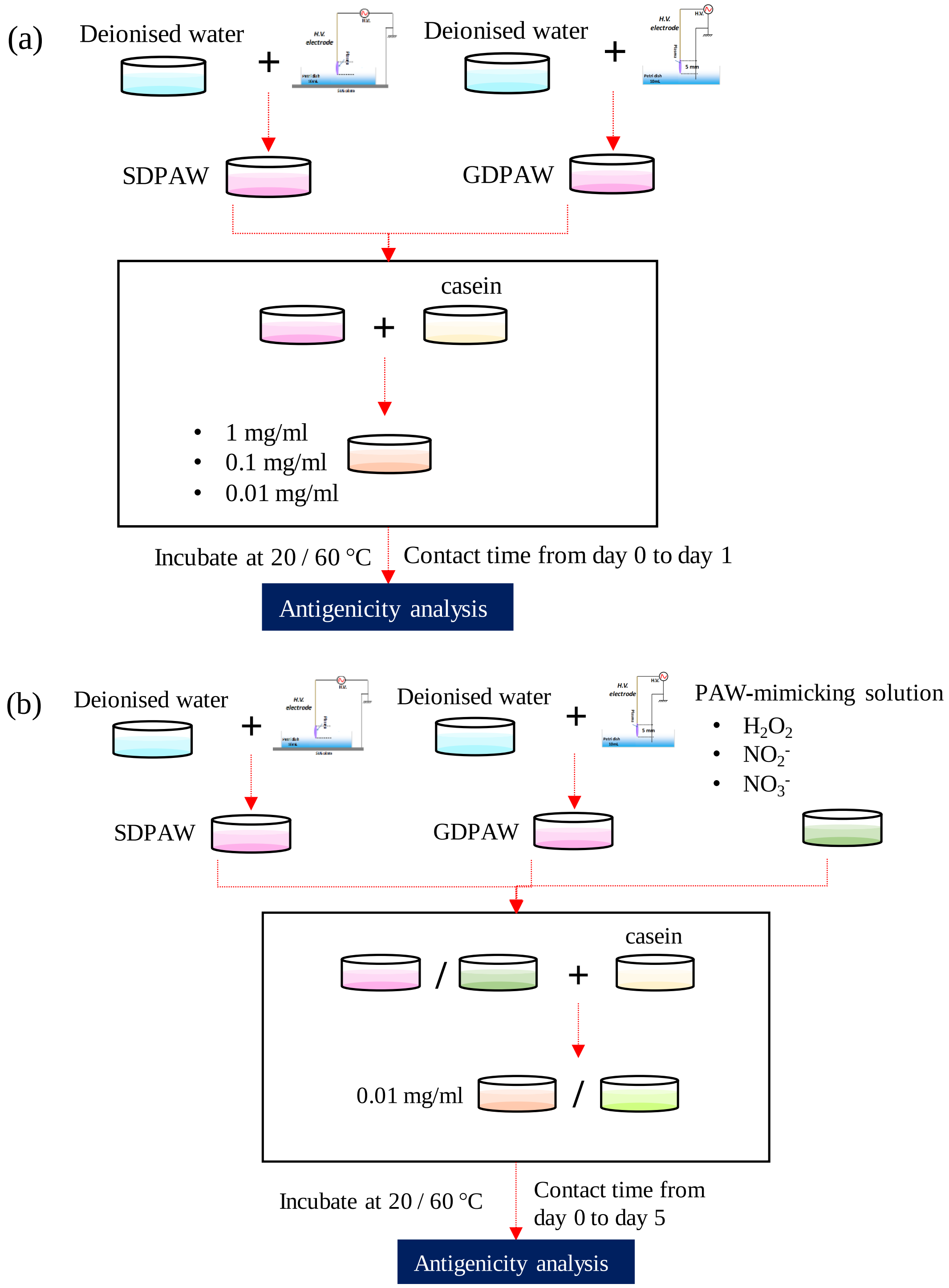


Figure 6.1 PAW treatment of milk casein – experimental design.

(a) PAW treatment on casein antigenicity in combination with mild heat treatment with respect to casein concentrations. (b) PAW and PAW-mimicking treatment on casein antigenicity

6.2 Results and Discussion

6.2.1 Chemical composition in SDPAW and GDPAW after 10 min treatment time

The RONS concentrations were quantitively measured and are shown in Figure 6.2. Ultrapure water served as a control. H_2O_2, NO_2^- and NO_3^- were determined by $TiOSO_4$, Griess reagent and Spectroquant® nitrate assay kit containing 2, 6-dimethyl phenol (DMP), respectively. 10 min SDPAW contained 993±87 µM of H_2O_2 and 4869±287 µM of NO_3^-; while 10 min GDPAW contained 1076±154 µM of NO_2^- and 913±107 µM of NO_3^-. The H_2O_2 and NO_3^- concentrations of SDPAW were similar to the findings in Chapter 3.2.3. The NO_3^- concentration of GDPAW was similar to the findings in previous chapter. However, the NO_2^- concentration in GDPAW was 2-fold higher than the findings in the previous chapter. The differences in NO_2^- concentration in GDPAW is likely due to differences in power supply used.

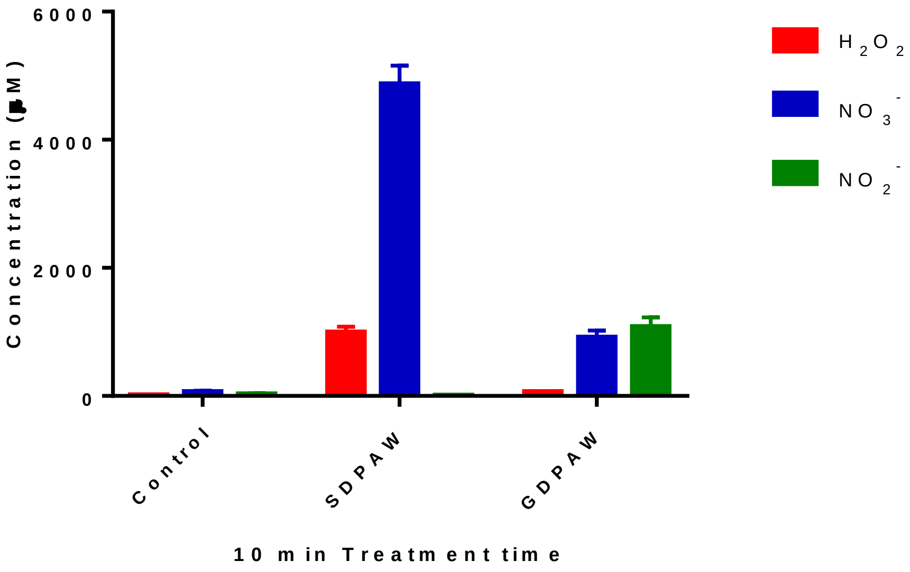


Figure 6.2 Chemical composition of SDPAW and GDPAW treated for 10 min. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

6.2.2 Effect of PAW treatment on casein antigenicity in combination with mild heat treatment with respect to casein concentrations

PAW was mixed with casein solutions and the casein concentration was adjusted to 1, 0.1 and 0.01 mg/ml. To maintain high concentrations of RONS after mixing, the ratios of PAW to casein were 75%, 95% and 99% in 1, 0.1 and 0.01 mg/ml PAW-casein/mimicking-casein mixtures, respectively. The antigenicity of casein-PAW mixture was studied from day 0 to day 1 under two different conditions of 20 °C and 60 °C. The efficiency of casein antigenicity reduction by PAW with respect to casein concentration was studied in Figure 6.3 and Figure 6.4. Figure 6.3A to C shows the effect of PAW on casein antigenicity with different casein concentrations of 1, 0.1 and 0.01 mg/ml at 20 °C. The casein antigenic responses of samples treated with either SDPAW or GDPAW at day 1 remained constant compared to day 0 in three different casein concentrations. Figure 6.4A to C shows the effect of PAW on casein antigenicity with different casein concentrations of 1, 0.1 and 0.01 mg/ml at 60 °C. There was a decrease of 30% of antigenic response of casein treated with SDPAW at 60 °C in day 1 at a concentration of 0.01 mg/ml while no effect was seen on the higher concentrations. As shown in Figure 6.2, SDPAW contained H_2O_2 and NO_3^-. H_2O_2 has a high potential of holding a central role in this antigenicity reduction process via oxidation of proteins (Finnegan et al., 2010; Hambly & Gross, 2009). Similarly, the effectiveness of denaturation of whey proteins by H_2O_2 at 75 °C has been reported by Marshall (2009). Nonetheless, the role of NO_3^- should not be excluded. There was no effect seen on the casein antigenic responses of samples treated with GDPAW, either at 20 °C or at 60 °C, at day 1 compared to day 0 in three different casein concentrations. In short, there are limitations to the casein denaturation and antigenicity reduction that can be achieved with high concentrations of allergenic residues using an indirect PAW based process.

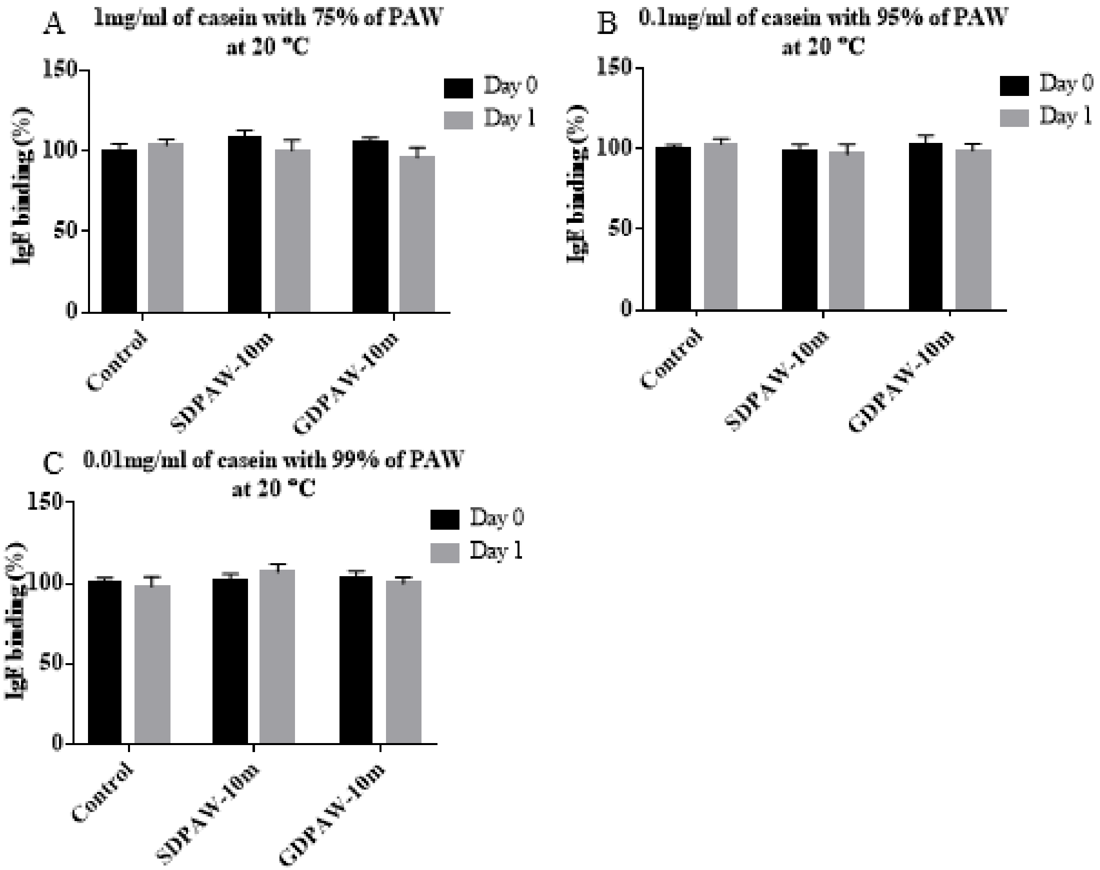


Figure 6.3 Bovine casein antigenic responses to SDPAW and GDPAW with different casein concentrations of 1, 0.1 and 0.01 mg/ml under conditions of 20 °C.

In the treatment condition of 20 °C, (A) 1 mg/ml of casein with 75% of PAW, (B) 0.1 mg/ml casein with 95% of PAW, (C) 0.01 mg/ml of casein with 99% of PAW. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

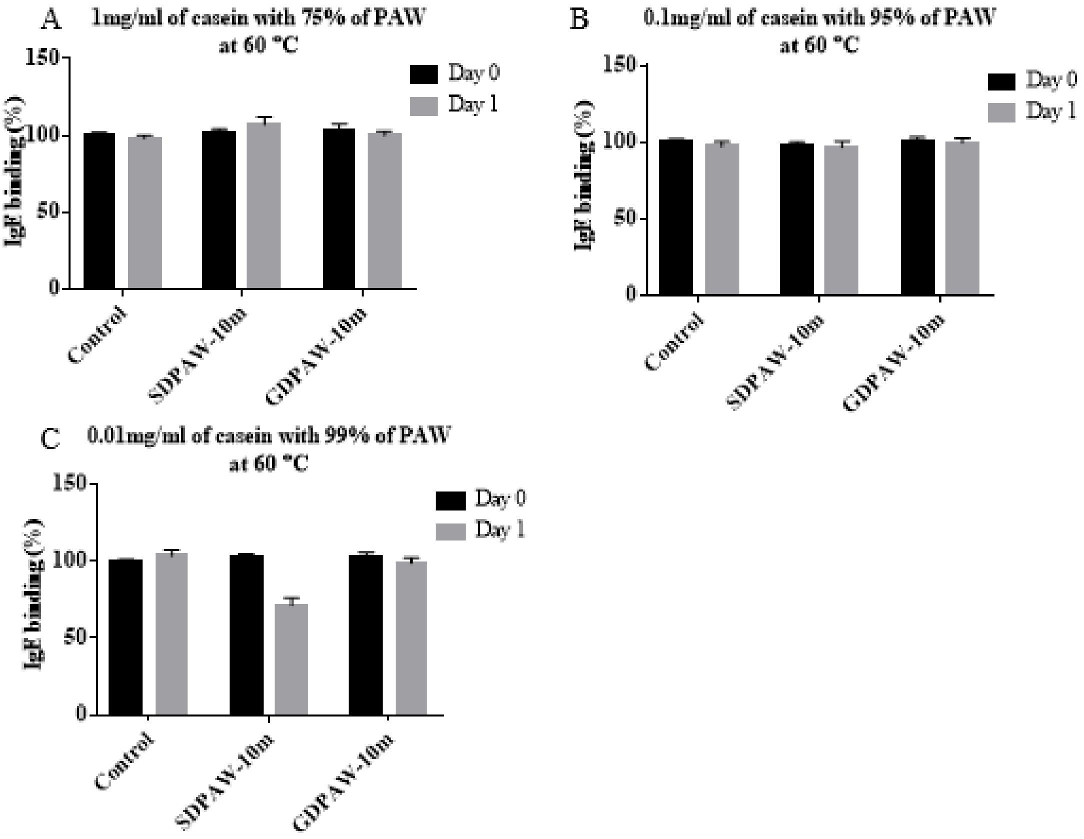


Figure 6.4 Bovine casein antigenic responses to SDPAW and GDPAW with different casein concentrations of 1, 0.1 and 0.01 mg/ml under conditions of 60 °C.

In the treatment condition of 60 °C, (A) 1 mg/ml of casein with 75% of PAW, (B) 0.1 mg/ml casein with 95% of PAW, (C) 0.01 mg/ml of casein with 99% of PAW. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

6.2.3 Effect of PAW and PAW-mimicking treatment on casein antigenicity

To understand the long-term effect of PAW on casein antigenicity, the experiment was carried out with increased contact time of 5 days combined with low casein concentration of 0.01 mg/ml (Figure 6.5). Figure 6.5A shows that the casein antigenicity of samples treated with SDPAW and GDPAW remained constant at 20 °C. In Figure 6.5B, the antigenicity of casein treated with SDPAW logarithmically decreased from day 0 to day 5 at 60 °C. The maximum reduction was 77% at day 5 at 60 °C. The GDPAW did not have any casein antigenicity reduction effect. The 18 % of reduction was caused by the mild heat treatment, which was shown by the control.

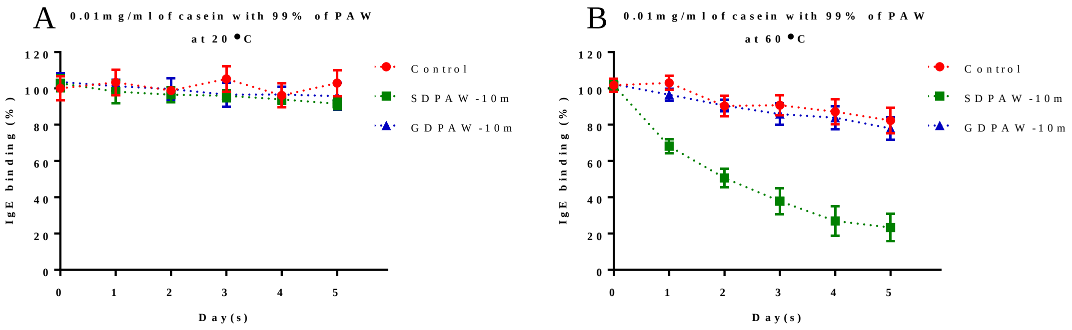


Figure 6.5 Bovine casein antigenic responses to SDPAW and GDPAW under conditions of (A) 20 °C and (B) 60 °C for contact treatment times from day 0 to day 5. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

To better understand the mechanism of casein antigenicity reduction by RONS in SDPAW and GDPAW, RONS mimicking solutions were artificially made according to the RONS concentration of SDPAW and GDPAW. Figure 6.6 shows the casein antigenicity reduction effects of PAW and RONS mimicking solutions at 60 °C. As shown in Figure 6.6A, the casein antigenicity gradually decreased from day 0 to day 5 when treated with H_2O_2 mimicking solution, however, NO_3^- had no effect. The mixture of H_2O_2 and NO_3^- mimicking solution had a similar antigenicity curve to the H_2O_2 mimicking solution. This showed that H_2O_2 plays the main role in this casein antigenicity reduction process.

Finnegan et al. (2010) reported that H_2O_2 was an oxidizing agent that could alter protein structure via oxidation. Hammadi, Ponton, and Belhadri (2012) reported that H_2O_2 with an increase in temperature induced a reduction in sludge from a water purification plant. GDPAW and its RONS mimicking solutions again demonstrated no casein antigenicity reduction effect. This showed that NO_2^- and NO_3^- was not effective for casein antigenicity reduction. Conversely, Feng et al. (2016) reported that NO_2^- had effects on protein oxidation by promoting the disulphide-crosslinks. Figure 6.7 showed that no RONS in SDPAW and GDPAW were lost during the casein-PAW reaction.

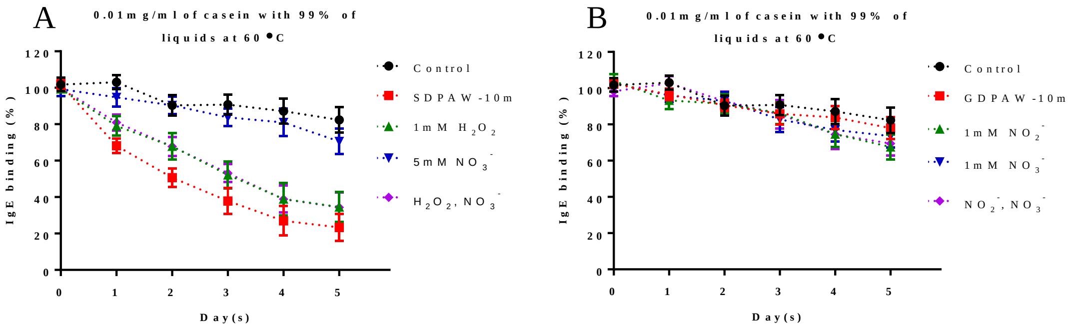


Figure 6.6 Bovine casein antigenic responses to PAW and RONS mimicking solutions at 60 °C. (A) effect of SDPAW and its mimicking solution on bovine casein antigenicity, (B) effect of GDPAW and its mimicking solutions on bovine casein antigenicity. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

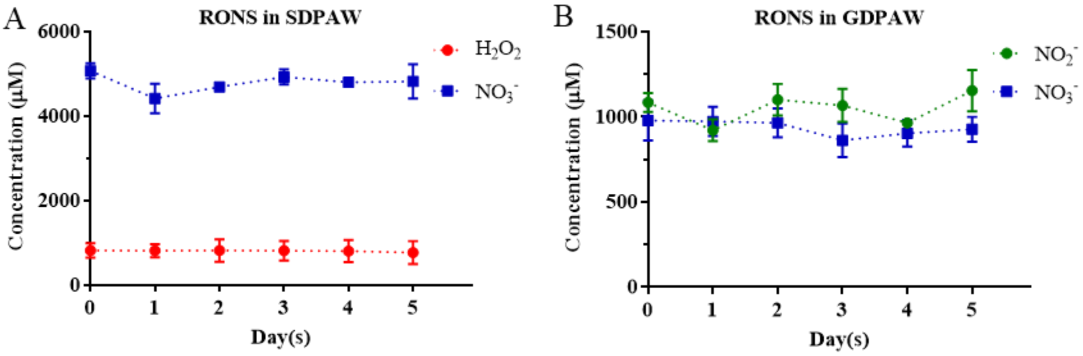


Figure 6.7 RONS concentrations of (A) SDPAW and (B) GDPAW during casein-PAW reaction at 60 °C. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

6.3 Conclusion

This study describes the bovine casein antigenicity reduction effects of PAW generated by SD and GD with different casein concentrations and with or without a mild heat treatment. SDPAW can oxidize low casein concentrations to reduce the casein antigenicity when combined with a mild heat treatment at 60 °C, where the mild heating can speed up the protein oxidation. GDPAW had no effect on casein antigenicity reduction. The ROS in PAW generated by SD contributed to the reduction in antigenicity of casein. The casein structure is most likely modified by the ROS, as an oxidizing agent, via oxidation. The reactive nitrogen species (RNS) in PAW generated by either SD or GD did not contribute to reducing the antigenicity of casein. This study shows potential for a novel processing protocol for reducing antigenicity of milk-derived products through the combination of indirect plasma treatment using PAW and heat treatment. However, to adapt cleaning processes in food processing environments, an improvement in efficacy of antigenicity reduction is required.

Chapter 7 Effect of DBD in-package plasma treatment on the antigenicity of milk and wheat proteins

7.1 Experimental Design

Two of the most prevalent dietary allergies are milk and wheat. The main allergens in bovine milk are casein and whey proteins. Wheat's main allergen is gliadin protein. In Chapter 5, the RSS system was employed for ACP treatment. The two modes of the RSS system continuously generated a single intense streamer discharge. In this study, a dielectric barrier discharge (DBD) in-package system was used for ACP treatment. The DBD system generated multiple filamentary micro-discharges from the planar electrodes, which was very different from the RSS system.

The influence of ACP treatment using DBD on the antigenicity of milk proteins (casein, β-lactoglobulin and α-lactalbumin) and wheat proteins (gliadin) was investigated. To gain a better understanding of the potential interaction of DBD treatment with these proteins for in-package antigenicity reduction, the influence of DBD treatment on the proteomic profile of casein, whey, and gluten proteins was determined. The effect of protein concentration on antigenicity reduction using ACP was assessed. The approach used is illustrated in Figure 7.1. The applied voltage and frequency were 70 kV rms and 50 Hz. 2 mg/ml of casein or whey solutions, and 10 mg/ml of gluten solution (considered as high concentration), as well as 0.2 mg/ml of casein or whey solutions, and 1 mg/ml of gluten solution (considered as medium concentration) were subjected to DBD treatment for a treatment time of 30 min. 0.02 mg/ml of casein or whey solutions, and 0.1 mg/ml of gluten solution (considered as low concentration) were subjected to DBD treatment for 10, 20, and 30 min.

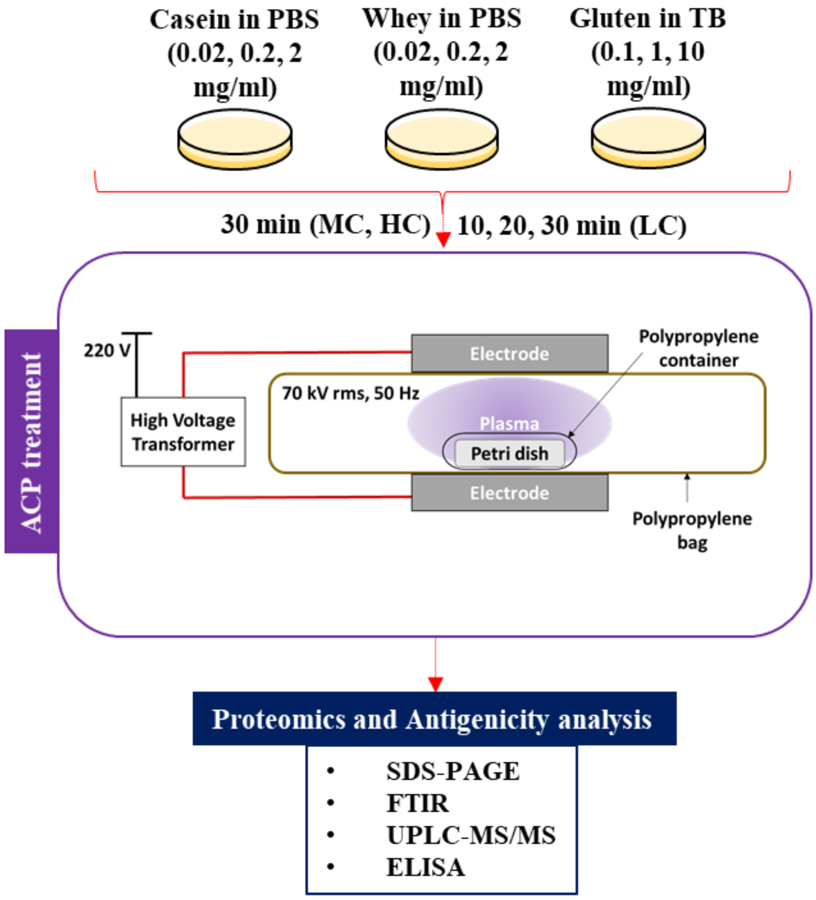


Figure 7.1 DBD treatment of casein, whey and gluten proteins – experimental design.

7.2 Results and Discussion

The in-package dielectric barrier discharge (DBD) system was previously described and characterised in Moiseev et al. (2014), Patil et al. (2014) and Milosavljević and Cullen (2017). The authors identified that a variety of reactive oxygen and nitrogen species (RONS) such as O_3, NO_2, NO_3, and N_2O_4 were generated within the package during treatment. This type of chemistry and the principal reactors generated is usually observed in plasmas generated using atmospheric air and the intensities of the plasma reactive species is a function of the input voltage level, treatment time, working gas and post treatment storage time. This system when used with air can give similar plasma reactive species to the those identified when using direct RSS approach as both use atmospheric air as the working gas to produce the plasma reactive species.

7.2.1 SDS-PAGE analysis

In the bovine milk studies, DBD treated caseins were analysed using SDS-PAGE under reducing conditions for their electrophoretic profiles, shown in Figure 7.2. The protein bands of casein were present around the protein marker of 25-35 kDa. The DBD treated casein samples showed notable changes in the protein profiles when compared to the control in lane 2. The high concentration (2 mg/ml) of casein after 30 min DBD treatment (shown in lane 3) was distinctly decreased in band intensity when compared to the control in lane 2. Moreover, a soluble aggregate was found in lane 3, showing high concentration (2 mg/ml) of casein treated for 30 min. This could be explained by formation of new, higher molecular weight aggregates via disulphide bonds formation, hydrophobic and electrostatic interactions and inter protein crosslinking (Tammineedi et al., 2013). The protein bands for medium (0.2 mg/ml) and low (0.02 mg/ml) concentrations of caseins after 30 min DBD treatment shown in lane 4 and 5, respectively, had disappeared. The band of low concentration casein (0.02 mg/ml) after 10 min DBD treatment in lane 7 had disappeared. This could indicate that the casein structure could be completely denatured within a 10 min treatment time or even lower when 10 ml of 0.02 mg/ml of casein were exposed to DBD treatment; or at a 30 min treatment time or even lower when 10 ml of 0.2 mg/ml of casein were exposed to DBD treatment. Furthermore, there were smaller intensities of soluble aggregates found in lanes 4, 5, 6 and 7 (casein medium concentration (0.2 mg/ml) of casein treated for 30 min and low concentration (0.02 mg/ml) of caseins treated for 30, 20 and 10 min, respectively), compared to the aggregate band in lane 3 (high concentration of casein treated for 30 min). This could be attributed to the modification of proteins by cross-linkage of free amino acids to the protein and protein-protein aggregation, resulting in insoluble aggregates (Yang et al., 2010; Zhenxing et al., 2007).

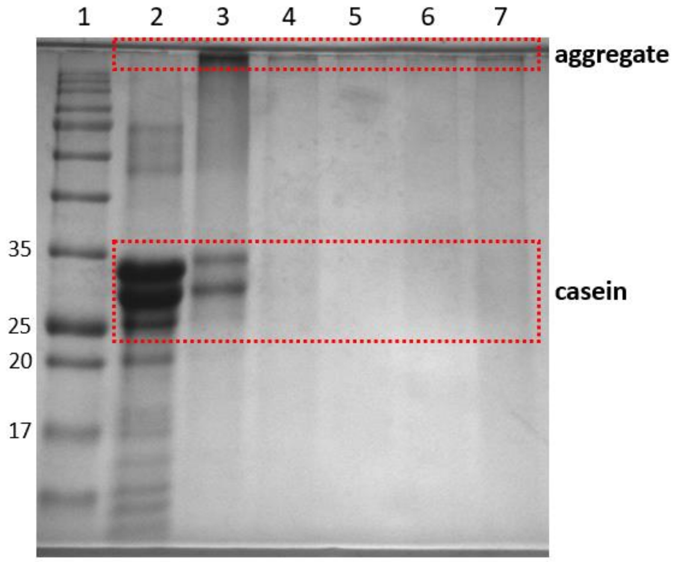


Figure 7.2 SDS-PAGE profiles of untreated and DBD treated caseins subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Casein control. Lane 3-4: high (2 mg/ml) and medium (0.2 mg/ml) protein concentrations, respectively, treated for 30 min. Lane 5-7: low (0.02 mg/ml) protein concentrations treated for 30, 20, 10 min, respectively.

SDS-PAGE profiles of DBD treated whey proteins under reducing conditions are shown in Figure 7.3. The α-lactalbumin and β-lactoglobulin are the major allergenic protein fragments in milk. The band at 11 kDa corresponds to α-lactalbumin. The band of the β-lactoglobulin monomer is located at 17 kDa; and the dimer is located approximately at 35 kDa. The DBD treated whey shows notable changes in the protein profiles when compared to the control in lane 2. The high concentration (2 mg/ml) of whey solution exposed to DBD treatment for 30 min is located in lane 3. In lane 3, the β-lactoglobulin dimer band intensity was similar to the control. However, its band was more smeared, which was different from the control. The band intensity of the β-lactoglobulin monomer in lane 3 was notably decreased, and α-lactalbumin had disappeared when compared to the control. The medium concentration (0.2 mg/ml) of whey solution exposed to DBD treatment for 30 min was located in lane 4. In lane 4, the bands of β-lactoglobulin dimer and α-lactalbumin had disappeared. The band of β-lactoglobulin monomer was much less intense, as shown in lane 4. The low whey concentrations (0.02 mg/ml) exposed to DBD treatment for 10, 20 and 30 min are located in lanes 7, 6 and 5, respectively. At a 10 min DBD treatment time, the band intensity of β-lactoglobulin monomer with low whey concentration (0.02 mg/ml) in lane 7 was much less intense. The bands of β-lactoglobulin dimer and α-lactalbumin had disappeared. There were no bands found in lane 5 and 6, showing whey treated for 30 and 20 min, respectively. In short, this could indicate the α-lactalbumin structure could be completely denatured at a 10 min treatment time or even lower when 10 ml of 0.02 mg/ml of whey were exposed to DBD treatment; or at a 30 min treatment time or even lower when 10 ml of 0.2 mg/ml of whey were exposed to DBD treatment. Simultaneously, the β-lactoglobulin dimer could be denatured at a 10 min treatment time when 10 ml of 0.02 mg/ml of whey were exposed to DBD treatment; or at a 30 min treatment time or even lower when 10 ml of 0.2 mg/ml of whey were exposed to DBD treatment. The β-lactoglobulin monomer could be denatured at a 20 min treatment time when 10 ml of 0.02 mg/ml of whey were exposed to DBD treatment. Meinlschmidt et al. (2016) observed a reduction in the band intensities of soy proteins treated with direct surface DBD plasma treatment. However, Tammineedi et al. (2013) reported that no visible change in the band intensities of α-casein, α-lactalbumin and β-lactoglobulin treated with nonthermal atmospheric plasma was found in the electrophoretic profiles.

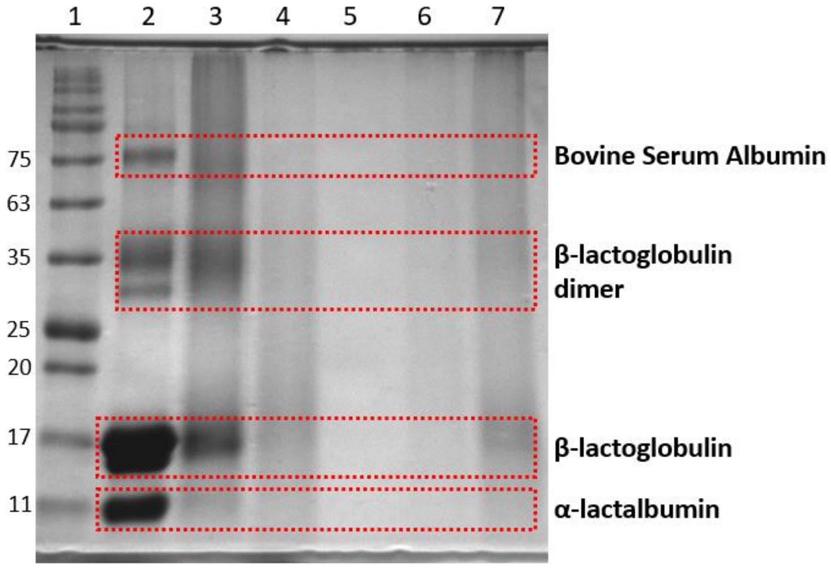


Figure 7.3 SDS-PAGE profiles of untreated and DBD treated whey proteins subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Whey control. Lane 3-4: high (2 mg/ml) and medium (0.2 mg/ml) protein concentrations, respectively, treated for 30 min. Lane 5-7: low (0.02 mg/ml) protein concentrations treated for 30, 20, 10 min, respectively.

In the gluten studies, Figure 7.4 shows SDS-PAGE results of DBD treated gluten under reducing conditions. The bands of high molecular weight (HMW)- and low molecular weight (LMW)-glutenin subunits appeared around the protein markers of 100-125 kDa and 45-55 kDa, respectively. The ω5-gliadin is represented as the band at 75 kDa. The ω1,2-gliadins are the bands that are 60-70 kDa in molecular weight. The α-/β-gliadins are bands with a molecular weight of 30-40 kDa. The band intensities of high concentrations (10 mg/ml) of gluten subjected to DBD treatment for 30 min in lane 3 were analogous to the control in lane 2. The band intensities of medium gluten concentration (1 mg/ml) treated with 30 min DBD treatment time and low gluten concentration (0.1 mg/ml) treated with 10 min DBD treatment time were significantly decreased. At a low gluten concentration (0.1 mg/ml) treated by DBD for 20 min and 30 min, no bands were observed in lane 6 and 5, respectively. This may imply that the gluten structure could be completely denatured when a 10 ml sample of 0.1 mg/ml of gluten was exposed to DBD treatment for 20 min.

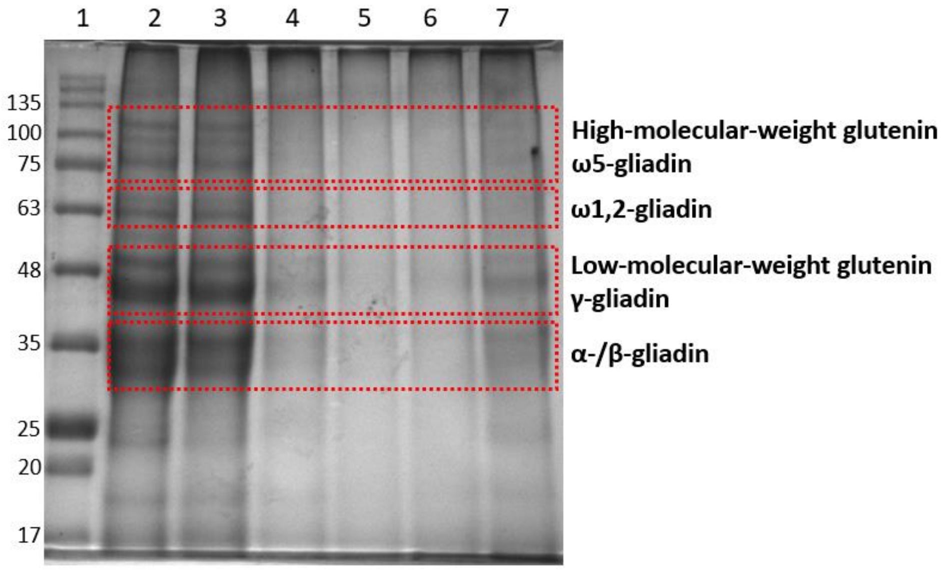


Figure 7.4 SDS-PAGE profiles of untreated and DBD treated glutens subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Gluten control. Lane 3-4: high (10 mg/ml) and medium (1 mg/ml) protein concentrations, respectively, treated for 30 min. Lane 5-7: low (0.1 mg/ml) protein concentrations treated for 30, 20, 10 min, respectively.

Atmospheric cold plasma is a good source of reactive species such as ozone, hydroxyl radicals, nitric oxide radicals as well as ultraviolet and electric fields. These species induce modification in protein structure. Several authors have observed that prolonged exposure of cold plasma resulted in reduced band intensity due to increased protein-to-protein interactions derived during protein unfolding and aggregation (Meinlschmidt et al., 2016; Venkataratnam et al., 2019). In this study, the electrophoretic results showed significant changes in casein, whey, and gluten protein profiles post in-package DBD treatment.

7.2.2 Secondary structural analysis

The extent of secondary structural changes in proteins subjected to cold plasma was determined using FTIR spectra. In the study of protein secondary structure, the deconvoluted amide I (1600-1700 cm^{-1}) area was employed. The fitting procedure of resultant peaks for α-helices, β-sheets, β-turns, and random coils, was followed as described in Chapter 5.

As shown in Figure 7.5, the FTIR analysis of the amide I band showed that in-package DBD treatment changed the secondary structure components of casein, particularly α-helices, β-sheets and β-turns. At a high concentration (2 mg/ml) of casein after 30 min DBD treatment, the β-turn content increased significantly while α-helix content decreased significantly ($p < 0.05$), compared to control (Figure 7.5a). However, the β-sheet and random coil contents of DBD treated casein did not change significantly ($p > 0.05$). In the lower casein concentrations subjected to DBD treatment, the change in their secondary structural components was more intense (Figure 7.5a). At the low casein concentration (0.02 mg/ml), the β-turn content of DBD treated casein increased, while the β-sheet content decreased with increased treatment time (Figure

7.5b). The α-helix content of DBD treated casein was not observed after 20 min DBD treatment.

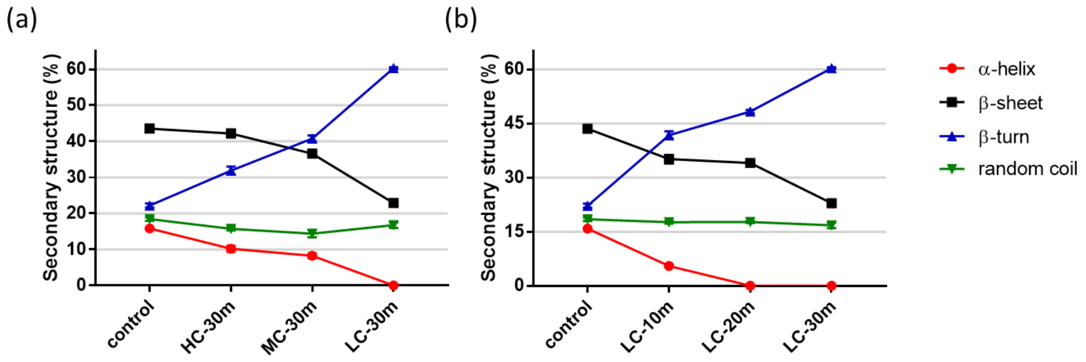


Figure 7.5 Secondary structures of DBD treated casein proteins (a) with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time (b) with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

The FTIR analysis of the deconvoluted amide I band illustrated that the secondary structural components of whey proteins were notably changed after in-package DBD treatment (Figure 7.6). At the high concentration (2 mg/ml) of casein after 30 min treatment, the secondary structure of treated whey was not considerably altered ($p > 0.05$) (Figure 7.6a). The change in secondary structure of DBD treated whey was more pronounced when lower whey concentrations were exposed. At the low whey concentration (0.02 mg/ml), the α-helix and β-sheet contents of DBD treated whey decreased by 6% and 36%, respectively, compared to the control. The β-turn and random coil contents of DBD treated whey increased by 39% and 3%, respectively, compared to the control. In Figure 7.6b, the change in secondary structure of DBD treated whey was more considerable when whey was subjected to prolonged plasma exposure. The β-sheet and α-helix contents of DBD treated whey were decreased while the β-turn and random coil contents were increased.

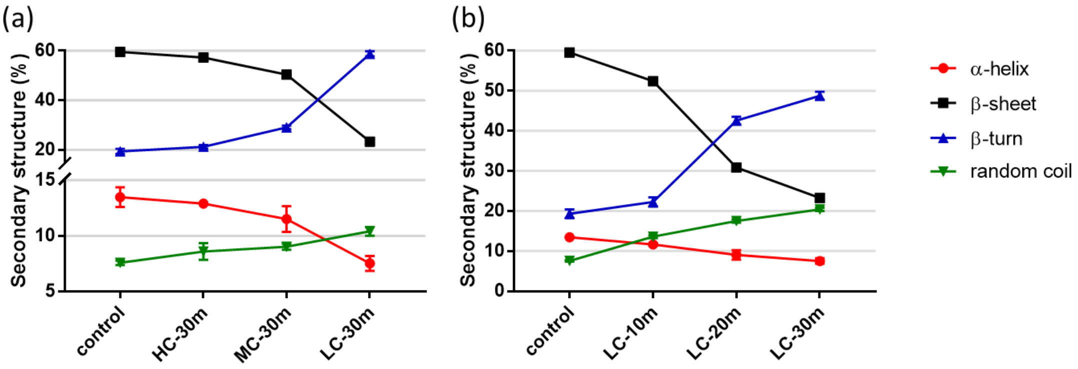


Figure 7.6 Secondary structures of DBD treated whey proteins (a) with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time (b) with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

The change in secondary structural components of gluten proteins is shown in Figure 7.7. At the medium (1 mg/ml) and high (10 mg/ml) gluten concentrations after 30 min DBD treatment, the secondary structures of gluten were not changed significantly ($p > 0.05$) (Figure 7.7a). For the low gluten concentration (0.1 mg/ml), the β-sheet content increased by 7.3 % while the α-helix and random coil contents were decreased by 2.5% and 4.9%, respectively, after 30 min treatment. In Figure 7.7b, the β-sheet content of DBD treated gluten was increased while the α-helix and random coil contents were decreased when the low gluten concentration (0.1 mg/ml) was subjected to prolonged treatment.

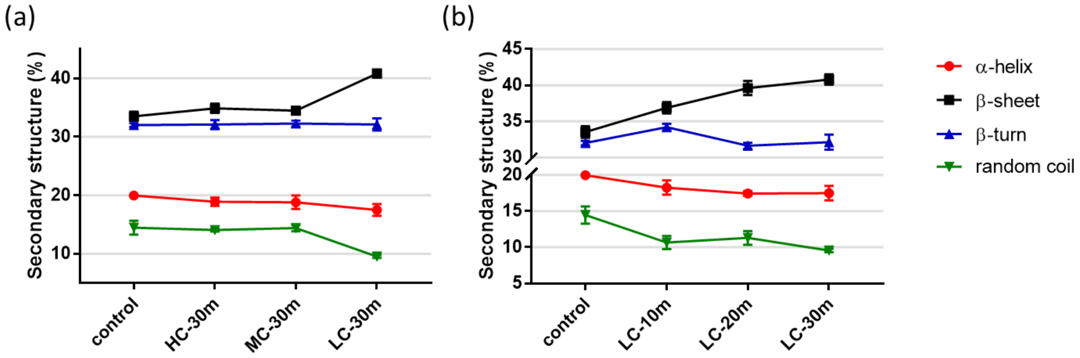


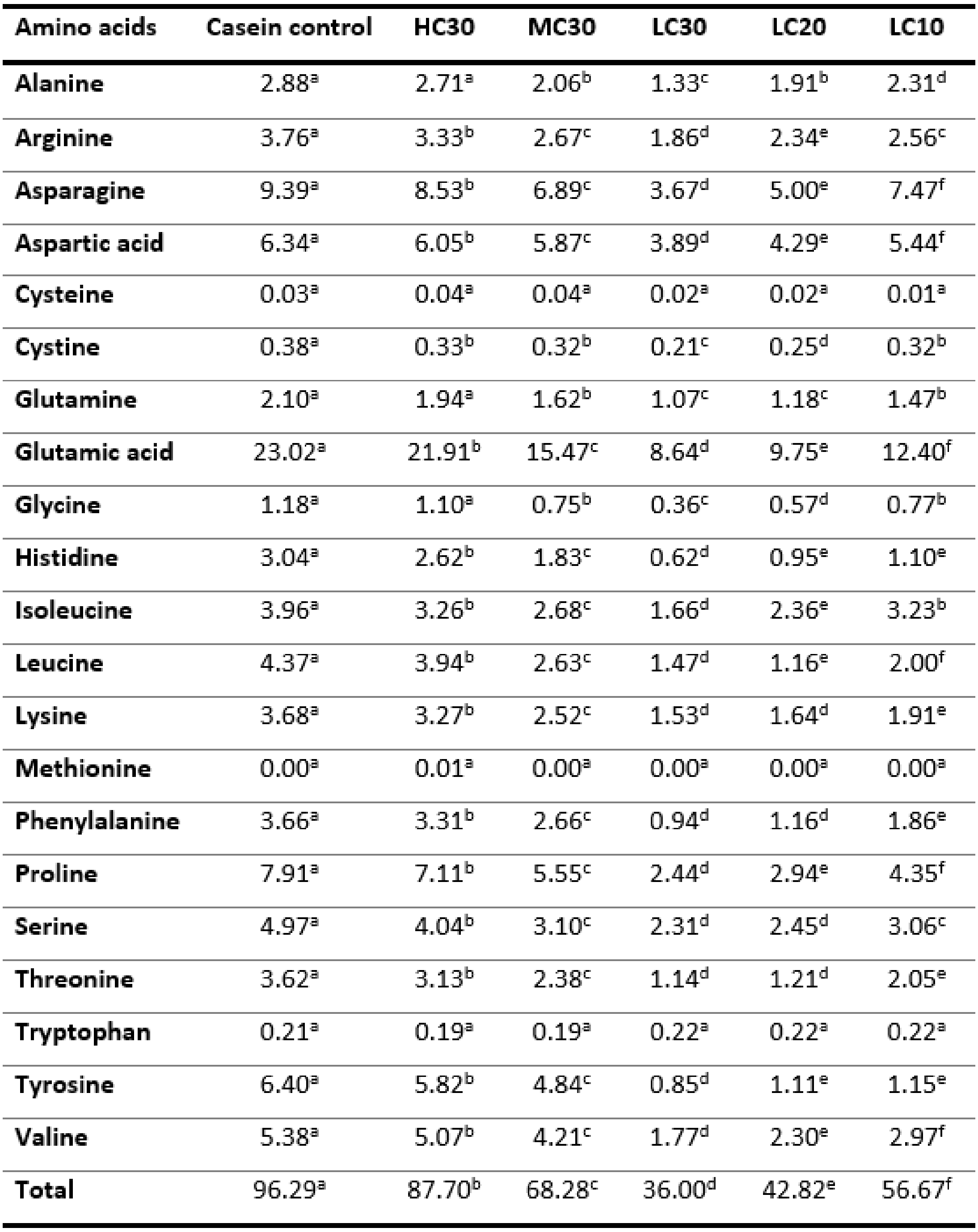
Figure 7.7 Secondary structures of DBD treated gluten proteins (a) with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time (b) with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

These results show that the in-package DBD treatment altered the secondary structures of proteins (casein, whey, and gluten) to different extents. An increase in β-turn content and decreases in α-helix and β-sheet contents were found when casein and whey were subjected to DBD treatment. Unlike DBD treated casein and whey, the β-sheet content increased while the α-helix and random coil contents decreased when gluten was subjected to DBD treatment. Venkataratnam et al. (2019) also reported variations in the secondary structures in peanut allergens using a DBD treatment. The interactions of plasma reactive species with protein could cause alterations in the secondary structures. The β strand structure correlated with the sheet-turn transformation with partial unfolding (Zhao et al., 2013). Additionally, the variation in the secondary structure could cause modification of the allergen epitopes and their binding capacity. This study indicates the importance of the native secondary structure of food allergens for atmospheric cold plasma induced effects.

7.2.3 Amino acid profile analysis

The amino acid compositions of in-package DBD treated casein samples were examined to assess the primary protein structure of casein post treatment (Table 7.1). Based on the results of low casein concentration (0.02 mg/ml), the total amino acid content of DBD treated casein decreased with increasing treatment time. When observing the results for high (2 mg/ml) and medium (0.2 mg/ml) casein concentrations, the total amino acid content of DBD treated casein decreased compared to casein control. Moreover, the results showed that for the lower casein concentrations, the decrease in the total amino acid content was more intense. Alanine, glutamic acid, leucine, lysine, proline, serine and valine are the common amino acids presented in IgE binding epitopes on casein (Busse et al., 2002; Chatchatee, Jarvinen, et al., 2001; Chatchatee, Järvinen, et al., 2001). At the low concentration (0.02 mg/ml) of DBD treated casein, the content of these amino acids decreased significantly with increasing treatment time (Amino acid: reduction of amino acid content for 30 min DBD treatment in percentage compared to the control = alanine: 54%; glutamic acid: 62%; leucine: 66%; lysine: 59%; proline: 69%; serine: 53%; valine: 67%, shown in Appendix 5). Simultaneously, the content of these amino acids of DBD treated casein with high (2 mg/ml) and medium (0.2 mg/ml) protein concentrations was decreased compared to casein control.

Table 7.1 Amino acid profile in untreated and treated casein proteins by DBD with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (g amino acid/100 g protein)

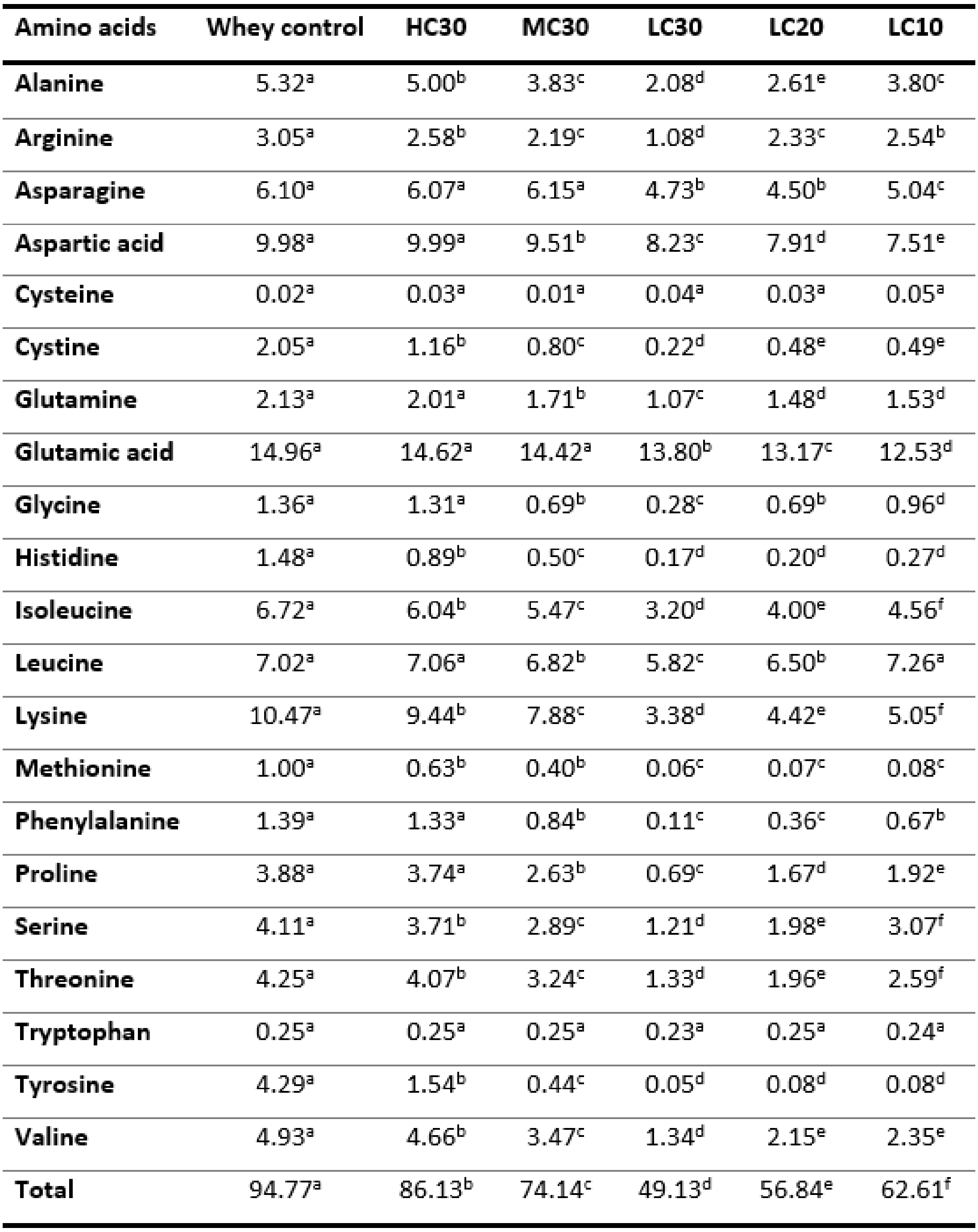


Amino acids	Casein control	HC30	MC30	LC30	LC20	LC10
Alanine	2.88^{a}	2.71^{a}	2.06^{b}	1.33^{c}	1.91^{b}	2.31^{d}
Arginine	3.76^{a}	3.33^{b}	2.67^{c}	1.86^{d}	2.34^{e}	2.56^{c}
Asparagine	9.39^{a}	8.53^{b}	6.89^{c}	3.67^{d}	5.00^{e}	7.47^{f}
Aspartic acid	6.34^{a}	6.05^{b}	5.87^{c}	3.89^{d}	4.29^{e}	5.44^{f}
Cysteine	0.03^{a}	0.04^{a}	0.04^{a}	0.02^{a}	0.02^{a}	0.01^{a}
Cystine	0.38^{a}	0.33^{b}	0.32^{b}	0.21^{c}	0.25^{d}	0.32^{b}
Glutamine	2.10^{a}	1.94^{a}	1.62^{b}	1.07^{c}	1.18^{c}	1.47^{b}
Glutamic acid	23.02^{a}	21.91^{b}	15.47^{c}	8.64^{d}	9.75^{e}	12.40^{f}
Glycine	1.18^{a}	1.10^{a}	0.75^{b}	0.36^{c}	0.57^{d}	0.77^{b}
Histidine	3.04^{a}	2.62^{b}	1.83^{c}	0.62^{d}	0.95^{e}	1.10^{e}
Isoleucine	3.96^{a}	3.26^{b}	2.68^{c}	1.66^{d}	2.36^{e}	3.23^{b}
Leucine	4.37^{a}	3.94^{b}	2.63^{c}	1.47^{d}	1.16^{e}	2.00^{f}
Lysine	3.68^{a}	3.27^{b}	2.52^{c}	1.53^{d}	1.64^{d}	1.91^{e}
Methionine	0.00^{a}	0.01^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Phenylalanine	3.66^{a}	3.31^{b}	2.66^{c}	0.94^{d}	1.16^{d}	1.86^{e}
Proline	7.91^{a}	7.11^{b}	5.55^{c}	2.44^{d}	2.94^{e}	4.35^{f}
Serine	4.97^{a}	4.04^{b}	3.10^{c}	2.31^{d}	2.45^{d}	3.06^{c}
Threonine	3.62^{a}	3.13^{b}	2.38^{c}	1.14^{d}	1.21^{d}	2.05^{e}
Tryptophan	0.21^{a}	0.19^{a}	0.19^{a}	0.22^{a}	0.22^{a}	0.22^{a}
Tyrosine	6.40^{a}	5.82^{b}	4.84^{c}	0.85^{d}	1.11^{e}	1.15^{e}
Valine	5.38^{a}	5.07^{b}	4.21^{c}	1.77^{d}	2.30^{e}	2.97^{f}
Total	96.29^{a}	87.70^{b}	68.28^{c}	36.00^{d}	42.82^{e}	56.67^{f}

* Values with different letters in a row are significantly different ($p < 0.05$). (mean of triplicate measurements from two independent experiments (n=6))

The amino acid profiles of DBD treated whey were evaluated in Table 7.2. In the results of low whey concentration (0.02 mg/ml), the total amino acid content of DBD treated whey decreased with increasing treatment time. In the results of high (2 mg/ml) and medium (0.2 mg/ml) whey concentrations, the total amino acid content of DBD treated whey decreased compared to whey control. In addition, the results showed that for the lower whey concentrations exposed to DBD treatment, the decrease in the total amino acid content was more intense. Isoleucine, lysine and valine are the common amino acids present in IgG-binding epitopes on α-lactalbumin (K.-M. Järvinen et al., 2001). The content of these amino acids of DBD treated whey with high (2 mg/ml) and medium (0.2 mg/ml) concentrations was decreased compared to whey control. At the low concentration (0.02 mg/ml) of DBD treated whey, the content of these amino acids decreased significantly after extended treatment (Amino acid: reduction of amino acid content for 30 min DBD treatment in percentage compared to the control = Isoleucine: 52%; lysine: 68%; valine: 73%, shown in Appendix 6). Aspartic acid, glutamic acid and leucine are the common amino acids present in IgE binding epitopes on β-lactoglobulin (K.-M. Järvinen et al., 2001). At the high (2 mg/ml) and medium (0.2 mg/ml) concentrations of whey treated with DBD for 30 min, the content of these amino acids did not change significantly. The reason why these amino acids did not decrease could be that the native β-lactoglobulin dimer is not susceptible to plasma treatment. This could be explained by combining these findings with the results in Figure 7.3. The β-lactoglobulin dimer bands were visible in lane 3 when a high concentration (2 mg/ml) of whey was exposed to DBD treatment for 30 min. The β-lactoglobulin dimers are rigid and cover these amino acids internally to isolate from plasma exposure. At the low concentration (0.02 mg/ml) of DBD treated whey, the content of these amino acids decreased significantly after extended treatment (Amino acid: reduction of amino acid content for 30 min DBD treatment in percentage compared to the control = aspartic acid: 18%; glutamic acid: 8%; leucine: 17%, shown in Appendix 6). The decrease in the contents of aspartic acid, glutamic acid and leucine could be attributed to the possibility that these amino acids were exposed externally when plasma treatment fully unfolded β-lactoglobulin dimers.

Table 7.2 Amino acid profiles in untreated and treated whey proteins by DBD with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (g amino acid/100 g protein)

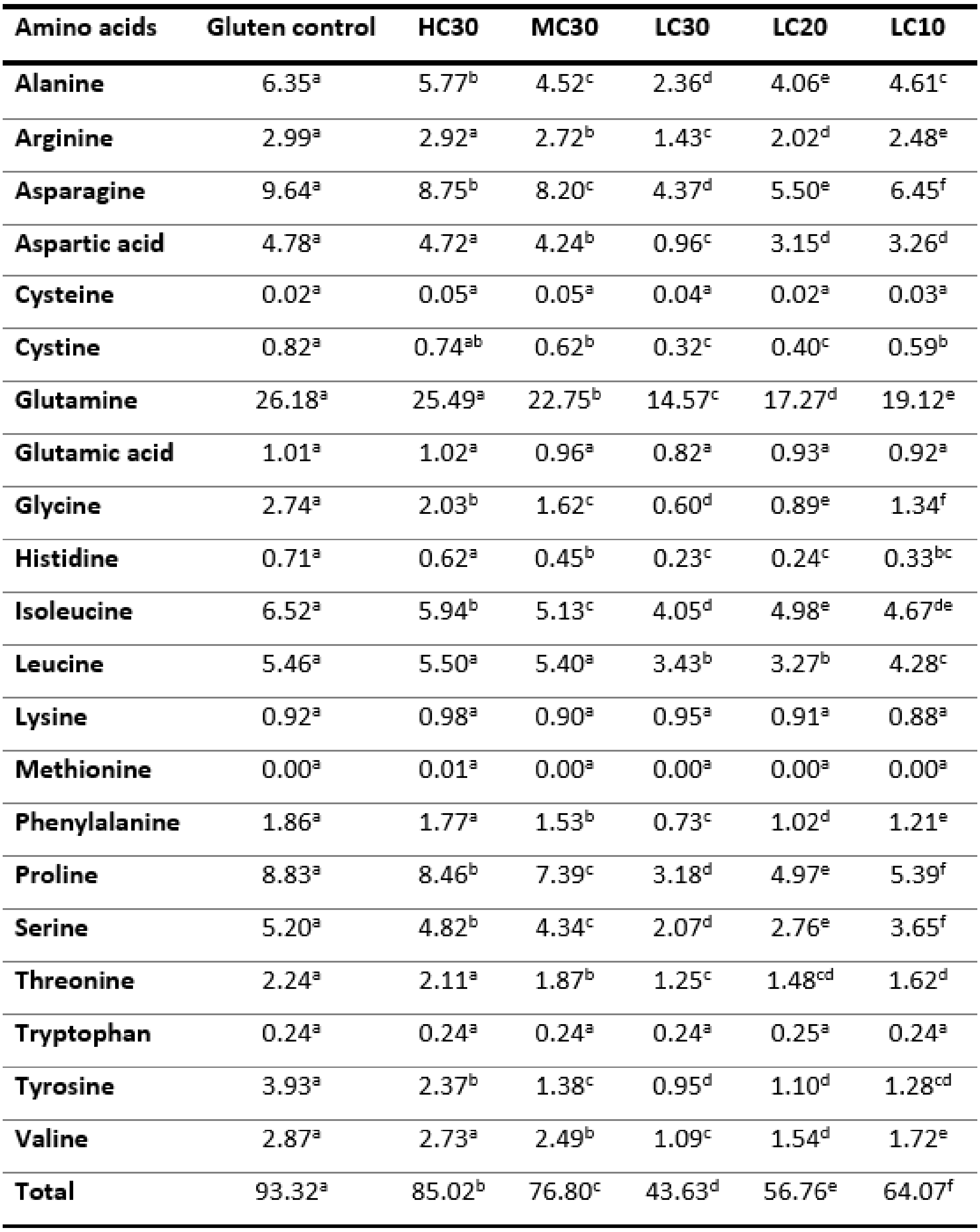


Amino acids	**Whey control**	**HC30**	**MC30**	**LC30**	**LC20**	**LC10**
Alanine	5.32^{a}	5.00^{b}	3.83^{c}	2.08^{d}	2.61^{e}	3.80^{c}
Arginine	3.05^{a}	2.58^{b}	2.19^{c}	1.08^{d}	2.33^{c}	2.54^{b}
Asparagine	6.10^{a}	6.07^{a}	6.15^{a}	4.73^{b}	4.50^{b}	5.04^{c}
Aspartic acid	9.98^{a}	9.99^{a}	9.51^{b}	8.23^{c}	7.91^{d}	7.51^{e}
Cysteine	0.02^{a}	0.03^{a}	0.01^{a}	0.04^{a}	0.03^{a}	0.05^{a}
Cystine	2.05^{a}	1.16^{b}	0.80^{c}	0.22^{d}	0.48^{e}	0.49^{e}
Glutamine	2.13^{a}	2.01^{a}	1.71^{b}	1.07^{c}	1.48^{d}	1.53^{d}
Glutamic acid	14.96^{a}	14.62^{a}	14.42^{a}	13.80^{b}	13.17^{c}	12.53^{d}
Glycine	1.36^{a}	1.31^{a}	0.69^{b}	0.28^{c}	0.69^{b}	0.96^{d}
Histidine	1.48^{a}	0.89^{b}	0.50^{c}	0.17^{d}	0.20^{d}	0.27^{d}
Isoleucine	6.72^{a}	6.04^{b}	5.47^{c}	3.20^{d}	4.00^{e}	4.56^{f}
Leucine	7.02^{a}	7.06^{a}	6.82^{b}	5.82^{c}	6.50^{b}	7.26^{a}
Lysine	10.47^{a}	9.44^{b}	7.88^{c}	3.38^{d}	4.42^{e}	5.05^{f}
Methionine	1.00^{a}	0.63^{b}	0.40^{b}	0.06^{c}	0.07^{c}	0.08^{c}
Phenylalanine	1.39^{a}	1.33^{a}	0.84^{b}	0.11^{c}	0.36^{c}	0.67^{b}
Proline	3.88^{a}	3.74^{a}	2.63^{b}	0.69^{c}	1.67^{d}	1.92^{e}
Serine	4.11^{a}	3.71^{b}	2.89^{c}	1.21^{d}	1.98^{e}	3.07^{f}
Threonine	4.25^{a}	4.07^{b}	3.24^{c}	1.33^{d}	1.96^{e}	2.59^{f}
Tryptophan	0.25^{a}	0.25^{a}	0.25^{a}	0.23^{a}	0.25^{a}	0.24^{a}
Tyrosine	4.29^{a}	1.54^{b}	0.44^{c}	0.05^{d}	0.08^{d}	0.08^{d}
Valine	4.93^{a}	4.66^{b}	3.47^{c}	1.34^{d}	2.15^{e}	2.35^{e}
Total	94.77^{a}	86.13^{b}	74.14^{c}	49.13^{d}	56.84^{e}	62.61^{f}

* Values with different letters in a row are significantly different ($p < 0.05$). (mean of triplicate measurements from two independent experiments (n=6))

The amino acid compositions of the glutens were assessed in Table 7.3. When observing the results of low gluten concentration (0.1 mg/ml), the total amino acid content of DBD treated gluten was reduced with increasing treatment time. When looking at the results of high (10 mg/ml) and medium (1 mg/ml) gluten concentrations, the total amino acid content of DBD treated gluten was reduced compared to gluten control. In addition, the results showed that for the lower gluten concentrations subjected to DBD treatment, the decrease in the total amino acid content of DBD treated gluten was more intense. Glutamine, isoleucine, leucine, phenylalanine, and proline are the fundamental amino acids of IgE binding epitopes on gluten (Battais et al., 2005; Denery-Papini et al., 2011). At the low gluten concentration (0.1 mg/ml), the content of these amino acids of DBD treated gluten decreased with increasing treatment time (Amino acid: reduction of amino acid content for 30 min DBD treatment in percentage compared to the control = glutamine: 44%; isoleucine: 38%; leucine: 37%; phenylalanine: 60%; proline: 64%, shown in Appendix 7). Additionally, these amino acid contents of DBD treated glutens with high (10 mg/ml) and medium (1 mg/ml) protein concentrations decreased compared to gluten control.

Table 7.3 Amino acid profile in untreated and treated glutens by DBD with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein)



Amino acids	Gluten control	HC30	MC30	LC30	LC20	LC10
Alanine	6.35^{a}	5.77^{b}	4.52^{c}	2.36^{d}	4.06^{e}	4.61^{c}
Arginine	2.99^{a}	2.92^{a}	2.72^{b}	1.43^{c}	2.02^{d}	2.48^{e}
Asparagine	9.64^{a}	8.75^{b}	8.20^{c}	4.37^{d}	5.50^{e}	6.45^{f}
Aspartic acid	4.78^{a}	4.72^{a}	4.24^{b}	0.96^{c}	3.15^{d}	3.26^{d}
Cysteine	0.02^{a}	0.05^{a}	0.05^{a}	0.04^{a}	0.02^{a}	0.03^{a}
Cystine	0.82^{a}	0.74^{ab}	0.62^{b}	0.32^{c}	0.40^{c}	0.59^{b}
Glutamine	26.18^{a}	25.49^{a}	22.75^{b}	14.57^{c}	17.27^{d}	19.12^{e}
Glutamic acid	1.01^{a}	1.02^{a}	0.96^{a}	0.82^{a}	0.93^{a}	0.92^{a}
Glycine	2.74^{a}	2.03^{b}	1.62^{c}	0.60^{d}	0.89^{e}	1.34^{f}
Histidine	0.71^{a}	0.62^{a}	0.45^{b}	0.23^{c}	0.24^{c}	0.33^{bc}
Isoleucine	6.52^{a}	5.94^{b}	5.13^{c}	4.05^{d}	4.98^{e}	4.67^{de}
Leucine	5.46^{a}	5.50^{a}	5.40^{a}	3.43^{b}	3.27^{b}	4.28^{c}
Lysine	0.92^{a}	0.98^{a}	0.90^{a}	0.95^{a}	0.91^{a}	0.88^{a}
Methionine	0.00^{a}	0.01^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Phenylalanine	1.86^{a}	1.77^{a}	1.53^{b}	0.73^{c}	1.02^{d}	1.21^{e}
Proline	8.83^{a}	8.46^{b}	7.39^{c}	3.18^{d}	4.97^{e}	5.39^{f}
Serine	5.20^{a}	4.82^{b}	4.34^{c}	2.07^{d}	2.76^{e}	3.65^{f}
Threonine	2.24^{a}	2.11^{a}	1.87^{b}	1.25^{c}	1.48^{cd}	1.62^{d}
Tryptophan	0.24^{a}	0.24^{a}	0.24^{a}	0.24^{a}	0.25^{a}	0.24^{a}
Tyrosine	3.93^{a}	2.37^{b}	1.38^{c}	0.95^{d}	1.10^{d}	1.28^{cd}
Valine	2.87^{a}	2.73^{a}	2.49^{b}	1.09^{c}	1.54^{d}	1.72^{e}
Total	93.32^{a}	85.02^{b}	76.80^{c}	43.63^{d}	56.76^{e}	64.07^{f}

* Values with different letters in a row are significantly different ($p < 0.05$). (mean of triplicate measurements from two independent experiments (n=6))

The primary concept of antigenicity reduction is the alteration of conformational and linear epitopes. The linear epitopes are composed of a linear sequence of amino acids (known as primary structure) that participate in antibody binding. In this study, the alteration of linear epitopes of proteins (casein, whey, and gluten) was studied by determining their amino acid profiles. These results show that DBD treatment modifies the primary structures of proteins. Takai et al. (2014) and R. Zhou et al. (2016) reported that the amino acid side chains were modified via oxidation, hydroxylation, dehydrogenation, nitration and dimerization induced by atmospheric cold plasma.

7.2.4 Antigenicity analysis

ELISA was performed to quantify the change in the IgE binding of casein, β-lactoglobulin and gliadin; as well as in the IgG-binding of α-lactalbumin after DBD treatment. Protein concentration and treatment time, as variables, are depicted in all the figures below.

The antigenicity of casein after DBD treatment is presented in Figure 7.8. The results showed that at lower concentrations of casein subjected to DBD treatment the decrease in the casein antigenicity was more intense. Moreover, the casein antigenicity of DBD treated casein decreased with increasing treatment time. The increased DBD treatment time is illustrated from right to left in the figure; the figure represented a ‘V’ trend. The middle trough is low concentration (0.02 mg/ml) of casein treated with DBD for 30 min. At the low concentration (0.02 mg/ml) of DBD treated casein, the casein antigenicity was reduced to values from 17.4% to 6.8% with the treatment times ranging from 10 min to 30 min.

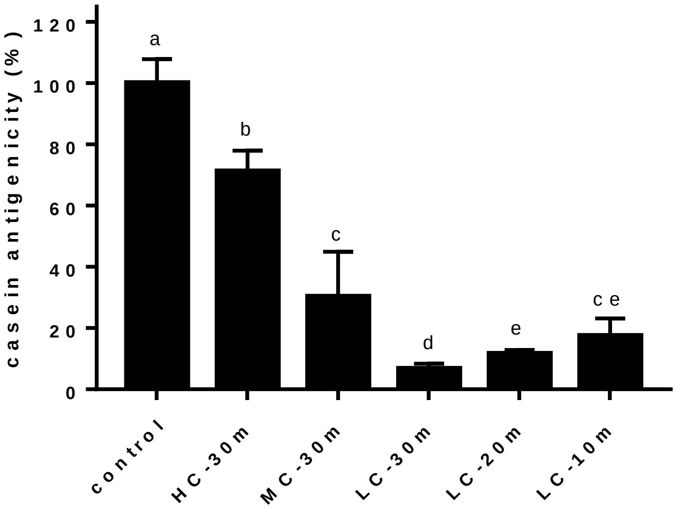


Figure 7.8 ELISA responses of casein subjected to DBD treatment against IgE binding activity (%). DBD treated casein proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml)

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

The antigenicity of α-lactalbumin of whey samples subjected to in-package DBD treatment is depicted in Figure 7.9. The results of α-lactalbumin antigenicity were analogous to the results of casein antigenicity. The α-lactalbumin antigenicity decreased gradually with decreasing concentration of whey subjected to treatment; or decreased as a function of treatment time when a low concentration of whey was DBD treated. In addition, the α-lactalbumin antigenicity was reduced by 90% or more when 10 ml of 0.02 mg/ml of whey sample was subjected to DBD treatment for 10 min.

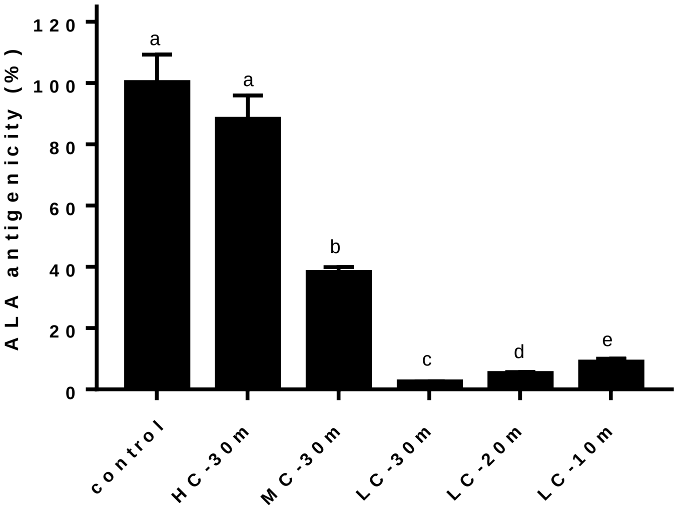


Figure 7.9 ELISA responses of α-lactalbumin (ALA) subjected to DBD treatment against IgG binding activity (%). DBD treated whey proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml)

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

The β-lactoglobulin antigenicity for whey samples exposed to DBD treatment is illustrated in Figure 7.10. At the high and medium concentrations of whey after 30 min DBD treatment, the β-lactoglobulin antigenicity was reduced to 53.7% and 91.5%, respectively. Notably, when the high concentration (2 mg/ml) of whey was treated, the β-lactoglobulin antigenicity was decreased by half. Such a decrease in β-lactoglobulin antigenicity was attributed to the plasma-based denaturation of the conformational epitopes of β-lactoglobulin that were exposed externally when combining the results of SDS-PAGE and amino acid profiles. Similarly, Rahaman, Vasiljevic, and Ramchandran (2015) suggested that a decrease in β-lactoglobulin antigenicity could be attributed to the destruction of some conformational epitopes due to intermolecular disulphide-mediated aggregation as well as a resultant compacting of structure of β-lactoglobulin that resulted in some inner epitopes becoming inaccessible. When the medium concentration (0.2 mg/ml) was treated, the β-lactoglobulin antigenicity was not significantly changed ($p > 0.05$). At the low concentration (0.02 mg/ml) of DBD treated whey, the β-lactoglobulin antigenicity increased gradually with increasing treatment duration. The increase in β-lactoglobulin antigenicity was attributed to the exposure of hidden epitopes as reported by (Ambrosi, Polenta, Gonzalez, Ferrari, & Maresca, 2016), where the in-package treatment could unfold the native β-lactoglobulin dimers, exposing the hidden epitopes which further increased the antigenicity detected.

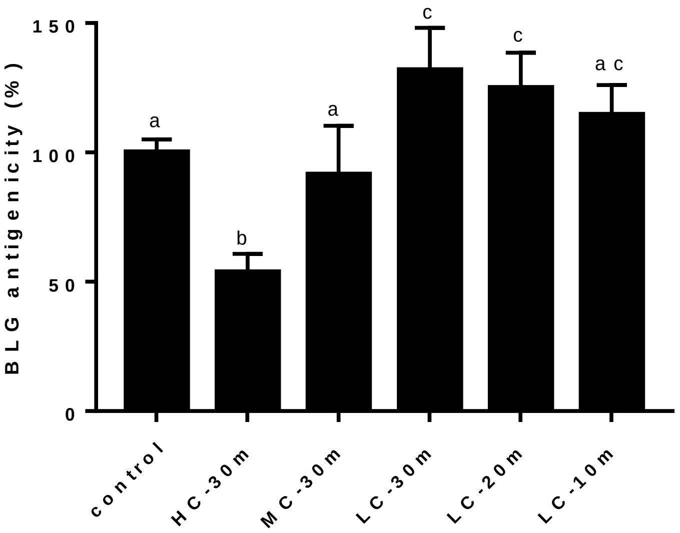


Figure 7.10 ELISA responses of β-lactoglobulin (BLG) subjected to DBD treatment against IgE binding activity (%). DBD treated whey proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml)

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

The gliadin antigenicity for gluten samples subjected to DBD treatment is presented in Figure 7.11. The results of gliadin antigenicity were similar to the results of casein antigenicity. The antigenicity of gliadin with high (10 mg/ml), medium (1 mg/ml), and low (0.1 mg/ml) concentrations after 30 min in-package treatment was reduced by 19%, 25% and 65%, respectively, compared to the control. The gliadin antigenicity decreased gradually at all protein concentrations but showed a much stronger reduction when a lower concentration of gluten was treated. Moreover, the gliadin antigenicity decreased gradually with increasing DBD treatment time. Similarly, Gojković Cvjetković, Marjanović-Balaban, Vujadinović, Vukić, and Rajić (2021) reported that the molecular weight distribution of wheat protein was changed after a surface barrier discharge (SBD) treatment applied with an input voltage of 200 V and a frequency of 50 Hz using atmospheric air and a decrease in the amount of proteins was observed compared to the untreated samples. The decrease in the amount of protein within the gluten fractions was responsible for allergenicity reduction after SBD treatment.

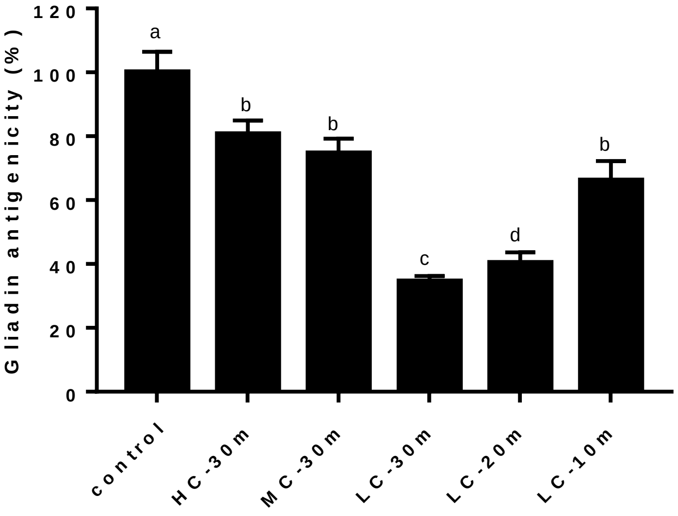


Figure 7.11 ELISA responses of gliadin subjected to DBD treatment against IgE binding activity (%). DBD treated gluten proteins with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time and with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml)

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

7.3 Conclusion

This study investigated the effect of dielectric barrier discharge (DBD) in-package treatment on the structures and antigenicity of milk protein (casein, β-lactoglobulin and α-lactalbumin) and wheat protein (gluten). The results clearly showed that the in-package DBD treatment was effective for reduction of casein, α-lactalbumin and gliadin antigenicity as assessed by ELISA. Notably, without unfolding β-lactoglobulin dimers by plasma at high concentrations of whey, β-lactoglobulin antigenicity was decreased. After prolonged DBD treatment, β-lactoglobulin dimers might unfold and expose the hidden epitopes. This may have caused the increase in β-lactoglobulin antigenicity observed at low concentrations. Moreover, DBD treatment resulted in changes in the content of secondary structures and changes in the amino acid compositions of milk and wheat proteins. These modifications in secondary and primary structures may collectively affect the antibody binding capacity of milk proteins and wheat proteins. Additionally, the differences in the reduction in antigenicity of these allergens are due to difference in the protein structure and composition. Wu et al. (2014) also reported that the mechanism of allergen reduction by cold plasma depends on the form of protein structure. In addition, the efficacy for allergenicity reduction is correlated with duration of plasma treatment, sample concentrations and type of sample. In summary, the findings suggest that in-package DBD treatment has the potential to reduce food allergen residues, however this specific set up and plasma process is inherently contained in nature as described in Chaple et al. (2020) and is thus practical for treatment of foods, in whole or part. This approach has been developed to pilot scale where in-package foods pass through 1 metre electrode processing channel, described in Ziuzina et al. (2020). It is possible that this approach can be adapted for surface residue treatment to mitigate cross contamination in food processing. The process input optimisation specific to allergen management for each food commodity was required prior to the introduction of this technology in food industry. The surface material impact on allergen control would also need to investigate the mode of application.

Chapter 8 Effect of direct surface treatment using plasma brush on the antigenicity of milk and wheat proteins

8.1 Experimental Design

Food allergens can be present on the equipment, lines, and utensil surfaces in the food chain. In this chapter, the ability of a commercial and scalable plasma brush (PB) for allergen mitigation on surfaces was assessed. One of the common material surfaces used in food industries is 304 stainless steel. The influence of ACP treatment using PB on the antigenicity of milk proteins (casein, β-lactoglobulin and α-lactalbumin) and wheat protein (gliadin) on stainless steel surfaces was investigated. The influence of PB treatment on the proteomic profile of casein, whey, and gluten proteins was studied to provide a better understanding of the potential interaction of PB treatment with casein, whey, and gluten proteins. The approach used is illustrated in Figure 8.1. The applied voltage and frequency used were 1 kW and 50 Hz. PB treatment was performed to expose the casein, whey, and gluten that were dried onto stainless steel surfaces. 2 mg/ml of casein or whey solutions, and 10 mg/ml of gluten solution (considered as high concentration), as well as 0.2 mg/ml of casein or whey solutions, and 1 mg/ml of gluten solution (considered as medium concentration) were subjected to PB treatment for a treatment time of 10 min. 0.02 mg/ml of casein or whey solutions, and 0.1 mg/ml of gluten solution (considered as low concentration) were subjected to PB treatment for 0.5, 1, 2, 5 and 10 min.

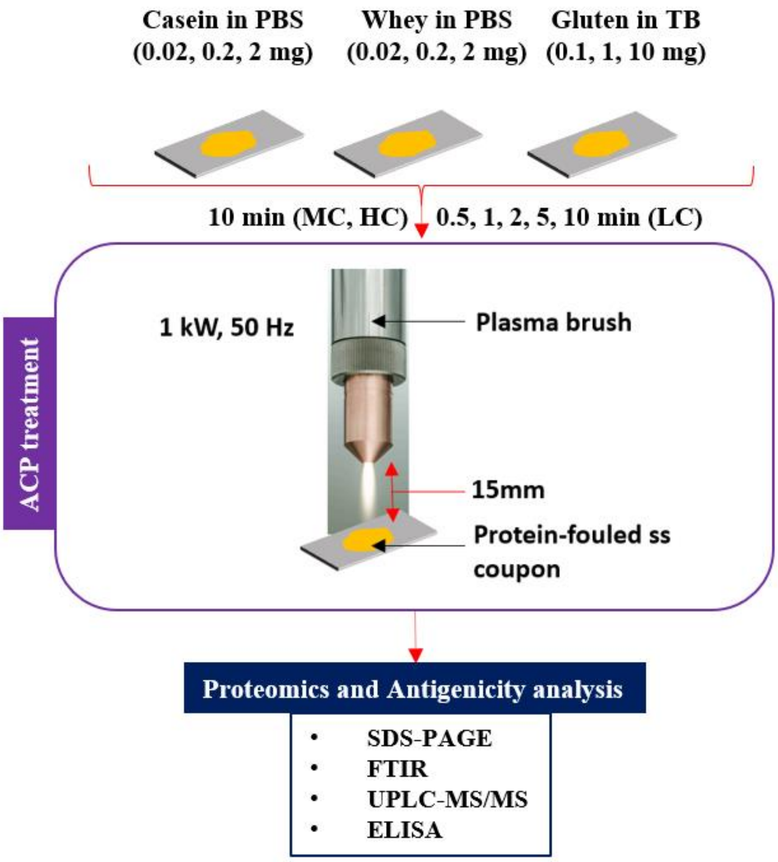


Figure 8.1 PB treatment of casein, whey, and gluten proteins – experimental design.

Low concentration (LC): 0.02 mg/ml of casein and whey, 0.1 mg/ml of gluten;

Medium concentration (MC): 0.2 mg/ml of casein and whey, 1 mg/ml of gluten;

High concentration (HC): 2 mg/ml of casein and whey, 10 mg/ml of gluten.

8.2 Results and Discussion

8.2.1 SDS-PAGE analysis

The electrophoretic profiles of PB treated caseins were evaluated under reducing conditions, as illustrated in Figure 8.2. The casein protein bands were present around the protein marker of 25-35 kDa. Compared to the control in lane 2, the high concentration (2 mg/ml) of casein after 10 min of PB treatment, did not show significant alterations in protein profiles. After 10 min PB treatment, the medium concentration (0.2 mg/ml) of treated casein in lane 4 was obviously decreased in band intensity while the band of low concentration (0.02 mg/ml) of treated casein in lane 5 had nearly vanished. This could indicate that for the lower protein concentration subjected to PB treatment, the degree of protein denaturation was more pronounced. The low casein concentrations (0.02 mg/ml) exposed to PB treatment for 0.5, 1, 2, 5 and 10 min are located in lanes 9, 8, 7, 6 and 5, respectively. There was no band visible when low casein concentrations were exposed to prolonged PB treatment.

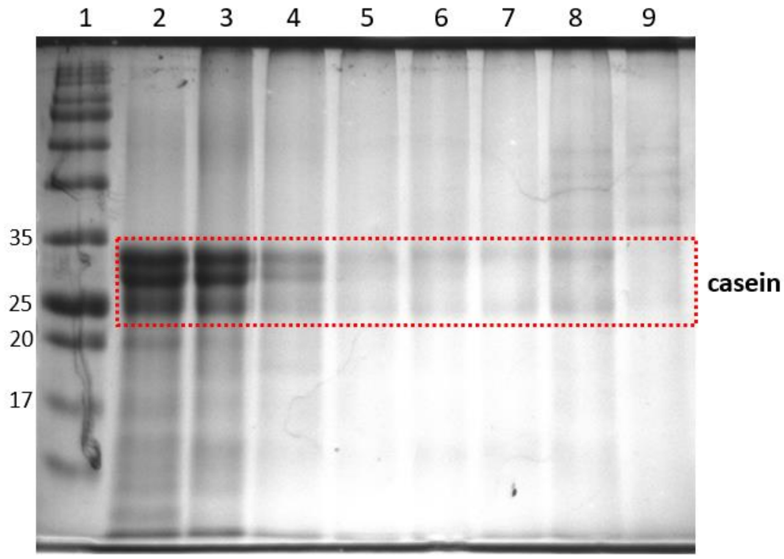


Figure 8.2 SDS-PAGE profiles of untreated and PB treated caseins subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Casein control. Lane 3-4: high (2 mg/ml) and medium (0.2 mg/ml) protein concentrations treated for 10 min, respectively. Lane 5-9: low (0.02 mg/ml) protein concentrations treated for 10, 5, 2, 1, 0.5 min, respectively.

Figure 8.3 shows SDS-PAGE patterns of PB treated whey proteins under reducing conditions. The α-lactalbumin and β-lactoglobulin are the allergenic protein fragments in whey. The α-lactalbumin is shown in the band at 11 kDa. The β-lactoglobulin monomer is around 17 kDa, whereas the dimer is approximately 35 kDa. Compared to the control (lane 2), the protein profiles of PB treated whey revealed significant differences. When observing the SDS-PAGE profiles of high (2 mg/ml) and medium (0.2 mg/ml) concentrations of whey treated with PB for 10 min shown in lanes 3 and 4, respectively, the band intensities of β-lactoglobulin monomer and α-lactalbumin decreased compared to the control, whereas the β-lactoglobulin dimer was not changed significantly and became smeared. The band intensity of β-lactoglobulin monomer and α-lactalbumin decreased compared to the control when the low whey concentration (0.02 mg/ml) was PB treated for 30 sec, shown in lane 9. The band intensity then remained unchanged as a function of treatment time, shown in lanes 8, 7, 6 and 5. The band of the β-lactoglobulin dimer had vanished after 30 sec PB treatment (lane 9).

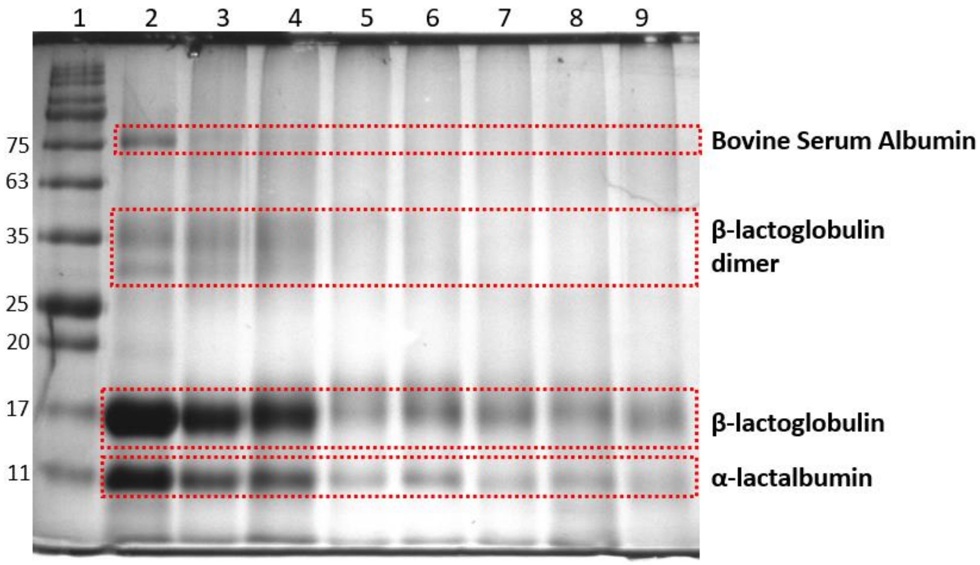


Figure 8.3 SDS-PAGE profiles of untreated and PB treated whey proteins subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Whey control. Lane 3-4: high (2 mg/ml) and medium (0.2 mg/ml) protein concentrations treated for 10 min, respectively. Lane 5-9: low (0.02 mg/ml) protein concentrations treated for 10, 5, 2, 1, 0.5 min, respectively.

In the gluten studies, Figure 8.4 displays SDS-PAGE profiles of PB treated glutens under reducing conditions. The high molecular weight (HMW)- and low molecular weight (LMW)-glutenin subunits occur in the bands at 100-125 kDa and 45-55 kDa, respectively. The ω5-gliadin is presented at 75 kDa. The ω1,2-gliadins are the bands with a molecular weight of 60-70 kDa. The α-/β-gliadins are shown in the band at 30-40 kDa. Compared to the control in lane 2, the band intensities of high concentration (10 mg/ml) of gluten treated with PB for 10 min were decreased (lane 3). The bands of medium concentration (1 mg/ml) of gluten treated with PB for 10 min (lane 4) had disappeared. Similarly, the bands of low concentration (0.1 mg/ml) of gluten after 30 sec PB treatment shown in lane 9 were faint and remained unchanged after extended treatment, shown in lanes 8, 7, 6 and 5.

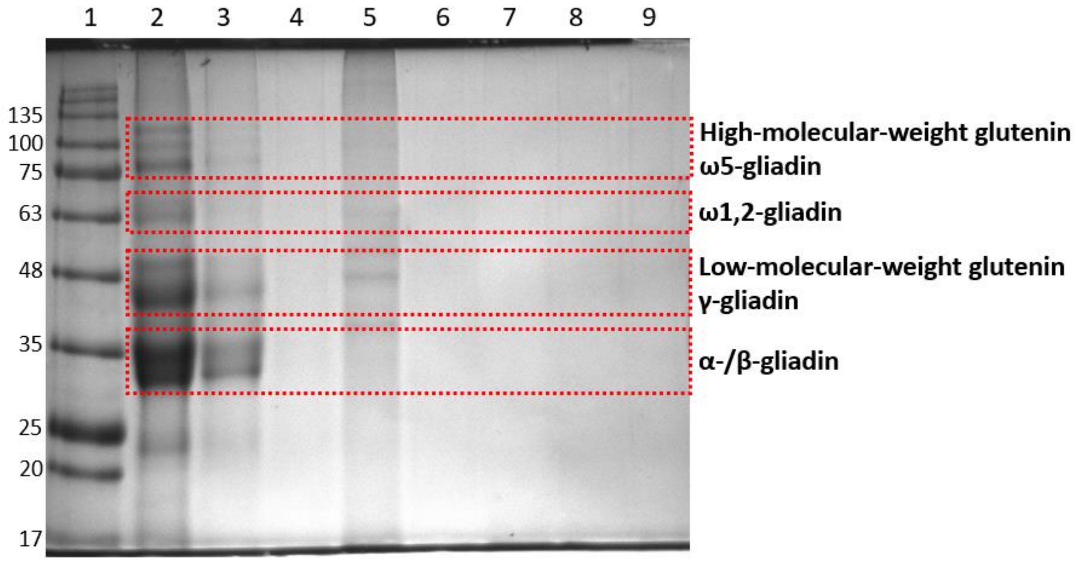


Figure 8.4 SDS-PAGE profiles of untreated and PB treated glutens subjected to different exposure times with different protein concentrations under reducing condition. Lane 1: the standard protein marker. Lane 2: Gluten control. Lane 3-4: high (10 mg/ml) and medium (1 mg/ml) protein concentrations treated for 10 min, respectively. Lane 5-9: low protein concentrations (0.1 mg/ml) treated for 10, 5, 2, 1, 0.5 min, respectively.

In summary, all these allergen structures could be denatured when 0.02 mg/ml of sample were subjected to PB treatment for 30 sec, except the β-lactoglobulin monomer and α-lactalbumin. The β-lactoglobulin dimer could be completely denatured when low whey concentration (0.02 mg/ml) was subjected to PB treatment. However, the band of the β-lactoglobulin dimer remained when the high (2 mg/ml) and medium (0.2 mg/ml) concentrations of whey were PB treated. This indicated that the covalent and intermolecular bonds of the dimer is resistant to breakdown by plasma treatment when a high protein concentration was subjected to PB treatment. N. Li et al. (2020) reported that the surface etching on the surface of rice is mainly due to the breakdown of covalent bonds induced by the bombardment of reactive species generated from plasma. The β-lactoglobulin monomer and α-lactalbumin could be partially denatured when whey was subjected to PB treatment. The gluten structure could be entirely denatured either when 1 mg/ml of gluten was subjected to PB treatment for 10 min, or 0.1 mg/ml of gluten was subjected to PB treatment for 30 sec or less. The studies on Gly m5, a major soy allergen, which has a molecular weight similarity with casein, showed a reduction in the band intensity after direct and remote cold atmospheric pressure plasma (Meinlschmidt et al., 2016). The decrease in band intensity may be related to interactions of the plasma reactive species with proteins, which can change the conformational structure and cleave peptide bonds, or the protein denaturation by oxidation of amino acids in proteins, or the new formation of aggregates via the reduction of protein solubility or intra/inter protein crosslinking (Ekezie, Cheng, & Sun, 2018; Shriver & Yang, 2011).

8.2.2 Secondary structural analysis

The secondary structure of proteins subjected to PB plasma treatment was evaluated by using FTIR analysis. The deconvoluted amide I (1600-1700 cm^{-1}) area was employed to determine the secondary structures of proteins. The fitting procedure of resultant peaks for α-helices, β-sheets, β-turns, and random coils, was followed as described in Chapter 6.

As shown in Figure 8.5, the secondary structural components of casein were changed after surface plasma treatment using plasma brush (PB), particularly α-helices, β-turns and random coil. At the high (2 mg/ml) and medium (0.2 mg/ml) concentrations of caseins after 10 min PB treatment, the secondary structural contents of PB treated casein did not change significantly ($p > 0.05$), compared to control (Figure 8.5a). At the low casein concentration (0.02 mg/ml) subjected to PB treatment, the β-turn content of PB treated casein increased by 5% while the α-helix and random coil contents of PB treated casein decreased by 4% and 3%, respectively, compared to control (Figure 8.5b). These secondary structural changes of PB treated casein were observed after 30 seconds PB treatment and remained constant thereafter.

At the high (2 mg/ml) and medium (0.2 mg/ml) concentrations of whey proteins after 10 min PB treatment, the secondary structural contents of PB treated whey did not change significantly ($p > 0.05$), compared to control (Figure 8.6a). At the low concentration (0.02 mg/ml) of whey proteins after 10 min PB treatment, the α-helix content of PB treated whey decreased by 3.5% while the random coil content increased by 3.6%. In Figure 8.6b, the α-helix content of PB treated whey decreased gradually while the random coil content increased gradually, when the low concentration (0.02 mg/ml) of whey proteins was subjected to prolonged PB treatment.

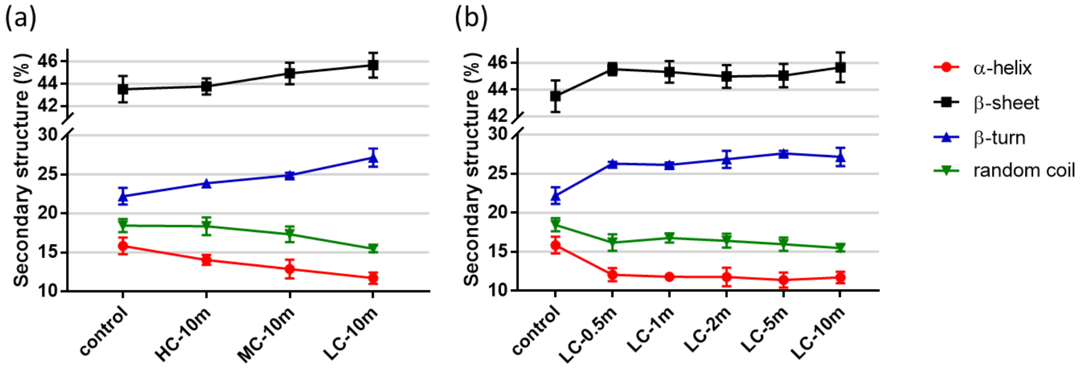


Figure 8.5 Secondary structures of PB treated casein proteins (a) with different protein concentration (2, 0.2, 0.02 mg/ml) at 10 min treatment time (b) with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

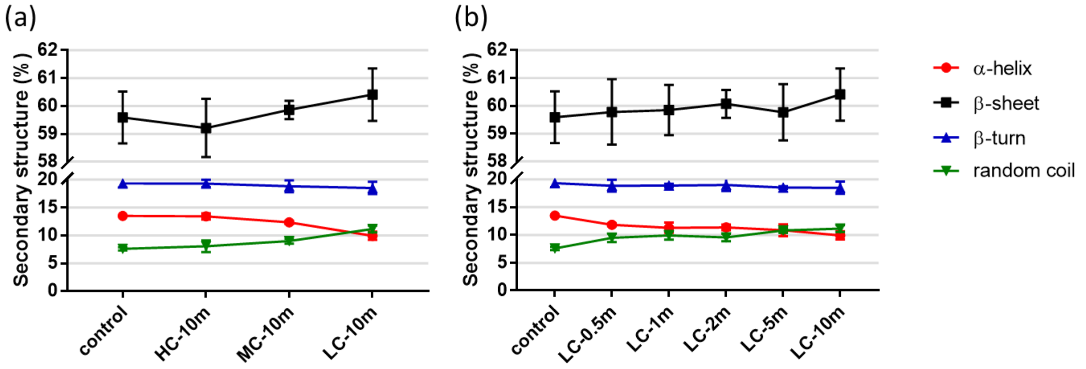


Figure 8.6 Secondary structures of PB treated whey proteins (a) with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time (b) with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml);

LC: low concentration (0.02 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

The change in secondary structural components of gluten proteins is shown in Figure 8.7. For the high gluten concentration (10 mg/ml), the secondary structures of PB treated gluten did not change significantly after 10 min ($p > 0.05$) (Figure 8.7a). The change in secondary structures (particularly α-helices, β-turns and random coils) of PB treated gluten proteins was more pronounced when lower gluten concentrations were subjected to plasma exposure. In Figure 8.7b, the α-helix and random coil contents of PB treated gluten decreased gradually from 10.4% to 5.9% and from 8.5% to 7.2%, respectively, when the low gluten concentration (0.1 mg/ml) was subjected to PB treatment for 10 min. The β-turn contents of PB treated gluten increased from 71.0% to 75.4%.

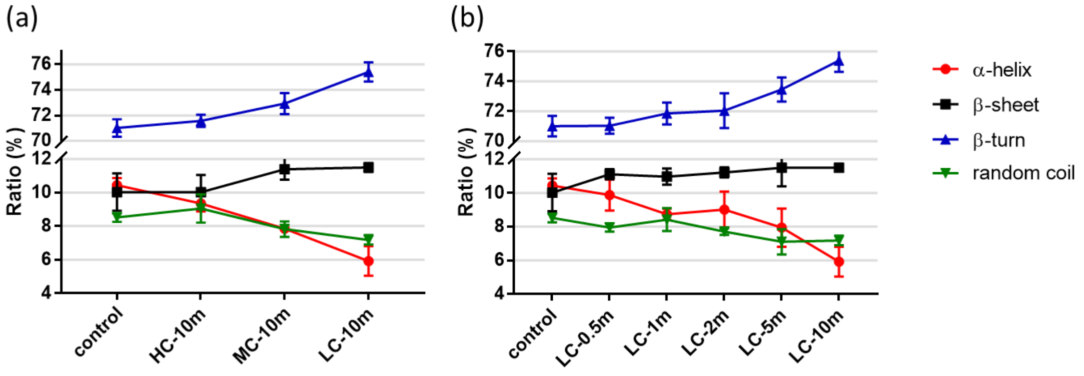


Figure 8.7 Secondary structures of PB treated gluten proteins (a) with different protein concentrations (10, 1, 0.1 mg/ml) at 10 min treatment time (b) with low protein concentration of 0.1 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml);

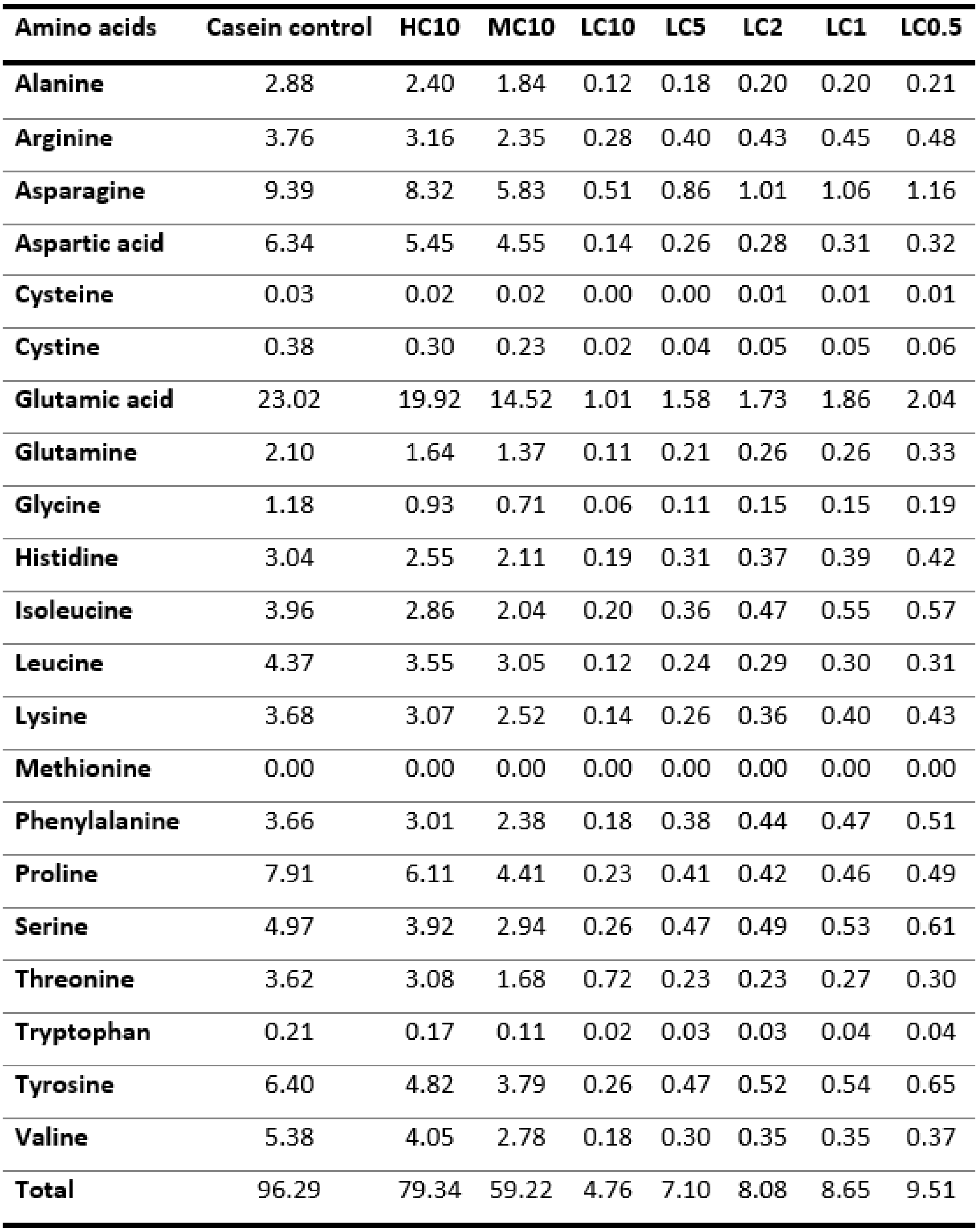
LC: low concentration (0.1 mg/ml) (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

There are multiple known mechanisms of action of cold plasma on the modification of protein structure. An ample amount of reactive species generated by cold plasma cleaves the protein into peptides and oxidizes the amino acid side chains, leading to the alteration in secondary structure. The reactive species can cleave the disulphide binds of the protein or oxidize the sulphur-containing amino acids and further lead to destruction of binding sites for antibodies. The change in the secondary structure could cause the modification of conformational epitopes and further affect their binding ability (Ji et al., 2017; Surowsky et al., 2013).

8.2.3 Amino acid profile analysis

The amino acid compositions of PB treated casein samples were evaluated to assess the primary protein structure of casein after PB treatment (Table 8.1). When observing the results of high (2 mg/ml), medium (0.2 mg/ml), and low (0.02 mg/ml) casein concentrations after 10 min PB treatment, the total amino acid content of PB treated casein decreased to 79%, 59% and 5%, respectively, compared to casein control (96%). When observing the results of low casein concentrations (0.02 mg/ml), the total amino acid content of PB treatment decreased sharply to 9.5% within the first 30 seconds and gradually decreased after prolonged treatment. Alanine, glutamic acid, leucine, lysine, proline, serine and valine are the common amino acids present in IgE binding epitopes on casein (Busse et al., 2002; Chatchatee, Jarvinen, et al., 2001; Chatchatee, Järvinen, et al., 2001). At the low concentration (0.02 mg/ml) of PB treated casein, the contents of these amino acids decreased sharply within the first 30 seconds and gradually decreased with increasing treatment time (Amino acid: control → 30 seconds PB treatment → 10 min PB treatment = alanine: 2.88 → 0.21 → 0.12; glutamic acid: 23.02 → 2.04 → 1.01; leucine: 4.37 → 0.31 → 0.12; lysine: 3.68 → 0.43 → 0.14; proline: 7.91 → 0.49 → 0.23; serine: 4.97 → 0.61 → 0.26; valine: 5.38 → 0.37 → 0.18). Moreover, the decrease in the content of these amino acids of PB treated casein was more pronounced when lower casein concentrations were subjected to PB treatment (Amino acid: control → high casein concentration → medium casein concentration → low casein concentration = alanine: 2.88 → 2.4 → 1.84 → 0.12; glutamic acid: 23.02 → 19.92 → 14.52 → 1.01; leucine: 4.37 → 3.55 → 3.05 → 0.12; lysine: 3.68 → 3.07 → 2.52 → 0.14; proline: 7.91 → 6.11 → 4.41 → 0.23; serine: 4.97 → 3.97 → 2.94 → 0.26; valine: 5.38 → 4.05 → 2.78 → 0.18).

Table 8.1 Amino acid profile in untreated and treated casein proteins by PB with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (g amino acid/100 g protein)

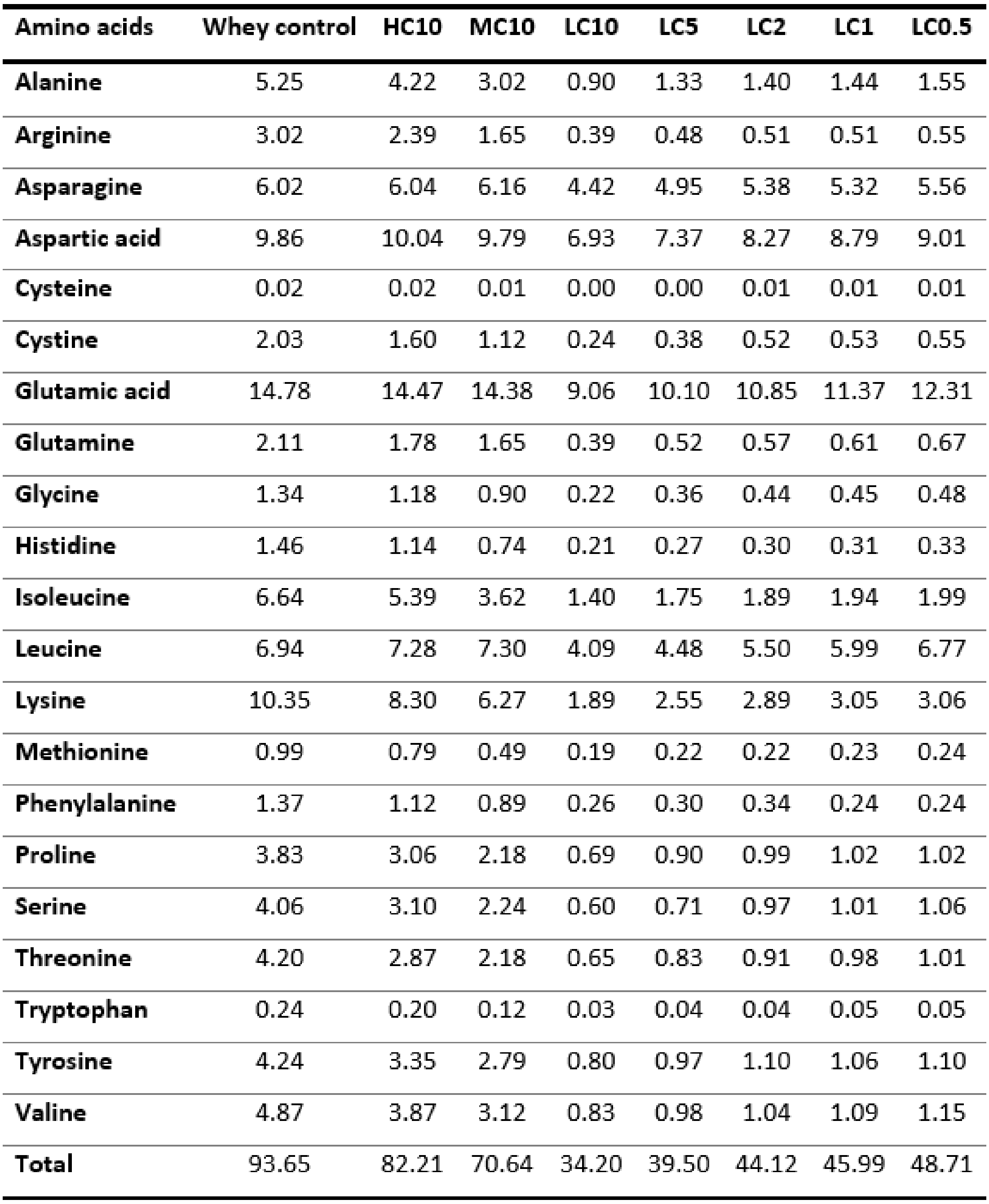


Amino acids	Casein control	HC10	MC10	LC10	LC5	LC2	LC1	LC0.5
Alanine	2.88	2.40	1.84	0.12	0.18	0.20	0.20	0.21
Arginine	3.76	3.16	2.35	0.28	0.40	0.43	0.45	0.48
Asparagine	9.39	8.32	5.83	0.51	0.86	1.01	1.06	1.16
Aspartic acid	6.34	5.45	4.55	0.14	0.26	0.28	0.31	0.32
Cysteine	0.03	0.02	0.02	0.00	0.00	0.01	0.01	0.01
Cystine	0.38	0.30	0.23	0.02	0.04	0.05	0.05	0.06
Glutamic acid	23.02	19.92	14.52	1.01	1.58	1.73	1.86	2.04
Glutamine	2.10	1.64	1.37	0.11	0.21	0.26	0.26	0.33
Glycine	1.18	0.93	0.71	0.06	0.11	0.15	0.15	0.19
Histidine	3.04	2.55	2.11	0.19	0.31	0.37	0.39	0.42
Isoleucine	3.96	2.86	2.04	0.20	0.36	0.47	0.55	0.57
Leucine	4.37	3.55	3.05	0.12	0.24	0.29	0.30	0.31
Lysine	3.68	3.07	2.52	0.14	0.26	0.36	0.40	0.43
Methionine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phenylalanine	3.66	3.01	2.38	0.18	0.38	0.44	0.47	0.51
Proline	7.91	6.11	4.41	0.23	0.41	0.42	0.46	0.49
Serine	4.97	3.92	2.94	0.26	0.47	0.49	0.53	0.61
Threonine	3.62	3.08	1.68	0.72	0.23	0.23	0.27	0.30
Tryptophan	0.21	0.17	0.11	0.02	0.03	0.03	0.04	0.04
Tyrosine	6.40	4.82	3.79	0.26	0.47	0.52	0.54	0.65
Valine	5.38	4.05	2.78	0.18	0.30	0.35	0.35	0.37
Total	96.29	79.34	59.22	4.76	7.10	8.08	8.65	9.51

(mean of triplicate measurements from two independent experiments (n=6))

The amino acid profiles of PB treated whey are shown in Table 8.2. In the results of high (2 mg/ml), medium (0.2 mg/ml), and low (0.02 mg/ml) whey concentrations, the total amino acid content of PB treated whey decreased to 82%, 71%, and 34%, respectively, compared to whey control (94%). In the results of low whey concentrations (0.02 mg/ml), the total amino acid content of PB treated whey decreased sharply to 49% within the first 30 seconds and decreased gradually to 34% with increasing treatment time. Isoleucine, lysine and valine are the common amino acids present in IgG-binding epitopes on α-lactalbumin (K.-M. Järvinen et al., 2001). At the low concentration (0.02 mg/ml) of PB treated whey, the content of these amino acids had a sharp decrease within the first 30 seconds and a gradual decrease after extended treatment (Amino acid: control → 30 second PB treatment → 10 min PB treatment = isoleucine: 6.64 → 1.99 → 1.4; lysine: 10.35 → 3.06 → 1.89; valine: 4.87 → 1.15 → 0.83). Simultaneously, a pronounced decrease was present in the content of these amino acids of PB treated whey when lower whey concentration was subjected to PB treatment (Amino acid: control → high whey concentration → medium whey concentration → low whey concentration = isoleucine: 6.64 → 5.39 → 3.62 → 1.4; lysine: 10.35 → 8.3 → 6.27 → 1.89; valine: 4.87 → 3.87 → 3.12 → 0.83). Aspartic acid, glutamic acid and leucine are the common amino acids present in IgE binding epitopes on β-lactoglobulin (K.-M. Järvinen et al., 2001). For the high (2 mg/ml) and medium (0.2 mg/ml) concentrations of whey treated with PB for 10 min, the content of these amino acids either remained the same or increased. This could be attributed to the hidden presence of these amino acids inside the native β-lactoglobulin dimers not subjected to plasma exposure, which was explained in Chapter 7.2.3. At the low concentration (0.02 mg/ml) of PB treated whey, the content of these amino acids decreased gradually after extended treatment (Amino acid: control → 10 min treatment = aspartic acid: 9.86 → 6.93; glutamic acid: 14.78 → 9.06: leucine: 6.94 → 4.09). The decrease in the content of these amino acids could be attributed to the modification of the chemical structure of these amino acids by plasma treatment after these hidden amino acids were exposed externally by the unfolding of the β-lactoglobulin dimers induced by plasma treatment.

Table 8.2 Amino acid profile in untreated and treated whey proteins by PB with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (g amino acid/100 g protein)



Amino acids	Whey control	HC10	MC10	LC10	LC5	LC2	LC1	LC0.5
Alanine	5.25	4.22	3.02	0.90	1.33	1.40	1.44	1.55
Arginine	3.02	2.39	1.65	0.39	0.48	0.51	0.51	0.55
Asparagine	6.02	6.04	6.16	4.42	4.95	5.38	5.32	5.56
Aspartic acid	9.86	10.04	9.79	6.93	7.37	8.27	8.79	9.01
Cysteine	0.02	0.02	0.01	0.00	0.00	0.01	0.01	0.01
Cystine	2.03	1.60	1.12	0.24	0.38	0.52	0.53	0.55
Glutamic acid	14.78	14.47	14.38	9.06	10.10	10.85	11.37	12.31
Glutamine	2.11	1.78	1.65	0.39	0.52	0.57	0.61	0.67
Glycine	1.34	1.18	0.90	0.22	0.36	0.44	0.45	0.48
Histidine	1.46	1.14	0.74	0.21	0.27	0.30	0.31	0.33
Isoleucine	6.64	5.39	3.62	1.40	1.75	1.89	1.94	1.99
Leucine	6.94	7.28	7.30	4.09	4.48	5.50	5.99	6.77
Lysine	10.35	8.30	6.27	1.89	2.55	2.89	3.05	3.06
Methionine	0.99	0.79	0.49	0.19	0.22	0.22	0.23	0.24
Phenylalanine	1.37	1.12	0.89	0.26	0.30	0.34	0.24	0.24
Proline	3.83	3.06	2.18	0.69	0.90	0.99	1.02	1.02
Serine	4.06	3.10	2.24	0.60	0.71	0.97	1.01	1.06
Threonine	4.20	2.87	2.18	0.65	0.83	0.91	0.98	1.01
Tryptophan	0.24	0.20	0.12	0.03	0.04	0.04	0.05	0.05
Tyrosine	4.24	3.35	2.79	0.80	0.97	1.10	1.06	1.10
Valine	4.87	3.87	3.12	0.83	0.98	1.04	1.09	1.15
Total	93.65	82.21	70.64	34.20	39.50	44.12	45.99	48.71

(mean of triplicate measurements from two independent experiments (n=6))

The amino acid compositions of the glutens are shown in Table 8.3. When observing the results of high (10 mg/ml), medium (1 mg/ml), and low (0.1 mg/ml) gluten concentrations, the total amino acid content of PB treated gluten was reduced to 75%, 53%, and 16%, respectively, compared to gluten control (93%). When looking at the results of low gluten concentration (0.1 mg/ml), the total amino acid content of PB treated gluten decreased sharply to 30% within the first 30 seconds and decreased gradually to 16% with increasing treatment time. Glutamine, isoleucine, leucine, phenylalanine, and proline are the fundamental amino acids of IgE binding epitopes on gluten (Battais et al., 2005; Denery-Papini et al., 2011). At the low gluten concentration (0.1 mg/ml), the content of these amino acids of PB treated gluten decreased with increasing treatment time (Amino acid: control → 30 seconds PB treatment → 10 min PB treatment = glutamine: 26.16 → 4.12 → 2.44; isoleucine: 5.8 → 3.25 → 2.13; leucine: 5.84 → 2.64 → 1.72; phenylalanine: 1.85 → 0.69 → 0.27; proline: 8.8 → 2.9 → 1.5). In addition, the decrease of the content of these amino acids for PB treated gluten was more pronounced for the lower gluten concentration (Amino acid: control → high gluten concentration → medium gluten concentration → low gluten concentration = glutamine: 26.16 → 19.54 → 13.83 → 2.44; isoleucine: 5.8 → 4.82 → 4.44 → 2.13; leucine: 5.84 → 4.36 → 3.85 → 1.72; phenylalanine: 1.85 → 1.64 → 0.95 → 0.27; proline: 8.8 → 7.37 → 4.72 → 1.5).

Table 8.3 Amino acid profile in untreated and treated glutens by PB with different protein concentrations (10, 1, 0.1 mg/ml) at 10 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein).

Amino acids	Gluten control	HC10	MC10	LC10	LC5	LC2	LC1	LC0.5
Alanine	6.41	5.51	3.83	0.95	1.21	1.34	1.48	1.54
Arginine	3.02	2.72	2.00	0.76	0.84	1.09	1.27	1.66
Asparagine	9.55	7.56	5.51	1.77	2.64	3.23	3.80	3.90
Aspartic acid	4.79	3.98	2.61	1.33	1.60	2.01	2.25	2.30
Cysteine	0.06	0.04	0.05	0.02	0.04	0.04	0.07	0.07
Cystine	0.81	0.65	0.49	0.21	0.24	0.33	0.39	0.43
Glutamine	26.16	19.54	13.83	2.44	2.81	4.08	4.09	4.12
Glutamic acid	1.02	0.91	0.62	0.29	0.29	0.31	0.35	0.36
Glycine	2.74	2.20	1.50	0.61	0.70	0.81	0.83	0.86
Histidine	0.71	0.68	0.37	0.11	0.15	0.20	0.27	0.27
Isoleucine	5.80	4.82	4.44	2.13	2.82	3.50	3.15	3.25
Leucine	5.84	4.36	3.85	1.72	1.72	2.12	2.63	2.64
Lysine	0.94	0.79	0.57	0.18	0.65	0.66	0.76	0.76
Methionine	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01
Phenylalanine	1.85	1.64	0.95	0.27	0.45	0.67	0.67	0.69
Proline	8.80	7.37	4.72	1.50	1.96	2.55	2.87	2.90
Serine	5.19	4.69	2.72	0.64	0.83	1.02	1.12	1.20
Threonine	2.15	1.65	1.17	0.40	0.50	0.67	0.71	0.81
Tryptophan	0.26	0.18	0.15	0.01	0.02	0.03	0.03	0.03
Tyrosine	3.92	3.56	2.26	0.66	0.57	0.79	1.02	1.06
Valine	2.87	2.50	1.74	0.52	0.81	1.07	1.15	1.23
Total	93.05	75.47	53.40	16.43	21.12	26.70	29.06	30.24

(mean of triplicate measurements from two independent experiments (n=6))

Changes in the primary structure of proteins can be inferred by using modification of amino acid sequence. Sulphur-containing amino acids, such as cystine and methionine, are particularly susceptible to plasma-induced reactive oxygen species (ROS) (Pal et al., 2016). It is possible that the disulphide bonds in cystine were oxidatively cleaved. Moreover, the side chains of amino acids could be oxidized preferentially to form various products. Aromatic amino acids, such as phenylalanine and tyrosine, are also susceptible to cold plasma treatment. Takai et al. (2014); R. Zhou et al. (2016) suggested that the sequence of susceptibility of amino acids to reactive species starts with sulphur-containing amino acid and aromatic amino acids. This could provide an indication to anticipate whether the proteins are susceptible to cold plasma treatment by evaluating their content of the amino acids.

8.2.4 Antigenicity analysis

ELISA was employed to quantify the change in the IgE binding of casein, β-lactoglobulin and gliadin; as well as in the IgG-binding of α-lactalbumin after PB treatment. Protein concentration and treatment time, as variables, are depicted in all the figures below.

Figure 8.8 shows the effect of PB treatment on casein antigenicity. The antigenicity of casein with high (2 mg/ml), medium (0.2 mg/ml), and low (0.02 mg/ml) concentration after 10 min PB treatment was reduced by 14%, 35% and 93%, respectively, compared to control. The results revealed that the reduction in casein antigenicity was more pronounced at a lower concentration of casein treated by PB. Furthermore, when the low concentration (0.02 mg/ml) of casein was treated by PB, the antigenicity of PB treated casein decreased sharply by 86% within the first 30 seconds of treatment, remained constant until 5 min treatment and was followed by an additional drop of 7% after extended treatment to 10 min.

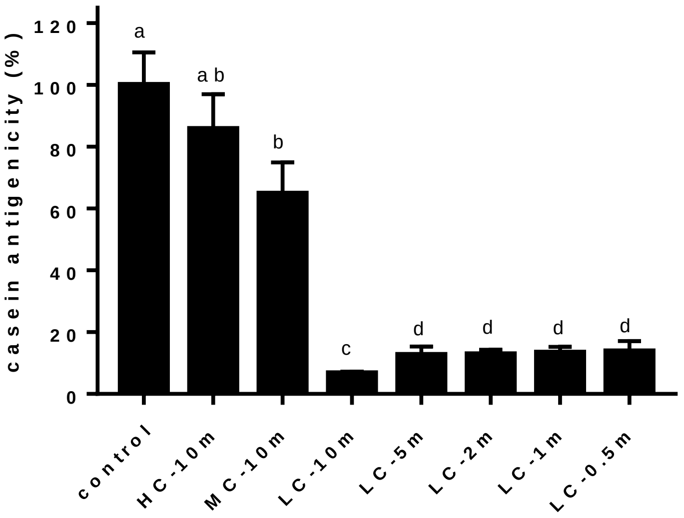


Figure 8.8 ELISA responses of casein subjected to PB treatment against IgE binding activity (%). PB treated casein proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml).

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

Figure 8.9 shows the antigenicity of α-lactalbumin in the whey samples treated with PB. The results of α-lactalbumin antigenicity were similar to the casein antigenicity results. The antigenicity of α-lactalbumin with high (2 mg/ml), medium (0.2 mg/ml), and low (0.02 mg/ml) concentrations of whey after 10 min

PB treatment was reduced by 42%, 91% and 99%, respectively, compared to control. The results indicated that the α-lactalbumin antigenicity decreased with decreasing concentrations of whey treated by PB. When the low concentration (0.02 mg/ml) of whey was treated by PB, the antigenicity of α-lactalbumin decreased sharply by 99% within the first 30 seconds of treatment and remained constant thereafter.

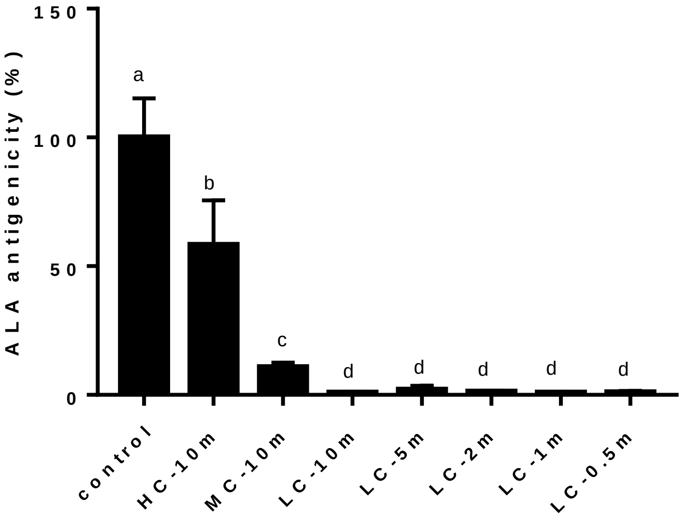


Figure 8.9 ELISA responses of α-lactalbumin (ALA) subjected to PB treatment against IgG binding activity (%). PB treated whey proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml).

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

Figure 8.10 depicts the antigenicity of β-lactoglobulin in whey samples treated by PB. The antigenicity of β-lactoglobulin with high (2 mg/ml), medium (0.2 mg/ml), and low (0.02 mg/ml) concentrations of whey treated with PB treatment increased by 16%, 22% and 78%, respectively, compared to control. The results showed that the β-lactoglobulin increased with increasing concentrations of whey treated by PB. The reason why the antigenicity of β-lactoglobulin increased is that the plasma treatment may have unfolded the native β-lactoglobulin dimers, and the hidden epitopes were exposed externally. When the low concentration (0.02 mg/ml) of whey was treated by PB, the antigenicity of β-lactoglobulin decreased to 75% within the first 30 seconds of treatment and increased gradually to 178% afterwards. The reason for the initial decrease in β-lactoglobulin antigenicity could be that the plasma treatment denatured the external epitopes of β-lactoglobulin. The reason for the following increase in β-lactoglobulin antigenicity is that the hidden epitopes were exposed externally by unfolding of the native dimers by plasma treatment (Meng et al., 2017).

Figure 8.11 illustrates the antigenicity of gliadin in gluten samples treated by PB. The antigenicity of gliadin with high (10 mg/ml), medium (1 mg/ml), and low (0.1 mg/ml) concentrations of gluten treated by PB decreased by 28%, 52% and 81%, respectively, compared to control. The results of gliadin antigenicity were similar to the results of casein antigenicity. The results showed a strong reduction in gliadin antigenicity when lower concentrations of gluten were subjected to PB treatment. When the low concentration (0.1 mg/ml) of gluten was treated by PB, the antigenicity of gliadin decreased sharply by 65% within the first 30 seconds of treatment, remained unchanged until 5 min treatment and was followed by an additional drop of 16% after extended treatment to 10 min.

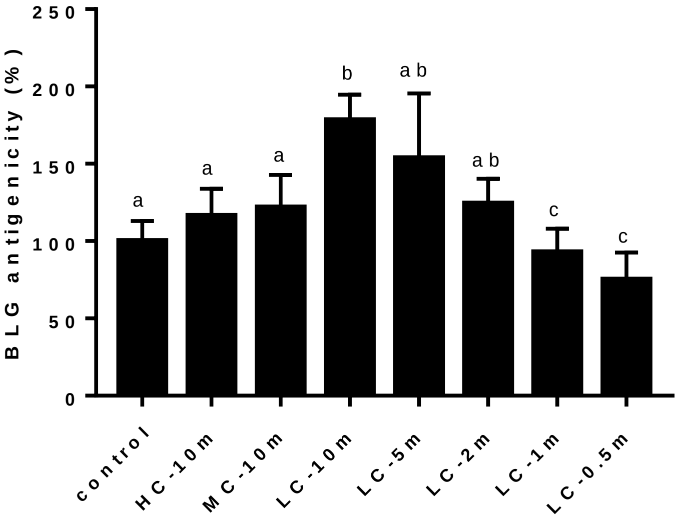


Figure 8.10 ELISA responses of β-lactoglobulin (BLG) subjected to PB treatment against IgE binding activity (%). PB treated whey proteins with different protein concentrations (2, 0.2, 0.02 mg/ml) at 10 min treatment time and with low protein concentration of 0.02 mg/ml at different treatment times (10, 5, 2, 1, 0.5 min). HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml)

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

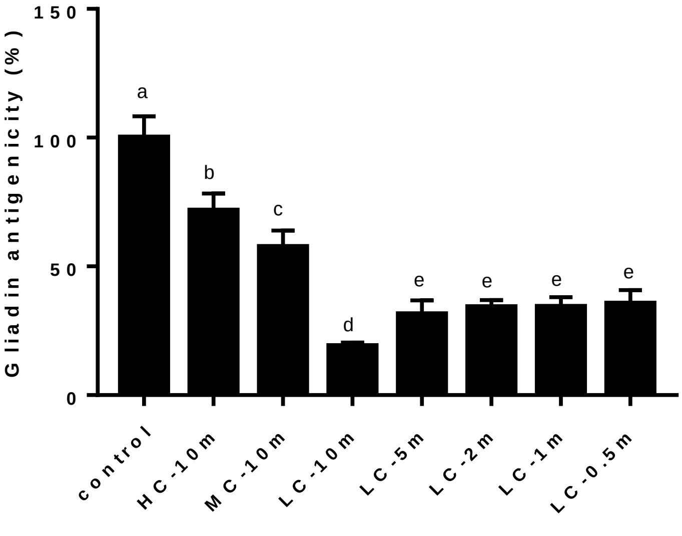


Figure 8.11 ELISA responses of gliadin subjected to PB treatment against IgE binding activity (%). PB treated gluten proteins with different protein concentrations (10, 1, 0.1 mg/ml) at 10 min treatment time and with low protein concentration of 0.1 mg/ml at different treatment times (10, 5, 2, 1, 0.5min). HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml)

*Different superscript letters indicate significantly different ($p < 0.05$) compared with the other. (mean ± standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

These results show that PB treatment significantly reduced the antigenicity of casein, α-lactalbumin and gliadin at the low concentration within a 30 sec. direct exposure. Similarly, Wu et al. (2014) reported that a dielectric barrier discharge (DBD) plasma treatment of atmospheric air using a voltage of 14 kV with a frequency of 10 kHz had a rapid airborne allergen inactivation. They observed that the allergenicity of Can f1 (dog allergen) decreased by 80% for 0.12 sec plasma exposure. Nooji (2011) suggested that DBD plasma treatment of air using 30 kV with frequency of 60 Hz decreased the wheat antigenicity by 37% for 5 min plasma exposure. A study on treating shrimp using DBD plasma treatment that was the same as Nooji (2011) for 5 min showed a reduction in antigenicity of tropomyosin by 76% as reported by Shriver (2011).

8.3 Conclusion

The study investigated the effect of direct surface plasma treatment using a plasma brush (PB) on the structures and antigenicity of milk protein (casein, β-lactoglobulin and α-lactalbumin) and wheat protein (gluten). The results clearly demonstrated that PB treatment can effectively reduce casein, α-lactalbumin and gliadin antigenicity as assessed by ELISA. However, the antigenicity of β-lactoglobulin was increased after PB treatment, in a similar trend to the previous set ups. The plasma brush is a plasma system designed for surface cleaning (Duan, Huang, & Yu, 2007). Particular attention should be noted for milk-derived products due to the enhancement of β-lactoglobulin antigenicity by PB treatment. Moreover, PB treatment caused changes in the protein profiles shown in SDS-PAGE, changes in the content of secondary structures and changes in the content of amino acids of milk and wheat proteins. This could collectively alter the conformational and linear epitopes and further affect the antigenicity of milk and wheat proteins. Additionally, the efficacy for allergenicity reduction is closely linked with duration of plasma treatment, sample concentration and type of sample. However, there are some limitations with the plasma brush in this study. The area of the treatment is given on the localised centre zone of high activity with the plasma brush treatment. It is possible to increase the area of treatment by employing an array of approaches which are commercially available and can be implemented in line within food processing environments. Hence, using multiple arrays may be necessary for large target surface areas while maintaining the efficiency of plasma generation for mitigating the allergen residues on surface. In summary, the findings suggest that the plasma brush treatment was effective and efficient for allergen mitigation and has the potential to be used as surface cleaning in food processing environments. The distance to samples (up to 15 mm) was investigated as this is within the manufacturer operating recommendations. A distance to surface of 15 mm or greater is feasible if the system is incorporated into arrays along process lines which may be mobile to a fixed array or vice versa.

Chapter 9 Overall discussion, conclusions, and future recommendations

9.1 Summary and major findings

Food allergies are increasingly recognised as a health and consumer issue throughout the world. Food allergens can pose a serious threat to food safety and have become a serious challenge for the food industry (Popping & Diaz-Amigo, 2018). In response, food business operators are increasingly focusing on allergen management to prevent allergen cross-contamination in food processing (Kupińska-Adamczyk et al., 2018). To date, the most effective method of preventing allergen cross-contamination is total avoidance via dedication-harmonisation or allergen wet-based cleaning (Jackson et al., 2008). However, avoidance is costly, time-consuming, and often difficult or even unachievable. It is possible that allergen residues can still unintentionally be present via cross-contamination during manufacturing and inefficiency of conventional decontamination techniques for shared equipment or centralised processing facilities. This underlines the importance of exploring prospective in-built technologies for allergen cross-contamination mitigation.

Recently, atmospheric cold plasma (ACP) has gained considerable interest as an alternative approach for allergen attenuation as well as for altering the functionality of food proteins via structural and conformational changes (Bourke et al., 2018; Segat et al., 2016). Overall, this work comprehensively investigated and advanced understanding of how ACP can be developed to mitigate allergen cross contamination in food processing environments, with regards to understanding the mechanism of allergen attenuation.

The performance of ACP is closely linked to its unique ability to generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Locke, Sato, Sunka, Hoffmann, & Chang, 2006). Plasma and liquid diagnostics are essential to gain an understanding of the underpinning physical and chemical processes at play, to provide insights that are essential for many applications (Gaisin & Son, 2010; Mezei, Cserfalvi, & Csillag, 2005).

Liquid diagnostics of the Reactive Species Specificity (RSS) system are presented in Chapter 3. The RSS system was used to generate plasma treated liquids by two modes: spark discharge (SD) and glow discharge (GD). It was found that plasma activated water (PAW) generated by the two RSS system discharge modes had different physicochemical properties. The PAW generated by SD or GD was an acidic, high oxidant solution with high conductivity. SDPAW predominantly contains NO_3^- and total oxidizing species with moderate to high ratios of H_2O_2 while GDPAW predominantly contains NO_2^- and NO_3^- at the initial stage and contains NO_2^-, NO_3^- and total oxidizing species without H_2O_2 at the late stage. If a liquid food model is treated by the RSS system, it might turn acidic and contain reactive species. The food model treated by ACP could be preserved longer from microbial and biochemical spoilage of foods (Illera et al., 2019; L. Xu, Garner, Tao, & Keener, 2017). Moreover, PAW can be an effective method for surface decontamination in food processing due to its inherent characteristics and multiple potential mechanisms of action against a wide range of contaminants. During processing, many foods are required to be processed in cold or mild temperature conditions. In this study, the RSS system has a minor thermal effect on the sample. The RSS system when applied directly to the samples does cause the temperature increase for each sample type studied. The temperature increase alone does not impact on the antigenicity reduction, as supported in Figure 6.4. In addition, the results demonstrated that the plasma performance was closely correlated with applied voltage and frequency. The plasma generation towards a high intensity of reactive oxygen and nitrogen species (RONS) concentration in the liquid is more efficient when a higher voltage and/or a lower frequency is applied.

Plasma diagnostics of the RSS system were described in Chapter 4, where it was observed that a cathode-directed streamer and an anode-directed streamer were ignited at the positive and negative polarity half-periods during SD generation. During GD generation, only anode-directed streamer was ignited at the negative polarity half-period. The cathode-direct streamer emitted a higher OH density than any of the anode-directed streamers. The production of H_2O_2 in the SDPAW might be attributed to the higher OH density emitted from the cathode-directed streamer in SD. The OH radical is a strong oxidant (Ehhalt et al., 2011), and it is assumed that the degree of oxidation induced by SD is higher than GD, due to the presence of the higher OH density. Illera et al. (2019) reported that SD inactivated the polyphenol oxidase in cloudy apply juice via oxidation.

Combining the results from both chapters 3 and 4, we can observe that the liquid chemistry and plasma chemistry are governed by the configurations of the plasma setup. Many studies on the applications of PAW across the food and medical sector are now reported (Abuzairi et al., 2018; Al-Sharify, Al-Sharify, al-Obaidy, & al-Azawi, 2020; Schnabel, Andrasch, et al., 2019; Schnabel et al., 2021; Schnabel et al., 2020), however, it is critical to provide an adequate description of the specific plasma setup, the process parameters and the resultant liquid chemistry. Hence, it is important to understand the liquid chemistry and plasma diagnostics to facilitate understanding and process control, and to provide the detailed descriptions in order to make informed decisions on suitable applications as well as insights for engineering scale-up.

The next stage involved assessing the effect of ACP on food allergens. To establish the basic interactions of the ACP with allergens, the protein profiles and the conformational structures of known allergens were examined. The different modes of ACP application were studied to understand how and what plasma system approach can be applied in food processing environments. Additionally, the universality of ACP across food proteins was evaluated by applying ACP to different allergen targets in terms of protein structures and compositions.

The effect of direct plasma treatment using the RSS system (SD and GD) on the antigenicity of milk and wheat proteins was described in Chapter 5. Either SD or GD was effective for antigenicity reduction of casein, α-lactalbumin and gliadin. However, the antigenicity of β-lactoglobulin was enhanced by direct ACP treatment, using either SD or GD. The protein profiles of casein, whey and gluten were changed after SD or GD treatment. The band intensity of casein, α-lactalbumin and gluten decreased after plasma treatment, whereas the β-lactoglobulin band appeared smeared and broad. Additionally, either SD or GD treatment resulted in changes in the amino acid compositions and changes in the content of secondary structures of milk and wheat proteins. The changes in antigenicity of milk and wheat proteins had a strong relationship with the modification of primary and secondary structures. The results obtained show the effectiveness of the RSS system against milk-derived allergens (such as casein, α-lactalbumin) and wheat-derived allergens (such as gliadin). This is important in the context of offering an alternative for thermo-resistant allergens, such as casein and gliadin. However, a potential risk of antigenicity enhancement was found for β-lactoglobulin, which is a complex protein due to its native dimer. The native dimeric form of β-lactoglobulin could hide parts of epitopes. The antigenicity enhancement after ACP treatment could be attributed to ACP treatment unfolding the dimer by breaking down the covalent bonds. Hence, this underlines the importance of assessing the universality of the ACP system against food allergens and the range of different structures and amino acid profiles. Additionally, higher mitigation efficacy of ACP against allergens was observed with extended plasma treatment time. The RSS system, as a liquid-based plasma application, can be applied to reduce the allergenicity of food allergens present in liquid whole food or in a liquid application to food processing environments.

To discover the possible modes of application for the RSS, indirect treatment using PAW was introduced (Chapter 6). The effectiveness of an ozonated water against milk-derived allergens has been reported by Guzel-Seydim et al. (2000). The mechanisms of PAW can be somewhat similar to ozonated water, which also acts as a strong oxidant. The effect of indirect treatment using plasma activated water (PAW) on bovine casein antigenicity was examined. PAW generated by SD (SDPAW) contains the reactive oxygen species (ROS) and reactive nitrogen species (RNS), whereas PAW generated by GD (GDPAW) contains only RNS. It was found that indirect SDPAW treatment in combination with mild heat (60 °C) can potentially reduce the antigenicity of bovine casein. However, SDPAW treatment alone was ineffective for significant antigenicity reduction of bovine casein. The ROS in SDPAW plays an important role in the antigenicity reduction of bovine casein. The mild heating can accelerate the ROS-induced oxidation and further affects the functionality of casein. GDPAW treatment, which contains RNS, did not aid in the antigenicity reduction of bovine casein. SDPAW in combination with mild heat treatment is effective for the antigenicity reduction of bovine casein by up to 77%, but in terms of practical application, the process time required to achieve up to 77% reduction would need to be improved or become part of a 'stay in place' treatment. Apart from antigenicity reduction, the removal of milk-derived proteins from food-contact surfaces using PAW can be investigated further. Overall, both direct RSS and indirect PAW treatments could represent a novel liquid mediated processing protocol for reducing antigenicity of bovine milk and process environments.

In food processing, allergen residues often present and adhere on working surfaces (such as stainless-steel surfaces). To explore the potential for surface cleaning of allergen residues in food processing, the commercially available industrial surface cleaning device - plasma brush (PB) from Relyon was utilised as described in Chapter 8. The results clearly demonstrated that PB treatment was effective for reducing the antigenicity of casein, α-lactalbumin and gliadin present on stainless-steel surfaces, however, the antigenicity of β-lactoglobulin was again mostly increased. The antigenicity of β-lactoglobulin was reduced by 25%, but only when a low concentration of whey was subjected to PB treatment within the first 30 seconds. Notably, the antigenicity of casein, α-lactalbumin and gliadin decreased by 86%, 99% and 65%, respectively, within the first 30 seconds of dry surface treatment. These results point to ACP application for rapid dry allergen cleaning that is compatible and can also be engineered and scaled for use on diverse surface geometries in food processing environments. Dry allergen cleaning can be challenging to successfully achieve since it is applied in facilities where dry products are produced, and minimal or no water contact is desired. At this point, the plasma brush, as a gaseous-based direct plasma, is a feasible approach for dry allergen cleaning on surfaces in food processing environments. A limitation of the work using this system in this study is the lack of experiment specific electrical and plasma diagnostics, which would further support process design. However, given the efficacy achieved against the protein targets on relevant surfaces, it was included. This system has been extensively characterised and described in the literature and by the manufacturer, and it is part of an ongoing experimental plan to develop electrical and plasma diagnostics to complement the protein denaturation study in collaboration with industry partner.

To investigate a suitable plasma system for industrial application to food products, the effects of a dielectric barrier discharge (DBD) ‘in-package’ plasma process for allergen mitigation was studied in Chapter 7. The results clearly showed that DBD treatment was effective for antigenicity reduction of casein, α-lactalbumin and gliadin. However, the antigenicity of β-lactoglobulin was again mostly increased, where the antigenicity of β-lactoglobulin was reduced only when the high concentration of whey was used.

The results suggest that the protein residue concentration plays an important role in the successful antigenicity mitigation achievable with ACP treatment. The very high concentrations of allergen residues tested represented protein levels actually found in foods, whereas the lower concentrations represented cross contaminants / residues in food processing environments. Significant antigenicity mitigation was achievable across the different plasma systems and processes when the lower challenge concentrations were used.

For the RSS system and the DBD system, the graphs show that the process optimization can be achievable. Their representative graphs in terms of time and concentration show a ‘V’ shape, indicating that the antigenicity reduction is correlated with the prolonged treatment and lower protein concentration samples treated. When this is applied to surface treatment, the graphs follow an ‘L” shape, primarily because it is far more effective and because of the intensity achievable with the industrial PB system. The high intensity achieved is due to the high output power of 1 kW which was applied in this work. This system uses air as the working gas and thus presents a sustainable process.

Furthermore, since the liquid samples of allergen with the same concentrations were used across the studies of RSS system (Chapter 5) and DBD system (Chapter 7), the antigenicity reduction efficacy between liquid-based plasma treatment and gaseous-based plasma was compared. At a high casein concentration, the antigenicity reduction of DBD treated casein (28.8%) was lower than SD treated casein (49.9%) and GD treated casein (91.9%). At a high concentration of whey, the α-lactalbumin antigenicity reduction of DBD treated whey (11.9%) was also lower than SD treated whey (49.5%) and GD treated whey (45.5%). In comparison to the results shown in Figure 5.10 and Figure 7.11, the overall gliadin antigenicity reduction of DBD treated gluten was lower than that achieved for SD treated gluten and GD treated gluten. It can be concluded that the in-package DBD treatment applied was less effective for milk-derived and wheat-derived allergenicity reduction by comparison with SD or GD RSS system. However, the in-package DBD as a gaseous-based dry plasma, could be applied for reducing the allergenicity of food allergens present in whole foods, dependent on the other quality characteristics of the whole food product. Air was the only working gas used in our study, however, there may be potential to enhance the results achievable by modulating the packaging gas to increase the RONS intensity.

It is clear that the protein structures, bonds and amino acid profiles influence the antigenicity modulation achievable. Therefore, the results of the relative susceptibility of amino acids resulting from plasma treatments were compared through a collation of the data across the different chapters and are shown in Tables 9.1, 9.2, 9.3, 9.4, 9.5, 9.6 and 9.7 for comparison. The high protein concentration samples (either 2 mg/ml for milk-derived allergen or 10 mg/ml for wheat-derived allergen) treated by either RSS plasma for 30 min or DBD plasma for 30 min or PB plasma for 10 min was chosen to facilitate the comparison across the plasma systems and the source proteins with their amino acid breakdown.

The amino acid contents that are shown in the top five rows of each table are considered as relatively susceptible to plasma treatment. The results showed that tyrosine, cystine, threonine and serine in the milk-derived allergens (casein and whey) are the most susceptible amino acids to the plasma treatments used (Table 9.1 and Table 9.2). The results showed that serine, aspartic acid, glycine, tyrosine, threonine and leucine are the most susceptible amino acids from wheat-derived allergens to plasma treatments (Table 9.3).

This indicates that aromatic amino acids (such as tyrosine) and sulphur-containing amino acids (such as cystine) are the most susceptible amino acids to plasma treatments. This is in good agreement with the results of R. Zhou et al. (2016). The amino acids containing a hydroxyl side chain (such as serine, threonine) were also susceptible to plasma treatments in this work. However, conversely, Takai et al. (2014) reported that there was no plasma-induced oxidation of serine or threonine when the individual amino acids were treated with a pin-discharge plasma using helium as the working gas. Combining and integrating the information from Tables 9.4, 9.5, 9.6 and 9.7, the results also showed that SD, GD, DBD and PB treatments can easily alter tyrosine and cystine. In addition, SD and GD can also easily alter methionine, threonine and serine. The susceptibility of amino acids to plasma treatment was different for the different plasma systems applied. The differences in plasma sources could generate various types and quantities of reactive species. The differences in types and quantities of reactive species may result in changes in the amino acid structure to various extents (Park et al., 2016). Cysteine, methionine, lysine, arginine, histidine and tryptophan are the common target amino acids of ROS in proteins (Takai et al., 2014; R. Zhou et al., 2016). The aromatic ring of Tyrosine has been reported to be easily nitrated by RNS in plasma (Lin et al., 2000; van der Vliet et al., 1994). R. Zhou et al. (2016) also reported that aromatic acids, such as phenylalanine and tyrosine, could be nitrated by RNS. However, Takai et al. (2014) observed that no nitration had occurred on the aromatic ring of tryptophan. It could be tryptophan has low reactivity to RNS. Leucine, isoleucine and valine were nitrated by RNS in plasma. In contrast, cysteine, methionine, proline, histidine, lysine, arginine, glutamine and glutamic acid were not modified via nitration by RNS. Hence, the generation of ROS or RNS rich plasma effluents could influence the susceptibility of specific amino acid structures to plasma treatment.

Table 9.1 Relative susceptibility of amino acids of casein resulting from different plasma treatments in descending order

	Spark	**Glow**	**DBD**	**PB**
1	Tyrosine	Tyrosine	Serine	Isoleucine
2	Cystine	Cystine	Isoleucine	Tyrosine
3	Methionine	Threonine	Histidine	Valine
4	Threonine	Methionine	Threonine	Proline
5	Serine	Phenylalanine	Cystine	Cystine
6	Proline	Serine	Arginine	Glutamic acid
7	Phenylalanine	Lysine	Lysine	Glycine
8	Histidine	Histidine	Tryptophan	Serine
9	Lysine	Proline	Proline	Tryptophan
10	Valine	Arginine	Leucine	Leucine
11	Alanine	Valine	Phenylalanine	Phenylalanine
12	Isoleucine	Leucine	Asparagine	Alanine
13	Arginine	Glutamic acid	Tyrosine	Lysine
14	Glutamic acid	Isoleucine	Glutamine	Histidine
15	Aspartic acid	Alanine	Glycine	Arginine
16	Leucine	Glycine	Alanine	Threonine
17	Glycine	Aspartic acid	Valine	Aspartic acid
18			Glutamic acid	Glutamine
19			Aspartic acid	Asparagine

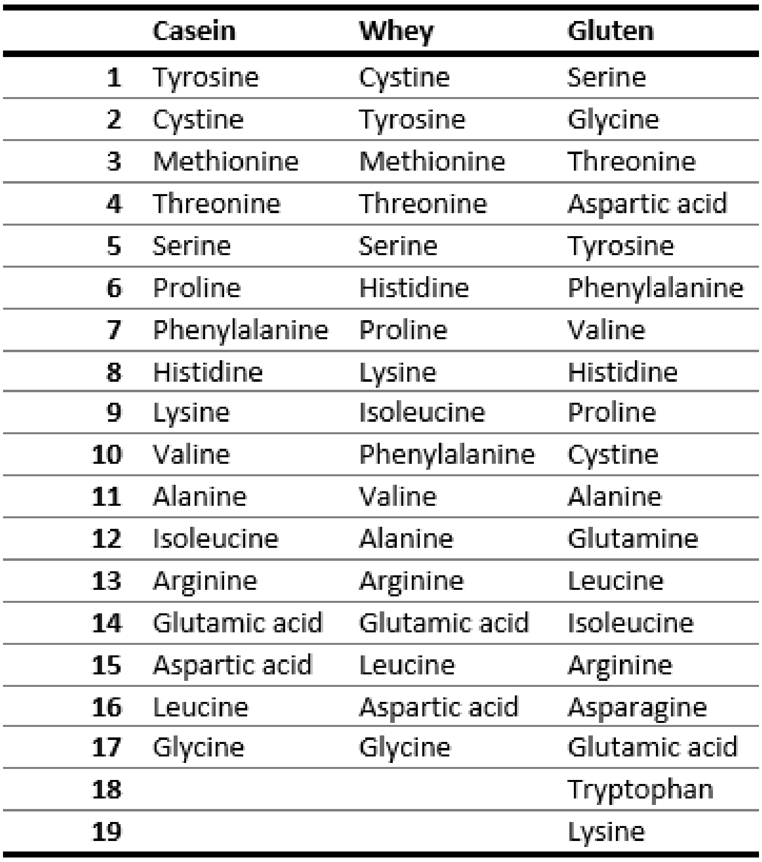
Table 9.2 Relative susceptibility of amino acids of whey resulting from different plasma treatments in descending order

	Spark	**Glow**	**DBD**	**PB**
1	Cystine	Cystine	Tyrosine	Threonine
2	Tyrosine	Tyrosine	Cystine	Serine
3	Methionine	Methionine	Histidine	Histidine
4	Threonine	Threonine	Methionine	Tyrosine
5	Serine	Serine	Arginine	Cystine
6	Histidine	Histidine	Isoleucine	Arginine
7	Proline	Lysine	Lysine	Valine
8	Lysine	Proline	Serine	Proline
9	Isoleucine	Isoleucine	Alanine	Methionine
10	Phenylalanine	Valine	Glutamine	Lysine
11	Valine	Phenylalanine	Valine	Alanine
12	Alanine	Arginine	Threonine	Isoleucine
13	Arginine	Alanine	Phenylalanine	Phenylalanine
14	Glutamic acid	Glutamic acid	Glycine	Tryptophan
15	Leucine	Aspartic acid	Proline	Glutamic acid
16	Aspartic acid	Leucine	Glutamic acid	Glycine
17	Glycine	Glycine	Asparagine	Glutamine
18			Aspartic acid	Asparagine
19			Leucine	Aspartic acid
20			Tryptophan	Leucine

Table 9.3 Relative susceptibility of amino acids of gluten resulting from different plasma treatments in descending order

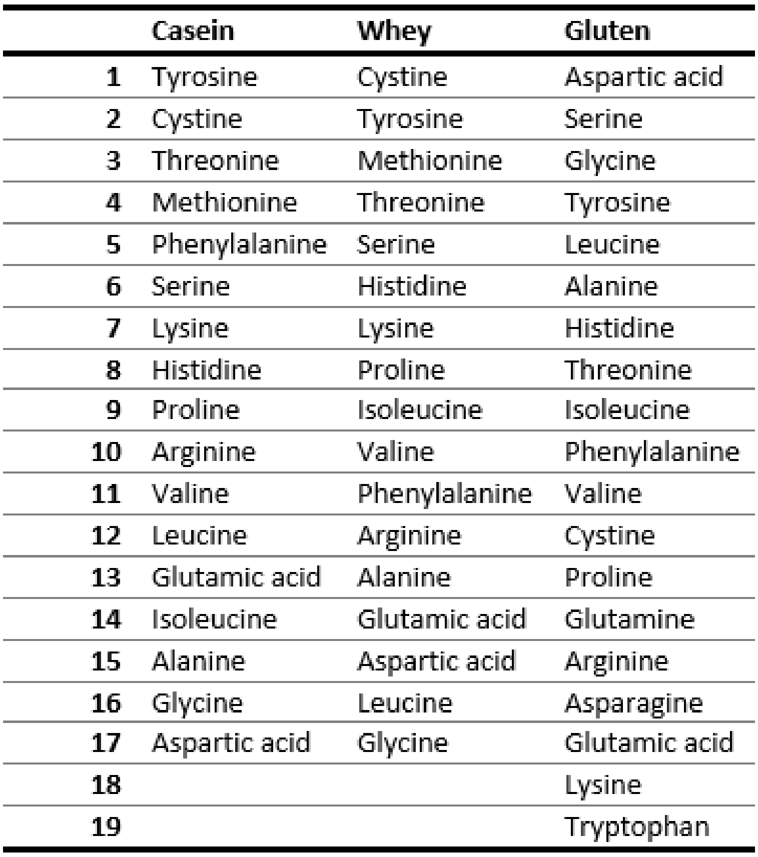
	Spark	**Glow**	**DBD**	**PB**
1	Serine	Aspartic acid	Aspartic acid	Tryptophan
2	Glycine	Serine	Tyrosine	Leucine
3	Threonine	Glycine	Glycine	Glutamine
4	Aspartic acid	Tyrosine	Histidine	Threonine
5	Tyrosine	Leucine	Cystine	Asparagine
6	Phenylalanine	Alanine	Alanine	Glycine
7	Valine	Histidine	Asparagine	Cystine
8	Histidine	Threonine	Isoleucine	Isoleucine
9	Proline	Isoleucine	Serine	Aspartic acid
10	Cystine	Phenylalanine	Threonine	Proline
11	Alanine	Valine	Valine	Lysine
12	Glutamine	Cystine	Phenylalanine	Alanine
13	Leucine	Proline	Proline	Valine
14	Isoleucine	Glutamine	Glutamine	Phenylalanine
15	Arginine	Arginine	Arginine	Glutamic acid
16	Asparagine	Asparagine	Tryptophan	Arginine
17	Glutamic acid	Glutamic acid	Leucine	Serine
18	Tryptophan	Lysine	Glutamic acid	Tyrosine
19	Lysine	Tryptophan	Lysine	Histidine

Table 9.4 Relative susceptibility of amino acids of different proteins (casein, whey and gluten) resulting from spark discharge treatment in descending order



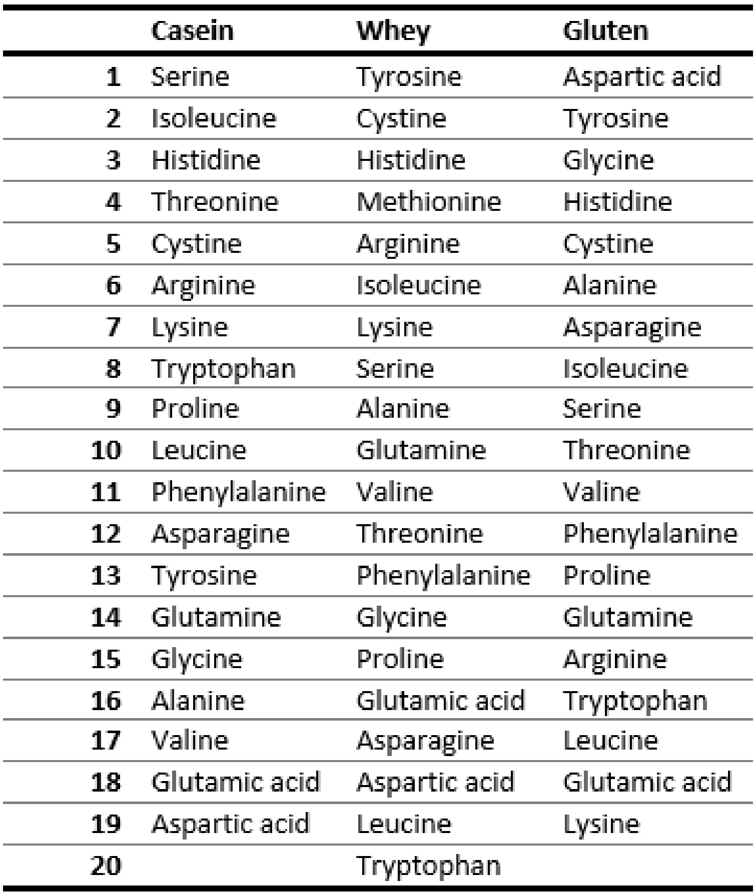
	Casein	Whey	Gluten
1	Tyrosine	Cystine	Serine
2	Cystine	Tyrosine	Glycine
3	Methionine	Methionine	Threonine
4	Threonine	Threonine	Aspartic acid
5	Serine	Serine	Tyrosine
6	Proline	Histidine	Phenylalanine
7	Phenylalanine	Proline	Valine
8	Histidine	Lysine	Histidine
9	Lysine	Isoleucine	Proline
10	Valine	Phenylalanine	Cystine
11	Alanine	Valine	Alanine
12	Isoleucine	Alanine	Glutamine
13	Arginine	Arginine	Leucine
14	Glutamic acid	Glutamic acid	Isoleucine
15	Aspartic acid	Leucine	Arginine
16	Leucine	Aspartic acid	Asparagine
17	Glycine	Glycine	Glutamic acid
18			Tryptophan
19			Lysine

Table 9.5 Relative susceptibility of amino acids of different proteins (casein, whey and gluten) resulting from glow discharge treatment in descending order



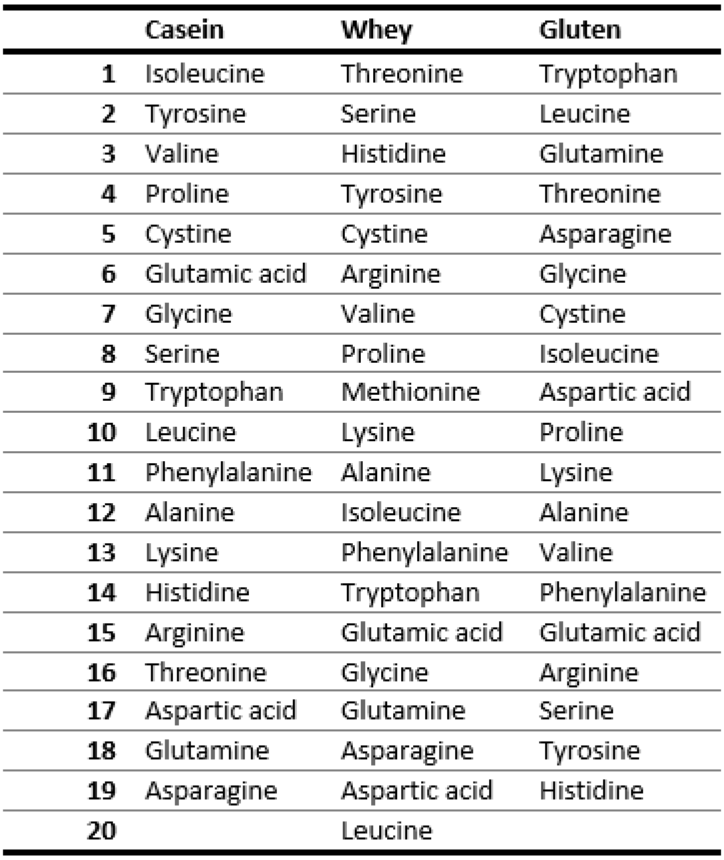
	Casein	Whey	Gluten
1	Tyrosine	Cystine	Aspartic acid
2	Cystine	Tyrosine	Serine
3	Threonine	Methionine	Glycine
4	Methionine	Threonine	Tyrosine
5	Phenylalanine	Serine	Leucine
6	Serine	Histidine	Alanine
7	Lysine	Lysine	Histidine
8	Histidine	Proline	Threonine
9	Proline	Isoleucine	Isoleucine
10	Arginine	Valine	Phenylalanine
11	Valine	Phenylalanine	Valine
12	Leucine	Arginine	Cystine
13	Glutamic acid	Alanine	Proline
14	Isoleucine	Glutamic acid	Glutamine
15	Alanine	Aspartic acid	Arginine
16	Glycine	Leucine	Asparagine
17	Aspartic acid	Glycine	Glutamic acid
18			Lysine
19			Tryptophan

Table 9.6 Relative susceptibility of amino acids of different proteins (casein, whey and gluten) resulting from dielectric barrier discharge plasma treatment in descending order



	Casein	Whey	Gluten
1	Serine	Tyrosine	Aspartic acid
2	Isoleucine	Cystine	Tyrosine
3	Histidine	Histidine	Glycine
4	Threonine	Methionine	Histidine
5	Cystine	Arginine	Cystine
6	Arginine	Isoleucine	Alanine
7	Lysine	Lysine	Asparagine
8	Tryptophan	Serine	Isoleucine
9	Proline	Alanine	Serine
10	Leucine	Glutamine	Threonine
11	Phenylalanine	Valine	Valine
12	Asparagine	Threonine	Phenylalanine
13	Tyrosine	Phenylalanine	Proline
14	Glutamine	Glycine	Glutamine
15	Glycine	Proline	Arginine
16	Alanine	Glutamic acid	Tryptophan
17	Valine	Asparagine	Leucine
18	Glutamic acid	Aspartic acid	Glutamic acid
19	Aspartic acid	Leucine	Lysine
20		Tryptophan	

Table 9.7 Relative susceptibility of amino acids of different proteins (casein, whey and gluten) resulting from plasma brush treatment in descending order



	Casein	Whey	Gluten
1	Isoleucine	Threonine	Tryptophan
2	Tyrosine	Serine	Leucine
3	Valine	Histidine	Glutamine
4	Proline	Tyrosine	Threonine
5	Cystine	Cystine	Asparagine
6	Glutamic acid	Arginine	Glycine
7	Glycine	Valine	Cystine
8	Serine	Proline	Isoleucine
9	Tryptophan	Methionine	Aspartic acid
10	Leucine	Lysine	Proline
11	Phenylalanine	Alanine	Lysine
12	Alanine	Isoleucine	Alanine
13	Lysine	Phenylalanine	Valine
14	Histidine	Tryptophan	Phenylalanine
15	Arginine	Glutamic acid	Glutamic acid
16	Threonine	Glycine	Arginine
17	Aspartic acid	Glutamine	Serine
18	Glutamine	Asparagine	Tyrosine
19	Asparagine	Aspartic acid	Histidine
20		Leucine	

In summary, atmospheric cold plasma treatment is effective in mitigating the antigenicity associated with the specific food allergens and has significant potential to mitigate allergen cross contamination in food processing environments. The allergen mitigation studies demonstrated significant correlation between the plasma performance, plasma design, allergen composition and allergen concentration. The extrinsic control parameters play an important role in allergen mitigation efficacy by producing various reactive species. The reactive species generated from plasma can induce certain interactions with proteins and change their functional properties (Takai et al., 2012; Tolouie et al., 2018). For the ACP studied here, there are multiple mechanisms that have been explained which can affect food allergen reactivity, but the underlying concept is modification to the IgE-binding epitopes. Conformational epitopes can be altered by the aggregation, crosslinking of proteins or changing of the secondary structure via folding and unfolding. Linear epitopes can be fragmented or masked by modification. From the microscopic level of allergen epitopes, the side chains of amino acids, which are present in binding sites, are modified by plasma treatment. It was found that tyrosine and cystine are the most susceptible to plasma treatment. The food allergens that are rich in these amino acids could provide a good template for plasma processing induced modification. The relationship between protein conformation and allergen reactivity is a principal concept that can be utilised to advance and develop scalable and suitable ACP systems for food allergen reduction, with fundamental insights provided here that demonstrate the limitations as well as the benefits which are dependent on the specific proteins.

9.2 Conclusions

Overall a number of conclusions regarding the use of atmospheric cold plasma (ACP) for allergen mitigation and its applications in foods and in food processing environments can be made from this study:

- The plasma system was characterised by plasma and liquid diagnostics to better understand the physical and chemical processes within plasma. The performance of ACP is correlated with the generation of reactive oxygen and nitrogen species (RONS) in gas and liquid phases, which can be modulated by using different plasma configurations. The extrinsic control parameters, such as voltage and frequency, also played an important role in ACP performance.
- Atmospheric cold plasma (ACP) treatment is effective in mitigating the antigenicity associated with specific food allergens and has significant potential to attenuate allergen cross contamination in food processing environments. ACP can alter the conformational and linear epitopes of allergens and further change their antigenicity.
- Allergen residue concentration and plasma treatment duration play a critical role in the efficacy of antigenicity reduction.
- To explore a suitable ACP system for food allergen reduction, different modes of ACP exposure, such as direct or indirect treatments and liquid-based or gaseous-based plasmas, were observed for allergen mitigation. The different ACP systems used in our study have significant reductions in antigenicity but differences in the effectiveness and efficiency of allergen mitigation. In the case of liquid-mediated plasma treatment,

direct treatment was more effective than indirect for antigenicity reduction.

- The differences in protein structure and composition affect antigenicity reduction. The antigenicity of casein, α-lactalbumin and gliadin decreased after plasma treatment. However, β-lactoglobulin had a potential risk of antigenicity enhancement by ACP associated with its native dimeric form.
- The analysis of amino acid contents provides fundamental insight for plasma processing mitigation of antigenicity where susceptible profiles have been identified.
- Atmospheric cold plasma can be performed to achieve the antigenicity reduction in different modes of applications to foods or in food processing environments. Allergen residues, suspended in liquid or dried on surfaces, can be mitigated by ACP.

9.3 Future recommendations

Atmospheric cold plasma (ACP) is a promising tool for attenuation of food allergens in food processing. Overall, the improved knowledge of ACP interactions with allergens represents a crucial first step for understanding the mechanism of action of plasma with allergens. The universality of ACP applied on allergen targets in term of protein structures and compositions is critical to observe due to the differences in susceptibility of allergens to ACP. Moreover, there is a potential risk of allergenicity enhancement by ACP. In certain circumstances, cold plasma treatment could enhance the allergenicity of some complex allergens by exposing the hidden epitopes via unfolding the proteins. These changes are highly complex and not easily predictable. Hence, this implies the importance of assessing the universality of ACP applied on allergen targets.

This study focused on the main allergen proteins for milk and wheat. The results showed antigenicity reduction when ACP was applied to allergen targets. Hence, ACP has great potential for use in foods and in food processing environments. However, one key protein where the antigenicity actually increased showed there is an inherent risk. This may pose a risk for any foods that contain these proteins where antigenicity may actually be increased. The populations that previously were not susceptible or not allergic to these food products might then fall into the category of being allergic because of the increased antigenicity after treatment. However, there is a limitation, where only milk and wheat proteins were studied comprehensively. All of the other categories of food allergens would also need to be explored as comprehensively to further understand where the more successful situations are going to be, but also fully understand the limitations which may emerge. However, we have provided some fundamental insights into this gap by investigating the proteomic profiles of the allergens in milk and wheat. The analysis of proteomic profiles, particularly amino acid contents in allergens, is comparable across the different food groups that have yet to be investigated.

To provide opportunity to apply ACP widely in food processing and environmental decontamination, different modes of ACP exposure should be developed for allergen mitigation. The present study has explored direct and indirect treatments as well as liquid-based and gaseous-based plasma systems. Each of the modes of ACP exposure has its pros and cons.

The reactive species specificity (RSS) system is a direct liquid-based plasma system that possesses high mitigation efficacy of ACP against food allergens. It could be applied in whole food or in a liquid mediated application to food processing environments. The RSS system is a small lab-based plasma device. Scaling-up the RSS system is required for large volumes of treatment needed for food processing as well as maintaining the capability of allergen mitigation.

The plasma activated water (PAW) treatment is an indirect treatment that is effective for allergen attenuation. However, it was not adequately efficient for the allergen attenuation using the current configuration. Despite this, the removal of allergenic proteins from food-contact surfaces using PAW should be further explored for the cleaning in place processes in food processing.

The dielectric barrier discharge (DBD) in-package system is a direct gaseous-based plasma that was effective for allergen attenuation. This approach has been successfully scaled up for bio-decontamination previously (Ziuzina et al., 2015), but would require process input optimisation specific to allergen management for each food commodity to retain physicochemical quality characteristics etc.

The plasma brush (PB) system is a direct gaseous-based plasma that was effective for allergen attenuation present on food-contact surfaces. It is designed for cleaning or polishing of industrial surfaces. The present study investigated the allergen attenuation on stainless-steel surface. The study should be extended to explore compatibility with different food-contact surfaces, such as textured plastic, maple wood, rubber, ceramics, or glass, in addition to combining the dry plasma process with the liquid mediated plasma process to provide a scalable clean in place alternative. In the RSS studies, we found antigenicity reduction in liquid mediated application. We also found the PB system had mitigation efficacy against dried allergen residues on surfaces. Combining both studies by hydrating the allergen residues on surfaces, it may provide an enhanced effect due to the liquid mediated effects that have been studied previously. It is also possible that there would be no enhanced effect on allergen mitigation due to the dissipation of liquid during plasma treatment. Hence, this needs to be explored to understand the mechanisms in a moist environment. The compatibility of surface materials needs to be explored to prevent adverse effects on surfaces.

It is important to assess the adaptability of the plasma system for integration to food processing environments. Combining and tailoring this knowledge to the plasma system requires validation industrially in food processing and environmental decontamination.

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List of Publications

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Ng, S. W., Tsoukou, E., Chaple, S., Boehm, D., Bourke, P. Plasma Treatment of Liquids. Book chapter in reference module in Food Science 2021.

Ng, S. W., Lu, P., Rulikowska, A., Boehm, D., O'Neill, G., & Bourke, P. (2020). The effect of atmospheric cold plasma treatment on the antigenic properties of bovine milk casein and whey proteins. *Food Chem*, 128283. doi: 10.1016/j.foodchem.2020.128283

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He, Z., Liu, K., Scally, L., Manaloto, E., Gunes, S., **Ng, S. W.**, ... & Tian, F. (2020). Cold Atmospheric Plasma Stimulates Clathrin-Dependent Endocytosis to Repair Oxidised Membrane and Enhance Uptake of Nanomaterial in Glioblastoma Multiforme Cells. *Sci Rep* 10, 6985. doi: 10.1038/s41598-020-63732-y

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Conferences

Ng, S. W., Boehm, D., Lu, P., Bourke, P. The effect of atmospheric cold plasma treatment on the allergenic properties of cereal allergens. Oral presentation at 8th International Conference on Plasma Medicine, 3-6 August 2021 (online)

Ng, S. W., Lu, P., O'Neill, G., Boehm, D., Bourke, P. The effect of atmospheric cold plasma treatment on the allergenic properties of bovine milk casein and whey proteins. Oral presentation at 49th Annual Food Science & Technology Conference, 15 December 2020 (online)

Ng, S. W., Lu, P., O'Neill, G., Boehm, D., Bourke, P. The effect of atmospheric cold plasma treatment on the allergenic properties of bovine milk casein and whey proteins. Poster presentation at SFI Summit 2020, 5-6 November 2020 (online)

Ng, S. W., Lu, P., O'Neill, G., Boehm, D., Bourke, P. The effect of atmospheric cold plasma treatment on the allergenic properties of bovine milk casein and whey proteins. Poster presentation at UCD Institute of Food and Health Research Day, 15 October 2020 (online)

Ng, S. W., Lu, P., O'Neill, G., Boehm, D., Bourke, P. The effect of atmospheric cold plasma treatment on the allergenic properties of bovine milk casein and whey proteins. Poster presentation at 11th NIZO Dairy Conference Milk Protein Functionality, 8-11 October 2019 (Papendal, The Netherlands)

Ng, S. W., Boehm, D., Lu, P., O’Neill, G., Bourke, P. The effect of atmospheric cold plasma treatment on the allergenic properties of bovine milk casein and whey proteins. Oral presentation at Frontiers in Redox Biology and Medicine – 8th Young Professionals Workshop on Plasma Medicine, 5-8 August 2019 (Greifswald, Germany)

Ng, S. W., Lu, P., Boehm, D., Han, L., Bourke, P. Determining the effect of atmospheric cold plasma on the allergenic properties of bovine milk casein. Oral presentation at Conference on Cold Plasma Sources and Applications 2018, 19-23 November 2018 (Ypres, Belgium)

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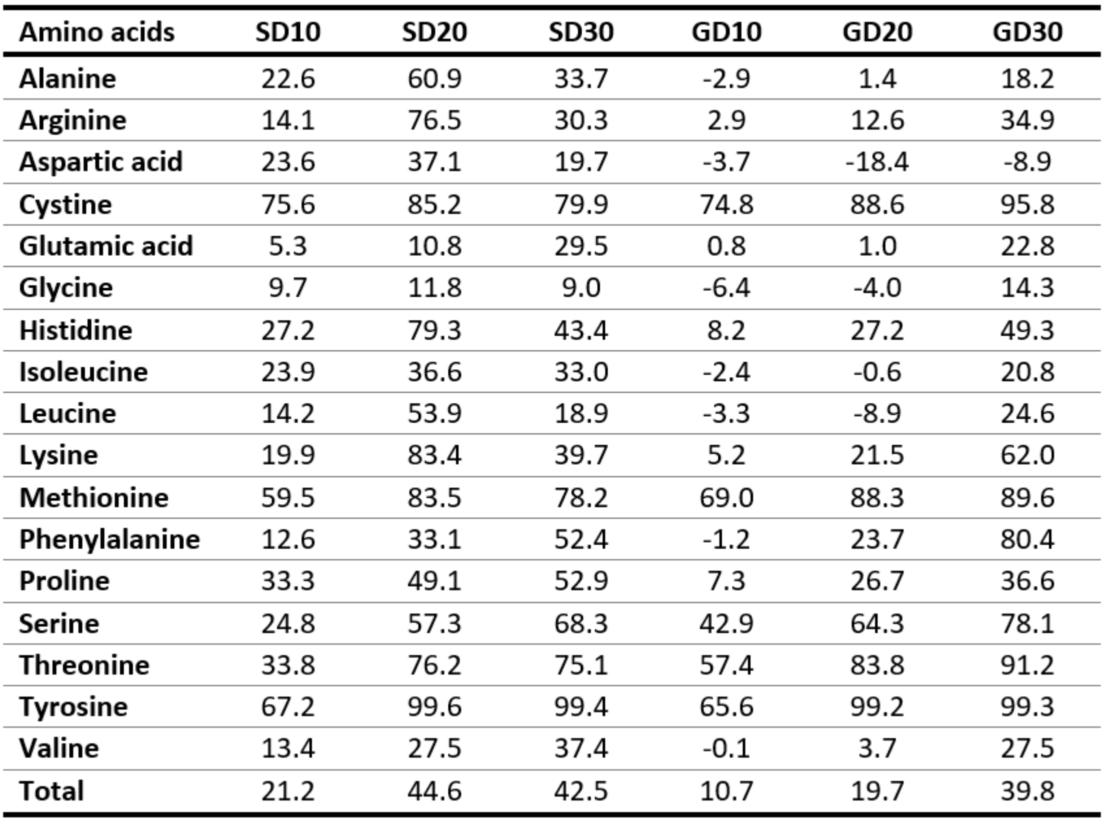
Appendices

The following appendices are not a new data. They are from a collation of the data in amino acid profile analysis across different chapter and are evaluated using the formula as below.

The reduction of amino acid contents of protein in percentage were calculated as (untreated amino acid content – treated amino acid content)/untreated amino acid content * 100, shown in Appendix 1 to Appendix 10.

The standard deviation of amino acid contents of protein in g amino acid/ 100 g protein were shown in Appendix 11 to Appendix 14.

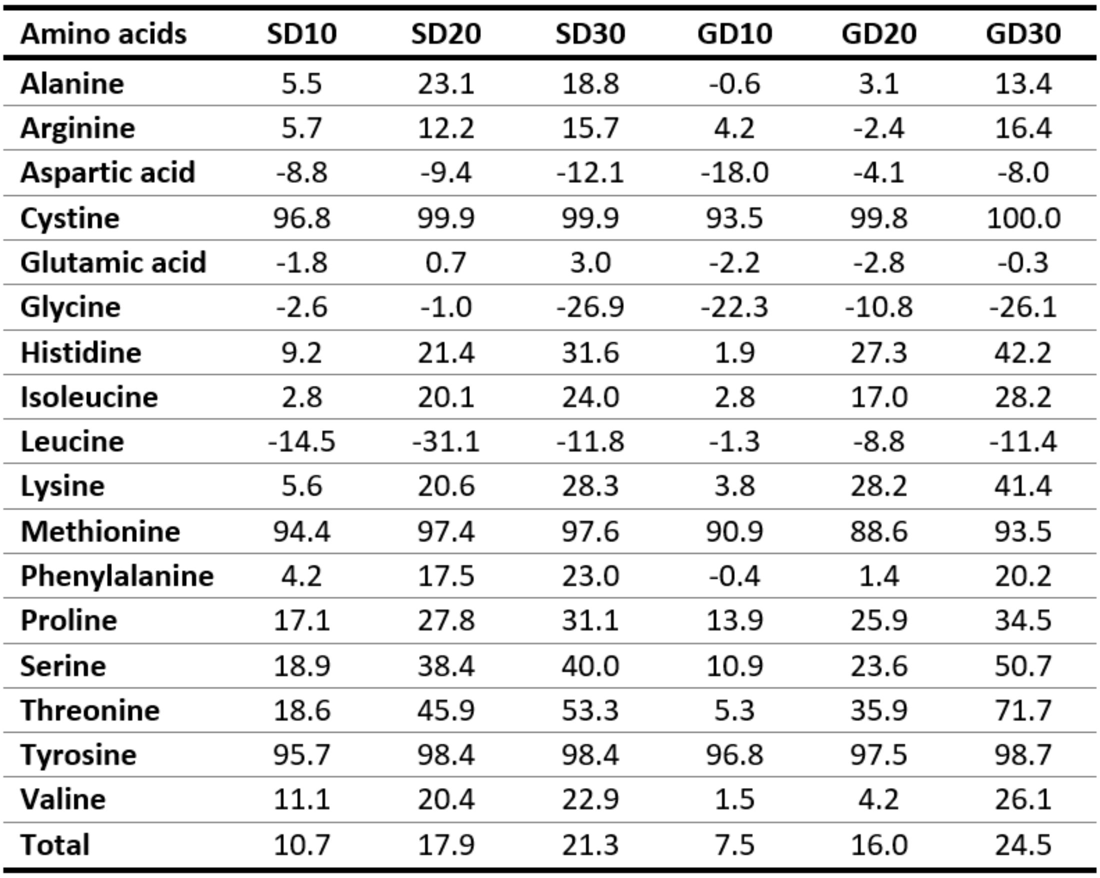
Appendix 1 Percentage reduction of amino acid contents of casein treated by spark discharge (SD) and glow discharge (GD) treatments at different treatment times (10, 20, 30 min) (%)



Amino acids	**SD10**	**SD20**	**SD30**	**GD10**	**GD20**	**GD30**
Alanine	22.6	60.9	33.7	-2.9	1.4	18.2
Arginine	14.1	76.5	30.3	2.9	12.6	34.9
Aspartic acid	23.6	37.1	19.7	-3.7	-18.4	-8.9
Cystine	75.6	85.2	79.9	74.8	88.6	95.8
Glutamic acid	5.3	10.8	29.5	0.8	1.0	22.8
Glycine	9.7	11.8	9.0	-6.4	-4.0	14.3
Histidine	27.2	79.3	43.4	8.2	27.2	49.3
Isoleucine	23.9	36.6	33.0	-2.4	-0.6	20.8
Leucine	14.2	53.9	18.9	-3.3	-8.9	24.6
Lysine	19.9	83.4	39.7	5.2	21.5	62.0
Methionine	59.5	83.5	78.2	69.0	88.3	89.6
Phenylalanine	12.6	33.1	52.4	-1.2	23.7	80.4
Proline	33.3	49.1	52.9	7.3	26.7	36.6
Serine	24.8	57.3	68.3	42.9	64.3	78.1
Threonine	33.8	76.2	75.1	57.4	83.8	91.2
Tyrosine	67.2	99.6	99.4	65.6	99.2	99.3
Valine	13.4	27.5	37.4	-0.1	3.7	27.5
Total	21.2	44.6	42.5	10.7	19.7	39.8

(mean of triplicate measurements from two independent experiments (n=6))

Appendix 2 Percentage reduction of amino acid contents of whey treated by spark discharge (SD) and glow discharge (GD) treatments at different treatment times (10, 20, 30 min) (%)



Amino acids	SD10	SD20	SD30	GD10	GD20	GD30
Alanine	5.5	23.1	18.8	-0.6	3.1	13.4
Arginine	5.7	12.2	15.7	4.2	-2.4	16.4
Aspartic acid	-8.8	-9.4	-12.1	-18.0	-4.1	-8.0
Cystine	96.8	99.9	99.9	93.5	99.8	100.0
Glutamic acid	-1.8	0.7	3.0	-2.2	-2.8	-0.3
Glycine	-2.6	-1.0	-26.9	-22.3	-10.8	-26.1
Histidine	9.2	21.4	31.6	1.9	27.3	42.2
Isoleucine	2.8	20.1	24.0	2.8	17.0	28.2
Leucine	-14.5	-31.1	-11.8	-1.3	-8.8	-11.4
Lysine	5.6	20.6	28.3	3.8	28.2	41.4
Methionine	94.4	97.4	97.6	90.9	88.6	93.5
Phenylalanine	4.2	17.5	23.0	-0.4	1.4	20.2
Proline	17.1	27.8	31.1	13.9	25.9	34.5
Serine	18.9	38.4	40.0	10.9	23.6	50.7
Threonine	18.6	45.9	53.3	5.3	35.9	71.7
Tyrosine	95.7	98.4	98.4	96.8	97.5	98.7
Valine	11.1	20.4	22.9	1.5	4.2	26.1
Total	10.7	17.9	21.3	7.5	16.0	24.5

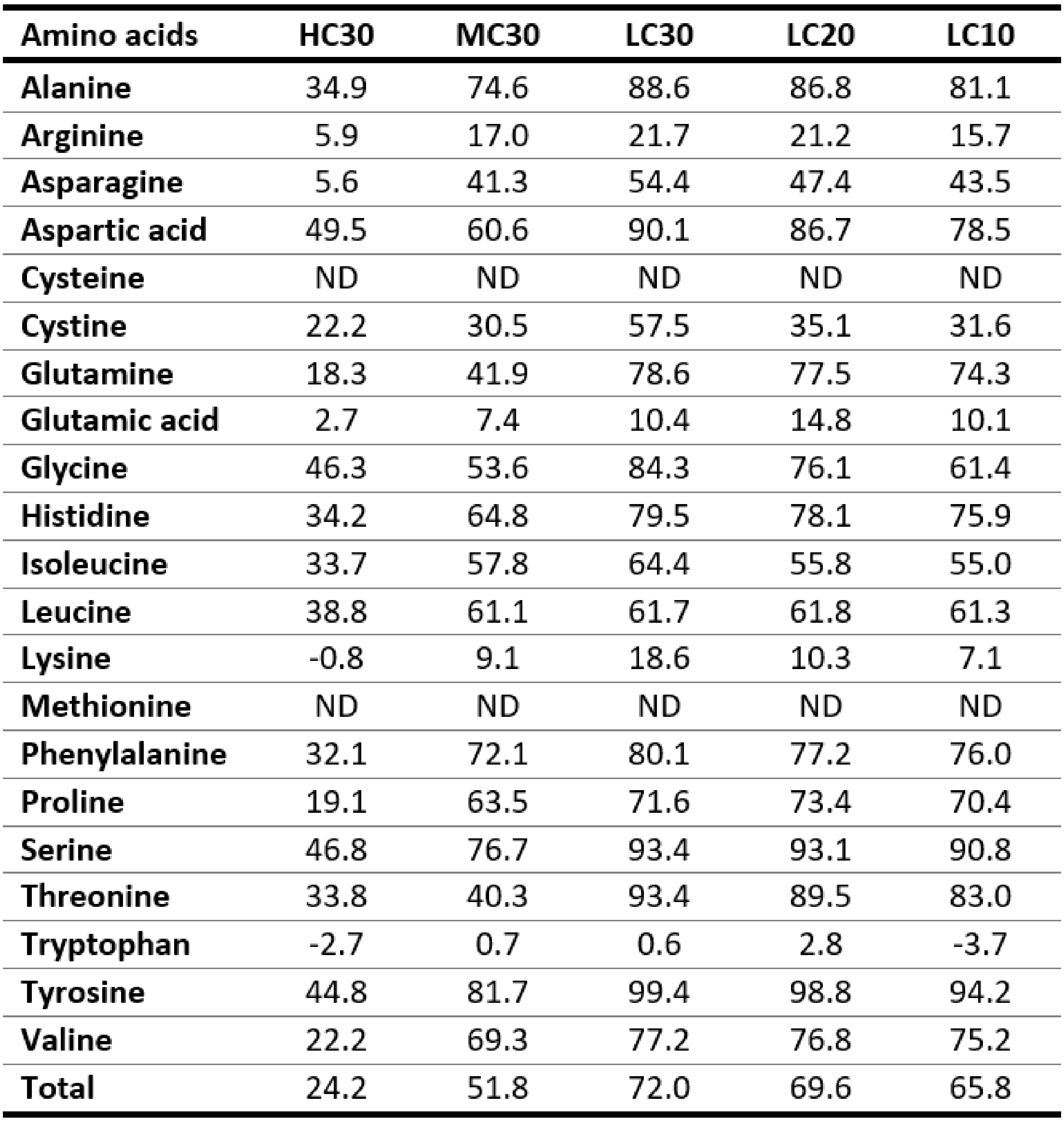
(mean of triplicate measurements from two independent experiments (n=6))

Appendix 3 Percentage reduction of amino acid contents of gluten treated by spark discharge treatment with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (%)

Amino acids	HC30	MC30	LC30	LC20	LC10
Alanine	25.7	37.0	90.7	87.0	73.5
Arginine	8.8	12.7	25.2	23.2	13.7
Asparagine	5.8	41.4	59.2	51.0	31.3
Aspartic acid	50.2	59.3	91.6	87.1	64.7
Cysteine	ND	ND	ND	ND	ND
Cystine	27.9	32.1	35.0	29.8	26.3
Glutamine	24.7	43.2	62.0	56.4	41.7
Glutamic acid	4.1	8.5	22.3	11.0	11.4
Glycine	57.2	71.7	75.5	54.8	51.3
Histidine	33.7	67.0	78.3	75.0	67.7
Isoleucine	20.7	49.3	67.4	63.6	46.9
Leucine	21.2	60.4	80.7	61.9	42.0
Lysine	-2.7	7.6	12.8	12.8	9.9
Methionine	ND	ND	ND	ND	ND
Phenylalanine	38.2	68.0	94.9	96.5	64.5
Proline	29.0	61.1	79.9	71.7	58.2
Serine	75.7	87.7	94.1	86.4	66.4
Threonine	54.4	72.7	83.9	87.0	65.7
Tryptophan	-1.0	-0.6	1.8	-0.7	2.2
Tyrosine	41.3	67.0	97.5	97.0	93.2
Valine	34.1	60.1	83.3	79.5	63.2
Total	28.8	50.7	71.4	65.1	49.8

(mean of triplicate measurements from two independent experiments (n=6))

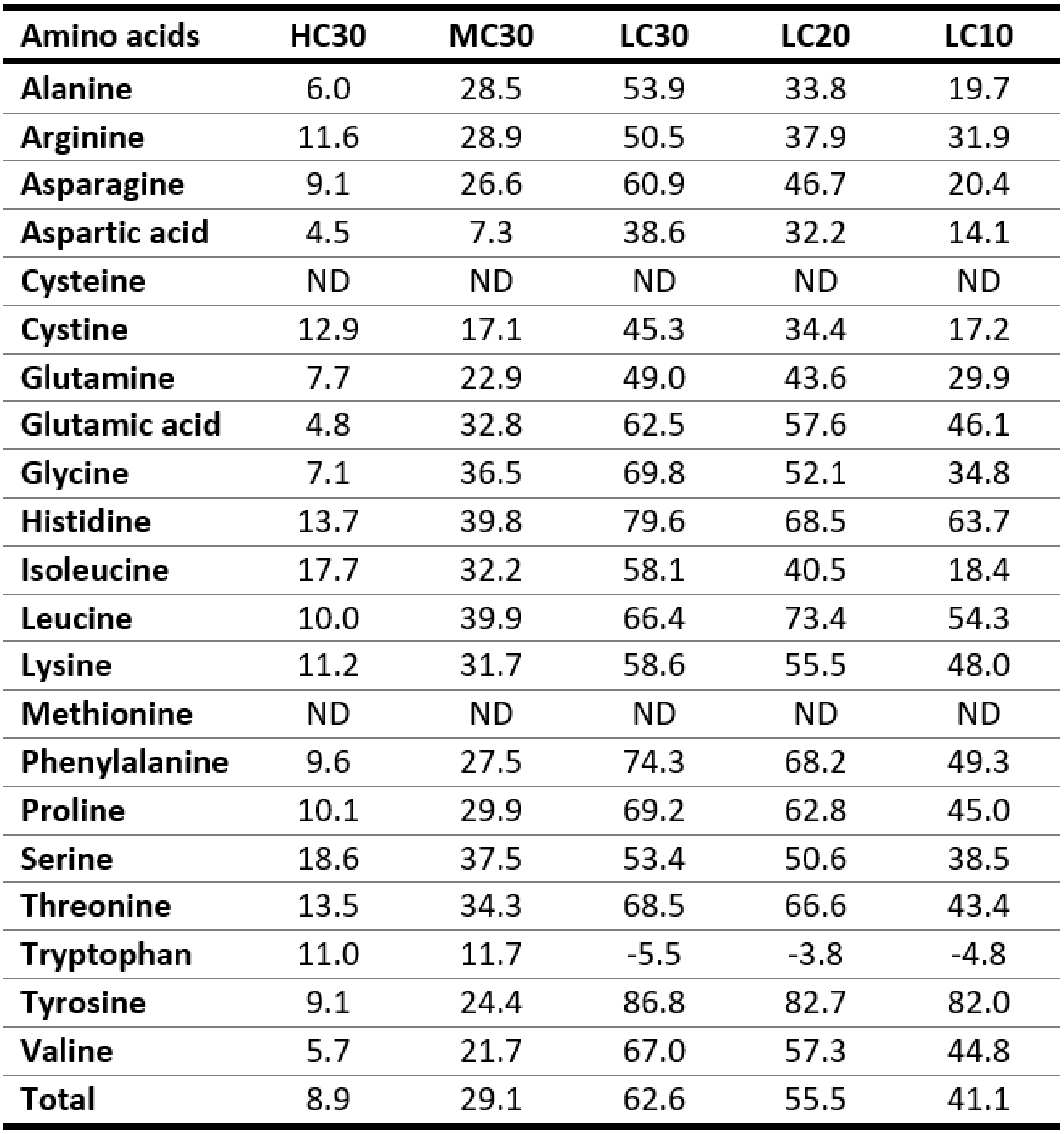
Appendix 4 Percentage reduction of amino acid contents of gluten treated by glow discharge treatment with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (%)



Amino acids	HC30	MC30	LC30	LC20	LC10
Alanine	34.9	74.6	88.6	86.8	81.1
Arginine	5.9	17.0	21.7	21.2	15.7
Asparagine	5.6	41.3	54.4	47.4	43.5
Aspartic acid	49.5	60.6	90.1	86.7	78.5
Cysteine	ND	ND	ND	ND	ND
Cystine	22.2	30.5	57.5	35.1	31.6
Glutamine	18.3	41.9	78.6	77.5	74.3
Glutamic acid	2.7	7.4	10.4	14.8	10.1
Glycine	46.3	53.6	84.3	76.1	61.4
Histidine	34.2	64.8	79.5	78.1	75.9
Isoleucine	33.7	57.8	64.4	55.8	55.0
Leucine	38.8	61.1	61.7	61.8	61.3
Lysine	-0.8	9.1	18.6	10.3	7.1
Methionine	ND	ND	ND	ND	ND
Phenylalanine	32.1	72.1	80.1	77.2	76.0
Proline	19.1	63.5	71.6	73.4	70.4
Serine	46.8	76.7	93.4	93.1	90.8
Threonine	33.8	40.3	93.4	89.5	83.0
Tryptophan	-2.7	0.7	0.6	2.8	-3.7
Tyrosine	44.8	81.7	99.4	98.8	94.2
Valine	22.2	69.3	77.2	76.8	75.2
Total	24.2	51.8	72.0	69.6	65.8

(mean of triplicate measurements from two independent experiments (n=6))

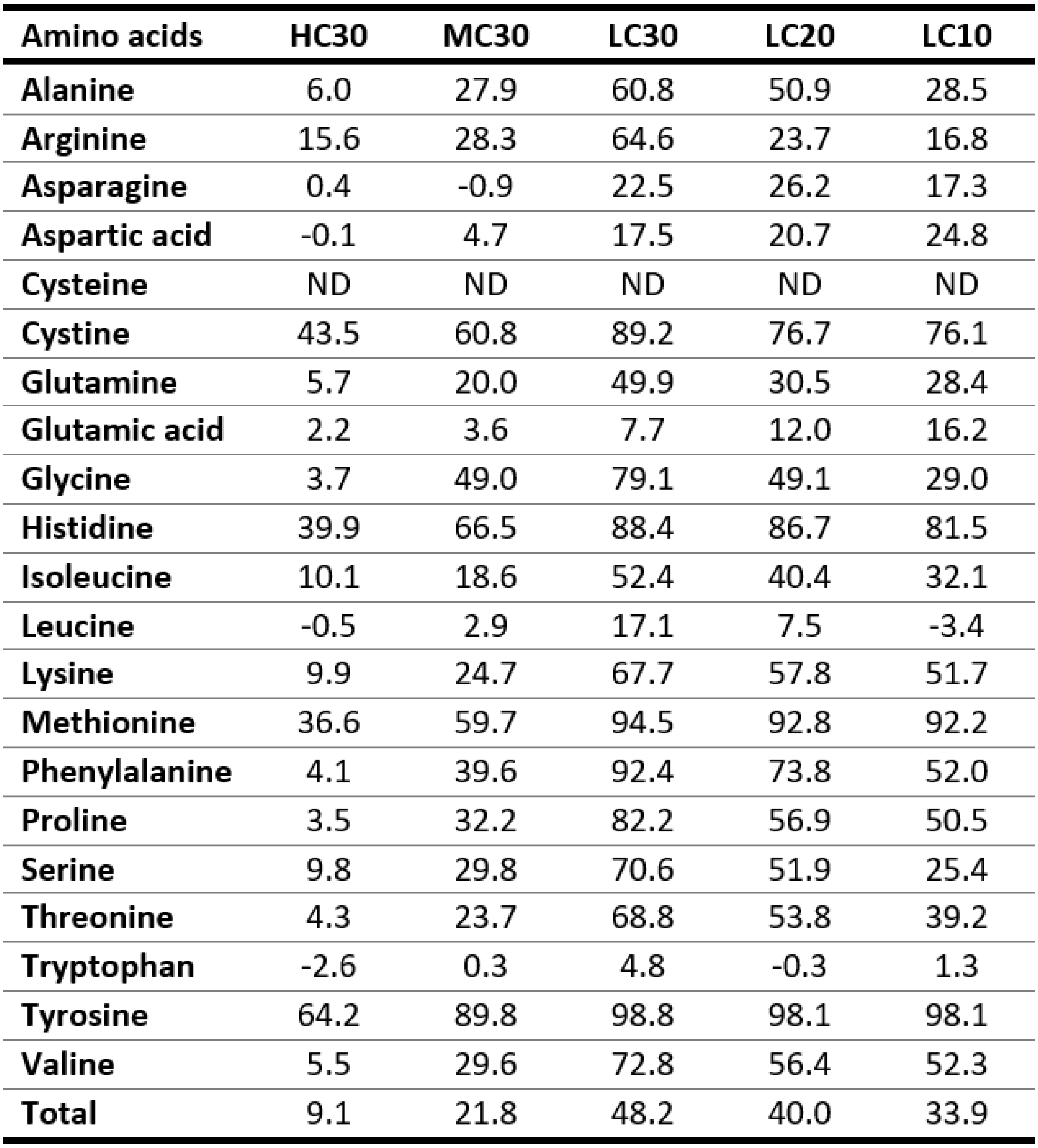
Appendix 5 Percentage reduction of amino acid contents of casein treated by dielectric barrier discharge treatment with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (%)



Amino acids	HC30	MC30	LC30	LC20	LC10
Alanine	6.0	28.5	53.9	33.8	19.7
Arginine	11.6	28.9	50.5	37.9	31.9
Asparagine	9.1	26.6	60.9	46.7	20.4
Aspartic acid	4.5	7.3	38.6	32.2	14.1
Cysteine	ND	ND	ND	ND	ND
Cystine	12.9	17.1	45.3	34.4	17.2
Glutamine	7.7	22.9	49.0	43.6	29.9
Glutamic acid	4.8	32.8	62.5	57.6	46.1
Glycine	7.1	36.5	69.8	52.1	34.8
Histidine	13.7	39.8	79.6	68.5	63.7
Isoleucine	17.7	32.2	58.1	40.5	18.4
Leucine	10.0	39.9	66.4	73.4	54.3
Lysine	11.2	31.7	58.6	55.5	48.0
Methionine	ND	ND	ND	ND	ND
Phenylalanine	9.6	27.5	74.3	68.2	49.3
Proline	10.1	29.9	69.2	62.8	45.0
Serine	18.6	37.5	53.4	50.6	38.5
Threonine	13.5	34.3	68.5	66.6	43.4
Tryptophan	11.0	11.7	-5.5	-3.8	-4.8
Tyrosine	9.1	24.4	86.8	82.7	82.0
Valine	5.7	21.7	67.0	57.3	44.8
Total	8.9	29.1	62.6	55.5	41.1

(mean of triplicate measurements from two independent experiments (n=6))

Appendix 6 Percentage reduction of amino acid contents of whey treated by dielectric barrier discharge treatment with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (%)



Amino acids	HC30	MC30	LC30	LC20	LC10
Alanine	6.0	27.9	60.8	50.9	28.5
Arginine	15.6	28.3	64.6	23.7	16.8
Asparagine	0.4	-0.9	22.5	26.2	17.3
Aspartic acid	-0.1	4.7	17.5	20.7	24.8
Cysteine	ND	ND	ND	ND	ND
Cystine	43.5	60.8	89.2	76.7	76.1
Glutamine	5.7	20.0	49.9	30.5	28.4
Glutamic acid	2.2	3.6	7.7	12.0	16.2
Glycine	3.7	49.0	79.1	49.1	29.0
Histidine	39.9	66.5	88.4	86.7	81.5
Isoleucine	10.1	18.6	52.4	40.4	32.1
Leucine	-0.5	2.9	17.1	7.5	-3.4
Lysine	9.9	24.7	67.7	57.8	51.7
Methionine	36.6	59.7	94.5	92.8	92.2
Phenylalanine	4.1	39.6	92.4	73.8	52.0
Proline	3.5	32.2	82.2	56.9	50.5
Serine	9.8	29.8	70.6	51.9	25.4
Threonine	4.3	23.7	68.8	53.8	39.2
Tryptophan	-2.6	0.3	4.8	-0.3	1.3
Tyrosine	64.2	89.8	98.8	98.1	98.1
Valine	5.5	29.6	72.8	56.4	52.3
Total	9.1	21.8	48.2	40.0	33.9

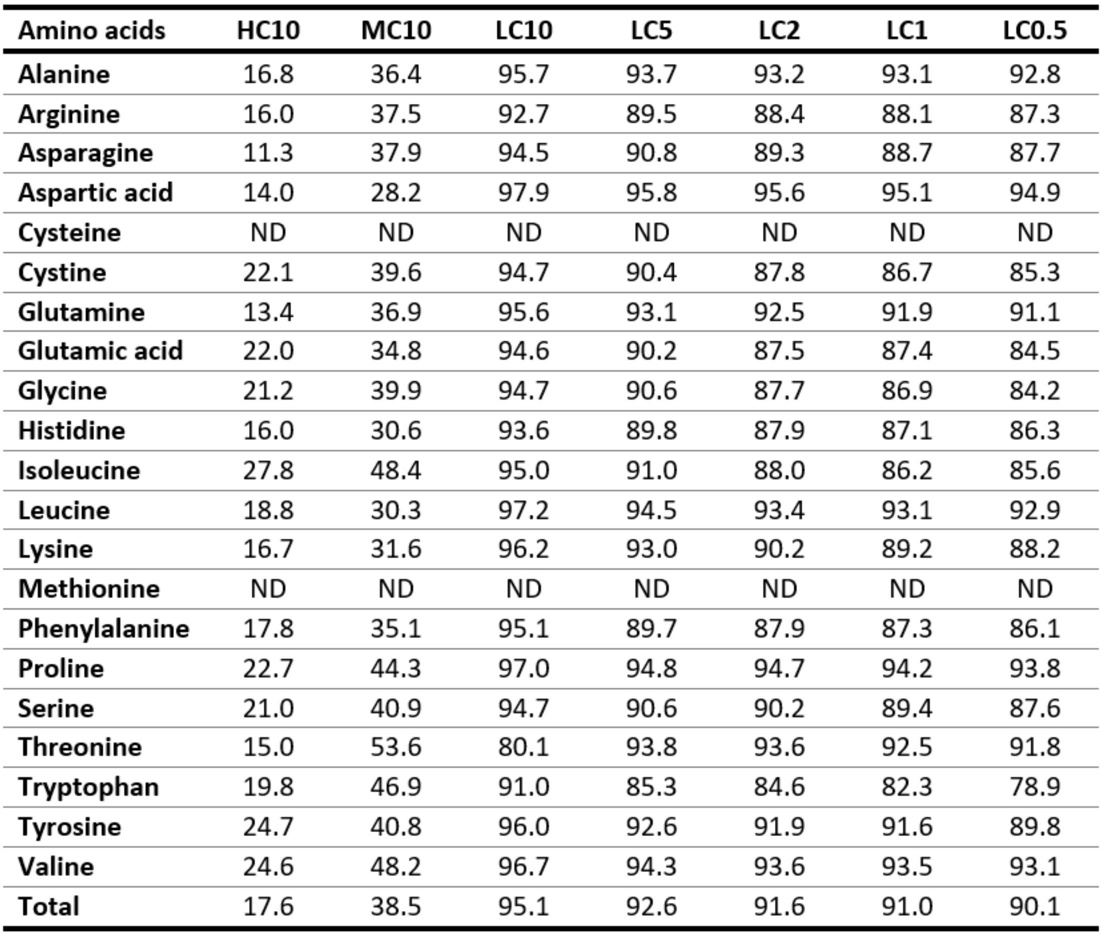
(mean of triplicate measurements from two independent experiments (n=6))

Appendix 7 Percentage reduction of amino acid contents of gluten treated by dielectric barrier discharge treatment with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (%)

Amino acids	HC30	MC30	LC30	LC20	LC10
Alanine	9.2	28.8	62.9	36.1	27.4
Arginine	2.5	9.2	52.2	32.4	17.1
Asparagine	9.2	15.0	54.7	42.9	33.1
Aspartic acid	43.2	11.3	80.0	34.1	31.9
Cysteine	ND	ND	ND	ND	ND
Cystine	9.5	23.7	60.7	51.3	27.8
Glutamine	2.6	13.1	44.4	34.0	26.9
Glutamic acid	-1.6	4.5	18.4	8.0	8.6
Glycine	26.0	40.9	78.0	67.6	51.2
Histidine	13.1	37.2	68.2	65.8	53.6
Isoleucine	8.9	21.3	37.9	23.7	28.3
Leucine	-0.6	1.2	37.2	40.1	21.8
Lysine	-5.9	3.2	-3.1	1.8	4.7
Methionine	ND	ND	ND	ND	ND
Phenylalanine	4.6	17.5	60.5	45.2	35.0
Proline	4.1	16.2	64.0	43.7	39.0
Serine	7.3	16.6	60.3	46.8	29.8
Threonine	5.9	16.7	44.2	34.1	27.9
Tryptophan	1.0	1.9	0.0	-1.8	0.1
Tyrosine	39.7	64.8	75.7	72.0	67.5
Valine	4.8	13.1	62.1	46.3	40.0
Total	8.9	17.7	53.2	39.2	31.3

(mean of triplicate measurements from two independent experiments (n=6))

Appendix 8 Percentage reduction of amino acid contents of casein treated by plasma brush treatment with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (%)



Amino acids	HC10	MC10	LC10	LC5	LC2	LC1	LC0.5
Alanine	16.8	36.4	95.7	93.7	93.2	93.1	92.8
Arginine	16.0	37.5	92.7	89.5	88.4	88.1	87.3
Asparagine	11.3	37.9	94.5	90.8	89.3	88.7	87.7
Aspartic acid	14.0	28.2	97.9	95.8	95.6	95.1	94.9
Cysteine	ND	ND	ND	ND	ND	ND	ND
Cystine	22.1	39.6	94.7	90.4	87.8	86.7	85.3
Glutamine	13.4	36.9	95.6	93.1	92.5	91.9	91.1
Glutamic acid	22.0	34.8	94.6	90.2	87.5	87.4	84.5
Glycine	21.2	39.9	94.7	90.6	87.7	86.9	84.2
Histidine	16.0	30.6	93.6	89.8	87.9	87.1	86.3
Isoleucine	27.8	48.4	95.0	91.0	88.0	86.2	85.6
Leucine	18.8	30.3	97.2	94.5	93.4	93.1	92.9
Lysine	16.7	31.6	96.2	93.0	90.2	89.2	88.2
Methionine	ND	ND	ND	ND	ND	ND	ND
Phenylalanine	17.8	35.1	95.1	89.7	87.9	87.3	86.1
Proline	22.7	44.3	97.0	94.8	94.7	94.2	93.8
Serine	21.0	40.9	94.7	90.6	90.2	89.4	87.6
Threonine	15.0	53.6	80.1	93.8	93.6	92.5	91.8
Tryptophan	19.8	46.9	91.0	85.3	84.6	82.3	78.9
Tyrosine	24.7	40.8	96.0	92.6	91.9	91.6	89.8
Valine	24.6	48.2	96.7	94.3	93.6	93.5	93.1
Total	17.6	38.5	95.1	92.6	91.6	91.0	90.1

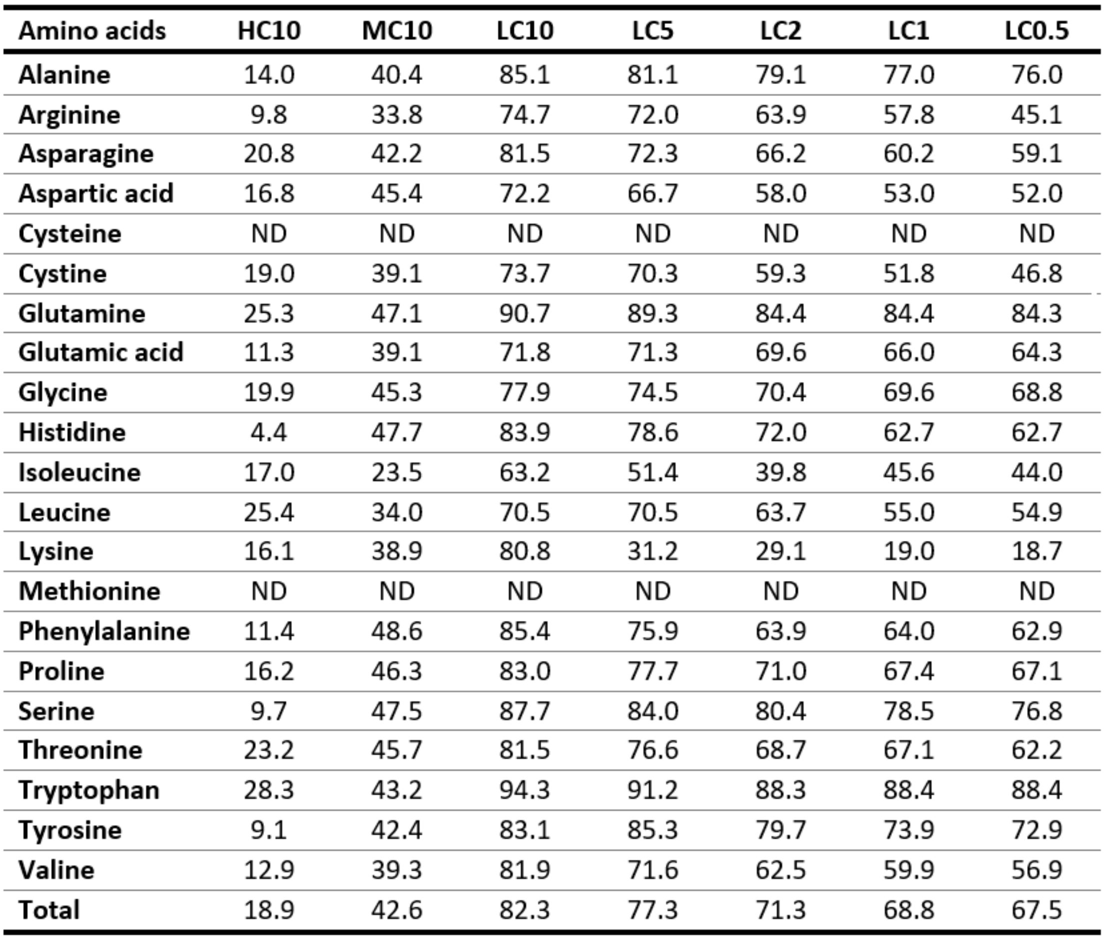
(mean of triplicate measurements from two independent experiments (n=6))

Appendix 9 Percentage reduction of amino acid contents of whey treated by dielectric barrier discharge treatment with different protein concentrations (2, 0.2, 0.02 mg/ml) at 30 min treatment time; with low protein concentration of 0.02 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (2 mg/ml); MC: medium concentration (0.2 mg/ml); LC: low concentration (0.02 mg/ml) (%)

Amino acids	**HC10**	**MC10**	**LC10**	**LC5**	**LC2**	**LC1**	**LC0.5**
Alanine	19.7	42.5	82.9	74.7	73.4	72.6	70.4
Arginine	20.8	45.1	86.9	83.9	83.2	83.1	81.7
Asparagine	-0.3	-2.2	26.7	17.8	10.7	11.6	7.8
Aspartic acid	-1.8	0.7	29.7	25.2	16.1	10.9	8.6
Cysteine	ND	ND	ND	ND	ND	ND	ND
Cystine	20.9	44.6	87.9	81.1	74.6	73.9	73.0
Glutamine	2.1	2.7	38.7	31.7	26.6	23.1	16.7
Glutamic acid	15.6	21.8	81.5	75.5	72.9	71.2	68.2
Glycine	12.1	33.2	83.7	73.5	67.5	66.4	64.2
Histidine	22.2	49.2	85.4	81.6	79.5	78.7	77.4
Isoleucine	18.8	45.5	78.9	73.6	71.6	70.8	70.1
Leucine	-4.9	-5.2	41.1	35.5	20.8	13.7	2.5
Lysine	19.8	39.4	81.8	75.3	72.0	70.6	70.4
Methionine	20.0	50.4	80.5	78.0	77.4	77.0	75.8
Phenylalanine	18.6	35.0	80.9	78.1	75.5	82.7	82.4
Proline	20.1	43.1	82.0	76.6	74.2	73.4	73.3
Serine	23.6	44.9	85.3	82.5	76.1	75.2	73.9
Threonine	31.7	48.2	84.6	80.1	78.4	76.6	76.0
Tryptophan	18.3	49.4	86.9	83.7	83.3	79.7	78.1
Tyrosine	21.1	34.2	81.0	77.2	74.0	75.0	74.2
Valine	20.6	35.9	82.9	79.8	78.6	77.7	76.5
Total	12.2	24.6	63.5	57.8	52.9	50.9	48.0

(mean of triplicate measurements from two independent experiments (n=6))

Appendix 10 Percentage reduction of amino acid contents of gluten treated by dielectric barrier discharge treatment with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (%)



Amino acids	HC10	MC10	LC10	LC5	LC2	LC1	LC0.5
Alanine	14.0	40.4	85.1	81.1	79.1	77.0	76.0
Arginine	9.8	33.8	74.7	72.0	63.9	57.8	45.1
Asparagine	20.8	42.2	81.5	72.3	66.2	60.2	59.1
Aspartic acid	16.8	45.4	72.2	66.7	58.0	53.0	52.0
Cysteine	ND	ND	ND	ND	ND	ND	ND
Cystine	19.0	39.1	73.7	70.3	59.3	51.8	46.8
Glutamine	25.3	47.1	90.7	89.3	84.4	84.4	84.3
Glutamic acid	11.3	39.1	71.8	71.3	69.6	66.0	64.3
Glycine	19.9	45.3	77.9	74.5	70.4	69.6	68.8
Histidine	4.4	47.7	83.9	78.6	72.0	62.7	62.7
Isoleucine	17.0	23.5	63.2	51.4	39.8	45.6	44.0
Leucine	25.4	34.0	70.5	70.5	63.7	55.0	54.9
Lysine	16.1	38.9	80.8	31.2	29.1	19.0	18.7
Methionine	ND	ND	ND	ND	ND	ND	ND
Phenylalanine	11.4	48.6	85.4	75.9	63.9	64.0	62.9
Proline	16.2	46.3	83.0	77.7	71.0	67.4	67.1
Serine	9.7	47.5	87.7	84.0	80.4	78.5	76.8
Threonine	23.2	45.7	81.5	76.6	68.7	67.1	62.2
Tryptophan	28.3	43.2	94.3	91.2	88.3	88.4	88.4
Tyrosine	9.1	42.4	83.1	85.3	79.7	73.9	72.9
Valine	12.9	39.3	81.9	71.6	62.5	59.9	56.9
Total	18.9	42.6	82.3	77.3	71.3	68.8	67.5

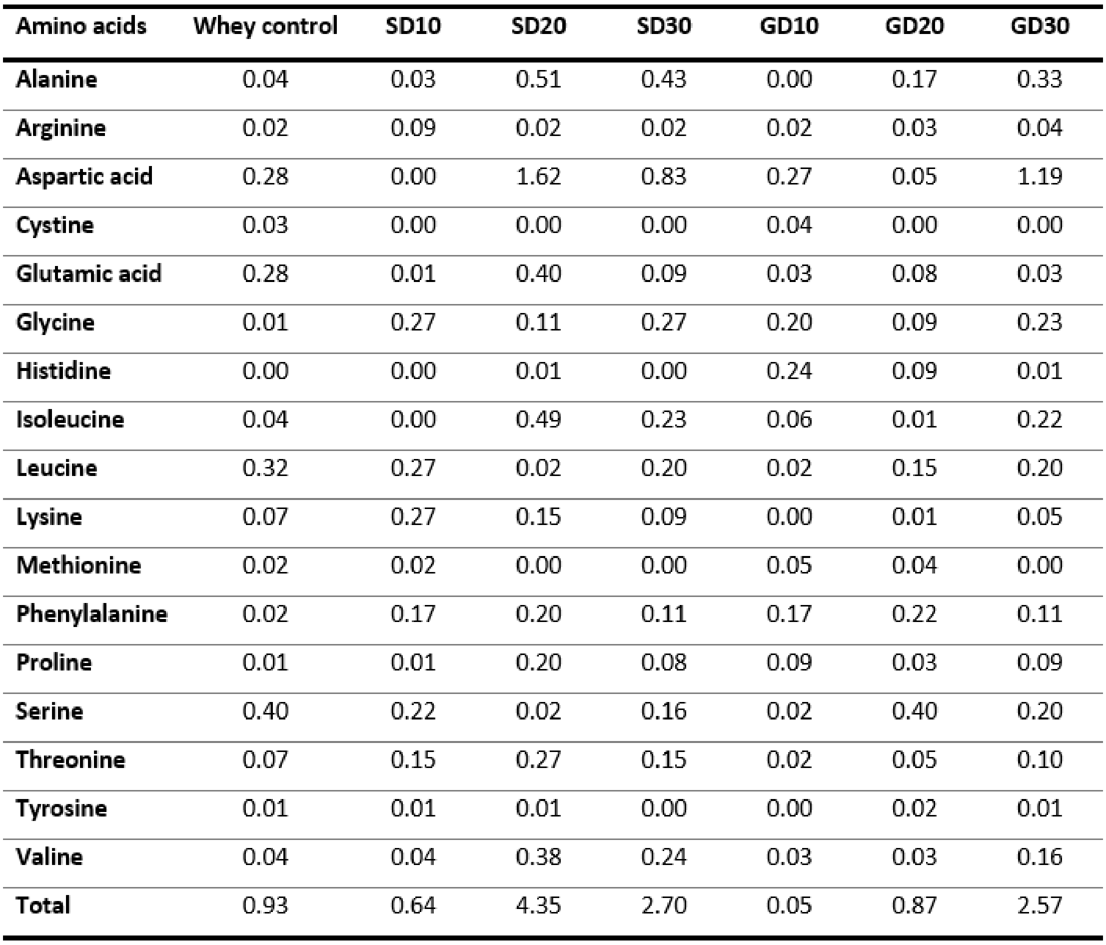
(mean of triplicate measurements from two independent experiments (n=6))

Appendix 11 Standard deviation of amino acid profile, shown in Table 5.3, in untreated and treated caseins by SD and GD at different treatment times (10, 20, 30 min) (g amino acid/100 g protein)

Amino acids	**Casein control**	**SD10**	**SD20**	**SD30**	**GD10**	**GD20**	**GD30**
Alanine	0.23	0.42	0.11	0.54	0.11	0.48	0.34
Arginine	0.23	0.01	0.19	0.12	0.04	0.17	0.03
Aspartic acid	0.28	0.04	2.55	1.26	0.11	1.10	0.83
Cystine	0.22	0.00	0.00	0.01	0.04	0.03	0.02
Glutamic acid	0.84	0.01	3.01	0.67	0.13	0.84	0.65
Glycine	0.04	0.00	0.54	0.48	0.02	0.32	0.18
Histidine	0.43	0.00	0.25	0.13	0.09	0.07	0.04
Isoleucine	0.26	0.01	1.80	0.86	0.08	0.58	0.50
Leucine	0.82	0.00	0.28	0.21	0.13	1.13	0.23
Lysine	0.02	0.01	0.06	0.08	0.06	0.25	0.04
Methionine	0.01	0.00	0.01	0.00	0.06	0.00	0.00
Phenylalanine	0.11	0.02	0.89	0.43	0.13	0.31	0.07
Proline	0.16	0.01	0.85	0.82	0.02	0.55	0.55
Serine	0.29	0.00	0.74	0.55	0.07	0.39	0.11
Threonine	0.53	0.00	0.07	0.22	0.03	0.01	0.05
Tyrosine	0.04	0.01	0.00	0.01	0.03	0.00	0.00
Valine	0.32	0.01	1.24	0.89	0.04	0.65	0.45
Total	0.74	0.15	0.45	7.26	1.18	4.16	3.63

(standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

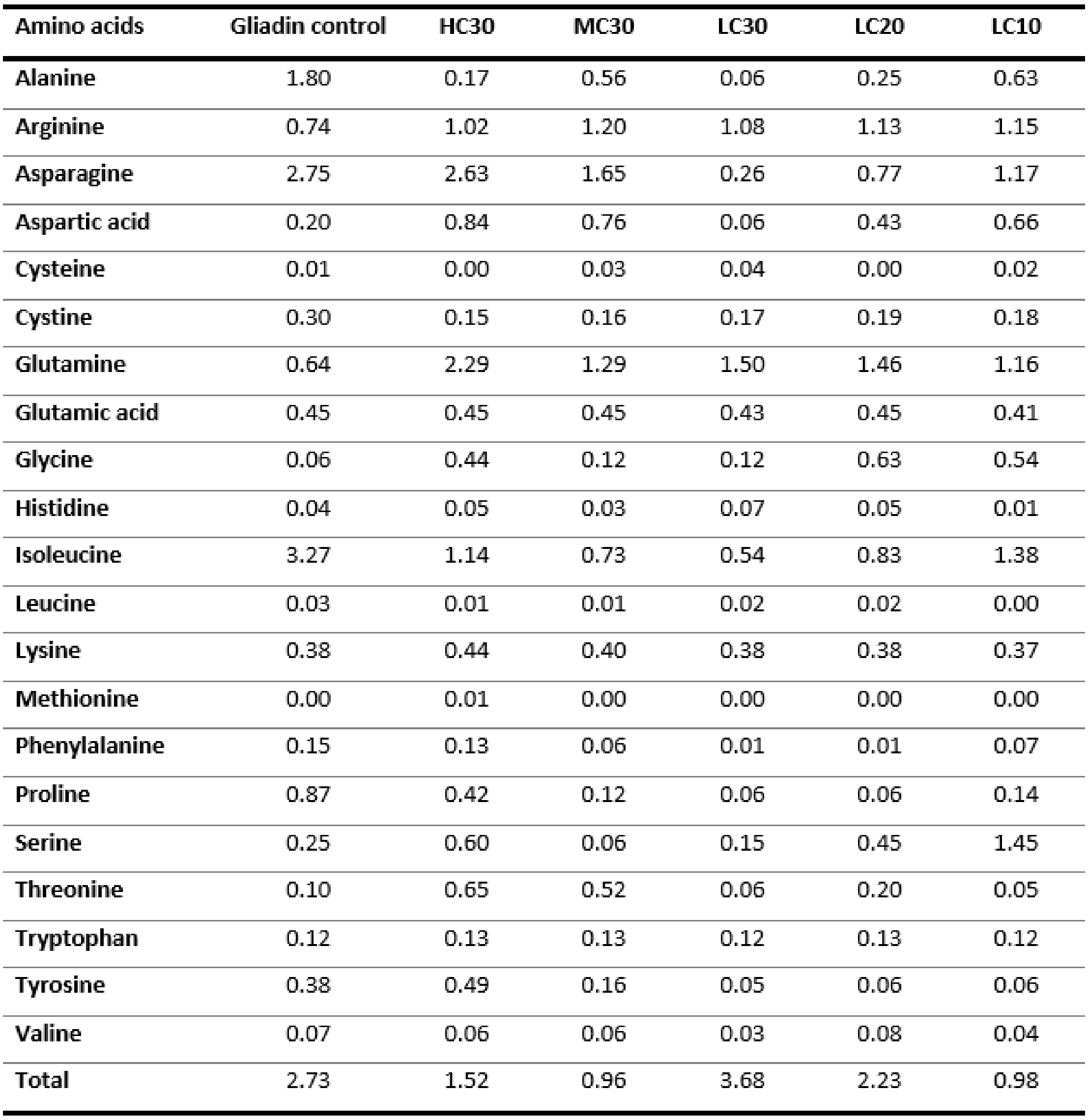
Appendix 12 Standard deviation of amino acid profile, shown in Table 5.4, in untreated and treated whey proteins by SD and GD at different treatment times (10, 20, 30 min) (g amino acid/100 g protein)



Amino acids	Whey control	SD10	SD20	SD30	GD10	GD20	GD30
Alanine	0.04	0.03	0.51	0.43	0.00	0.17	0.33
Arginine	0.02	0.09	0.02	0.02	0.02	0.03	0.04
Aspartic acid	0.28	0.00	1.62	0.83	0.27	0.05	1.19
Cystine	0.03	0.00	0.00	0.00	0.04	0.00	0.00
Glutamic acid	0.28	0.01	0.40	0.09	0.03	0.08	0.03
Glycine	0.01	0.27	0.11	0.27	0.20	0.09	0.23
Histidine	0.00	0.00	0.01	0.00	0.24	0.09	0.01
Isoleucine	0.04	0.00	0.49	0.23	0.06	0.01	0.22
Leucine	0.32	0.27	0.02	0.20	0.02	0.15	0.20
Lysine	0.07	0.27	0.15	0.09	0.00	0.01	0.05
Methionine	0.02	0.02	0.00	0.00	0.05	0.04	0.00
Phenylalanine	0.02	0.17	0.20	0.11	0.17	0.22	0.11
Proline	0.01	0.01	0.20	0.08	0.09	0.03	0.09
Serine	0.40	0.22	0.02	0.16	0.02	0.40	0.20
Threonine	0.07	0.15	0.27	0.15	0.02	0.05	0.10
Tyrosine	0.01	0.01	0.01	0.00	0.00	0.02	0.01
Valine	0.04	0.04	0.38	0.24	0.03	0.03	0.16
Total	0.93	0.64	4.35	2.70	0.05	0.87	2.57

(standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

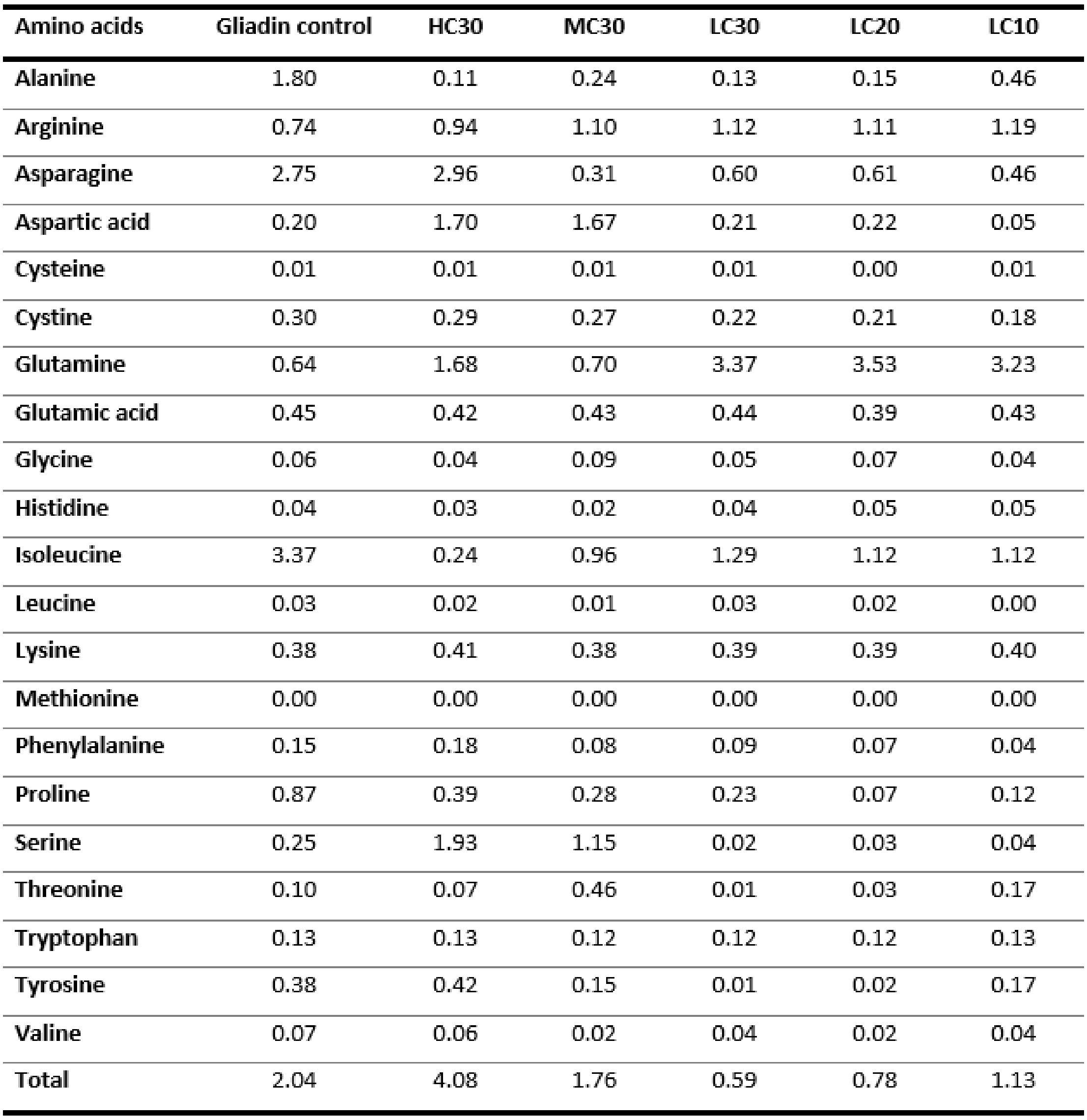
Appendix 13 Standard deviation of amino acid profile, shown in Table 5.5, in untreated and treated glutens by SD with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein).



Amino acids	Gliadin control	HC30	MC30	LC30	LC20	LC10
Alanine	1.80	0.17	0.56	0.06	0.25	0.63
Arginine	0.74	1.02	1.20	1.08	1.13	1.15
Asparagine	2.75	2.63	1.65	0.26	0.77	1.17
Aspartic acid	0.20	0.84	0.76	0.06	0.43	0.66
Cysteine	0.01	0.00	0.03	0.04	0.00	0.02
Cystine	0.30	0.15	0.16	0.17	0.19	0.18
Glutamine	0.64	2.29	1.29	1.50	1.46	1.16
Glutamic acid	0.45	0.45	0.45	0.43	0.45	0.41
Glycine	0.06	0.44	0.12	0.12	0.63	0.54
Histidine	0.04	0.05	0.03	0.07	0.05	0.01
Isoleucine	3.27	1.14	0.73	0.54	0.83	1.38
Leucine	0.03	0.01	0.01	0.02	0.02	0.00
Lysine	0.38	0.44	0.40	0.38	0.38	0.37
Methionine	0.00	0.01	0.00	0.00	0.00	0.00
Phenylalanine	0.15	0.13	0.06	0.01	0.01	0.07
Proline	0.87	0.42	0.12	0.06	0.06	0.14
Serine	0.25	0.60	0.06	0.15	0.45	1.45
Threonine	0.10	0.65	0.52	0.06	0.20	0.05
Tryptophan	0.12	0.13	0.13	0.12	0.13	0.12
Tyrosine	0.38	0.49	0.16	0.05	0.06	0.06
Valine	0.07	0.06	0.06	0.03	0.08	0.04
Total	2.73	1.52	0.96	3.68	2.23	0.98

(standard deviation (SD) of triplicate measurements from two independent experiments (n=6))

Appendix 14 Standard deviation of amino acid profile, shown in Table 5.6, in untreated and treated glutens by GD with different protein concentrations (10, 1, 0.1 mg/ml) at 30 min treatment time; with low protein concentration of 0.1 mg/ml at different treatment times (30, 20, 10 min) HC: high concentration (10 mg/ml); MC: medium concentration (1 mg/ml); LC: low concentration (0.1 mg/ml) (g amino acid/100 g protein).



Amino acids	Gliadin control	HC30	MC30	LC30	LC20	LC10
Alanine	1.80	0.11	0.24	0.13	0.15	0.46
Arginine	0.74	0.94	1.10	1.12	1.11	1.19
Asparagine	2.75	2.96	0.31	0.60	0.61	0.46
Aspartic acid	0.20	1.70	1.67	0.21	0.22	0.05
Cysteine	0.01	0.01	0.01	0.01	0.00	0.01
Cystine	0.30	0.29	0.27	0.22	0.21	0.18
Glutamine	0.64	1.68	0.70	3.37	3.53	3.23
Glutamic acid	0.45	0.42	0.43	0.44	0.39	0.43
Glycine	0.06	0.04	0.09	0.05	0.07	0.04
Histidine	0.04	0.03	0.02	0.04	0.05	0.05
Isoleucine	3.37	0.24	0.96	1.29	1.12	1.12
Leucine	0.03	0.02	0.01	0.03	0.02	0.00
Lysine	0.38	0.41	0.38	0.39	0.39	0.40
Methionine	0.00	0.00	0.00	0.00	0.00	0.00
Phenylalanine	0.15	0.18	0.08	0.09	0.07	0.04
Proline	0.87	0.39	0.28	0.23	0.07	0.12
Serine	0.25	1.93	1.15	0.02	0.03	0.04
Threonine	0.10	0.07	0.46	0.01	0.03	0.17
Tryptophan	0.13	0.13	0.12	0.12	0.12	0.13
Tyrosine	0.38	0.42	0.15	0.01	0.02	0.17
Valine	0.07	0.06	0.02	0.04	0.02	0.04
Total	2.04	4.08	1.76	0.59	0.78	1.13

(standard deviation (SD) of triplicate measurements from two independent experiments (n=6))