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Methodological Analysis of an Improved Gluten Quantitation Aptamer-based Biosensor

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Methodological Analysis of an Improved Gluten Quantitation Aptamer-based Biosensor

by

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Abstract

Fluorescence resonance energy transfer based aptamer are currently being studied in many research groups due to their potential contribution as an alternative gluten standard for gluten-quantitation. The current gold standard withholds critical limitations due to the nature of the system, hence this aptamer-based sensor (or aptasensor for short) application represents one of the alternatives; yet, drawbacks such as low signal-to-noise ratio and reliability are in the scope of research groups aiming to overcome them.

In this study, multiple variations of the protocol are assessed based on correctly classifying food samples as their actual concentration of gluten, this is coined as the accuracy of the biosensor. The study also aims to overcome the current limitation of the gold standard in fermented samples by including soy sauce and malt vinegar in the tests. And different additives aiming to help overcoming the limitation were implemented into the protocol and assessed.

This approach allowed the biosensor to classify the products with 98.28% of accuracy, and 0% of error in classifying gluten-rich products (false-negatives) within the first 3 days of bioassay preparation; yet, this bioassay needs to be studied further as only 18 different off-the-shelf products were tested (over 800 tests in total). Additionally, after the first week, false-negatives increased to around 5% and remained that way until the end of the first month.

The cause of this relies mostly on the decomposition of the conjugate reduced graphene oxide and polyethylene glycol that are implemented in the system. This implies that further research aiming solely at additives or alternative reagents that increase the lifespan or stability of the conjugate would augment the overall performance.

Preface

For a better quantitation of gluten in food samples, J. Carlos Kuri has endeavored his time to present an outstanding alternative that may compete among others for the gold standard title in this purpose. The research is heavily based on previous work from S. N. Diaz and N. Suresh among many others. Dr. Orly Yadid-Pecht has allowed me to get involved in the subject at hand, whilst Dr. Raymond Turner, Dr. Varun Vij, and Basma Akhter for having unconditionally supported and conveyed knowledge in the subject.

My previous research experience has given me the confidence and knowledge to lead the project according to unexpected outcomes. For that, I'm grateful to structure multiple research reports for which at least one is expected to be published.

Acknowledgments

I would love to thank my family for all the support and encouragement. Also, I'd like to thank Dr. Yadid-Pecht for the opportunity given to me for this research, Dr. Turner for the guidance and knowledge bestowed upon me, Dr. Vij for his dedication and patience, Dr. Murari, and Dr. Dalton for their professionalism. And once again, for all those who pushed me forward and helped me know how to help others with knowledge and dedication.

I'd like to particularly thank my fiancée, Brenda Ruiz, who has accompanied me on this journey from over 4000 km away in a virtual manner. I'd like to thank Mario Chavez for his support, friendship, and his encouragement. I'd like to thank Joel and Edgar Briones for their knowledge and introduction to the research community. And, every single fellow who has been part of my life positively and negatively.

To my future self who sees my naivety, yet holds the burden of my endless goals.

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List of abbreviations and nomenclature

NaH_2PO_4 . . sodium phosphate monobasic

Na_2HPO_4 . . sodium phosphate dibasic

N_2H_4 . . . . hydrazin

6-FAM . . . 6-carboxyfluorescein

AuNP . . . . gold nanoparticle

CAGR . . . . compound annual growth rate

CD . . . . . celiac disease

CFU . . . . . colony forming unit

CI . . . . . confience interval

CMOS . . . complementary metal-oxide-semiconductor

CNT . . . . . carbon nanotube

CV . . . . . coefficient of variation

DF . . . . . dilution factor

DI . . . . . deionized water

DMAP . . . 4-dimethylaminopropyridine

DMSO . . . dimethyl sulfoxide

DNA . . . . . deoxyribonucleic acid

EDC . . . . . carbodiimide hydrochloride

ELISA . . . . enzyme-linked immunosorbent assay

EtOH . . . . ethanol

FRET . . . . fluorescence resonance energy transfer

GF . . . . . gluten-free

GMR . . . . giant magnetoresistance

GO . . . . . graphene oxide

GR . . . . . gluten rich

HPLC . . . . high performance liquid chromatography

IBM . . . . . International Business Machines

Ig . . . . . immunoglobulin

LC . . . . . liquid chromatography

LED . . . . . light-emitting diode

LFIA . . . . . lateral flow immunoassay

LOD . . . . . limit of detection

LOQ . . . . . limit of quantification

MNP . . . . magnetic nanoparticles

MRM . . . . multiple reaction monitoring

MS . . . . . mass spectrometry

NaCl . . . . sodium chloride

PBS . . . . . phosphate saline buffer

PCR . . . . . polymerase chain reaction

PEG . . . . . polyethylene glycol

PVP . . . . . polyvinypyrrolidone

QD . . . . . quantum dot

QQQ . . . . triple quadrupole

rGO . . . . . reduced graphene oxide

RM . . . . . reference material

RNA . . . . . ribonucleic acid

RT . . . . . room temperature

SELEX . . . systematic evolution of ligands by exponential enrichment

SPR . . . . . surface plasmon resonance

SRM . . . . . selected reaction monitoring

TE . . . . . tris-EDTA

Epigraph

成功の大きさは、願望の強さや理想の高さ、成功を収める過程で希望を失ったとき、どのように対処してきたかによって評価される。

Robert Kiyosaki

The size of your success is measured by the strength of your desire; the size of your dream; and how you handle disappointment along the way.

Robert Kiyosaki

Chapter 1

Introduction

Biological applications have revolutionized many industries during the last decades. The humanity has learnt over time the nature of the world by science. Initially, the human technology was limited by the tools and knowledge of the molecular behaviour. One of the very first nanotechnological application, although not understood at that time, is the lycurgus cup [1]. Despite the fact they didn't understood how the cup color changed depending on the position of the light, they knew how to replicate that behaviour. Over time, the understanding of nature increased, allowing us to be able to control with precision the location of atoms like International Business Machines (IBM) did [2]. As I write this, I'm sure breathtaking technological advances are being developed.

This understanding of nature has allowed the humanity to discover the inner composition of organisms by understanding deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and many other parts of the body. Now, DNA and RNA can be tailored using clever selection techniques like systematic evolution of ligands by exponential enrichment (SELEX) to use them as molecular probes [3]. These long/short single stranded DNA/RNA are called aptamers. Aptamer's potential to specifically bonds to a target has shown advantages over other detection methods [4]. Its specificity, selectivity, simplicity, rapid-testing and thermal stability properties have been attractive for aptamer-based detection methods [5, 6].

I2Sense has worked with aptamers for over 6 years, revolving its studies around one aptamer-application: quantification of gluten concentration in gluten-free (GF) and gluten rich (GR) (or regular) food samples. MSc Suresh et al., a former student in I2Sense Group, developed a hybrid aptamer combining: reduced graphene oxide (rGO); as the fluorescence quencher, polyethylene glycol (PEG); as the blocking agent, and the 6-carboxyfluorescein (6-FAM) attached at one of the 2 ends of the aptamer; as the fluorescent molecule [7].

The endeavours of this work aimed to implement the I2Sense aptamer proof of concept into a realistic environment. This is achieved when local grocery off-the-shelf products and reference material (RM) are accounted in the assessment. In order to further improve the accuracy of the aptamer, an overhaul of the protocol is included in the study.

1.1 Thesis objectives

Primary objective

To study the performance of the I2Sense aptamer-based method for gluten quantification on off-the-shelf products and RM. And contrast its performance to the current globally standardize method: enzyme-linked immunosorbent assay (ELISA).

Secondary objective

To improve the accuracy of the aptamer by overhauling the protocol into a more consistent, stable and reliable protocol that accuracy quantifies gluten on GF and GR food samples.

Third objective

To provide an analysis and 3d render of the prototype for the previously mentioned aptamer application.

1.2 Hypotheses

Hypothesis #1

The addition of a secondary blocking agent for cacao in the I2Sense aptamer-based sensor improves the overall accuracy on GF and GR samples.

Hypothesis #2

The I2Sense aptamer-based sensor can measure gluten concentrations in fermented food samples.

Hypothesis #3

The overall testing speed can be increased without additional reagents nor compromising the accuracy of the I2Sense aptamer-based sensor

Chapter 2

Literature Review

2.1 Background Introduction

2.1.1 Celiac disease

Celiac disease (CD) is an immune system reaction triggered by ingesting gluten in susceptible individuals. The repercussions are fatigue, nausea, diarrhea, headache, and many more [8]. Gluten refers to the whole protein found in wheat, rye, and barley; gliadin refers to the alcohol-soluble fraction of gluten. A 33-mer peptide is the amino-acid part of gliadin responsible for triggering the immune reaction in CD patients.

The disease is presented worldwide, including in developing countries like North Africa, the Middle East, India, and China. The disease effects have been recognized as nearly twice as prevalent in female humans over males [9]; nevertheless, this study may be subject to incomplete data from asymptomatic patients or developing countries without the data.

The incidence of CD has been increasing every year by 7.5% over the last decades; affecting around 0.7% to 1.4% of the total population. Developed countries like Netherlands, Denmark, Canada, Italy, and the USA are not exempt from it, having an average incidence of 4.0%, 6.5%, 7.9%, 10.0%, and 13.6% respectively [10]. Considering that 25 g of bread contains approximately 1.6 g of gluten, for people suffering from CD 10 to 50 mg per day is enough to cause damage

to the intestinal mucosa over time [9]. Should CD not be treated, further complications are associated: cancer, osteoporosis, neurologic disorders, infertility, and recurrent miscarriage [9]. Therefore, adapting to a lifelong gluten-free (GF) diet is currently the most accepted treatment for CD [11].

2.1.2 Gluten-free labeling regulation

The Codex Alimentarius regulation was established in order to serve as a standardized labeling regulation of GF food products, which must contain no more than 20 mg in each kg of the product [12]. In order to comply with the regulation, enzyme-linked immunosorbent assay (ELISA) has been granted the title of 'the gold standard' for gluten quantification in food samples among the food industry and food-control agencies [13].

2.1.3 Cross-contamination

The current food industries that manufacture both GF and gluten rich (GR) products in the same machine or machines in close proximity are precursors of cross-contaminated products; leading to miss-labeling GF products. Rigorous and thorough sanitation procedures from the staff; appropriate filtered ventilation; and completely GF isolated milling, storage, and manufacture for the least possible cross-contamination are utterly important for all non-CD gluten sensitive, gluten allergic, and CD individuals. A systematic review from Dr Falcomer, Araujo et al. state the lack of adoption of good manufacturing practices, an standardized GF preparation protocol, and adequately trained personnel; which attribute to fewer contamination levels in foods prepared at home; then, higher contamination levels at industrialized settings; and the highest at non-industrialized food services [14].

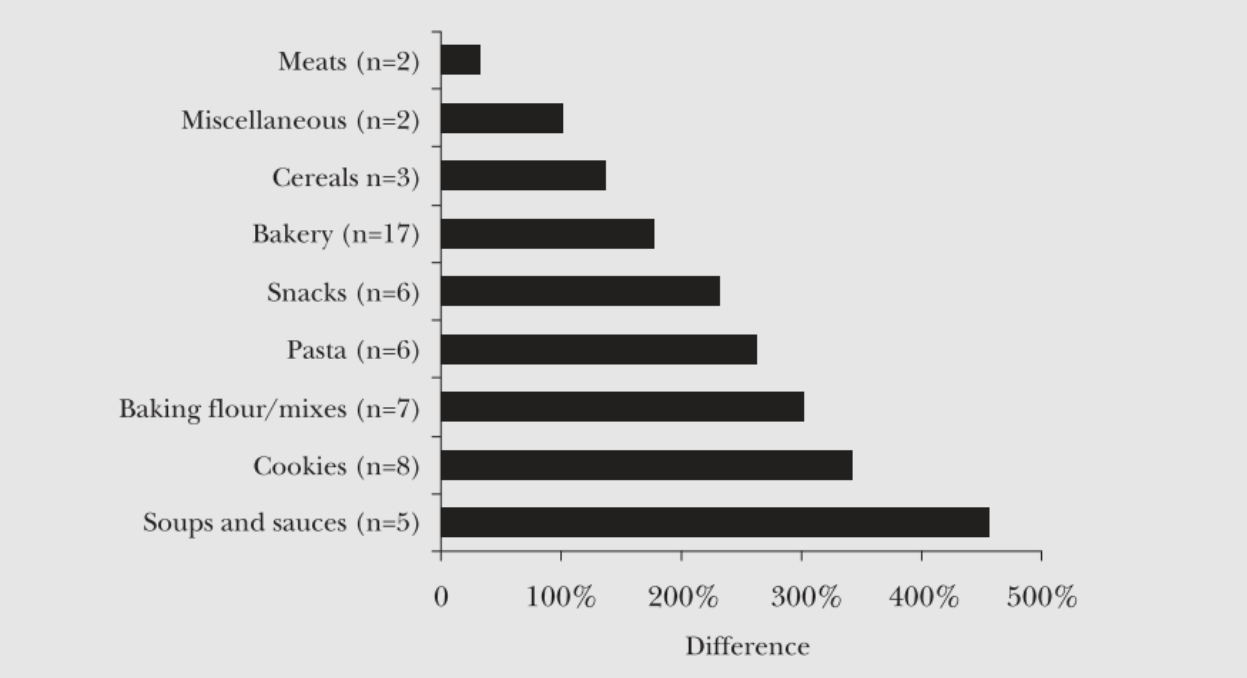

Figure 2.1: Price relation of gluten-free products found in grocery stores in UK relation to its regular version [17].

2.1.4 Availability and cost limitations on gluten-free products

Cross-contamination has been increasing in the last years; thus, several limitations are attached to this lifestyle. In 2011, Dr Singh and Whelan studied stores in the UK that sell GF products and found that 90% of regular supermarkets, 49% of health food shops, 9% of budget supermarkets, and 1.8% of corner shops sold them; although, the variety was limited [15]. In Brazil, 50% of the interested population for GF diet were unable to follow it; 12% rely on the high cost of products, and the other 38% on the lack of alternatives [16].

Dr Stevens and Rashid found that the GF products were 242% more expensive in Canada than their regular counterpart. As shown in Fig. 2.1, the increased price was mostly dependent on the type of GF food; meats were slightly more expensive, and soups & sauces were over 4 times more expensive [17]. In the UK, the prices of GF products ranged from 76% to 518% more expensive [15]. Similarly, in the United States, the average cost of GF foods was 240% more expensive [18]. In a recent study in 2022, Dr Demirkesen and Ozkaya demonstrated that the increased price is not influenced by geographical location [19].

2.1.5 Gluten-free diet awareness and adherence limitations

Although awareness has increased in the past years, various restaurants now include labels indicating GF dishes [20]. Unfortunately, 32% of the GF labeled dishes in United States restaurants tested positive; mostly pizza and pasta [21]. In 2010, two interested Brazilian researchers tested 185 GF labeled products; 13% tested positive [22, 23]. 5 years later, Dr Thompson and Simpson did the same experiment with 158 GF labeled products sold in the United States; 5.1% tested positive [24].

On top of that, COVID-19's impact affected the adherence to a GF diet due to the self-perception of the pandemic risks, anxiety, and depression. As shown in the study by Dr Bascuñán, Rodríguez, Osben et al. 331 of the participants, 87% were affected by the shortage and the increased price, 53.8% consumed GR foods as a lack of alternative, 36.6% failed to obtain medical assistance when needed. This attributed to 29% more gluten-related symptoms, 28% more anxiety, and 40.40% more depression; resulting in 2.3 times less adherence to a GF diet regardless of their efforts [25].

Nevertheless, some reactions may even occur in naturally GF food; contradictory results express that whilst some CD patients won't trigger an immune reaction to oat [26–29], others do [27, 30–32]. It has been shown in recent systematic reviews that CD is still under-diagnosed worldwide [33, 34]

2.1.6 Market value

Despite the cross-contamination and the mislabeling of GF products by food industries, the demand for GF food products is in constant growth; it has become more popular not only for gluten-sensitive or CD people but for its representation of a healthy lifestyle and the benefits that are attached to it [11]. The market value of gluten-free products was estimated to be around 3.84 billion USD [35] in 2017. Then, the same market value has been estimated to be around 6.1 billion USD out of the total of 19.5 billion USD in the food safety market [36]. Now, the GF market value has been estimated to be above 10 billion USD after 2029 [37, 38]

ELISA is not only employed in gluten-related detection but generally as the gold standard for foodborne allergens, meaning it conveys a great impact to the overall food safety market. Thus, the overall global market value of the gold standard has increased rapidly in the last years; being valued at 21.4 billion USD in 2020 with a compound annual growth rate (CAGR) of 6.7% consequence of the COVID-19 outbreak [39]. Google trend reports an increasing number of searches for the term gluten-free, which has tripled since the COVID-19 outbreak [40].

2.1.7 ELISA implications and limitations

The increase in the price mentioned in the section 2.1.4 is mostly driven by the process of making the food items; including production, certification, sterile environment, the regulatory validation, among others [41, 42].

It has been discussed in the section 2.1.5 the inaccuracies of GF labeled products and how hard it is to stick to a GF diet. As discussed in the section 2.1.4, one root cause is its availability of and the absurd increased cost of GF labeled products in comparison to its regular version. The other root cause is the inaccurate GF labeled products that directly relate to cross-contamination. It has been discussed in the section 2.1.3 that cross-contamination indicates the lack of appropriate practices of sanitation in a gluten-sterile environment; however, the ELISA protocol itself is so complex and time-sensitive that it also contributes to the inaccuracies of GF labeled products, and ultimately, the end consumer. Nevertheless, ELISA is still in a growing demand for its monopoly as the gold standard for allergen detection; as described in the section 2.1.6.

Part of the inaccuracy is contributed by one ELISA limitation among others: it is unable to quantify gluten concentrations in fermented and hydrolyzed food due to the high variability of proteins and peptide profiles, and the complexity of the fermentation process [43].

Briefly, ELISA uses an antibody (R5 in this case) to recognize the topography of its target/analyte (known as antigen); more specifically, a part of the antigen called the interaction "epitope" [44]. However, due to the nature of proteins, a very long chain may have similar or

partially identical epitopes to that of the target epitope in gluten. These similarities cause the R5 ELISA to bond non-targeted proteins giving false positives. As for the most important: false negatives take place when R5 ELISA is unable to recognize the target epitope in gluten. In this case, fermentation's complexity causes the protein/peptide profiles to change but yet still trigger CD symptoms [45, 46].

Furthermore, ELISA have several other limitations, such as easy denaturation, short shelf-life, temperature sensitivity, time-consuming, and time-sensitive results; additionally, it is limited to only the range of gluten concentration within 5 ppm to 80 ppm [47]. Accounting for all these said implications that are attached to ELISA, it is astonishing to see it still accepted as the "gold standard" for several years without a partial or complete replacement yet. Therefore, possible solutions are listed:

1. Changing the functionality of ELISA.

2. Nominating another method as the new gold standard.

As for the first solution, a different G12 antibody targeting the key 33-mer peptide was raised to detect the triggering section of CD similarly to the R5 antibody [48, 49]. A study from Dr Comino, Bernardo, Bancel et al. showed G12 antibody reacts to large and small peptides that are responsible for CD complications; whilst the R5 antibody responded to only 25% of those in barley beer (describing its augmented performance on hydrolyzed material) [45].

Generally, the G12 antibody has shown potential in overcoming the limitation of detecting gluten in hydrolyzed foods [43, 50]. Furthermore, Dr Panda et al. have endeavored their time over several years in studying the complexity of fermented and hydrolyzed foods; and reported the protein/peptide complexity evolving over the fermentation of yogurts for calibration and accurate quantitation of gluten [46]. However, until further studies show reliable results indicating the consistent fidelity in hydrolyzed and fermented foods, a regulation has to dwell among most food-safety industries. This regulation indicates all gluten-free ingredients have to be tested negative before the complex process of fermentation [51].

Therefore, researchers have opted for the second solution (an alternative method) and endeavored their time in the extensive search for a new gold standard for allergen detection.

2.2 Alternative sensors

In order to understand the possible alternative sensors, the main tools that compose a sensor have to be understood. It is important to know that many sensing techniques and recognition elements may be combined; after all, that is what research is: a free for all field where knowledge and understanding is the main goal. With that, a useful application can be achieved. There is a handful of sensing techniques and recognition elements; however, only those which are closest to food allergen detection are presented; Electrochemical, optical, and mass spectrometry.

2.3 Techniques

2.3.1 Electrochemical

This technique mainly uses the chemical interaction of an assay and an agent. The agent could be anything from proteins, nucleic acid molecules or chemical reagents, as long as the interaction between the assay and the agent is reproducible. Then that interaction characterizes the behaviour of the assay by gathering the change of pH, temperature, current, resistance or voltage as output of the interaction. The output is expected to be correlated to any controlled input perturbation, thus, characterizing the behaviour of the assay. Normally when volts are are being quantified in some way or another it is referred as voltammetric [52–54]; and when amperes are quantified, these systems are called amperometric [54–56]. In some instances, proteins or antibodies are immobilized on a surface, the interaction causes between the recognition element and the target-molecule inside the assay causes changes in the assay that can be quantified. For food safety, the application should be the allergen concentration in a food sample assay [57, 58].

2.3.2 Optical

This study focuses in review only those techniques that optic-based sensors are employed for allergen detection. In this field of study, the light-matter interaction is studied based on the change of light frequency, intensity, and polarization. The most common applications for food allergens are: surface plasmon resonance (SPR), fluorescence, or absorbance.

Surface plasmon resonance (SPR)

SPR utilizes an incident light source that targets an optical-sensitive surface at a certain angle called resonance angle or at a certain wavelength. Then, depending on the interactions of the recognition element that is immobilized onto the optical-sensitive surface, the resonance angle or wavelength changes [59].

Fluorescence

This technique takes advantage of the material properties that emit light due to the electron absorption and relaxation within the electron states. In summary, an electron absorbs energy from emitted light, part of that energy is lost in heat; the inner electrons relax to its ground state by giving up energy in form on fluorescence (in the scale of nanoseconds); or in phosphorescence in case of a triplet state (lasts longer than nanoseconds). lastly, as the electrons gave up energy in heat, the energy of the emitted light is lower than the excitation light, which wavelength is greater [60].

Although the fluorescence properties are not present in all materials, dyes like florescein are utilized; such as 'turn-on' and 'turn-off' arrangements. As for the 'turn-on', the fluorescent agent is attached to a molecule that identifies and bond to the target. When that interaction takes place the fluorescent agent is freed from the quenching agent; then, emission light is gathered. On the other hand, 'turn-off' takes place when the analyte is already attached to a quencher; and, once the fluorescent molecule is carried to the analyte, fluorescence is hindered [61].

Fluorescent molecules tend to change its structure after radiation; thus, photobleaching reduce the overall fluorescence intensity over time. These molecules also demand the use of lens arrangement and/or filters, which increases the complexity of the system and the cost, but reduces the signal-to-noise ratio. One example is by adding a chromatic filter before the photoreceptor, which blocks the excitation light and allows the emission light of the dye to by picked up by the photoreceptor. One way of eluding fluorescent molecules and all the said drawbacks is by using only quenching molecules attached to the recognition element; and after washing off unbound quenching molecules to the analyte, the excitation light is absorbed by the quencher; thus, determining the number of interactions within the assay [62].

Colorimetry

This technique utilizes recognition elements like enzymes, antibodies, or peptides to quantify the analyte concentration based on the change of color of the assay at different concentrations. A clever way of changing the color based on the analyte concentration is by making the substrate to change the color. And then remove all the substrate from the assay that did not interacted with the analyte, so that the concentration of substrate is as close as to a linear correlation to the concentration of analyte. Hence, a recognition element takes the role of carrying the substrate and attaching to the analyte inside the assay. The number of interactions between the recognition element to the analyte defines the amount of light absorbed absorbed. In order to quantify the concentration, a standard curve with no concentration and pre-quantified concentrations allow for interpolation measurements [63].

2.3.3 Mass spectrometry

For food allergen detection and quantitation, mass spectrometry (MS) has been employed using the mass-to-charge ratio of a precursor. This method is better known as selected reaction monitoring (SRM) or multiple reaction monitoring (MRM), which is heavily based on the theory of MS [64]. This method allows for the separation of the precursor by changing the

charges of quadrupoles at a certain frequency, for which only the precursor with the correct mass-to-charge ratio would be able to go through a tunnel to the next quadrupole array. The whole concept consists of 3 quadrupole sections, also known as triple quadrupole (QQQ). Each section is focused on filtering different properties of the whole sample, resulting in a quantitative or qualitative relation of the peptide in the sample [65].

Liquid chromatography (LC) or high performance liquid chromatography (HPLC) are usually used to separate the precursor by its compounds. Subsequently, the separated compounds go through QQQ sections as previously described in the method of SRM; this is known as LC-MS or LC-MS/MS [66]. This allows for multiplexed detection, which is very appealing for many applications; however, the high-profile equipment level and the required expertise of the staff make this method out of the reach for many routine uses [64].

2.4 Recognition elements

Food allergens are usually detected using a recognition element. By any means, if the allergic protein is being recognized by any element, it can be used cleverly to serve as a probe or detector. The most common methods for allergen detection use protein-based or deoxyribonucleic acid (DNA)-based methods as the recognition element. In the case of protein-based methods, antibodies, and enzymes are leading the field; whereas, DNA-based methods use polymerase chain reaction (PCR) and aptamers [13]. Similarly, The physical interactions between matter-matter or its physical properties allow for the use of nanomaterials.

2.4.1 Antibody-based techniques

When an individual gets an allergic reaction, normally the immune system creates immunoglobulin (Ig)M as the first response, and then IgG as long-term system protection [67]. Then the antibodies can be extracted from the human blood serum for research. Although there are applications with IgY extracted from egg yolks and IgE from food-specific allergic individuals [68].

Then, these antibodies can be used for the detection of the target (or analyte) they were created for. Antibodies are principally useful to bond to a specific analyte based on the composition of the analyte. The method introduces the use of antibodies in a complex sample containing the analyte; and the number of bonds between antibody-analyte determines the concentration of the analyte in the assay [67]. In order to quantify the concentration of the analyte a label molecule is utilized; however, there are clever techniques like SPR [69] and electrochemical [70] which elude the drawbacks like photobleaching and more complex setup that is required in labeling. Nevertheless, the use of a label allows for a low-budget consumer-friendly lateral flow immunoassay (LFIA) [71,72]. In this application, the ambient light becomes highly important; thus, future research for a portable is currently a topic to strive for [73].

2.4.2 PCR-based techniques

DNA have been proposed as a specific, sensitive and reliable alternative for the enzyme-based technique: ELISA. DNA is able to maintain their integrity better than proteins [74]. Originally, PCR was a qualitative method; however, over time, quantitative methods were achieved by labeling their probes [75] or internal standards [76]. Dr Sun et al. studied a dually labeled DNA able to detect peanut allergen. By using a stem-loop probe, the loop is closed when there is absence of the target and opened when the target interacts with the probe. The number of interaction is characterized by the changes of impedance in electron-transfer efficiency when doing electrochemical impedance spectroscopy. This method reached limit of detection (LOD) of 0.35 fM [74]. Dr Ernest and Anklam found that usually, DNA-based methods like PCR can detect lower concentrations of the target than protein-based methods [77]. Nevertheless, similar to ELISA, the DNA degradation in highly processed or hydrolyzed samples is a critical limitation for quantitation of gluten by DNA-based methods [78].

2.4.3 Nanomaterial-based techniques

Nanotechnology has also printed its foot as a potential candidate for biomedical applications having many advantages such as lower cost of testing and detection time [79]. Magnetic nanoparticless (MNPs) can be used in conjunction with a recognition element that targets the analyte and separates them from the assay [80–82]; quantum dots (QDs) can be used as fluorescent molecules with higher signal-to-noise ratio; carbon nanotubes (CNTs) as quenchers in fluorescence spectroscometry settings [83]; nanowires as conductive thin walls that quantify concentration in cyclic voltammetry; and giant magnetoresistances (GMRs) techniques quantifies the changes of its resistance when labelled MNP bond to the analyte [84].

The length of nanowires were calibrated for optimal sensitivity during electrodeposition of nanoporous membranes; reaching LOD of 0.81 $mAcm^{-2}$ and limit of quantification (LOQ) of 2.71 $mAcm^{-2}$ [85]. Additionally, carbon nanowires <100 nm in diameter have been employed as label-free electronic biosensors for the detection of *Salmonella* with LOD of 10 CFU mL^{-1} [86][1]. Although, there is no paper showing the results on gluten sensors, there is certainly an application of electrochemistry using nanowires to implicitly sense the concentration of gluten by the change of impedance. I'm looking forward to reading one paper using that application.

QDs as fluorescent molecules have demonstrated they overcome common fluorescence problems like photobleaching and chemical degradation [87]. QDs utilized with gold nanoparticles (AuNPs) allow the detection of foodborne bacteria in samples [88, 89]. Nevertheless, the toxic nature of QDs limits their biological applications [90].

2.4.4 Aptamers-based techniques

Aptamers are peptides or more commonly single-stranded oligonucleotides that are composed of short or long synthetic ribonucleic acid (RNA) or DNA. They have been characterized to be highly sensitive, selective, and lower cross-reactive [78]. This advantages has been promoted due to its preparation protocol called systematic evolution of ligands by exponential enrich-

[1] Colony forming unit (CFU)

ment (SELEX). SELEX is a common method recognized to provide a high-affinity selection of a target within a large pool of randomized RNA [6]. Its sensitivity [91–94], selectivity [7, 95, 96], pH resistance [97], and temperature stability [98] over other methods have attracted many researchers [4, 97, 99–102] to endeavor their time into electrochemistry-based [53, 103–106], magnetic-based [107–110], SPR [111, 112], and fluorescence-based methods [7, 62, 90, 113–117] for allergen detection. Despite the shortcomings of photo-bleaching or background noise in fluorescent-based aptamer, researchers have found its competitive edge in portability, cost effectiveness, simplicity, and quick-testing attributes of the optic-based aptamer [117–119].

2.5 Current biosensor: rGO-PEG Aptamer

Gli4 aptamer was developed to target 33-mer peptide, the peptide responsible for CD reactions, rather than the whole protein [106] back in 2015. Gli4 performance was compared to its other aptamer sequences gli1, gli12, gli3, gli4, and glisq04; for which, gli4 excelled among others [120]. Thereafter, the I2Sense lab at the University of Calgary endeavored their time into implementing gli4 to their system: an aptamer-based fluorescent biosensor.

Before the setup of the aptamer in this application is shown, the basics of its functionality and the theory behind are described.

2.5.1 Aptamer-based fluorescence biosensor

As previously explained in section 2.3.2, the main functionality of a fluorescent biosensor is the fluorophore or the fluorescent molecule. Ideally, the number of fluorescent molecules emitting light at a specific moment would indicate the number of target molecules present in the sample; however, this process is not trivial. The aptamer is in charge of carrying a fluorescent molecule and bonding to its target. Once the aptamer bonds, the fluorescent molecule should be able to emit light, and not otherwise. In this manner, only those fluorescent molecules that can indirectly bond to the target would emit light, and those which didn't would not emit anything

at all; this is known as 'turn-on' [95, 96, 121]. This is achieved by the use of a quencher and the physics involved in the fluorescence resonance energy transfer (FRET) phenomenon.

FRET phenomenon

Also known as Förster energy transfer or Förster resonance energy transfer is a phenomenon that describes the behavior of energy transfer between molecules. The one that serves electronic excited states is considered the donor, and the one that receives those states is known as the acceptor. The donor may only transfer energy within a certain spectrum characteristic of each molecule. Similarly, the acceptor may only receive energy within a certain spectrum characteristic of each molecule. Once a donor meets an acceptor, the donor transfers a certain range of energy that matches the acceptor's spectrum of energy. Taking advantage of this behavior, the fluorescent molecules that are being excited due to the excitation light would donate their energy to the acceptor rather than converting it into emitting light; effectively, quenching the fluorescence [83, 122].

2.5.2 Current biosensor: Setup

MSc Suresh et al. has endeavored in a gluten quantitation application implementing gli4 aptamer [7]. He used a 6-carboxyfluorescein (6-FAM) as a fluorescent molecule attached to the 3' prime end of the aptamer. Taking advantage of the FRET phenomenon, he implemented graphene oxide (GO) as quencher or acceptor.

The FRET-based fluorescence-aptasensor functions as expected; however, GO adsorption to non-specific proteins reduces the overall performance of the biosensor. This is overcome by implementing a blocking agent. Taking advantage of polyethylene glycol (PEG) that covalently bonds to available functional groups of GO, PEG took the role of the blocking agent. Additionally, He increased the performance even further by reducing the non-specific interactions of the epoxy groups of the GO; effectively reducing it (reduced graphene oxide (rGO)).

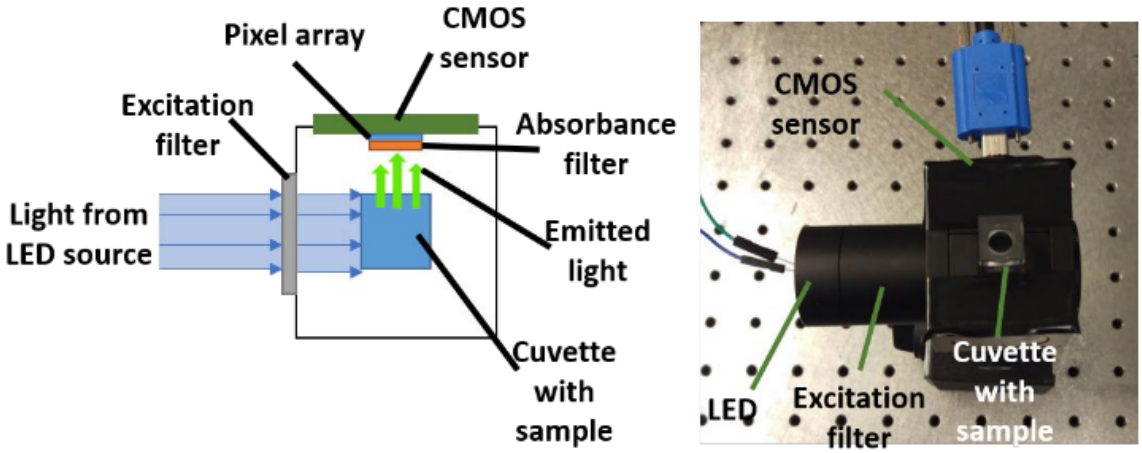

Figure 2.2: MSc Diaz et al. setup for a turn-on fluorescence gluten quantitation sensor based on the FRET effect and utilizing the gli4 aptamer [111].

As shown in Figure 2.2, MSc Diaz et al proposed a setup that included a light-emitting diode (LED) as excitation light, an excitation filter, and lenses to achieve collimated light, a sample containing a cuvette, an emission filter, and a complementary metal-oxide-semiconductor (CMOS) as a photoreceptor [115]. MSc Suresh's setup implemented the biosensor in a microfluidic system with immobilized aptamers, but the main idea of the setup remained [7].

With that setup, he studied the molecular interaction of different solvents and buffers (70% ethanol (EtOH) , phosphate saline buffer (PBS),), and assessed their performance in extracting gluten from different food samples to each other [7]. His study lead to a proof-of-concept starting-point protocol, which is described, studied and improved in Chapter 3.

2.5.3 Current biosensor: limitations

Initially, the limitations of gli4 have been indicated previously [123]. In her paper, Dr Amaya Diaz et al. reported Gli4 is unable to accurately quantify gluten concentrations in chocolate-rich samples even though fish gelatin or polyvinypyrrolidone (PVP) were added. MSc Suresh et al. have also exposed the limitations of their set-up, the aptamer-based biosensor they proposed is unable to accurately measure gluten concentrations in chocolate-rich samples either. For which, he suggested the addition of skimmed milk powder so cocoa and skimmed

milk powder aggregate, as ELISA tackles the same problem with that. The correct carrier that allows the extraction of gluten in chocolate samples is sought in this study.

2.5.4 The approach of this study

For several years, antibodies were introduced as a novel biosensor for the industry as its selectivity and sensitivity advantages over other methods at that time [124]. Years later, the combination of antibodies with enzymes allowed for the detection of allergens, from that point ELISA arose as the gold standard for allergen detection [125]. CD gathered attention during the same period as the incidence and the population of CD patients was roughly quantified [8, 10]; henceforth, the CODEX Alimentarius regulation defined gluten-free labeled foods containing no more than 20 ppm (or $mg\,kg^{-1}$). Over time, several disadvantages and limitations of ELISA were discovered as discussed in section 2.1.7 and alternatives were sought as described in section 2.2 and 2.3.

Over the years, aptamers gathered a lot of attention as described in the section 2.4.4 due to numerous advantages over other techniques. However, the instability and photo-bleaching in fluorescent-based aptamers represent a major concern for its further development [4]; Dr Röthlisberger and Hellenstein further support this concern [5]. Dr Weng and Neethirajan combated the limitations of photobleaching and background noise of low-intensity fluorescent dyes by implementing QDs as the fluorescent agent, achieving LOD of $56\,ng\,mL^{-1}$ [95]. However, further research is yet required to reduce the cost of QDs for budget and portable applications. On the other hand, the aptasensor is found to have numerous advantages which include being selective and specific; yet, at the same time unstable. This indicates the available room for study. Therefore, this study presents aims to find its reliability over time in the Chapter 4.

It has been expressed [78] that the reference material (RM) is required when assessing the reliability of an allergen quantitation method; henceforth, RM is included in the study. In order to assess the accuracy of the bioassay, the protocol of the aptamer is optimized and calibrated to the RM for augmented accuracy. Lastly, a summary is described in Chapter 5.

Chapter 3

Aptasensor protocol overhaul

3.1 Introduction

FRET-based biosensors, in general, suffer from photo-bleaching and instability [4]. Although QDs have shown a great advantage as an alternative fluorescent molecule due to their increased signal-to-noise ratio [87–89], their current price of production limit budget biological applications; including portable ones [90]. Hence, MSc Suresh et al. and MSc Diaz et al. have seen potential in using a 6-FAM as the labeling agent attached to the 3' prime end of the aptamer [7, 115].

As explained in the section 2.5.1, this phenomenon requires an acceptor and a donor. In this setup, 6-FAM behaves as the donor once it is excited by light at 490 nm. The acceptor part of the phenomenon, on the other hand, is conducted by rGO. The wide range of quenching properties from rGO provides the flexibility of being paired with multiple fluorescent molecules; however, its absorptive behavior on non-specific proteins is undesired. Hence, it leaves but only one alternative: the addition of a blocking agent. MSc Suresh et al. found PEG suffices this role [7].

Lastly, buffers are advantageous as they provide stability of pH within the solution. Although aptamers are known to be stable at pH perturbations during the complex molecular interaction, their performance may vary from buffer to buffer. MSc Suresh studied and compared 70%

EtOH, tris-EDTA (TE) and PBS performance as buffers; PBS excelled as a working buffer, as EtOH had lower hydrogen bonding between unquenched fluorophores; TE as dilution and storing buffer due to its long-term stability [7].

3.1.1 Specifications and characteristics

This experiment works with low concentrations of gluten in samples. Therefore, in order to keep the results as constant as possible, it is important to avoid any environmental or personal perturbation entering the system. Hence, gloves, hairnet, face-mask, coat, and glasses were the minimum required equipment for this purpose. In addition to that, prewashed hands, and thoroughly cleaned work areas with 70% EtOH are the daily routine. The samples were weight in a gram micro-scale with 3 decimals of accuracy and remained within ±3% of the intended weight.

The reusable equipment was autoclaved whenever possible; for example, 96 well-plate. Eppendorf's, falcon tubes, nor pipette tips were used more than once. All the fluorescence measurements were done in the same 96-well plate reader every time and the colorimetric measurements were in a photospectrometer specific for that purpose. The measurements were consistent at 9.5 mm away from the 200 μl 96 well-plate after 5 seconds of gentle shaking. As shown in Figure 3.1, the system is composed of a monochromator at 490 nm exciting the assay. As shown in Figure 3.2, the 6-FAM fluorophore absorbs around those wavelengths, which peak is at 495 nm; and emits at 520 nm. The emission and excitation light go through an emission filter, where only emission light reaches the optic receiver.

As shown in the Figure 3.2 from the database of ThermoFisher [126], the excitation maximum is at 495 nm, and the emission maximum is at 520 nm. In this study, all the excitation and emission light is centered at 490 nm and 520 nm, respectively.

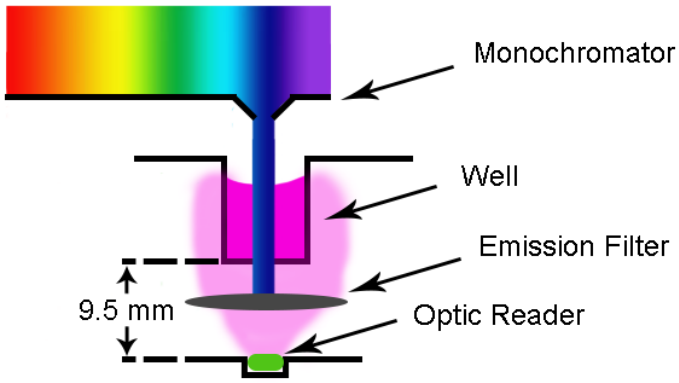

Figure 3.1: Graphical representation of the optical setup of the 96-well plate reader used for all experiment results

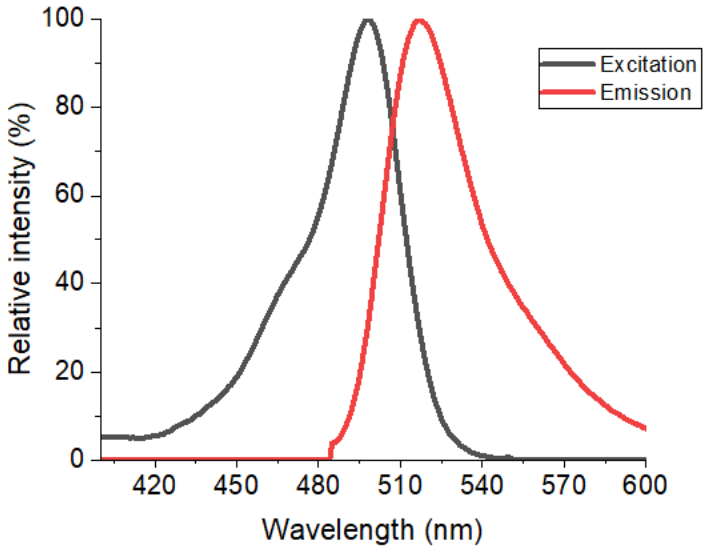

Figure 3.2: Excitation and emission wavelengths of 6-FAM, in which excitation peaks at 495 nm, and emission peaks at 520 nm.

3.1.2 Purpose

MSc Suresh et al. have shown the initial proof-of-concept setup does work based on their hypotheses; however, this study is far from realistic applications as it has to provide constant accuracy every time it is done. Thus, the purpose of this study is to find the protocol that: accurately quantifies gluten concentrations, the results are reliable within the first days of stock preparation, and the results are constant every new stock is made.

The preparation of rGO-PEG 6-FAM aptamer has already been studied by MSc Suresh et al. and it is not the focus of this chapter. here in this chapter presents the overhaul of the extraction protocol.

Although MSc Diaz et al. [115] and Suresh et al. [7] have shown different optical setups using an array of lenses, fiber optics, filters, LEDs, cuvettes, microfluidic system, and CMOSs; in contrast, this study setup uses a 96 well-plate and a normal fluorescence well-plate reader for fewer system variation.

3.2 Materials and methods

3.2.1 Materials

The gli4 DNA aptamer sequence: 5'-CCAGTCTCCCGTTTACCGCGCCTACACATGTCTGAATGCC-3'. The 6-FAM aptamer labeled in the 3' prime end was obtained from Integrated DNA Technologies (IDT www.idtdna.com) in a dried form. The 6-FAM-aptamer is diluted in TE buffer (100 μM) and stored at 253 K. TE buffer is from IDT.

The R5 antibody sandwich ELISA kit is obtained from r-Biopharm (QQPFP epitope - RIDASCREEN Gliadin R7001).

The reference material (RM) is obtained from Trilogy Labs, measured with r-Biopharm RIDASCREEN Gliadin R7001. This study refers the RM with a mean of 6.2 ± 1.5 ppm of gluten and a coefficient of variation (CV) of 24.8% as "6ppm" (Lot N°121110), the RM with a mean of

15.7 ± 3.0 ppm of gluten and a CV of 19.1% as "15ppm" (Lot N°121109), the RM with a mean of 25.0 ± 3.1 ppm of gluten and a CV of 12.2% as "25ppm" (Lot N°121103), the RM with a mean of 43.6 ± 5.7 ppm of gluten and a CV of 13.1% as "43ppm" (Lot N°121111). The GF and regular samples were collected from a local grocery store

The phosphate saline buffer (PBS), graphene oxide (GO), gliadin, molecular sieves, 4-dimethylaminopropyridine (DMAP), sodium chloride (NaCl), dimethyl sulfoxide (DMSO), carbodiimide hydrochloride (EDC), hydrazin (N_2H_4), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), and ethanol 95% are from Sigma Aldrich.

3.3 Protocols

The following protocols are referred and explained during the results section. This protocol section serves as the database for the thesis to be organized.

3.3.1 rGO-PEG

1. 50 ml of dimethyl sulfoxide (DMSO) and 15 ml of GO are sonicated for 15 minutes.
2. GO and DMSO are added in a crystal flask with a magnetic stirrer (stir is off).
3. The flask is sealed using septum and parafilm.
4. The oxygen is removed out of the flask using a syringe-tipped vacuum pump.
5. Nitrogen gas is injected into the sealed flask. This was archived using a nitrogen-filled balloon with a syringe attached and sealed with parafilm.
6. After 5 minutes, the nitrogen gas is left attached and the stirrer is turned on for 10 minutes.
7. In the meanwhile, weight 150 mg of PEG, 300 mg of 4-dimethylaminopropyridine (DMAP), and 195 mg of carbodiimide hydrochloride (EDC).

8. The PEG is dissolved in 2.5 ml of deionized water (DI) and the EDC in 2.5 ml of DI in different containers.
9. The diluted PEG and DMAP are added to the flask using a syringe.
10. The Nitrogen balloon is removed with vacuum for 5 minutes, then the vacuum is removed with the nitrogen balloon for another 5 minutes.
11. The previous step is repeated 2 more times.
12. The flask is unsealed and EDC is added.
13. The flask is sealed back with septum and parafilm.
14. The oxygen is removed out of the flask using vacuum for 5 minutes and a nitrogen balloon is injected overnight.
15. The flask is unsealed and 21 μl of hydrazin (N_2H_4) is added followed by 1 hour of stirring between 343 K to 373 K
16. The flask is set at room temperature to cool down for 10 minutes.
17. The content of the flask is added in 2 falcon tubes of 50 ml in equal quantities.
18. The falcon tubes are centrifuged for 10 minutes at 5000 rpm
19. The supernatant is removed
20. 7.5 ml of DI is added to each falcon tube and vortex for 30 sec.
21. The steps from 18 to 20 are repeated 2 more times.
22. The falcon tubes are joined in a single falcon tube and labeled "rGO-PEG".
23. At this point, the rGO-PEG is stored at 253 K.

3.3.2 rGO-PEG-Aptamer: Hybrid

1. The day before the testing day, rGO-PEG is taken out of the freezer and sonicated for 20 minutes.
2. The 6-FAM-aptamer that should be stored at
3. 1000 μl of rGO-PEG is added to a 1.5ml Eppendorf along with 100 μl of 6-FAM-aptamer.
4. The Eppendorf is gently mixed up and down overnight.
5. The Eppendorf is centrifuged for 10 minutes at 2000 rpm
6. The supernatant is removed
7. 1000 μl of TE buffer is added and vortex.
8. The steps from 5 to 7 are repeated 2 more times.
9. The Eppendorf is labeled "rGO-LED 6-FAM aptamer" or "hybrid".

3.3.3 The first gluten extraction protocol

1. The work area and tools are thoroughly cleaned with 70% EtOH
2. The food sample is ground with a mortar and pestle
3. 750 mg or 750 ml of food sample is added to a 15 ml falcon tube.
4. 3 ml of 70% EtOH is added to the falcon tube and vortex for 3 minutes; giving a current dilution factor (DF) of 5.
5. Incubate at 305K for 1 minute.
6. The compound is centrifuged at 5k rpm for 10 minutes.
7. The supernatant is collected and filtered using a syringe filter (0.45 μm).
8. 50 μl of the filtered supernatant is diluted in 450 μl of EtOH for a final 50x DF.

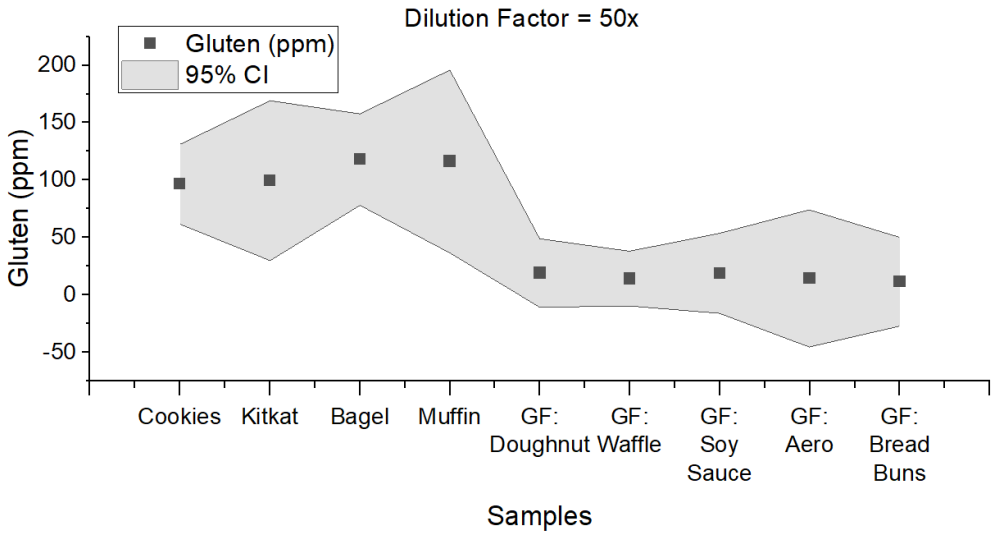

Figure 3.3: The gluten concentration of 4 GR samples (on the left side) and 5 GF samples (on the right side) are tested using the starting gluten extraction protocol.

3.4 Results

As shown in Figure 3.3, the starting protocol described in the section 3.3.3 provided the gluten concentration on off-the-shelf samples with a clear discrepancy of 90 ppm between GF (around 107 ppm) and GR samples (around 17ppm). It is not ideal to evaluate the overall gluten concentrations based solely on the discrepancy between measured fluorescence intensity of GF and GR samples, as it is required to have a solid interpretation of the fluorescence intensity with actual gluten standards to compare with. Although this could be easily automated by a numerical algorithm, the process crumbles when a single type of sample is being tested.

3.4.1 First extraction protocol: initial assessment

Although Figure 3.3 indicates that the scale of the measurements corresponds to the type of food sample, this data was not reproducible. The measurements given in Figure 3.3 refer to the protocol 1 (50x) as shown in Figure 3.4. Their accuracy were obtained by repeating the first protocol on 9 different samples, including GF and GR; this process was repeated 281 times. The average, standard deviation, and confience interval (CI) of each 9 food types were gathered and averaged to obtain the graph shown in Figure 3.4. It is improtant to note that the CV is defined

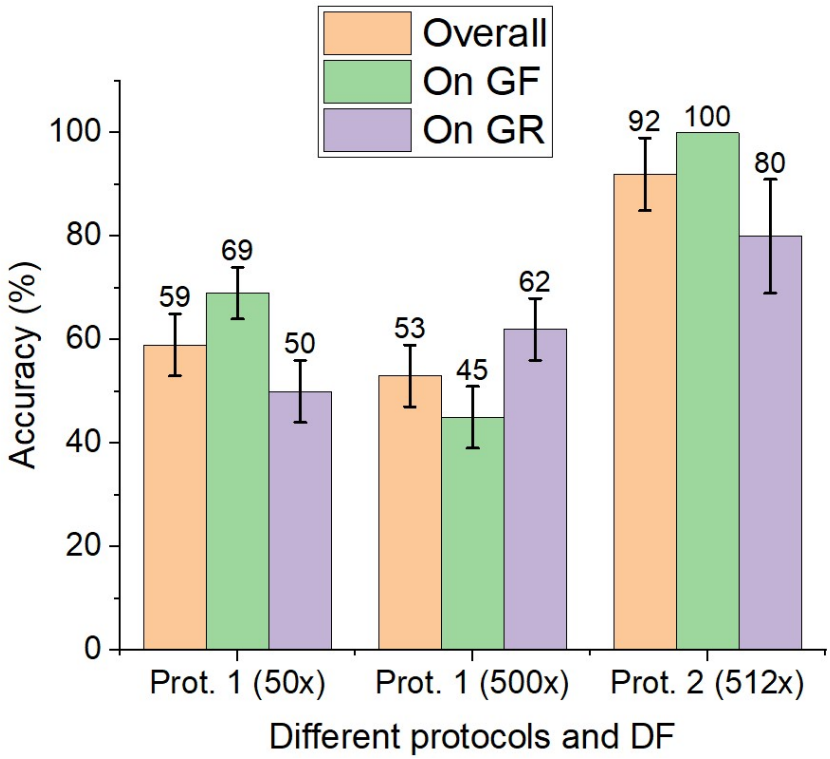

Figure 3.4: The accuracy (95% CI) of each protocol is displayed; for Prot.1 refers to the first protocol used in the study where the dilution factor (DF) is 50x and 500x; for Prot.2 refers to the second protocol based on ELISA, which has a DF of 512x. The tests are applied to 9 different off-the-shelf samples.

as the standard deviation over the average, and CI is always 95% in all repeatable experiments.

This inconsistency represents the numerous cross-reaction in the assay due to the insufficient blocking agents compared to the amount of the food sample. Hence, a further dilution was explored. As shown in Figure 3.4, a 500x DF reduced the overall accuracy. Nevertheless, the variation remains the same at 6% regardless of the DF, which indicates that the inconsistent denature of the outer layers of the agglomerated food is taking relevant in the study at hand. Resulting in an accuracy of 59% (95% CI: 53%-65%) and 53% (95% CI: 47%-59%) for the 50x and 500x DF, respectively. The accuracy quantifies from a binary response whether the bioassay correctly quantified the food sample as GF or GR based on the 20 ppm threshold. Although, it is shown that when the DF increases using protocol 1, the average measures gluten concentration is higher; thus, lower accuracy on GF. This problem is tackled by implementing an already explored protocol for gluten extraction, as explained in the next subsection.

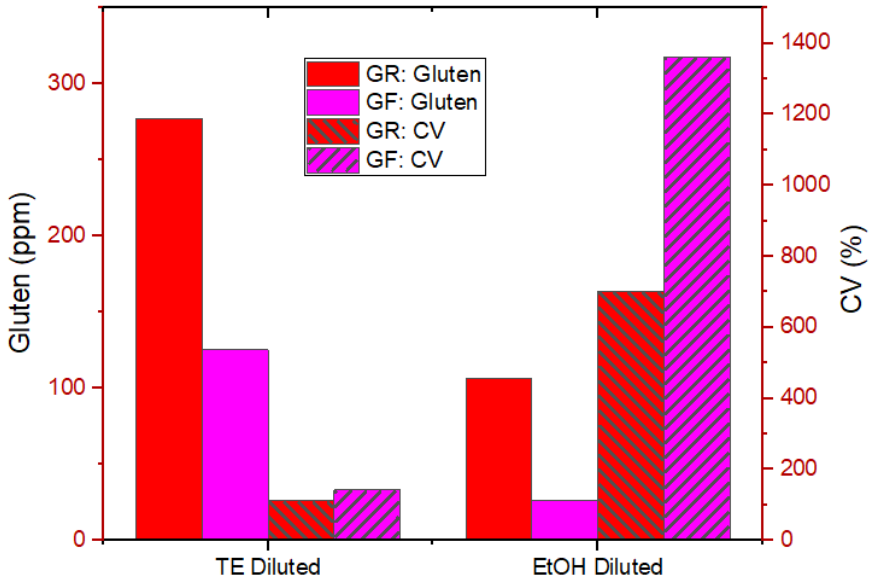

Figure 3.5: Based on the starting gluten extraction protocol, the gluten concentration and CV are assessed by changing the diluent of the step 8; where TE buffers as diluent assessment are on the left, and EtOH on the right.

3.4.2 Second extraction protocol: increasing dilution factor

The EtOH-based gluten extraction protocol in the study of Dr Fallahbaghery, Zou, Byrne et al. [127] showed that at least 85% of each gliadin peptide was extracted when 60 minutes of incubation is considered in the protocol. Additionally, the gluten extraction protocol of an ELISA kit [128] included 60 minutes of rotational shaking. It has been shown that TE buffer can provide 6-days-long stability of the substance [7],

Hence, considering the required salt concentrations the second extraction protocol was born. In this case, TE buffer is used as diluent due to the stability. Therefore, Figure 3.5 indicates the stability of quantified gluten concentrations on off-the-shelf samples; where TE buffer substitutes EtOH as the diluent in step 8 of the gluten extraction protocol described in the section 3.3.3.

In the same Figure 3.5, the gluten concentration and the CV of GF and GR samples are averaged. In the case of TE buffer as the diluent, the difference in gluten concentrations between the GF and GR is greater than EtOH as diluent. As long as this discrepancy remains

at greater dilutions, the room for false negatives should be lower; this case would only apply if the measured gluten concentrations behaves linearly to the actual gluten concentration. Nevertheless, now using the TE buffer, the gluten measurements indicate values above 100 ppm. Hence further dilutions using TE buffer ought to be used to reduce the gluten measurements, so they match the correct value using RM with fixed gluten concentration.

The second extraction protocol with a final DF of 512x has shown an overall accuracy of 92% (95% CI: 85%-99%), as they are shown in Figure 3.4. The second protocol is as follows:

The second gluten extraction protocol

1. The work area and tools are thoroughly cleaned with 70% EtOH
2. The food sample is ground with a pill pulverizer machine
3. 250 mg of powdered food sample or 250 μl of the liquid sample is added to a 15 ml falcon tube
4. 2.5 ml of ethanol 70% is added and vortex for 30 seconds (DF of 11)
5. The falcon tube is sealed and incubated in a water bath for 10 minutes at 323 K
6. The falcon tube is set outside the water bath for 5 minutes at room temperature (RT)
7. The falcon tube is unsealed and 7.5 ml of TE buffer is added and vortex for 30 seconds (DF of 3.7)
8. The falcon tube is sealed and centrifuged for 10 minutes at 5000 rpm
9. The supernatant is collected
10. 80 μl of the supernatant is diluted in 920 μl of TE buffer for an additional 12.5 DF (for a total DF of 512.5)

Implementing 40 minutes of incubation and 60 minutes of rotational stirring in the protocol has improved the results by reducing the overall variability of the gluten concentration

measurements. However, at this point, the further improvements and assessments cannot be based on the off-the-shelf samples, as they contribute with more variables; such as the expiration date, the store it was taken from, and the process of pulverization. These variables represent a risk to the fidelity of the protocol overhaul and further assessments. Henceforth, RMs are used for the protocol overhaul and analysis instead of the off-the-shelf samples until stated otherwise.

3.4.3 Homogeneous section of sample dilution

In the case of RM, the extraction protocol described in the section 3.3.3 is followed except for the step 2, as they are powdered samples.

The first step is to ensure the process is not subject to concentration variability, and only collecting the samples from regions of constant gluten concentration. This was attempted to be tackled by vortex for 5 seconds before any step before collecting or diluting; except when the sample was previously filtered by centrifuging (step 9 of the second extraction protocol). In summary, the supernatant is extracted right after the centrifuge step, then it is vortex for 5 seconds and the process is followed as shown in the second gluten extraction protocol 3.4.2. This process is repeated over 20 times to get the standard deviation and CV. Nevertheless, as shown in Figure 3.6, the gluten concentration is higher at the bottom due to the density and gravitational force. At higher points of the falcon tube, the CV of gluten concentration remains relatively constant around 8%; whereas, at lower points beyond the middle, the concentration reaches above 25%.

This study using 25 ppm and 43 ppm RM samples demonstrated that even though the sample is being vortex, the weight and bonding interactions contribute to the dissimilar concentration before the sample is collected. Although this could be combated by diluting the whole falcon tube altogether, the consumption of the reagents, the cost, and the environmental impact overweight the benefits of this approach. Hence, the measurements were preferably taken between the middle and the top of the sample.

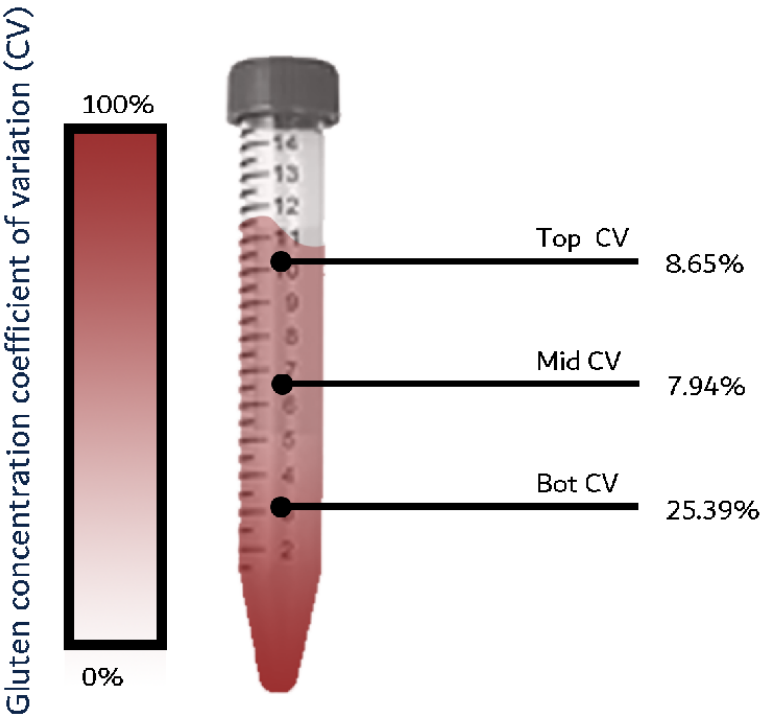

Figure 3.6: The coefficient of variation for gluten concentration inside a falcon tube of 25 ppm and 43 ppm reference materials. Where the least variation in found in the upper middle section of the falcon tube.

3.4.4 Tuning RM sample quantity for accuracy

Considering the variation is mostly contributed by the nature of the sample and the inconsistencies within the sample, both have been already tackled by changing the sample to RM and collecting from the most homogeneous part of the sample, respectively. This subsection is dedicated to identifying the food quantity that is being measured according to its labeled specification; i.e. 25ppm labeled samples should be measured to have 25 ppm on average.

A series of quantities (q): 15 mg, 20 mg, 25 mg, 30 mg, 40 mg, and 50 mg of each RM were measured and analyzed. The analysis is based on getting the average of the measured gluten concentration for each RM at a certain sample quantity. As shown in Figure 3.7, for each quantity of RM the average measured gluten concentration was contrasted to its expected concentration according to the provider of the RMs. Where $E(L)$ is the expected concentration; $\rho_{q,L}$ is the measured concentration based on the purchased RM specifications; and, L is the

labeled RM gluten concentration.

In the same Figure 3.7 when q= 15 mg, the number of bonds among the analyte and the aptamer do not match the expected standard curve; hence, the fluorescence in all cases RM concentrations is the minimum. On the other hand when q= 50 mg, the number of bonds overwhelms all the available aptamers, giving a flat and nearly constant curve for its maximum possible fluorescence. In between, the measured concentration was closest to the expected one when q= 25mg; however, only below 25 ppm are similar to the expected value. Meaning this concentration is more adequate on lower ppm samples.

The overall performance considering the accuracy and variation of all concentrations is studied. Before that, it is important to state that $\Delta\rho_{q,L}$ defines the difference between each measured sample to its corresponding expected value (E) as shown in Figure 3.8a.

Then, in Figure 3.8b, all samples are attached around their corresponding expected value $E(L)$ for each RM. According to the amount of sample (q), the average gluten concentration increments linearly as shown in Figure 3.8c, but its CV is lower in a faster motion. Concluding that assessing the performance as accuracy over $\Delta\sigma$ times CV, the assessment indicates q = 20 or 25 mg (DF of 6262 and 5012, respectively) provide the most accurate results for these settings (as shown in Figure 3.8d. Nevertheless, the overall accuracy on off-the-shelf samples decreased from 92% (95% CI: 85%-99%) when DF is 512 to 54% (95% CI: 42%-66%) when DF is 5012 as shown in Figure 3.4 and Figure 3.9, respectively. This inaccuracy is influenced by the cross-reactivity of ethanol on chocolate-rich samples; hence, this cross-reactivity is tackled by a carrier as further explained in Chapter 4.

Descriptively, food agglomeration represents a higher influence at greater DFs; hence, the overall effective area of gluten extraction reaches a constant limit. Resulting in a minimal difference of analyte recognition among all the tested RM samples as shown in Figure 3.7, where the measured gluten concentration is nearly constant at all q's. For this reason, the amount sampled is not reliable at concentrations of 50 mg (greater than 2512 DF) per sample.

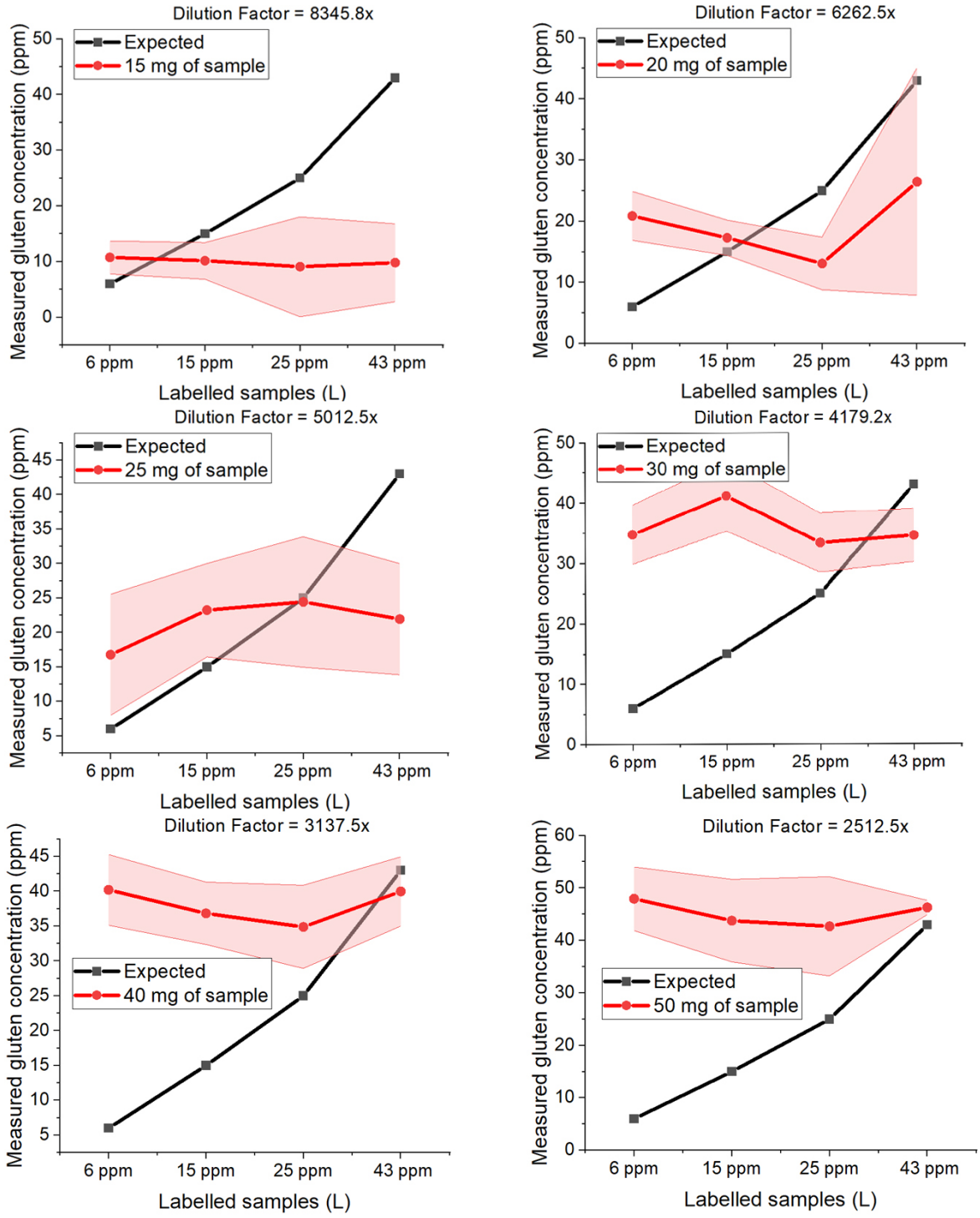

Figure 3.7: The measured gluten concentration (95% CI) was contrasted to what is expected from each labeled reference material (L). This is done 6 times with different quantities (q) of reference material.

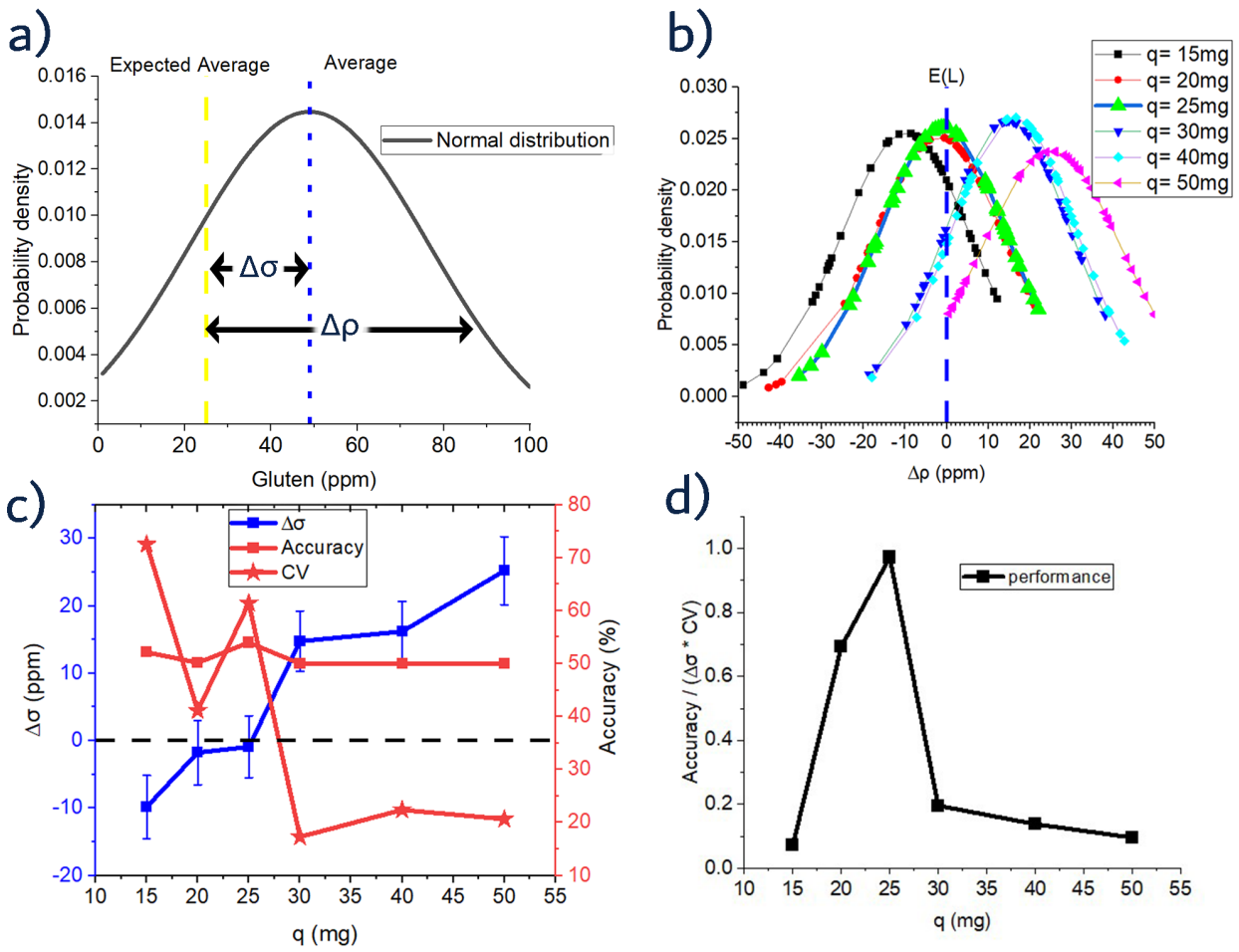

Figure 3.8: Where L is the labeled gluten concentration (in ppm, 95% CI) of the RM sample; q is the quantity (in mg) used of the RM; Average is the most probable measured gluten concentration (in ppm); $E(L)$ is the expected average gluten concentration; $\Delta\rho$ is the difference between any of the measured RM and the according expected gluten concentration; and, $\Delta\sigma$ is the difference between the measured average and the according expected gluten concentration.

3.4.5 Third extraction protocol: optimizing time

Going back to the second protocol using 250 mg (DF 512x), this time, as shown in Figure 3.9, the accuracy on 15 off-the-shelf samples is 71% (95% CI: 63%-79%). Although this is lower than what was shown previously in Figure 3.4, this graph includes 6 more types of samples.

Nevertheless, the protocol is the same; in this instance, the accuracy is lower due to the complex denature of the rGO-PEG over time. In Chapter 4, the decaying accuracy over time is detailed.

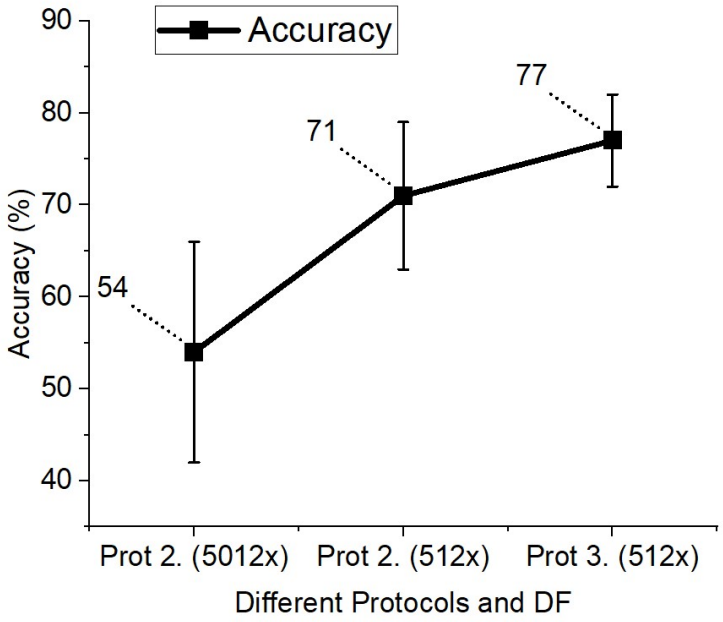

Figure 3.9: The accuracy of protocol 2 and protocol 3 (95% CI) at different DFs are illustrated. Whereas for lower DF, the accuracy is higher. The tests are applied to 15 different off-the-shelf samples.

Before that, the protocol can be optimized and enhanced by reducing the time the ethanol has to extract gluten from the sample by studying the incubation time and stirring time. The reliability of RMs are employed in this study. this time, 0, 10, 20, 30, and 40 minutes of incubation; and, 0, 15, 30, 45, and 60 minutes of stirring are assessed. As shown in Figure 3.10a, employing the previously described $\Delta\sigma$, the difference between the average gluten concentration and the expected gluten concentration is illustrated; where the lower the difference, the better. In Figure 3.10b, the standard deviation is studied, and 3.10in c, an assessment of both 3.10a and 3.10b is illustrated; where at 10 minutes of incubation and 0 minutes of stirring, the Std.dev times $\Delta\sigma$ is the lowest.

As shown in Figure 3.9, by reducing the ethanol-gliadin interaction time, low gluten-concentrated samples are less likely to be fully extracted; thus, only gluten-rich samples have enough gluten to interact with the aptamer within the given window of time. Resulting in a 6 times faster protocol, and 10% more accurate results compared to its slower version. In this case, the third protocol is the same as protocol 2 described in the section 3.4.2, but the

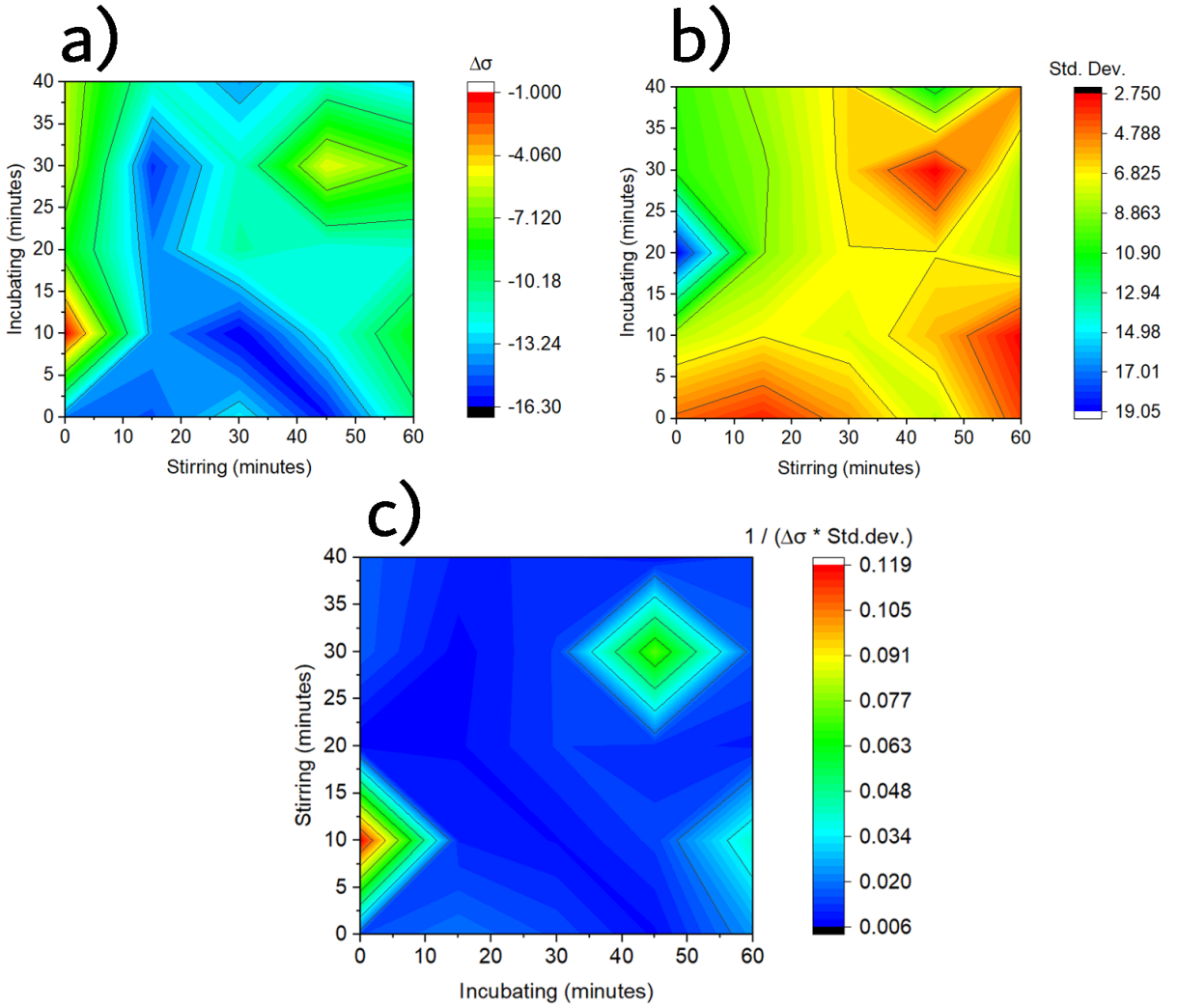

Figure 3.10: A color map of different stirring and incubation times; representing the $\Delta\sigma$ (ppm) as the difference of the gluten concentration from its expected concentration, their standard deviation (ppm), and the inverse of their product.

incubation time is changed from 40 minutes to 10, and the stirring time is changed from 60 minutes to 0.

3.5 Conclusion

RMs represent a major role in this chapter due to their reliability of the low CV in their gluten concentration. The accuracy has been changing in each version of the protocol and each DF; from 59% (95% CI: 53%-65%) in the initial protocol (version 1), 92% (95% CI: 85%-99%) in the

second version, and 77% (95% CI: 72%-82%) in the third version. Let us not be mistaken, as the accuracy decays over time. This is explained in Chapter 4. It is known that 6-FAM-aptamer biosensors are not reliable at lower concentrations [115, 116], hence QDs allow for a lower LOD [95]. Overall, the accuracy of protocol 3 was 10% and 30% more accurate than protocol 2 and 1, respectively. The most reliable DF was found to be at 512 (250 mg of the sample). In addition to that, the first and second protocols are time-consuming, and the third protocol tackles that by removing less relevant steps and achieving around 6 times faster than the other protocols, taking around 30 minutes in total.

Chapter 4

Aptasensor evaluation and comparison to standard ELISA assay

4.1 Introduction

Fluorometric aptasensor has shown great potential due to their portability and rapid-testing capabilities within a window of 10 minutes and detection limit of 0.45 $ng * ml^{-1}$, as Yildirim, Long, He et al. showed in their study [118].

NIMA, EZ Gluten, 3M Gluten Rapid Kit, and 3-D for Gluten are one of the few gluten-sensing kits or devices that have been commercialized for the end-user experience. The Canadian Celiac Association's Professional Advisory Council has declared they do not recommend the NIMA sensor as a gluten quantitation device suitable for celiac disease (CD) or any gluten-sensitive person to determine the gluten status of their food. Although their references are not clearly stated, it is assumed that they have declared their statement due to the previous studies done on NIMA. Taylor, Nordlee, Jayasena, and Baumert [129] studied the NIMA sensor and found that 75.9% of 10 ppm samples were correctly classified as gluten-free (GF), and 5.9% false negatives on gluten rich (GR) samples . Wolf, Green, Lee et al. [130] have demonstrated that although NIMA is easy to use, the waiting time of testing made them anxious. On top of

that 87% of adults reported NIMA indicated "gluten found" in products, they thought to be GF. In addition to that, they found the device and the single-use capsules to be expensive.

On the other hand, Ez Gluten takes 20 to 25 minutes in each test; representing a higher inconvenience for the end-user in a rapid-testing environment [129]. 3M Gluten Rapid Kit takes up to 12 minutes [131] and reveals 3-D for Gluten costs over 270 USD every 10 tests. All these hindrances are represented as hills stacked one on top of each other leading to a seemingly endless path of never finding the proper gluten detection device that is reliable, affordable, and trustworthy for the end-user. Hence, this study strives to develop the aptamer-based bioassay as one reliable, affordable, reusable, trustworthy, and rapid bioassay for gluten detection.

4.1.1 Purpose

Addressing false positives and false negatives

As shown in Figure 3.4, most of the inaccuracies are derived from GR samples. Hence, this chapter explores which samples represent the major concern and principal agent of false positives and negatives (overestimation and underestimation, respectively). Previously studied DNA-based aptamers have shown limitations on chocolate-rich samples; as they contain non-targeted proteins that are very similar to those we aim to quantify. In order to avoid cross-reaction, additives that isolate, filter, or react with the proteins responsible for this behavior have to be considered in the extraction protocol. Skimmed milk powder [7] or sodium chloride [132]; even polyvinypyrrolidone (PVP) and fish gelatin [106] were used to assist in the extraction of gluten in chocolate samples, yet the expected yield of extraction still behaved abnormally. Hence, this chapter includes the accuracy when different additives addressing the chocolate samples are included in the protocol.

Accuracy over time

In Chapter 3, the aptamer-based biosensor has improved from its initial accuracy of 59% (95% CI: 53%-65%) to 92% (95% CI: 85%-99%) as shown in Figure 3.4. The protocol time was reduced

to 30 minutes, yet its accuracy was reduced along too to 77% (95% CI: 72%-82%) as shown in Figure 3.9. The speed could have been increased even further if the ethanol and buffers been preheated; this approach was not studied. The concentration of the sampled food was diluted up to dilution factor (DF) of 5012, yet its accuracy was only 54% (95% CI: 42%-66%); thus, such DFs were left aside.

The same protocol 2 and the same concentration (512x) were applied to the same samples, yet the accuracy dropped from 92% (95% CI: 85%-99%) to 71% (95% CI: 63%-79%, as shown in Figures 3.4 and 3.9, respectively); only one significant variation between these 2 measurements, time. The literature indicates single-stranded deoxyribonucleic acid (DNA) based aptamers have been previously reported to be stable when stored in blood serum for more than a week at room temperature (RT) [133]; or in tris-EDTA (TE) buffer for more than 6 days at cooler temperatures (temperature not specified) [7]. The measurements providing an accuracy of 92% (95% CI: 85%-99%) were gathered within the 1st and 2nd week from the preparation of the new reduced graphene oxide (rGO)-polyethylene glycol (PEG) stock, and 8th month after the preparation of the new 100μM 6-carboxyfluorescein (6-FAM)-aptamer stock; whilst, the ones providing an accuracy of 71% (95% CI: 63%-79%) were gathered between the 8th week and 10th week after rGO-PEG preparation, and within the 2nd month from the 6-FAM-aptamer preparation. This suggests the accuracy is not dependent on the 6-FAM-aptamer, but somewhat dependent on the rGO-PEG conjugate decomposition. Hence, this chapter explores the accuracy over time since the preparation of the rGO-PEG.

4.1.2 Final extraction protocol

1. The work area and tools are thoroughly cleaned with 70% ethanol (EtOH)
2. The food sample is ground with a pill pulverizer machine
3. 250 mg of powdered food sample or 250 μl of the liquid sample is added to a 15 ml falcon tube

4. 100 mg of activated charcoal/molecular sieves or 250 mg of skimmed milk powder is added in the same tube
5. 2.5 ml of ethanol 70% is added and vortex for 30 seconds (DF of 11)
6. The falcon tube is sealed and incubated in a water bath for 10 minutes at 323 K
7. The falcon tube is set outside the water bath for 5 minutes at RT
8. The falcon tube is unsealed and 7.5 ml of TE buffer is added and vortex for 30 seconds (DF of 3.7272)
9. The falcon tube is sealed and centrifuged for 10 minutes at 5000 rpm
10. The supernatant is collected
11. 80 μl of the supernatant is diluted in 920 μl of TE buffer for an additional 12.5 DF (for a total DF of 512.5)

4.2 Results

In chapter 3, the protocol overhaul was augmented from 59% (95% CI: 53%-65%) to 77% (95% CI: 72%-82%); yet, malt vinegar, GF soy sauce, nor GR soy sauce were tested. Thus, in the following results, these samples are included in the analysis. Considering the previous motioned samples into the overall assessment, the accuracy falls from 77% (95% CI: 72%-82%) to 72% (95% CI: 65%-79%), as shown in Figure 3.9 and Figure 4.2, respectively.

That said accuracy is only achieved due to the tuning of the DF and sample quantity. As explained before, the more sample is within the assay, the faster the reaction between the ethanol and the gluten. As shown in Figure 4.1, the measured values were tuned at difference quantities and DFs; where 250 mg showed the closest values to the expected according to the reported values from the reference material. It is important to note, that there are not enough

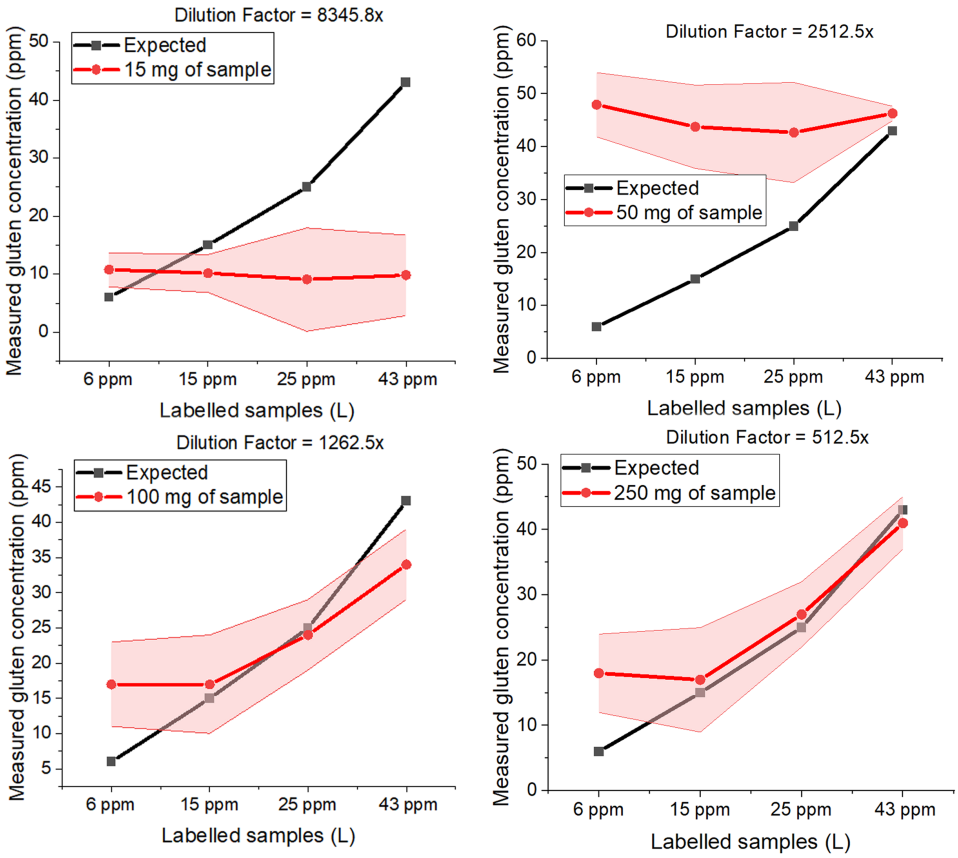

Figure 4.1: The gluten concentration (95% CI) of reference material contrasted to each other. From 15 mg, 50 mg, 100mg to 250 mg. Where the measured values are tuned and found that when 250 mg and the dilution factor is 512.5, the results lay within the expected value according to the reported reference material.

experiments to conclude only 250 mg is the best value. Hence, further tests ought to be done to find a better results, principally at lower concentrations (6 ppm).

4.2.1 Reduced false-negatives by additive integration

Activated charcoal

Activated charcoal has been used to reduce the bio-availability of peanut protein by adsorbing the proteins into the porous sites of the activated charcoal surface within the first minute [134], at any pH between 2 and 7 [135]. Hence, should the chocolate protein interact with activated charcoal more than the target proteins, then activated charcoal could be filtered out and

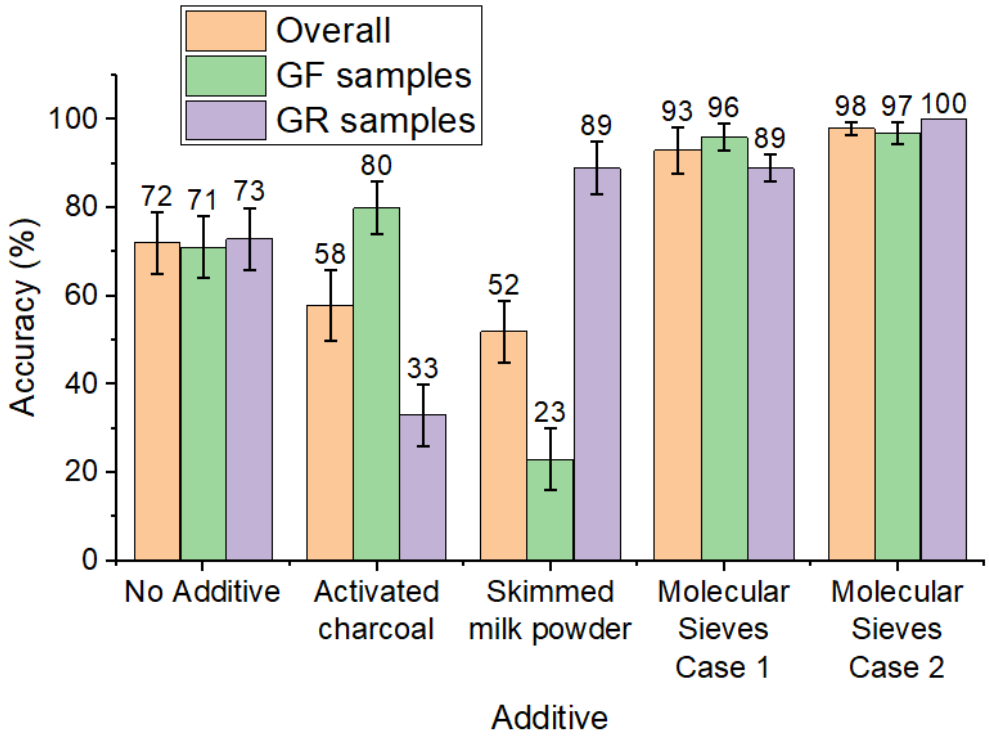

Figure 4.2: The accuracy (95% CI) of correctly classifying gluten-free and gluten-rich samples accordingly has been assessed when different additives are included in the final gluten extraction protocol. In the case of Molecular sieves case 1, molecular sieves were included indiscriminately; and in case 2, molecular sieves were included in all samples but crackers.

increase the overall accuracy of both GF and GR samples. Nevertheless, as shown in Figure 4.2, the accuracy is 58% (95% CI: 50%-66%) when it is used as an additive. In this case, the activated charcoal bonds indiscriminately with all gluten-related proteins, including those intended to quantify. Considering false-negatives are critical for gluten-sensitive people, the accuracy of GR samples is the most important result. Hence, activated charcoal has been set aside.

Skimmed milk powder

It has been reported that enzyme-linked immunosorbent assay (ELISA) allergen detection in chocolate food samples is augmented with skimmed milk powder or fish gelatin [136]. R-Biopharm has included in their R5 ELISA kit that chocolate, coffee, cocoa, chestnut flour, buckwheat, millet, and spices are to be sampled along with skimmed milk powder to reliably measure gluten in food samples [128]. Dr Amaya Diaz et al. have worked with the sample

aptamer of this study (gli4), and concluded that polyvinypyrrolidone (PVP) nor fish gelatin augmented the gluten extraction [106]. MSc Suresh et al. concluded that gluten extraction from polyphenols and tannins such as chocolate, spices, coffee, and chestnut flour can be assisted by 50% w/w of skimmed milk powder [7].

As shown in Figure 4.2, the addition of 50% w/w skim milk powder in the protocol 4.1.2 does increase the measured gluten concentrations on both GF and GR samples. Leading to reduced false negatives, but increased false positives. It can be considered an improvement, yet not ideal. Hence, skimmed milk powder may work for ELISA due to their target epitope, but it certainly does not improve the accuracy of the DNA-based aptamer (gli4).

Molecular sieves

Molecular sieves have not received enough attention in allergen detection applications; yet Dr Poms and Anklam have implemented molecular sieves as membranes able to filter out molecules by their molecular weight; thus, retaining molecules >50 kDa [137].

In Figure 4 (Case 1) molecular sieves were given a chance to shine in this allergen detection application and excelled by giving an overall accuracy of 93% (95% CI: 88%-98%), yet false negatives are still a concern. As shown in Table 4.1, the addition of molecular sieves 4A (pore size 4 angstromgs) does reduce the standard deviation and corrects the expected gluten concentration in both GF and GR samples. However, GR crackers behaved differently, avoiding molecular sieves when testing crackers is necessary.

Thus, defining case 2 as the use of molecular sieves on all samples but crackers results in an augmented accuracy of 100% (95% CI: 100%-100%) on GR samples, and 97% (95% CI: 94%-100%) on GF samples (Figure 4). Using molecular sieves cleverly on the aptamer bioassay (and disregarding the measurements of soy sauce as their values are extreme), the average deviation is 2.18 ppm on GF samples, and 4.82 ppm on GR samples; whereas, ELISA average deviation is 4.60 ppm on GF samples, and 10.18 ppm on GR samples (disregarding the measurements of crackersas they are quite extreme). It is important to emphasize that the results are based

on the 18 tested off-the-shelf samples described in Table 4.1; including samples that ELISA has difficulty with, soy sauce, and malt vinegar. ELISA measured GR soy sauce and GR malt vinegar as GF, which indicates the potential implications for gluten-sensitive people.

Molecular sieves, in this case, have been employed not only to filter out the complex based on the molecular weight but also to further smash into smaller pieces the food samples due to their rough composition during vortex steps. Then, their dimensions allowed for a simple filtration step of the sieves.

4.2.2 Accuracy over time

Considering only the preparation day of rGO-PEG, every day counted towards the assessed accuracy as shown in the x-axis of Figure 4.3. During 8 weeks of each run, 4 runs were assessed and averaged to quantify the accuracy of the aptasensor over time. The settings of each run and each test were not the same, as this was not the scope of the study. The correlation of the accuracy over time was found at the late stage of the study due to the inconsistent correlation of the accuracy in the same settings but at different times.

This was found and described in the chapter 3, where the accuracy decreased from 92% (95% CI: 85%-99%) to 71% (95% CI: 63%-79%) using the same samples, same protocol, and same concentrations, but several weeks apart from each other.

Week 0 is considering the accuracy of the first 3 days. Each week considers all 7 days of the week, and are counted since the preparation day of the rGO-PEG. It is perceived in Figure 4.3 that in the first 4 weeks, the accuracy on off-the-shelf samples remains around 85% during the first 4 weeks before reaching 63% at the 5^{th} week; whereas, the accuracy on reference material (RM) quickly falls after the 1^{st} week from 90% to 63% in the 3^{rd} week. Nevertheless, when testing off-the-shelf samples, false-negatives remain around 5% in most of the weeks before reaching 31% in the 5^{th} week.

It is important to reiterate that the focus study was not revolving around finding the accuracy over time; thus, results are altered by the variation of the protocol used at certain points of

Table 4.1: Gluten quantitation of reference material, gluten-free and regular food samples using aptamer-based sensor and ELISA

Gluten concentration of each sample				
Samples		**Aptasensor**		**ELISA**
		Without Sieves	**With Sieves**	**Without Skim milk**
		Gluten (ppm)	***Gluten (ppm)***	***Gluten (ppm)***
RM	6ppm	18 ± 6	19 ± 6	6 ± 1
	15ppm	24 ± 12	17 ± 12	19 ± 6
	25ppm	23 ± 7	27 ± 9	25 ± 1
	43ppm	38 ± 10	41 ± 8	53 ± 12
Gluten Free Samples	Crackers	4 ± 2	6 ± 1	5 ± 4
	Buns	5 ± 3	4 ± 1	7 ± 3
	Blueberry Waffle	3 ± 4	4 ± 5	25 ± 27
	Waffle	3 ± 3	1 ± 2	3 ± 2
	Bagel	5 ± 2	5 ± 3	1 ± 1
	Chocochip cookie	22 ± 15	13 ± 1	4 ± 3
	Choco doughnut	14 ± 11	1 ± 2	9 ± 5
	Aero	14 ± 11	18 ± 3	11 ± 10
	Soy sauce	111 ± 129	16 ± 2	5 ± 1
Regular Samples	Crackers	36 ± 7	12 ± 1	313 ± 121
	Annas	35 ± 6	37 ± 2	192 ± 30
	Hotdog bun	28 ± 10	34 ± 7	131 ± 23
	White bread	27 ± 9	36 ± 5	121 ± 14
	Beagle	35 ± 16	49 ± 6	135 ± 19
	Chocobar	44 ± 9	55 ± 8	2 ± 2
	Kitkat	29 ± 9	28 ± 3	196 ± 25
	Soy sauce	8240 ± 6093	15261 ± 640	3 ± 3
	Malt Vinegar	27 ± 15	32 ± 1	3 ± 1

the study. Yet, the graph should provide a broad idea of the behavior, and open the door to an expected analysis that describes each aptamer's reliability over time.

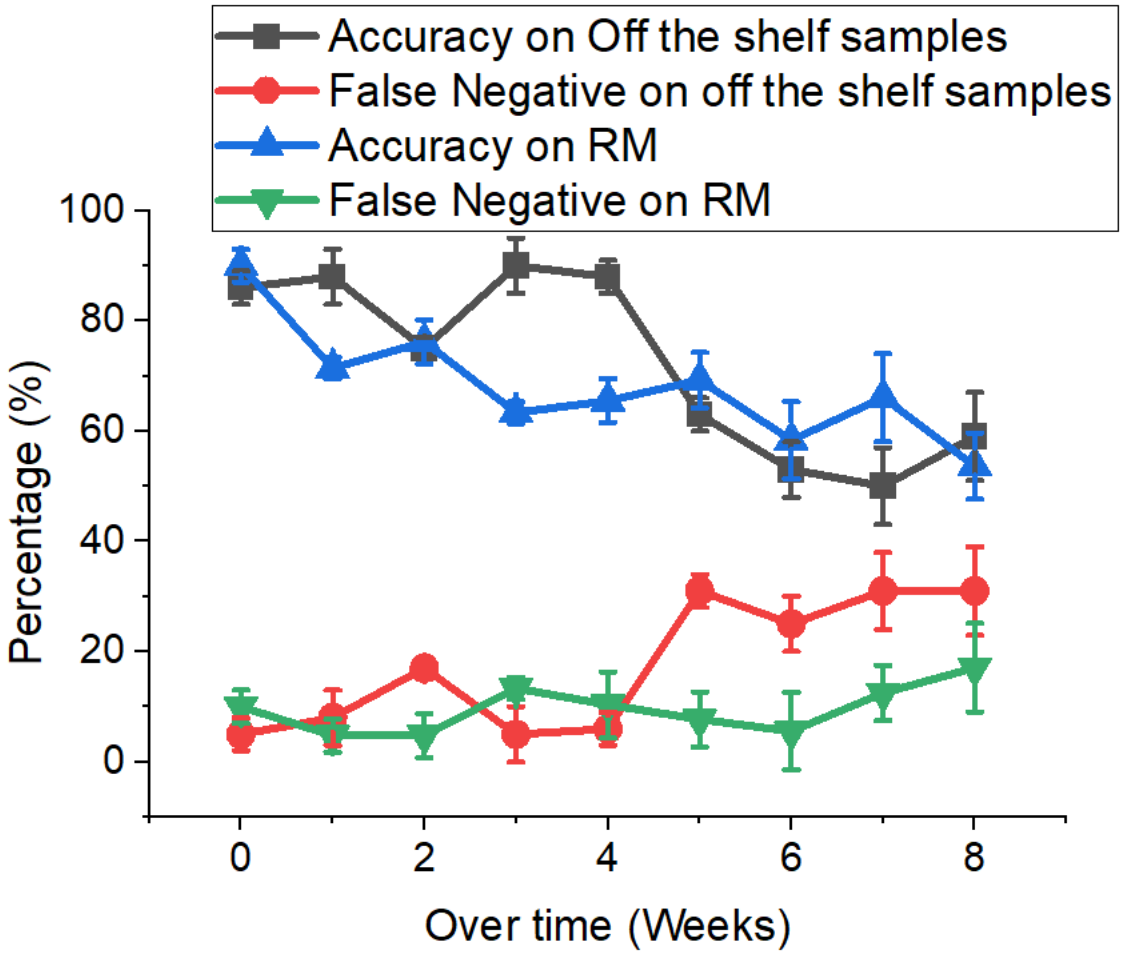

Figure 4.3: 836 subsamples were tested and assessed by the accuracy of the measurement (95% CI). Aptasensor's accuracy over time (in weeks) were tested on: Reference material (RM) and Off the shelf samples; the false negatives of each one were also included. The first 3 days since the synthesis of the rGO-PEG were considered as week 0.

4.3 Conclusion

This chapter explored different additives addressing the inaccurate measurements of gluten concentration in chocolate samples: activated charcoal, skimmed milk powder and molecular sieves.

- Activated charcoal was employed due to its porous surface capable of trapping proteins; yet, an increase of false-negatives resulted from its implementation.
- Skimmed milk has been utilized by ELISA for several years as it has shown results of

increased affinity; yet, cross-reaction was prominent in both GF and GF.

- Lastly, molecular sieves were utilized as membranes filtering the complex by its molecular weight; an augmented overall accuracy of 98.28% is perceived; where false-negatives were reduced to 0% if no molecular sieves were implemented in crackers. Additionally, molecular sieves were able to allow the correct measurement of not only chocolate samples but also soy sauce and malt vinegar; samples R5 ELISA cannot. The unexpected behavior of crackers with molecular sieves suggests there is room to explore before stating this additive and rule can be applied to all samples.

During the study, the accuracy was found to drastically change even in the same settings; thus, an additional study aiming to find the overall behavior of the aptamer during 8 weeks was displayed. This study showed that the aptasensor was reliable during the first 4 weeks around 85% before decreasing to 63%; and, the false-negatives behaved accordingly in the first 4 weeks around 5%. However, this study in particular used a combination of all the different settings used over 8 months. Thus, the illustrated graph indicates only the broad idea of the aptasensor; yet, opens the door for a future study revolving around the reliability over time of each aptamer.

Chapter 5

Summary

This study reflects the metaphorical correlation of the dark matter to the yet unknown field that we know exists but represents most of the knowledge. Acknowledging the technological advances from times were machinery was a delusional dream to the modern civilization, knowledge has allowed us to use as an instrument the very composition carrying ancient information: deoxyribonucleic acid (DNA). Such technologies have evolved to numerous applications that can be considered breathtaking from a naive perspective, such as myself; after all, the very desire for knowledge and understanding is one of the main reasons why humans are human; yet, this study focuses only on DNA aptamer, and its biomedical application for gluten quantitation in food samples.

The aptamer was subject to an application based on thefluorescence resonance energy transfer (FRET). Reduced graphene oxide (rGO) is utilized as the acceptor of excited states; polyethylene glycol (PEG) conjugates rGO for reduced non-specific bonding; and 6-carboxyfluorescein (6-FAM) is both, the donor of the excited states, and is labeling the aptamer for fluorescence quantitation. The overall system is often referred to as a 'turn-on' fluorescence aptasensor.

Although a gold standard for gluten quantitation currently dwells among food-safety industries and regulatory laboratories, false-negatives on fermented, hydrolyzed, colorized and heated samples, the lack of gluten-free (GF) products in convenience stores, and the lack of GF

version of the gluten rich (GR) ingredients are the major concerns that are inherited due to the limitations of the current gold standard. Few alternatives have been released attempting to satisfy the needs of gluten-sensitive people as an end-user device; yet, third-party assessments of the devices have proven questionable performance from a perspective of the trustworthiness and reliability value it conveys. For those that are brave enough to rely on a device that is currently striving for accuracy, these devices have been employed to rather provide a second validation of the currently consumed GF products.

Hence, this study is fueled by the importance of the reliability and long-term stability of the application. In chapter 3, the protocol was torn apart and studied to find the proper values of each step so that the overall accuracy and false-negatives were ideal. Dilution factors (DFs) ranging from 50 to over 5000 were explored, for which 512 was found to be optimal. A matrix of different incubation times and stirring times revealed stirring had not a big contribution to the overall performance, but rather a burden as a time-consuming step. Only 10 minutes of incubation would suffice for the ethanol to extract the gluten from the denatured food; yet, this process time could be suppressed should the ethanol and buffers were preheated; this was not studied, but rather speculation for future research. This study is not finished yet, even if the accuracy was improved to 92% (95% CI: 85%-99%) from its initial 59% (95% CI: 53%-65%).

In chapter 4, additives were employed to elude cross-reaction to non-targeted proteins. Activated charcoal interacted with all targeted proteins due to its porous surface, thus increasing false-negatives. Skimmed milk powder is usually included in some gluten-quantitation kits; yet, the aptamer reacted unfavorably with it. Molecular sieves were implemented as membranes filtering the sampled complex by its molecular weight, where 50>kDa molecules are filtered out. This approach was tested on 18 different off-the-shelf samples, composed of 9 GF and 9GR products including soy sauce and malt vinegar. Molecular sieves excelled at their employment providing a remarkable 0% false-negatives and 97% (95% CI: 94%-100%) accuracy on GF products. Where the accuracy is considered as the ratio of which the biosensor is correctly classifying the product; and, false-negatives as the ratio of wrongfully classifying GR products

as GF.

The impressive performance is soon set into trial after drawbacks are found. First, it is utterly important to affirm the study is far from completion, the biosensor has to be limit-tested with all types of food samples. Currently, the biosensor may use molecular sieves as additives for augmented accuracy; yet, they should not be used with crackers, according to the results from this study. Lastly, the biosensor accuracy decreases after the first week from its outstanding performance to 63%, and the conjugate rGO-PEG is the main indicator of this drawback. Thus, further research has to be employed in finding an alternative or additive to the conjugate for a larger lifespan and stability. Nevertheless, false-negatives remain around 5% during the first 4 weeks.

Hence, going back to the Hypotheses:

1. The first hypothesis talks about adding a secondary blocking agent for cacao so the overall accuracy improves. In this case, molecular sieves worked as filters rather than blocking agents. And as stated before, molecular sieves did improve the overall accuracy on GF and GR samples, and removed cacao cross-reaction from the assay. In conclusion, the first hypothesis is correct.

2. The second hypothesis states the aptamer-based biosensor can measure gluten concentrations in fermented food samples. Although, this was achieved using molecular sieves on soy sauce, this does not imply the bioassay is capable of sensing gluten in all the other fermented food samples. A complex and larger experimentation aiming to test all possible combinations of fermented samples ought to be tested before affirming it works with them. In conclusion, there is not enough data to judge it.

3. Lastly, the third hypothesis states the overall testing speed can be increased without addition additional reagents nor compromising the accuracy of the I2Sense aptamer-based sensor. The overall testing speed was reduced from the protocol v1 to the protocol v3, in which instead of taking 4+ hours, it takes 30 minutes. Although later on the process

was also altered with molecular sieves, for higher accuracy, the overall accuracy was increased without molecular sieves. In conclusion, the hypothesis is correct.

My contributions convey in a smaller though of increasing the accuracy of the bioassay and reducing the time by cleverly tuning the parameters of the protocol, which is sometimes overlooked. The process if relatively longer than expected, taking over 8 months of testing; analyzing data, changing parameters 1 by 1, comparing the alternatives just as machine learning would do. Nevertheless, machine learning can do that simultaneously, while this is much slower and resources ought to be taken into consideration. I would not be surprised if the exposed protocol is improved, in fact, I encourage everyone to do it. I was faced with the fact time goes by fairly quick and I was to utilize the time I had the most I could by assuming based on patterns of where would be the improvement.

I am proud to say this study has shown that a portable application using FRET-based aptasensor for all types of samples is a viable course of action. After all, being part of the research community exploring the battlefield of the unknown to find an application for the greater good is...

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