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DIPLOMARBEIT

Microfluidic Protein Analysis of Human Breast Milk – From Colorimetric Assays to Lab-on-a-Chip Analysis

ausgeführt am

Institut für Chemische Technologien und Analytik

der Technischen Universität Wien

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ABSTRACT

Human breast milk and its feeding are generally recognized to be the best form of nutrition for infants and neonates, as it is exhibiting many hundreds to thousands of constituents that do not only play a nutritional role but are also contributing to a lot of different immunologic and growth promoting properties. Human breast milk constituents are protecting against infection and inflammation and are furthermore contributing to immune maturation, organ development, and healthy microbial colonialization. As the composition of human breast milk is not constant over time but highly affected by various factors, a sufficient nutrient intake of the baby is not necessarily guaranteed by breastfeeding. Especially the extraordinary requirements of preterm infants, which are particularly vulnerable combined with the fact that the mother's milk is not fully developed because of the preterm birth, have to be nourished by an enrichment of human breast milk with infant formulas. As there are several studies reporting disadvantages in feeding infant formulas, starting with a substandard risk to be contracted by necrotizing enterocolitis, up to diverse unequal neurological and immunological outcomes in comparison with infants that were exclusively fed human breast milk. For fast routine analysis for the estimation of macronutrients in human breast milk is required directly at the point-of-care, to ensure a careful handling of infant formula.

The Neonatology of the AKH Vienna is in need for an easy, fast and accurate possibility for the quantification of macronutrients in human breast milk for personalized fortification to meet in particular the special needs of preterm infants. To handle these requirements the Institute of Electrical Engineering and IT wants to develop a microfluidic device based on a microchip design developed at the same institute.

During this work three different methods for the analysis of proteins in human breast milk were compared not only against each other but also to a commercial human milk analyzer called MIRIS. UV/Vis spectroscopy based on the Bromophenol Blue and the Bradford Assay as well as a capillary gel electrophoresis based method – the Agilent Bioanalyzer P230 Assay – were tested as alternatives. This work is revealing differences between the individual methods as well as potential cross-sensitivities with other human breast milk constituents, eventually disturbing protein quantification. A further subject of this work is the transfer of the Bradford Assay onto the microfluidic device.

KURZFASSUNG

Muttermilch gilt allgemein anerkannt als die beste Form der Ernährung für Säuglinge und Frühgeborene. Die zu Assayerten bis tausendenden enthaltenen verschiedenen Bestandteile, spielen nicht nur in der Ernährung der Säuglinge eine entscheidende Rolle sondern erfüllen darüber hinaus unzählige verschiedenen Eigenschaften die zum Wachstum und der Entwicklung des kindlichen Immunsystems beitragen. Die einzelnen Muttermilchbestandteile dienen unter anderem dem Schutz vor Infektionen und Entzündungen, des Weiteren regen sie die Reifung des Immunsystems sowie Organentwicklung an und tragen zu einer gesunden mikrobiellen Kolonisierung bei. Da die Zusammensetzung von Muttermilch über die Zeit nicht konstant ist und darüber hinaus durch vielfältige Faktoren beeinflusst wird, ist eine ausreichende Nährstoffzufuhr des Kindes im Zuge des Stillens nicht notwendigerweise garantiert. Insbesondere die speziellen Bedürfnisse von Frühgeborenen, welche zum einen besonders verletzlich sind, gepaart mit der Tatsache dass die Muttermilch bedingt durch den zu frühen Zeitpunkt der Geburt noch nicht vollständig entwickelt ist, müssen durch eine Anreicherung der Muttermilch mit Nahrungsergänzungsmitteln für Säuglinge gewährleistet werden. Vielfältige Studien zeigen Nachteile der Ernährung von Kindern durch Säuglingsnahrungen auf, angefangen bei einem erhöhten Risiko der Erkrankung an Nekrotisierende Enterokolitis, bis hin zu diversen neurologischen und immunologischen Störungen. Um diesen Herausforderungen sowie dem sorgfältigen Umgang mit Säuglingsnahrung gerecht zu werden ist ein schnelles, möglichst genaues System zur Routineanaylse von Nährstoffen in Muttermilch erforderlich.

Die Neonatologie des AKH Wien benötigt ein solches System, zur einfachen, schnellen und genauen Quantifizierung von Nährstoffen in Muttermilch um sicherzustellen, dass eine gezielte personalisierte Anreicherung erfolgen kann. Zu diesem Zweck möchte das Institut für Elektrotechnik und IT einen mikrofluidischen Chip entwickeln, basierend auf einem mikrofluidischen Design welches an demselben Institut konzipiert wurde.

Im Zuge dieser Arbeit wurden drei verschiedene Methoden für die Analyse von Proteinen in Muttermilch nicht nur untereinander sondern auch gegen einen kommerzieller Muttermilchanalysator MIRIS verglichen. UV/Vis-Spektroskopie basierende Methoden (Bromphenolblau/Bradford Assay) sowie eine Kapillargelelektrophorese basierende Methode - der Agilent Bioanalyzer P230 Assay wurden als Alternativen getestet. Diese Arbeit befasst sich darüber hinaus mit den Unterschieden der einzelnen Methoden sowie möglichen Querempfindlichkeiten der Methoden mit anderen Muttermilchbestandteilen, welche die Proteinquantifizierung stören. Ein weiterer Gegenstand dieser Arbeit befasst sich mit dem Transfer des Bradford Assays auf den mikrofluidischen Chip.

DANKSAGUNG

An dieser Stelle möchte ich mich gerne bei Prof. Dr. Martina Marchetti-Deschmann bedanken, zum einen für die Aufnahme in ihre Arbeitsgruppe Omics Technologies sowie zum anderen für ihre freundliche, intensive sowie produktive Unterstützung und Betreuung während dieser Arbeit. Mein Dank gebührt des Weiteren Victor U. Weiss, der mir ebenfalls bei der Durchführung dieser Arbeit immer mit Rat und Tat zur Seite stand sowie für seine Geduld und Mühe.

Ein weiteres großes Dankeschön geht an alle Mitglieder der Arbeitsgruppen Omics Technologies sowie Bio- und Polymeranalytik von Associate Prof. Martina Marchetti-Deschmann sowie Univ.Prof Günter Allmaier für die nette Atmosphäre in der Gruppe, für die gute Zusammenarbeit, für Ratschläge sowie Hilfestellungen und für die schönen Momente die wir zusammen erlebt haben. Ich danke besonders Christina Bliem, Gerhard Krejcy, Rudolf Bednar und Victoria Dorrer für eure fachliche und persönliche Unterstützung.

Auch Anna Haller sowie dem Institut für Elektrotechnik und IT möchte ich an dieser Stelle ein herzliches Dankeschön aussprechen, für die Möglichkeit an ihrem interessanten und innovativen Projekt mitarbeiten zu dürfen.

Ebenfalls bedanken möchte ich mich bei der Neonatalogie des AKH Wiens, insbesondere bei Prof. Dr. Nadja Haiden und Dr. Alexandra Kreissl, für die reibungslose Zusammenarbeit und für das zur Verfügung stellen des MIRIS Systems sowie von zahlreiche Muttermilchproben.

Ganz besonders bedanken möchte ich mich bei meiner Familie und Freunden, insbesondere bei meinen Eltern sowie bei meinem großen Bruder, für ihre unermüdliche Unterstützung, ihren Rückhalt und ihren Glauben an mich.

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
BMPM	Bovine milk protein mix
$CaCl_2$	Calcium chloride
CMC	Critical Micellar Concentration
DNA	Deoxyribonucleic acid
DTT	Dithiotreitol
EOF	Electroosmotic flow
FDA	Food and Drug Administration
H_3PO_4	Phosphoric acid
HMA	Human Milk Analyzers
HAMLET	Human α-lactalbumin Made Lethal to Tumor Cells
HMO	Human Milk Oligosaccharides
HMPM	Human milk protein mix
HMPP	2-hydroxy-2-methypropiophenone
IDF	International Dietary Federation
IgA	Immunoglobuline A
IgG	Immunoglobuline G
IgM	Immunoglobuline M
IR	Infrared
ISO	International Organization for Standardization
LBW	low-birth-weight infants
LOD	Limit of Detection
LOQ	Limit of Quantification
NEC	Necrotizing Enterocolitis
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG-DA	polyethylenglycol diacrylate
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
sIgA	secretory immunoglobuline A
UHQ	Ultra-high quality water
VLBW	very-low-birth-weight infants

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1. Introduction

1.1 Motivation – Aim of the work

Breastfeeding is recognized to be the best form of nutrition for neonates and infants, providing an ideal basis for the physical, mental and emotional development of the child. Human breast milk is a unique, species-specific, highly complex and dynamic nutritive fluid containing Assayreds to thousands of nutritional and bioactive constituents that protect against infection and inflammation and contribute to immune maturation, organ development, and healthy microbial colonialization [1].

However, due to a lot of different factors affecting the composition of human breast milk, a sufficient nutrient intake of the baby is not necessarily guaranteed by breastfeeding. Therefore often supplements have to be added to human breast milk to ensure an adequate nutrition of the child. Especially in the case of preterm infants, the mother's milk is often not fully developed and therefore does not provide the quantities of nutrients that preterm infants actually need. But to get an idea about how much of the supplement is actually needed, a fast routine analysis for the estimation of macronutrients in human breast milk is required.

In collaboration with the Neonatology of the AKH Vienna and the Institute of Electrical Engineering and IT first studies for an easy, fast and accurate possibility for the quantification of macronutrients in human breast milk were performed, to be able to do personalized instead of blind fortification to meet in particular the special nutritional and developmental supporting needs of preterm infants.

To cope with this requirement the Institute of Electrical Engineering and IT wants to develop a microfluidic device suitable for point-of-care diagnostics in clinical applications based on a setup that was developed at the same institute.

1.2 Human Breast Milk composition

Human breast milk is a system designed to deliver nutrients and nonnutritive messages to the neonate and therefore contains a number of complex proteins, lipids, carbohydrates and other biologically active components. The composition changes during the different stages of lactation as well as during a single feed. In table 1 the average nutrient composition of human breast milk in three different stages of lactation is given [2]. However, it is obvious that the composition of human breast milk is not homogenous but highly variable over time and also among women. The composition is changing in response to the infant's requirements according to its age and other characteristics.

Table 1: Average nutrient composition of human breast milk (g/100 mL; mg/100 mL, µg/100 mL and kcal/100 mL) in three different stages [2]

Nutrient	***Human Milk, Colostrum***	***Human Milk, transitional***	***Human Milk, mature***
Water, g	88.2	87.4	87.1
Protein, g	2.0	1.5	1.3
Fat, g	2.6	3.7	4.1
Carbohydrate, g	6.6	6.9	7.2
Energy, kcal	56	67	69
Na, mg	47	30	15
K, mg	70	57	58
Ca, mg	28	15	34
Mg, mg	3	3	3
P, mg	14	16	15
Fe, mg	0.07	0.07	0.07
Cu, mg	0.05	0.04	0.04
Zn, mg	0.6	0.3	0.3
Cl, mg	-	86	42
Mn, mg	Traces	traces	traces
Se, µg	-	2	1
I, µg	-	-	7
Retinol, µg	155	85	58
Carotene, µg	135	37	24
Vit D, µg	-	-	0.04
Vit E, mg	1.3	0.48	0.34
Thiamin, mg	traces	0.01	0.02
Riboflavin, mg	0.03	0.03	0.03
Niacin, mg	0.1	0.1	0.2
Vit B6, mg	traces	traces	0.01
Vit B12, µg	0.01	traces	traces
Folate, µg	2	3	5
Pantothenate, mg	0.12	0.20	0.25
Biotin, µg	traces	0.2	0.7
Vit C, mg	7	6	4

Nutrition is not the only function of human breast milk. The many included antimicrobial and immunomodulatory components suggest it compensates the initial weakness of the neonate's immune system. This can be seen by the fact, that carbohydrates, lipids and proteins do not only act nutritive but are also implementing versatile beneficial properties.

Human breast milk consists of a number of different carbohydrates, with lactose being the most abundant by far, making up about 40 % [3] of all carbohydrates present. Lactose in human milk has the highest concentration compared to other species, corresponding to the high energy demand of the human brain. Human milk oligosaccharides (HMO) are also representing a significant fraction of carbohydrates in human breast milk. These are a family of structurally diverse glycans that are unique to human breast milk and are assembled by the monosaccharides L-fucose, D-glucose, D-galactose, N-acetylglucosamine and N-acetylneuraminic acid [4]. HMO`s were found to act as prebiotics, stimulating and encouraging the growth of beneficial bacteria, particularly the colonialization of *Bifidobacterium infantis* [5] within the infant's gastrointestinal tract. There is accumulating evidence suggesting that HMO`s are operating as antiadhesive antimicrobials preventing the attachment of pathogenic bacteria to the infant mucosal surfaces, by acting as receptor decoys [5].

The lipid fraction is the largest source of energy in human breast milk consisting mainly of triacylglycerides, diacylglycerides, monoacylglycerides, free fatty acids, phospholipids and cholesterol [3] with triacylglycerides making up the vast majority with about 98%. Oleic, palmitic and linoleic acid [6] are the fatty acids predominantly found in human breast milk. Human breast milk lipids are essential for the development of the brain, retina and the nervous system, the absorption of fat-soluble vitamins and are representing the primary calorie source. Furthermore, breast milk lipids have been shown to inactivate a number of pathogens in vitro [7], suggesting that lipids provide an additional protection to prevent infections of the gastrointestinal tract.

Non-protein nitrogen-containing compounds make up about 25% [8] of the human milk nitrogen content and are consisting mainly of urea, uric acid, creatine, creatinine, amino acids and nucleotides [8]. This fraction as well contains a lot of bioactive molecules involved in different cell processes as well as in the development of the gastrointestinal tract, microbiota and the immune system [3].

Human breast milk contains approximately 400 different proteins [3] with many different properties and physiologically effects that will be pointed out later. This work focuses on the major constituents.

1.2.1 Length of lactation

Human breast milk is commonly classified into three different types corresponding to gradual alteration throughout lactation. Pre-milk also called colostrum, is produced during the first days (1-7) after birth and differs from the other two lactation stages as it is exceptionally rich in proteins, vitamins, trace elements and immunoglobulins. Colostrum is a yellowish fluid that is excreted also during pregnancy and provides passive immunity to the baby due to its high amount of different antibodies. The second stage of lactation is transitional milk, which is the transition from colostrum to mature milk that is produced during day 8-20 after birth. Finally, mature milk is produced from day 20 and onwards, differing from colostrum in a higher concentration of fat and carbohydrates, but a lower amount of protein and immunologic factors [9].

In figure 1 the transformation of nutrients within human breast milk concerning proteins, carbohydrates and fat is given as a function of lactation time. Whereas the protein content decreases (b), the content of carbohydrates (a) and lipids (c) is increasing over lactation time.

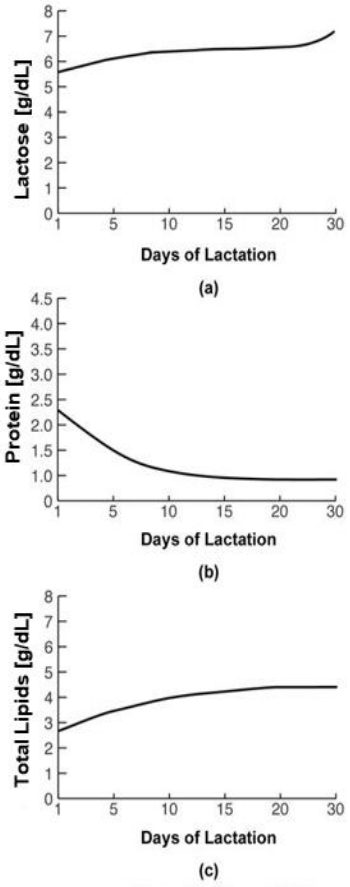


Figure 1: a) Lactose, b) protein and c) total lipid concentrations in human breast milk throughout lactation [3]

The colostrum composition suggests that its primary role is not a nutritional one, but an immunological, protecting the baby as it emerges from the relatively sterile environment of the womb to being exposed to many environmental pathogens [3]. As colostrum is also containing a number of growth factors like the endothelial growth factor, the hepatic growth factor and the epidermal growth factor [10], human milk is appearing also as some kind of growth promoter. Due to its low quantity of fat and carbohydrates, the colostrum is low-calorie, therefore can be digested more easily hence helps the digestive system of the baby to grow and function properly.

1.2.2 Other influencing factors

The composition of human breast milk is not uniform and the concentrations of many of its constituents are changing during the lactation period and are differing between individual mothers. There are several factors that are known to influence the concentrations of breast milk constituents, including the stage of lactation, breastfeeding routine, parity, age and other maternal characteristics, regional differences and in some situations season of the year and maternal diet. The composition is changing in terms of the length of lactation, resulting in an increase of fat concentration and therefore energy content [11] for mothers lactating longer than one year compared to mothers lactating less

than 6 months. The age of the mother also seems to be a parameter influencing human breast milk composition in terms of protein and fat concentration. Protein concentration is highest in mothers aged 20-30 [3]. Human breast milk of older mothers contain higher concentrations of fat than that of younger mothers. This increase in fat content obtained from mothers with advanced age may be due to increased fat synthesis and excretion in milk, reduced water content of milk, or a combination of both [12]. The composition may be influenced also by the parity. As demonstrated in a study of Gambian mothers: young, primiparous mothers have higher concentrations especially of fat, protein, and immunoglobulins, whereas older mothers of very high parity (nine or more children) tend to produce milk of reduced quality [13-14]. Also, a correlation between maternal weight gain during pregnancy and the fat content of human breast milk was reported. The influence of maternal diet on the composition of human breast milk is much more complex [3], depending on the type of nutrient, there can be almost no or a significantly high impact. Recently no convincing relationship between the maternal nutritional stage (regarding body mass index, BMI) and either breastmilk output or energy content was observed [15].

1.3 Infant formula

Breastfeeding will normally provide all the nutrient requirements of a baby for the first four to six months of life. After this time some nutrients may become limited, affecting not only dietary energy but also the intake of essential minerals such as zinc and iron. For that reason, a mixed diet is recommended after six months [16].

Although breastfeeding is strongly recommended and many mothers hope to breastfeed their infants, in a lot of cases it is not feasible and therefore children have to be nourished by giving them infant formula. Especially preterm infants require special consideration and advice should be sought from the dietitian and medical team [16] that are caring for it.

Manufacturers of infant formulas state that its composition is designed to be roughly based on a human mother's milk at approximately one to three months postpartum and there are significant differences between different products [17]. Most formula are using whey and casein as a protein source, vegetable oils as a fat source, lactose as a carbohydrate source and a vitamin-mineral mixture.

There are several different formula available for infants younger than twelve months who are not drinking breast milk, regulated by the Food and Drug Administration (FDA) [18]:

- *Cow's milk-based formula:* include the bovine counterparts of macronutrients of human breast milk that have been recognized to resemble breast milk.
- *Soy-based formula:* these kind of formula are highly suitable if the exclusion of animal proteins is necessary due to miscellaneous reasons like intolerances or allergies to bovine milk proteins or lactose.

- *Protein-hydrolysate formula:* there are partially- and extensively-hydrolyzed formulas available, in which cow's milk protein is broken into more or less smaller pieces. Proteins can, therefore, be digested more easily. Also babies intolerant or allergic to bovine proteins can benefit.
- *Amino acid-based formula (elemental formula):* these formula are made of individual amino acids and are some kind of hypoallergenic formula. Due to the fact that amino acids are the simplest form of protein, it is easy for the body to process and digest.

In addition, there are several different specialized formulas available for premature infants and babies who have specific medical conditions [19].

It is highly recognized that several outcomes of breastfed infants are superior to those of formula-fed infants. For formula-fed infants a different growth pattern, a particular higher gaining of body fat and weight and higher concentrations of serum amino acids, insulin, and blood urea nitrogen [17] have been recognized than to breastfed infants. These variables may be associated with the higher risks of obesity, type 1 and 2 diabetes and cardiovascular disease later in life, as documented in several reports [1].

Standard infant formula are complex mixtures of proteins, carbohydrates, fats, vitamins and trace elements, but a plenty of different immunologic and growth promoting factors present in human breast milk are either completely missing or just present in insufficient quantities. Manufactures are often changing the composition of formula, adding substances while trying to mimic human breast milk composition. However, the addition of ingredients is still difficult and hazardous concerning bioavailability, digestibility and toxicity [20]. But the goal of all changes in infant formula is to achieve maybe not the composition of human breast milk but at least the performance of breastfeeding [21].

Breast milk contains a large variety of bioactive proteins, which are involved in the defense against infections, immune function, and development. Some bovine milk proteins are similar to human milk proteins, albeit not identical, and may provide some of the bioactivities [21] of their human counterparts. Moreover, because human breast milk contains many unique bioactive components like the epidermal growth factor, the insulin- like growth factors I and II, amylases, proteases erythropoietin and lactoferrin [21]; alternative sources of such components need to be explored and possibly incorporated into infant formula.

1.4 Benefits of breastfeeding – Health and development

The American Academy of Pediatrics (AAP, 1997), the World Health Organization (WHO, 2002) and many other health organizations are declaring and describing a lot of different advantages for a healthy outcome and development of the children being exclusively breastfed for at least their first six months of life, predicting that breastfeeding results in improved infant and maternal health outcomes [22].

They are stating that children that were partially fed human breast milk or were fed infant formula have a fourfold higher risk to contract pneumonia [22]. Breastfeeding for the first six months after birth of the child is also associated with an extensive reduction in the incidence of gastrointestinal tract infections like necrotizing enterocolitis (NEC) [23]. A reduction of allergic diseases, asthma, dermatitis, obesity, type 1 diabetes mellitus and also childhood leukemia and lymphoma is reported correlated with exclusively breastfed children [22]. Consistent differences in neurodevelopmental outcome between breastfed and commercial infant formula-fed infants have been reported [24]. Evidence was provided that adjust outcomes of intelligence scores are significantly higher and also the total brain volume [24] is increased in case of exclusively breastfed infants.

Especially in the case of preterm infants, several significant short- and long-term beneficial effects of feeding human breast milk have been reported. Lucas investigated in a multicenter study on 926 preterm infants that among babies born at more than 30 weeks of gestation necrotizing enterocolitis was rare in those whose diet included breast milk; it was 20 times more common in those fed formula only [25]. Furthermore, not only a reduction of the mortality rate caused by NEC was reported but also lower long-term growth failure and fewer neurodevelopmental disabilities [26]. Long-term studies of preterm infants also suggest that human milk feeding is associated with lower rates of metabolic syndrome, and it is associated with lower blood pressures in adolescence [27].

1.5 Premature infants

A premature infant is an infant born before week 34 of gestation and any infant weighing less than 2.5 kg is termed premature [28]. Infants with a birth weight below 2.5 kg are considered as low-birth-weight infants (LBW), and those weighing less than 1.5 kg are considered as very low-birth-weight infants (VLBW). These infants have increased incidences of complications and mortality. According to estimations about 15 million children are born too early every year and about 1 million of these children die annually due to complications originated from their preterm birth [28].

Compared with term infants, preterm infants are more frequently affected by respiratory distress, temperature instability, hypoglycemia, kernicterus, apnea, seizures, and feeding problems, as well as higher rates of rehospitalization [29]. Many survivors face a lifetime of disability, including learning disabilities and visual and hearing problems.

The survival rates of preterm infants around the world are significantly unequal; in low-income settings, half of the babies born at or below 32 weeks die due to a lack of feasible, cost-effective care, such as warmth, breastfeeding support, and basic care for infections and breathing difficulties. In high-income countries, almost all of these babies survive [28].

Most premature infants tolerate breast milk, which is providing a number of different nutritional-, immunologic- and growth-promoting factors; feeding colostrum or transitional milk improves immune protection and promotes gut maturation. However, breast milk does not necessarily provide sufficient

amounts of some nutrients like calcium, phosphorus and proteins for very low-birth-weight infants (below 1.5 kg). In order to meet the unique nutritional requirements of VLBW infants and preserve the singular benefit of breastfeeding, human milk should be fortified to allow adequate growth and bone mineralization [30].

Due to the many described advantageous effects of breastfeeding, a system for accurate quantification of macronutrients in human breast milk is required directly at point-of-care, to enable personalized fortification and cautious handling of infant formulas.

1.6 Microfluidic chip – how to get from colorimetric Assays to Lab-on-a-Chip analysis? [31]

Microfluidic chips, illustrated in figure 2, are fabricated on microscope glass slides by hot roll lamination of a dry film resist (Ordyl SY300). Up to 16 devices can be fabricated on one slide. The chambers and pressure barriers are structured by using standard photolithography, furthermore, the inlet and outlet are powder-blasted into the top glass layer. The upper and bottom glass slides are subsequently aligned by eye and finally bonded by hot roll lamination.

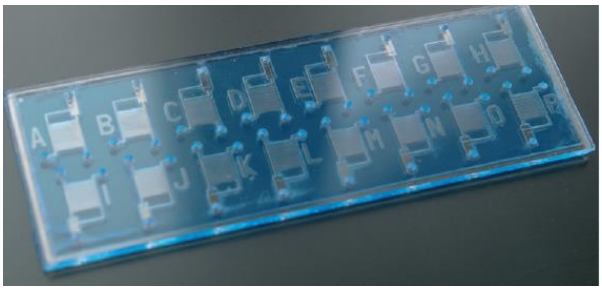


Figure 2: Microfluidic chips [31] fabricated in microscope glass slides by hot roll lamination of Ordyl SY300, standard photolithography

The device consists of a chip in which a biosensing hydrogel can be incorporated, so that the hydrogel contains the sensing reagent in a three-dimensional matrix and allows the diffusion of the analyte through the pores of the gel to be able to react with the sensing reagent. The dimensions of the hydrogel structures have to be relatively small (in µm range) to enable reactions within a reasonable short time. Furthermore, the pore size of the gel has to be large enough to facilitate diffusion.
The experimental design of Puchberger-Enengl, given in figure 3, is based on polyethylenglycol diacrylate (PEG-DA), which is a biocompatible hydrogel and is polymerized by UV exposure (365 nm) and after addition of 2-hydroxy-2-methypropiophenone (HMPP) as a photoinitiator.
PEG-DA and HMPP are mixed and introduced into the chip only into predefined regions. The regions of the gel and the analyte are defined by capillary pressure barriers within the chip. An advancing fluid meniscus is pinned to a barrier and propagates along it, instead of crossing it. The device is prepared by filling the sensing hydrogel into the chip via one inlet, subsequently, the gel is cured and the analyte is introduced via the other inlet, fills the chamber interdigitated to the gel and can be detected either

by using UV/Vis- or fluorescence spectroscopy. Only 1 µl of the sensing hydrogel is needed to fill the chip and the same amount of sample is needed.

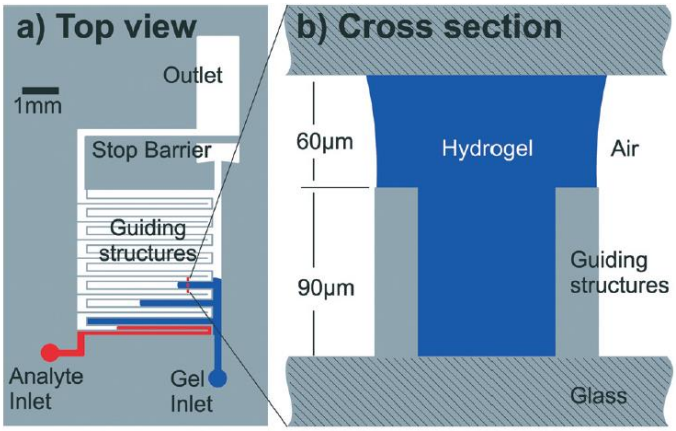


Figure 3: Illustration of the chip design [31]

First experiments about colorimetric protein detection using the color shift of Bromophenol Blue from yellow to blue were already published [31].

The combination of several analytical tests on a single glass slide containing up to 16 chip positions offers the possibility to obtain multiparametric analysis directly at point-of-care. Consequential this microfluidic application, which is user-friendly, time-saving and applicable on different analytical problems, seems to be highly suitable for the analysis of macronutrients in human breast milk, concerning at least proteins and some of the trace elements like calcium and phosphate which are essential nutrients for a healthy development. Another advantage of this analytical miniaturization is that the actual quantity of human breast milk is limited with about 25-50 mL per feed. To ensure that the baby actually receives enough the problem of conventional analyzers is that there are only small sample volumes available.

To answer the question, whether the experimental design of Puchberger-Enengl for protein quantification based on Bromophenol Blue is suitable, three different analytical methods were compared.

First of all, an infrared spectroscopy based instrument dedicated for human milk analysis, the MIRIS, has been proposed as efficient in terms of routine analysis in neonate units and milk banks.

The MIRIS system was compared to UV/Vis spectroscopy because detection on the lab-on-a-chip approach is based on this method. The major issue about the previously published system is simply that the Bromophenol Blue Assay is not a generally accepted method for protein quantification, so the generally accepted Bradford Assay was tested for suitability. A reference material was defined, consisting of a mixture of high-abundance proteins in human and bovine milk and compared the analytical performance of this reference to BSA, a protein usually used for spectroscopy based quantification approaches.

The third tested method was capillary gel electrophoresis-on-a-chip, an instrument named Bioanalyzer, that is used with a dedicated protein quantification Assay, the P230 Assay. It was of interest to see if the analytical performance can be enhanced by protein separation and fluorescence detection. Furthermore, the influence of other human breast milk constituents on protein quantification, for example fat and calcium, was investigated, substantiated by the fact that these two constituents are major components in human breast milk. The scheme of the experimental design and research approach is given in figure 4.

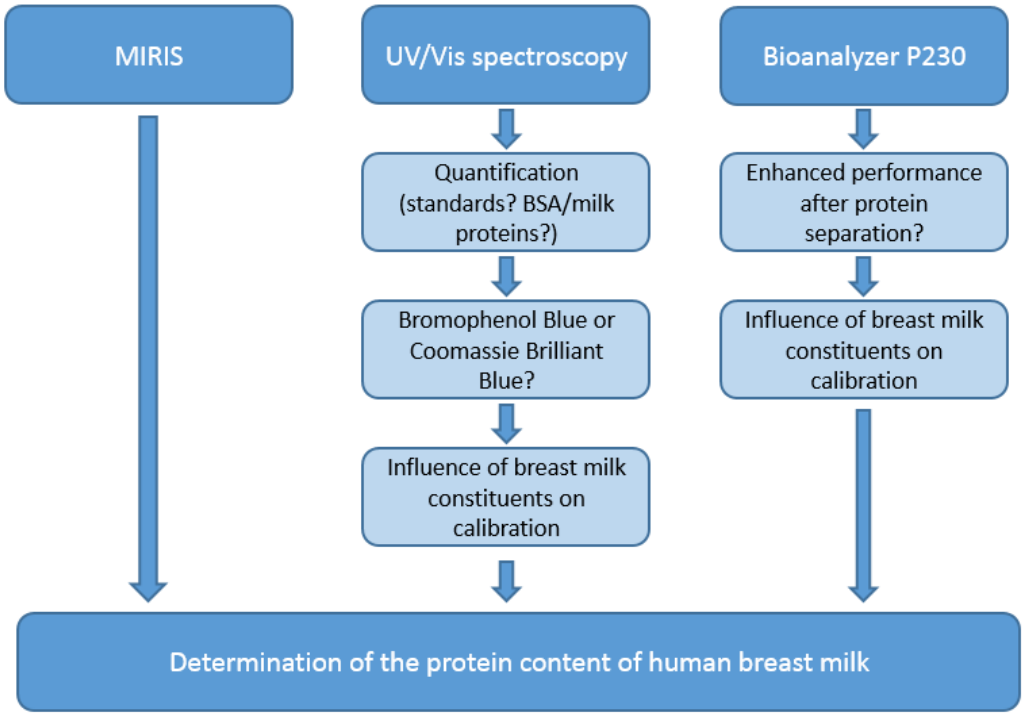


Figure 4: Experimental approach

2. Theoretical Background

2.1 Proteins in human breast milk

Human breast milk contains about over 400 different proteins [3], performing a variety of different functions from providing nutrition and facilitate digestion and uptake of other nutrients to possessing antimicrobial and immunomodulatory activities. Most of the human breast milk proteins are synthesized in the mammary gland [30], with only a few exceptions like serum albumin, which are derived from the mother's blood. Human milk proteins are classified in general into three different types of proteins according to their solubility: mucins, whey proteins and caseins [32], with whey proteins present in solution, while caseins are present in the form of insoluble micelles. Mucins, also known as milk fat globule membrane proteins, are proteins surrounding the lipid globules in human milk and contribute only to a small percentage of the total protein content of human milk. Although whey proteins are representing the main fraction of proteins in human milk, there is no fixed ratio of whey to casein, caused by changes during lactation. The frequently cited ratio of 60:40 is an approximation of the ratio during the normal course of lactation, but it does vary from 80:20 in early lactation to 50:50 in late lactation. [32]

Approximately two thirds of the proteins present in human breast milk are whey proteins, with significant quantities of α-lactalbumin, lactoferrin, sIgA, serum albumin and lysozyme. For caseins three different types are present in human breast milk: α-, β- and κ-casein. The total protein content concerning caseins is the lowest in human breast milk than in any other studied species, corresponding to the slow growth rate of human infants [3].

2.1.1 α-Lactalbumin

α-lactalbumin is one of the major proteins present in human breast milk and has been described to contribute to several different physiologic functions in the neonatal period. Besides its nutritional role, it is participating as a co-enzyme in lactose synthesis as it is part of the enzyme lactose synthase, creating some kind of an osmotic drag to facilitate milk production and secretion [33]. In human milk α-lactalbumin has a molecular weight of 14.7 kDa [34] and is a non-glycosylated protein. It was noticed that α-lactalbumin is able to bind Ca^{2+} [35] and Zn^{2+} as well [36], so it is supposed that α-lactalbumin is having a positive effect on mineral absorption, especially acting as Ca^{2+} carrier in human breast milk. α-lactalbumin was furthermore found to have antimicrobial activity against *E. coli, Klebsiella pneumoniae, Staphylococcus aureus, Staphylococcus epidermis, Streptococci and C. albicans* [37].

HAMLET (Human α-lactalbumin Made Lethal to Tumor Cells) is a protein-lipid complex consisting of α-lactalbumin and oleic acid that was found to induce apoptosis-like death of tumor cells [38], while it leaves healthy, fully differentiated cells unaffected. HAMLET was shown to kill tumor cells in vitro and the activity is retained in vivo [39], so that is why it is believed to act as chemotherapeutic agent. Multimeric α-lactalbumin was found in the insoluble casein fraction of human milk, to be able to

partially unfold in the acidic environment of the stomach and therefore being able to release calcium and bind oleic acid instead [40]. This mechanism is therefore supposed to contribute to the protection of breastfed babies against cancer.

2.1.2 Casein

Casein is not a single protein unit, but a group of protein subunits associated with organic and inorganic components [32] so they are almost exclusively present in micellar form. Beside the protein subunits, phosphate is present in these micelles. These negatively charged phosphoproteins were found to be able to bind essential cations like calcium, zinc and copper [41-43] and are capable of enhancing mineral bioavailability by being better absorbed [44]. In human milk β-casein is the major casein subunit, while α- and κ-casein are only minor components.

β-casein with a molecular weight of 24 kDa is phosphorylated to various degrees at several positions in the polypeptide chain, therefore giving rise to a number of different casein species and was found to be able to complex Ca^{2+}[45] keeping it soluble and therefore facilitate absorption.

κ-casein with a molecular weight of 19 kDa is a casein subunit that is highly glycosylated and present in significantly lower concentrations in human breast milk than β-casein . This protein is supposed to inhibit the adhesion of *Helicobacter plyori* to the human gastric mucosa and minimize the growth, proliferation and adhesion of this pathogen in cooperation with other proteins present in human breast milk like lactoferrin and sIgA [45]. α-casein (molecular weight: 23 kDa) is apparently absent in human breast milk whereas it makes up a large portion of casein in cow's milk [45], where it was reported to exhibit antioxidant and antiradical properties.

2.1.3 Lactoferrin

Lactoferrin is a red-colored iron binding protein in human breast milk with a molecular weight of 77 kDa. It has two carbohydrate antennas [46] and each molecule of lactoferrin has two iron-binding sites. The biological function of lactoferrin is supposed to be a promoter for iron absorption, explaining the high bioavailability of iron in human breast milk compared to that of cow's milk. On the other hand, a bacteriostatic function of lactoferrin is assumed because it is not only present in human milk but as well in many other body fluids like tears, saliva and the pancreatic juice [47].

Several antimicrobial activities have been described for lactoferrin especially in terms of *E. coli* infections, where it is supposed that due to its high affinity to iron, lactoferrin is able to withhold iron from iron-requiring pathogens [45] prohibiting the growth of these pathogens.

In addition, antiviral properties, associated with lactoferrin, were observed for a number of different viruses like, for example, herpes simplex virus 1, hepatitis C virus or rotavirus [48]. The mechanisms involved in this connection are, lactoferrin prohibiting the docking of virus particles to the target cell by either binding directly to the viral particle or by blocking its docking position.

Lactoferrin might furthermore play an important role in the pathogenesis of diseases caused by various retroviruses. Its enzymatic activity is that of ribonucleases, and therefore it is able to prevent reverse transcription of the retrovirus RNA genome [48], associated to cause breast cancer.

2.1.4 Immunoglobulins

After birth, infant survival depends on a transition to a phase of immune activation. During this period, infants are extremely vulnerable to infections and often have suboptimal responses to vaccination. Therefore, infants rely on maternal immune protection through antibodies initially acquired transplacentally and subsequently via breast milk.

Several immunoglobulins present in serum are also found in human breast milk and especially colostrum is rich in immunoglobulins like sIgA, IgA, IgG and IgM [35]. Although the concentration of immunoglobulins in human milk decreases [35] after the first days of lactation, they still constitute a significant part of the total protein concentration in mature milk. So the mother's immunity against several different pathogens can be passed on to the baby and its immune system can be strengthened by the already existing immunity of the mother.

The major type found in human milk is secretory IgA which is a dimer of IgA linked together with a secretory component and a joining chain [35]. Besides its well-known and documented capacity to protect the intestinal epithelium from toxins, viruses and pathogenic bacteria [36], sIgA antibodies affect bacterial pathogens such as *Escherichia coli*, *Vibrio cholerae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Clostridium difficile,* and *Salmonella*. Immunity conveyed viruses such as rotavirus, cytomegalovirus, HIV, influenza virus, and respiratory syncytial virus; and against yeasts such as *Candida albicans* which have been found in breast milk [35] illustrate the breadth of this defense.

2.1.5 Serum albumin

In contrast to other proteins present in human breast milk, serum albumin is not produced in the mammary gland [30] but it is reasonable to believe that serum albumin is transported from the blood into the milk due to its identical form and properties. In human breast milk no significant biological function was determined for serum albumin, besides its function as a provider of amino acids and a being a carrier protein [32].

2.2 Proteins in cow's milk

One of the major differences between cow's milk and human breast milk is the fact that the total protein content of human milk is about threefold lower [4] than that of cow's milk. Part of the reason for this difference is that the nature of the proteins is different. As already mentioned the whey to casein ratio in human breast milk is altering through lactation (between 80:20 and 50:50), but in cow's milk the situation looks completely different, the ratio is constantly 20:80 [4]. Furthermore, the

dominant whey protein in human breast milk is α-lactalbumin, whereas the dominant whey protein in cow's milk is β-lactoglobulin [53], a protein usually not found in human milk.

The composition of subunits in caseins vary among different species, so in contrast to human breast milk, cow's milk contains a large proportion of α-casein that is found only in very low concentrations in human milk. Casein micelles were furthermore found to be significantly larger in cow's milk compared to human milk (83 nm vs 43 nm [51]), due to the different subunit composition or maybe also because of a higher permeability of larger micelles by the lacteal ducts of the bovine mammary gland as compared to the human breast. The κ-casein subunit in cow's milk was furthermore found to be lesser glycosylated than its human counterpart [52].

2.2.1 β-Lactoglobulin

β-Lactoglobulin is the major whey protein in a lot of mammalian species like cows and sheep with α-lactalbumin present in minor concentrations. Several genetic variants have been identified [51], the main ones in cows being labeled A and B. The molecular mass of this protein is about 18.4 kDa and under physiological conditions, it is primarily existing as a dimer [53]. Beside its high nutritional value, β-lactoglobulin has some peculiar properties as it binds hydrophobic molecules like free fatty acids and triacylglycerides, conjugated molecules and other macromolecules [54] suggesting its role in the transport of these molecules. Furthermore, this protein was shown to possess unique vitamin binding properties [55-56] emphasizing its role in the transport of fat-soluble vitamins A, D, E and K.

β-lactoglobulin is suspected to be one of the most significant allergens in cow milk. It belongs to the protein family lipocalins, possessing binding pockets for iron accommodation via so-called siderophores. It was shown that empty molecules of β-lactoglobulin are able to activate Th2 lymphocytes. As a consequence, the production of IgE antibodies against the milk protein is stimulated, the patient gets sensitized and may develop an allergic reaction to milk [57].

It is also important to remark that β-lactoglobulin can be found in breast milk of mothers consuming a lot of cow's milk and its products [58], indicating that this protein can be absorbed by the gastrointestinal tract and transported into breast milk. Also the fact that β-lactoglobulin is an ingredient of various cow's milk-based infant formulas is particularly important for infants with cow's milk allergies.

2.3 Theoretical Background on Instrumentation and Applied Spectroscopic Methods

2.3.1 Spectroscopy [59]

Spectroscopy is the study of the interaction between matter and electromagnetic radiation. All spectroscopic methods rely on the same basic principle: electromagnetic radiation of a certain intensity and wavelength is interacting with an object and is consequently absorbed, scattered and/or reemitted with different intensity and sometimes altered wavelength. Photometric measurements are therefore comparing the intensity (I) and wavelength (λ) of the original electromagnetic radiation with the one after interaction with an object.

The nature of light can be understood in two terms: in terms of particles and also in terms of waves, called wave-particle duality. Electromagnetic radiation can be seen as transverse waves, consisting of an electric (E) and a magnetic (H) component and are formed by the vibrations of these electrical and magnetic fields. These two fields are perpendicular to one another in the direction the wave is traveling. Both fields depend on space *x* and time *t* with the frequency *v*, the wavelength λ and ϑ with the phase of the wave.

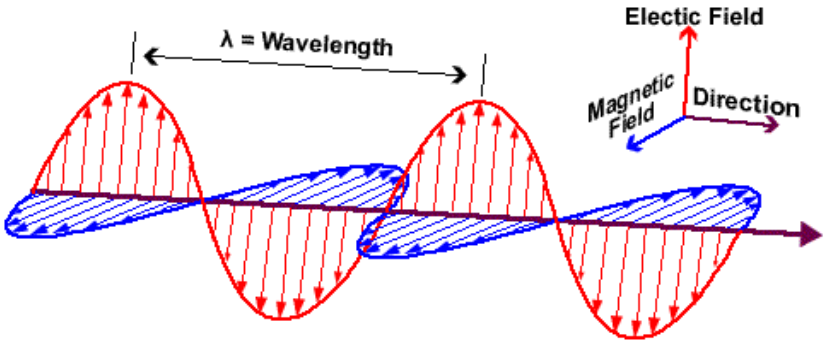


Figure 5: Electric E(x,t) and magnetic field H(x,t) of electromagnetic radiation [60]

$$E(x,t) = E_0 \cdot \cos[2\pi\left(vt - \frac{x}{\lambda}\right) + \theta] \qquad \textit{equation 1}$$

$$H(x,t) = H_0 \cdot \cos[2\pi\left(vt - \frac{x}{\lambda}\right) + \theta] \qquad \textit{equation 2}$$

The relationship between frequency and wavelength is given in equation 3, with *c* as the velocity of light that is dependent on the particular medium in which the light is propagating, accounting to c_0=2.9979·10^{-8} m·s^{-1}in vacuum.

$$c = v \cdot \lambda \qquad \textit{equation 3}$$

The description of light as particles is described as a flow of light particles called photons, that are moving with the velocity of light c and whose energy is calculated by equation 4. This equation displays

that the energy of light is not transferred constantly but in the form of small energy portions (photons) revealing the quantization of light.

$$E = h \cdot v \qquad \textit{equation 4}$$

h…Planck's constant

Considering that the binding energy of atoms in a molecule is in the range of some electron volt, the absorption of ultraviolet, visible and infrared light is not rich enough in energy to break these chemical bonding but to excite electronic states. By the absorption of a photon, electrons of an occupied molecular orbital can be excited to an empty molecular orbital.

The total energy of a molecule is composed of different types of energies; the electrical energy E_{el}, the vibrational energy E_{vib} of the atomic nuclei, the rotation energy E_{rot} of atoms or groups of atoms around an axis and magnetic properties of the nuclei and the electron shell E_{magn}.

$$E_{ges} = E_{el} + E_{vib} + E_{rot} + E_{magn} \qquad \textit{equation 5}$$

Depending on the energy of the absorbed photon transitions between different electrical and vibrational stages can be stimulated. While the absorption of infrared light excites transitions in between vibrational and rotational states, the absorption of electromagnetic radiation in the ultraviolet and the visible spectrum causes electronic transitions.

2.3.1.1 MIRIS - Infrared human milk analyzer

Commercial human milk analyzers (HMA) are based on infrared spectroscopy (IR spectroscopy) and have been proposed as efficient in terms of routine analysis of the macronutrient content of human breast milk in neonate units and milk banks. In figure 6 the working principle of an infrared based human milk analyzer is given, consisting of an emitter, a sample cuvette and a detector. This system allows the simultaneous quantification of human breast milk composition concerning its content of proteins, carbohydrates, fat, energy and dry matter.

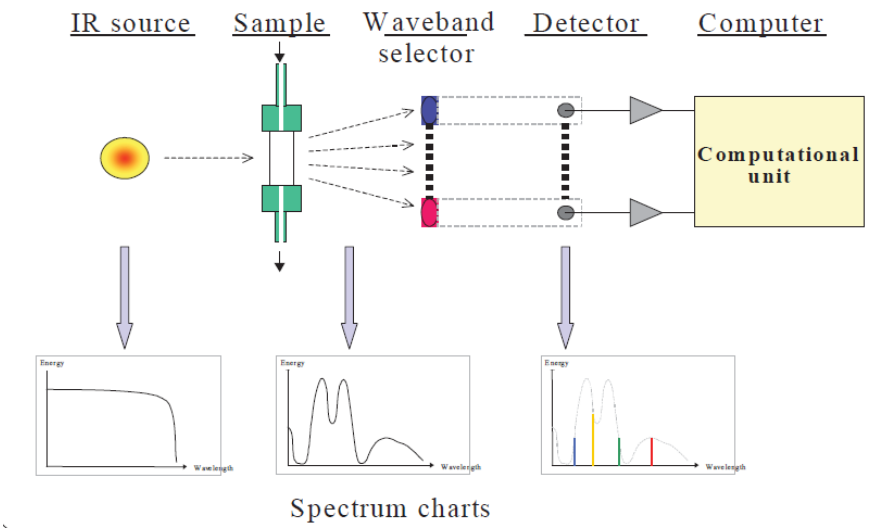


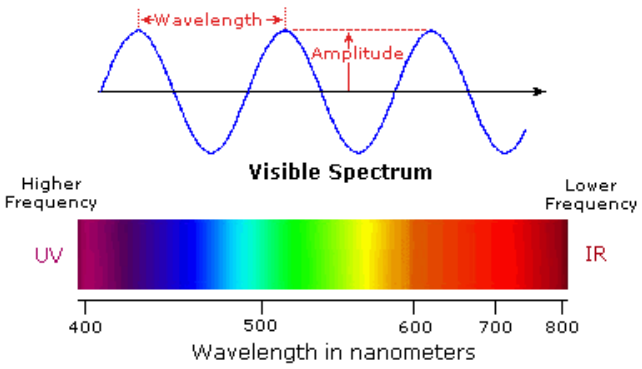
Figure 6: Working principle of an infrared based human breast milk analyzer [61]

The infrared portion of the electromagnetic spectrum is usually divided into three different regions: near- (760 nm - 3000 nm), mid- (3000 nm - 30 µm) and far-infrared (30 µm - 1000 µm) [62]. Infrared light imposed on a molecule will not create electronic transitions but it does contain enough energy to interact with a molecule, causing vibrational and rotational changes. IR-spectroscopy is used to determine functional groups in molecules as it measures the vibrations of atoms. Based on this it is possible to determine the functional groups. The transmission values are converted to concentrations by using different calibration models that are predicted from pure breast milk samples with known concentrations. The human milk analyzer is calibrated against standard reference methods that are ISO certified and IDF recommended [61]. Vibrational modes that can be used for protein quantification via IR-spectroscopy are: vibration of the polypeptide backbone, vibrations of the amino acid side chains or vibration of potentially existing cofactor. The most dominant mode in the absorption spectrum of proteins are vibrations caused by the peptide bond, especially the amid I and II band [63], which is mainly attributed to the C=O stretching vibration.

These kind of analyzers are offering an opportunity for direct determination of the nutritional content of breast milk with the benefits of a small sample size (1-3 mL), no additional chemicals, and fast and easy analysis within 60 seconds. But the disadvantage concerning the nutritional requirements of preterm infants is that the analysis of trace elements is not possible by using this system. Especially calcium and phosphate, which are fundamental for a healthy development of especially preterm infants, cannot be analyzed by MIRIS.

2.3.1.2 UV/Vis spectroscopy

Ultraviolet-visible spectroscopy (UV/Vis spectroscopy) refers to absorption spectroscopy in the ultraviolet (200-400 nm) and visible (400-800 nm) [64] spectral region. The wavelengths of what we perceive as particular colors in the visible portion of the spectrum are displayed and listed below in figure 7.



Violet: 400-420 nm

Indigo: 420-440 nm

Blue: 440-490 nm

Green: 490-570 nm

Yellow: 570-585 nm

Orange: 585-620 nm

Red: 620-780 nm

Figure 7: Visible electromagnetic spectrum [64]

While interactions with infrared light cause molecules to undergo vibrational transitions, the shorter wavelength and therefore higher energy of the visible and ultraviolet electromagnetic spectrum causes many organic molecules to undergo electronic transitions. Molecules containing π-eletrons or non-bonding electrons are able to absorb energy in form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular orbitals. The higher the gap between the HOMO- and LUMO-orbitals [59] the lower the wavelength of the absorbed light, due to the fact that wavelength and energy are indirectly proportional (equation 3).

UV/Vis spectroscopy is routinely used in analytical chemistry in case of quantification of different analytes like organic molecules or biological macromolecules as well as for measurements of the enzyme activity of certain enzymes. Quantification via UV/Vis spectroscopy is based on the law of Lambert-Beer given in equation 6, stating that the absorbance of a solution is directly proportional to the concentration of the analyte in this solution and the path length.

$$A = -\log(T) = -\log\left(\frac{I}{I_0}\right) = \varepsilon \cdot c \cdot d \qquad \textit{equation 6}$$

A…Absorbance

T…Transmission

I…Intensity of the incident light

I_0…Intensity of the transmitted light

ε…Molar absorption coefficient

c…Concentration of the analyte

d...Path length

The law of Lambert-Beer presumes certain requirements for its validity [65]:

- For validity, monochromatic radiation is required to verify that the molar absorbance coefficient is constant
- The analytes solution have to be strongly diluted (usually < 0.1 mol/L) because at higher concentrations the molecules are interacting with each other and therefore provoking deviations
- Furthermore, the absorbing molecules have to be equally distributed
- The dimensions of scattering and reflection have to be negligible small to ensure that the attenuation of light is caused invariably by absorption (requires clear solutions, no turbidity)

If there are any of these conditions not fulfilled, deviations from the law of Lambert-Beer will be obtained.

The experimental set-up of a commercial UV/Vis-spectrometer is illustrated in figure 8. A beam of an UV- or visible light source (H_2, D_2, W, W-halogen or Cd) [65] is separated into its individual wavelengths by using a prism or a diffraction grating. The corresponding wavelength is subsequently selected by the slit, passes a filter and is then guided through the sample or rather the reference cuvette containing only the solvent in which the analyte has been dissolved. During the incident, light emitted by the source is passing through the sample and a fraction of light is absorbed by the molecules in the sample solution, inducing a decrease of the lights intensity. The intensity of these light beams passing the sample and the reference cuvette are measured by electronic detectors and compared to each other.

There are double-beam spectrometers available, like shown in figure 8 and also single-beam spectrometers, where the sample and the reference are not measured simultaneously but one after another.

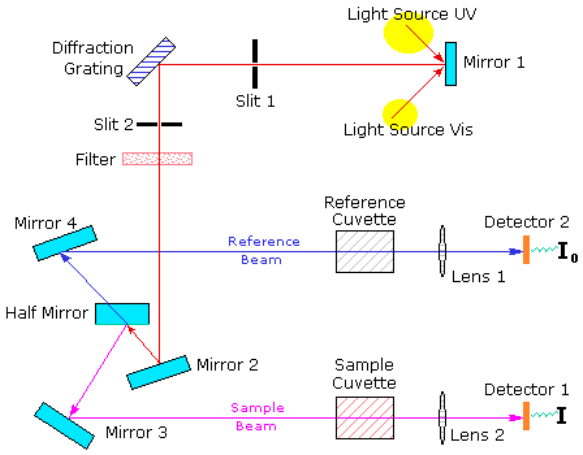


Figure 8: Experimental set-up of a standard UV/Vis spectrometer [61]

2.3.1.2.1 Bromophenol Blue Assay

Bromophenol Blue is usually used as a pH indicator, a color marker or a dye. As an acid-base indicator, it is changing its color in a reversible reaction from yellow (pH 3.0) to blue (pH 4.6). As a color marker, Bromophenol Blue is usually used in cases of monitoring agarose- or polyacrylamide- gel electrophoresis, at moderate pH, the molecules are slightly charged negatively and therefore migrate in the same direction as DNA or proteins in gel electrophoresis [67].

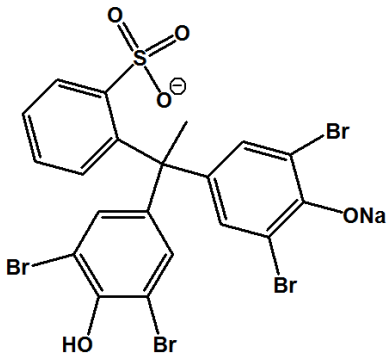


Figure 9: Chemical structure of Bromophenol Blue

Furthermore, it is supposed that Bromophenol Blue is interacting under acidic conditions with acidic and basic amino acid side chains of the protein [37], inducing a color shift from yellow (410 nm) to blue at 605 nm. The problem about this Assay for protein quantification is that Bromophenol Blue is highly prone to interferences and this Assay is therefore not generally accepted for protein quantification.

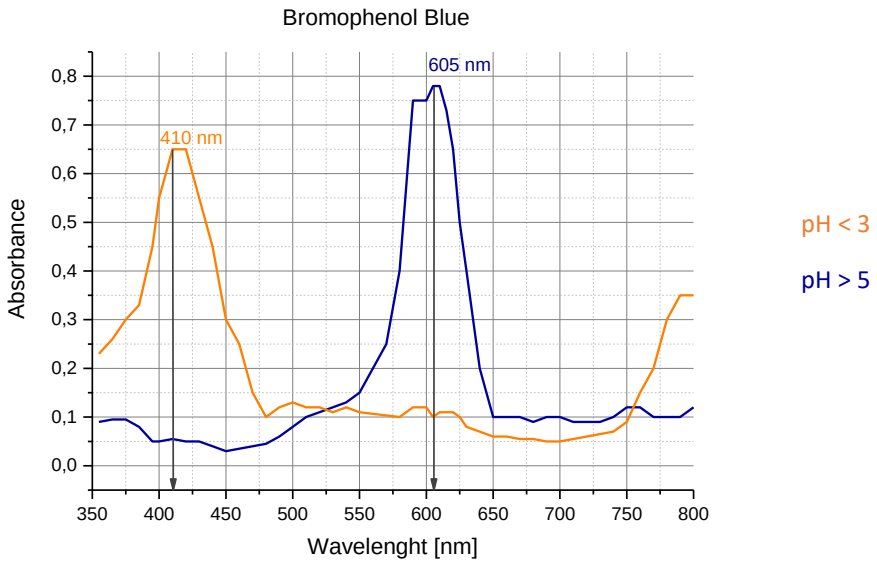


Figure 10: Absorption spectrum of Bromophenol Blue at different pH

2.3.1.2.2 Bradford Assay

The Bradford Assay is a colorimetric, spectroscopic analytical procedure for the quantification of proteins in a solution. The Assay was developed by Marion M. Bradford in 1975 [68] and is based on the interaction of the dye Coomassie Brilliant Blue G250 with hydrophobic, basic and aromatic amino acid side chains of the protein under acidic conditions.

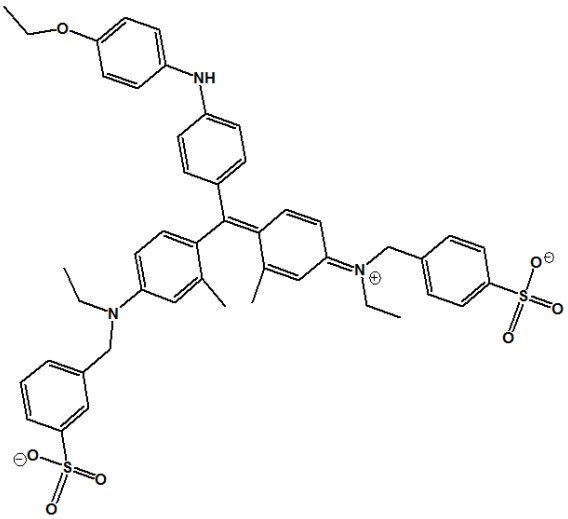


Figure 11: Chemical structure of Coomassie Brilliant Blue G250

Under acidic conditions, the red form of the dye is converted into its blue form caused by the interaction with the protein that is Assayed. During the formation of the protein-dye complex, the dye first of all transfers its free electron to ionizable groups of the protein inducing a disruption of the protein's native state so that hydrophobic groups of the protein are exposed and therefore able to interact with the hydrophobic regions of the dye [68]. The binding of the dye to the protein causes a

shift of the absorbance maximum from 465 nm to 595 nm. It's the increase in absorbance at 595 nm that is measured. The protein-dye complex is stable for about 60-90 minutes at room temperature.

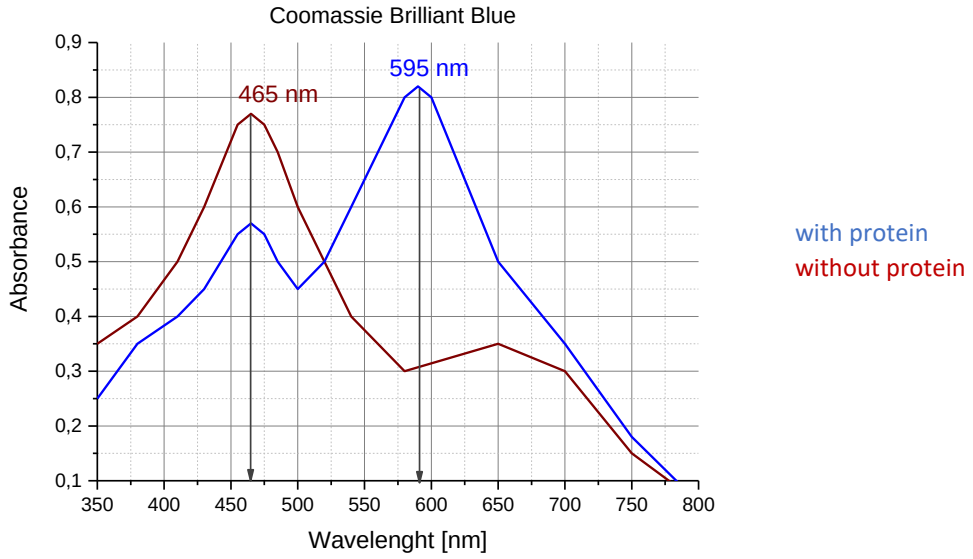


Figure 12: Absorption spectrum of free Coomassie Brilliant Blue G250 and after complexation with a protein

The Bradford Assay is a simple, rapid and accurate method for quantification of proteins with a linearity up to 2000 µg/mL [68] (depending on the analyte) and is also a very sensitive technique.

The dye seems to bind most strongly to arginine and lysine residues of the proteins but also but to histidine and aromatic amino acid residues (phenylalanine, tyrosine and tryptophan) [69]. The binding behavior is attributed to Van der Waals forces and hydrophobic interactions. This ability of the dye to bind to certain amino acids is the principle drawback of this method because this can lead to variations in the response of the Assay especially by analyzing different proteins in standards and in the sample.

Besides its simple handling and easy throughput another advantage of the Bradford Assay is its low susceptibiity to interferences with various other chemicals or components that can be present in the sample. An exception that should be mentioned is the interaction of the dye with some detergents like sodium dodecyl sulfate (SDS) that is however strongly dependent on the concentration of SDS. Concentrations below the critical micellar concentration (CMC) of the detergent tends to bind strongly to the protein and therefore is occupying binding sites for the dye, leading to an underestimation of the actual protein concentration. In the case of SDS concentrations above the CMC, the detergent is strongly interacting with the dye producing preferentially the blue form, leading to an overestimation of the protein content [70].

2.3.2 Electrophoresis [71]

Electrophoresis is the motion of charged particles in a medium under the influence of an applied electrical field. Different mobility's of diverse particles are caused by disparities in size and charge and can, therefore, be used to separate a mixture of different substances.

In an electrical field two different kinds of forces are affecting the migration of charged particles; an accelerating electrical force F_e that is based on the charges of the particles, and a dragging frictional force F_R that is caused by the movement of particles in the presence of solvent molecules.

$$F_e = q \cdot E \quad with \quad q = z \cdot e \qquad \textit{equation 7}$$

$$F_R = f_c \cdot v \quad with\, f_c = 6 \cdot \pi \cdot \eta \cdot r \qquad \textit{equation 8}$$

If these two forces are in balance, the substances are migrating with constant velocities in the electrical field. This equilibrium is resulting in the electrophoretic mobility *u* that is, therefore, substance specific and dependent on the electrical field strength, the charge of the particles and matrix or solvent properties like viscosity or resistance. But furthermore, it is dependent on the size, shape and the pK values of the charged molecules as well as on temperature, the pH and the ionic strength of the solvent which is most often a buffer.

$$F_e = F_R \rightarrow q \cdot E = f_c \cdot v \rightarrow \; v = \frac{q \cdot E}{f_c} = u \cdot E \qquad \textit{equation 9}$$

$$u = \frac{q}{f_c} = \frac{z \cdot e}{6 \cdot \pi \cdot \eta \cdot r} \qquad \textit{equation 10}$$

F_e…accelerating force

F_R…frictional force

E…electrical field strength

q…charge of the particle

z…charge number

e…elementary charge

f_c...coefficient of friction

v…migration velocity of the particle

η…dynamic viscosity

r… Stokes radius

u…electrophoretic mobility

M…molecular weight

A correlation of the electrophoretic mobility and the molecular weight of the particle was found empirically for non-globular particles (equation 11).

$$u = \frac{q}{M^{2/3}} \qquad \textit{equation 11}$$

2.3.2.1 Capillary electrophoresis [72]

Electrophoresis can be performed either in a free solution or in a supporting matrix, which is most often a gel (most commonly used are agarose or acrylamide).

Capillary electrophoresis is a separation carried out in thin capillaries in µm range or in micro- and nanofluidic channels. The capillary can be packed with a buffer solution or with a solid matrix like a gel. So several different types of electrophoresis can be performed, like capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), and capillary isoelectric focusing (CIEF) or capillary isotachophoresis (CITP), to name a few.

The general setting of a capillary electrophoresis is consisting of a capillary (fused silica, borosilicate, etc.), a high voltage power supply (30 kV), two electrodes, buffer reservoirs and an on-column detector.

The crucial point is that the capillary material has to be transparent for UV light for the purpose of detection. The capillary has also to be thin enough to enable efficient heat dissipation. Common capillary diameters are in a range of 50-100 µm and are cladded with a coating of polyimide to enhance mechanical stability. The detection of the analyte can be performed by using UV- or fluorescence spectroscopy, conductivity detection, refraction index detection, mass spectrometry or nuclear magnetic resonance spectroscopy to give some examples.

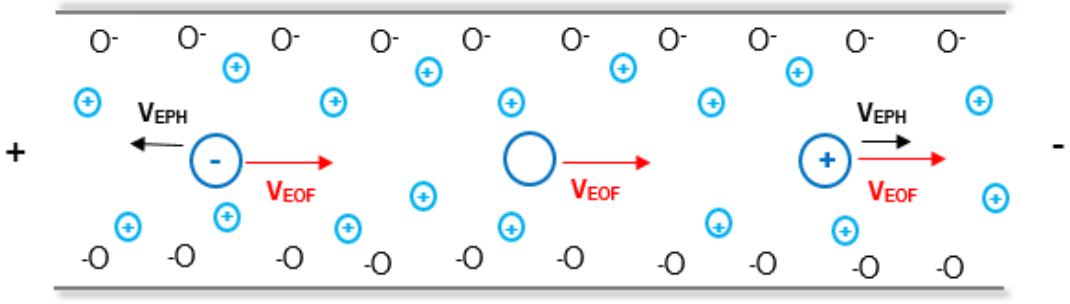


Figure 13: Principle of capillary zone electrophoresis, electroosmotic flow (EOF). V_{EOF} = electroosmotic velocity, V_{EPH} = electrophoretic velocity

Usually, fused-silica capillaries and a background electrolyte with basic pH are used, producing negatively charged silanol groups at the inner surface of the capillary. For this reason, not only the

electrophoretic velocity of the analyte ions have to be considered, but the electroosmotic flow (EOF) as well, depending on the pH of the background electrolyte and the dissociation of the silanol groups. In case of basic pH, the EOF is usually higher than the velocity of the analyte ions, so for this reason, not only cations and neutral molecules but anions are additionally carried to the anode and are detected there [72]. The velocity of the analyte ions is therefore the sum of electroosmotic (v_{EOF}) and electrophoretic (v_{EPH}) velocities. The EOF is furthermore strongly influenced by temperature and the ionic strength of the buffer, due to this, these parameters should be kept constant during the experiment.

2.3.2.2 Agilent *Bioanalyzer P230 Assay*

Capillary electrophoresis can furthermore be downscaled to a chip-format. The company *Agilent (Waldbronn, Germany)* is fabricating such chip systems, which enable sizing, quantification and quality control of proteins, DNA, RNA and even intact cells, directly by an electrophoretically separation on the chip. Different kits for different problems concerning proteins, DNA, RNA and intact cells are available.

The Agilent Bioanalyzer P230 Kit is used for the separation of proteins up to an apparent molecular mass of 230 kDa. The separation of proteins in these systems takes place in principle in some kind of SDS-PAGE like manner, downscaled on a chip.
In Figure 14 you see a schematic presentation of the chip system used in the Bioanalyzer P230 Assay. Each protein chip is containing 10 sample wells, 4 wells for a mixture of a sieving gel and a fluorescent dye (Gel-Dye), one well for an external protein ladder for molecular weight determination, and a last well for the destaining solution.

The Agilent Bioanalyzer 2100 system possesses two different excitation sources, a light-emitting diode (LED) (λex/em = 470/525 nm) and a red laser (λex/em = 635/685 nm). Detection of proteins with the Bioanalyzer 2100 system is based on fluorescence. For this purpose, the proteins can either form protein-SDS micelles in which the fluorescent dye is intercalated during separation, like in the Bioanalyzer P230 Assay or alternatively the proteins can be labeled covalently previously the separation, like it is done in the Bioanalyzer HSP 250 Assay.

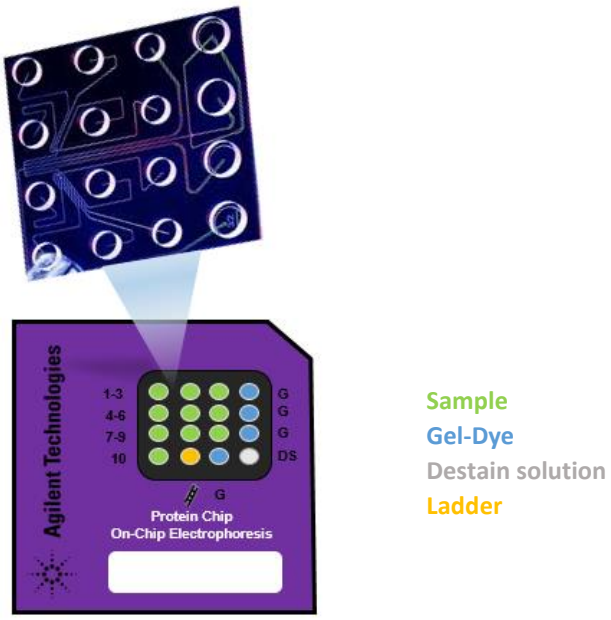


Figure 14: Bioanalyzer P230 Assay protein chip

Micro-channels are fabricated from glass to create an interconnected network among all of these wells. During chip preparation, these micro-channels are filled with the sieving gel, which is containing a fluorescent dye.

Samples are denaturated by adding a solution containing sodium dodecyl sulfate (SDS), dithiothreitol (DTT) and also the fluorescent dye heating for 5 minutes at 95 °C. During denaturation and SDS treatment, the proteins are forming micelles with SDS and within this protein-SDS micelles, the fluorescent dye can be intercalated.

In addition, the sample buffer also contains a lower (4.5 kDa) and an upper molecular mass marker (240 kDa) as an internal standard to compensate minor migration disparities.

Once all sample wells are filled, the chip becomes an integrated electrical circuit due to the fact that 16-pin electrodes of a cartridge, which are all connected to an independent power supply, are arranged so that they exactly fit into the wells of the chip. Denatured protein-SDS complexes are loaded on a chip and bind additional the fluorescent dye as they are migrating through the separation channel. At the end of the separation channel, an intersection is used to dilute the SDS below its critical micellar concentration before the detection point. This strongly reduces the background signal due to diluting also the local concentration of dye molecules bound to SDS micelles and therefore increases the peak amplitude. The principle of staining and detection is illustrated in figure 15.

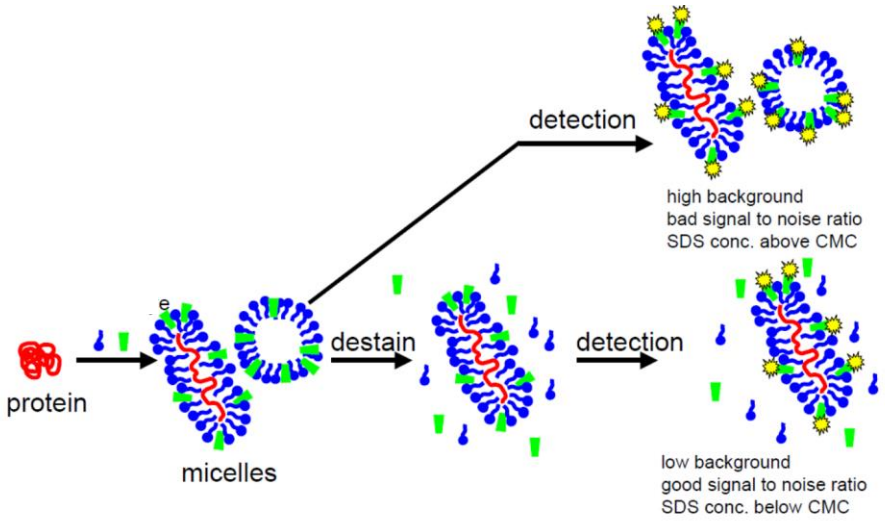


Figure 15: Scheme for protein staining and detection using an intercalating fluorescent dye (Bioanalyzer P230 Assay) [74]

The charged proteins are electrophoretically driven by a voltage gradient and because of the SDS treatment they all have a constant mass-to-charge ratio so that they are separated according to their size in presence of the sieving polymer. The data is subsequently translated by the Bioanalyzer 2100 Experts software into gel-like images (bands) and electropherograms (peaks). A typical electropherogram of a protein separation that is obtained from the P230 Assay in a commercial way and the corresponding gel images are illustrated in figure 16 and 17.

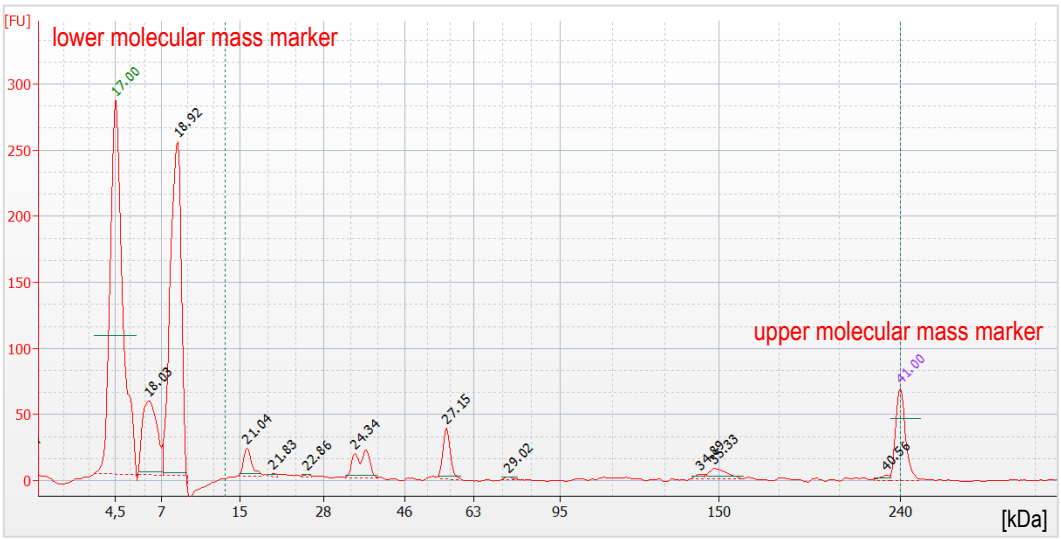


Figure 16: Electropherogram of a protein sample separated on the Bioanalyzer using the P230 Assay under reducing conditions

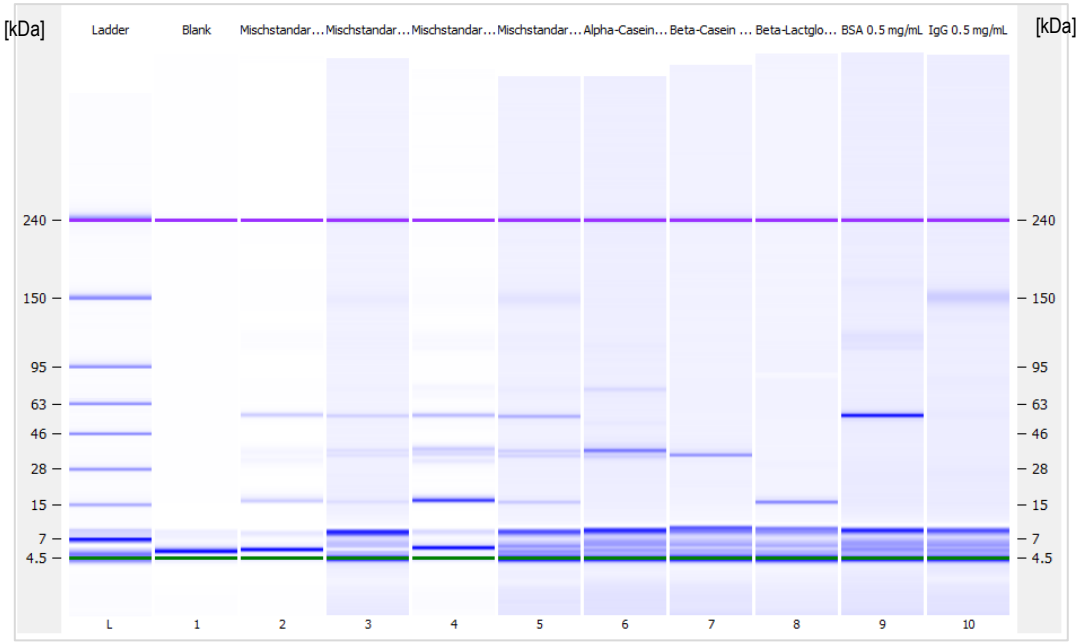


Figure 17: Gel-like images of a protein sample separated on the Bioanalyzer using the P230 Assay

These kind of chip systems are highly suitable for the use of routine processes like fermentation. Products can be monitored easily and fast. Advantages are found in the minimal need of chemicals and reagents, a marginal generation of waste and a low expenditure of time, about 25 minutes for the analysis of 10 samples including the whole sample preparation. The separation *per se* is finished within seconds.

3. Materials and Methods

3.1 Materials

Table 2 is providing information of all chemicals and proteins that were used during the work on this thesis.

Table 2: Materials

Product	***Abbreviation***	***Purity***	***Product #***	***Company***
Bovine serum albumin	BSA	≥ 96 %	A8022	Sigma Aldrich, Steinheim, Germany
α-Lactalbumin type III from bovine milk	α-Lactalbumin	≥ 85%	L6010	Sigma Aldrich, Steinheim, Germany
α-Casein from bovine milk	α-Casein	≥ 70 %	22084	Sigma Aldrich, Steinheim, Germany
β-Casein from bovine milk	β-Casein	≥ 98 %	C6905	Sigma Aldrich, Steinheim, Germany
κ-Casein from bovine milk	κ-Casein	≥ 70 %	C0406	Sigma Aldrich, Steinheim, Germany
β-Lactoglobulin A from bovine milk	β-Lactoglobulin A	≥ 90 %	L7880	Sigma Aldrich, Steinheim, Germany
β-Lactoglobulin B from bovine milk	β-Lactoglobulin B	≥ 90 %	L8005	Sigma Aldrich, Steinheim, Germany
Lactoferrin from bovine milk	Lactoferrin	≥ 85%	L9057	Sigma Aldrich, Steinheim, Germany
γ-Globulins from bovine blood	IgG	≥ 99 %	G5009-G5	Sigma Aldrich, Steinheim, Germany
Polyethyleneglycol diacrylate	PEG-DA	-	455008	Sigma Aldrich, Steinheim, Germany
2-hydroxy-2-methylpropiophenone	HMPP	≥ 97 %	405655	Sigma Aldrich, Steinheim, Germany
Coomassie Brilliant Blue G250	-	-	27815	Fluka Chemika, Steinheim Germany
Bromophenol Blue	-	-	B-8026	Sigma Aldrich, Steinheim, Germany

H_3PO_4	-	≥ 85 %	438081	Sigma Aldrich, Steinheim, Germany
Citric acid	-	≥ 99.5 %	251275	Sigma Aldrich, Steinheim, Germany
$CaCl_2$	-	-	10043-52-4	Merck, Darmstadt, Germany
Castor oil	-	-	3140931	Aponorm
Methanol	-	≥ 99.9 %	200-659-6	Merck, Darmstadt, Germany
Ethanol	-	≥ 99.8 %	200-578-6	Merch, Darmstadt, Germany
Phosphate buffered Saline PBS pH 7.4	PBS	-	P-5368	Sigma Aldrich, Steinheim, Germany
Ultrapure water (ultra-high quality, UHQ)	UHQ	Specific resistivity ≥ 18 Ω*cm (25 °C)	-	Millipore, Billerica, MA, USA
Bioanalyzer P230 Assay reagents		-	5067-1517	Agilent Technologies, Waldbronn, Germany
Dithiothreitol	DTT	-	D0632	Sigma Aldrich Steinheim, Germany
MIRIS Check	-	-	-	MIRIS Holding AB, Uppsala, Sweden
MIRIS Clean	-	-	-	MIRIS Holding AB, Uppsala, Sweden
Aptamil FMS	-	-		Milupa, Bad Homburg vor der Höhe, Hessen, Germany

3.2 Instruments

Table 3: Instruments

Instrument	***Company***
Nanophotometer	Implen
Bioanalyzer 2100	Agilent Technologies
MIRIS Human Milk Analyzer	MIRIS Holding AB
Thermomixer comfort	Eppendorf AG
Ultrasonic bath (USC200TH)	VWR

pH meter MP220	Mettler Toledo
Laboratory centrifuge	Sigma Aldrich

3.3 Methods

3.3.1 Preparation of protein stock solutions and dilution series

For the preparation of all solutions/dilutions ultra-high quality water (UHQ, specific resistivity ≥ 18 Ω*cm (25 °C)) or Phosphate buffered Saline (PBS 0.1 M, pH 7.4) was utilized. 0.1 M PBS was prepared by dissolving the provided powder (Sigma Aldrich) in 1 L UHQ.

Due to the fact that α-lactalbumin, β-casein and lactoferrin are the proteins that are counted as the most frequently occurring proteins in human breast milk, stock solutions of these proteins with a concentration of 3000 µg/mL in PBS were prepared. Furthermore, β-Lactoglobulin is a major component present in bovine milk. For the purpose of comparing human and bovine milk, a stock solution of this protein was prepared. In addition, bovine serum albumin, a protein from bovine blood that is principally used as a reference material in protein quantification, was utilized.

- 500 µl of PBS were provided in 1.5 mL Eppendorf tubes
- The proteins were weight in the caps of Eppendorf tubes and the solid matter was afterwards transferred into the tube by centrifugation
- The Volume of PBS was adapted to the weight protein powder to receive the favored final concentration

Table 4: Preparation of protein stock solutions

Protein	**Initial weight [µg]**	**Volume [µL]**	**Concentration [µg/mL]**
α-lactalbumin	3016	1003.3	3000
β-casein	3383	1127.7	3000
Lactoferrin	2813	937.7	3000
β-lactoglobulin B	2928	976.0	3000
BSA	3128	1042.7	3000

For all samples PBS (0.1 M, pH 7.4) was used as solvent if not otherwise stated.

- Stock solutions of α-lactalbumin, β-casein, lactoferrin and β-lactoglobulin B were diluted to concentrations of 600; 500; 400; 300; 200; 100; 50 and 25 µg/mL
- BSA stock solution was diluted to concentrations of 3000; 2500; 1500; 1250; 1000; 750; 500; tw50; 125 and 62.5 µg/mL

In addition *protein mix standards* were prepared for:

a) *Human breast milk:* α-lactalbumin, β-casein and lactoferrin (Human milk protein mix -HMPM)
b) *Bovine milk:* α-lactalbumin, β-casein, lactoferrin and β-lactoglobulin B (Bovine milk protein mix - BMPM)

- For the human milk mix and the bovine milk mix standards were prepared with concentrations of 600; 500; 400; 300; 200; 100; 25 and 50 mg/mL perprotein

3.3.2 Bradford Assay

The Bradford reagent was prepared as provided in table 5. Coomassie Brilliant Blue G250 was entirely dissolved in methanol, afterwards 85 % H_3PO_4 was added and the solution was adjusted to a volume of 100 mL by addition of UHQ.

Table 5: Bradford reagent

Bradford reagent (100 mL in total)	**Amount**
Coomassie Brilliant Blue G250	10.0 mg
Methanol	5.0 mL
85 % phosphoric acid	10.0 mL
UHQ	85.0 mL

- 15 µl of standards/samples were mixed with 750 µL of the freshly filtrated Bradford reagent by vortexing, filtration was performed by a glass funnel through a standard filter paper
- The mixtures were incubated for 10 minutes at room temperature in cuvettes
- The determination of the protein concentration was performed at 595 nm by using the Implen Nanophotometer after blank correction (15 µL UHQ and 750 µL Bradford reagent)
- The data was recorded manually

3.3.3 Bromophenol Blue Assay

The Bromophenol Blue reagent was prepared as provided in table 6. Bromophenol blue was entirely dissolved in ethanol, 10 mL of a 10 % citric acid solution was added to get a final concentration of 1 % (in a total volume of 100 mL) afterwards the solution was adjusted to a volume of 100 mL by addition of UHQ.

Table 6: Bromophenol Blue reagent

Bromophenol Blue reagent (100 mL in total)	**Amount**
Bromophenol Blue	10.0 mg

Ethanol	15.0 mL
10 % citric acid	10.0 mL
UHQ	75.0 mL

- 25 µl of standards/samples were mixed with 225 µL of the freshly filtrated Bromophenol Blue reagent by vortexing, filtration was performed by a glass funnel through a standard filter paper
- The mixtures were incubated for 10 minutes at room temperature in cuvettes
- The determination of the protein concentration was performed at 605 nm by using the Implen Nanophotometer after blank correction (25 µL UHQ and 225 µL Bromophenol Blue reagent)
- The data was recorded manually

3.3.4 Human Breast Milk Sample Preparation

Twenty human breast milk samples (A-T) were provided by the Neonatology of the AKH Vienna from 20 different mothers of preterm infants. The samples were received under cooled conditions and afterwards equilibrated to room temperature. For homogenization, the samples were warmed to 40 °C and treated in the ultrasonic bath for 15 minutes. Human breast milk samples were subsequently aliquoted in 500 µL samples and frozen at -20 °C until further examination.

Samples were analyzed in triplicates, so 3 of the 500 µl aliquots were thawn and the homogenization procedure was repeated before analysis. Repeated homogenization is needed for frozen human breast milk samples due to the fact that casein micelle aggregation and/or fat separation can occur upon thawing and to improve repeatability and accuracy. After thawing and homogenization, samples were diluted either with UHQ or PBS.

- UV/Vis Spectroscopy: dilution of the samples with UHQ
  - Bradford Assay:
    - 1:50: 20 µL sample + 980 µL UHQ
    - 1:40: 20 µL sample +780 µL UHQ
  - Bromophenol Blue Assay:
    - 1:15: 20 µL sample + 280 µL UHQ
    - 1:40 20 µL sample + 780 µL UHQ
- Bioanalyzer P230 Assay: dilution of the samples with PBS
  - 1:50: 20 µL sample + 980 µL PBS
  - 1:60: 20 µL sample + 1180 µL PBS
  - 1:70: 20 µL sample + 1380 µL PBS
  - 1:80: 20 µL sample +1580 µL PBS
- MIRIS: samples were analyzed without further dilution

3.3.5 MIRIS

The human milk analyzer based on IR-spectroscopy is consisting of an emitter, a sample cuvette and a detector. To load the sample cuvette there is an inlet (blue) and an outlet (red), the outlet is connected via a sleeve to a waste jar. Prior the analysis of human breast milk samples the device has to be cleaned and subsequently calibrated by using solutions called MIRIS Clean and MIRIS Check that are provided by the manufacturer and are prepared as specified in table 7.

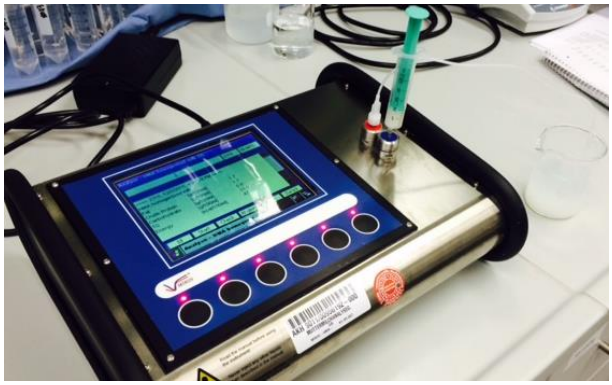


Figure 1818: Human milk analyzer (MIRIS)

Table 7: Preparation of MIRIS working solutions

Description	***Solution***	***Water***	***Storage***
MIRIS Clean	1 tube (10 mL)	490 mL	Room temperature
MIRIS Check	1 tube (10 mL)	90 mL	4 °C

Working protocol:

- *Cleaning:*
  - To purify the sample cuvette, the MIRIS Clean solution is warmed to 40 °C by using a water bath. 15 mL are injected through the outlet (red), afterwards, 10 mL are injected through the inlet (blue).
- *Calibration:*
  - For calibration of the instrument, 6 mL of the MIRIS Check solution that was previously equilibrated to room temperature are injected.
  - Calibration #1 for homogenized milk is selected and the calibration is performed by pressing “Check”.
  - If the check fails (“Adjustment needed!”) the device has to be cleaned carefully again and the check has to be repeated. If it fails again an adjustment of the instrument has to be carried out by pressing “Adjust”.
- *Analysis of human breast milk samples:*

- After purification and calibration, the instrument is ready to analyze human milk samples. The samples are thawed at room temperature, homogenized and warmed at 40 °C for 15 minutes by using a commercial ultrasonic bath. After gentle shaking, 6 mL of the sample are taken up with a syringe and injected into the instrument. For each measurement at least 1.5 mL are needed and each sample is analyzed three times from the syringe filling.
- The analysis itself takes about one minute and the results concerning the content of fat, protein, carbohydrates, dry matter and energy are displayed immediately.

- After the analysis of each sample the instrument has to be cleaned and the calibration has to be carried out again.
- After all samples are analyzed, the instrument is cleaned once more with the cleaning solution and subsequently with water. Finally, the cuvette is dried by the injection of air.
- The data can be saved to an USB stick or can also be recorded manually

3.3.6 Investigation of the influence of fat and calcium

For the investigation of interferences of the Bromophenol Blue, the Bradford and the Bioanalyzer P230 Assay, three different concentrations of fat, in terms of castor oil, and calcium, in terms of calcium chloride, were added to each standard of the BSA dilution series, the human milk protein mix (HMPM) and the bovine milk protein mix (BMPM).

Concerning the concentrations of these constituents present in human breast milk, the following three concentrations were chosen:

- *Fat:* in terms of castor oil
  - 10 mg/mL
  - 20 mg/mL
  - 30 mg/mL
- *Calcium:*
  - 0.76 mg $CaCl_2$/mL (≅ 2.5 mmol Ca^{2+}/L)
  - 1.53 mg $CaCl_2$/mL (≅ 5.0 mmol Ca^{2+}/L)
  - 2.30 mg $CaCl_2$/mL (≅ 7.5 mmol Ca^{2+}/L)

Concerning BSA also experiments were performed in which fat and calcium were added simultaneously as illustrated in table 8.

Table 8: Simultaneous addition of fat and calcium, n.a. = not analyzed

	Fat		
Calcium	***10 mg/mL***	***20 mg/mL***	***30 mg/mL***
2.5 mmol/L	x	n.a	X
5.0 mmol /L	n.a	X	n.a
7.5 mmol/L	x	n.a	X

3.3.7 *Agilent* Bioanalyzer P230 Assay

For analysis the *Agilent* Protein 230 Kit was used, the reagents and materials that are required for this purpose, including the fluorescent dye, the gel matrix, sample buffer and chips are summarized in figure 19.

Protein 230 Kit (reorder number 5067-1517)	
Protein Chips	**Protein 230 Reagents (reorder number 5067-1518) & Supplies**
25 Protein Chips	● (red) Protein 230 Gel-Matrix (4 vials)
1 Electrode Cleaner	● (blue) Protein 230 Dye Concentrate*
	❍ (white) Protein 230 Sample Buffer (4 vials)
Syringe Kit	● (yellow) Protein 230 Ladder
1 Syringe	4 Spin Filters

Figure 19: Agilent Bioanalyzer Protein 230 Kit [73]

Preparation of the Gel-Dye Mix and the Destaining solution:

- *Gel-Dye Mix:* 25 µL of the fluorescent dye are added to 650 µL of the Protein 230 gel matrix
- *Destaining solution:* 650 µL gel matrix
- Both solutions are centrifuged through a spin filter for 15 minutes and 2500 g

Preparation of the denaturing solution:

- 7 µL of 1M DTT was added to 200 µL of sample buffer and vortexed carefully

Preparation of the samples/standards:

Standard dilution series of the reference proteins (α-lactalbumin, β-casein, lactoferrin and β-lactoglobulin B) were prepared in a concentration range of 600-25 µg/mL; in addition the mixed standards of these proteins (HMPM and BMPM) were analyzed with concentrations 600-25 µg/mL for each protein in the respective. Human breast milk samples were diluted with PBS as already mentioned in chapter 3.3.4.

- 4 µL standard/sample and 2 µL denaturing solution were mixed by vortexing and spun down subsequently
- Standards/samples and the protein ladder were heated up for 5 minutes to 95 °C by using a thermomixer
- The tubes containing samples/standards/ladder were afterwards placed on ice to cool down and spun down once again
- 84 µL UHQ were added to each sample/standard/ladder, then they were vortexed and centrifuged for 15 seconds
- Until loading the samples/standards onto the chip, the tubes were left on ice and in the dark to protect the fluorescent dye from light

Cleaning of the instrument:

- The cleaning of the instrument is done by loading 380 µL UHQ to the cleaning chip that is provided by the manufacturer. It is placed it into the instrument, the lid is closed and after about 5 minutes the cleaning chip is removed. To dry the pin electrodes after cleaning, the lid of the instrument is left open for about 30 seconds.

Setting up the chip priming station:

In the case of the priming station, the base plate has to be adjusted to position A and the syringe clip has to be placed into the middle position.

Loading of the Chip:

Figure 20: Preparation of the chip

- 12 µL of the prepared gel-dye mix are pipetted in the first well G
- The plunger is put on 1 mL and the chip priming station is closed
- The plunger is pressed until it is held by the clip, after 60 seconds it is released
- After the plunger is no longer moving anymore, it can be pulled back to 1 mL and the priming station can be opened again
- 12 µL of the gel-dye mix are pipetted into the left G well
    - 12 µL of the destaining solution are pipetted into the well labeled with DS
- 6 µL of the protein ladder is pipetted into the according well (yellow)
- Finally, 6 µL of each sample is loaded into the sample wells 1-10

Performing the measurement:

After each well of the chip is filled with either gel-dye mix, destaining solution, protein ladder or samples, the chip is shortly vortexed and placed into the instrument. The lid is closed carefully, the program Protein 230 Series II is selected and the run is started.

3.3.8 Data evaluation and processing – Bioanalyzer P230 Assay

Each standard/sample was prepared and analyzed in triplicate, the resulting data was exported as txt.file and further processed in Microsoft Excel 2013 and OriginPro 9.1. For the peak integration OriginPro 9.1 was used with the parameters illustrated in figure 21. Based on the signal height of the upper marker, acting as internal standard, a correction factor for signal height was calculated and averaged for each analysis. After the normalization of the different measurements, the average peak area was plotted against the concentration of the standards.

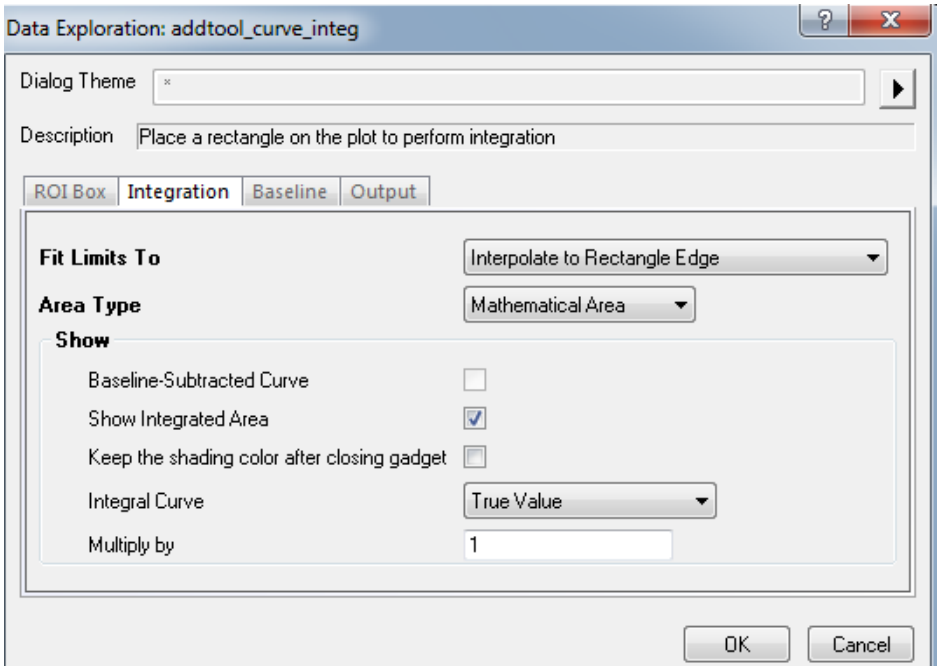


Figure 21: Parameters for the peak integration with OriginPro 9.1

4. Results and Discussion

4.1 *Agilent* Bioanalyzer P230 Assay

4.1.1 Human milk reference proteins

For the purpose of quantification of proteins in human breast milk, a reference material is essential for accurate analytical measurements and quality control. However, there are certified standards and reference materials available for a vast number of substances supporting a wide range of sectors including food and beverage, environmental, pharmaceutical, forensic, clinical, industrial, life science and the petrochemical sector. These high-quality products are pure but nevertheless or just because of this fact very expensive. Beyond that, a certified reference material for human breast milk is not available. For this reason, it waster intention to mix standard proteins to have some kind of reference material at hand. The most frequently occurring proteins in human breast milk were chosen, namely α-lactalbumin, β-casein and Lactoferrin.

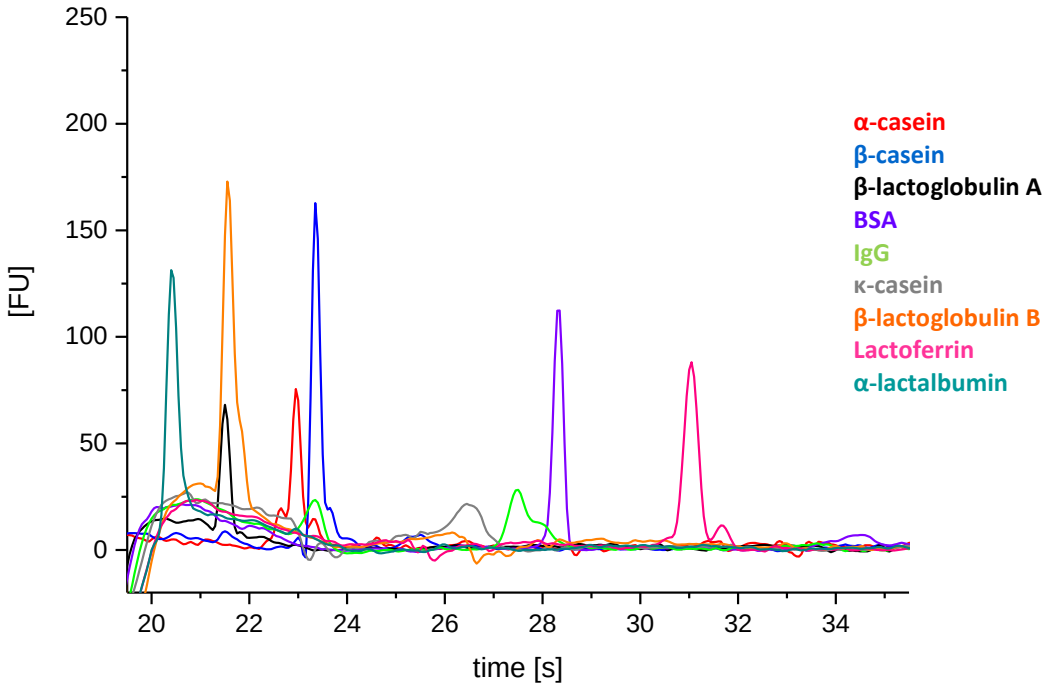


Figure 22: Overlay of separate electropherograms of proteins present in human and bovine milk using the Agilent Bioanalyzer P230 Assay under reducing conditions, 200 µg/mL per protein

In figure 22 the electropherograms of frequent proteins present in human and bovine milk are shown in an overlay. The separation was performed on the Bioanalyzer P230 Assay under reducing conditions. Overlapping signals are already visible (e.g. lactoglobulin signals, α-csein and BSA) and some proteins had only very low signal intensities for the rather high concentrations (e.g. IgG, κ -casein). The major

part of proteins in human breast milk is whey protein and the most frequent are α-lactalbumin, lactoferrin, serum albumin, secretory IgA (sIgA) and lysozyme. The most frequent casein in the case of human breast milk is β-casein, κ-casein is only present in minor concentrations, whereas α-casein is almost absent in human breast milk. In contrast to that, bovine milk contains a higher amount of casein, once again β-casein makes up the majority followed by α-casein. Beside α-lactalbumin, the genetic variants of β-lactoglobulin A and B make up the largest part of whey proteins in bovine milk, a protein that is not present in human breast milk at all.

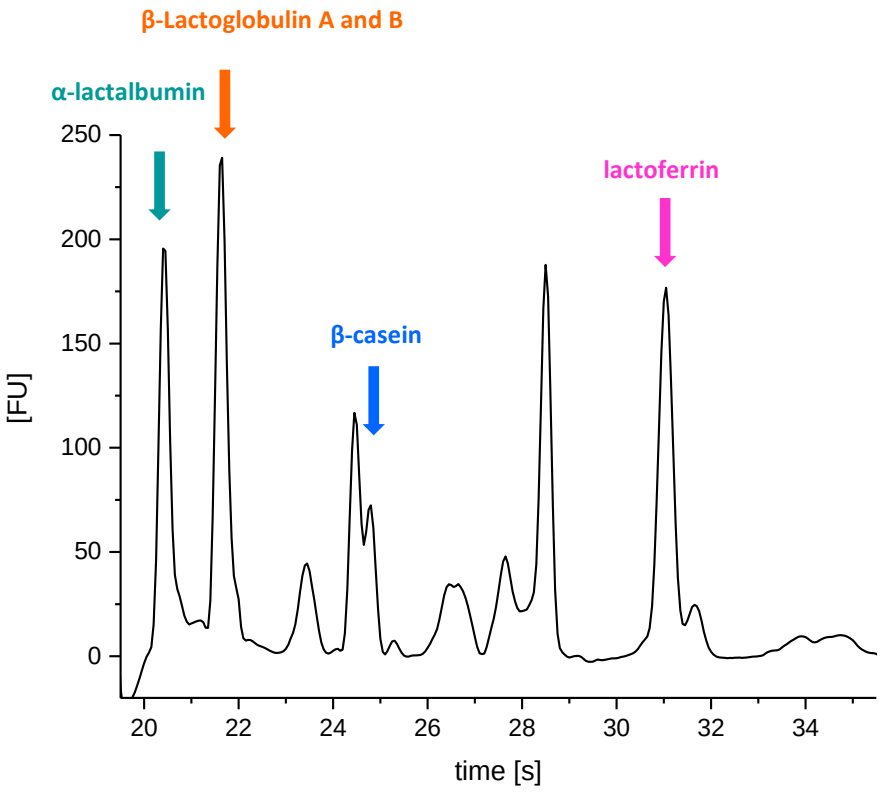


Figure 23: Selection of proteins for an external milk standard, mixture of proteins present in human breast milk and cow's milk, Bioanalyzer P230 Assay under reducing conditions, concentrations: 200 µg/mL per protein

Due to the milk composition and also because of cost efficiency, a mixture of three proteins was chosen to represent an artificial human milk protein standard (HMPM) and a mixture of four as bovine milk protein standard (BMPM).

- Human breast milk: α-lactalbumin, β-casein, lactoferrin
- Bovine milk: α-lactalbumin, β-casein, lactoferrin and β-lactoglobulin B

As presented in figure 24, the chosen proteins, α-lactalbumin, β-casein, lactoferrin and β-lactoglobulin B, can be well separated by using the Bioanalyzer P230 Assay, supporting the suitability of these proteins as reference standard for protein quantification.

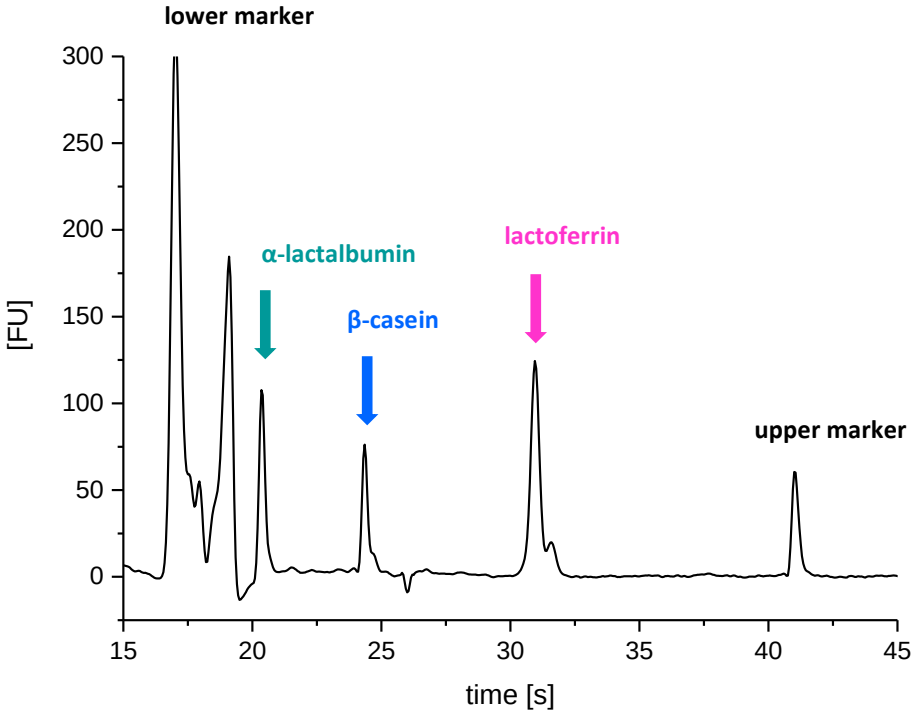


Figure 19: Represantative separation of the HMPM using the Bioanalyzer P230 Assay under reducing conditions, concentrations: 300 µg/mL per protein

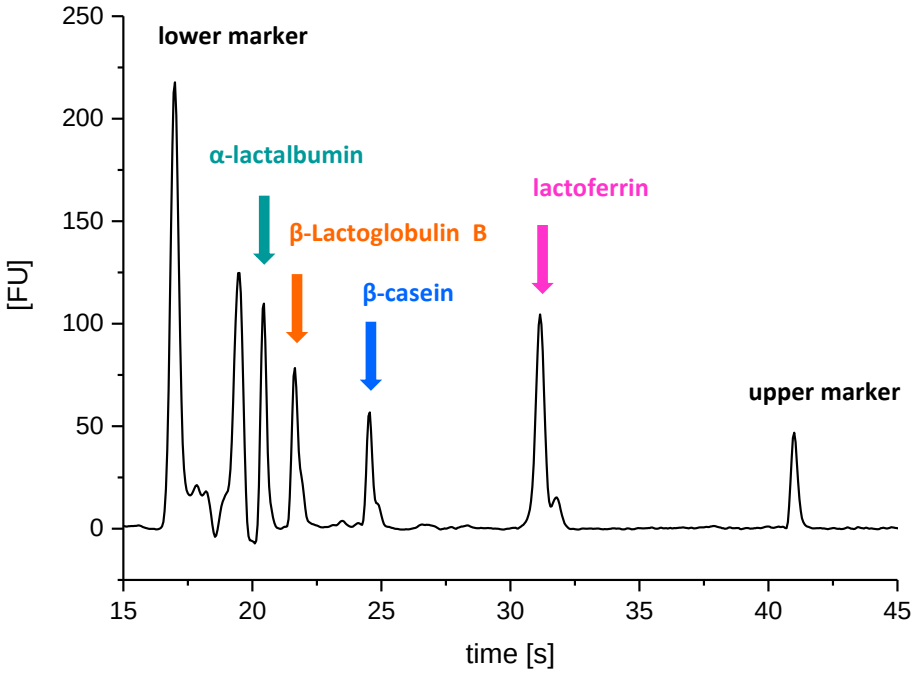


Figure 20: Representative separation of the BMPM using the Bioanalyzer P230 Assay under reducing conditions, concentrations: 300 µg/mL per protein

4.1.2 Data evaluation and processing

Whereas no or at least only slight shifts (1-2 seconds) of the three different analysis respective the x-axis were observed, a significant variability of the fluorescence signal in the y-axis occurred. These differences are making it very difficult to produce reproducible results and to compare the electropherograms for quantification purposes. Based on the signal height of the upper marker, acting as the internal standard for aligning multiple electropherograms that also acts as molecular weight marker, a correction factor for signal height was calculated and averaged for each analysis. For this purpose, the signal heights of the upper marker of each analysis were determined, the arithmetic mean was calculated and subsequently, for each measurement a correction factor f was estimated. The fluorescence signal was now multiplied by this factor and therefore normalized.

$$Average_{[FU]} = \frac{([FU]_{1^{st}run} + [FU]_{2^{nd}run} + [FU]_{3^{rd}run})}{3} \qquad \textit{equation 12}$$

$$f = \frac{[FU]}{Mean} \qquad \textit{equation 13}$$

$$[FU]_{normalized} = f \cdot [FU] \qquad \textit{equation 14}$$

The results for this normalization are displayed in figure 26, where triplicate measurements of HMPM at a concentration of 200 µg protein/mL for each protein were measured under reducing conditions.

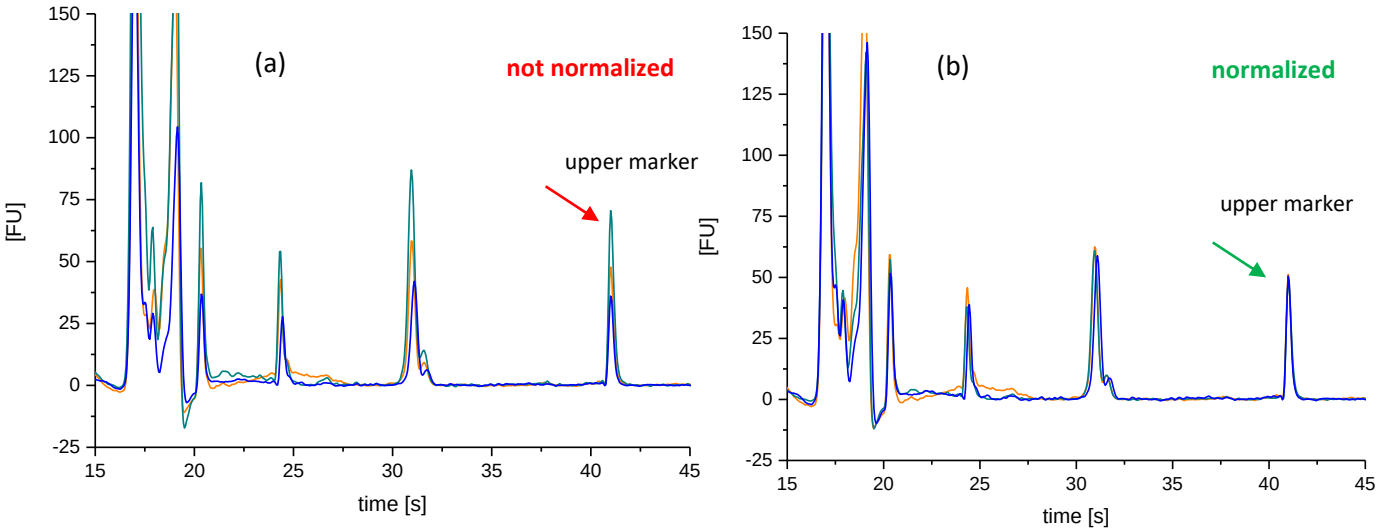


Figure 26: Electropherograms of three human milk protein standards containing 200 µg protein/mL for each protein. Results are shown (a) before and (b) after normalization correcting

4.1.3 Determination of LOD and LOQ

4.1.3.1 Protein standards

For the determination of the limits of detection (LOD) and the limits of quantification (LOQ) for each single milk component, stock solutions of the four chosen proteins, α-lactalbumin, β-casein, lactoferrin and β-lactoglobulin B with a concentration of 3000 µg/mL were prepared. Based on these stock solutions, standard dilution series were prepared in concentrations between 600 and 25 µg/mL and analyzed under reducing conditions as described in chapter 3.3.7. The calibration curves obtained after normalization (chapter 3.3.8) of the different standard proteins are illustrated in figure 27.

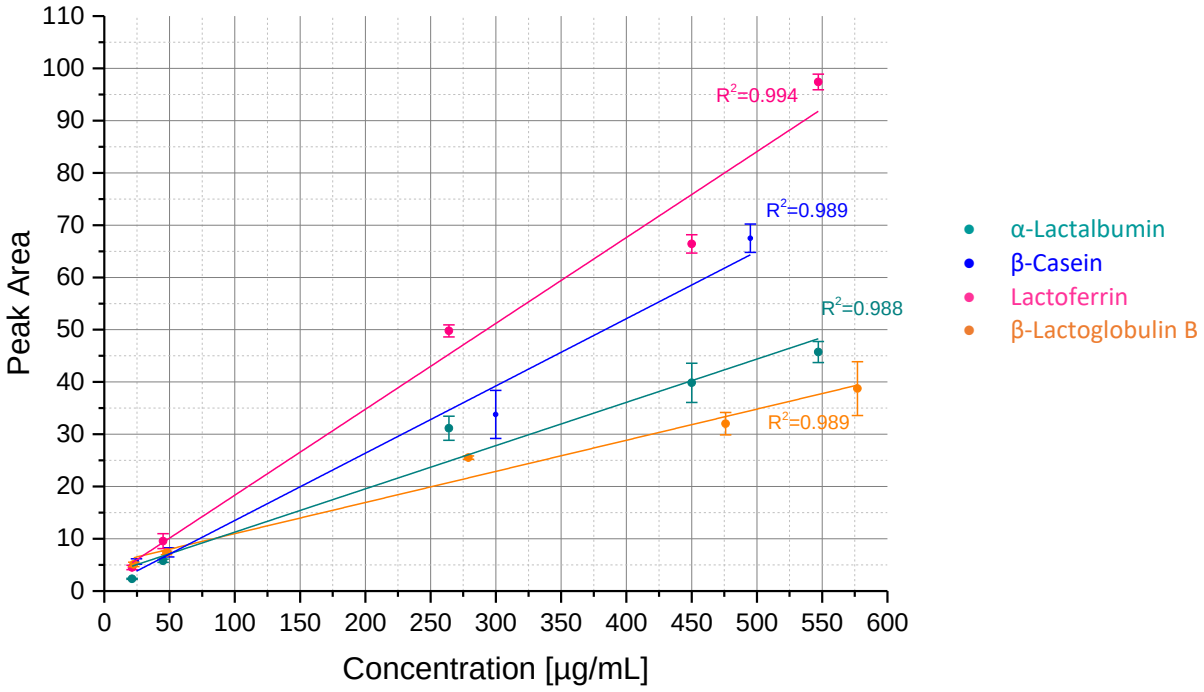


Figure 27: Calibration curves of α-lactalbumin, β-casein, lactoferrin, β-lactoglobulin B, Determination of LOD and LOQ in a concentration range between 600 and 25 µg/mL, after separation using the Bioanalyzer P230 Assay under reducing conditions

Table 9: Linear equations of the calibration curves of the protein standards α-lactalbumin, β-casein, lactoferrin, β-lactoglobulin B

Proteinstandard	Linear equation	R^2
α-lactalbumin	y = 0.08·x + 3.00	0.988
β-casein	y = 0.13·x + 0.64	0.989
Lactoferrin	y = 0.16·x + 1.91	0.994
β-lactoglobulin B	y = 0.06·x + 5.03	0.989

Linear ranges for all of the four proteins were determined in a range between 25 and 600 µg/mL or at least between 25 and 500 µg/mL for β-casein, with good correlation coefficients between 0.98 and 0.99. LOD and LOQ were calculated by equation 15 and 16 according to [75], with *y* as the standard deviation of the lowest standard and *k* as the slope of the resulting calibration curve.

$$Limit\ of\ Detection\ (LOD) = \frac{3 \cdot y_{lowest\ standard}}{k} \qquad \textit{equation 15}$$

$$Limit\ of\ Quantification\ (LOQ) = \frac{10 \cdot y_{lowest\ standard}}{k} \qquad \textit{equation 16}$$

The results of the determination of LOD and LOQ for the four different proteins are summarized in table 10.

Table 10: LOD and LOQ of α-lactalbumin, β-casein, lactoferrin, β-lactoglobulin B, using the Agilent Bioanalyzer P230 Assay under reducing conditions

Protein standards		
Protein	***LOD***	***LOQ***
α-lactalbumin	3.8 µg/mL	12.8 µg/mL
β-casein	12.3 µg/mL	40.8 µg/mL
Lactoferrin	7.7 µg/mL	25.7 µg/mL
β-lactoglobulin B	21.1 µg/mL	70.4 µg/mL

Values for LOD and LOQ were found in the lower µg/mL range for all of the investigated proteins, with the lowest values for α-lactalbumin and the highest values in case of β-lactoglobulin B. The highest sensitivity is given in the case of lactoferrin. In the case of β-casein the calibration curve is steeper than it is in the case of α-lactalbumin, but also here the limited linear range has to be considered. Furthermore, due to the normalization of the signal height of the protein peaks on the height of the internal standard, the runs became more reproducible. This is reflected in the relatively low experimental standard deviations of the individual measuring points (RSD: 1-8%).

4.1.3.2 Human milk protein mix (HMPM)

In addition to the standard proteins, dilution series of the mixture of frequent proteins present in human breast milk were prepared, to investigate potential interactions or influences among these different proteins. These parallel experiments are required because the situation, in which the proteins are available in the same matrix, is more similar to the actual situation in milk. Calibrations curves were determined for the individual proteins as well as for the total protein present in the mixture by separate integration of the different protein peaks and by integration over the whole peak area. This was done by integration of the area between the lower and the upper marker.

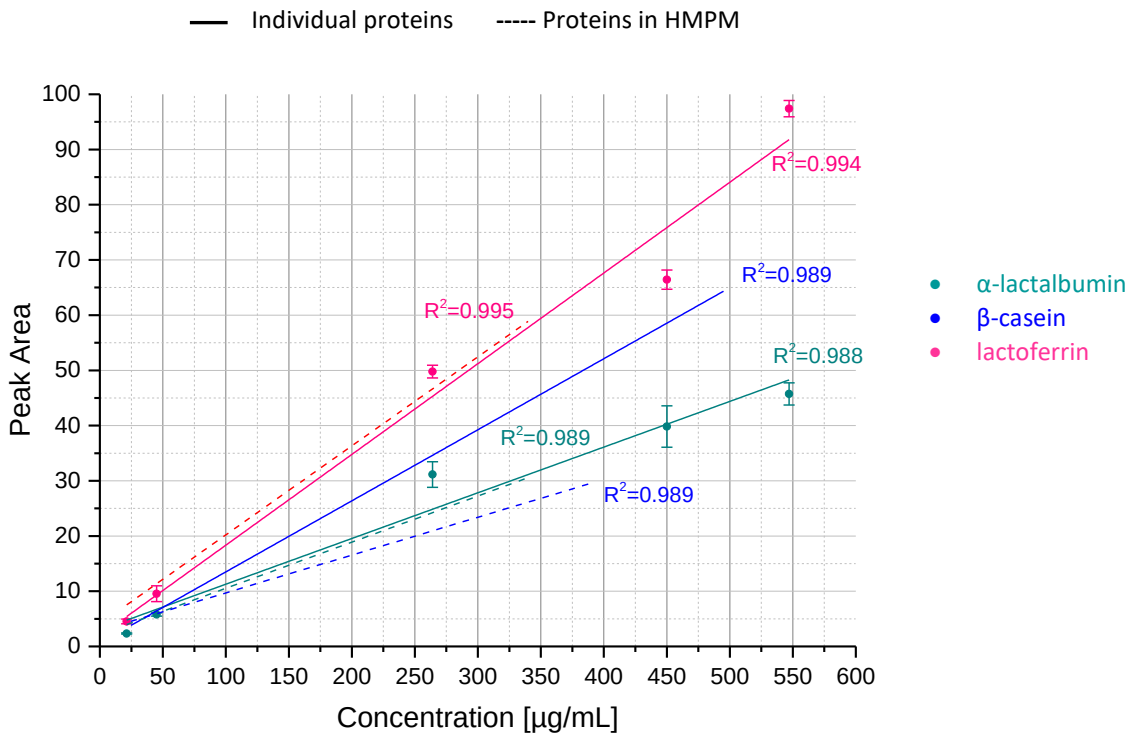


Figure 21: Determination of LOD and LOQ in HMPM, calibration curves of the individual proteins α-lactalbumin, β-casein and lactoferrin in a concentration range from 25 to 600 µg/mL, separation with Agilent Bioanalyzer P230 Assay under reducing conditions

Table 11: Linear equations of the calibration curves of the proteins α-lactalbumin, β-casein and lactoferrin in the HMPM

Protein in HMPM	Linear equation	R^2
α-lactalbumin	y = 0.16·x + 4.10	0.988
β-casein	y = 0.08·x + 2.20	0.989
lactoferrin	y = 0.07·x + 2.86	0.994
total protein	y = 0.10·x + 26.28	0.977

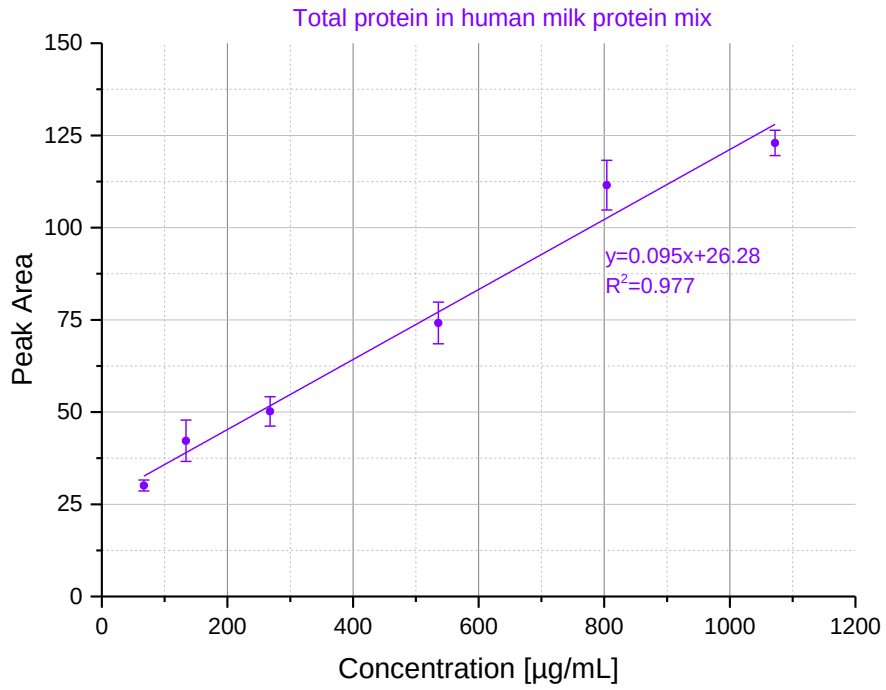


Figure 29: Peak areas determined for the total protein content in HMPM, for LOD and LOQ determination, concentration range from 65 to 1080 µg/mL, separation with Agilent Bioanalyzer P230 Assay under reducing conditions

In the figures 28 and 29 calibration curves of the three different proteins α-lactalbumin, β-casein and lactoferrin, as well as the calibration curve of the total protein present in the mixture, are given. The solid lines stand for the protein standards whereas the dashed lines are marking the particular proteins in the mixture. Corresponding values for LOD and LOQ are given in table 12.

Table 12: LOD and LOQ of the proteins in HMPM α-lactalbumin, β-casein, lactoferrin, Agilent Bioanalyzer P230 Assay

Human milk protein mix (HMPM)		
Protein	***LOD***	***LOQ***
α-lactalbumin	7.2 µg/mL	23.8 µg/mL
β-casein	12.9 µg/mL	43.1 µg/mL
Lactoferrin	1.5 µg/mL	5.0 µg/mL
Total protein	47.0 µg/mL	156.6 µg/mL

Comparing the calibration curves of the protein standards and the ones obtained from the protein mixture, it is seen that in the cases of α-lactalbumin and lactoferrin the slope of the calibration curve is practically not shifted but the linear ranges are reduced from 25-600 µg/mL to 25-400 µg/mL. Values for LOD and LOQ are altered in both cases, for α-lactalbumin higher values were detected and in the case of lactoferrin even lower values. Due to the fact that the slopes are almost not affected, this can

be explained by the higher or respectively lower standard deviations of the lowest standard that is used for calculation. The higher standard deviation can be explained by the interaction of different proteins in the mixture, influencing and affecting the migration of each other. In the case of β-casein even higher values for LOD and LOQ were detected and additionally the slope of the calibration curve is declined, resulting in a strong decrease in sensitivity. Except in the case of lactoferrin values for LOD and LOQ were increasing in the presence of other proteins, suggesting an effect of the proteins on each other.

By integration over the whole peak area of the electropherograms, information about the total protein can be obtained which is later required for the quantification of the total protein content of human breast milk samples. For the total protein in the HMPM, low values for the parameters of the sensitivity of the Assay were calculated, with LOD of 47 µg/mL and LOQ of about 157 µg/mL. The offset on the y-axis is constituted by the fact that by integration over the whole peak area also other proteins from impurities are recorded.

4.1.3.3 Bovine milk protein mix (BMPM)

Due to the same reasons as described in chapter 4.1.3.2 dilution series of the BMPM including α-lactalbumin, β-casein, lactoferrin and β-lactoglobulin B were prepared and analyzed in the same way as the HMPM.

In the case of BMPM illustrated in figure 30, it is seen that the slopes of the calibration curves are altered concerning all four proteins. Upon analysis of the mixture for α-lactalbumin, lactoferrin and β-lactoglobulin B an increase of the slope was detected, especially in the case of lactoferrin. For β-casein, a decrease of the slope was recognized. LOD and LOQ were determined with higher values for α-lactalbumin, β-casein and lactoferrin and with lower values for β-lactoglobulin B in the mixture compared to the protein standards.

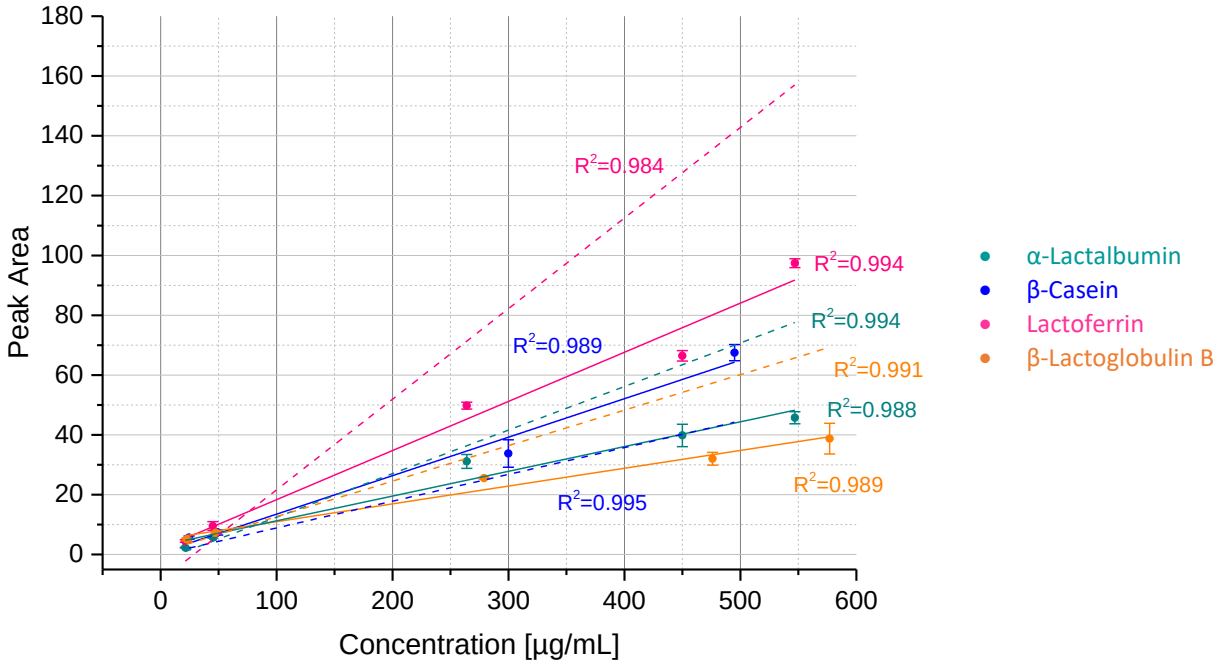


Figure 22: Calibration curves for the BMPM to determine LOD and LOQ for the individual proteins, α-lactalbumin, β-casein, lactoferrin and β-lactoglobulin B; concentration range: 600-25 µg/mL, separation with Agilent Bioanalyzer P230 Assay under reducing conditions

Table 13: Linear equations of the calibration curves of the proteins α-lactalbumin, β-casein, lactoferrin and b-lactoglobulin B in the BMPM

Proteinstandard	Linear equation	R^2
α-lactalbumin	y = 0.15·x – 2.07	0.994
β-casein	y = 0.09·x – 0.04	0.995
Lactoferrin	y = 0.30·x – 8.65	0.984
β-lactoglobulin B	y = 0.12·x + 0.80	0.991
total protein	y = 0.13·x + 10.93	0.996

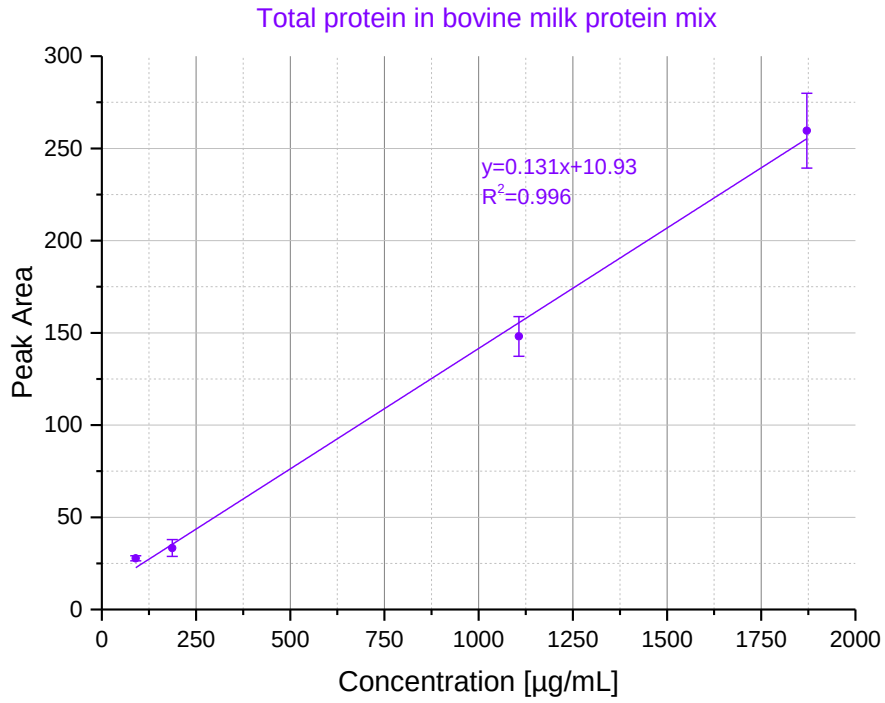


Figure 31: Peak areas determined for the total protein content in BMPM for LOD and LOQ determination, concentration range from 90 to 1900 µg/mL, separation with Agilent Bioanalyzer P230 Assay under reducing conditions

In the case of the BMPM, the lowest values for LOD and LOQ in the case of β-lactoglobulin B were evaluated , followed up by α-lactalbumin and lactoferrin. The highest values were here also found for β-casein, indicating the lowest sensitivity of the Assay for this protein. For the total protein in the BMPM, values for LOD and LOQ were found to be 31.9 µg/mL for LOD and with 106.4 µg/mL for LOQ.

Table 14: LOD and LOQ of the proteins in BMPM, α-lactalbumin, β-casein, lactoferrin, and β-lactoglobulin B, Agilent Bioanalyzer P230 Assay

Bovine milk protein mix (BMPM)		
Protein	***LOD***	***LOQ***
α-lactalbumin	6.3 µg/mL	21.1 µg/mL
β-casein	18.3 µg/mL	60.9 µg/mL
Lactoferrin	7.0 µg/mL	23.3 µg/mL
β-lactoglobulin B	2.2 µg/mL	7.3 µg/mL
Total protein	31.9 µg/mL	106.4 µg/mL

4.1.2.4 Comparison of the human (HMPM) and bovine (BMPM) milk protein mix

In the HMPM, it was detected that the sensitivity in the case of α-lactalbumin and lactoferrin was almost not affected by the presence of other proteins. In the case of β-casein, a reduction in sensitivity

was found, so it can be assumed that β-casein is influenced by the other proteins. β-casein is a protein very likely to form micelles and this micellar state is its natural conformation in milk. Due to the fact that the detection principle of the Agilent Bioanalyzer P230 Assay is based on the formation of protein-SDS-fluorescent-dye complexes, in which the fluorescent dye is intercalating, it appears reasonable that β-casein could be a problematic protein for this Assay. It can be assumed that β-casein, in mixture with other proteins, is able to form micelles with just those or probably with available impurities or ions present in the sample buffer, changing its migration behavior.

Furthermore, by the analysis of human breast milk samples with the Bioanalyzer P230 Assay, it was observed that the peak attributed to casein does not show constant peak pattern, which is illustrated in figure 32. In contrast to the remaining investigated proteins, the peak pattern of the casein peak in the electropherograms is significantly harder to predict. Furthermore, it is conceivable that the structure of casein in milk can be altered due to the many other constituents, therefore changes in the migration pattern and the peak shape can also be attributed to this circumstance. This observation is as well suggesting complications for the analysis of casein proteins with the Bioanalyzer P230 Assay.

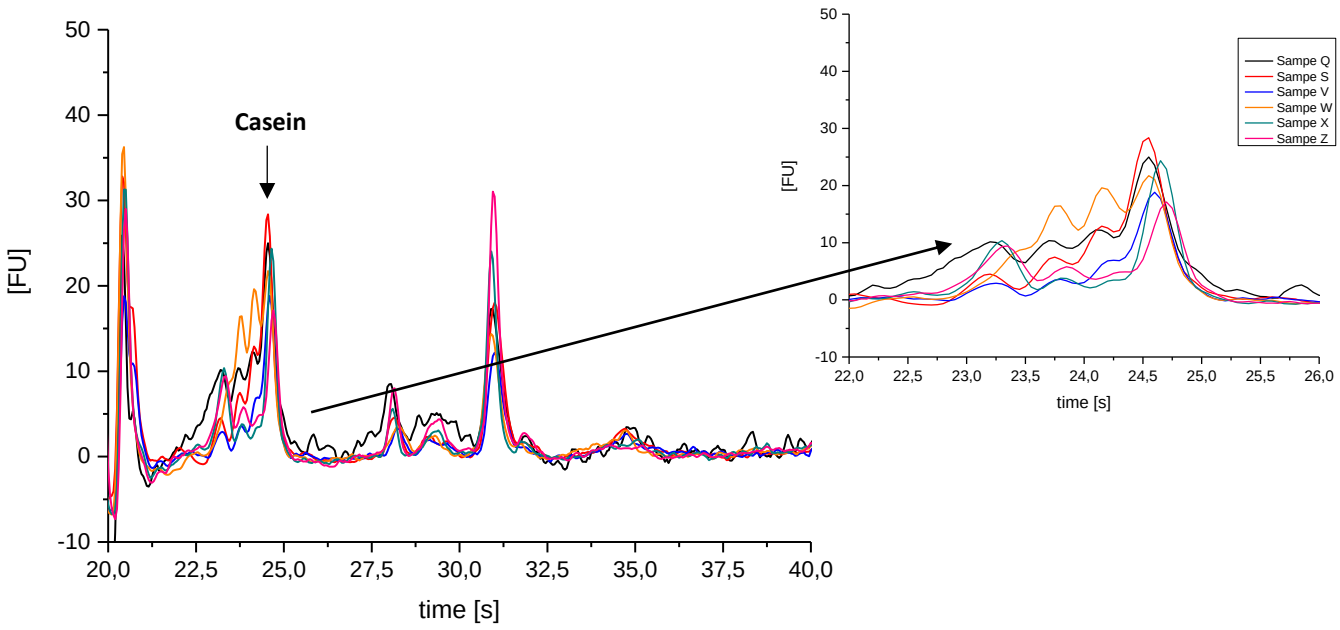


Figure 32: Peak pattern of different human breast milk samples with the analyzed with the Agilent Bioanalyzer P230 Assay under reeducng conditions, the inset shows details of the casein peak pattern having significantly higher variability compared with other protein peaks

Comparing the protein standards, the HMPM and the BMPM, the lowest sensitivity was found for β-casein, reflected in the highest values of LOD and LOQ in all three cases.

In the BMPM, an alteration in sensitivity was seen in all four cases and therefore an influence of all of the proteins on each other. Higher sensitivities were found for α-lactalbumin, lactoferrin and β-

lactoglobulin B, and also a lower sensitivity for β-casein was detected. So here again the influence on β-casein caused by the remaining proteins in a negative way but also an influence on α-lactalbumin and lactoferrin which is therefore supposed to be induced by β-lactoglobulin B, was found.

4.2 UV/Vis spectroscopy

In terms of UV/Vis spectroscopy, two different spectroscopic Assays were compared, the Bradford Assay, which is a golden standard in protein quantification, and the Bromophenol Blue Assay, which was applied by Puchberger-Enengl when establishing the microfluidic system and provided data on the first experiments on protein quantification. Furthermore, three different calibration models were compared. One used BSA for calibration and protein determination, the other used the established HMPM and the last used the BMPM. In contrast to the Bioanalyzer approach in which proteins are separated before quantification in UV/Vis spectroscopy, only the total protein content can be investigated.

4.2.1 Determination of LOD and LOQ

For UV/Vis spectroscopy LOD and LOQ were calculated in the same way as already explained for the analysis with Bioanalyzer P230 Assay according to equation 15 and 16.

4.2.1.1 BSA

The calibration curves of the Bradford and the Bromophenol Blue Assay obtained by a BSA standard dilution series are presented in figure 33, the results of the calculation of LOD and LOQ are combined in table 15.

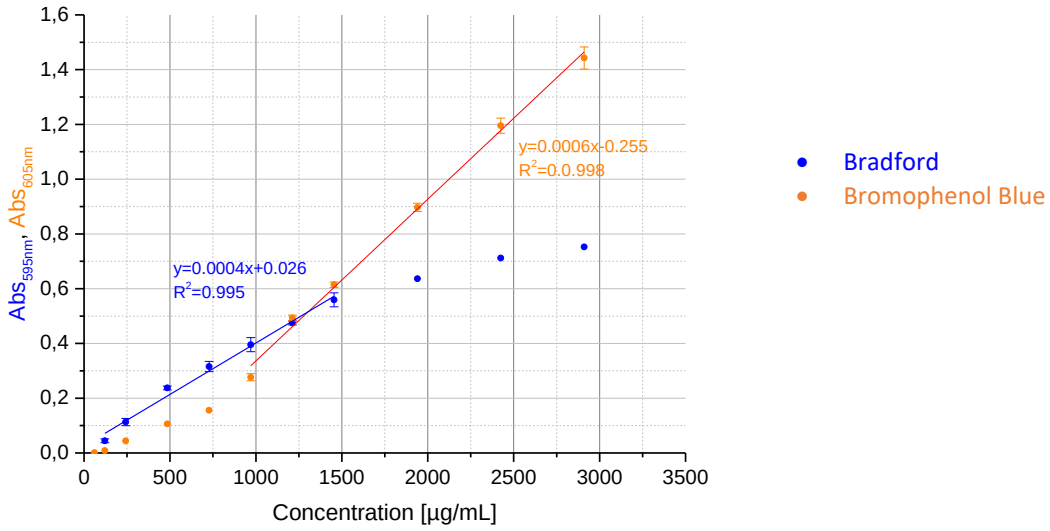


Figure 33: Determination of LOD and LOQ for BSA using the Bradford and the Bromophenol Blue Assay by UV/Vis spectroscopy

Different linear ranges for both Assays were founf between 62.5 and 1500 µg/mL for the Bradford Assay and 1000-3000 µg/mL for the Bromophenol Blue Assay. Both Assays showed excellent correlation coefficients of 0.99 and better. For the Bradford Assay, a deviation from linearity was found

for concentrations beyond 1500 µg/mL and in the case of the Bromophenol Blue Assay a deviation from linearity was observed for concentrations below 1000 µg/mL. Although the Bromophenol Blue Assay shows a broader linear concentration range, the linear range of the Bradford Assay seems to be more suitable for quantification of proteins in human breast milk. Furthermore, for the Bradford Assay a higher sensitivity (steeper calibration curve), as well as lower values for LOD and LOQ, were determined.

Table 15: Determination of LOD and LOQ for BSA, UV/Vis spectroscopy

	Bradford		***Bromophenol Blue***	
	LOD	*LOQ*	*LOD*	*LOQ*
BSA	57 µg/mL	189 µg/mL	66 µg/mL	219 µg/mL

4.2.1.2 Human milk protein mix (HMPM)

Figure 34 is displaying the calibration curves of the HMPM of the Bradford and the Bromophenol Blue Assay.

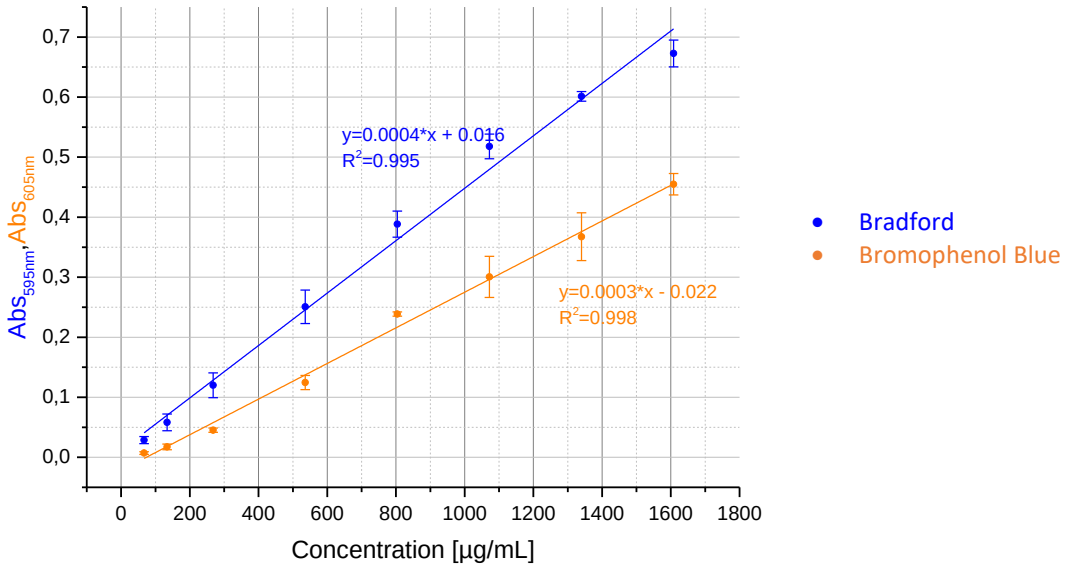


Figure 34: Determination of LOD and LOQ for the HMPM using the Bradford and the Bromophenol Blue Assay by UV/Vis spectroscopy

Also for the HMPM a higher sensitivity, as well as lower LOD and LOQ, were determined in the case of the Bradford Assay. But in contrast to BSA calibration, the two colorimetric Assays are showing the same linearity in the concentration range from 65 and 1600 µg/mL, indicating that the interaction of the dyes with proteins is dependent on the type of protein. As already pointed out in chapter 2.5.1.2.2,

Coomassie Brilliant Blue binds the most strongly to arginine, lysine, histidine and aromatic amino acid side chains of the protein, causing differences in the response of the Assay due to different abundances of these amino acids in different proteins. Although the interaction between Bromophenol Blue and proteins is not definitely identified, it seems to be reasonable that it is interacting with acidic and basic amino acids. Additionally, differences in the primary structure of proteins can cause variations in the response of the Assay.

Furthermore, the calculated values for LOD and LOQ, illustrated in table 16, were determined to be lower than in the case of the BSA calibration. However, the sensitivity expressed in the slope of the calibration curve was found to be nearly the same for BSA and the HMPM calibration for the Bradford Assay (k=0.0004).

Table 16: Determination of LOD and LOQ for the HMPM, UV/Vis spectroscopy

	Bradford		***Bromophenol Blue***	
Total protein	*LOD*	*LOQ*	*LOD*	*LOQ*
HMPM	40 µg/mL	134 µg/mL	49 µg/mL	162 µg/mL

4.2.1.3 Bovine milk protein mix (BMPM)

For the BMPM, linear ranges were found for the Bradford and the Bromophenol Blue Assay between 90 and 1400 µg/mL with excellent correlation coefficients of 0.99 and better, given in figure 35. Like in the case of the HMPM, no differences in the linear ranges of the two Assays were observed. Likewise to the both already discussed calibration models, a significantly higher sensitivity resulting in lower LOD and LOQ was again found for the Bradford Assay than for the Bromophenol Blue Assay.

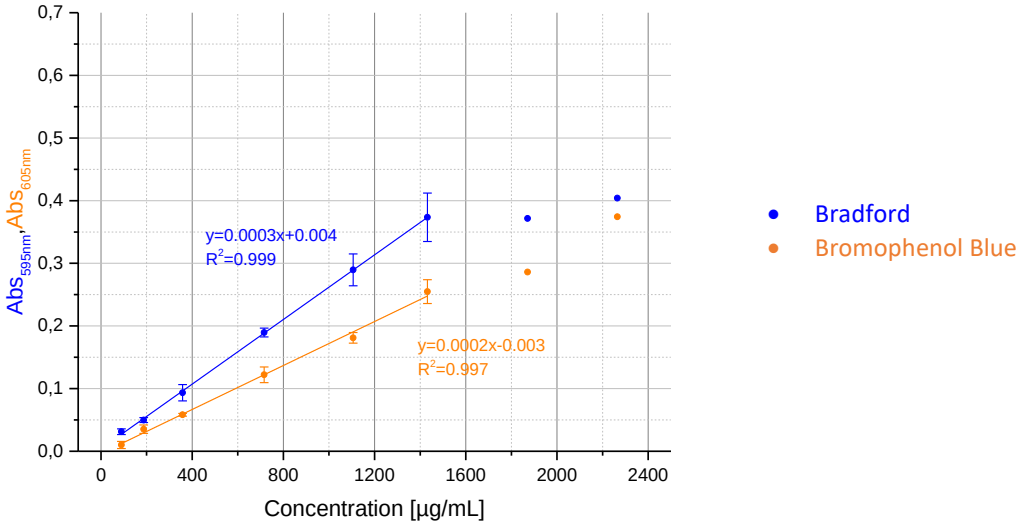


Figure 35: Determination of LOD and LOQ for the BMPMusing the Bradford and the Bromophenol Blue Assay by UV/Vis spectroscopy

Table 17: Determination of LOD and LOQ for the BMPM, UV/Vis spectroscopy

	Bradford		***Bromophenol Blue***	
Total protein	*LOD*	*LOQ*	*LOD*	*LOQ*
BMPM	53 µg/mL	177 µg/mL	97 µg/mL	325 µg/mL

4.2.1.4 Comparison of results using BSA, HMPM or BMPM as calibrations

Comparing the three different standard calibrations, one can see that in all three cases lower values for LOD and LOQ, as well as higher sensitivity in the case of the Bradford Assay than in the case of the Bromophenol Blue Assay, were found. For the BSA calibration, the highest LOD/LOQ values were recognized, in the case of the HMPM the lowest for both Assays. Comparing the BMPM to the other two calibrations, it was observed that the sensitivity was slightly reduced. For the Bradford Assay, for all calibrations nearly the same linear ranges were investigated, whereas for the Bromophenol Blue Assay the linear range for BSA is significantly differing from the other two reference materials. For the Bromophenol Blue Assay the highest values for LOD and LOQ were found in the case of the BMPM. Also, here an extenuated sensitivity given in the reduced slope of the calibration curve was recognized.

4.3 Influences of other human breast milk constituents

As already explained in chapter 3.3.6, fat and calcium were added to the protein standards to investigate if other substances present in human breast milk and also bovine milk interfere with the quantification of proteins. The influence of fat and calcium was tested for the Bioanalyzer P230 Assay (HMPM) and for UV/Vis spectroscopy (HMPM, BMPM and BSA) based on the Bradford and the Bromophenol Blue Assay. Experiments about the simultaneous addition of fat and calcium were done only for UV/Vis spectroscopy.
Interferences of other human breast milk constituents would be represented in a shift of the slope of the calibration curve, either to higher or lower values for absorbance. Another possibility is a complete loss of linearity of the investigated Assays.

4.3.1 Bioanalyzer P230 Assay

The influence of the addition of fat and calcium was investigated for the HMPM, as well as for the individual proteins in the mixture, by integration the whole peak area and by separate integration of the individual signals. The resulting electropherograms with additional fat and calcium were normalized against the upper marker as described in chapter 4.1.2.

4.3.1.1 Influence of fat

Three different concentrations of castor oil were added to simulate the fat content in human breast milk. 10 mg/mL to represent a low fat content, 20 mg/mL for medium fat concentrations and 30 mg/mL for high fat content were chosen. The results for the the Bioanalyzer P230 Assay are illustrated in figure 36.

The addition of fat to the HMPM did not cause total loss of linearity but the slope of the resulting calibration curve was shifted to higher values because of larger values for the respective peak areas. The changes were not significant for lower concentrations. At higher concentration values deviated significantly from the results presented in chapter 4.1.3.1. The single protein standards (α-lactalbumin, β-casein, lactoferrin) and the total protein content showed significant absorbance shifts at the highest concentrations (400 µg/mL protein and 1100 µg/mL for the total protein respectively).

The highest variation was found for 30 mg/mL fat, the lowest in the case of 20 mg/mL. The influence of 10 mg/mL fat was found to be between these values. The higher peak areas can possibly be explained by the theory that fat is able to attach to the proteins and is, therefore, promoting the formation of protein micelles. The formation of micelles on the other hand perhaps can favor the emplacement of the fluorescent dye provoking higher signal intensities.

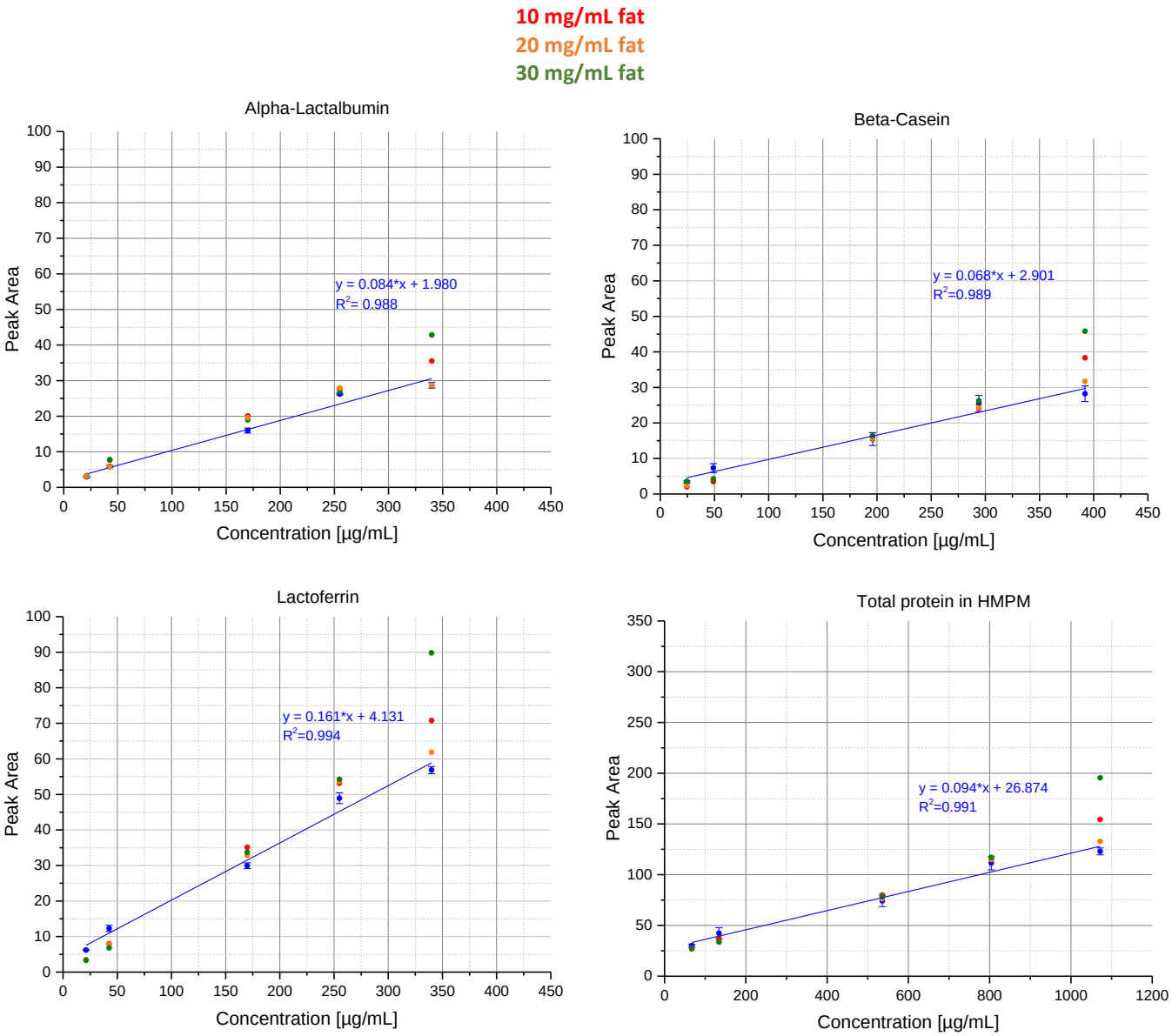


Figure 36: Determination of the Influence of fat (castor oil) on the quantification of human milk proteins α-lactalbumin, β-casein, lactoferrin and the total protein using the Agilent Bioanalyzer P230 Assay

4.3.1.2 Influence of calcium

Like for the addition of fat, three different concentrations of calcium were chosen: 2.5 mmol/L, 5.0 mmol/L and 7.5 mmol/L. The results of the investigation of the influence of calcium addition are illustrated in figure 37.

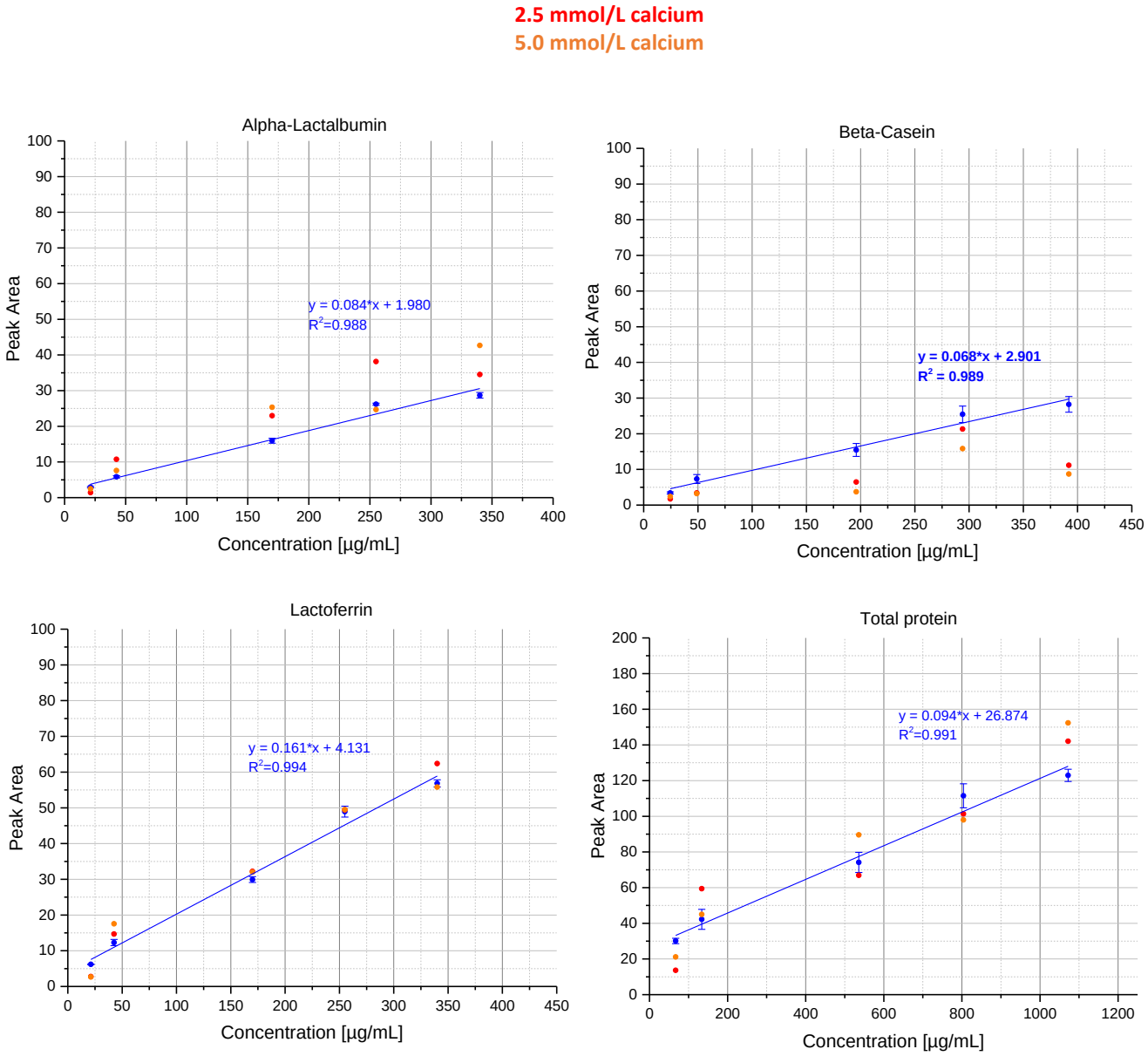


Figure 37: Determination of the Influence of calcium ($CaCl_2$) on the quantification of human milk proteins α-lactalbumin, β-casein, lactoferrin and the total protein using the Agilent Bioanalyzer P230 Assay

In contrast to the addition of fat, the addition of calcium was observed to be problematic for the quantification of human breast milk proteins. For the different proteins, diverse effects were noticed. While the addition of calcium apparently did not cause any effect on lactoferrin an interference of calcium with α-lactalbumin, β-casein and therefore with the total protein as well, was seen. For α-lactalbumin, a shift of the calibration curve up to higher absorbance values was detected, while in the case of β-casein a decline in the slope of the calibration curve was noticed. For β-casein as well a loss of linearity was observed. The addition of calcium was also detected to be more influential at high protein concentrations, while at lower concentrations almost no significant influence was observed.

For calcium, the actual concentration seems to be a parameter whose impact is ascending with increasing concentration. As already described in chapter 2.1 the ability to bind Ca^{2+} was reported for α-lactalbumin as well as for β-casein. This ability is furthermore reflected in the results exhibited by the fact that the calibrations of those two proteins are altered. These findings are indicating an interferences on protein quantification in human breast milk, caused by calcium.

Another problem that was occurring by monitoring the influence of calcium on protein quantification was that the instrument is not able to separate samples of high ion concentration, therefore separation of milk samples containing calcium concentrations of 7.5 mmol/L failed. The reason for this is the fact that free Ca^{2+} ions cause a higher electrical current flow, generating heat and therefore resulting in a melting of the gel matrix.

4.3.2 UV/Vis spectroscopy

The influence of the addition of fat and calcium was investigated for calibrations using BSA, HMPM and the BMPM. Furthermore, experiments about the influence of the simultaneous addition of fat and calcium were performed for BSA and the HMPM.

4.3.2.1 Influence of fat

The results of the investigation of the influence of available fat in milk are illustrated in figure 38-40, showing diverse impacts on the different calibration models.

While for the BSA calibration almost no significant influence of fat was detected for both calorimetric Assays, a significant loss of sensitivity (reduced slope of the calibration curve) was observed for the Bromophenol Blue Assay using the HMPM. Furthermore, for the BMPM, it was observed that the impact of the addition of fat showed stronger effects in the case of the Bradford Assay, resulting in an increased slope of the calibration curve. In summary, it was observed that the influence of fat is not linearly increasing with the absolute concentration of fat but it tends to get more distinct with increasing protein concentration.

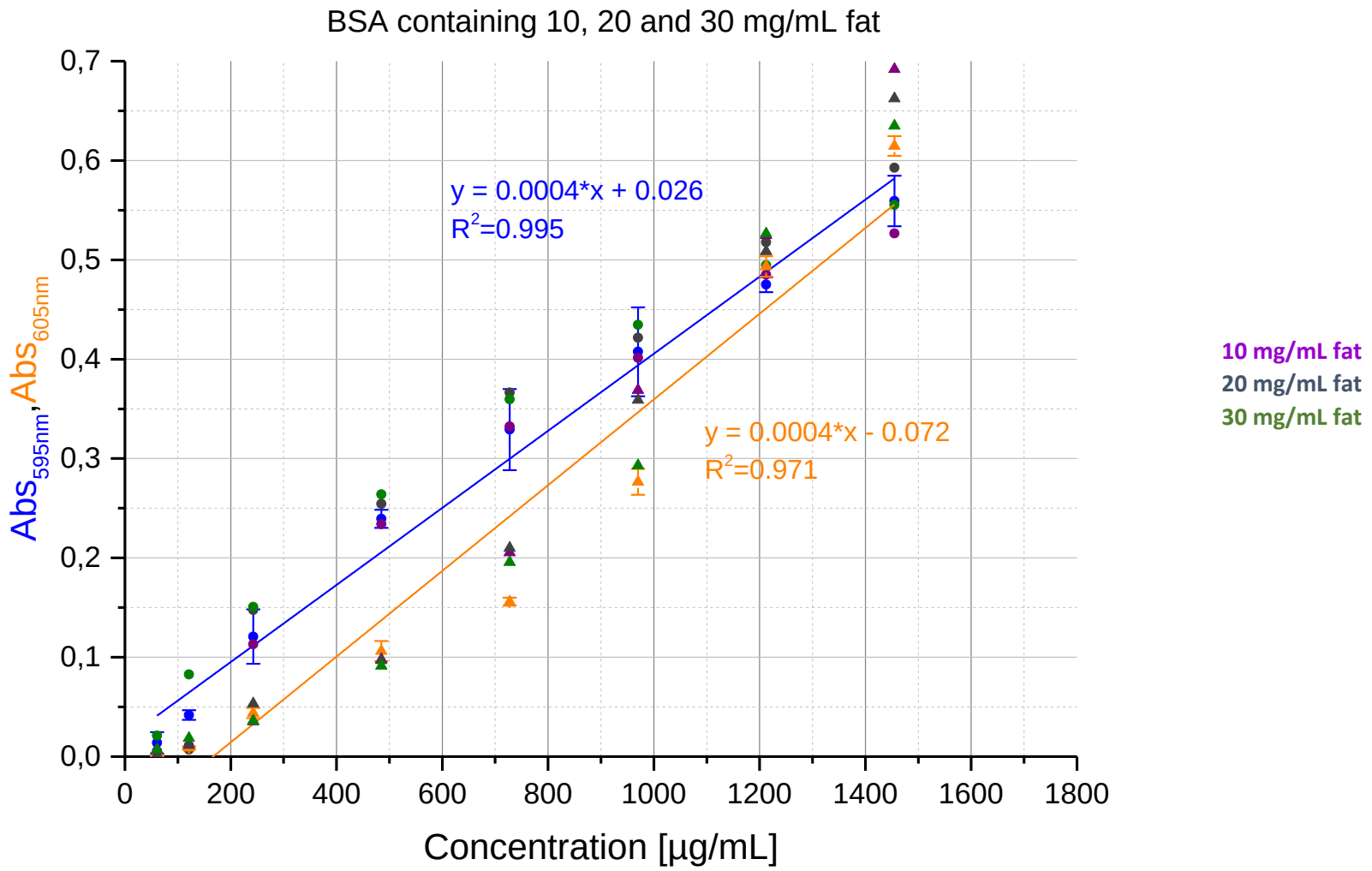


Figure 23: Determination of the influence of fat on BSA calibration using the Bradford and Bromophenol Blue Assay by UV/Vis spectroscopy

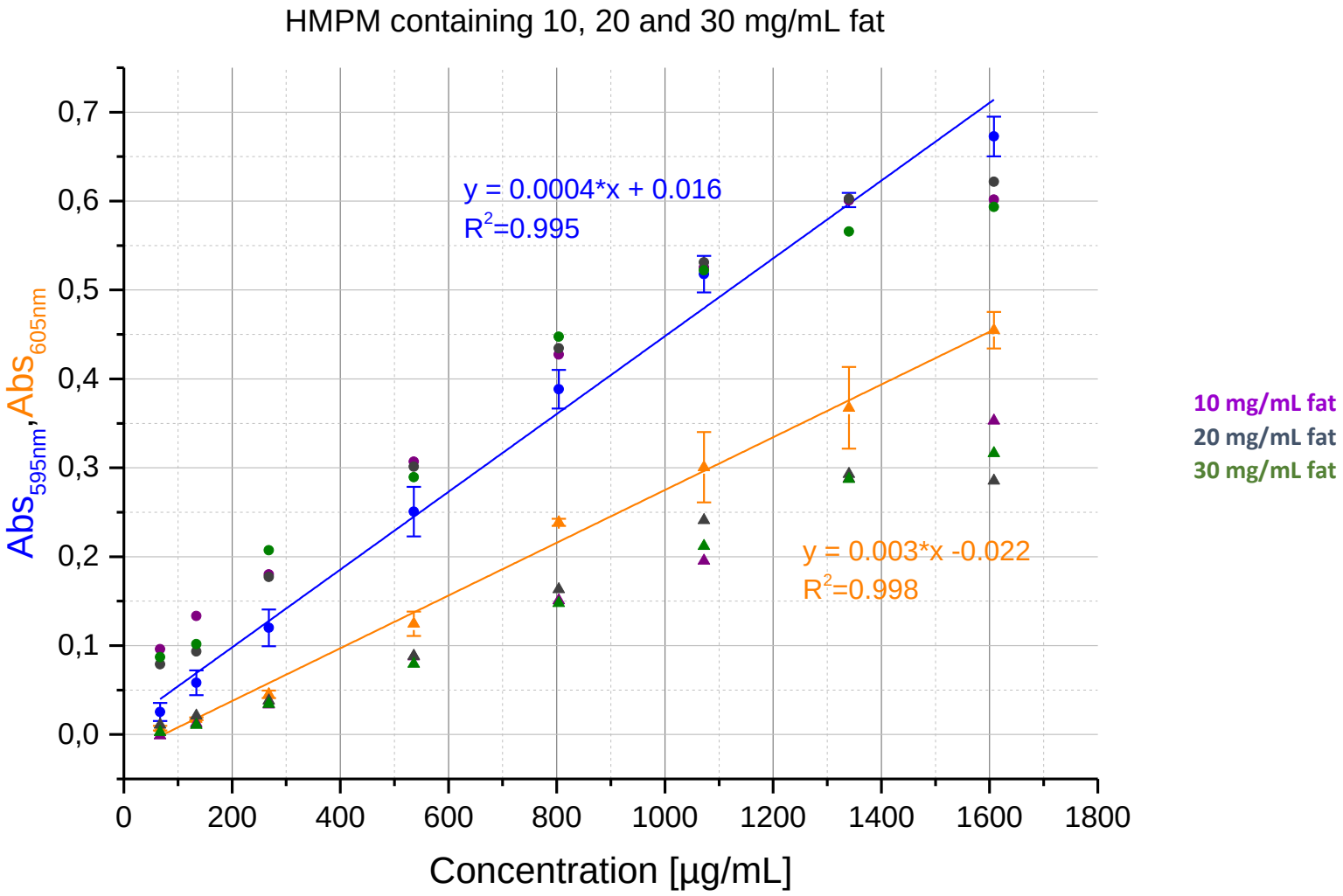


Figure 24: Determination of the influence of fat on HMPM calibration using the Bradford and Bromophenol Blue Assay by UV/Vis spectroscopy

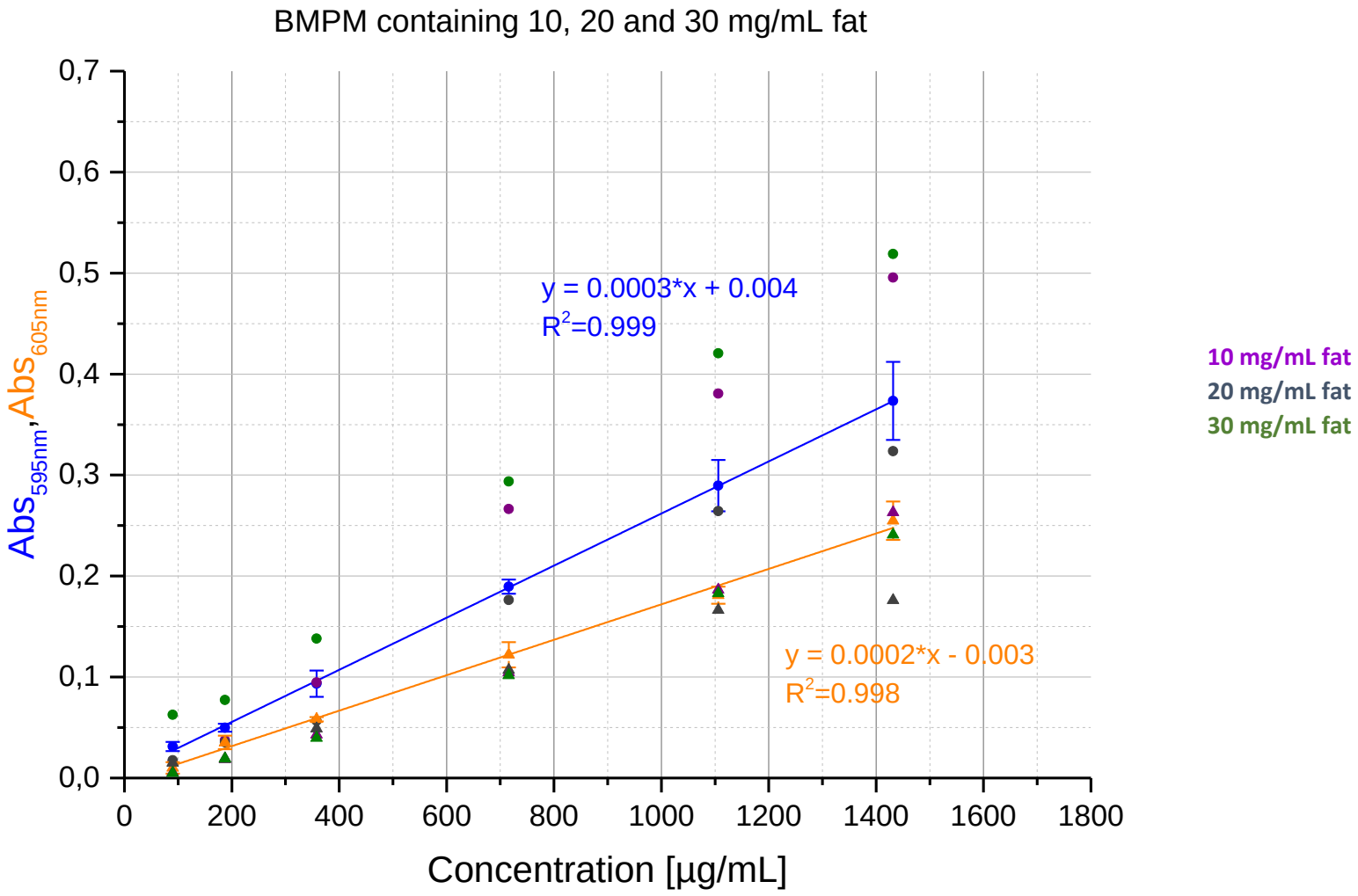


Figure 40: Determination of the influence of fat on BMPM calibration using the Bradford and Bromophenol Blue Assay by UV/Vis spectroscopy

4.3.2.2 Influence of calcium

In like manner, a different behavior of the three calibration models for the addition of calcium was detected. Resulting calibration functions are given in figure 41-43. In contrast to the addition of fat, a significant impact of calcium was noticed on BSA when measured by the Bradford Assay that was recognized to be the most significant at the final concentration of 7.5 mmol Ca^{2+}/L. For the BSA calibration, a shift to higher absorbance values induced by calcium was observed. The HMPM, as well as the BMPM, showed almost the same behavior as for the addition of fat. The Bradford Assay using the BMPM showed a steeper calibration curve.

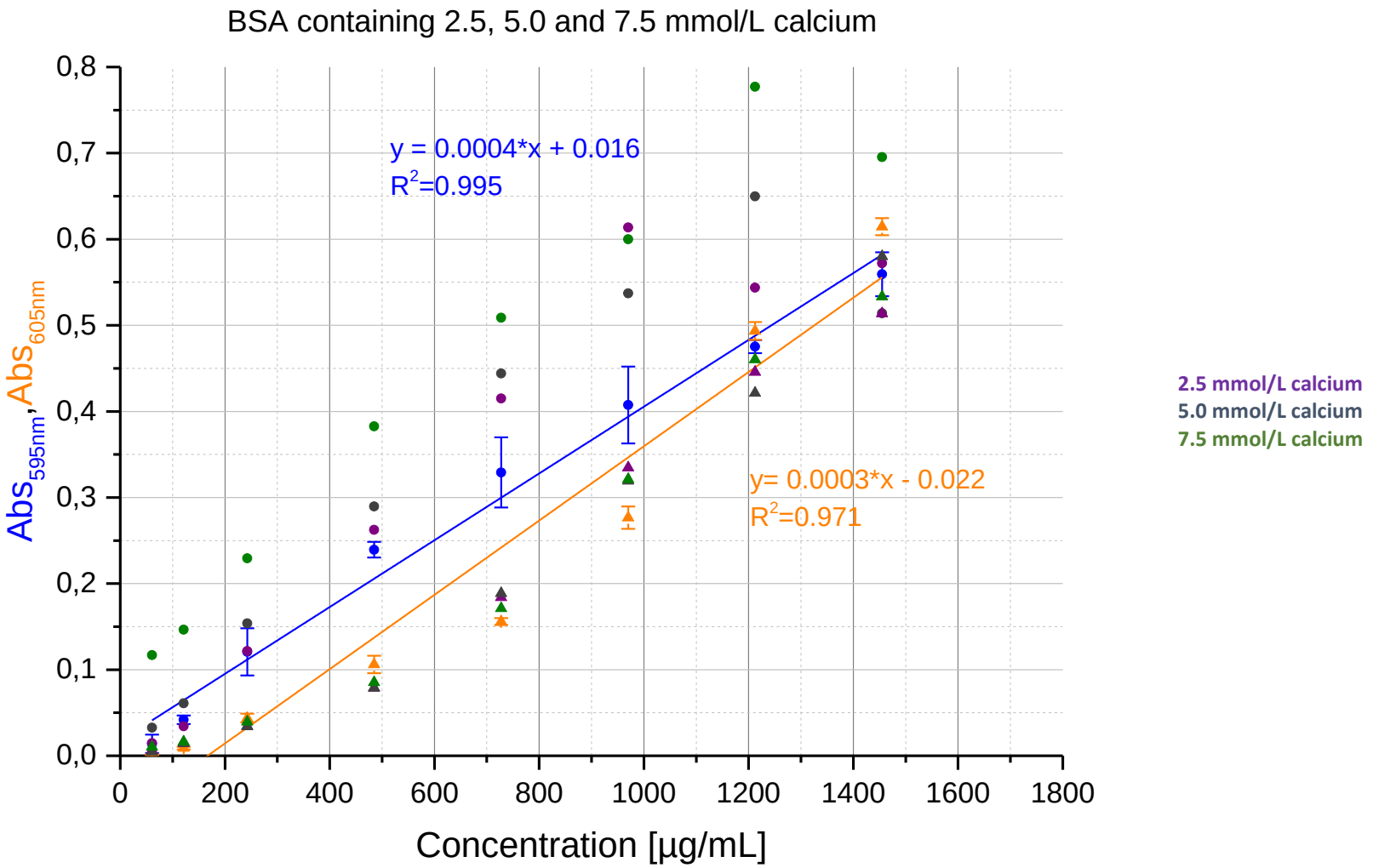


Figure 41: Determination of the influence *of calcium on BSA calibration using the Bradford and Bromophenol Blue Assay by UV/Vis spectroscopy*

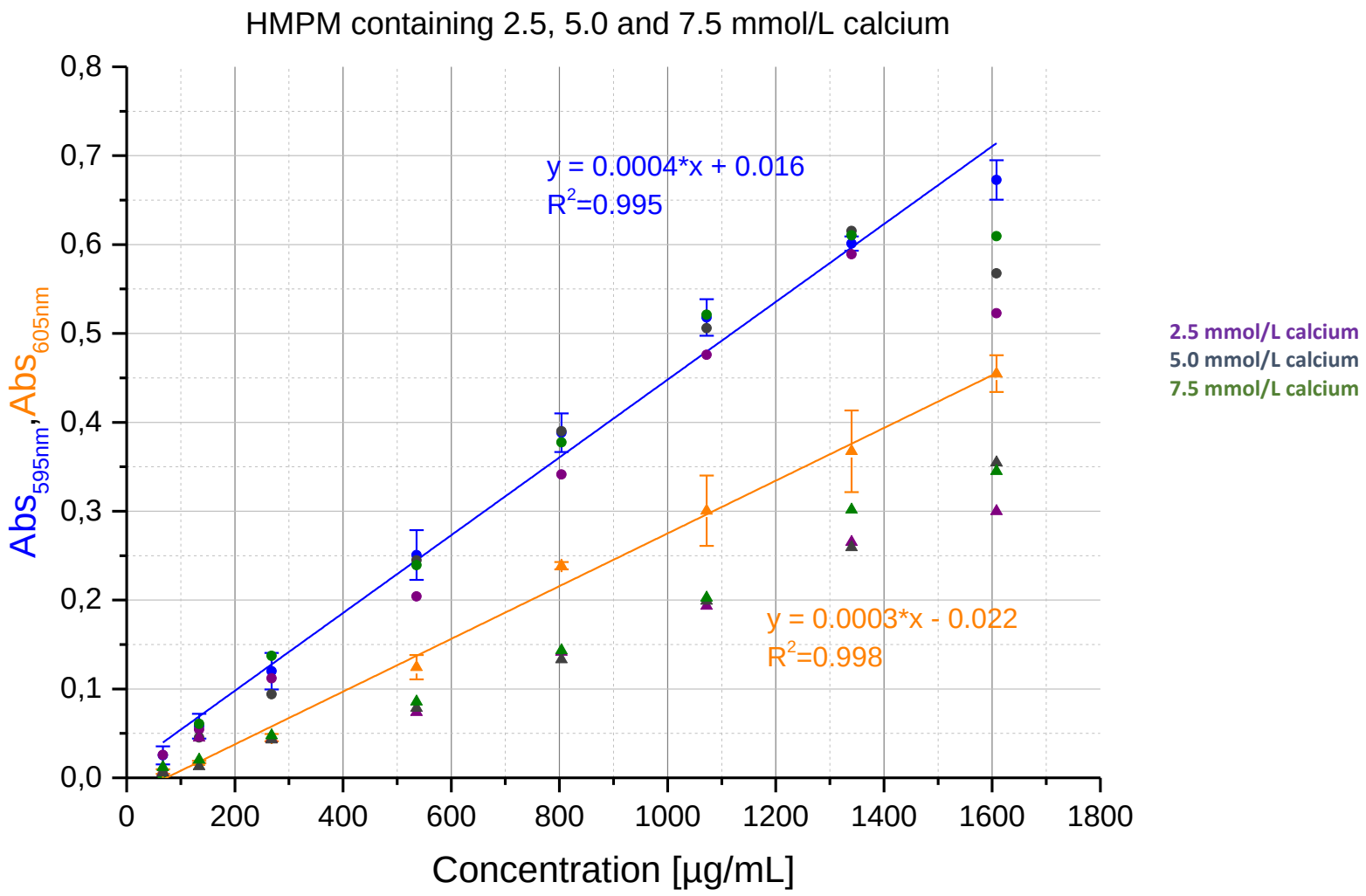


Figure 42: Determination of the influence of calcium on HMPM calibration using the Bradford and Bromophenol Blue Assay by UV/Vis spectroscopy

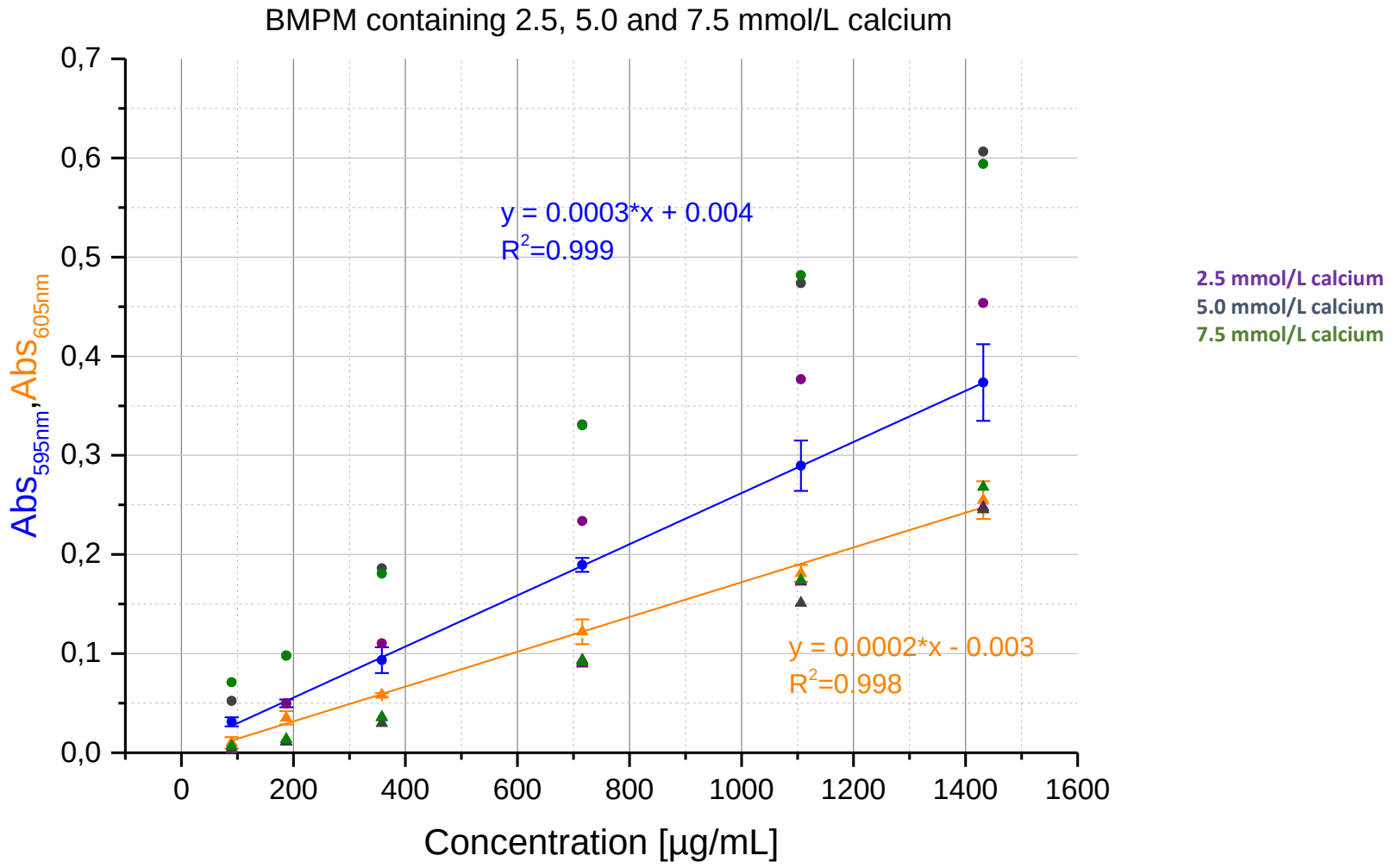


Figure 43: Determination of the influence of calcium on BMPM calibration using the Bradford and Bromophenol Blue Assay by UV/Vis spectroscopy

4.3.2.3 Influence of fat and calcium

Furthermore, the influence of the simultaneous addition of fat and calcium for the BSA calibration was investigated. Five combinations of different concentrations were evaluated. For the HMPM, experiments for the mean values of 20 mg fat/mL and 5.0 mmol calcium/L were performed.

For the Bradford Assay using BSA as calibration, no significant influence of fat but a considerable impact for the addition of calcium was observed. The simultaneous addition of both, fat and calcium, compensated these significant interferences. For the Bromophenol Blue Assay, it was detected that the BSA calibration model was significantly more influenced by the simultaneous addition, whereas for the separate experiments almost no interferences were observed.

For the HMPM calibration, in contrast, an influence on both, the Bradford and the Bromophenol Blue Assay, was observed, both resulting in a decrease of the slope of the calibration curve, resulting in a loss of sensitivity.

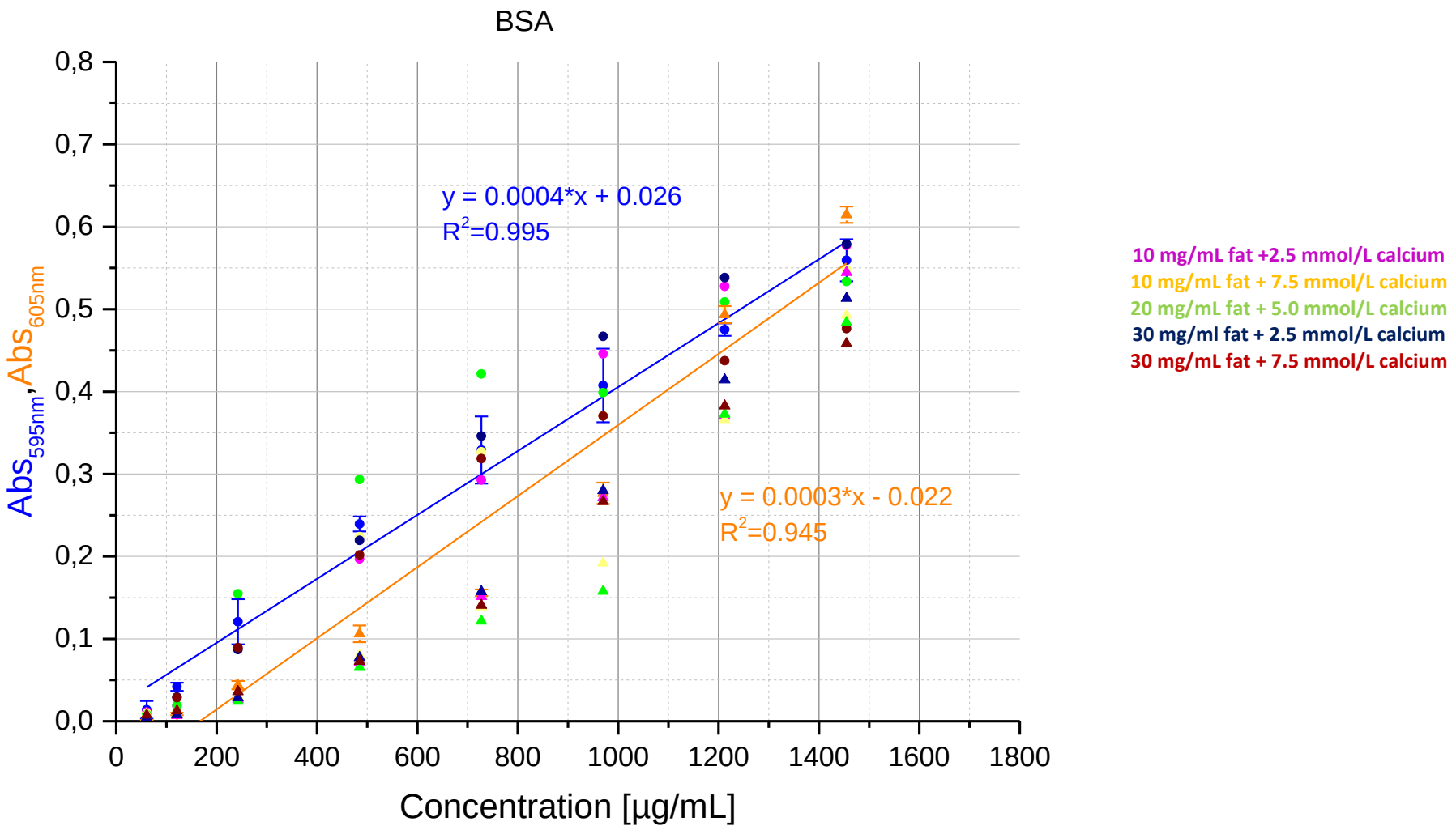


Figure 44: Determination of the influence of fat and calcium on BSA calibration by using the Bradford and Bromophenol Blue Assay by UV/Vis spectroscopy

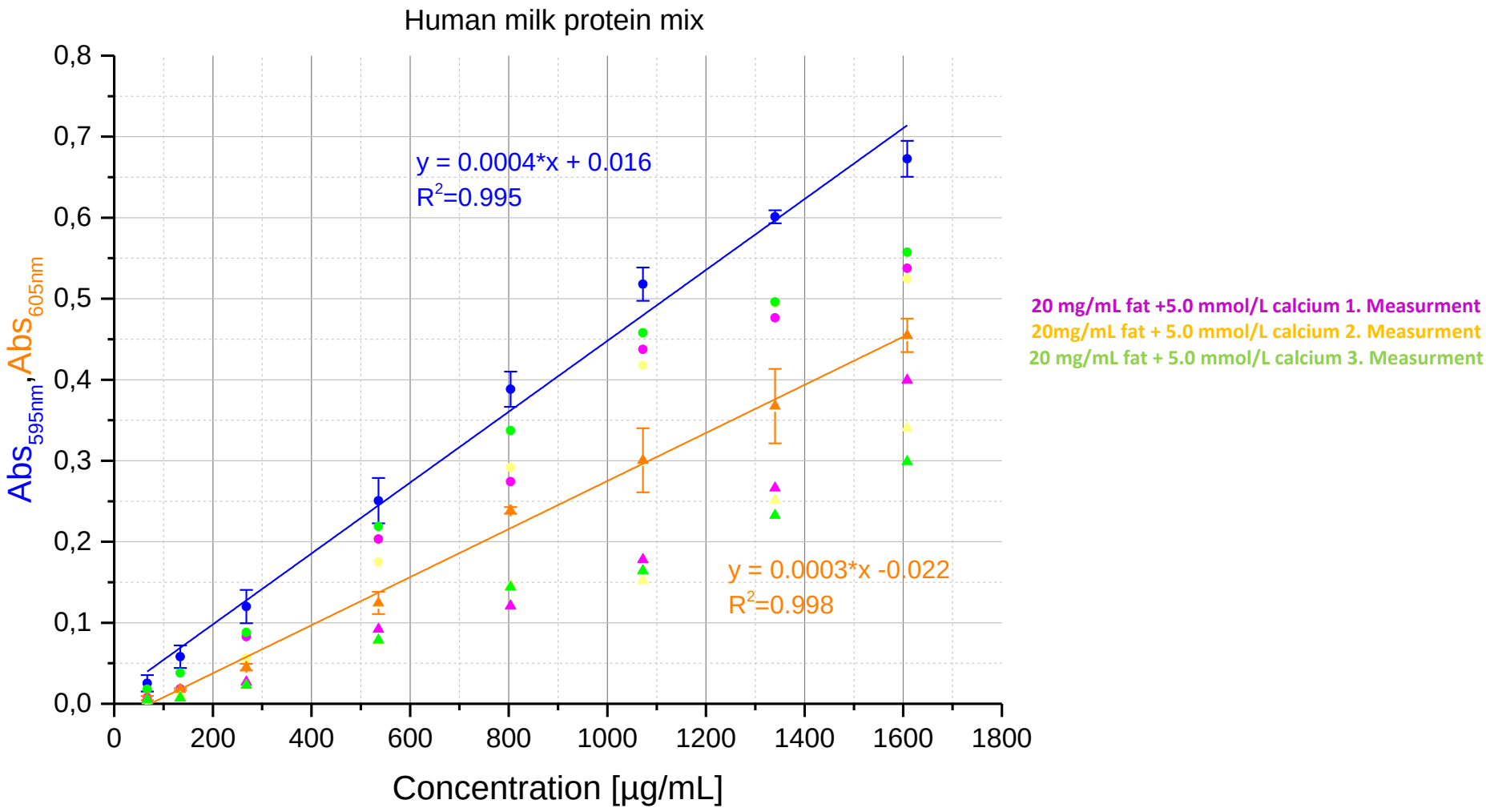


Figure 45: Determination of the influence of fat and calcium on HMPM calibration by using the Bradford and Bromophenol Blue Assay by UV/Vis spectroscopy

4.3.2.4 Influence of milk constituents – Summary

For the Bioanalyzer P230 Assay, no significant influence of fat was observed but significant deviation from previous results was observed for the addition of calcium.

For UV/Vis spectroscopy an expected lower proneness of interferences for the Bradford Assay compared to the Bromophenol Blue Assay was concluded, which was seen at least for the addition of fat for the BSA and the HMPM calibration and for the addition of calcium to the HMPM. The fact that the impact of calcium on BSA calibration was compensated by the concurrent addition of fat also affirms that the Bradford Assay is the more robust Assay. For the HMPM calibration, furthermore a significant impact was observed when adding fat and calcium in the case of the Bromophenol Blue Assay.

Despite the lower error-proneness of the Bradford Assay, also the linear range of the Bradford Assay, which was found for BSA in a lower concentration range than for the Bromophenol Blue Assay, seems to be more suitable. In cases of all three calibration models higher sensitivities were observed for the Bradford Assay.

Table 18: Summary – Investigation of the influence of other human breast milk constituents, n.a. = not analyzed

		UV/Vis Spectroscopy	
BSA	**Bioanalyzer P230 Assay**	**Bromophenol Blue**	**Bradford**
fat	n.a	Impact	no impact
calcium	n.a	no impact	Impact
fat and calcium	n.a	Impact	no impact
		UV/Vis Spectroscopy	
HMPM	**Bioanalyzer P230 Assay**	**Bromophenol Blue**	**Bradford**
fat	no impact	Impact	no impact
calcium	impact	Impact	no impact
fat and calcium	n.a	Impact	no impact
		UV/Vis Spectroscopy	
BMPM	**Bioanalyzer P230 Assay**	**Bromophenol Blue**	**Bradford**
fat	n.a	no impact	Impact
calcium	n.a	no impact	Impact
fat and calcium	n.a	n.a	n.a

4.4 Investigation of the protein content of human breast milk samples

Twenty human breast milk samples were provided by the Neonatology of the AKH Vienna to check for the suitability of the different methods for protein determination. The analysis of these samples was performed using the MIRIS system, the Bioanalyzer P230 Assay and UV/Vis spectroscopy applying the Bradford and the Bromophenol Blue Assay. The different methods were revealing different results for the protein content of human breast milk samples, which are summarized in the following chapters.

4.4.1 MIRIS

The results of the analysis of macronutrients in human breast milk samples with the MIRIS system, based on IR-spectroscopy, which is general accepted and certified for analysis of human breast milk, are illustrated in table 19. The results are indicating the fluctuating composition of human breast milk that is dependent on several different parameters pointed out in the previous chapters.

Table 19: Results of the analysis of macronutrients in human breast milk samples, MIRIS, number of measurements n = 3

Sample	*Fat [mg/ml]*	***Protein [mg/ml]***	*Carbohydrate [mgml]*	*Dryweight [mg/ml]*	*Energy [kcal/100ml]*
A	34 ± 0.82	**9 ±0.47**	7 ±0.47	122 ±1.25	63 ± 0.47
B	26 ± 0.47	**9 ±0**	73 ±0.47	115 ±0.94	63 ± 0.47
C	44 ± 1.25	**10 ±0.94**	69 ±0.47	133 ±2.16	56 ± 0.94
D	26 ±0	**8 ±0.47**	73 ±0	114 ±0.47	74 ± 1.41
E	42 ±0	**9 ±0.47**	69 ±0	129 ±0	55 ± 0
F	35 ± 0.47	**16 ±0.47**	7 ±082	130 ±1.25	71 ± 0
G	44 ±0.47	**10 ±0**	69 ±0.47	132 ±0	67 ± 0.47
H	37 ±1.25	**8 ±0**	67 ±0.47	122 ±1.63	74 ± 0
I	27 ±0.47	**14 ±0**	69 ±0.47	120 ±0.47	65 ± 1.24
J	15 ±0.47	**10 ±0**	70 ±0.47	103 ±0	57 ± 0.47
K	45 ±0.94	**6 ±1.41**	67 ±6.13	128 ±5.43	44 ± 0
L	20 ±0	**7 ±0.47**	75 ±0	108 ±0.47	73 ± 2.36
M	20 ±0.94	**9 ±0**	73 ±0.47	108 ±1.25	48 ± 0.47
N	19 ±0	**8 ±0.47**	74 ±0	107 ±0.47	50 ± 0.94
O	20 ±0.47	**8 ±0**	73 ±0.47	107 ±0.94	48 ± 0
P	19 ±0.47	**9 ±0.47**	73 ±0.47	107 ±0.94	49 ± 0.47
Q	34 ±0.47	**9 ±0**	65 ±2.62	120 ± 0	49 ± 0.47
R	25 ± 1.25	**8 ±0.47**	62 ±0	105 ±1.25	62 ± 0.47
S	22 ± 0.47	**15 ±0.47**	66 ±0.47	113 ±1.63	42 ±15.3
T	17 ± 0.82	**9 ±0.47**	61 ±1.25	98 ±0.82	52 ±0 82

The protein content of the twenty samples is illustrated in figure 46. Protein concentrations were determined in a range between 6 mg/mL and 16 mg/mL.

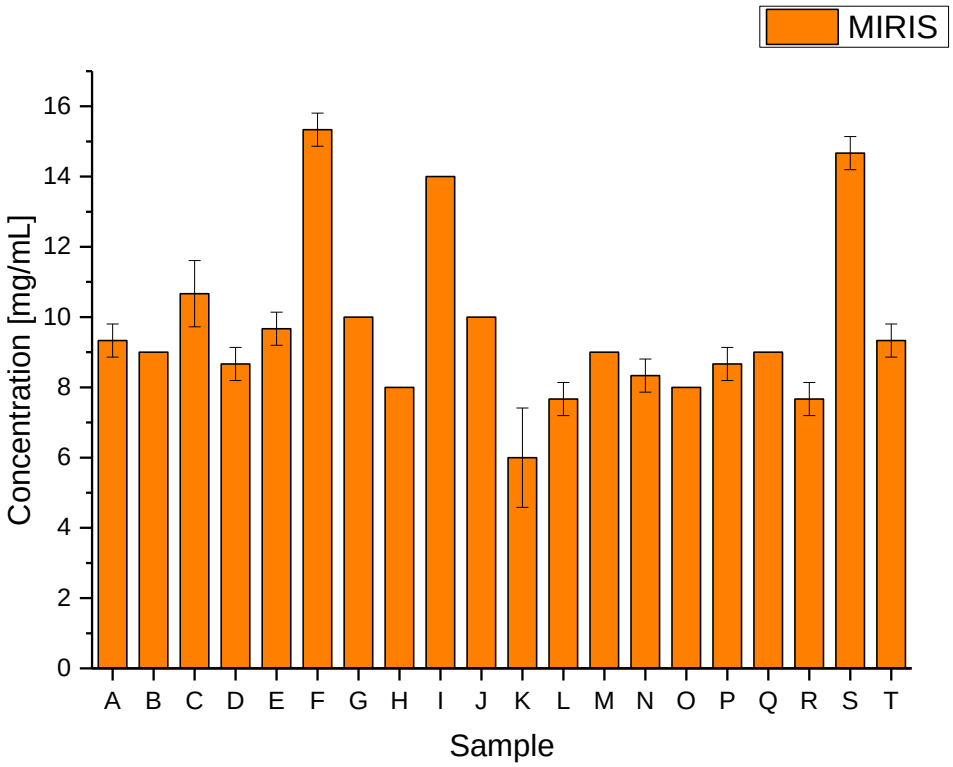


Figure 46: Results of the determination of the protein content of human breast milk samples by using MIRIS

4.4.2 Bioanalyzer P230 Assay

Concerning the Bioanalyzer P230 Assay, the protein content of human breast milk samples was calculated from calibration functions using either the human or the BMPM. Results are illustrated in figure 47, the concentrations of the individual human breast milk samples were determined in a range between 15 mg/mL and 40 mg/mL protein.

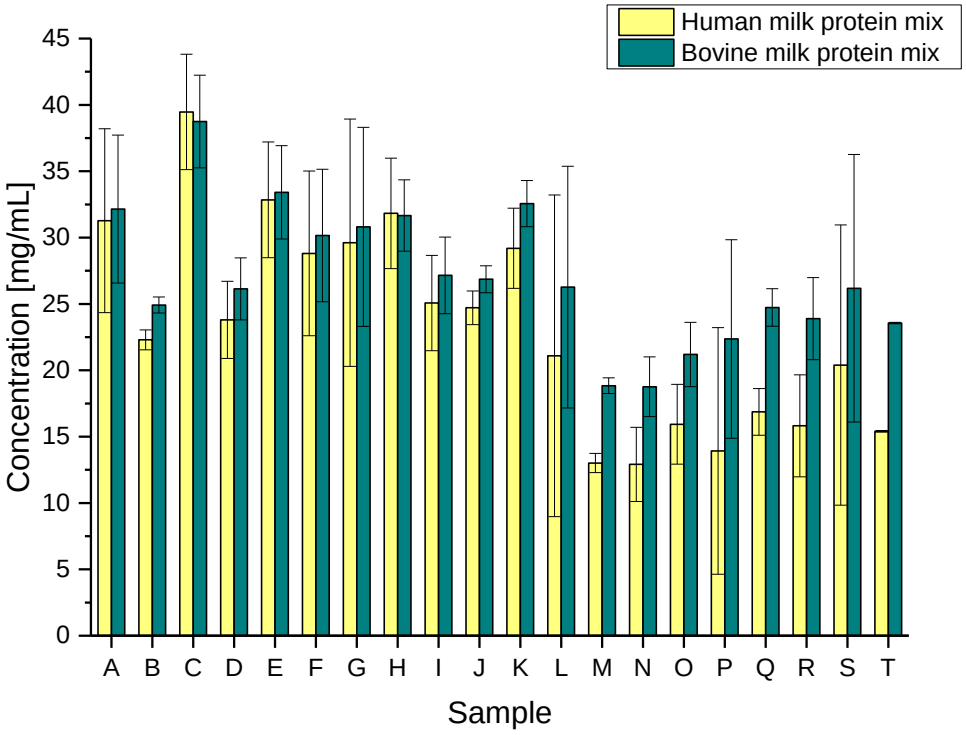


Figure 47: Results for the determination of the total protein content of human breast milk samples usig the HMPM and the BMPM calibration with the Agilent Bioanalyzer P230 Assay

In almost all cases higher concentrations were detected with the calibration of the BMPM. Furthermore, a variability between the individual human breast milk samples was found, illustrating the composition variability among different women and most likely also lactation stage. Also, significant standard deviations between the individual measurements were perceived, suggesting problems with sample homogenization and/or reproducible sampling. Another possibility is that casein causes problems during separation, which was pointed out already earlier in chapter 4.1.2.4.

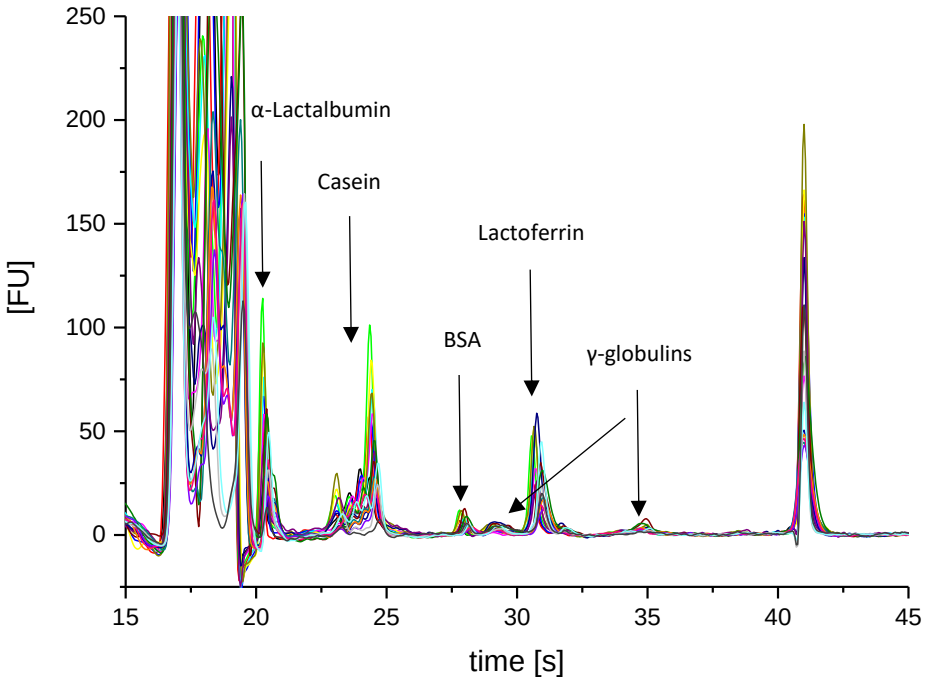


Figure 48: Analysis of human breast milk samples A-T with the Bioanalyzer P230 Assay under reducing conditions. Without normalization because of different samples

Figure 48 is showing the separation of all twenty human breast milk samples on the Bioanalyzer. According to the molecular weight, the peaks can be assigned to the particular proteins. α-lactalbumin with an average molecular weight of 14 kDa is followed by the casein fraction with molecular weights between 19 and 24 kDa (α-, β- and κ-casein). The third peak, with an average molecular mass of 63 kDa, can be assigned to serum albumin, and lactoferrin can be assigned to the peak with an average molecular weight of about 89 kDa. The remaining two peaks most likely correspond to γ-globulins present in human breast milk, whereas light and heavy chains of the globulins are dissociating under reducing conditions (SDS, DTT).

4.4.3 UV/Vis spectroscopy

4.4.3.1 Background Correction

During analysis of human breast milk samples with UV/Vis spectroscopy, it was discovered that human breast milk itself shows absorbance at the wavelengths relevant for protein quantification, 595 nm and 605 nm respectively. For this reason, blank correction was necessary. For this the instrument was set to zero by diluting the sample in the respective reagents not containing the interacting dye. It is therefore necessary to do two different blank corrections, one for the instrument (reagent and water) and one for the sample itself (sample and reagent without the dye).

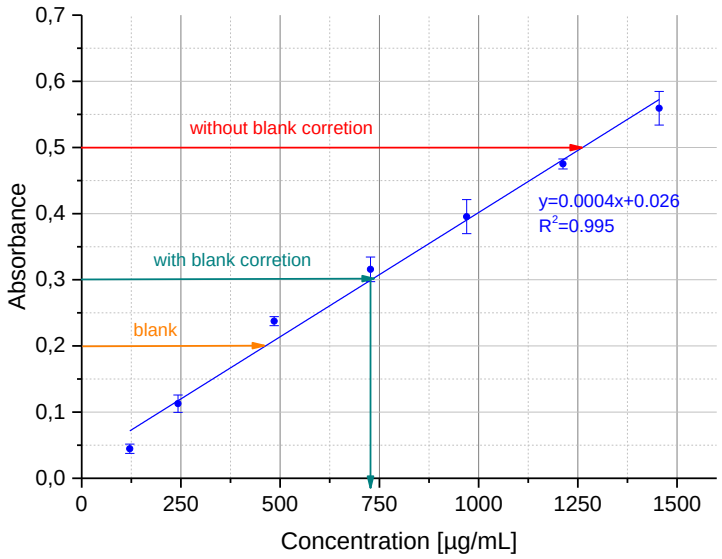
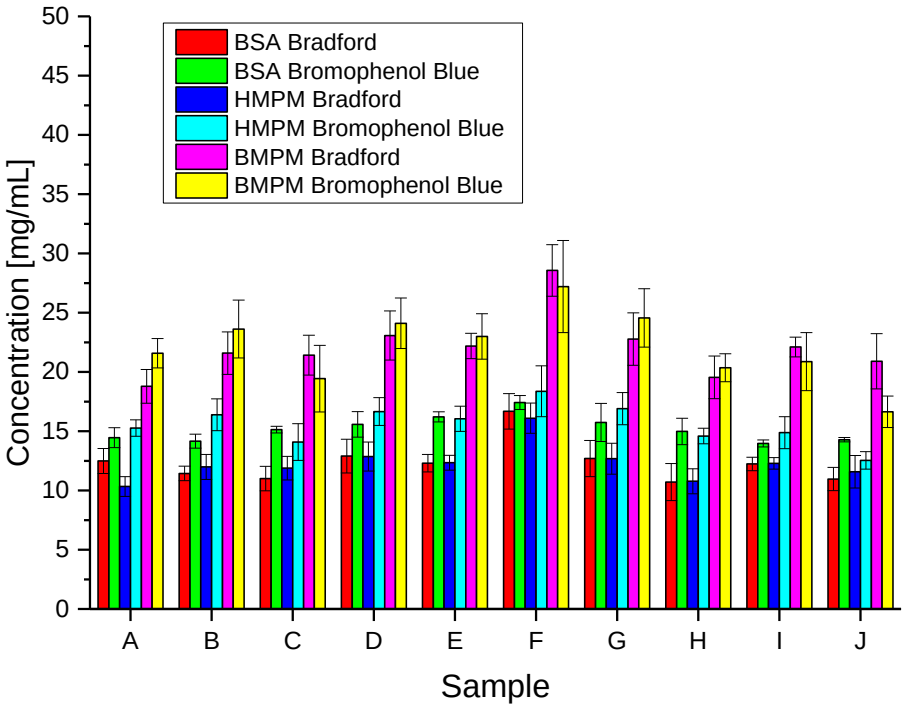


Figure 49: Scheme for the effect of blanks, background correction and sample measurement for human breast milk samples using the Bradford Assay

4.4.3.2 Determination of the protein content

The results of determined protein concentrations of the blank corrected human breast milk samples are illustrated in figure 50, including the findings of calculations based on BSA, HMPM and BMPM calibration models. As already seen before for the Bioanalyzer P230 Assay approach, the highest results obtained from calculations based on the BMPM calibration were recognized here, whereas the BSA and the HMPM calibration were yielding significantly lower values. While BSA and HMPM calibration yielded protein concentrations between 7 and 18 mg/mL, significantly higher values of 18 to 45 mg/mL were detected with the BMPM calibration. Like in the case of the Bioanalyzer, significant variations among different samples as well as in between the different colorimetric Assays were detected. The Bromophenol Blue Assay was providing the highest results independent of the actual calibration model.



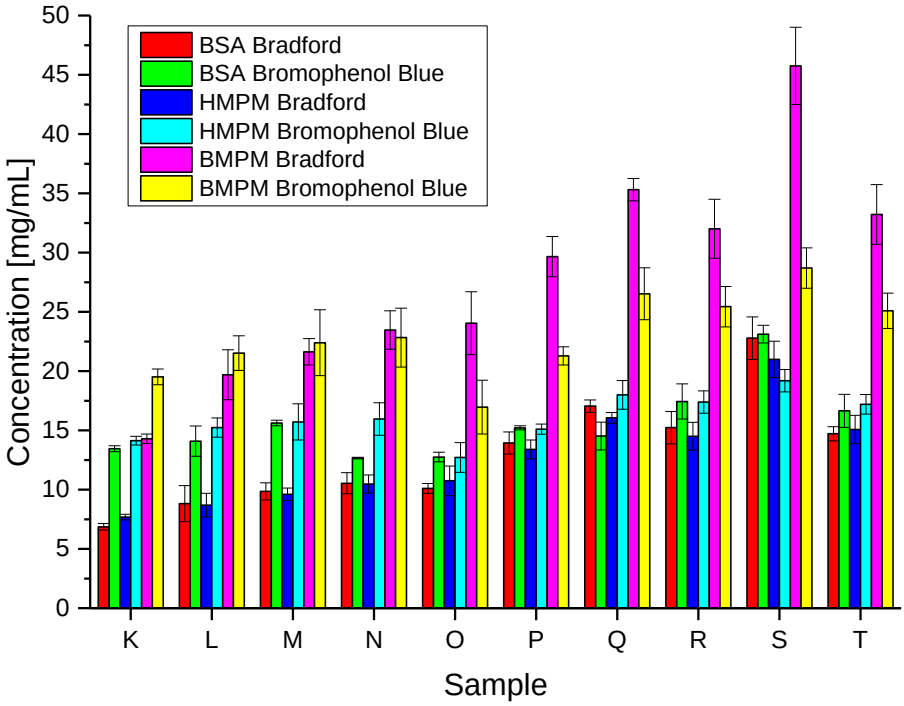


Figure 50: Results for the determination of the protein content of twenty human breast milk samples (A-T) using the Bradford and the Bromophenol Blue Assay by UV/Vis spectroscopy

4.4.4 Comparison of different methods for quantification of the protein content of human breast milk samples

Figure 51 is representing a comparison of the results obtained by the different applied methods. The significantly higher concentration findings obtained from the bovine milk protein are suggesting it to be not a convenient reference material for the purpose of protein quantification in human breast milk. Although this mix is containing three characteristic human breast milk proteins, β-Lactoglobulin is not present in human breast milk at all and somehow seems to disturb the measurement. Furthermore, this calibration model was generating the highest results for protein concentration, giving on average 25 mg/mL for UV/Vis spectroscopy and 27 mg/mL for the Bioanalyzer system. These high values that are not reported in literature. Even for colostrum which is exceptionally rich in proteins, a maximum protein concentration of 20 mg/mL [2] was reported.

But also the HMPM used with the Bioanalyzer P230 Assay was yielding significantly higher results than the other methods. The determined averaged value of 23 mg/mL, suggests that the Bioanalyzer P230 system, seems to be an inappropriate system in the context of this analysis. Additionally, casein micelle formation led to severe problems for detecting the correct protein content. Same was observed for fat and calcium.

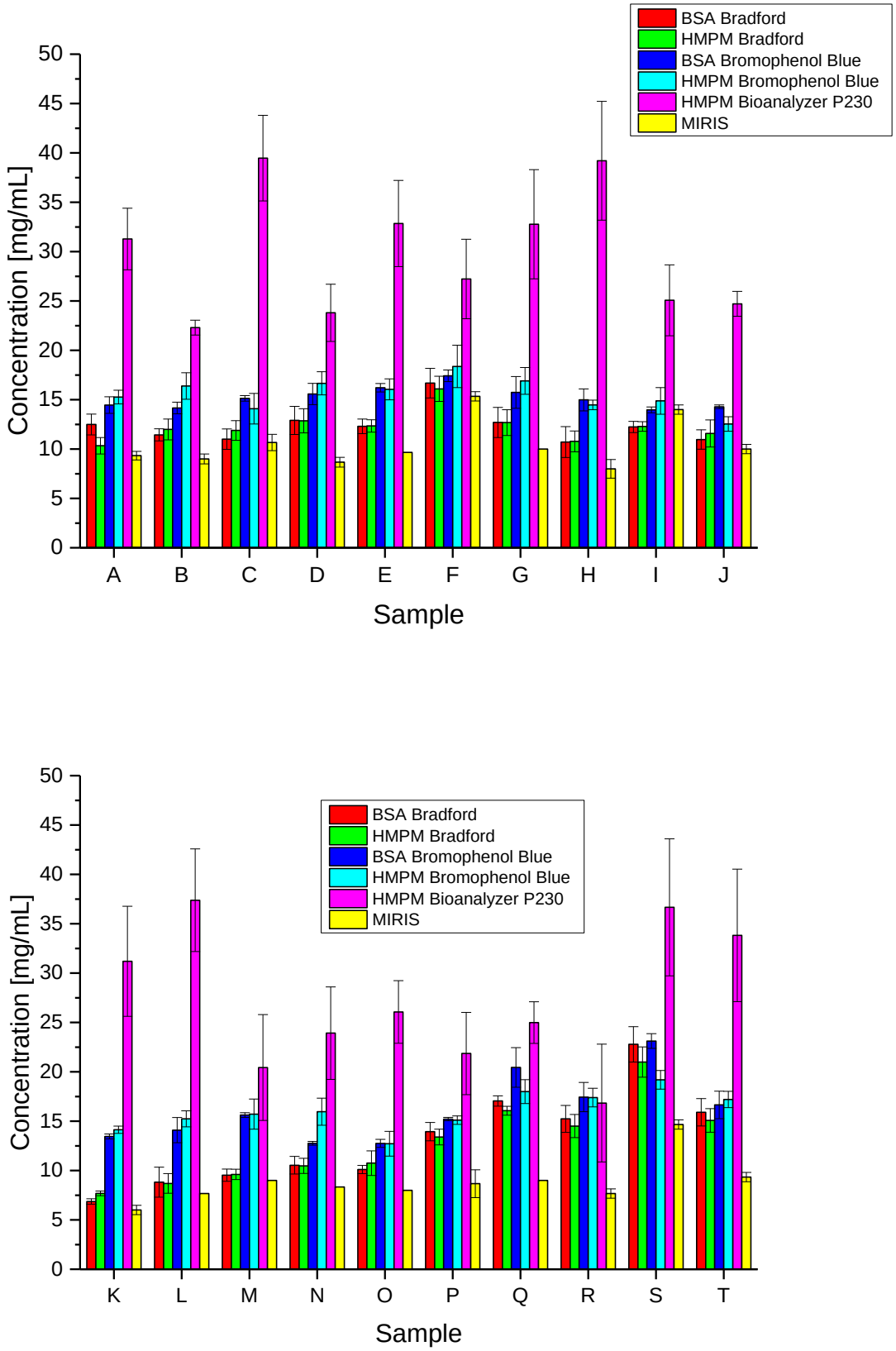
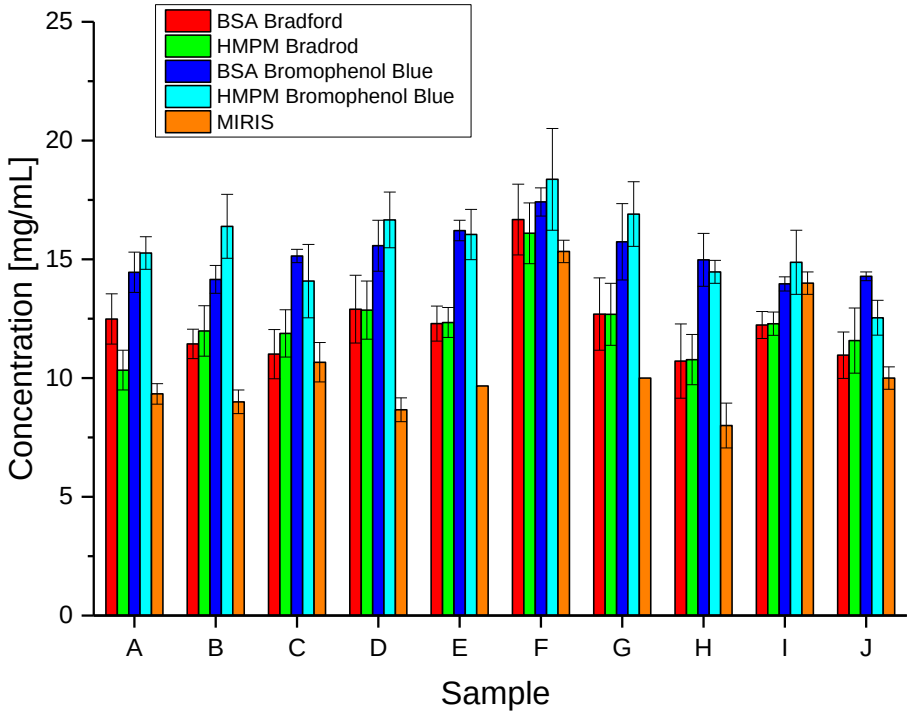


Figure 51: Results of protein determination of human breast milk samples A-T, using the MIRIS system, the Bioanalyzer P230 Assay and UV/Vis spectroscopy Assays, the Bradford and Bromophenol Blue

Comparing only UV/Vis spectroscopy and the MIRIS system, it was observed that these methods are revealing different results (illustrated in figure 52). The Bromophenol Blue Assay yields significantly higher concentrations for most samples, whereas the MIRIS system is providing the lowest values.



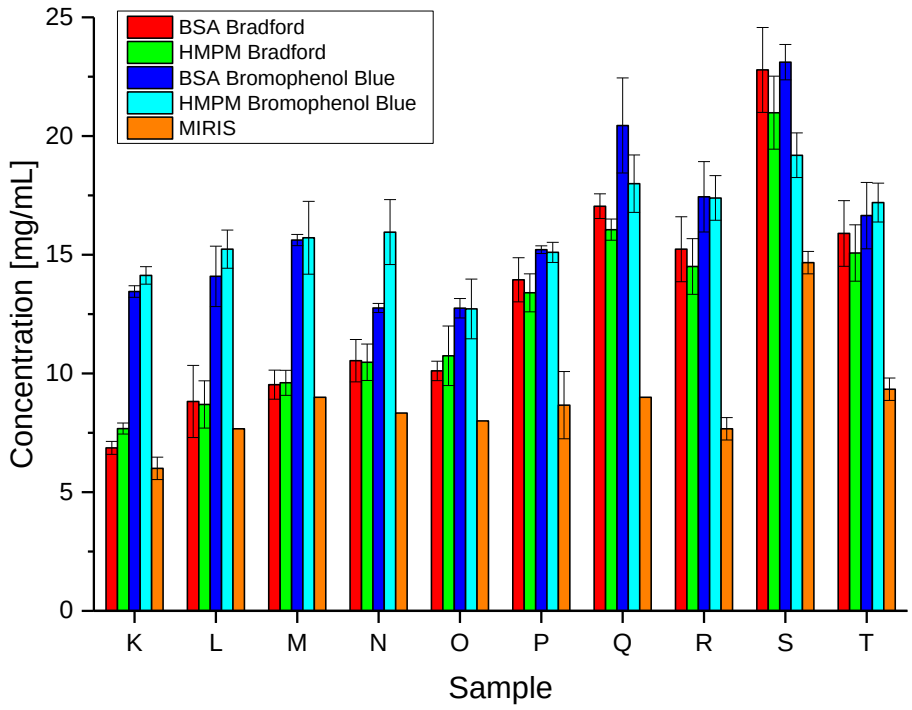


Figure 52: Results for determination of the protein content of human breast milk samples A-T, Comparison of MIRIS and the Bradford and Bromophenol Blue Assays (UV/Vis spectroscopy)

Even though it is hard to say which method is actually giving correct results, the MIRIS system is a method accepted for routine analysis of human breast milk. The Bradford Assay is a generally accepted method for protein quantification. However, it was found that the Bromophenol Blue Assay is more influenced by other milk constituents the Bradford Assay (Chapter 4.3.2). From this it can be said when comparing the results of the MIRIS system and the Bradford Assay (figure 52), one can see that in all cases (except sample I) MIRIS is producing significantly lower results. However, there are samples in which the results of these two methods are showing comparable results (C,F, J, K, L and M). However there are other samples (P, Q, R, S and T) exhibiting significant differences.

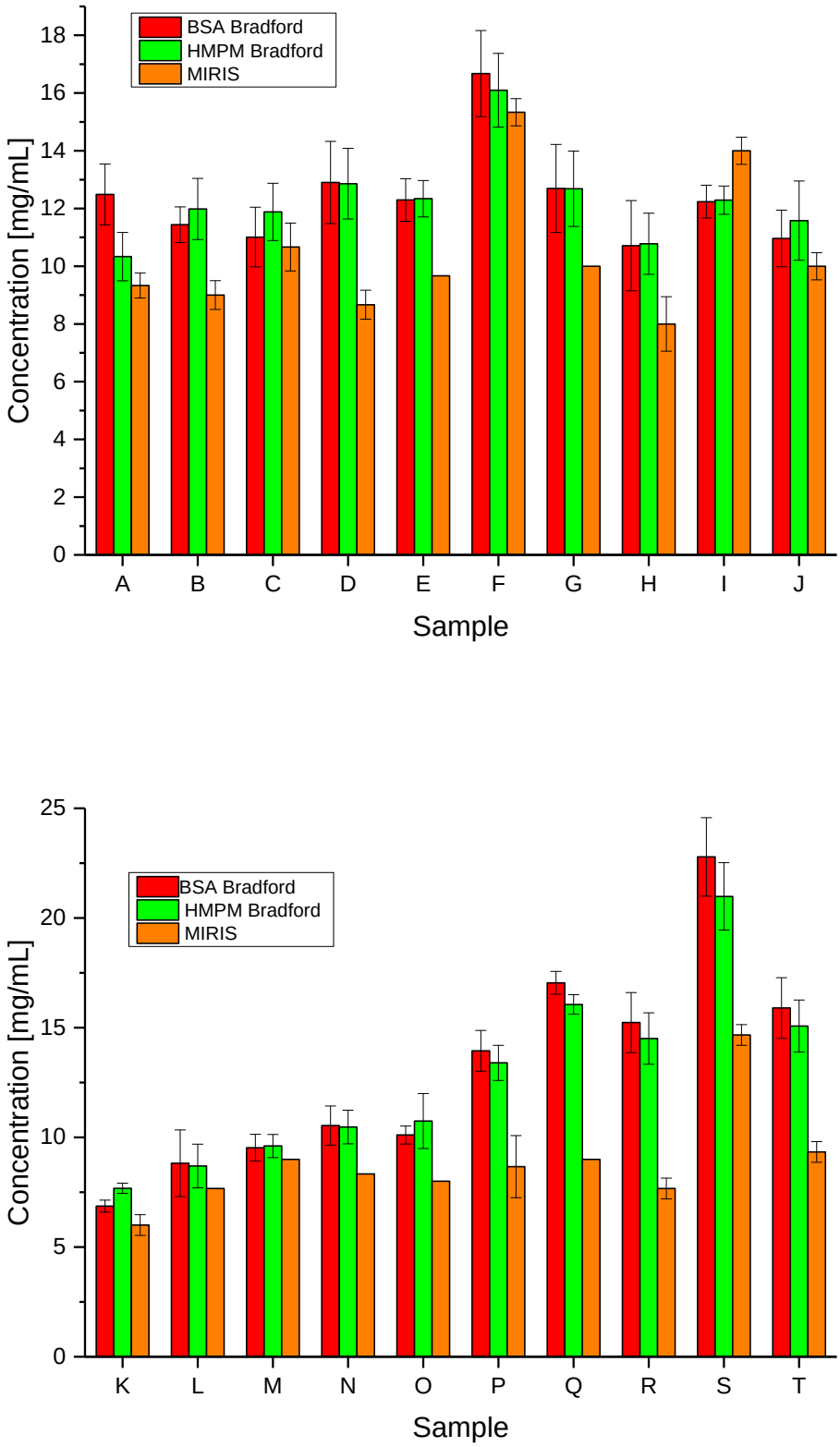
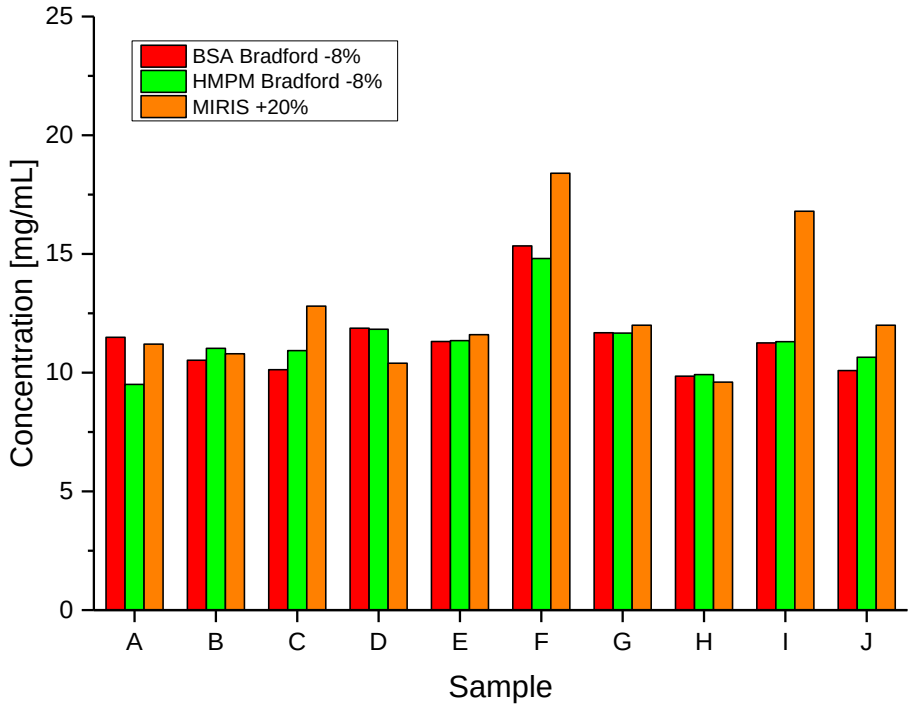


Figure 53: Results of the determination of the protein content of human breast milk samples A-T, Comparison of the results of the MIRIS system and and the Bradford Assay (UV/Vis spectroscopy)

For investigation of the reliability of the different methods, BSA standards with a known concentration of 10 mg/mL were measured three times and the concentration was recalculated by the BSA and the human milk protein mix calibration models. On average, an overestimation of the protein content of about 8 % was found for the Bradford Assay for both calibration models. This standard was also analyzed with the MIRIS system, where an underestimation of the actual concentration of about 20 % was recognized. All results were corrected for these under- and overestimations. Figure 54 shows that by this the differences between these two methods become significantly smaller and the results become more comparable.



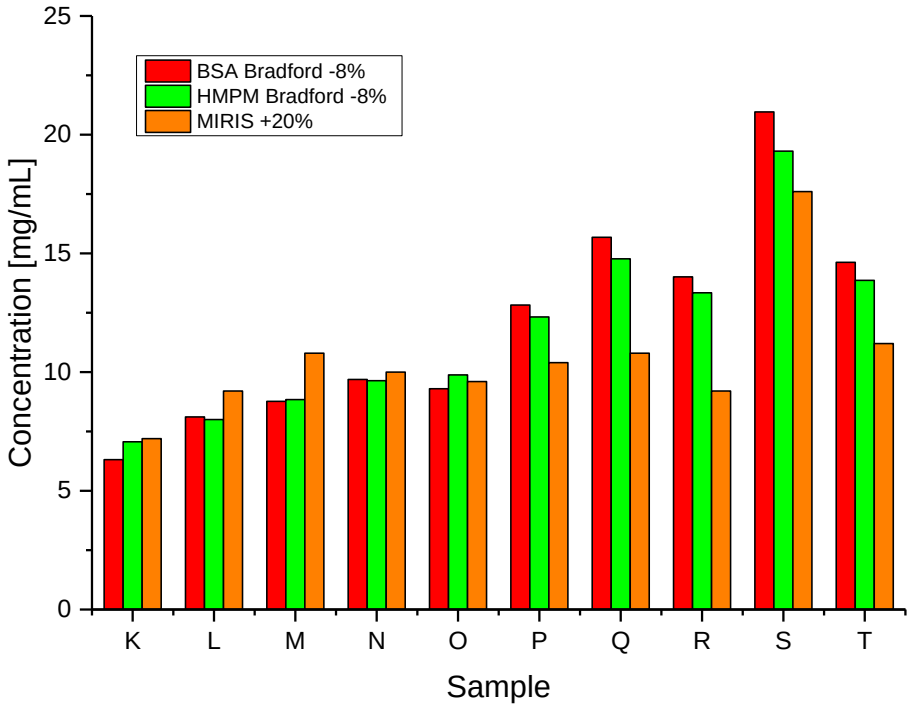


Figure 54: Results for the determination of the protein content of human breast milk samples A-T, Comparison of the results of the MIRIS system and the Bradford Assay (UV/Vis spectroscopy) after correction

4.4.5 Investigation of the protein content of human breast milk samples – Summary

It was found that the BMPM seems to be not applicable as a reference material for the quantification of proteins in human breast milk, assuming that β-lactoglobulin - a protein present in cow's milk but not in breast milk – is interfering with analysis. Beyond that, the Bioanalyzer P230 system was found as to be not suited for quantification, due to interference observed for fat and calcium constituents.

Based on the fact that MIRIS is an accepted and certified method for quantification of macronutrients in human breast milk for the daily use in milk banks and neonate units, it is believed that the Bradford Assay applied on the microfluidic chip of Puchberger-Enengl [31] could represent an alternative for protein quantification at the point-of-care.

Comparing the BSA and the HMPM calibration models, it is not definitely possible to decide which model is actually qualified and appropriate. Whereas the HMPM mix was shown to be lesser influenced by other human breast milk constituents, for the BSA calibration was found that the impacts of fat and calcium were almost neutralizing each other. For the HMPM, in contrast, a divergence from linearity was seen at higher protein concentrations, in case of fat and calcium were present at the same time.

4.5 Method Transfer

Due to the fact that the Bradford Assay is the generally accepted method for protein quantification and based on the findings of this work, it is suggested to replace the Bromophenol Blue Assay. For this purpose, further experiments about the transfer of the Bradford Assay onto the microfluidic chip were performed.

The matrix used in the beginning was PEG-DA, a hydrogel compatible with the Bromophenol Blue Assay. Yet, the Bradford Assay requires very low pH, about 0.7, to ensure that Coomassie Brilliant Blue is presented in its charged form and therefore able to interact with the protein.
On the microfluidic chip, the ratio of reagent to hydrogel is 1:4. Mixing the reagent with the used gel showed a pH-shift of ΔpH 0.18, indicating no significant influence in the formation of the protein-dye complex.
Furthermore, the influence of the coloring agent on the polymerization process of the hydrogel was studied, but no problems were observed.
Yet in the Coomassie Brilliant Blue reagent of the Bradford Assay immediately turned blue when mixed with the hydrogel. The structure of polyethylenglycol diacrylate is given in figure 55, showing a lot of functional groups most likely interacting with the dye.

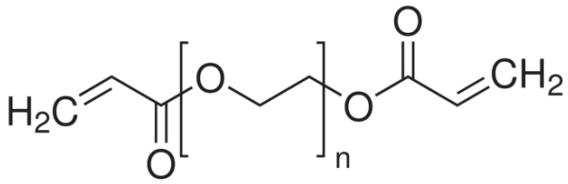


Figure 55: Chemical structure of polyethylenglycol diacrylate (PEG-DA) [76]

Figure 56: Interaction between Coomassie Brilliant Blue and PEG-DA

A second problem that was arising is that the polymerization of PEG-DA is performed under UV exposure, catalyzed by the photoinitiator HMPP. Coomassie Brilliant blue seems not to be light-resistant. Experiments were pointing out that the blue coloration of the dye-hydrogel mixture was vanishing after 2 minutes of UV irradiation and was not reappearing after the addition of protein, suggesting that the dye is irreversibly damaged by UV exposure (Figure 57).

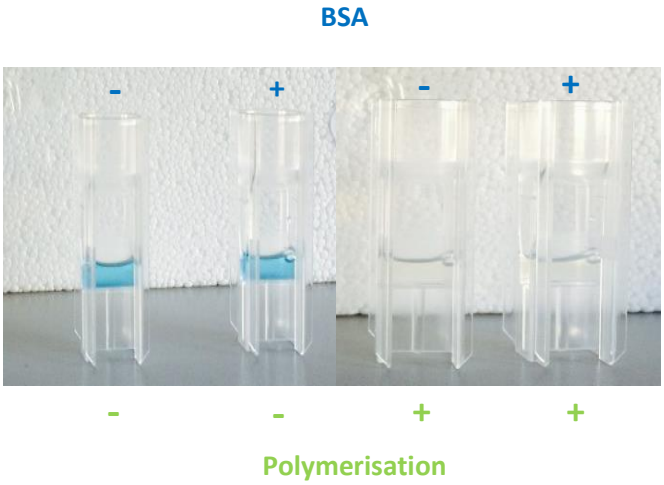


Figure 57: Polymerization of PEG-DA and Bradford reagent, before (left) and after polymerization (right) caused by UV irradiation, left cuvette without protein, right cuvette containing BSA

Furthermore, kinetic experiments (UV/Vis: 595 nm, 60 min, 10 sec intervals) about the diffusion of the protein through the pores of the hydrogel were performed. Two different strategies were applied:

a) PEG-DA and the Bradford reagent were cured under UV light. BSA was added later and the diffusion into the polymer was investigated

b) PEG-DA and BSA were cured under UV light. Bradford reagent was added later and the diffusion into the polymer was investigated

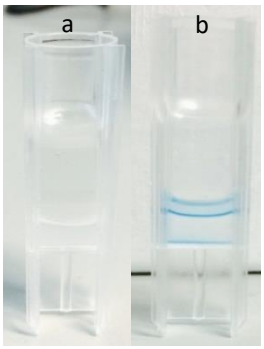


Figure 58: Kinetic experiments (UV/Vis: 595 nm, 60 min, 10 sec intervals). Simulation of the diffusion of proteins into the hydrogel, left cuvette a) containing PEG-DA and the Bradford reagent, right cuvette b) containing PEG-DA and BSA

In both cases, no blue coloration for the polymerized hydrogel was observed, not even after three hours. Moreover, no significant absorbance was recorded over the whole period of analysis, indicating that the diffusion of proteins into the hydrogel is not responsible for the coloration of the chip as observed in previous analysis. The blue coloration on the borders of the gel when the Bradford reagent was added to the cured PEG-DA containing BSA, can therefore be contributed to the PEG-DA-Coomassie Brilliant Blue interaction, but not to the one between the protein and the dye.

These results confirm that the coloring of the chip is not induced by the diffusion of the protein into the hydrogel but by the interaction of the dye molecules with the protein at the gel/protein solution interface.

Based on the result that the hydrogel is giving a strong background signal when interacting with the Bradford reagent it was concluded that the gel matrix has to be changed. Furthermore the UV-enhanced polymerization process is destroying the dye. Carboxymethylcellulose and agarose were proposed as alternatives. The latter is used as a sieving medium in gel electrophoresis indicating good permeability for proteins. Both materials are prepared by dissolving them in water under heating, forming gels by cooling down to room temperature, so no UV enhanced polymerization is necessary. With carboxymethylcellulose, the same problem as with PEG-DA was observed. Strong coloration of the mixture occurred, suggesting again an interaction between carboxymethylcellulose and Coomassie Brilliant Blue. Agarose, in contrast, was apparently not interacting with the dye, as the mixture of them remained transparent. As this finding seems quite promising, further experiments should be done for the combination of the Bradford Assay and agarose as a medium directly on the chip.

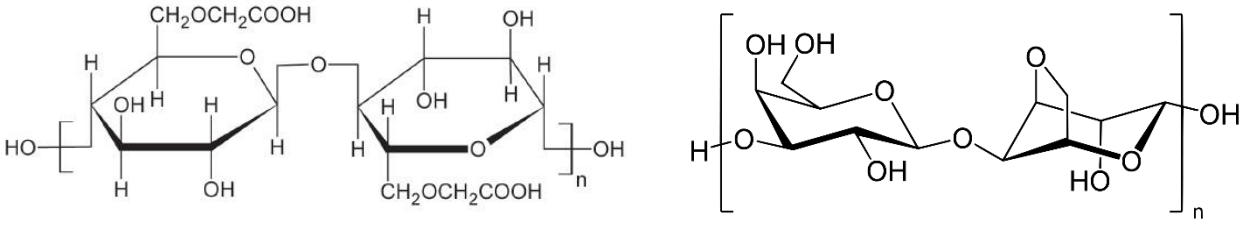


Figure 59: Chemical structures of carboxmethylcellulose [77] (left) and agarose (right) [78]

5. Conclusion

The aim of this work was to find out if the microfluidic chip design based on the Bromophenol Blue Assay for protein quantification [31] is suitable for protein quantification in human breast milk. The Assay was compared to the well accepted Bradford Assay, a IR-based method, the MIRIS system, and the Bioanalyzer P230 Assay which is based on capillary gel electrophoresis and fluorescence detection.

A further aim was to find a suitable reference material for the quantification of proteins in human breast milk. For that reason, three different calibration models were compared concerning sensitivity, linearity and interferences with other human breast milk constituents. Values for LOD and LOQ were determined for the single protein standards, for the proteins in the mixture as well as for the total protein in the mixture in the lower µg/mL range.

Interferences of fat and calcium, two prominent human breast milk constituents were analyzed for the Bioanalyzer and the UV/Vis spectroscopy approach, revealing a greater impact on the Bioanalyzer system For UV/Vis spectroscopy, advantages of the Bradford Assay over the Bromophenol Blue Assay were found, the latter to be more robust. Whereas the BMPM was proven to be an inappropriate standard for quantification, BSA and an artificial HMPM were found to be suitable and nearly equivalent.

The analyses of twenty human breast milk samples with MIRIS, Bioanalyzer P230 Assay and UV/Vis spectroscopy with the Bradford and the Bromophenol Blue Assay were providing significantly different results for every method. Due to the fact that the Bioanalyzer P230 system was producing significantly higher values than reported in literature, this system was concluded to be hardly suitable for this analysis. The Bradford Assay - in contrast - was producing similar, albeit higher results than the MIRIS system, latter being a general accepted and certified method for analysis of human breast milk. However, one has always to bear in mind that the accurate values for the protein content of these samples is not known.

Based on the results of this work it was concluded that the Bradford Assay using the BSA or the HMPM calibration model is the preferred method for protein quantitation in human breast milk. The detection principle of the microfluidic chip, applied at the point-of-care analysis of human breast milk should, therefore, be based on this Assay.

The transfer of the Bradford Assay onto the microfluidic chip design was proven to be problematic, because of PEG-DA-Coomassie Brilliant Blue interaction and the instability of the dye under UV exposure.

6. Outlook

Amongst the information gained in the course of this thesis, there are serval points left to be done, concerning the transfer of the Bradford Assay onto the microfluidic chip [31] in particular.

As interactions between Coomassie Brilliant Blue and the hydrogel consisting of polyethyleneglycol diacrylate were reported, it is obvious that the hydrogel has to be exchanged by another material that is not interacting with the dye and therefore not producing a huge background signal inducing a lower sensitivity of the Assay. Furthermore, a material is needed forming a gel independently of UV exposure. As agarose is an expected alternative to PEG-DA, further experiments have to be done concerning the introduction of agarose onto the microfluidic chip. In detail:

- An adaptation of the agarose concentration is necessary, which is needed to create pores in the size range required for the diffusion of proteins into the gel.
- Furthermore the ratio of agarose: Bradford reagent has to be adapted to ensure the saturation of the formation of the protein-dye complex. For this purpose, the Coomassie Brilliant Blue concentration has to be eventually multiply increased, eventually causing problems with solubility and the adjustment of the required low pH.
- In addition, problems about the handling and the filling of predefined regions on the chip with agarose, without a preterm solidification, have to be solved.

Besides this, of course, it would be important to analyze the protein content of more human breast milk samples, to do an extensive statistical evaluation of the results of the compared methods to gain accuracy and reliability.

Another point is the investigation of a certified reference material for human breast milk that was unfortunately not available during this thesis. As there are certified reference materials available for cow's milk, potentially this should be analyzed additionally to compare it to the results obtained from the self-made reference materials containing human and bovine milk proteins.

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8. Appendix

8.1 Data – Numeration according to the corresponding chapter

4.1.3.1 Protein standards – Bioanalyzer P230 Assay

α-Lactalbumin	*Average*		**Beta-Casein**	*Average*	
c [µg/mL]	***Peak Area***	***SD***	***c [µg/mL]***	***Peak Area***	***SD***
21.30	2.34	0.11	24.60	5.65	0.53
45.00	5.79	0.30	49.30	7.41	0.87
264.00	31.14	2.32	299.90	33.79	4.60
450.00	39.84	3.74	495.00	67.50	2.70
547.00	45.74	2.01	596.00	46.25	1.71
Lactoferrin	*Average*		***β-Lactoglobulin B***	*Average*	
c [µg/mL]	***Peak Area***	***SD***	***c [µg/mL]***	***Peak Area***	***SD***
21.30	4.53	0.42	22.60	5.09	0.42
45.10	9.54	1.44	47.70	7.30	0.34
264.00	49.77	1.15	279.00	25.52	0.34
450.00	66.43	1.75	476.00	32.03	2.13
547.00	97.40	1.48	577.00	38.73	5.14

4.1.3.2 Human milk protein mix (HMPM) – Bioanalyzer P230 Assay

α-Lactalbumin	*Average*		***β-Casein***	*Average*	
c [µg/mL]	***Peak Area***	***SD***	***c [µg/mL]***	***Peak Area***	***SD***
21.25	2.94	0.20	24.50	3.39	0.30
42.50	5.83	0.33	49.00	7.31	1.26
85.00	9.82	0.52	98.00	9.45	2.04
170.00	15.97	0.70	196.00	15.46	1.81
255.00	26.20	0.27	294.00	25.45	2.30
340.00	28.68	0.79	392.00	28.23	2.20
425.00	69.17	5.40	490.00	64.97	6.12
510.00	72.38	5.70	588.00	69.05	6.97
Lactoferrin	*Average*		**Total protein**	*Average*	
c [µg/mL]	***Peak Area***	***SD***	***c [µg/mL]***	***Peak Area***	***SD***
21.25	6.19	0.08	67.00	30.09	1.49
42.50	12.28	0.87	134.00	42.22	5.61
85.00	17.71	1.03	268.00	50.16	3.98
170.00	29.92	0.82	536.00	74.15	5.64
255.00	48.93	1.53	804.00	111.51	6.74
340.00	56.82	1.00	1072.00	122.96	3.43

425.00	151.81	12.20	1340.00	302.19	23.29
510.00	157.68	11.22	1608.00	314.28	23.50

4.1.3.3 Bovine milk protein mix (BMPM) – Bioanalyzer P230 Assay

α-Lactalbumin	*Average*		***β-Casein***	*Average*	
c [µg/mL]	***Peak Area***	***SD***	***c [µg/mL]***	***Peak Area***	***SD***
21.30	2.96	0.31	24.60	3.09	0.54
45.00	4.95	0.81	49.30	4.64	0.54
264.00	33.54	2.29	299.90	23.92	3.66
450.00	58.95	2.95	495.00	45.91	3.21
547.00	82.62	4.64	596.00	77.65	2.91
Lactoferrin	*Average*		***β-Lactoglobulin B***	*Average*	
c [µg/mL]	***Peak Area***	***SD***	***c [µg/mL]***	***Peak Area***	***SD***
21.30	5.99	0.71	22.60	5.83	0.09
45.10	6.82	1.14	47.70	7.00	0.92
264.00	56.91	1.79	279.00	29.94	2.15
450.00	116.03	4.93	476.00	53.48	2.13
547.00	173.17	7.26	577.00	74.31	1.87
total protein	*Average*				
c [µg/mL]	***Peak Area***	***SD***			
89.80	27.76	1.39			
187.10	33.34	4.59			
1106.90	148.06	10.75			
1871.00	259.62	20.31			
2267.00	452.42	20.38			

4.2.1.1 BSA – UV/Vis spectroscopy

Bradford			***Bromophenol Blue***		
BSA	*Average*		***BSA***	*Average*	
c [µg/mL]	***Abs_{595nm}***	***SD***	***c [µg/mL]***	***Abs_{605nm}***	***SD***
2910.00	0.75	0.01	2910.00	1.42	0.04
2425.00	0.71	0.02	2425.00	1.20	0.03
1940.00	0.64	0.01	1940.00	0.88	0.02
1455.00	0.56	0.03	1455.00	0.63	0.01
1212.50	0.48	0.01	1212.50	0.51	0.01
970.00	0.40	0.03	970.00	0.27	0.01
727.50	0.32	0.02	727.50	0.16	0.00

485.00	0.24	0.01	485.00	0.11	0.01

4.2.1.2 Human milk protein mix (HMPM) - UV/Vis spectroscopy

Bradford			***Bromophenol Blue***		
HMPM	*Average*		***HMPM***	*Average*	
c [µg/mL]	*Abs_{595nm}*	*SD*	*c [µg/mL]*	*Abs_{605nm}*	*SD*
1608.00	0.67	0.02	1608.00	0.45	0.02
1340.00	0.60	0.01	1340.00	0.37	0.04
1072.00	0.52	0.02	1072.00	0.30	0.03
804.00	0.39	0.02	804.00	0.24	0.00
536.00	0.25	0.03	536.00	0.12	0.01
268.00	0.12	0.02	268.00	0.05	0.00
134.00	0.06	0.01	134.00	0.02	0.00
67.00	0.03	0.01	67.00	0.01	0.00

4.2.1.3 Bovine milk protein mix (BMPM) - UV/Vis spectroscopy

Bradford			***Bromophenol Blue***		
BMPM	*Average*		***BMPM***	*Average*	
c [µg/mL]	***Abs595nm***	***SD***	***c [µg/mL]***	***Abs595nm***	***SD***
2265.00	0.40	0.01	2265.00	0.37	0.01
1871.00	0.37	0.01	1871.00	0.29	0.02
1431.70	0.37	0.04	1431.70	0.25	0.02
1106.00	0.29	0.03	1106.00	0.18	0.01
715.85	0.19	0.01	715.85	0.12	0.01
357.93	0.09	0.01	357.93	0.06	0.00
187.20	0.05	0.00	187.20	0.04	0.01
89.80	0.03	0.00	89.80	0.01	0.01

4.3.1.1 Influence of fat – Bioanalyzer P230 Assay

α-Lactalbumin	*10 mg/mL fat*	*20 mg/mL fat*	*30 mg/mL fat*	***β-Casein***	10 mg/mL fat
c [µg/mL]	***Peak Area***	***Peak Area***	***Peak Area***	***c [µg/mL]***	***Peak Area***
21.25	3.05	3.12	3.29	24.50	1.99
42.50	7.65	5.73	7.76	49.00	3.47
170.00	20.06	19.59	18.96	196.00	16.31
255.00	27.64	27.92	27.04	294.00	24.07
340.00	35.49	28.73	42.85	392.00	38.32

Lactoferrin	*10 mg/mL fat*	*20 mg/mL fat*	*30 mg/mL fat*	**total protein**	*10 mg/mL fat*
c [µg/mL]	***Peak Area***	***Peak Area***	***Peak Area***	***c [µg/mL]***	***Peak Area***
21.25	3.46	3.46	3.29	67.00	26.96
42.50	7.94	7.72	6.80	134.00	37.30
170.00	35.09	32.84	33.73	536.00	79.90
255.00	53.10	53.61	54.27	804.00	114.80
340.00	70.78	61.86	89.81	1072.00	154.34

4.3.1.2 Influence of calcium – Bioanalyzer P230 Assay

α-Lactalbumin	*2.5 mmol/L Ca*	*5 mmol/L Ca*	*β-Casein*	*2.5 mmol/L Ca*	*5 mmol/L Ca*
c [µg/mL]	***Peak Area***	***Peak Area***	***c [µg/mL]***	***Peak Area***	***Peak Area***
21.25	1.43	2.47	24.50	1.73	2.40
42.50	10.73	7.59	49.00	3.40	3.22
170.00	22.96	25.35	196.00	6.46	3.73
255.00	38.14	24.68	294.00	21.31	15.84
340.00	34.51	42.65	392.00	11.16	8.71
Lactoferrin	*2.5 mmol/L Ca*	*5 mmol/L Ca*	**total protein**	*2.5 mmol/L Ca*	*5 mmol/L Ca*
c [µg/mL]	***Peak Area***	***Peak Area***	***c [µg/mL]***	***Peak Area***	***Peak Area***
21.25	2.78	2.66	75.00	13.66	21.13
42.50	14.68	17.55	150.00	59.40	44.99
170.00	32.22	32.00	600.00	66.92	89.59
255.00	49.42	49.47	900.00	101.31	97.95
340.00	62.38	55.80	1200.00	142.05	152.32

4.3.2.1.1 Influence of fat – UV/Vis spectroscopy

Bradford				**Bromophenol Blue**			
BSA	*10 mg/mL fat*	*20 mg/mL fat*	*30 mg/mL fat*	***BSA***	*10 mg/mL fat*	*20 mg/mL fat*	*30 mg/mL fat*
c [µg/mL]	***Abs_{595nm}***	***Abs_{595nm}***	***Abs_{595nm}***	***c [µg/mL]***	***Abs_{605nm}***	***Abs_{605nm}***	***Abs_{605nm}***
1455.0	0.527	0.593	0.555	1455.000	0.692	0.662	0.635
1212.5	0.485	0.518	0.495	1212.500	0.525	0.509	0.526
970.0	0.401	0.422	0.435	970.000	0.369	0.359	0.293
727.5	0.332	0.366	0.360	727.500	0.205	0.210	0.196
485.0	0.234	0.255	0.264	485.000	0.098	0.097	0.091
242.5	0.113	0.148	0.151	242.500	0.035	0.053	0.036
121.3	0.011	0.007	0.083	121.250	0.012	0.012	0.019
60.6	0.006	0.003	0.021	60.625	0.002	0.001	0.007

Bradford				Bromophenol Blue			
HMPM	*10 mg/mL fat*	*20 mg/mL fat*	*30 mg/mL fat*	**HMPM**	*10 mg/mL fat*	*20 mg/mL fat*	*30 mg/mL fat*
c [µg/mL]	**Abs_{595nm}**	**Abs_{595nm}**	**Abs_{595nm}**	**c [µg/mL]**	**Abs_{605nm}**	**Abs_{605nm}**	**Abs_{605nm}**
1608.0	0.602	0.383	0.593	1608.000	0.353	0.322	0.316
1340.0	0.601	0.316	0.566	1340.000	0.288	0.254	0.287
1072.0	0.525	0.382	0.522	1072.000	0.195	0.245	0.212
804.0	0.427	0.257	0.447	804.000	0.151	0.206	0.148
536.0	0.307	0.108	0.289	536.000	0.088	0.067	0.079
268.0	0.180	0.041	0.207	268.000	0.034	0.025	0.034
134.0	0.133	0.026	0.102	134.000	0.013	0.013	0.011
67.0	0.096	0.000	0.087	67.000	-0.001	0.000	0.003

Bradford				Bromophenol Blue			
BMPM	*10 mg/mL fat*	*20 mg/mL fat*	*30 mg/mL fat*	**BMPM**	*10 mg/mL fat*	*20 mg/mL fat*	*30 mg/mL fat*
c [µg/mL]	***Abs_{595nm}***	***Abs_{595nm}***	***Abs_{595nm}***	***c [µg/mL]***	***Abs_{605nm}***	***Abs_{605nm}***	***Abs_{605nm}***
2265.0	0.613	0.609	0.619	2265.000	0.405	0.414	0.411
1871.0	0.578	0.585	0.601	1871.000	0.361	0.366	0.362
1431.7	0.496	0.511	0.519	1431.700	0.263	0.282	0.241
1106.0	0.381	0.415	0.421	1106.000	0.186	0.192	0.183
715.9	0.266	0.256	0.294	715.850	0.105	0.099	0.102
357.9	0.095	0.120	0.138	357.925	0.043	0.037	0.040
187.2	0.037	0.047	0.077	187.200	0.019	0.018	0.019
89.8	0.016	0.008	0.063	89.800	0.006	0.006	0.005

4.3.2.1.2 Influence of calcium – UV/Vis spectroscopy

Bradford				Bromophenol Blue			
BSA	*2.5 mmol/L Ca*	*5.0 mmol/L Ca*	*7.5 mmol/L Ca*	**BSA**	*2.5 mmol/L Ca*	*5.0 mmol/L Ca*	*7.5 mmol/L Ca*
c [µg/mL]	***Abs_{595nm}***	***Abs_{595nm}***	***Abs_{595nm}***	***c [µg/mL]***	***Abs_{605nm}***	***Abs_{605nm}***	***Abs_{605nm}***
1455.0	0.572	0.514	0.695	1455.000	0.514	0.580	0.533
1212.5	0.544	0.650	0.777	1212.500	0.446	0.421	0.460
970.0	0.614	0.537	0.600	970.000	0.335	0.319	0.321
727.5	0.415	0.444	0.509	727.500	0.184	0.189	0.171
485.0	0.262	0.290	0.383	485.000	0.079	0.079	0.085
242.5	0.122	0.154	0.229	242.500	0.034	0.034	0.039

121.3	0.034	0.061	0.146	121.250	0.015	0.014	0.016
60.6	0.015	0.033	0.117	60.625	0.007	0.005	0.010

Bradford				***Bromophenol Blue***			
HMPM	*2.5 mmol/L Ca*	*5.0 mmol/L Ca*	*7.5 mmol/L Ca*	***HMPM***	*2.5 mmol/L Ca*	*5.0 mmol/L Ca*	*7.5 mmol/L Ca*
c [µg/mL]	***Abs_{595nm}***	***Abs_{595nm}***	***Abs_{595nm}***	***c [µg/mL]***	***Abs_{605nm}***	***Abs_{605nm}***	***Abs_{605nm}***
1608.0	0.523	0.568	0.609	1608.000	0.300	0.355	0.345
1340.0	0.589	0.615	0.610	1340.000	0.265	0.259	0.302
1072.0	0.476	0.506	0.521	1072.000	0.193	0.199	0.203
804.0	0.341	0.390	0.377	804.000	0.142	0.133	0.143
536.0	0.204	0.245	0.239	536.000	0.074	0.079	0.086
268.0	0.112	0.094	0.137	268.000	0.044	0.043	0.048
134.0	0.054	0.045	0.061	134.000	0.045	0.013	0.020
67.0	0.026	0.005	0.000	67.000	0.006	0.006	0.012

Bradford				***Bromophenol Blue***			
BMPM	*2.5 mmol/L Ca*	*5.0 mmol/L Ca*	*7.5 mmol/L Ca*	***BMPM***	*2.5 mmol/L Ca*	*5.0 mmol/L Ca*	*7.5 mmol/L Ca*
c [µg/mL]	***Abs_{595nm}***	***Abs_{595nm}***	***Abs_{595nm}***	**c [µg/mL]**	***Abs_{605nm}***	***Abs_{605nm}***	***Abs_{605nm}***
2265.0	0.592	0.734	0.703	2265.000	0.382	0.430	0.450
1871.0	0.522	0.684	0.663	1871.000	0.341	0.337	0.385
1431.7	0.454	0.606	0.594	1431.700	0.248	0.245	0.268
1106.0	0.377	0.474	0.482	1106.000	0.172	0.151	0.174
715.9	0.234	0.331	0.330	715.850	0.090	0.094	0.092
357.9	0.110	0.186	0.181	357.925	0.036	0.030	0.036
187.2	0.050	0.098	0.098	187.200	0.011	0.011	0.014
89.8	0.004	0.052	0.071	89.800	0.003	0.004	0.007

4.3.2.3 Influence of fat and calcium – UV/Vis spectroscopy

Bradford					
BSA	*10 mg/mL fat + 2.5 mmol/L Ca*	*10 mg/mL fat + 7.5 mmol/L Ca*	*20 mg/mL fat + 5 mmol/L Ca*	*30 mg/mL fat + 2.5 mmol/L Ca*	*30 mg/mL fat + 7.5 mmol/L Ca*
c [µg/mL]	***Abs_{595nm}***	***Abs_{595nm}***	***Abs_{595nm}***	***Abs_{595nm}***	***Abs_{595nm}***
2910.000	0.746	0.748	0.762	0.786	0.744
2425.000	0.685	0.724	0.725	0.711	0.705

1940.000	0.648	0.665	0.697	0.697	0.573
1455.000	0.577	0.548	0.534	0.579	0.476
1212.500	0.528	0.505	0.509	0.538	0.437
970.000	0.446	0.371	0.399	0.467	0.370
727.500	0.293	0.327	0.421	0.346	0.319
485.000	0.197	0.224	0.293	0.219	0.202
242.500	0.088	0.087	0.155	0.087	0.089
121.250	0.018	0.012	0.019	0.029	0.029
60.625	0.011	0.010	0.008	0.002	-0.002
Bromophenol Blue					
BSA	*10 mg/mL fat + 2.5 mmol/L Ca*	*10 mg/mL fat + 7.5 mmol/L Ca*	*20 mg/mL fat + 5 mmol/L Ca*	*30 mg/mL fat + 2.5 mmol/L Ca*	*30 mg/mL fat + 7.5 mmol/L Ca*
c [µg/mL]	***Abs_{605nm}***	***Abs_{605nm}***	***Abs_{605nm}***	***Abs_{605nm}***	***Abs_{605nm}***
2910.000	1.132	1.137	1.200	1.212	1.135
2425.000	1.061	0.961	0.953	1.037	1.052
1940.000	0.744	0.757	0.743	0.839	0.784
1455.000	0.545	0.493	0.484	0.513	0.458
1212.500	0.370	0.366	0.373	0.414	0.383
970.000	0.271	0.192	0.158	0.280	0.267
727.500	0.151	0.139	0.122	0.157	0.141
485.000	0.071	0.080	0.065	0.077	0.073
242.500	0.027	0.030	0.024	0.029	0.036
121.250	0.007	0.013	0.008	0.008	0.013
60.625	0.003	0.009	0.006	0.004	0.008

Bradford	***1. Run***	***2. Run***	***3. Run***
HMPM	*20 mg/mL fat + 5 mmol/L Ca*	*20 mg/mL fat + 5 mmol/L Ca*	*20 mg/mL fat + 5 mmol/L Ca*
c [µg/mL]	***Abs_{595nm}***	***Abs_{595nm}***	***Abs_{595nm}***
1.608	0.538	0.525	0.557
1.340	0.476	0.495	0.496
1.072	0.437	0.418	0.458
0.804	0.274	0.292	0.337
0.536	0.203	0.175	0.219
0.268	0.083	0.056	0.088
0.134	0.018	0.009	0.038
0.067	0.008	0.004	0.018

Bromophenol Blue	*1. Run*	*2. Run*	*3. Run*
HMPM	*20 mg/mL fat + 5 mmol/L Ca*	*20 mg/mL fat + 5 mmol/L Ca*	*20 mg/mL fat + 5 mmol/L Ca*
c [µg/mL]	*Abs_{605nm}*	*Abs_{605nm}*	*Abs_{605nm}*
1.608	0.400	0.341	0.299
1.340	0.266	0.252	0.233
1.072	0.178	0.153	0.164
0.804	0.121	0.146	0.144
0.536	0.092	0.078	0.079
0.268	0.027	0.023	0.023
0.134	0.008	0.008	0.007
0.067	0.005	0.004	0.005

4.4.1 Investigation of the protein content of human breast milk samples –MIRIS & Bioanalyzer P230 Assay

MIRIS			**Bioanalyzer P230 Assay**			
			HMPM		**BMPM**	
	Average		**Average**		**Average**	
Sample	**c[mg/mL]**	**SD**	**c[mg/mL]**	**SD**	**c[mg/mL]**	**SD**
A	9.33	0.47	31.28	6.93	32.15	5.578
B	9	0	22.3	0.75	24.92	0.61
C	10.67	0.94	39.47	4.34	38.74	3.5
D	8.67	0.47	23.8	2.9	26.13	2.34
E	9.67	0.47	32.85	4.36	33.41	3.51
F	15.33	0.47	28.81	6.2	30.16	4.99
G	10	0	29.61	9.32	30.81	7.5
H	8	0	31.83	4.16	31.66	2.69
I	14	0	25.07	3.59	27.15	2.89
J	10	0	24.71	1.26	26.86	1.02
K	6	1.41	29.19	3.02	32.56	1.73
L	7.67	0.47	21.09	12.12	26.27	9.11
M	9	0	13.01	0.73	18.84	0.58
N	8.33	0.47	12.91	2.79	18.76	2.25
O	8	0	15.93	3.01	21.12	2.42
P	8.67	0.47	13.92	9.29	22.37	7.48
Q	9	0	16.87	1.75	24.74	1.41
R	7.67	0.47	15.82	3.85	23.89	3.1
S	14.67	0.47	20.39	10.56	26.18	10.08

T	9.33	0.47	15.4	0.065	23.56	0.05

4.4.3 Investigation of the protein content of human breast milk samples – UV/Vis spectroscopy

Sample	Bradford					Bromophenol Blue				
	BSA					BSA				
	Abs_{595nm}	Blank	Abs_{595nm}-Blank	c[mg/mL]	SD	Abs_{605nm}	Blank	Abs_{605nm}-Blank	c[mg/mL]	SD
A	0.262	0.162	0.099	12.49	1.06	1.085	0.771	0.315	14.45	0.85
B	0.259	0.167	0.091	11.44	0.62	0.952	0.649	0.303	14.15	0.59
C	0.207	0.168	0.040	11.00	1.03	0.881	0.539	0.342	15.14	0.28
D	0.279	0.086	0.193	12.90	1.43	0.813	0.454	0.359	15.57	1.07
E	0.258	0.124	0.134	12.30	0.74	0.913	0.529	0.384	16.21	0.43
F	0.275	0.143	0.133	16.67	1.49	1.198	0.766	0.431	17.42	0.59
G	0.247	0.112	0.135	12.69	1.52	0.884	0.519	0.365	15.73	1.61
H	0.219	0.101	0.119	10.71	1.56	0.717	0.382	0.335	14.98	1.11
I	0.277	0.161	0.116	12.24	0.57	1.315	1.019	0.295	13.97	0.30
J	0.153	0.082	0.071	10.96	0.98	0.389	0.081	0.308	14.28	0.18
K	0.182	0.133	0.049	6.87	0.27	1.556	1.280	0.275	13.45	0.25
L	0.182	0.054	0.128	8.82	1.52	0.849	0.549	0.300	14.09	1.27
M	0.179	0.053	0.126	9.85	0.72	0.901	0.541	0.360	15.62	0.24
N	0.180	0.047	0.132	10.54	0.89	0.916	0.672	0.244	12.67	0.07
O	0.173	0.062	0.111	10.10	0.41	1.034	0.786	0.247	12.75	0.40
P	0.192	0.065	0.127	13.94	0.93	0.984	0.639	0.345	15.21	0.16
Q	0.273	0.091	0.182	17.04	0.52	1.267	0.950	0.317	14.52	1.17
R	0.241	0.057	0.183	15.23	1.37	0.854	0.422	0.432	17.44	1.48
S	0.351	0.076	0.275	22.78	1.79	1.300	0.644	0.656	23.11	0.75
T	0.232	0.055	0.176	14.71	0.59	0.784	0.383	0.401	16.65	1.40

Sample	Bradford					Bromophenol Blue				
	HMPM			Average		HMPM			Average	
	Abs_{595nm}	Blank	Abs_{595nm}-Blank	c[mg/mL]	SD	Abs_{605nm}	Blank	Abs_{605nm}-Blank	c[mg/mL]	SD
A	0.282	0.181	0.101	10.33	0.84	0.270	0.179	0.092	15.27	0.69
B	0.279	0.164	0.115	11.98	1.06	0.235	0.136	0.100	16.39	1.35
C	0.276	0.162	0.114	11.88	0.99	0.240	0.157	0.083	14.08	1.55
D	0.209	0.086	0.123	12.86	1.22	0.222	0.120	0.102	16.66	1.17
E	0.243	0.124	0.118	12.34	0.63	0.227	0.129	0.097	16.04	1.06
F	0.294	0.143	0.151	16.10	1.28	0.309	0.195	0.115	18.37	2.14

G	0.234	0.112	0.121	12.68	1.31	0.224	0.120	0.104	16.91	1.36
H	0.207	0.103	0.105	10.78	1.06	0.182	0.096	0.087	14.59	0.65
I	0.279	0.161	0.118	12.29	0.49	0.297	0.209	0.089	14.88	1.35
J	0.191	0.079	0.112	11.58	1.37	0.074	0.003	0.071	12.54	0.73
K	0.211	0.133	0.078	7.68	0.23	0.220	0.137	0.083	14.13	0.37
L	0.163	0.058	0.106	8.69	0.99	0.206	0.115	0.091	15.24	0.80
M	0.168	0.053	0.116	9.61	0.53	0.239	0.144	0.095	15.72	1.53
N	0.172	0.047	0.125	10.47	0.77	0.219	0.122	0.097	15.95	1.37
O	0.183	0.055	0.128	10.75	1.25	0.203	0.130	0.073	12.72	1.26
P	0.222	0.065	0.157	13.40	0.80	0.230	0.140	0.090	15.10	0.42
Q	0.277	0.091	0.186	16.06	0.45	0.253	0.141	0.112	17.99	1.21
R	0.226	0.057	0.169	14.50	1.17	0.217	0.110	0.107	17.39	0.94
S	0.316	0.076	0.240	20.98	1.54	0.327	0.206	0.121	19.19	0.94
T	0.231	0.055	0.175	15.07	1.19	0.222	0.116	0.106	17.20	0.82

Sample	**Bradford**					**Bromophenol Blue**				
	BMPM			**Average**		**BMPM**			**Average**	
	Abs_{595nM}	**Blank**	**Abs_{595nm}-Blank**	**c[mg/mL]**	**SD**	**Abs_{605nm}**	**Blank**	**Abs_{605nm}-Blank**	**c[mg/mL]**	**SD**
A	0.282	0.181	0.101	18.79	1.42	0.270	0.179	0.092	21.58	1.25
B	0.279	0.164	0.115	21.59	1.80	0.235	0.136	0.100	23.62	2.44
C	0.276	0.162	0.114	21.41	1.69	0.240	0.157	0.083	19.43	2.81
D	0.209	0.086	0.123	23.07	2.08	0.222	0.120	0.102	24.10	2.13
E	0.243	0.124	0.118	22.19	1.07	0.227	0.129	0.097	22.99	1.92
F	0.294	0.143	0.151	28.56	2.17	0.309	0.195	0.115	27.20	3.89
G	0.234	0.112	0.121	22.77	2.22	0.224	0.120	0.104	24.55	2.46
H	0.207	0.103	0.105	19.54	1.79	0.182	0.096	0.087	20.36	1.18
I	0.279	0.161	0.118	22.10	0.83	0.297	0.209	0.089	20.87	2.45
J	0.191	0.079	0.112	20.90	2.33	0.074	0.003	0.071	16.63	1.33
K	0.211	0.133	0.078	14.29	0.40	0.220	0.137	0.083	19.51	0.67
L	0.163	0.058	0.106	19.69	2.11	0.206	0.115	0.091	21.52	1.46
M	0.168	0.053	0.116	21.63	1.11	0.239	0.144	0.095	22.39	2.78
N	0.172	0.047	0.125	23.46	1.63	0.219	0.122	0.097	22.83	2.48
O	0.183	0.055	0.128	24.04	2.65	0.203	0.130	0.073	16.96	2.28
P	0.222	0.065	0.157	29.66	1.69	0.230	0.140	0.090	21.28	0.77
Q	0.277	0.091	0.186	35.30	0.94	0.253	0.141	0.112	26.52	2.19
R	0.226	0.057	0.169	32.01	2.49	0.217	0.110	0.107	25.44	1.71
S	0.316	0.076	0.240	45.75	3.26	0.327	0.206	0.121	28.70	1.71
T	0.231	0.055	0.175	33.22	2.52	0.222	0.116	0.106	25.08	1.49

8.2 Additional experiments

8.2.1 Analysis of the Aptamil FMS infant formula

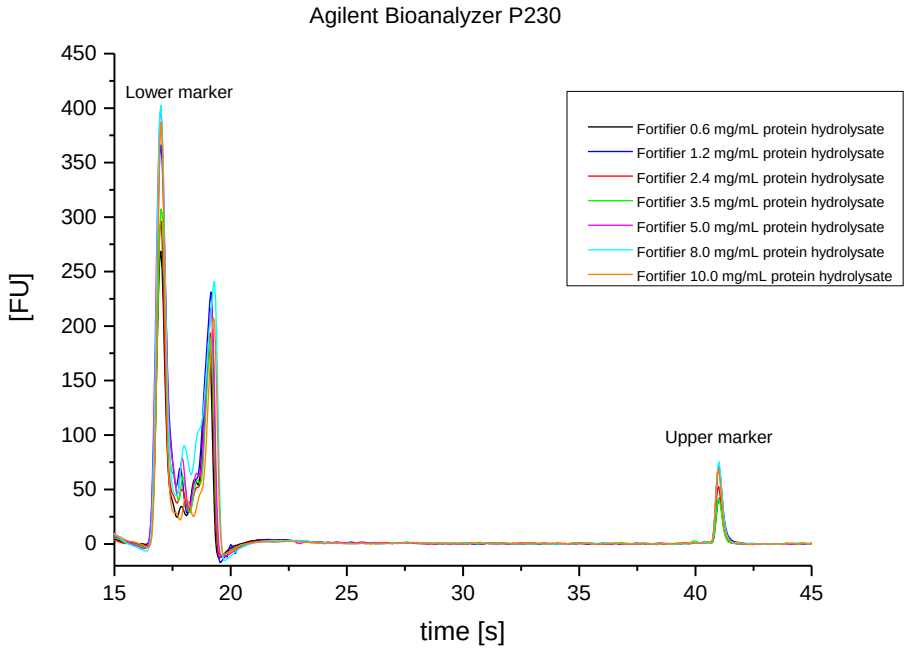

Figure 60: Analysis of the Aptamil FMS infant formula, Agilent Bioanalyzer P230 Assay

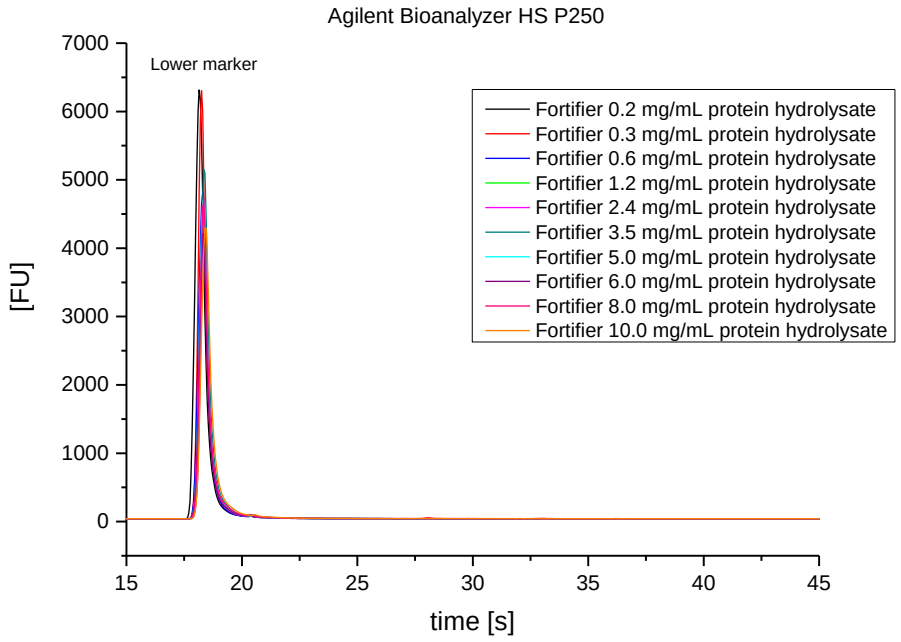

Figure 61: Analysis of the Aptamil FMS infant formula, Agilent Bioanalyzer HSP 250 Assay

By the analysis of a standard infant formula (Aptamils FMS) with the Bioanalyzer P230 Assay as well as with the P250 HSP Assay no results were obtained.