UPTEC X 11 015
Examensarbete 30 hp
Mars 2011

Studies of the BCA assay for determination of total protein in allergen extracts

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UPTEC X 11 015	**Date of issue 2011-03**

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Title (English)

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Title (Swedish)

Abstract

The aim of this thesis has been to evaluate and to optimize the BCA protein assay for quantification of total protein. This was done by analyzing the precision, accuracy, linearity and range of the method. Since complex solutions, for example allergen extracts, contain a vast range of non-protein substances that may interfere with the BCA assay, the degree of interference from different substances was evaluated. It was shown that protein precipitation with deoxycholate (DOC) and trichloroacetic acid (TCA) is a suitable way of eliminating these substances before BCA analysis and in that way increase the reliability of the results. Also, the DOC-TCA precipitation step showed promising results in increasing the sensitivity of the assay and lowering the limit of detection by concentrating dilute samples.

Keywords

BCA protein assay, allergen extracts, total protein, interfering substances, precipitation

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Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **42**

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Populärvetenskaplig sammanfattning

Inom biokemisk forskning och produktion kan det ofta vara viktigt att veta exakt hur mycket protein en lösning innehåller innan nästa steg i en process påbörjas. Det finns olika metoder och tekniker där vissa av proteinernas egenskaper kan utnyttjas för att kunna bestämma mängden protein i en lösning. Examensarbetet handlar om att utvärdera och förbättra en sådan metod: BCA-metoden. I BCA metoden sker en kemisk reaktion som gör att färg uppkommer om protein finns närvarande. Intensiteten på färgen kan mätas och motsvarar då mängden protein. Metoden fungerar bäst när rent protein studeras, men man vill även ha möjlighet att analysera mer komplexa lösningar, till exempel allergenextrakt, som bland annat innehåller proteiner som orsakar allergier. Olika betingelser i metoden har därför studerats för att kunna förbättra metoden genom att göra den mer precis och noggrann. Komplexa lösningar innehåller oftast också andra substanser som, trots att de inte är protein, kan bidra till färgutvecklingen i BCA-metoden. Eftersom detta inte är önskvärt har ett preparativt steg för att ta bort icke-protein från en lösning tagits fram och utvärderats. Genom att kombinera BCA-metoden med tekniken för att avlägsna icke-protein blir skattningen av proteinmängden mer korrekt.

Examensarbete, 30 hp
Civilingenjörsprogrammet Molekylär bioteknik
Uppsala Universitet mars 2011

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Abbreviations

BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CV	Coefficient of Variation
DOC	Deoxycholate
LOD	Limit of Detection
LOQ	Limit of Quantification
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
SDS	Sodium Dodecyl Sulfate
TCA	Trichloroacetic Acid
UV	Ultraviolet

1 Introduction

Today, there are a number of commonly used assays available for determination of protein concentration (Olson and Markwell 2007). Quantification of protein is important because it is usually performed before further analysis, e.g. chromatography or immunochemical studies (Thermo Scientific Pierce 2009). It can also be used as process or quality controls. In general, the assays used to quantify protein concentration are based on a comparison of a solution of unknown amount of protein to different known protein concentrations forming a standard curve. When deciding which protein assay to use there are several issues to consider including the existing amount of protein, sensitivity and specificity of the method, interfering substances which may have an effect on the reliability of the results and the number of pre-treatments of the samples that has to be done (Stoschek 1990, Lovrien and Matulis 1998, Thermo Scientific Pierce 2009).

1.1 Background

The following sections include a brief description of four assays for determination of protein concentration: the BCA assay that this thesis discusses, the Lowry assay, the Bradford assay and UV spectroscopy. Also information about how interfering substances may affect protein assays and advantages as well as disadvantages of the different assays are included.

1.1.1 The BCA assay

The BCA protein assay, an assay to determine protein concentration with the use of bicinchoninic acid (BCA), was first described by Smith *et. al.* in 1985. In common with the Lowry assay (Lowry *et. al.* 1951), the well known biuret reaction is the first step in the reaction that takes place in the BCA assay. In this reaction, protein reduces Cu^{2+} to Cu^{1+} in an alkaline environment (Smith *et. al.* 1985). In the second step of the reaction, BCA reacts with the newly formed Cu^{1+}-ions to form a purple coloured BCA-Cu^{1+} complex that has an absorbance at 562 nm. In figure 1, the two-step reaction can be seen (Smith *et. al.* 1985, Thermo Scientific Pierce 2009).

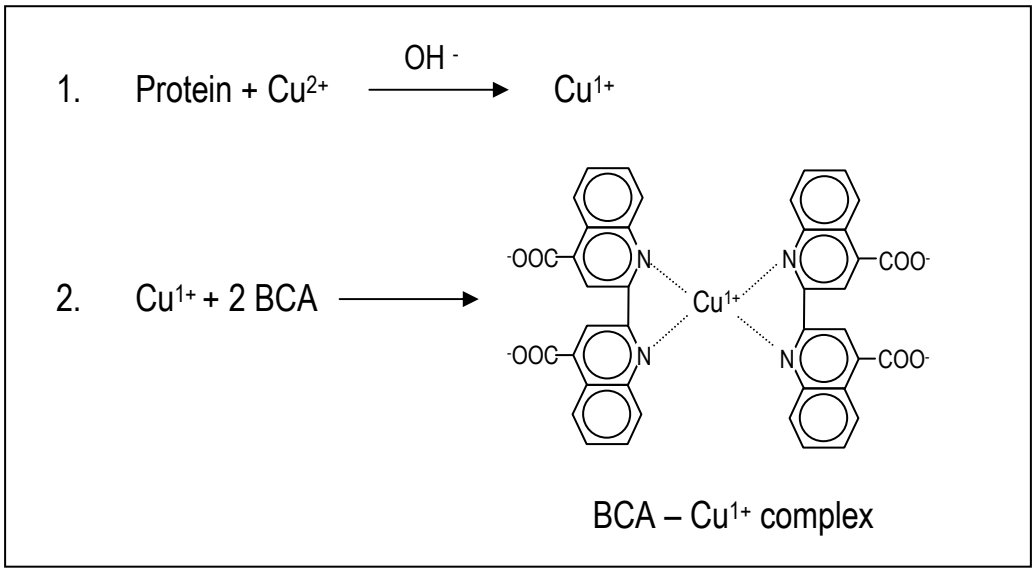

Figure 1: Chemistry of the BCA protein assay. 1: Protein reduces Cu^{2+} to Cu^{1+} in an alkaline environment. 2: Cu^{1+} forms a complex with two BCA molecules resulting in colour formation.

The intensity of the colour that is measured is proportional to the number of Cu^{1+}-ions forming the BCA-Cu^{1+} complex, which in turn is proportional to the number of Cu^{2+}-ions being reduced by the protein. Consequently the amount of protein is quantified (Lovrien and Matulis 1998, Smith *et.al.* 1985).

The capability of proteins to reduce Cu^{2+} to Cu^{1+} is caused by the peptide bonds as well as by some of the amino acids; cysteine, tryptophan and tyrosine. Due to the reducing ability of these amino acids, protein-to-protein variation occurs when assaying different proteins as a result of the varying amino acid compositions. This effect can to some extent be reduced by increasing the temperature. The colour contribution from the peptide bonds are more significant at higher temperatures, hence, the protein-to-protein variations are decreased (Weichelman *et.al.* 1988).

Once protein comes in contact with the BCA reagents the reaction starts and the colour development can be monitored. The reaction proceeds at room temperature, but if incubated at higher temperatures acceleration in the colour development is seen. This gives BCA analysis some flexibility (Smith *et. al.* 1985). Furthermore, the BCA assay is not an end-point reaction, i.e. there is no reaction to stop the colour development, and therefore the colour development will continue as long as there are reagents present (Thermo Scientific Pierce 2009). For that reason a standard curve should always be run in parallel with the unknown samples for each assay to achieve greatest accuracy and to minimize errors (Thermo Scientific Pierce 2009,

Olson and Markwell 2007). In addition, the absorbances of all samples in an assay need to be read in the shortest possible period of time (Smith *et. al.* 1985).

1.1.2 The Lowry assay

Another assay for determination of small amounts of protein in solutions is the Lowry assay, described by Lowry *et. al.* in 1951. The first step in the Lowry assay is similar to that in the BCA assay, the Biuret reaction, where Cu^{1+}-ions are formed when protein reduces Cu^{2+}-ions. The produced complex of reduced Cu^{1+}-ions and amide bonds then reduces the so called Folin-Ciocalteu reagent that is added in the second step. The reduced Folin-Ciocalteu reagent is coloured blue and therefore detectable (Olson and Markwell 2007, Thermo Scientific Pierce 2009).

1.1.3 The Bradford assay

The Bradford assay uses the binding of Coomassie Brilliant Blue G-250 to protein. This simple and rapid assay was first described by Bradford in 1976. When the red coloured Coomassie Brilliant Blue G-250 binds to protein its colour is converted to blue and the absorbance maximum is shifted from 465 to 595 nm. The absorbance increase at 595 nm is monitored and thus the total protein amount is measured (Bradford 1976). The dye binds to protein in acidic environments at some of the basic amino acids, primarily arginine (Olson and Markwell 2007, Thermo Scientific Pierce 2009).

1.1.4 UV spectroscopy

Protein concentrations can be measured with ultraviolet (UV) absorption at 280 nm since aromatic amino acids present in proteins absorb light at this wave length. This is an easy and fast method, since no reagents need to be added (Stoschek 1990). However, this method is insensitive and often produces results that are not correct since other substances also absorb UV light and for example the pH of a solution and the tertiary structure of proteins can affect UV detection. To be able to accurately determine protein concentration using UV spectroscopy one needs to know the extinction coefficient (ε) and have a pure protein (Olson and Markwell 2007).

1.1.5 Interfering substances

One usually encountered problem when performing protein assays is the presence of interfering substances in the samples to be analyzed. Complex solutions containing protein, e.g.

allergen extracts that are considered in this thesis, most often also consist of a vast range of other substances that may interfere with the assay. Also the buffer used might contain molecules that cause interference. The interfering substances can have an effect on the result by either increase or decrease the response, or raise the background signal (Thermo Scientific Pierce 2009).

The above mentioned assays are sensitive to different kinds of substances; therefore if it is possible to choose among different assays, one that is compatible with the samples to be tested can be used (Olson and Markwell 2007, Thermo Scientific Pierce 2009). Another option is to remove interfering substances in a complex solution. One commonly used strategy is to precipitate the protein prior to assay analysis, and subsequently resuspend the precipitate in a buffer compatible with the assay of choice (Noble and Bailey 2009, Olson and Markwell 2007).

1.1.6 Advantages, disadvantages and the assay of choice

When the BCA assay is compared to the Lowry assay the former has benefits over the latter since samples containing detergents are to a certain extent totally compatible with the BCA assay while detergents and some salts can interfere with the detection reagents used in the Lowry assay (Olson and Markwell 2007, Smith *et. al.* 1985). Another advantage of the BCA assay over the Lowry assay is the one-step procedure in the BCA assay (the chemical two-step reaction starts immediately after adding the BCA reagent) compared to the Lowry assay with the need of adding two reagents at different times. Also, the detection reagent, Folin-Ciocalteu, used in the Lowry assay is not as sensitive and stable as the BCA reagent; therefore the BCA assay procedure is more flexible since incubation times and temperatures can be varied (Smith *et. al.* 1985).

The Bradford assay is simple and quite sensitive but the BCA assay and the Lowry assay are in most cases superior to the Bradford assay concerning protein-to-protein variations (Brown *et. al.* 1989). The variation in absorbance between proteins that can be seen in the Bradford assay is due to the different capacity of dye-binding among proteins. Also, since the degree of arginine residues differs between proteins, running different proteins as standards might be needed (Olson and Markwell 2007, Lovrien and Matulis 1998).

However, there are some disadvantages with the BCA assay. One is the fact that reducing agents can contribute to the colour formation by reducing Cu^{2+} ions. The reduced ions then form complexes with the BCA molecules, which cause the response to be falsely positive (Smith *et. al.* 1985, Thermo Scientific Pierce 2009). There exist commercial kits where reducing agents can be modified and thus the BCA assay can be used to analyze samples containing these interfering substances (Thermo Scientific Pierce 2009). This application is most often used when the protein sample buffers contain known amounts of reducing agents, however, this is not the case in complex solutions like allergen extracts, and therefore a reducing agent compatible kit has not been used in this study.

Using UV spectroscopy to establish protein concentration is probably the quickest method to make a rough determination of a pure protein sample (Olson and Markwell 2007). Though, in the case of complex solutions, UV spectroscopy will not produce reliably results since a lot of other substances also absorb UV light (Stoschek 1990).

The BCA assay is altogether less sensitive to interfering substances than the comparable early used protein assays, e.g. the Lowry assay (Wiechelman *et. al.* 1988, Lovrien and Matulis 1998). This benefit together with its ease of use is why it nowadays has become one of the most frequently used total protein assays (Olson and Markwell 2007). These are also the reasons why the BCA assay is the assay of choice in this thesis. Since the method is most consistent when analyzing pure protein samples, which is not the limited usage in the frame of this study, the method is optimized to achieve best possible estimates of protein contents in complex solutions.

1.2 Phadia

This project was performed at Phadia, a company that develops, manufactures and markets complete blood test systems for allergy and autoimmune disease diagnostics (Phadia 2011). At Phadia, protein assays are used in the manufacturing process as well as in the area of research and development. The BCA assay, performed at different conditions, is used for determination of protein concentrations in pure protein solutions as well as in complex allergen extracts. Also, protein-coated polystyrene particles are analyzed with the BCA assay. One of the responsibilities at Bioreagent - the section where this project was performed - is to purify components out of complex allergen extracts and in this procedure the BCA assay can be used when protein concentrations are to be determined.

1.3 Objective of the study

The aim of this thesis has been to evaluate and to optimize the BCA protein assay for quantification of total protein in allergen extracts. This was done by analyzing the precision, accuracy and range of the method. In addition, studies of how interfering substances affect the BCA assay and evaluation of different ways to eliminate these substances were made. The results from this thesis will be used in the validation and in the future use of the BCA protein assay.

1.3.1 Specific aims

The following aspects were studied in the evaluation and optimization of the BCA assay:

- Incubation protocol conditions
- Drift in absorbance over time
- Evaluation of regression models
- Limit of detection and limit of quantification
- The precision of the method was estimated
- Evaluation of the conditions for proper analysis of protein-coated particles

In order to determine how interfering substances affect the BCA assay and how to eliminate these substances two protein precipitation protocols were tested. The following were studied:

- Protein recovery after precipitation for some different proteins
- BCA analysis in the presence of interfering substances to examine some substances' degree of interference
- Precipitation prior to BCA analysis in the presence of interfering substances in order to evaluate the effectiveness in removing interfering substances
- Analysis of diluted samples where the protein contents are concentrated through the precipitation step with the intention of improving the sensitivity

Finally;

- The optimized assay together with the precipitation step was applied on allergen extracts to study the effect of interfering substances.

2 Materials and methods

2.1 Chemicals and reagents

A commercial BCA Protein Assay Kit (Thermo Scientific Pierce) has been used. The kit contains BCA reagent A (sodium bicarbonate, bicinchoninic acid and sodium tartare in 0.1 sodium hydroxide), BCA reagent B (4% cupric sulfate) and Albumin Standard Ampoules (bovine serum albumin (BSA) at 2.0 mg/ml in 0.9% saline and 0.05% sodium azide). Bovine gammaglobulin was obtained from Thermo Scientific Pierce. Trichloroacetic acid (TCA), acetone, sodium hydroxide (NaOH), ammonium sulfate, urea and gelatin were obtained from Merck. Deoxycholate (DOC), conalbumin, ovomucoid, glucose and guanidine hydrocholoride were obtained from Sigma-Aldrich. NaCl, sodium azide, beta galactosidase, ferritin, catalase were supplied by Phadia. Sodium dodecyl sulfate (SDS) was obtained from GE Healthcare. Allergen extracts were supplied by Phadia. DNA was given as a kind gift from Inger Jonasson at Uppsala Genome Centre.

2.2 Standard BCA protocol

The optimization of the BCA assay resulted in the following protocol:

2.2.1 Preparation of standards and samples

BSA standards were prepared by diluting BSA ampoules (2.0 mg/ml) with 0.9 % NaCl using a dual syringe diluter (Hamilton Microlab 530B). The concentration of the BSA ampoules have been checked (by the manufacturer) against a National Institute of Standards & Technology traceable BSA standard, NIST # 927d (National Institute of Standards and Technology 2010). Samples were diluted in 0.9 % NaCl.

2.2.2 Preparation of BCA working reagent

BCA working reagent was prepared fresh daily by mixing 50 parts BCA reagent A and one part BCA reagent B (Thermo Scientific Instructions).

2.2.3 BCA analysis procedure

50 µl of each standard or sample was mixed with 1 ml of BCA working reagent in 1.5 ml Eppendorf tubes. Samples were incubated at 60 ºC for 30 minutes in a heating block (Grant BT3). Next, the samples were cooled to room temperature for 20 minutes prior to measure-

ments of the absorbance (Shimadzu UV-1700 spectrophotometer) at 562 nm in disposable plastic cuvettes (Kartell).

2.2.4 Evaluation

After BCA analysis the concentration of the BSA standards were plotted against absorbance responses and a second order curve was fitted to the data. The concentrations of the unknown samples were calculated from the corresponding absorbance responses and the standard curve using Prism (GraphPad Prism version 4.03).

2.3 Experiments in the assay optimization

2.3.1 Incubation protocols

Incubation times and temperatures were varied to establish what conditions that gave the best result in terms of signal-to-background ratios, range and absorbance levels. The signal-to-background ratio was defined as the signal obtained from a sample containing protein divided by the background signal (BSA at 0 µg/ml).

2.3.2 Drift in absorbance

The increase in absorbance over time was investigated by repeated measurements combined with the incubation protocol experiment above. The drift was calculated as increase in percentage per minute for all temperature/time combinations using the slope from absorbance-time plots (for the temperatures 60 ºC and 80 ºC slopes from the last 40 minutes were used).

2.3.3 Evaluation of regression models

BSA standards at 18 different concentrations were tested according to the standard BCA protocol. Regression analysis was performed by GraphPad Prism to find the equation that best describes the relationship between concentration and absorbance response. Different curve-fitting models were compared using the F-test, a method based on hypothesis testing and ANOVA (analysis of variance) (GraphPad Prism, Regression Book). The following curve-fitting models were evaluated: linear, second, third and fourth order and four parameter logistic curve fit. Furthermore, GraphPad Prism was used to back calculate concentrations that correspond to obtained absorbance values according to the curve fits of the linear, the second order and the third order equation. These obtained concentrations from each model were compared to the actual tested concentrations. The accuracy for each concentration was calculated as the deviation from the actual concentration in percentage [Equation 1]:

$$\left(\left(\frac{obtained\ conc}{actual\ conc} \right) * 100 \right) - 100 = accuracy\ (\%) \qquad \text{[Equation 1]}$$

2.3.4 Limit of detection and limit of quantification

Limit of detection (LOD) was defined as the mean signal obtained from the blank (BSA at 0 µg/ml) plus three standard deviations and limit of quantification (LOQ) was defined as the mean signal obtained from the blank (BSA at 0 µg/ml) plus ten standard deviations (Armbruster *et. al.* 1994). The corresponding concentrations were estimated by evaluation against BSA concentrations between 0 µg/ml and 20 µg/ml according to the standard BCA protocol.

2.3.5 BCA analysis of protein-coated particles

To be able to use the BCA assay to analyze polystyrene particles coated with protein, centrifugation is needed in order to avoid particles in the light path during absorbance measuring. The centrifugation time that was necessary to make all particles settle was investigated. Particles were mixed with BCA reagent A and centrifuged at 15000×g for 15 minutes, 30 minutes or 45 minutes before the absorbance was measured.

To study any possible impacts on the colour development caused by centrifugation, two BSA standard curves were analyzed according to the standard BCA protocol, with the exception that one curve was centrifuged 45 minutes after the heat incubation and the other one was simultaneously cooled on bench for 45 minutes.

2.4 Elimination of interfering substances

2.4.1 TCA-acetone precipitation protocol

A trichloroacetic acid (TCA) – acetone precipitation protocol was used to remove interfering substances from samples (Olson and Markwell 2007). 40 µl TCA (100 %, w/v) was added to 960 µl sample to a final concentration of 4 % in 1.5 ml Eppendorf tubes. The samples were incubated on ice for 30 minutes and centrifuged at 15000×g (Eppendorf centrifuge 5415D) for 10 minutes at 4 ºC. The supernatant was decanted and an equal volume of 80 % cold acetone was added (acetone wash). The samples were vortexed and centrifuged as above. The super-

natant was decanted and acetone wash was repeated four times. Prior to resuspension of the pellets in 0.9 % NaCl, they were dried inverted for 30 minutes.

2.4.2 DOC-TCA precipitation protocol

A protein precipitation protocol with deoxycholate (DOC) and TCA (Bensadoun and Weinstein 1975, Brown *et. al.* 1989) was also used to precipitate the protein. 50 µl of the samples and 950 µl of ultra pure water were mixed in 1.5 ml Eppendorf tubes. After adding 100 µl of DOC (0.15 %, w/v) the samples were incubated in room temperature for 10 minutes. 100 µl of TCA (72 %, w/v) was added and the samples were centrifuged at 15000×g at room temperature for 15 minutes (Eppendorf centrifuge 5415D). Following centrifugation the supernatant was decanted by aspiration using vacuum. Subsequently 50 µl of sodium dodecyl sulfate (SDS) (5%, w/v) containing 0.1 M NaOH was added to resuspend the precipitate.

2.4.3 Experiments

2.4.3.1 Protein recovery

Protein recovery after precipitation was studied for BSA at concentrations over the whole range. Protein recovery was also studied for some other proteins.

2.4.3.2 Test in the presence of interfering substances

The effectiveness of the precipitation protocol was studied by mixing 50 µl of BSA at 500 µg/ml, 50 µl of different possibly interfering substances and 900 µl of ultra pure water. Precipitated samples (according to the above mentioned procedure with DOC and TCA) were run in parallel with unprecipitated samples with the standard BCA protocol.

2.4.3.3 DOC-TCA precipitation protocol including concentration of diluted samples

DOC-TCA precipitation was applied on samples that were diluted 600 times to study the possibility to concentrate dilute samples through the precipitation step. 3 ml of DOC (0.15 %, w/v) was added to 30 ml of the 600 times diluted samples in 40 ml centrifuge tubes. Samples were incubated at room temperature for 10 minutes before 3 ml of TCA (72 %, w/v) was added. Then, the samples were centrifuged at 15000×g at room temperature for 30 minutes (Beckman Coulter centrifuge Avanti J-20). Following centrifugation the supernatant was decanted by aspiration. The precipitate was resuspended in 50 µl of sodium dodecyl sulfate (SDS) (5%, w/v) in 0.1 M NaOH. The experiment was repeated in 50 ml Falcon tubes as above except for a centrifugation at 3000×g for 2 hours (Beckman GPR).

2.5 Analysis of allergen extracts

Allergen extracts were analyzed according to the standard BCA protocol combined with DOC-TCA precipitation. In addition, PD-10 Desalting columns (GE Healthcare) were used to separate low molecular weight substances from proteins in allergen extracts. Columns were equilibrated using 0.9 % NaCl prior to adding 2.5 ml of sample and discarding of the flow-through. The samples were eluted using 0.9 % NaCl and fractions were collected. Three fractions were collected; one protein fraction (Fraction I: 3 ml), one "border fraction" in between (Fraction II: 1 ml) and one low molecular weight fraction (Fraction III: 4 ml), in order to analyze the contents of the extracts.

3 Results

3.1 Assay optimization

3.1.1 Incubation protocols

Incubation protocols were studied and signal-to-background ratios were calculated for all of the tested temperature/time combinations. As seen in figure 2, optimal signal-to-background ratios were observed when incubating the samples at 60 ºC for 30 minutes or longer, or at 80 ºC for 15 minutes. The results are similar at 50 µg/ml BSA as well as at 500 µg/ml BSA. BSA at 2000 µg/ml was also tested, but most of the obtained absorbance responses were over the range of the spectrophotometer and therefore could not be properly measured (data not shown).

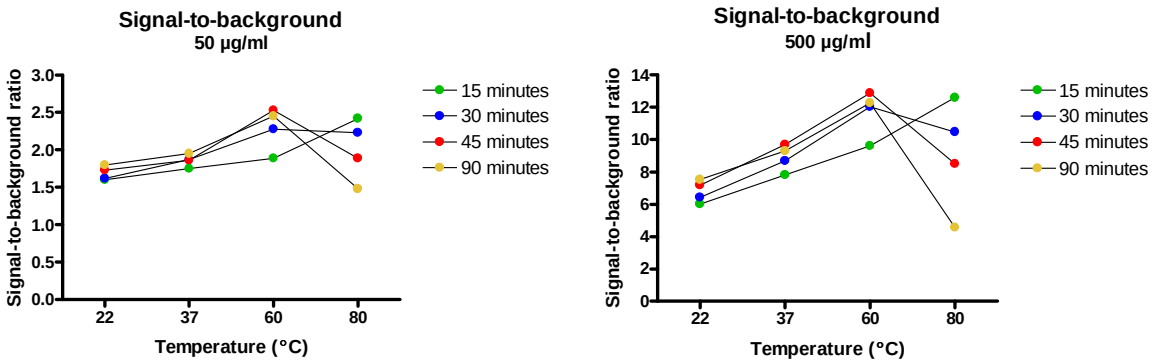

Figure 2: Signal-to-background ratios for the tested temperature/time combinations. Four different incubation temperatures (22 ºC (room temperature), 37 ºC, 60 ºC and 80 ºC) and four different incubation times (15 minutes, 30 minutes, 45 minutes and 90 minutes) were tested. To the left: BSA concentration: 50 µg/ml. To the right: BSA concentration 500 µg/ml.

3.1.2 Drift in absorbance

The increase in absorbance over time for the temperatures 60 ºC and 80 ºC was not linear, as can be seen in figure 3. The drift was faster the first 20 minutes compared to the last 40 minutes.

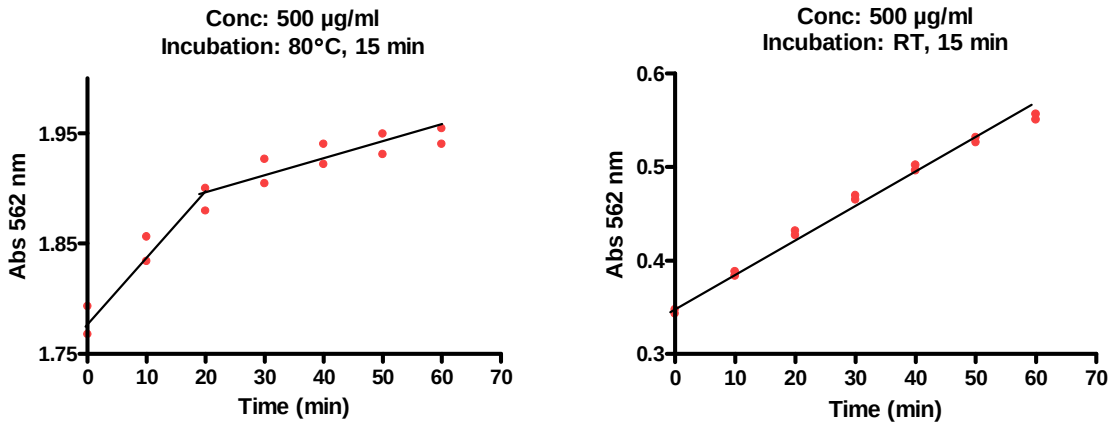

Figure 3: Drift in absorbance over time. The absorbance was measured straight after heating incubation in the BCA protocol and then repeatedly every ten minutes for an hour. To the left: incubation at 80 ºC for 15 minutes, the drift is not linear. To the right: incubation at room temperature for 15 minutes, the drift is linear.

The drift decreased with elevated temperature. At lower temperatures the incubation time influenced the drift more than can be seen at 60 ºC and 80 ºC, figure 4. The absorbance increased with as much as 0.5-1 % per minute at low incubation temperatures combined with short incubation times in contrast to an increase of less than 0.1 % per minute when incubating the samples at high temperatures.

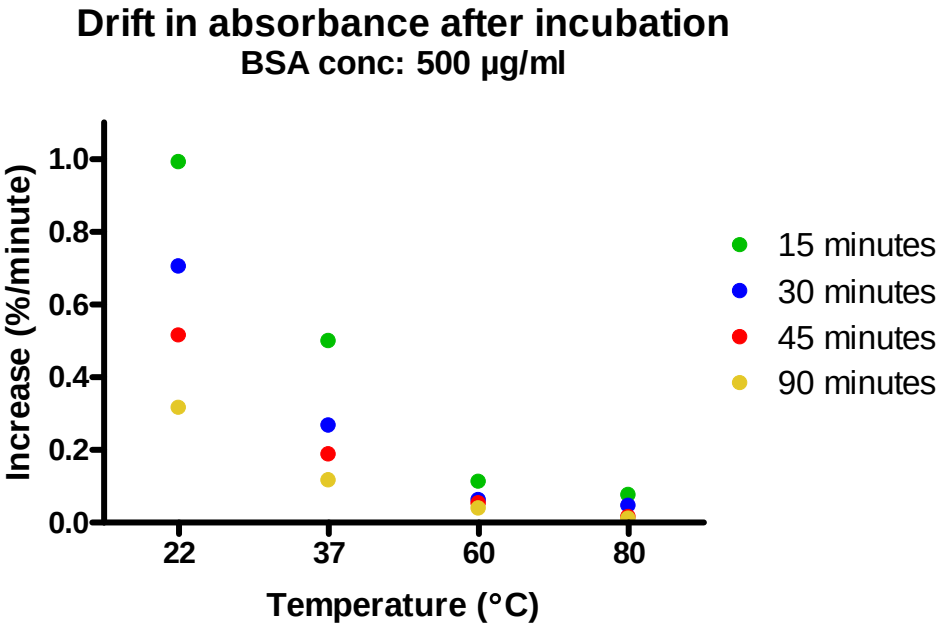

Figure 4: The drift in absorbance after incubation. The drift is calculated as increase in percentage per minute for all the tested temperature/time combinations. Results are shown for a BSA concentration at 500 µg/ml. For example, when incubating the samples at 22 ºC for 15 minutes, the absorbance increases with 1 % per minute.

3.1.3 Evaluation of regression models

According to the F-test the third order equation fitted the data better than both the linear and the second order model. Also, the third order equation was preferred over the forth order since this model did not fit the data significantly better than the simpler third order equation. Evaluation with a four parameter logistic curve was not possible since the model did not fit the data at all.

When the obtained concentrations were compared to the actual concentrations the use of a third order equation gave most accurate results, as can be seen in table 1. The differences between the models are in particular seen in the lower range.

Table 1: Comparison of linear, second order and third order equations as standard curve. The accuracy is calculated according to Equation 1.

Conc BSA	Accuracy (%)		
(µg/ml)	Linear	Second order	Third order
25	124,9	26,2	9,0
50	30,4	-3,4	-5,5
100	5,8	-1,0	1,7
150	-6,9	-5,0	-1,5
200	-9,5	-4,6	-1,5
250	-4,6	1,0	3,4
300	-9,9	-3,7	-2,3
350	-6,4	-0,6	0,1
400	-2,9	2,3	2,4
450	-3,8	0,6	-0,1
500	-4,1	-0,8	-2,0
550	-2,1	0,1	-1,2
600	0,6	1,9	0,6
650	2,7	3,0	1,9
700	0,8	-0,9	-1,2
750	3,0	0,1	0,5
800	2,8	2,6	2,4

< 5 %
< 10 %
< 20 %
> 20 %

Altered subset combinations of the tested BSA concentrations were evaluated with the third order regression model to choose how many standard points to include and where to place

them on the standard curve. The total accuracy for the subset combinations was calculated. There was a somewhat better accuracy when a standard at a concentration close to the quantification limit was included. Also, it appeared that the accuracy increased when the standards were equally covering the range. Therefore, the following BSA standard concentrations were chosen to define the standard calibration curve: 0 µg/ml, 25 µg/ml, 150 µg/ml, 300 µg/ml, 450 µg/ml, 600 µg/ml and 750 µg/ml.

However, in regular use with only the seven selected standards, it was found that the third order equation did not give significant better fit than the second order equation. Consequently, the use of a second order equation is probably good enough and was therefore decided to be used further on.

3.1.4 Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) for the optimized method were calculated. The corresponding concentrations were estimated to around 12 µg/ml for LOD and approximately 17 µg/ml for LOQ, which can be seen in figure 5.

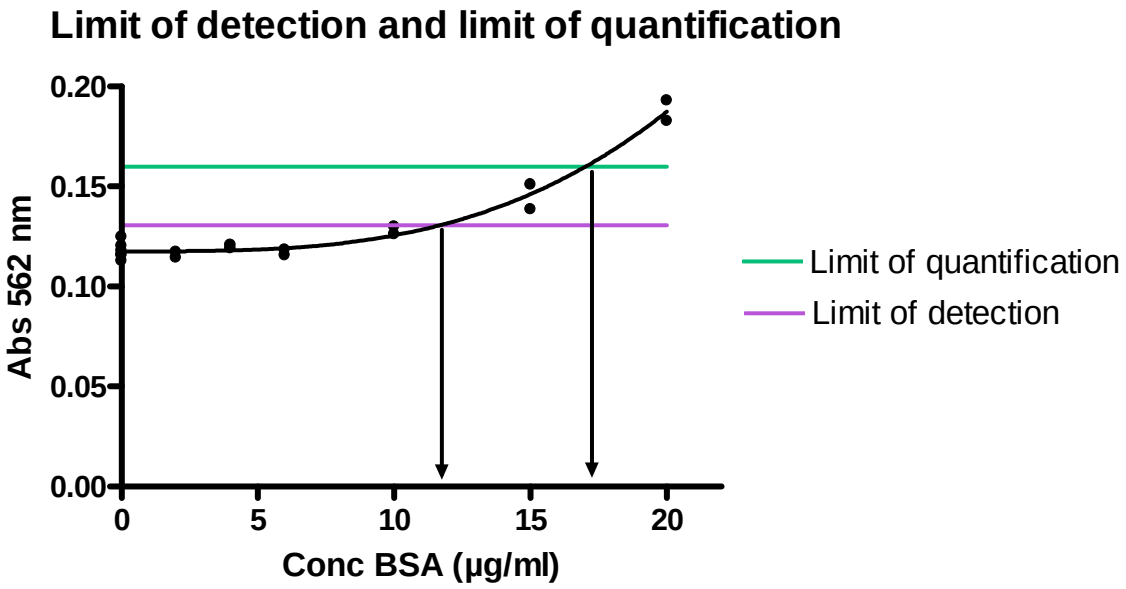

Figure 5: Determination of the concentrations that correspond to the limit of detection and the limit of quantification. Limit of detection was estimated to 12 µg/ml, and limit of quantification to 17 µg/ml.

3.1.5 BCA analysis of protein-coated particles

When analyzing protein-coated particles with the BCA assay a centrifugation step is necessary. As seen in figure 6, a centrifugation time of >30 minutes at 15000×g is required to avoid a contribution in response from particles in the light path.

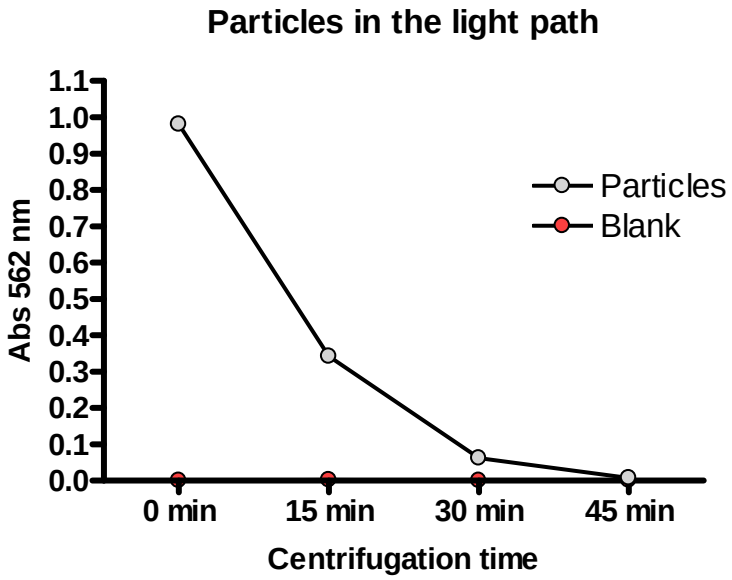

Figure 6: Absorbance response versus centrifugation time. Particles were centrifuged for different times and the absorbance responses were measured to study the effect of particles in the light path. A centrifugation time of >30 minutes is necessary to avoid a contribution in response from particles in the light path.

Any possible effects on the colour development caused by centrifugation were investigated; results are seen in figure 7. No considerably differences were seen in the colour intensity between two standard curves where one of them was centrifuged. This indicates that the centrifugation itself does not affect the colour development.

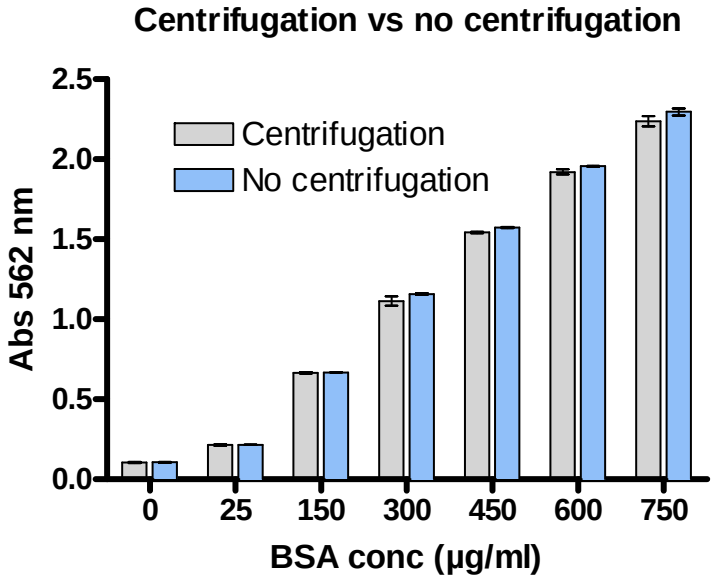

Figure 7: BSA standard curves analyzed with the BCA assay. One standard curve was centrifuged 45 minutes after the heat incubation period, whereas the other one was cooled on bench.

3.1.6 Precision

The intermediate precision of the BSA standards in the BCA assay was estimated using 22 determinations of each concentration (11 different standard curves with 2 replicates for each concentration). As can be seen in table 2, CV (%) of the mean obtained concentration varies between 0.96 and 6.22, and CV (%) of the mean obtained absorbance responses varies between 1.24 and 4.95.

Table 2: Intermediate precision, N=22.

Expected BSA conc (µg/ml)	Mean obtained conc (µg/ml)	CV (%)
0	-	-
25	25.9	6.22
150	152.1	1.43
300	301.5	1.46
450	448.5	1.03
600	596.7	1.52
750	752.7	0.96

Expected BSA conc (µg/ml)	Mean Abs 562 nm	CV (%)
0	0.122	4.77
25	0.221	4.95
150	0.645	2.13
300	1.108	2.34
450	1.520	1.43
600	1.892	1.82
750	2.238	1.24

3.2 Elimination of interfering substances

3.2.1 TCA-acetone precipitation

When the BCA assay was performed on a BSA standard curve precipitated according to the protocol containing TCA and acetone the precipitates that were seen after adding of TCA disappeared after acetone wash. This resulted in no signals at all.

3.2.2 DOC-TCA precipitation

3.2.2.1 Protein recovery

Detected concentration after DOC-TCA precipitation versus starting concentration is plotted in figure 8. The detected concentrations correspond well with the starting concentrations, indicating a good precision of the DOC-TCA precipitation protocol.

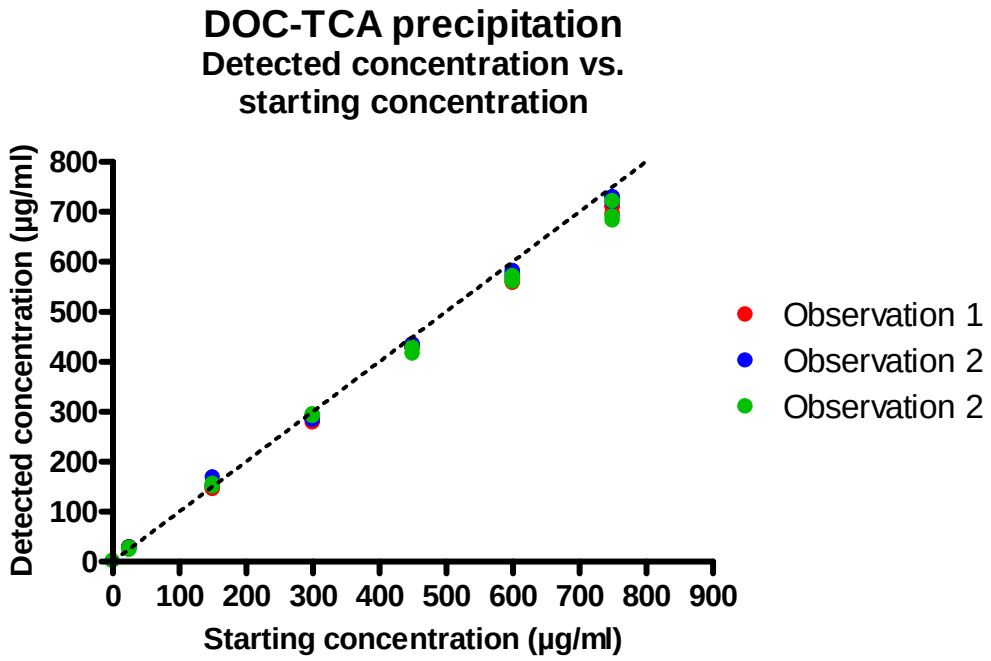

Figure 8: Detected concentration after DOC-TCA precipitation versus starting concentration. Results from three different observations are shown.

The percentage of protein recovered after DOC-TCA precipitation is seen in figure 9. Approximately 95 % protein was recovered over the whole range and the variation between runs was low. CV of the mean for each concentration was less than 6 %.

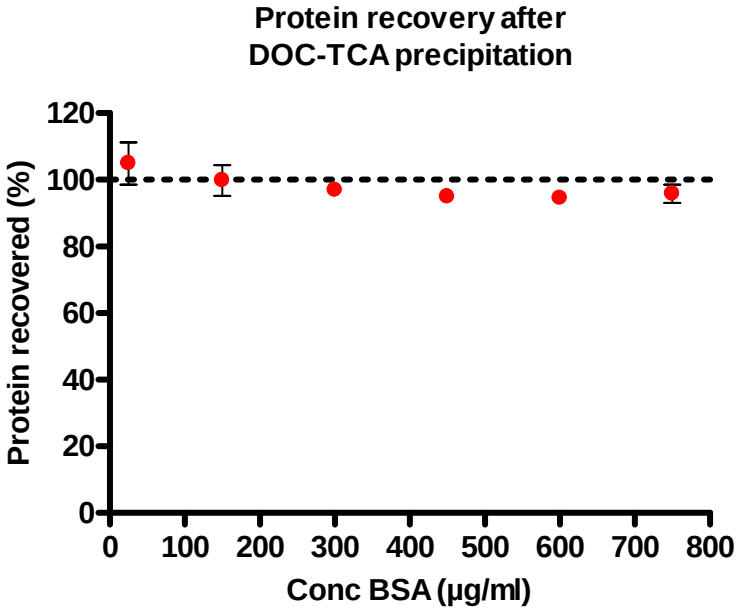

Figure 9: Protein recovery after DOC-TCA precipitation. Results from three different observations are shown. The recovered protein is approximately 95 % over the whole range.

Some other proteins were precipitated according to the DOC-TCA protocol prior BCA analysis to confirm that the protocol was reliable for other proteins than BSA. Protein recovery after precipitation was calculated and the results can be seen in figure 10.

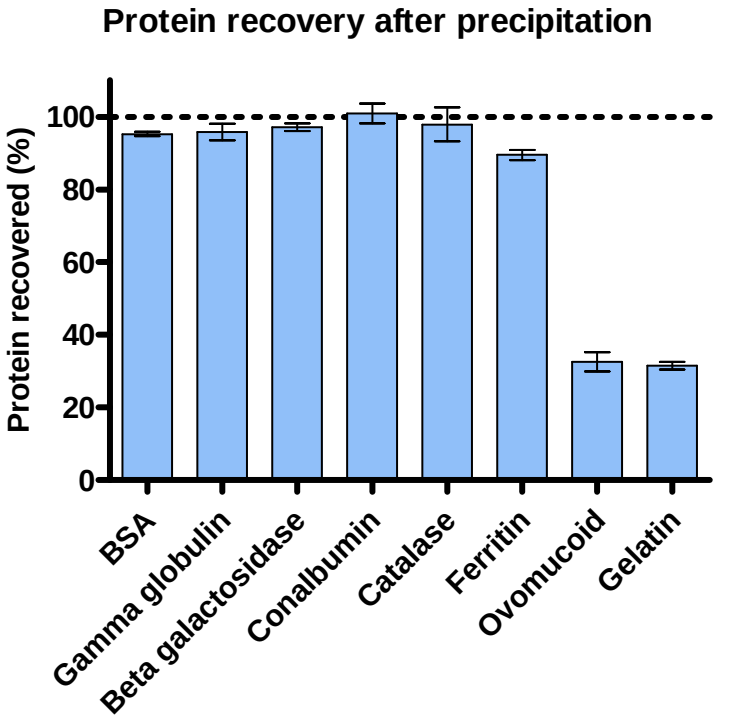

Figure 10: Protein recovery after DOC-TCA precipitation for different proteins. Mean percentages of three replicates are shown and the error bars represents the standard deviation of the mean.

The obtained protein recovery was above 85 % for BSA, gamma globulin, beta galactosidase, conalbumin, catalase and ferritin. A recovery of about 30 % was seen for ovomucoid and gelatin.

3.2.2.2 Test in the presence of interfering substances

As can be seen in figure 11, ammonium sulfate, guanidine hydrochloride, glucose and DNA interfered with the BCA assay, but after DOC-TCA precipitation these substances were efficiently removed.

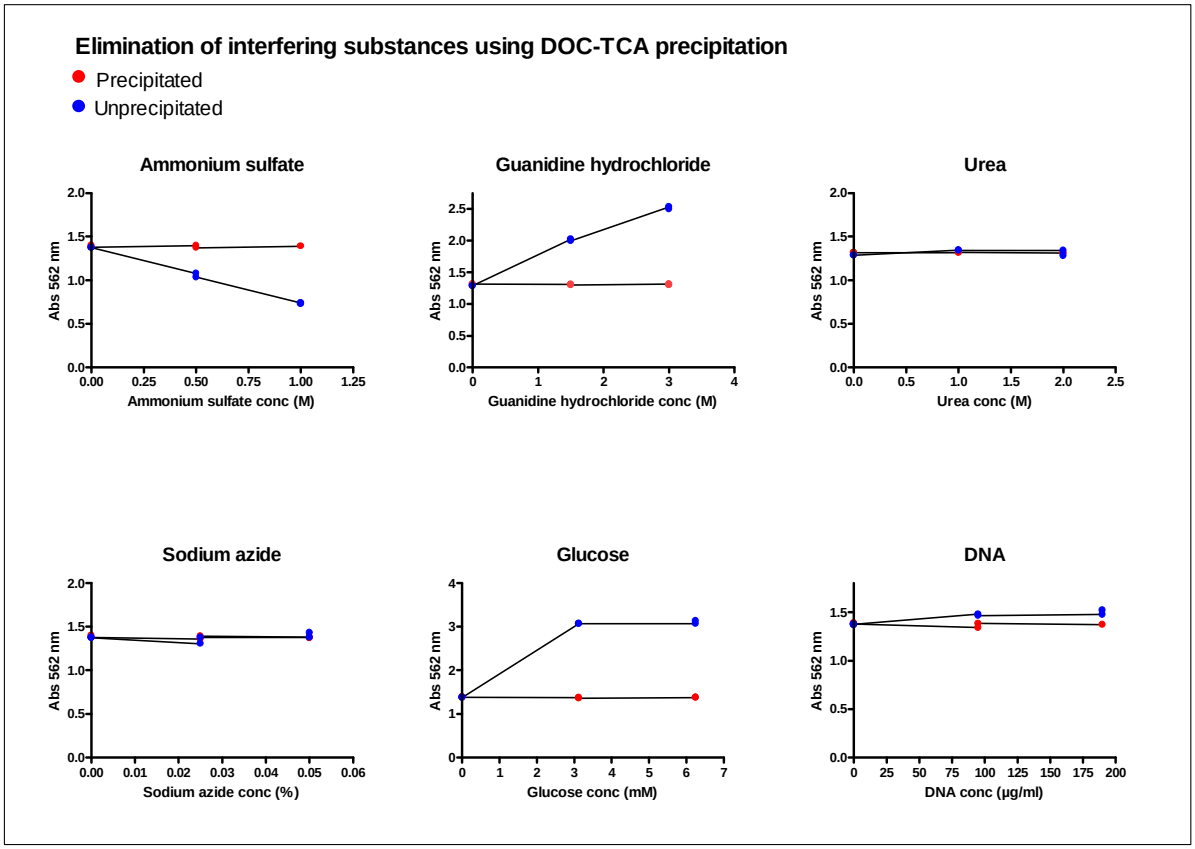

Figure 11: Elimination of interfering substances using DOC-TCA precipitation. Different interfering substances were added to the standard BCA assay and precipitated samples were run in parallel with untreated samples.

Ammonium sulfate has a decreasing effect on the absorbance while guanidine hydrochloride, glucose and DNA increase the absorbance responses. For example, when 50 µl of 1 M ammonium sulfate was present the obtained response was approximately 50 % of the signal when no interfering substances were present. The signal obtained when 50 µl of 3 M guanidine hydrochloride was present corresponds to approximately 200 % of the signal when no

interfering substances were present. The tested concentrations of urea and sodium azide on the other hand did not interfere with the BCA assay.

Since the DOC-TCA protocol does not include any wash steps to remove remaining TCA, experiments to confirm that TCA on its own does not interfere with the BCA assay were performed. As can be seen in figure 12, 50 µl of 10 % TCA did not have an effect on the BCA assay at a BSA concentration of 500 µg/ml. It was estimated that the amount of remaining TCA is less than 50 µl of 10 % TCA.

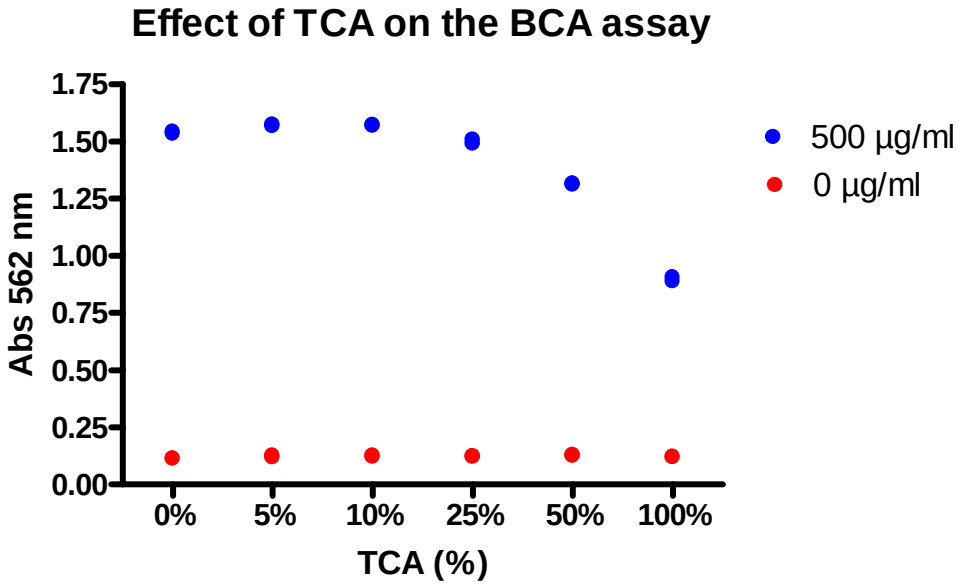

Figure 12: Effect of TCA on the BCA assay. Absorbance responses when 50 µl increasing concentrations of TCA was added to the standard BCA protocol are seen. Blue dots represent the responses when the BSA concentration is 500 µg/ml and red dots are background responses. An effect is seen when the TCA concentration added is more than 10 %.

3.2.2.3 DOC-TCA precipitation including concentration of diluted samples

BSA standards were diluted 600 times before subjected to the DOC-TCA precipitation protocol including concentration of the samples. Results when using 40 ml centrifuge tubes are seen in figure 13. The detected concentrations compared to the concentration before dilution in the lower range are questionable high and the deviation between replicates is high (CV of the mean < 57 %). The results in the upper range show better correlation between detected concentration and concentration before dilution.

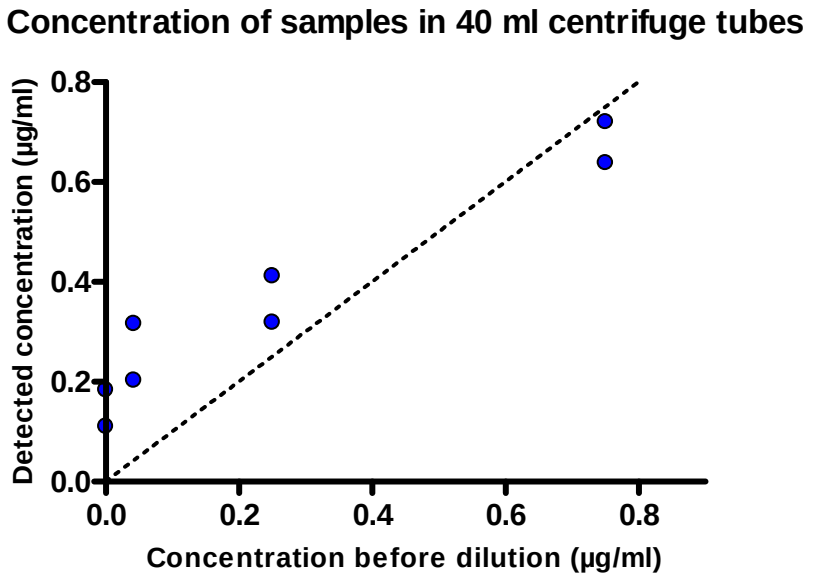

Figure 13: Concentration of samples in 40 ml centrifuge tubes. Detected concentration after precipitation versus concentration before dilution is plotted. The dotted line shows a recovery of 100 %.

To investigate the cause of high background signals, a comparison was made between the background signals of these tubes and the background signals of tubes that were extra cleaned (using 1 M NaOH). The background signals of disposable Falcon tubes were compared as well. NaCl and BCA reagent was mixed in the tubes and the absorbance was read. The background levels detected from the Falcon tubes corresponded to "normal" blanks, whereas the mean absorbance levels detected from both the extra cleaned and the normal cleaned tubes corresponded to concentrations of 40-50 µg/ml.

When the experiment with sample concentration using DOC-TCA precipitation was repeated in 50 ml Falcon tubes, the results improved, which is seen in figure 14. The background signal was lowered and the precision was better (CV of the mean < 19 %).

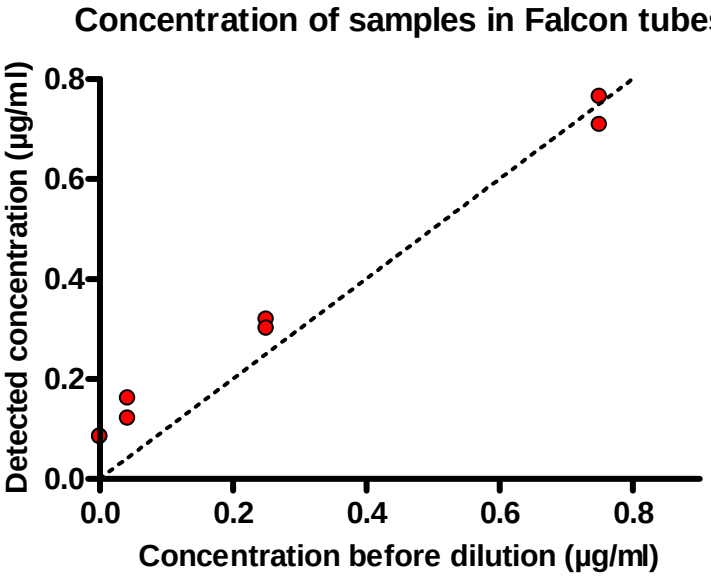

Figure 14: Concentration of dilute samples through precipitation in 50 ml Falcon tubes. Detected concentration after precipitation versus concentration before dilution is plotted. The dotted line shows a recovery of 100 %.

3.3 Analysis of allergen extracts

Several allergen extracts (see table 3) were analyzed according to the DOC-TCA precipitation protocol followed by the standard BCA protocol.

Table 3: Allergen extracts that were tested.

Extract:	Explanation:
e5	Dog dander
i8	Moth
d1	Dermatophagoides pteronyssinus
t17	Japanese cedar
m10	Stemphylium herbarum
f2	Cow's milk
g2	Bermuda grass
t3	Common silver birch
w18	Sheep sorrel

Signal recovery after DOC-TCA precipitation was calculated, as can be seen in figure 15. For all extracts with the exception of f2 (~70 % recovery), very low recoveries were obtained (less than 30 %).

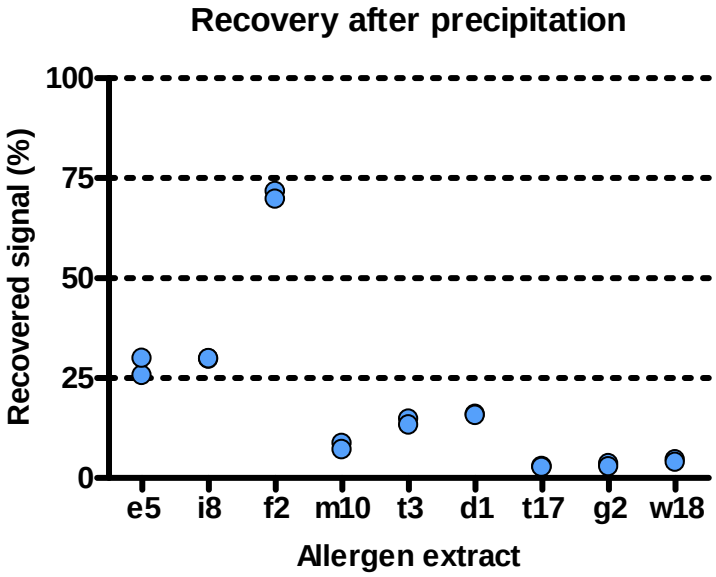

Figure 15: Signal recovery after precipitation of nine different allergen extracts. The f2 extract gave a recovery of about 70 % while all the other extracts gave a recovery of less than 30 %.

Additionally, the extracts were fractionated by the use of PD-10 columns. The collected fractions were analyzed according to the DOC-TCA precipitation protocol followed by the standard BCA protocol. The recovered signal in relationship to the unprecipitated extract ("100 %") was calculated in both the precipitated starting extracts and in the collected fractions (Fraction I-III). Figure 16 summarizes this.

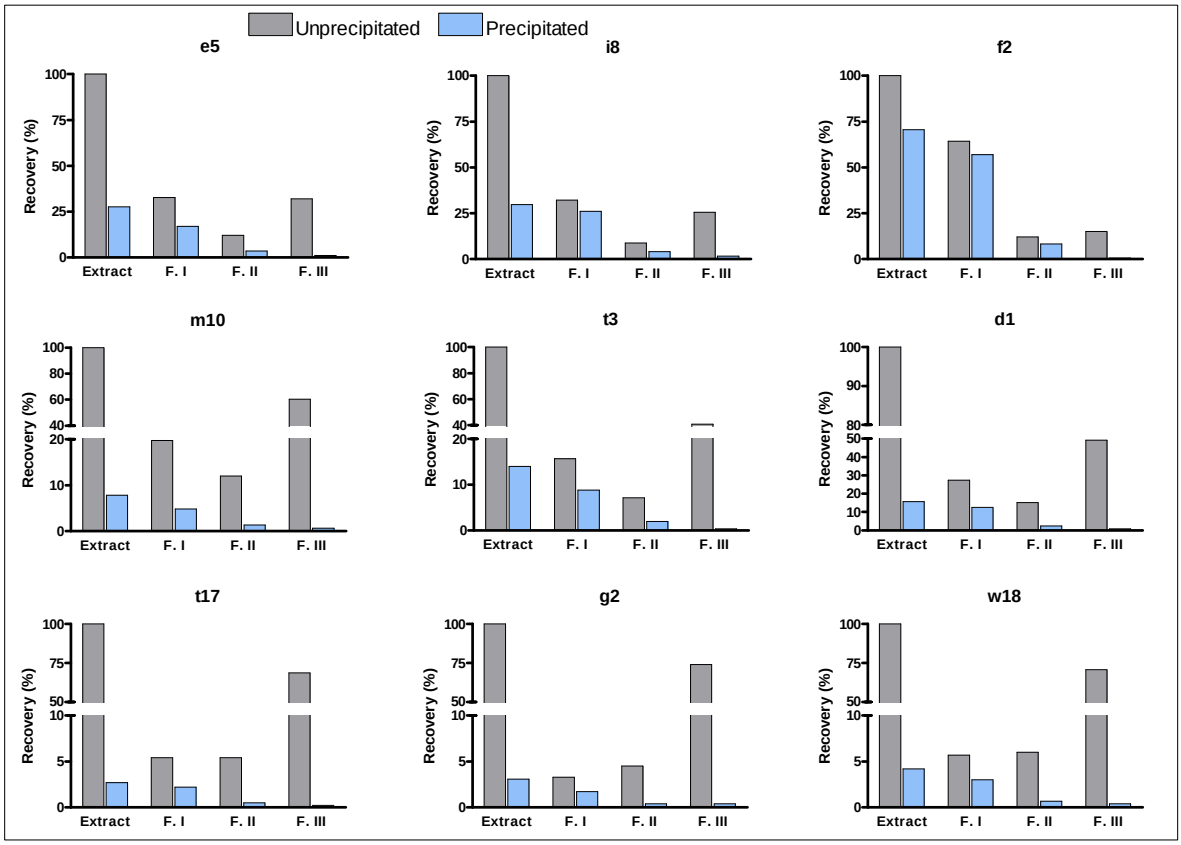

Figure 16: Signal recovery in fractionated extracts. Extracts were fractionated by PD-columns and/or precipitation. Signals were compared to the response of unprecipitated extracts (=100%). F. I = fraction I (protein), F. II = fraction II ("border fraction"), F. III = fraction III (low molecular weight substances).

Overall, high signals were obtained in the unprecipitated fraction III, which contains low molecular weight substances. In those extracts with the lowest recovery seen in figure 15 (t17, g2 and w18) the highest signals in fraction III were obtained. When fraction III was precipitated these signals more or less disappeared in all tested extracts.

Furthermore, the signals in the precipitated extracts correlate in most of the cases fairly well with the unprecipitated fraction I.

4 Discussion

4.1 Assay optimization

4.1.1 Incubation protocols

The fact that the colour reaction in the BCA assay is not an end-point reaction means that the reaction continues as long as there are non-reduced cupric ions present for the protein to reduce. The reduced ions in turn, form complexes with BCA molecules and increase the intensity of the colour. Consequently, two absorbance measurements of the same sample at two different times will not give rise to the same signal; the signal will drift over time. When the incubation protocol was chosen a low drift in absorbance over time was desirable. Also, high signal-to-background ratios were wanted.

Overall the BCA method was shown to be fairly robust concerning changes in incubation temperatures and times. As can be seen in figure 2, an incubation temperature of 60 ºC generated good signal-to-background ratios when the incubation time was 30 minutes or more. Similar values were obtained when incubating the samples at 80 ºC for 15 minutes. In general, the ratios for 60 ºC were best and 15 minutes incubation gave low ratios for all temperatures below 80 ºC. Furthermore, an incubation time of 30 minutes was less sensitive to changes in temperature than the other incubation times that were tested. Additionally, when increase in absorbance over time was studied it was shown that the drift decreased with elevated incubation temperatures. Also, at lower temperatures the incubation time influenced the drift more than at 60 ºC and 80 ºC. For these reasons the chosen incubation conditions are 30 minutes at 60 ºC. The drift in absorbance was not linear when the temperature was 60 ºC or more, as can be seen in figure 3. The absorbance increased faster the first 20 minutes compared to the last 40 minutes. Therefore, if the absorbance measurements are made after 20 minutes cooling to room temperature the drift is avoided as far as possible.

When choosing the incubation protocols all of the above mentioned aspects were taken into account. Also, it is desirable to have a short total assay time. Since the drift in absorbance at 60 ºC was similar for all the tested incubation times, the longer incubation times were not considered in order to reduce the assay time.

The chosen protocol is the following:

1. Incubation at 60 ºC for 30 minutes
2. Cooling at room temperature for 20 minutes
3. Absorbance measuring at 562 nm

Even though the drift in absorbance at the chosen conditions is very small (less than 0.1 % per minute), it is important to read the absorbance of all tubes in a short time interval and to perform all steps in the same order for all the test tubes, in order to minimize any effects from the absorbance drift.

4.1.2 Evaluation of regression models

When comparing the accuracy between different curve-fitting models, it is clear that a non-linear regression fits the data better than a linear one. This is mostly obvious when studying the lower working range, where a concentration obtained from a linear regression can correspond to an error of more than 100 %, when compared to the actual concentration. These results show that even though a linear regression usually generates a fairly good R^2-value, which sometimes is used as a measurement of how good a model fits some data points, the R^2-value is not enough when choosing regression model.

A third order equation has more flexibility over a second order equation since it contains more parameters that can adjust to the data. However, if this should be useful there has to be many data points, otherwise the simpler model is usually good enough. This is the probable explanation of the difference between the initial results with 18 standards compared to the results after re-evaluation with the seven selected standards. Hence, in the standard BCA protocol a second order equation is used for evaluation.

BSA was used as standard. This has some limitations when total protein is to be measured since BSA does not respond in exactly the same way as the protein mixture in the samples. However, BSA is commonly used as standard and it is considered a suitable choice for most protein assays (Olson and Markwell 2007). It was chosen to use six standards (excluding blanks) equally covering the range, which goes in line with the recommendations for bioanalytical method validation by FDA (U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) 2001). These recommendations also include a suggestion to place

the standard of the lowest concentration near the limit of quantification (LOQ). The LOQ of the method was estimated to 17 µg/ml and the lowest standard is 25 µg/ml.

The BSA standards are prepared from BSA ampoules of a known concentration checked against a NIST standard. When diluting the BSA standards it is important that an exact amount of BSA and buffer are used, since the results of the assay rely on a correct standard curve. Pipettes do have good repeatability when a single person uses the very same pipette, though the accuracy is not always satisfactory, resulting in shifting of the standard curve. An alternative to using pipettes are Hamilton syringes, which have good precision as well as accuracy. In the optimized assay Hamilton syringes will be used to prepare a new standard curve before every new analysis. Furthermore, since the precision of the method is good, with CV of the mean absorbance responses of less than 5 %, it may be possible to specify certain response intervals in order to accept the standard curve of an assay run.

4.1.3 BCA analysis of protein-coated particles

To be able to use the BCA assay to analyze the amount of protein coated to polystyrene particles, exceptions from the standard BCA protocol need to be made. A centrifugation time of 45 minutes at 15000×g was needed to make sure that no particles were in the light path. This is 25 minutes longer than the cooling period in the standard protocol. Since the drift in absorbance over time is low, this will only lead to a small increase in absorbance that does not have an effect on the test result. Furthermore, the number of samples can be limited by the capacity of the centrifuge, but the centrifugation itself does not seem to have an effect on the colour development, which means that the standard curve (with no particles) does not have to be centrifuged in parallel with particle-containing samples.

4.2 Elimination of interfering substances

4.2.1 TCA-acetone precipitation

The first protein precipitation protocol to be used was a TCA-acetone protocol since this seemed to be one of the most common protein precipitation protocols (Olson and Markwell 2007). During acetone wash a problem raised, namely the visible precipitates were solubilised. This caused the protein to be washed away and no signals were obtained. This was found to be in accordance with the described solubility of BSA precipitated with TCA in

acetone (Levine 1954, Schwert 1956). Studies even exist where the main goal with the TCA-acetone precipitation protocol was to remove albumin from serum (Chen *et. al.* 2005).

4.2.2 DOC-TCA precipitation

4.2.2.1 Protein recovery

In total the protein recovery after DOC-TCA precipitation was good. It was approximately 95 % for BSA at different concentrations. Also, more than 85 % protein recovery was seen for most of the other tested proteins, including gamma globulin, beta galactosidase, conalbumin, catalase and ferritin.

The recovery of 30 % generated when ovomucoid and gelatin was precipitated may to a certain extent be explained by the unknown purity of the substances used. It is consequently difficult to make an evaluation when the starting point is uncertain. Also, ovomucoid and gelatin contain lower amounts of hydrophobic amino acid residues than most of the other proteins (Shimada and Matsushita 1980). The limited accessibility of hydrophobic residues is likely to cause difficulties in forming aggregates. In addition, the precipitate of ovomucoid was not completely settled, which probably caused some of the precipitate to be removed by the aspiration. The recovery might have been better if a longer centrifugation time or higher speed was used, which is something that may be further tested.

4.2.2.2 Test in the presence of interfering substances

Ammonium sulfate, guanidine hydrochloride, glucose and DNA interfered with the BCA assay. When the ammonium sulfate concentration was increased the absorbance levels decreased. These results correspond with those of Brown *et. al.* in 1989, whom suggest this is caused either by oxidation of Cu^{1+} back to Cu^{2+}, or by blocking the formation of BCA-Cu^{1+} complexes. When guanidine hydrochloride, glucose and DNA on the other hand were added, the absorbance increases, which probably is due to the fact that these substances themselves is capable of reducing Cu^{2+} to Cu^{1+} (Brown *et. al.* 1989). This ability hence results in more BCA-Cu^{1+} complexes than those that actually are proportional to the amount of protein. Urea and sodium azide did not cause any changes to the response signals; indicating that these substances can be used in buffers with no need for elimination before BCA analysis, at least at those concentrations that have been tested.

Furthermore, it was shown that elimination of interfering substances by the use of DOC-TCA precipitation is useful and works well regardless of the interfering mechanism. The results even indicate that it is possible to remove DNA from a sample, however; this result can not be seen as absolutely certain, since the composition of the DNA sample is not known, but the results still show a potential of the precipitation protocol.

4.2.2.3 DOC-TCA precipitation including concentration of diluted samples

An interesting application of the DOC-TCA precipitation protocol combined with the BCA assay is to use the precipitation step to concentrate samples. By this, the sensitivity of the method can be improved and the limit of detection can be lowered since concentrations below the normal range can be analyzed with the standard BCA assay. This could be a useful application for example in cleaning qualifications when very low concentrations need to be detectable. There is a commercial micro BCA assay that has been developed to analyze dilute protein samples and therefore has lower detection limit (Thermo Scientific Pierce 2009). Although a drawback with this micro assay is the increased influence from interfering samples. This is because a larger proportion of the sample compared to the regent is needed than in the standard assay, thus the contribution in colour development from interfering substances are increased. The hopes by using the precipitation to concentrate samples were to achieve as good sensitivity as with a micro assay and at the same time eliminate interfering substances.

In theory, the detected concentrations should equal the concentrations before dilution. The obtained results, however, differ from this theory. The experiment was first performed using washed reusable 40 ml centrifuge tubes. When samples containing BSA at 0.04 µg/ml were analyzed the detected concentrations were way over the starting concentration. Also, for samples with no added protein, signals were detected. One possible reason explaining these elevated background signals might be if the 40 ml centrifuge tubes that were used were contaminated. Though, if this was the case, the contaminations would have been removed after the extra wash with 1 M NaOH.

The recovery for samples containing BSA at 0.75 µg/ml was around 90 %. However, if all of the tested 40 ml tubes have a contribution from elevated background signals, the real recovery of the added protein is most probably lower. The inside of the tubes were rather uneven causing trouble for some of the precipitate to settle during centrifugation, which most likely con-

tributed to lower recovery either if parts of the precipitate were lost during aspiration or if the precipitate was not completely resuspended.

The results improved when Falcon tubes were used; the deviation between the replicates was lowered and the detected concentrations were closer to the starting concentrations. Also, the recovery for samples containing BSA at 0.75 μg/ml increased. Though, there was still a contribution from elevated background signals even though disposable Falcon tubes were used.

Overall, the use of DOC-TCA precipitation to increase the sensitivity and lower the LOD of the method shows potential. If the source of the background signals is found this technique might be useful.

4.3 Analysis of allergen extracts

The impact of interfering substances in complex solutions on BCA assay analysis was studied in some allergen extracts. When the extracts were precipitated, very low (3-30 %) signal recovery were obtained for almost all of the tested allergens extracts. Thus, there is most likely a significant contribution from non-protein molecules in the colour development when unprecipitated extracts are analyzed according to the standard BCA protocol.

The extracts were separated by the use of PD-10 columns to confirm the presence of non-protein substances that contribute to the increment in response in the BCA assay. High signals were obtained from the unprecipitated fraction III even though this fraction contains low molecular weight substances. This explains why the protein recovery seemed to be very low - large parts of the appeared protein in the unprecipitated samples are actually not proteins. This was again verified when fraction III was precipitated and more or less no signals were obtained in all of the tested extracts. This means that applying the BCA assay on unprecipitated unpurified extracts may result in huge errors.

The protein amount in the precipitated extracts correlates in most of the cases with the unprecipitated fraction I. By this, separation on PD-10 columns supports the effectiveness of the DOC-TCA precipitation protocol. Precipitation has also some advantages over gel filtration including its ease of use and low costs. Therefore, DOC-TCA precipitation is a suitable way of eliminating interfering substances in complex solutions and by that increase the reliability of the results.

5 Conclusions

In the optimized BCA protocol an incubation period for 30 minutes at 60 ºC is suggested. These conditions have been shown to give good signal-to-background ratios and the drift in absorbance after incubation is fairly low. To further avoid the drift, samples are cooled for 20 minutes at room temperature before the absorbance is measured at 562 nm. The working range for the optimized method is 25 µg/ml – 750 µg/ml. When the assay is performed, concentrations of unknown samples are determined by evaluation against a standard curve fitted by a second order equation, which has shown to be satisfactory regarding precision and accuracy. The intermediate precision for the method has been shown to be good, with CV less than 6.3 %. If the lowest standard is excluded, CV is less than 1.6 %.

Furthermore, elimination of interfering substances from complex samples has been shown to be possible by the use of DOC-TCA precipitation before BCA analysis. Protein recovery using DOC-TCA precipitation has been discovered to be approximately 95 % for BSA concentrations covering the working range. Also, more than 85 % protein recovery was seen for some other proteins. The DOC-TCA precipitation has been tested in the presence of interfering substances that successfully could be removed.

Allergen extracts contain a vast range of non-protein substances that interfere with the BCA assay. Applying the standard BCA assay protocol on untreated extracts hence results in huge errors in the obtained protein amount. It has here been shown that DOC-TCA precipitation is a suitable way of eliminating these substances and by that increase the specificity and reliability of the results. In addition, the DOC-TCA protocol has shown promising results in increasing the sensitivity of the assay and lowering the limit of detection by concentrating dilute samples.

6 Future perspectives

The method has to be fully validated before it can be used in the daily work. Part of the validation has been done by this work and other parts still need to be done. A summary of commonly considered parameters in method validation are found in the appendix.

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8 Appendix

8.1 Validation

When a laboratory starts using a newly developed method or an optimized, already existing method, it has to be validated to confirm its suitability for the intended application. Some parameters that are most often studied during method validation are: accuracy, precision, selectivity, robustness, range, linearity, limit of detection, limit of quantification and stability (U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) 2001). For the above optimized BCA assay, some of the validation parameters have already been studied and some still need to be considered.

Accuracy

The accuracy of the method demonstrates how close to the true value the mean test results are. The deviation from the true value is calculated. In the case of total protein assays it is difficult to find a real true value. Accuracy is sometimes established by spiking samples with known amounts of protein and calculating the O/E ratio (Obtained/Expected).

Precision

The precision of the method shows how closely the results of repetitive measurements are placed. Both the precision between runs and during a single run are investigated. The coefficient of variation (CV %) is calculated.

Selectivity

A method should only measure the analytes of interest. In the case of the BCA assay, it is known that there exist several substances that do interfere with the assay, thus the selectivity of the assay is not constrained to proteins. However, a precipitation step removing interfering substances has been developed.

Robustness

Different incubation protocols in the BCA assay have been evaluated, thus the robustness regarding time and temperature is known. Also, the drift in absorbance has been studied. The robustness concerning preparing the BCA working reagent, which are prepared in the proportions 50:1, reagent A:B, may also be studied.

Range

The range is defined by a concentration interval knowing to meet certain conditions, including accuracy and precision. The range of the optimized BCA protocol is 25-750 µg/ml.

Linearity/Standard curve

The linearity of a method is the relationship between concentration and response. The simplest regression model that best (determined by statistical tests) describes the relationship should be used. In the optimized BCA assay a second order equation is used.

Limit of detection & Limit of quantification

The limit of detection (LOD) is the lowest concentration that can be detected, i.e. the lowest concentration that can be differentiated from blank samples. The limit of quantification (LOQ) is the lowest concentration that can be determined quantitatively. For the optimized BCA assay, the LOD was determined to around 12 µg/ml, and the LOQ to approximately 17 µg/ml.

Stability

Short time stability tests of the BCA working reagent and the BSA standards can be made to investigate the stability of these reagents if prepared several hours or days before usage.