Chong *et al. Chem. Biol. Technol. Agric.* (2022) 9:32
https://doi.org/10.1186/s40538-022-00297-0

Chemical and Biological Technologies in Agriculture

METHODOLOGY

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Higher resolution protein band visualisation via improvement of colloidal CBB-G staining by gel fixation

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Abstract

Background: Gel staining is a crucial step that allows the visualisation of proteins separated through SDS-PAGE. Colloidal Coomassie Brilliant Blue-G (CBB-G) staining is among the commonly used visualisation methods due to several factors such as compatibility with mass spectrometry (MS) analysis, sensitivity, reproducibility, and simplicity of the staining process. However, the standard colloidal CBB-G staining has a drawback: the resolution of protein bands is compromised because of diffusion of proteins during the washing step.

Results: A modification to an established colloidal CBB-G staining method, which greatly increases the resolution of protein bands, is described. The addition of a fixation step, which prevents the diffusion of proteins during the washing step, is shown to increase protein band resolution.

Conclusion: The fixation step is fast, flexible, and also retains all the advantages of the standard colloidal CBB-G staining methods. As there are no drawbacks, incorporating this fixation step into the standard colloidal CBB-G staining is an easy way to improve protein visualisation in SDS-PAGE.

Keywords: SDS-PAGE, Protein staining, Colloidal, Coomassie, Gel fixation, Proteomics

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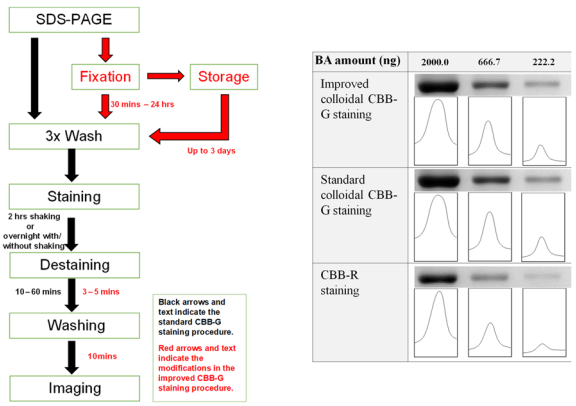
Graphical Abstract



Introduction

Polyacrylamide gel electrophoresis of proteins is the cornerstone of any proteomic study. Proteins are separated and resolved on polyacrylamide gels and are visualised through gel staining. Different staining methods have been reported, such as Coomassie blue, silver, and fluorescent stains [1]. Choosing the correct staining method is crucial as different methods have different sensitivities, advantages, and disadvantages. Coomassie blue stain is the most commonly used stain because of its compatibility with MS analysis, sensitivity, reproducibility, and simplicity [1, 2]. Despite having less sensitivity than silver and fluorescent stains [2], it is still the preferred stain for most applications.

Coomassie Brilliant Blue (CBB) is a triphenylmethane dye. The anionic CBB molecules bind to proteins by binding to the positively charged basic arginine and lysine residues of proteins [3]. CBB molecules also bind to aromatic groups through hydrophobic interactions [4]. This dye has two variants: CBB R-250, which has a

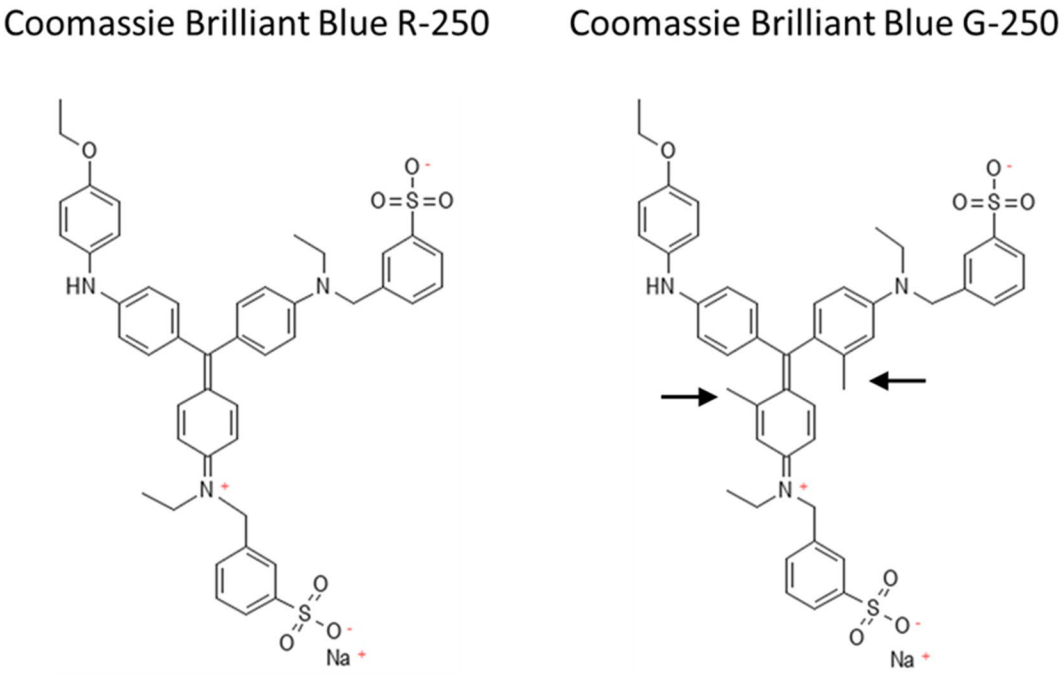


Fig. 1 The chemical structures of CBBR-250 and CBBG-250. The differences in the two dyes can be attributed to the addition of two methyl groups in CBB G-250, as indicated by the arrows in the diagram. Chemical structures were drawn using PubChem Sketcher [7]

reddish tint, and CBB G-250 which has a greenish tint [5]. The chemical structure of CBB G-250 differs from CBB R-250 by two additional methyl groups [6] as shown in Fig. 1 [7]. These dyes exhibit colloidal properties in the presence of ammonium or aluminium sulfate in acidic alcoholic media [8].

First described by Neuhoff et al. [9], colloidal CBB-G staining has emerged as the most sensitive of the CBB stains [8]. Colloidal CBB-G staining is said to be more reproducible, selective, and sensitive towards proteins, down to a detection limit of 1 ng per protein band compared with 200 ng per protein band for CBB-R staining [9–11]. In colloidal CBB-G staining, dye molecules are aggregated into colloidal particles [8]. Background staining is reduced due to the colloidal nature of the dye particles being less permeable in electrophoresis gels [12]. Therefore, the destaining step may be omitted in colloidal CBB-G staining [12]. While not as sensitive as silver staining, colloidal CBB staining is less laborious to perform and introduces less background staining [10]. Nevertheless, it should be noted that CBB-G has different dye binding capacity to different proteins [9].

Two major improvements have been made to the original method described by Neuhoff et al. [9], increasing sensitivity to 1 ng/band. Firstly, the percentages of the components were optimised by Candiano et al. [1], and this protocol is now widely used in various studies [13]. In another modification by Kang et al. [8], ammonium sulfate and methanol are replaced with aluminium sulfate and ethanol, respectively. This protocol was shown to have a sensitivity of 1 ng/band, approaching that of silver staining [11]. Since then, there has not been much improvement in staining protocols despite the importance of staining.

In this paper, a modification to the colloidal CBB-G staining method, which increases protein band resolution is introduced. The modification is done through the addition of a fixation step prior to washing and staining. The results of the modified and improved CBB-G method were compared with the standard colloidal CBB-G and CBB-R staining methods. To our knowledge, this is the first description to compare the effects of fixation on colloidal CBB-G staining, producing improved protein gel resolution for subsequent application.

Materials and methods

Evaluation of band sharpness, resolution and sensitivity

The band resolution and sensitivity of the three staining procedures were evaluated. For the evaluation of the resolution of the staining procedure, a total cell protein extract from late dough stage Taichung 65 rice endosperm was used. Rice endosperms were ground into powder and proteins were extracted using imidazole-HCl buffer [14]. The extracted proteins were quantified using the Bradford assay [15]. Then, 20.0 µg and 6.7 µg of protein samples were run on SDS-PAGE. For evaluation of the staining sensitivity, bovine albumin (BA) (Amresco) was used. The BA stock solution of 10 mg/mL was prepared. The stock solution was diluted to a series of concentrations and the following amounts of BA were run on SDS-PAGE: 2000.0 ng, 666.7 ng, 222.2 ng, 74.1 ng, 24.7 ng, and 8.2 ng.

SDS-PAGE was run using the standard method by Laemmli [16], using 10% separating gels and 4% stacking gels, and a 1 mm-thick mini-gel format with 0.5-cm wells [Biorad Mini Protean II system]. Gels were run at 90 V for 30 min, then 150 V for about 60 min or until the dye front reached the end of the gel. After SDS-PAGE, the gels were stained with one of the three staining methods described henceforth. Triplicate gels were run for each staining method.

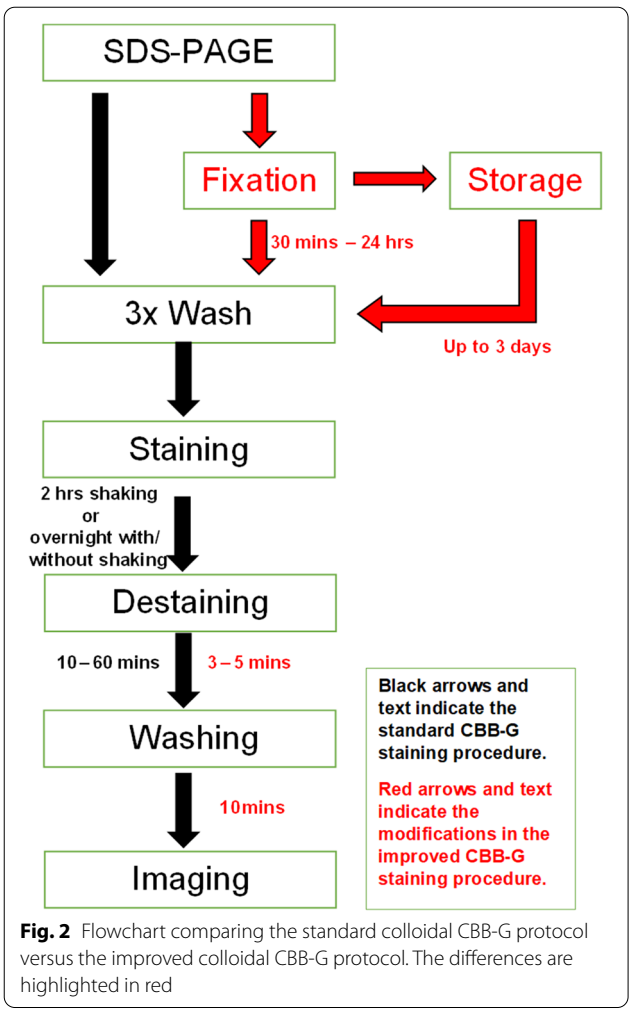


Fig. 2 Flowchart comparing the standard colloidal CBB-G protocol versus the improved colloidal CBB-G protocol. The differences are highlighted in red

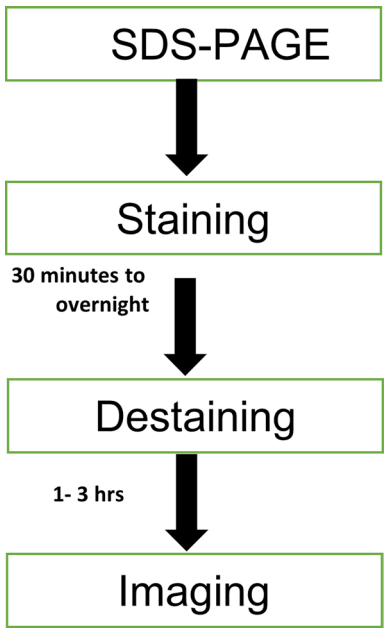


Fig. 3 Flowchart showing the standard CBB-R staining protocol

The improved colloidal CBB-G method was also evaluated for MS compatibility. Rice endosperm protein extracts were subjected to isoelectric focusing using non-linear pH 3–10 IPG strips (Biorad #1632016). After focusing, IPG strips were equilibrated, and the second dimension was run using 11% mini-gels. After gel imaging and analysis, 49 differentially expressed spots were selected for MS identification. Each spot was pooled from three to six gels, excised, trypsin digested and analysed using LC–MS/MS (Thermo™ Q Exactive Plus Hybrid Quadrupole-Orbitrap).

Staining methods

Standard colloidal CBB-G staining method

The standard colloidal CBB-G staining method was performed as described by Dyballa and Metzger [11]. After SDS-PAGE, the gel was rinsed three times with ultrapure water by shaking on a platform shaker at 80 rpm for 10 min each time. Then, the ultrapure water was decanted and the gel was incubated in CBB-G staining solution 0.02% (w/v) CBB G-250 (Sigma), 5% (w/v) aluminium sulfate (Bendosen), 10% (v/v) ethanol (HmbG), 2% (v/v) orthophosphoric acid (Merck) for 2 h with shaking at 80 rpm, or overnight, with or without shaking. If the staining solution turned a bright blue, the staining solution was replaced with a fresh solution. For maximum sensitivity, gels were stained overnight.

For destaining, the gels were rinsed twice with ultrapure water, and destained in CBB-G destain solution (10% ethanol, 2% orthophosphoric acid) for 10–60 min with shaking. After that, the gels were rinsed twice with ultrapure water.

The improved colloidal CBB-G staining method

For the improved colloidal CBB-G method, the staining protocol by Dyballa and Metzger [11] was slightly modified with an additional fixation step and a simplified destaining step. After the SDS-PAGE run, the gel was transferred to a plastic box and fixed with fixation solution [40% methanol (Merck), 10% acetic acid (Bendosen)] for 30 min, with shaking at 80 rpm. For convenience, this fixation step could be extended overnight or up to several days. After fixation, the gel was rinsed briefly with ultrapure water. The subsequent steps followed the colloidal CBB-G stain protocol described previously. Figure 2 illustrates the differences between the improved and standard protocols.

For the destaining process, the gel was rinsed briefly with ultrapure water, then destained in CBB-G destain solution by shaking on a platform shaker at 80 rpm for roughly 3–5 min. The gel was then rinsed briefly with ultrapure water, then washed with ultrapure water by shaking on a platform shaker at 80 rpm for 10 min. The water was then decanted, and the gel was briefly rinsed till all colloidal particles were removed from the staining box as colloidal particles may interfere with gel imaging. The gels were stored in ultrapure water at 4 °C.

CBB-R staining

For the CBB-R staining method, a standard staining protocol was used as shown in Fig. 3 (Bio-Rad manual). Briefly, the gel was immersed in CBB-R staining solution [0.1% CBB R-250 (Bio-Rad), 40% methanol, 10% acetic acid] overnight. The following morning, the gel was rinsed with water and destained with CBB-R destaining

(See figure on next page.)

Fig. 4 Comparison of the resolution of CBB staining methods for rice endosperm total cell protein extracts. **A** Comparison of the protein profiles of gels stained using the three methods. The gel stained using the improved colloidal CBB-G staining shows sharper and better separation of proteins bands. **B** The ImageJ plot profiles for the lanes loaded with 20.0 µg total proteins as indicated by the rectangular boxes. The sections circled and labelled H, M, and L are the high, medium, and low molecular weight sections. **C** Close-up view of the high, medium, and low molecular weight sections. The arrows indicate the peak differences that can be observed. The improved colloidal CBB-G staining method plot profile shows peaks which are sharper and more distinct, indicating improved protein band resolution

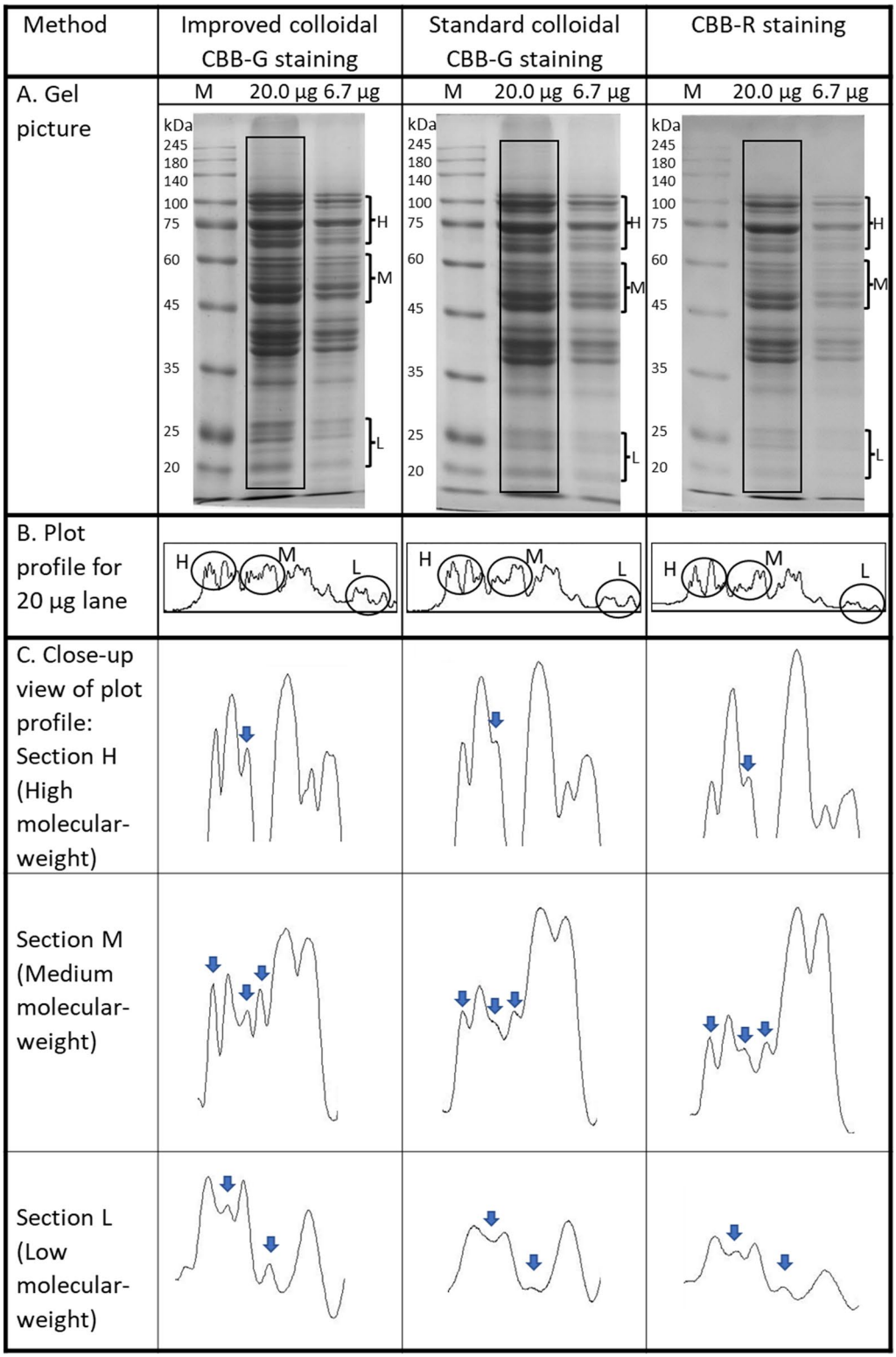


Fig. 4 (See legend on previous page.)

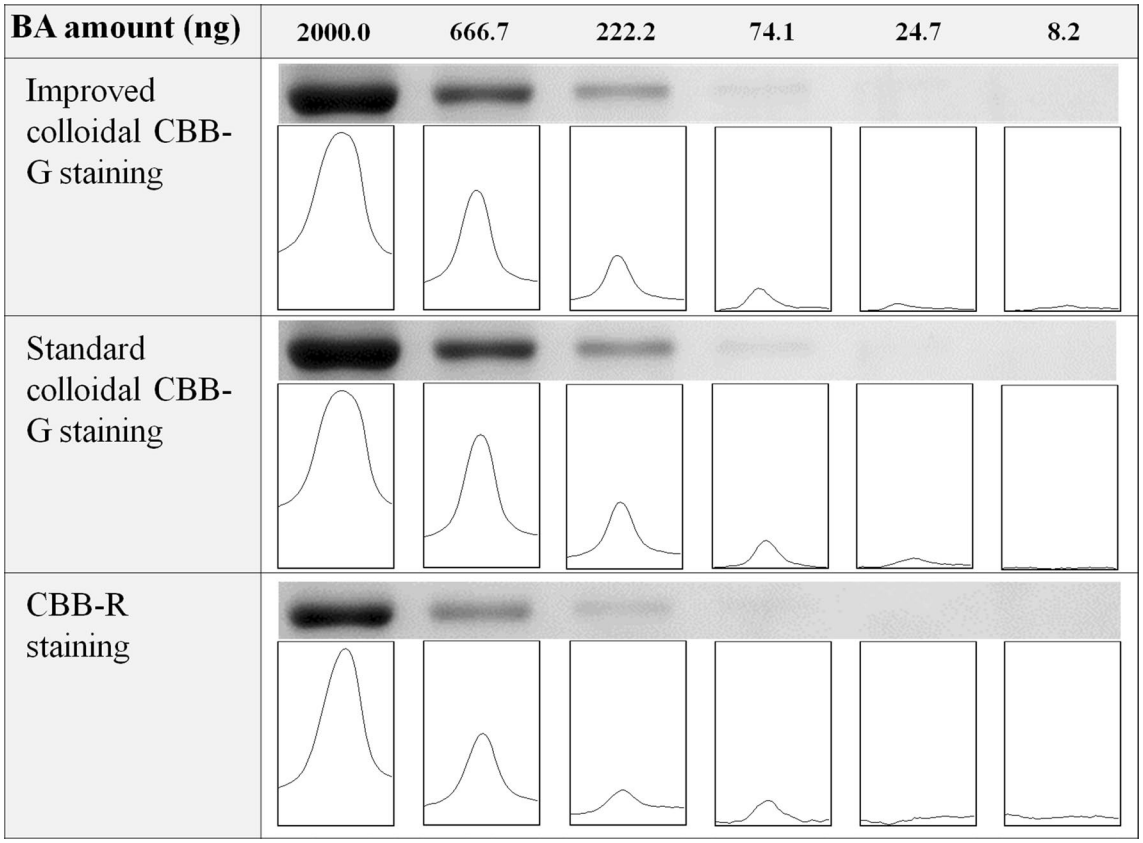


Fig. 5 Comparison of the sensitivity of CBB staining methods using BA as a standard. From naked-eye observation, a band can be observed up to 74.1 ng for all staining methods. However, through ImageJ analysis, a peak can be observed in the plot profile up to 24.7 ng for both the improved and standard colloidal CBB-G staining methods. In contrast, for CBB-R staining, a peak can be observed only up to 74.1 ng. For the three staining methods, the plot profiles of the 2000.0 ng, 666.7 ng, and 222.2 ng bands were vertically scaled to 1.0, whereas the plot profiles of the 74.1 ng, 24.7 ng, and 8.2 ng bands were vertically scaled to 2.0

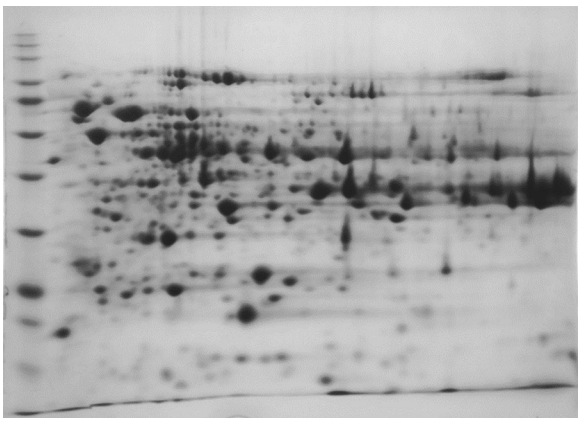


Fig. 6 A 2D-PAGE gel of rice endosperm proteins stained using the improved colloidal CBB-G method. In this differential expression study, 49 spots showing differential expression were excised and identified through MS. The spots selected for identification are shown in Additional file 1: Fig. S1

solution (40% methanol, 10% acetic acid) by shaking on a platform shaker at 80 rpm for several hours, with two to three changes of destaining solution.

Evaluation of resolution and sensitivity

Evaluation of resolution was conducted by visual observation of gels as well as through analysis of plot profiles generated using ImageJ software [17]. For sensitivity analysis, the presence of the BA bands were indicated by a distinct peak at the correct position in the plot profile. The areas under the peak (corresponding to band intensity) were calculated, and statistical analysis was conducted using the unpaired *t*-test.

Results

Evaluation of resolution/sharpness

Protein band resolution was compared by performing a visual comparison as well as through ImageJ analysis. Through visual observation, the improved colloidal CBB-G staining method had sharper, more distinct bands

compared to the other staining methods. This observation was confirmed through ImageJ software analysis. As shown in Fig. 4, the lane plot profiles showed that the improved colloidal CBB-G staining method had peaks which were more distinct with better resolution compared to the standard colloidal CBB-G staining and the CBB-R staining. This was evident for all proteins in the total cell protein extract. Proteins of high (~65–110 kDa), medium (~45–60 kDa), and low (~20–30 kDa) molecular weight were better resolved by the improved colloidal CBB-G staining.

Evaluation of sensitivity

For all staining methods, the BA protein band could be seen with the naked eye up to the third serial dilution (74.1 ng). However, using ImageJ software analysis, a peak was detected up to the fourth serial dilution (24.7 ng) for both colloidal CBB-G staining protocols, while only detected up to the third serial dilution (74.1 ng) using CBB-R stain. These results are summarised in Fig. 5. Analysis of the band intensity showed no significant difference between the intensity of the 24.7 ng BA bands for both improved and standard colloidal CBB-G staining protocols, with a *P*-value of 0.743417 (Additional file 3: Table S1).

Compatibility with 2D-PAGE and subsequent MS analysis

The improved colloidal CBB-G protocol was also used to stain 2D-PAGE gels, showing its applicability for a differential expression study. In total, more than 600 spots were detected in the stained gel (Fig. 6). A total of 49 spots showing differential expression were excised from replicate gels. All spots were successfully identified (unpublished data), indicating that the fixation step did not affect MS compatibility.

Discussion

The addition of the fixation step prior to the washing steps of the standard colloidal CBB-G staining procedure has greatly improved the sharpness and resolution of protein bands. In the standard colloidal CBB-G staining procedure, the fixation and staining are combined [11]. However, since the gel is unfixed during the washing step, diffusion of proteins occur, leading to the loss of resolution. This necessitates the introduction of the fixation step which prevents the diffusion of proteins during washing. Fixation is achieved using an acid/alcohol wash which limits diffusion of proteins from the gel matrix [18]. The fixation step only requires an additional 30 min and serves to precipitate the proteins in the polyacrylamide matrix at the point of resolution, which is immediately after electrophoresis separation [19]. The direct fixation of proteins in the gel after SDS-PAGE separation is therefore essential to prevent protein diffusion, particularly smaller proteins that are more susceptible to diffusion. Furthermore, the fixation step also helps to remove SDS from the proteins [19]. After the fixation, the fixative solutions needs to be removed through a washing step. The washing step also removes the SDS binding to the proteins, which interferes with the binding of the dye to the proteins [11]. Additionally, the fixation step also provides flexibility as fixation can be extended overnight, even up to several days. This may serve as a pause point as subsequent washing and staining can be continued the next day. On the other hand, the standard colloidal CBB-G staining requires the gel to be washed and stained immediately after SDS-PAGE separation.

Protein staining should not cause any loss of resolution of protein bands or spots. However, this inadvertently occurs in the standard colloidal CBB-G protocols, which combine the fixation and staining step. The most highly cited protocols describing the standard colloidal CBB-G lack a fixation step prior to the washing step [1, 8, 9, 11]. Therefore, it is not surprising that most experiments using colloidal CBB-G do not include a fixation step before the washing step. Indeed, there are only several reported experiments where a fixation step is performed prior to washing and staining with colloidal CBB-G. In one protocol, a 2-h to overnight fixation step using 30% ethanol and 10% acetic acid prior to the washing and staining step was used [20]. In another protocol, gels were fixed overnight in 10% methanol and 7% acetic acid, and stained using colloidal CBB-G [21]. In other staining protocols described by Dyballa and Metzger [18], gels can be fixed for 1 to 2 h using 12% trichloroacetic acid, or 30–50% ethanol and 2% phosphoric acid, though this fixation step is said to be optional. However, in all protocols, the fixation step was longer than the half-hour fixation step introduced here. Currently, the fixation step in colloidal CBB-G staining is optional, and even though it has been strongly recommended by Deng et al. [20], it is

Table 1 Comparison of the total time needed to perform the three CBB staining methods

Step	Staining method		
	CBB-R	Standard CBB-G	Improved CBBG
Fixation	–	–	30 min
3× Wash	–	10 min × 3	10 min × 3
Staining	30 min to overnight	2 h to overnight	2 h to overnight
Destaining	1–3 h	10–60 min	3–5 min
Washing	–	Rinse	10 min
Fastest total time	1 h 30 min	2 h 40 min	3 h 13 min

frequently omitted in most research. Based on the results shown here, the fixation step is not optional and should be incorporated into the standard colloidal CBB-G staining protocol.

Protein resolution is a crucial aspect in the analysis of protein gels. The application of improved protein resolution can be seen in gel-based comparative proteomic studies. The ability to resolve complex mixtures of proteins through SDS-PAGE or 2D-PAGE allows subsequent identification of thousands of proteins through MS [22]. In differential expression studies, the improved resolution keeps the spots distinct and may improve software analysis. As an added advantage, distinct spots are easier to excise, with less cross-contamination from neighbouring spots, improving MS identification of proteins. Furthermore, the increased resolution may aid detection/differentiation of proteins with more precise determination and small changes in molecular weight or pI resulting from post-translation modification [23].

The sensitivity of colloidal CBB-G stain has been reported to vary greatly, from between 1 ng/band [1, 8], 4 ng/band [11, 24], 8 ng/band [25], and 50 ng/band [26]. For 2D-PAGE, 2 ng/spot has been reported [27]. In this work, the sensitivity was found to be 24.7 ng/band, lower than reported [11]. This could be due to the quality of the imager or other factors. It has been reported that the width of the loading wells and the sensitivity of the acquisition device also play a role [28]. Indeed, 2D-PAGE spots and narrow wells allow higher sensitivity since the proteins are concentrated, allowing a higher signal to noise ratio. In this work, an ImageQuant 400 Imager was used to capture the images. Therefore, the lower sensitivity could also be attributed to the low resolution (75 dpi) of the images.

Additionally, the quality of the BA used may have affected the sensitivity analysis. SDS-PAGE separation showed that the BA used in this experiment consisted of a dominant band at 66 kDa and several other faint bands (Additional file 2: Fig. S2). Since the sensitivity analysis was performed using the dominant 66 kDa band, the actual amount of the 66 kDa band is slightly less than the assigned amount (ng), leading to a slight under-estimation of the sensitivity in this experiment. Nevertheless, the emphasis here is that the addition of the fixation step did not negatively impact the sensitivity of the stain. This is evident as the statistical analysis based on the plot profiles generated through ImageJ software showed no significant difference in sensitivity of both the improved and standard colloidal CBB-G staining methods. Meanwhile, colloidal CBB-G staining was more sensitive than CBB-R staining as reported by others [29].

The addition of the fixation step does not change the advantages of the colloidal CBB-G method over the CBB-R method. This includes the staining speed, increased resolution and sensitivity, better reproducibility, and low background stain [9, 11]. Furthermore, it has also been shown that the addition of the fixation step does not alter the compatibility with MS analysis. Apart from that, CBB-G gels were easier to handle and image compared to CBB-R stained gels, which tended to warp once removed from the destaining solution. The only drawback of the improved colloidal CBB-G staining method over the standard method is the additional time spent, which is about half an hour (Table 1).

Conclusion

The staining of SDS-PAGE gels is a critical step in any proteomic analysis. The addition of the half-hour fixation step to the standard colloidal CBB-G staining has been shown here to increase the resolution of protein bands by preventing diffusion of proteins during the washing step. At the same time, the improved colloidal CBB-G staining method retains all the advantages of the standard colloidal CBB-G staining method. In conclusion, the improved colloidal CBB-G staining method is suitable as a routine laboratory staining method and can improve the quality of proteomic studies.

Abbreviations

CBB: Coomassie Brilliant Blue; BA: Bovine albumin; IPG: Immobilized pH gradient; MS: Mass spectrometry; PAGE: polyacrylamide gel electrophoresis; SDS: Sodium dodecyl-sulfate.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-022-00297-0.

Additional file 1: Fig. S1. 2D-PAGE gel showing the 49 differentially expressed spots which were identified through MS

Additional file 2: Fig. S2. SDS-PAGE separation of BA (Amresco). Lane 1: BA consisted of a dominant band estimated at approximately 66 kDa, as well as several other faint bands. M: Ladder used was SMOBIO PM2610

Additional file 3: Table S1. Areas under the peak calculated from the plot profile generated from the BA band (24.7 ng)

Acknowledgements

This work was supported by the Ministry of Education Malaysia FRGS Grant (FRGS/1/2017/STG04/UNIMAS/02/1). NFMC is the recipient of the Universiti Malaysia Sarawak PhD Study Fund F07(DPP38)/1256/2015(13).

Author contributions

NFMC planned and performed the gel-staining experiments, performed data analysis, and the main author of the manuscript. HH designed the overall study, major contributor in data analysis and manuscript preparation. DHSW performed the gel-staining experiments and was involved in manuscript writing. NR performed trypsin digestion of spots, while BYCL performed LC–MS/MS analysis. NEH, MN and WJY were involved in data analysis and manuscript writing. All authors read and approved the final manuscript.

Funding
Open Access funding provided by Universiti Malaysia Sarawak. This study was supported and funded by Ministry of Higher Education Malaysia's Fundamental Research Grant Scheme FRGS/1/2017/STG04/UNIMAS/02/1 [F07/FRGS/1611/2017].

Availability of data and materials
Additional data are provided as additional material.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 2 February 2022 Accepted: 14 April 2022
Published online: 11 May 2022

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