

Article

Shifting Focus in the Bradford Assay: Interfering Compounds Re-Examined

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Abstract

Since its introduction in 1976, the Bradford assay has served as a gold standard for protein quantification across a wide range of applications. While its limitations—including protein-to-protein variation in dye binding, challenges in selecting a representative calibration standard, and susceptibility to matrix interferences—are recognized, the relevant information remains scattered throughout the literature, with little quantitative guidance available for assay optimization. Here, we review interfering compounds reported in the literature during nearly 50 years and report a systematic characterization of a panel of potential interfering compounds, evaluating the effects of 29 different substances in the presence or absence of the protein analytes. Our findings revealed that 12 of the tested compounds induce significant artefacts in the Bradford assay, with minimal interfering concentrations varying widely across compounds. Detergents were confirmed as the most problematic interference; furthermore, two novel groups of interfering compounds were identified, represented by the transfection reagents and oligoarginine peptides with molecular weight below 3 kDa. Importantly, the resulting artefacts were also observed in complex biological matrices. While these compounds also affected the Lowry assay, the magnitude of the artefacts was substantially lower than that observed in the Bradford assay. This study will provide a valuable resource for researchers working in proteomics and related fields, offering practical insights for improving the reliability of Bradford-based protein quantification.

Keywords: Bradford assay; Coomassie Brilliant Blue G-250; interfering compound; transfection reagent; oligoarginine



Academic Editor: Shile Huang

Received: 27 April 2026

Revised: 19 June 2026

Accepted: 23 June 2026

Published: 25 June 2026

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1. Introduction

The Bradford assay measures total protein content in simple or complex mixtures using the Coomassie Brilliant Blue G-250 (CBBG) dye [1]. The assay is based on the binding of the dye to the protein(s), which results in formation of a dye–protein complex with a shifted UV-Vis absorbance spectrum relative to the free dye. Typically, the assay is performed at acidic pH, at which the free dye is protonated and absorbs at 465 nm in solution; upon interaction with proteins, a metachromatic shift occurs, resulting in the emergence of deprotonated species with absorbance maxima in the range from 650 nm (neutral dye) to 595 nm (anionic dye) [2]. The binding of CBBG and the change in its optical properties occur very fast, enabling nearly immediate measurement of the absorbance

after mixing of CBBG and the sample of interest. Few works have assessed the temporal stability of the signal in the Bradford assay, but the available reports indicate that the signal is relatively stable within a 5–120 min time-frame in the case of both high (200 µg/mL) or low concentrations (2 µg/mL) of bovine serum albumin (BSA) [3].

The nature of the protein can also affect the absorbance spectrum of the formed complex. For instance, for the N-termini of the glycine transporters 1 and 2, a somewhat exceptional shift in the CBBG absorbance spectrum was reported where an increase in absorbance at 300 nm and 700 nm occurs [4]. Still, for quantification of the protein content in the sample, either just absorbance at 595 nm or the ratio of absorbances at 595 nm and 465 nm is reported in most studies [5,6]. According to previous review articles [5,6], although there is no direct correlation between the affinity of the dye for the analyte and the spectral change, the major interactions between CBBG and proteins include ion pairs (between the sulphonic acid groups of the dye and lysine or arginine residues of the protein) as well as hydrophobic interactions (the latter also being important for binding of a similar dye, Coomassie Brilliant Blue Red-250 aka CBBR to proteins). Interestingly, differences in the sensitivity of CBBG to different amino acid residues have even been used in forensic analysis to distinguish between male and female fingerprints due to the sex-specific variation in the amino acid content in bodily fluids [7].

The tendency of CBBG to bind preferentially to certain amino acids translates into the fact that the Bradford assay measurement window is analyte-dependent [1,2,5,8–12]. The mechanistic complexity behind the readout is further exacerbated by the fact that protein aggregation and the post-translational modifications of proteins (e.g., carbamoylation, glycosylation) also affect binding of CBBG, whereas the measured outcome depends on the specific protein (and, putatively, the site of modification) [6,13,14]. Consequently, the total protein quantification by CBBG can only be performed reliably relative to a standard protein, and the calibration curve is further necessary since the relationship between the protein concentration and the absorbance at 595 nm becomes non-linear at the elevated concentrations of the standard. Several studies have compared different protein analytes in the context of choice of proteins suitable for calibration [6,15–20]—with the arginine-rich BSA being among the most popular candidates.

The effect of protein size on the assay response has also been explored. In 1977, it was reported that peptides with a molecular weight of less than 3000 Da could not form a complex with CBBG [21], a finding that has since become widely accepted in the literature. More recent studies showed that the peptidic agonist of GLP-1 receptor, exendin-4 (molecular weight of ca 4200 Da) or amyloid beta 1–42 peptide (molecular weight of ca 4500 Da) could be quantified using the Bradford assay [22,23]. Furthermore and in contrast with the previous findings, application of CBBG for quantification of the pentapeptide Gln-Arg-Phe-Ser-Arg (693 Da) has been reported following production as multimer, enzymatic cleavage, and purification [24]; to our knowledge, this remains the only study that has accomplished detection of such a short peptide by the Bradford assay.

The reports on the importance of protein folding for CBBG binding also remain somewhat controversial. Given that CBBG is used for protein detection in the denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method, the absence of high-order structures in proteins should be well-tolerated also in the Bradford assay. This conclusion is supported by early studies which reported good performance of the assay in a large range of pH values [25], absence of negative interference from urea (up to 8 M) when using BSA as the analyte [26], and improvement of assay performance relative to variation of analytes by addition of a low concentration of SDS to the analyzed mixture [27]. Still, a recent study reported interference of CBBG and CBBR with aggregation

of α -synuclein into fibrils [28], thus inferring that binding of dyes can alter the pattern of interactions formed by the proteins and, putatively, the spatial structure of the proteins.

Comparisons of the efficacy and sensitivity of the Bradford assay to other well-known methods for measuring total protein content have yielded variable results depending on the system analyzed. In the case of purified BSA or porcine kidney extract, the Bradford method was reported as the most sensitive assay (limit of detection of 0.006 mg/mL) and had the widest range of detectability (0.006 to 100 mg/mL) when compared to several other methods (including direct spectrophotometric quantitation, bicinchoninic acid (BCA), Biuret, Lowry, and ninhydrin assays) [29]. The superiority of the Bradford assay to the BCA assay was also reported in the case of origin milk and retentate samples [30] and in the case of beer samples [31] when Kjeldahl values were used as the reference. On the other hand, a study focused on the quantification of the digestible protein in the exoskeleton or the full body of arthropods indicated that among the Bradford assay, BCA assay, and Lowry assay, the latter correlated best to the amino acid analysis results used as the reference, while the Bradford assay was the second choice [32]. Furthermore, the Bradford assay was outperformed by the BCA method during estimation of residual protein content in hydroxypropyl chitin [33] and estimation of protein content in plankton [6]. Finally, a very problematic performance of the Bradford assay has been reported in a study of *Hymenoptera* venoms [30] and in an analysis of protein content in soils [34].

The issues associated with poor performance of the Bradford assay can originate not only from the sample matrix, but also from auxiliary reagents and vessels used during the sample preparation and subsequent steps. The interfering effects of detergents such as SDS and Triton X-100 have already been reported in the original work [1]; standard protocols for the Bradford assay emphasize that plastic and glassware should be detergent-free [5]. Additionally, the use of quartz (silica) cuvettes is discouraged due to the non-specific binding of CBBG to the material [5]. Studies have further demonstrated that CBBG can adsorb to glass cuvettes [35] as well as a range of plastic materials [36,37], presenting challenges for implementing the assay in microplate formats. Still, given a sufficient optical pathway length, the measurement of absorbance can be carried out in polystyrene and polypropylene multi-well plates.

Several modified versions of the Bradford assay have been reported, aimed at reduction of the artefacts caused by detergents and other interfering compounds. Such modifications usually involve drying of the sample or coprecipitation of the protein analyte, followed by exhaustive washing stages; while these steps make the assay significantly more time-consuming [38–42], in several cases, the calibration curve remains shifted as compared to the control [38,39]. Alteration of assay buffer to achieve alkaline conditions (pH of 8.2 [25], pH of 9 [43]) or strongly acidic conditions (11% phosphoric acid content in the dye [44]) has also been suggested to reduce the artefacts, but the mildly acidic version of the assay remains most widely used due to its compatibility with the buffer pH range commonly used during lysis of biological specimens. Spiking of the protein sample with a known amount of BSA (so-called standard addition) has also been suggested to establish possible negative or positive interferences in the case of complex matrices [45].

Despite the widespread use of the Bradford assay, many reports on interfering substances date back several decades, and potential assay-disrupting compounds are often overlooked in contemporary experimental workflows. In this study, we not only systematically consolidate and contextualize all previously reported interfering agents but extend the field by investigating several previously unexamined classes of compounds—including synthetic peptides, endocrine disruptors, and cationic transfection reagents—selected based on chemical principles predicting their likelihood to perturb the assay. By combining literature-based systematization with targeted experimental validation, we aim to highlight

a broader and more chemically diverse landscape of assay interference than previously recognized. Our findings underscore the need for renewed attention to auxiliary reagents and sample treatments that can generate misleading signals, reinforcing the importance of critically evaluating assay conditions even for methods as widely used and seemingly robust as the Bradford assay.

2. Materials and Methods

2.1. Chemicals and Apparatus

The assays were carried out in 96-well clear flat bottom plates (Nunc™ 269620, Thermo Fisher Scientific; Waltham, MA, USA). For the Bradford assay, Pierce™ Coomassie Plus Assay Reagent (Thermo Fisher Scientific, catalogue number 23238; Waltham, MA, USA) was used; this formulation is modified to improve calibration curve linearity and reduce protein-to-protein variation relative to classical Bradford reagents, yet is not detergent-compatible.

Phosphate-buffered saline (PBS) was used as the assay buffer (pH 7.45); the Ca^{2+} and Mg^{2+} -supplemented formulation was from Sigma-Aldrich (St. Louis, MO, USA), and the non-supplemented version from VWR (Fontenay-sous-Bois, France). As standard proteins and mixtures of proteins, BSA (Roche, Germany), bovine γ -globulin (BGG; BioRad, Hercules, CA, USA) and non-fat milk powder (Frisolac Gold, Amersfoort, The Netherlands) were used. Prior to each independent experiment, a fresh solution of BSA in PBS was prepared (20 mg/mL by weight), and the non-fat milk powder was dissolved in MilliQ water (also 20 mg/mL by weight); the protein content of the reconstituted milk powder was 20% from nominal weight as assessed by SDS-PAGE. The lyophilized BGG powder was reconstituted in MilliQ water according to the manufacturer's instructions, yielding protein content of 1.48 mg/mL.

The substances explored as interfering compounds were from the following sources: SDS, dodecyl- β -D-maltopyranoside (DDM), L-arginine, L-tryptophan, digitonin—Sigma (St. Louis, MO, USA); glycerol and Nonidet P-40 (NP-40)—AppliChem GmbH (Darmstadt, Germany); dimethyl sulfoxide (DMSO)—Fisher Scientific (Waltham, MA, USA); dithiothreitol (DTT)—Fisher (Darmstadt, Germany); dimethylformamide (DMF)—Merck KGaA (Darmstadt, Germany); Triton X-100—Ferak (Berlin, Germany); glycine—Fluka (Neu-Ulm, Germany); Tween-20—Naxo (Tartu, Estonia); 3-[Dimethyl [3-(3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amido)propyl]azaniumyl]propane-1-sulfonate (CHAPS)—Calbiochem (Burlington, MA, USA); Fugene® 6—Promega (Madison, WI, USA); Lipofectamine® 2000—Invitrogen (Waltham, MA, USA); TurboFect—Thermo Scientific (Vilnius, Lithuania). MilliQ water (for detergents and DTT) or PBS (for amino acids) was used for preparation of the stock solutions.

The endocrine-disrupting compounds (EDCs) 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE), hexachlorobenzene (HCB), bisphenol A (BPA), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), 2,2',3,3',4,4',5-heptachloro-1,1'-biphenyl (PCB170), 2,2',3,4,4',5,5'-Heptachloro-1,1'-biphenyl (PCB180), perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) were purchased from Sigma-Aldrich (St. Louis, MO, USA); bisphenol F (BPF) and mono(2-ethylhexyl) phthalate (MEHP) were provided by Prof. Christian Lindh (Lund University, Sweden). Stock solutions of all EDCs were prepared as described previously [46,47] and diluted to 1 mM nominal concentration using DMSO; the stocks were stored in borosilicate glass vials at $-20\text{ }^{\circ}\text{C}$. ARC-902 and ARC-1041 (structures shown in the Supplementary Figure S1) were synthesized in-house as previously reported [48,49] and stored as lyophilized powders at $4\text{ }^{\circ}\text{C}$; prior to the assay, the compounds were dissolved in DMSO.

Human lung adenocarcinoma cell lines HCC-44 and A549 as well as human bone osteosarcoma cell line U2OS were obtained from the Leibniz Institute DSMZ (German

Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). The solutions and growth medium components for the cell culture were purchased from the following sources: phosphate-buffered saline (PBS), fetal bovine serum (FBS), L-glutamine, Dulbecco's Modified Eagle's medium (for the lung adenocarcinoma cell lines), McCoy's 5A medium (for the osteosarcoma cell line)—Sigma-Aldrich (Steinheim, Germany); a mixture of penicillin, streptomycin, and amphotericin B—Capricorn (Ebsdorfergrund, Germany).

The cells were grown at 37 °C in 5% CO₂ humidified incubator (Sanyo; Osaka, Japan) as adherent cultures on Falcon 75 cm² canted neck tissue culture-treated flasks with vented caps (Corning; Durham, NC, USA). For lysis of cells, HEPES and NaCl from Calbiochem (Darmstadt, Germany), Triton X from Ferak (Berlin, Germany), EDTA-containing cOmplete™ protease inhibitor cocktail from Roche (Basel, Switzerland) and PMSF from AppliChem (Darmstadt, Germany) were used; the lysis buffer contained 1% Triton X by volume. The cells were lysed on ice for 1 h; subsequently, the membranes were pelleted by centrifugation (20,000× *g*, 20 min, 4 °C), and the supernatant (lysate) was collected.

The reagents for the Lowry assay were obtained from the following sources: sodium carbonate (Na₂CO₃) and potassium sodium tartrate (KNaC₄H₄O₆)—Sigma-Aldrich (Saint Louis, MO, USA); sodium hydroxide (NaOH)—AppliChem GmbH (Darmstadt, Germany); copper(II) sulphate pentahydrate (CuSO₄·5H₂O)—BioTop (Tartu, Estonia), and Folin–Ciocalteu phenol reagent—Merck (Darmstadt, Germany).

2.2. Literature Search

The initial search for reports on compounds interfering with the Bradford assay was performed in the Clarivate Web of Science (All databases) bibliographic collection on 19 May 2025. For the search, Web of Science AI assistant was used, and the query was formulated as “please list publications reporting compounds interfering with Bradford assay”; the keywords chosen by the AI assistant were as follows: interfering, interference, intervene, impair, impairment, false positive, false negative, Coomassie, Bradford, colorimetry. The keywords “disruption”/“disrupting” and “inhibition”/“inhibitor” were manually removed to avoid multiple hits reporting use of the Bradford assay for systems where EDCs or inhibitors of enzymes were in fact explored.

An additional manual search was performed in the NIH PubMed bibliographic database search on 17 July 2025 to include more recent publications. The following query was used for the search: “Bradford assay interfering”.

Based on the two searches, a list of 171 publications was generated and analyzed. Of these articles, the full text of 12 reports was not accessible, and 1 article mentioned the Bradford assay only as a part of its reference section. The data from the other publications are summarized below.

2.3. Bradford Assay

For the experiments conducted in the presence of the protein analyte, 2-fold dilutions of BSA or non-fat milk were prepared in PBS on a separate multi-well plate. Initially, both Ca²⁺ and Mg²⁺-supplemented formulations as well as a non-supplemented version of PBS were tried; as the difference in outcomes for the initial tests and both analytes were marginal (see Supplementary Figures S2–S4), subsequent measurements were carried out in the Ca²⁺ and Mg²⁺-supplemented formulation of PBS only. The human cell line lysates were diluted in the Ca²⁺ and Mg²⁺-containing PBS, with the dilution of lysate starting from 13.5% by volume.

The dilutions were then transferred to a measurement plate containing PBS (same formulation as used for the analyte dilution) and a fixed concentration of interfering compound was added to some of the wells. Prior to addition of CBBG, the working volume

in each well of the measurement plate was 75 μL , and the final total concentrations of the components were as follows: for BSA and non-fat milk, starting from 133 $\mu\text{g}/\text{mL}$; for lysates, 1.35% or 0.675% or 0.338% v/v (the final concentration of the Triton X-100 of 0.0135%, 0.00675% and 0.00338%, respectively); for SDS, 1% or 0.01% v/v ; for Triton X-100, Tween-20 and NP-40, 2% or 0.02% v/v ; for glycine and L-arginine, 10 mM; for glycerol, 5% v/v ; for DTT, 5 mM; for DMSO and DMF, 1% v/v ; for CHAPS and DDM, 0.25% v/v ; for L-tryptophan, 5.6 mM; for digitonin, 50 μM ; for Fugene[®] 6, Lipofectamine[®] 2000 and TurboFect, 1% v/v or 0.3% v/v ; for ARC-902 or ARC-1041, 1 μM or 0.2 μM or 0.1 μM ; and for EDCs, 5 μM . Finally, 75 μL of CBBG solution was added to each well.

For the experiments conducted in the absence of the protein analyte, 2-fold or 3-fold dilutions of interfering compounds were prepared in PBS directly on the measurement plate. Prior to addition of CBBG, the working volume in each well of the measurement plate was 75 μL , and the final total concentrations of the components were as follows: for SDS, CHAPS, DDM, DMSO and DMF, starting from 1% v/v ; for Triton X-100, Tween-20, NP-40, Fugene[®] 6, Lipofectamine[®] 2000 and TurboFect, starting from 2% v/v ; for L-arginine and L-tryptophan, starting from 20 mM; for digitonin, starting from 100 μM ; and for ARC-902 or ARC-1041, starting from 133 μM . For the following compounds, only one concentration was tested: glycine, 10 mM; DTT, 5 mM; glycerol, 5% v/v ; and EDCs, 5 μM . Finally, 75 μL of CBBG solution was added to each well.

The absorbance at a single wavelength (590 ± 10 nm) was measured 3–5 min after addition of CBBG with a PHERAstar multi-mode reader (BMG Labtech; Ortenberg, Germany), with the following parameters: filter block ABS 608A, 20 flashes per well, focal height 10.5 mm, and optical pathway correction for volume of 150 μL . A selection of the obtained dose-response curves in the absence of the protein analyte is shown in Supplementary Figure S5. The absorbance spectra were recorded in the same samples 15–30 min after addition of CBBG with NEO or Cytation 5 multi-mode readers (BioTek; Winooski, VT, USA) within the range of 300 to 700 nm (step of 5 nm) using a monochromator; the examples are shown in Supplementary Figures S6 and S7. The suggested detailed measurement protocol is provided under Supplementary Methods.

2.4. Lowry Assay

Three stock solutions were prepared and stored at 4 $^{\circ}\text{C}$ until use. Reagent A consisted of 5% Na_2CO_3 and 0.1 N NaOH in MilliQ water, Reagent B consisted of 1% $\text{KNaC}_4\text{H}_4\text{O}_6$ in MilliQ water, and Reagent C consisted of 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in MilliQ water. The working reagent (Reagent E) was prepared immediately before use by mixing Reagents A, B, and C in a ratio of 48:1:1 ($v/v/v$).

Calibration curves in the presence or absence of the interfering compounds were prepared in Ca^{2+} and Mg^{2+} -supplemented PBS as in the case of the Bradford assay (final volume of 75 μL). Subsequently, 150 μL of Reagent E was added to each well, and the plate was immediately mixed and shaken for 10 min on a rotating shaker. Finally, 20 μL of Folin–Ciocalteu phenol reagent (1:1 dilution with MilliQ water) was added, followed by incubation for 30 min in the dark. Absorbance was measured using a Synergy Neo multimode plate reader (BioTek; Winooski, VT, USA) by recording absorbance spectra using a monochromator scan over the range of 700–800 nm with 1 nm increments.

2.5. Data Analysis and Software

For general data analysis, GraphPad Prism 6 or 10 (Boston, MA, USA) and Excel 2016 (Microsoft Office 365; Redmon, WA, USA) were used. For the Bradford assay, absorbance values measured at 590 ± 10 nm were used for all quantitative analyses. For the Lowry assay, absorbance spectra recorded between 740 and 760 nm were integrated

to calculate the area under the curve (AUC), which was used as the primary quantitative readout. Interference effects were evaluated either by comparing signal intensities obtained at different protein concentrations or by linear regression analysis of the calibration curves, with differences in slope used to assess changes in assay response. The effect of each interfering compound was established in $N \geq 2$ independent experiments (each performed in duplicate, triplicate or quadruplicate), and raw data were pooled for all experiments. For datasets containing a sufficient number of observations, the signal profiles were generally consistent with a normal distribution, as indicated by at least one of the four normality tests applied (D'Agostino–Pearson, Shapiro–Wilk, Anderson–Darling, or Kolmogorov–Smirnov); for the negative and the positive controls, all tests indicated normal distribution. Therefore, the grouped comparisons relative to the negative control (PBS) or positive controls (different concentrations of the analytes) were then carried out using one-way ANOVA with Dunnett's test for multiple comparisons. In all statistical tests, the significance of comparisons is indicated as follows: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

A heatmap and PCA plot were generated using the ClustVis 2.0 web tool [50]. For analysis, positive and negative controls as well as compounds that statistically significantly altered CBBG absorbance at 590 ± 10 nm relative to the negative control were chosen (except for L-arginine, which affected the assay only at the highest concentration tested). The absorbance spectra of the chosen compounds at the highest concentration tested in the presence of CBBG were averaged and normalized for each independent experiment to minimize uncertainty originating from variations of the CBBG amount in different experiments (maximal absorbance set to 100%).

3. Results and Discussion

3.1. Review of the Interfering Compounds Reported So Far

Based on the literature search in bibliographic databases Clarivate Web of Science and NIH PubMed, a total of 158 full-text articles were found reporting on the Bradford assay or use of CBBG/CBBR dye and exploring interfering compounds. The search covered the period from the original description of the Bradford assay in 1976 through June 2025. No retracted publications were identified among the articles included in the review. The data from articles which reported artefacts in the context of the Bradford assay in particular are summarized in Table 1.

Among the articles not listed in the table, several reports focused on the development of novel assays (e.g., refs. [9,10,51–62] for proteins generally, or refs. [63–66] for a certain specific protein) and compared these assays to Bradford, including characterization of the assay performance in the presence of interfering compounds known at the time. One publication referred to flavonoids as interfering compounds for the Bradford assay but explored effects of flavonoids on the Lowry and BCA assays instead [67]. One conference abstract reported application of the Bradford assay for quantification of proteins in hair, and the authors claimed the assay to be relatively robust towards lipids, cationic surfactants and EDTA, yet no experimental data were presented to verify such claims [68]. Finally, several articles utilized the Bradford assay as an example for educational purposes [19,20,69] and aimed at more user-friendly signal detection (e.g., by utilizing smartphone), yet interfering compounds were mentioned as the assay limitations only generally, without further experimental exploration. In the case of ref. [70], smartphone-mediated detection of protein-induced changes in the CBBG absorbance spectrum was reported, but interference was addressed in the context of artefacts caused by external light during the imaging.

Furthermore, numerous publications used the Bradford assay for various purposes, yet the interfering compounds were either not identified (i.e., artefacts were described

as just matrix effects) or were not addressed relative to the Bradford assay specifically (i.e., those were mentioned in the context of other methods). For instance, the Bradford assay has been used alone or in parallel with other methods as a part of biomarker discovery [71–75]; characterization of the proteome of various biological systems [3,18,30,32,34,76–79]; assessment of effects of pathological alterations [80–85] or (putative) therapeutic interventions [23,86–101]; or optimization of protocols for various protein-related analyses [102–107] and technological processes [108–114]. References [14,115–122] report the application of CBBG or CBBR staining as a part of a newly developed assay (in one case, such staining was not related to protein detection but to electrochemical detection of an organophosphorus pesticide triazophos [123]), and references [124–127] report CBBG-mediated precipitation of proteins from and detection of proteins in complex samples.

Table 1. Compounds interfering with Bradford assay performance according to reports published in 1976–2025.

Interfering Compound	Class of Compound ^a	Sample Dilution Buffer	Protein Present or Not	Lowest Concentration of Compound with Interfering Effect	Reported Type of Interference	Ref(s)
Cellobiose	Carbohydrate	Water	Present	150 mg/mL	Decrease in absorbance at 595 nm	[128]
Glucose	Carbohydrate	Water	Present	600 mg/mL	Decrease in absorbance at 595 nm	[128]
Mannose	Carbohydrate	Water	Present	600 mg/mL	Decrease in absorbance at 595 nm	[128]
Melibiose	Carbohydrate	Water	Present	100 mg/mL	Decrease in absorbance at 595 nm	[128]
Tween-20 (Polysorbate-80)	Alcohol, detergent, incorporates polymer chain	Water	Absent	0.2%	Absorption maximum at 650 nm	[129]
		Water	Absent	5 g/L	Absorbance at 595 nm	[11]
		Water	Present	5 g/L	Increase in the intercept of the protein curve	[11]
Tween-80 (Polysorbate-80) ^b	Alcohol, detergent, incorporates polymer chain	PBS pH 7.4	Present	1%	Decrease in absorbance at 595 nm	[43]
		Not reported	Present	2 mg/mL	Increase in absorbance at 595 nm	[42]
DNA (bovine, salmon, shrimp, kiwi) ^c	Carbohydrate, aromatic compound	Water	Absent	13 µg (assay volume not specified)	Absorbance at 595 nm	[130]
Metrizamide	Alcohol, aromatic compound, density gradient reagent	Water	Absent	1%	Absorbance at 672 nm	[131]
			Present	4%	Decrease in the slope of the protein curve	
NP-40 (Nonidet P-40) ^d	Alcohol, aromatic compound, detergent	Water	Absent	4%; 5 g/L	Absorbance at 595 nm	[11,44]
		Water	Absent	0.2%	Absorbance at 650 nm	[129]
		Sample buffer (undisclosed)	Present	>0.5%	Decrease in the slope of the protein curve	[26]
		Water	Present	5 g/L	Increase in the intercept of the protein curve	[11]
Heparin	Polysaccharide, complex forming reagent	PBS	Present	5 µg/mL	Decrease in the color yield at 595 nm	[132]

Table 1. Cont.

Interfering Compound	Class of Compound ^a	Sample Dilution Buffer	Protein Present or Not	Lowest Concentration of Compound with Interfering Effect	Reported Type of Interference	Ref(s)
Glycerol	Alcohol, density gradient reagent	150 mM NaCl	Absent	99%	Equivalent of BSA	[1]
		Water	Absent	100 g/L	Absorbance at 595 nm	[11]
		Water	Present	Not specified	Decrease in absorbance at 600 nm	[5]
Sucrose ^e	Carbohydrate, density gradient reagent	150 mM NaCl	Absent	1 M	Equivalent of BSA	[1]
		Water	Present	600 mg/mL	Decrease in absorbance at 595 nm	[128]
Ethanol ^f	Alcohol, organic solvent	Water	Present	10.56%	Stabilizes neutral form of the dye	[133]
Carboxy methyl cellulose	Polysaccharide	Water	Present	50 mg/mL	Increase in absorbance at 595 nm	[128]
Chitosan	Polysaccharide	PBS pH 7.4	Present	Not quantified (chitosan nanoparticle supernatant)	Decrease in absorbance at 595 nm	[43]
Ficoll	Polysaccharide	Water	Present	150 mg/mL	Decrease in absorbance at 595 nm	[128]
Hydroxypropyl chitin (HPCH)	Polysaccharide	Not reported	Present	1 mg/mL	Decrease in the slope of the protein curve	[33]
Hydroxypropyl starch, Reppal PES	Polysaccharide	Water	Present	5%	Decrease in the slope of the protein curve	[134]
Mannan	Polysaccharide	Water	Present	20 mg/mL	Decrease in absorbance at 595 nm	[128]
Dithiothreitol (DTT)	Alcohol, reducing agent	Water	Present	100 g/L	Increase in the intercept of the protein curve	[11]
2-mercaptoethanol ^g	Alcohol, reducing compound	150 mM NaCl	Absent	1 M	Equivalent of BSA	[1]
Chlorophyll extract	Aromatic compound	0.1 M NaOH	Present	Not quantified	Increased absorbance readings	[135]
RNA (baker's yeast)	Carbohydrate, aromatic compound	Water	Absent	17 µg (assay volume not specified)	Absorbance at 595 nm	[130]

Table 1. Cont.

Interfering Compound	Class of Compound ^a	Sample Dilution Buffer	Protein Present or Not	Lowest Concentration of Compound with Interfering Effect	Reported Type of Interference	Ref(s)
Humic acid ^h	Aromatic compound, complex mixture, organic acid, phenol, polymer	30 mM trisodium citrate at pH 7.0	Present	Spiked to sample at 0.25 g/kg	Increase in the intercept of the protein curve	[136]
		Water	Present	10 mg Cl ⁻¹	Decrease in the slope of the protein curve	[137]
		PBS	Present	100 ppm	Increase in the intercept of the protein curve	[138]
Triton X-100 ⁱ	Aromatic compound, detergent, incorporates polymer chain	150 mM NaCl	Absent	0.1%	Equivalent of BSA	[1]
		150 mM NaCl	Absent	4%	Absorbance at 595 nm	[44]
		Water	Absent	0.2%	Absorption maximum at 650 nm	[129]
		Water	Absent	5 g/L	Absorbance at 595 nm	[11]
		Water	Present	5 g/L	Increase in the intercept of the protein curve	[11]
Tannic acid ^j	Aromatic compound, organic acid, phenol	30 mM trisodium citrate at pH 7.0	Present	Spiked to sample at 0.25 g/kg	Increase in the intercept of the protein curve	[136]
Apigenin	Aromatic compound, phenol	Water or low percentage of DMSO in water	Absent	10 mM	Absorption maximum at 650 nm	[2]
Chrysin	Aromatic compound, phenol	Water or low percentage of DMSO in water	Absent	10 mM	Absorption maximum at 650 nm	[2]
Endogenous wine phenolic compounds ^f	Aromatic compound, phenol	Water	Present	200 mg/L	Decrease in the slope of the protein curve	[133]
Epicatechin	Aromatic compound, phenol	0.2 M, pH 4.0 sodium acetate buffer containing 5% ethanol	Present	100 mg/L	Increase in the intercept of the protein curve	[16]
Fisetin	Aromatic compound, phenol	Water or low percentage of DMSO in water	Absent	10 mM	Absorption maximum at 650 nm	[2]
Flavone	Aromatic compound, phenol	Water or low percentage of DMSO in water	Absent	10 mM	Absorption maximum at 650 nm	[2]

Table 1. Cont.

Interfering Compound	Class of Compound ^a	Sample Dilution Buffer	Protein Present or Not	Lowest Concentration of Compound with Interfering Effect	Reported Type of Interference	Ref(s)
Kaempferol	Aromatic compound, phenol	Water or low percentage of DMSO in water	Absent	10 mM	Absorption maximum at 650 nm	[2]
Myricetin	Aromatic compound, phenol	Water or low percentage of DMSO in water	Absent	10 mM	Absorption maximum at 650 nm	[2]
Phenol ^k	Aromatic compound, phenol	150 mM NaCl	Absent	5%	Equivalent of BSA	[1]
Quercetin	Aromatic compound, phenol	Water or low percentage of DMSO in water	Absent	10 mM	Absorption maximum at 650 nm	[2]
Quercetrin	Aromatic compound, phenol	Water or low percentage of DMSO in water	Absent	10 mM	Absorption maximum at 650 nm	[2]
Rutin	Aromatic compound, phenol	Water or low percentage of DMSO in water	Absent	10 mM	Absorption maximum at 650 nm	[2]
Polyquinones	Aromatic compound, polymer	Saline and 0.1 N NaOH	Present	Not quantified	Increase in absorbance at 595 nm	[139]
Reduced polyquinones	Aromatic compound, polymer	Cys-containing assay buffer	Present	Not quantified	Increase in the intercept of the protein curve	[139]
Glycine	Buffering compound, organic acid	Water	Present	100 g/L	Increase in the intercept of the protein curve	[11]
IPG buffer (pH 4–7)	Buffering compound	Water	Absent	2%	Absorbance at 595 nm	[44]
		8 M urea	Present	0.5%	Decrease in the slope of the protein curve	[140]
Tris (tris(hydroxymethyl)-aminomethane) ^l	Buffering compound	150 mM NaCl	Absent	2 M	Equivalent of BSA	[1]
		Water	Absent	100 g/L	Absorbance at 595 nm	[11]
		Water	Present	100 g/L	Increase in the intercept of the protein curve	[11]
2-D lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% (v/v) ampholytes (pH 3–10), 120 mM DTT, and 40 mM Tris-base)	Buffering compound, complex mixture, detergent	Water	Present	100% (dissolved in buffer, compared to dilutions in water)	Increase in absorbance at 595 nm	[29]

Table 1. Cont.

Interfering Compound	Class of Compound ^a	Sample Dilution Buffer	Protein Present or Not	Lowest Concentration of Compound with Interfering Effect	Reported Type of Interference	Ref(s)
Laemmli's buffer (2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue, 60 mM Tris-HCl; pH 6.8)	Buffering compound, complex mixture, detergent	Water	Present	100% (dissolved in buffer, compared to dilutions in water)	Decrease in absorbance at 595 nm	[29]
Citrate buffer pH 4.5	Buffering compound, salt	Water	Present	1%	Decrease in the slope of the protein curve	[134]
Citrate buffer pH 7.0		Water	Present	1%	Decrease in absorbance at 595 nm	[141]
Phosphate-buffered saline (PBS) ^m	Buffering compound, salt	Water	Present	5%	Decrease in absorbance at 595 nm	[141]
Sodium acetate	Buffering compound, salt	Water	Present	0.5%	Decrease in the slope of the protein curve	[134]
Sodium bicarbonate	Buffering compound, salt	Water	Present	1%	Decrease in the slope of the protein curve	[134]
Sodium carbonate	Buffering compound, salt	Water	Present	0.5%	Decrease in the slope of the protein curve	[134]
Sodium citrate	Buffering compound, salt	Water	Present	0.5%	Decrease in the slope of the protein curve	[134]
Ethylenediaminetetraacetic acid (EDTA) ⁿ	Chelator, complex forming reagent	150 mM NaCl	Absent	0.1 M	Equivalent of BSA	[1]
		Water	Present	10 mM	Decrease in absorbance at 600 nm	[5]
Protamine sulphate	Complex forming reagent	Sample buffer containing 9 M urea, 4% Nonidet P-40, 2% ampholine, and 2% 2-mercaptoethanol	Present	1.6 mg/mL	Decrease in the slope of the protein curve	[8]
Algal compounds in fish gut fluid	Complex mixture	Water	Present	Not quantified	Decrease in the slope of the protein curve	[142]
Components of diapers	Complex mixture	IPG rehydration buffer	Present	Not quantified	Decrease in absorbance at 595 nm	[143]

Table 1. Cont.

Interfering Compound	Class of Compound ^a	Sample Dilution Buffer	Protein Present or Not	Lowest Concentration of Compound with Interfering Effect	Reported Type of Interference	Ref(s)
Compounds co-extracted with glomalin-related soil protein	Complex mixture	Citrate solution (pH 7–8)	Present	1:5 dilutions of the extract	Decrease in the slope of the protein curve; increase in absorbance at 615 nm and 740 nm	[45,144]
Clay particles	Complex mixture, salt	Water	Present	0.435 mg/mL	Decrease in the slope of the protein curve	[145]
Urea ^o	Denaturing compound	Water	Absent	100 g/L	Absorbance at 595 nm	[11]
		Not reported	Present	8 M	Elevated absorbance at 595 nm	[146]
Dextran sulphate ^P	Density gradient reagent, polysaccharide	Water	Present	0.1 g/L	Decrease in the slope of the protein curve	[11]
Cetyltrimethylammonium bromide, CTAB	Detergent	Water	Absent	400 µg/mL	Absorbance at 650 nm, which gradually shifts to 800–950 nm	[147]
			Present	400 µg/mL	Increase in absorbance at 595 nm	
CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate) ^q	Detergent	Water	Absent	4%	Absorbance at 595 nm	[44]
		Water	Absent	0.2%	Absorbance at 650 nm	[129]
		8 M urea	Present	2%	Decrease in the slope of the protein curve	[140]
DDM (n-dodecyl-β-D-maltoside)	Detergent	Water	Absent	4%	Absorbance at 595 nm	[44]
Hexyl-β-D-glucopyranoside	Detergent	Water	Absent	0.2%	Absorbance at 595 nm	[129]
			Present	0.5%	Systematically increased absorbance at 595 nm	
OGP, OG (octyl-β-D-glucopyranoside)	Detergent	Water	Absent	4%	Absorbance at 595 nm	[44]
		Water	Absent	0.2%	Absorbance at 650 nm	[129]

Table 1. Cont.

Interfering Compound	Class of Compound ^a	Sample Dilution Buffer	Protein Present or Not	Lowest Concentration of Compound with Interfering Effect	Reported Type of Interference	Ref(s)
Sodium dodecyl sulphate (SDS) ^r	Detergent	150 mM NaCl	Absent	0.1%	Equivalent of BSA	[1,5]
		Water	Absent	10 mM; 0.2%	Absorption maximum at 650 nm	[2,129]
		Water	Absent	5 g/L	Absorbance at 595 nm	[11]
		Sample buffer containing 9 M urea, 4% Nonidet P-40, 2% ampholine, and 2% 2-mercaptoethanol	Present	0.1%	Decrease in the slope of the protein curve	[8]
		Saline	Present	0.004%	Decrease in the slope of the protein curve	[148]
		Water	Present	5 g/L	Increase in the intercept of the protein curve	[11]
		Water	Present	2%	Overestimation of the analyte (milk protein) as compared to no SDS conditions	[149]
Brij-35	Detergent, polymer	Water	Absent	0.2%	Absorption maximum at 650 nm	[129]
Citric acid	Organic acid	Water	Present	100 g/L	Increase in the intercept of the protein curve	[11]
				1%	Decrease in the slope of the protein curve	[134]
Oxalic acid	Organic acid	Water	Present	25 g/L	Increase in the intercept of the protein curve	[11]
Tartaric acid	Organic acid	Water	Present	100 g/L	Increase in the intercept of the protein curve	[11]
Acetone	Organic solvent	150 mM NaCl	Absent	100%	Equivalent of BSA	[1]
<i>N,N</i> -dimethylformamide, DMF	Organic solvent	In polyacrylamide gel matrix	Present	1.29 M in the loaded sample	Decrease in band intensity	[150]
Ampholine (pH 3.5–10)	pH gradient reagent	Water	Absent	2%	Absorbance at 595 nm	[44]
Ampholine (pH 4–6)	pH gradient reagent	Water	Absent	2%	Absorbance at 595 nm	[44]

Table 1. Cont.

Interfering Compound	Class of Compound ^a	Sample Dilution Buffer	Protein Present or Not	Lowest Concentration of Compound with Interfering Effect	Reported Type of Interference	Ref(s)
Pharmalyte (pH 3–10)	pH gradient reagent	Water	Absent	2%	Absorbance at 595 nm	[44]
Pharmalyte (pH 5–8)	pH gradient reagent	Water	Absent	2%	Absorbance at 595 nm	[44]
Poly(ethylene glycol), PEG (600, 1000, 3350 or 10,000 Da)	Polymer	5 mM Tris-HCl buffer, pH 7.5	Present	20% <i>w/w</i>	Decrease in the slope of the protein curve	[151]
(4000, 8000 or 20,000 Da)		Water	Present	10% <i>w/w</i>	Decrease in the slope of the protein curve	[134]
PEGylation of a protein extendin-4 with PEG20000		Not reported	Present	20% PEGylated protein in mixture with non-modified protein	Decrease in absorbance at 595 nm	[22]
PEGylation of a protein uricase from <i>Bacillus fastidiosus</i> with PEG5000		100 mM sodium phosphate buffer	Present	5 mg/mL of PEG5000	Decrease in absorbance at 595 nm	[152]
poly(lactide-co-glycolide), PLG	Polymer	DMSO	Absent	5 mg/mL	Absorbance at 595 nm	[12]
Polyvinylpyrrolidone, PVP	Polymer	Water	Present	5%	Decrease in the slope of the protein curve	[134]
UCON (a random copolymer of 50% ethylene oxide and 50% propylene oxide)	Polymer	Water	Present	5%	Decrease in the slope of the protein curve	[134]
Glycosylation	Post-translational modification	PBS, pH 7.4	Present	Not quantified	Decrease in the slope of the protein curve	[13]
Ammonium sulphate ^s	Salt	Water	Present	100 g/L	Decrease in the slope of the protein curve	[11]
Ammonium sulphate	Salt	Water	Present	0.5%	Decrease in the slope of the protein curve	[134]
Lithium sulphate	Salt	Water	Present	1%	Decrease in the slope of the protein curve	[134]
Magnesium sulphate	Salt	Water	Present	0.5%	Decrease in the slope of the protein curve	[134]

Table 1. Cont.

Interfering Compound	Class of Compound ^a	Sample Dilution Buffer	Protein Present or Not	Lowest Concentration of Compound with Interfering Effect	Reported Type of Interference	Ref(s)
Sodium chloride ^t	Salt	Water	Present	4 M	Decrease in absorbance at 600 nm	[5]
		Water	Present	0.5–3.5%	Non-monotonous change in the slope of the protein curve	[153]
Sodium orthovanadate	Salt	pH 1.8 or below	Present	25 µM	Decrease in absorbance at 595 nm and concomitant shift in the A _{max} to 405 nm	[154]
Sodium sulphate	Salt	Water	Present	1%	Decrease in the slope of the protein curve	[134]
Hemosol	Salt mixture	150 mM NaCl	Absent	0.1%	Equivalent of BSA	[1]
Amoxicillin	Small-molecular-weight drug	Phosphate buffer	Absent	10 g/L	Absorbance at 595 nm	[155]
Chlorpromazine	Small-molecular-weight drug	0.1 M sodium phosphate buffer, pH 7	Absent	1 mg/mL	Absorbance at 595 nm	[156]
		Water	Absent	1 g/L	Absorbance at 595 nm	[11]
		Phosphate buffer	Absent	5 g/L	Absorbance at 595 nm	[155]
		0.1 M sodium phosphate buffer, pH 7	Present	1 mg/mL	Increase in the intercept of the protein curve	[11,156]
Fluphenazine	Small-molecular-weight drug	Phosphate buffer	Absent	10 g/L	Absorbance at 595 nm	[155]
		Water	Absent	0.5 g/L	Absorbance at 595 nm	[11]
Prochlorperazine	Small-molecular-weight drug	Phosphate buffer	Absent	10 g/L	Absorbance at 595 nm	[155]
Promazine	Small-molecular-weight drug	Phosphate buffer	Absent	10 g/L	Absorbance at 595 nm	[155]
		Water	Absent	1 g/L	Absorbance at 595 nm	[11]
Thioridazine	Small-molecular-weight drug	Water	Absent	0.5 g/L	Absorbance at 595 nm	[11]
		Water	Present	0.5 g/L	Increase in the intercept of the protein curve	[11]

Table 1. Cont.

Interfering Compound	Class of Compound ^a	Sample Dilution Buffer	Protein Present or Not	Lowest Concentration of Compound with Interfering Effect	Reported Type of Interference	Ref(s)
Trifluoperazine	Small-molecular-weight drug	Water	Absent	0.5 g/L	Absorbance at 595 nm	[11]
		Water	Present	0.5 g/L	Increase in the intercept of the protein curve	[11]
Triflupromazine	Small-molecular-weight drug	Water	Absent	1 g/L	Absorbance at 595 nm	[11]
		Water	Present	1 g/L	Increase in the intercept of the protein curve	[11]

The table is sorted alphabetically by the “Class of compound” and then by the “Interfering compound”. ^a The keywords were assigned based on the chemical structure of the compound or mixture and its common role in assays. ^b By contrast, addition of Tween-80 (at 0.1%) was claimed to sensitize detection of proteins in forest litter [157]. ^{c,d} Ref. [17] reported 0.25 mg DNA (volume not specified) and 0.5% NP-40 as non-interfering compounds (not specified whether in the presence or absence of protein). ^e At lower concentration (up to 100 mg/mL), ref. [42] reported sucrose, trehalose and mannitol to have no effect on the absorbance spectra of CBBG/CBBR dyes. ^f Non-additive interference from ethanol and phenolic compounds in wine was also reported in [158], but the full text of this publication was not accessible for us. In the absence of protein, ref. [1] reported 95% ethanol to have no effect on the absorbance spectra of CBBG. ^g Ref. [159] reported 5% 2-mercaptoethanol as a non-interfering compound in the presence of protein. ^h An interfering effect of humic substances during BSA measurement was also reported in [160]. ⁱ Ref. [17] reported 0.125% Triton X-100 as a non-interfering compound (not specified whether in the presence or absence of protein). At lower concentration (0.008%), Triton X-100 was claimed to sensitize detection of proteins in [148]. ^j Ref. [16] reported tannic acid to have no effect on the measurement of hordein using the Bradford assay. ^k In the case of helminth parasite phenols and catecholamines, ref. [161] reported no interference in the presence of protein. ^{l,m,n} Ref. [17] reported 2 M Tris, undiluted PBS and 100 mM EDTA as non-interfering compounds (not specified whether in the presence or absence of protein). ^o Ref. [159] reported no interference in the presence of protein for 4–8 M urea, and ref. [26] reported no interference from pH or urea (up to 8 M) when using BSA as the standard. ^p Ref. [151] reported no interference from dextran at 20% *w/w* in the presence of protein. ^q Ref. [17] reported 5% CHAPS as a non-interfering compound (not specified whether in the presence or absence of protein). ^r At lower concentration (0.0035%), SDS was claimed to sensitize detection of collagens in [162], but the authors noted that detection of other proteins was desensitized. Ref. [17] reported 0.125% SDS as a non-interfering compound (not specified whether in the presence or absence of protein). ^s In the absence of protein, ref. [1] reported 1 M ammonium sulphate to have no effect on the absorbance spectra of CBBG; ref. [17] also reported 1 M ammonium sulphate as a non-interfering compound (not specified whether in the presence or absence of protein). ^t In the absence of protein, ref. [1] reported 5 M NaCl, 1 M KCl and 1 M MgCl₂ to have no effect on the absorbance spectra of CBBG.

The compounds listed in the table represent structurally diverse classes: apart from detergents already mentioned earlier, various alcohols, carbohydrates, phenols (including humic acids and flavonoids) and inorganic salts have been reported to interfere with the assay either in the presence or in the absence of the analyte, or in both formats. On the other hand, several publications report no interference from the substances at the assay settings explored. Such examples include melanin if added to the reaction system (at a mass equal to the mass of the protein analyzed) and then removed by centrifugation prior to the measurement [163]; melamine and cyanuric acid if added to the reaction system at a mass nearly double the mass of the protein analyzed [164]; low (0.004 mg/mL) to high levels (0.05 mg/mL) of urobilin in the presence of BSA [165]; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at 200 mg/mL [12]; and a range of other compounds including buffers, salts and reducing agents [17] or widely used small-molecular-weight drugs [156].

Additionally, within the analyzed set of publications, some conflicting data can also be found regarding the effects of certain compounds (see footnotes under Table 1): some report a substance as interfering, whereas other report no disturbance of the Bradford assay in the presence of the same substance. Such discrepancies can be explained by the presence or absence of the analytes, the nature of the analytes, the use of different concentrations of the interfering compounds or CBBG, the application of different buffers or sample matrices, and even the pipetting precision that affects the estimation of the statistical significance.

3.2. Construction of the Validation Set and Characterization of Interfering Effects in the Presence of Analytes

Building on the literature analysis presented in the previous sections, we sought to extend the landscape of known Bradford assay-interfering substances by systematically examining compound classes that, to our knowledge, have not been previously evaluated in this context. Guided by chemical principles governing CBBG–analyte interactions, we selected several additional categories of molecules that are increasingly common in proteomics workflows yet remain uncharacterized with respect to their potential assay-disrupting effects. For example, EDCs were included due to their typically low molecular weight, moderate-to-high hydrophobicity, and frequent incorporation of aromatic structures that are known to interfere with CBBG binding. Transfection reagents, characterized by their large size and positive charge, were also tested in light of the affinity of CBBG for positively charged functional groups. In addition, we examined oligoarginine-containing synthetic conjugates (ARC-902 and ARC-1041, structures shown in Supplementary Figure S1), given scattered reports suggesting that CBBG may interact with small peptides below 5000 Da. By introducing and experimentally probing these novel and chemically diverse classes of potential interfering compounds, our study aimed to identify previously unrecognized vulnerabilities relevant to contemporary proteomics workflows.

The validation set comprised 29 chemically diverse substances, each screened for its potential to interfere with the Bradford assay in the presence or absence of protein analytes. To ensure methodological robustness and internal consistency with established knowledge, we included several well-documented interfering agents—ionic detergents, non-ionic detergents, and reducing agents—as positive controls for assay disruption. The remaining compounds were selected based on mechanistic reasoning derived from the known principles of CBBG–analyte interactions (electrostatic and hydrophobic interactions) and their frequent use in contemporary proteomics workflows. Concentrations were chosen to reflect realistic exposure levels in applications such as cell lysis, membrane solubilization, protein extraction, and sample storage. For example, non-ionic detergents (Triton X-100, NP-40, Tween-20) and mild zwitterionic detergents (DDM, CHAPS) were tested near concentrations commonly used for membrane protein solubilization (0.02–2% *v/v*), while SDS concentrations (0.01–1% *v/v*) encompassed the lower ranges at which

ionic detergents are typically present after sample dilution or carryover from denaturing lysis buffers. Reducing agents (DTT, 5 mM) and cryoprotectants (glycerol, 5% *v/v*) were evaluated at concentrations routinely used during protein stabilization or storage. Positively charged reagents—cationic transfection agents (Fugene[®] 6, Lipofectamine[®] 2000, TurboFect) and oligoarginine-containing synthetic conjugates (ARC-902, ARC-1041)—were included because their high cationic charge density makes them plausible competitors for CBBG binding. Their tested concentrations (0.1–1 μ M for ARCs; 0.3–1% *v/v* for transfection reagents) reflect levels likely to remain in lysates following transfection or reagent washout, based on manufacturer protocols and empirical observations of incomplete reagent removal; while oligo-L-arginine peptides can be degraded intracellularly, the utilized compounds represented proteolytically stable oligo-D-arginine peptides. The selected endocrine-disrupting chemicals (EDCs) were tested at 5 μ M, a concentration aligned with exposure levels often used in cell-based assays and sufficient to interrogate their potential hydrophobic or aromatic interactions with CBBG. Finally, three amino acids—glycine, arginine, and tryptophan—were intentionally tested at supraphysiological concentrations (10 mM) to explicitly challenge the long-standing assumption that small molecules below ~3000 Da cannot meaningfully interfere with the Bradford assay. This design allowed us to probe whether chemical functionality, rather than molecular weight alone, determines interference potential.

As representative protein standards, we selected two biologically and chemically distinct models: BSA, a purified arginine-rich protein commonly used in assay calibration, and reconstituted non-fat milk, a complex protein mixture predominantly composed of glutamate-rich caseins. PBS served as the assay buffer, tested in two formulations—with or without calcium and magnesium ions—to assess the possible influence of divalent cations on assay performance. Initial absorbance measurements were conducted at 590 nm, a wavelength within the typical range for CBBG–protein complex detection. The interfering effects of compounds were systematically assessed for two concentrations of each analyte: 16.6 μ g/mL or 33.3 μ g/mL BSA (representing the concentration in the mid-linear range or at the end of the linear range), and 33.3 μ g/mL or 133 μ g/mL non-fat milk (one concentration was chosen for enabling comparison with BSA, and another concentration was the highest tested).

The results for compounds that showed significant interference with the assay under these conditions are summarized in Figure 1 and Supplementary Figures S2 and S3. In the paired experiments comparing conditions with and without divalent metal ions, the presence of these cations had only a minor impact on the assessment of compound interference. While the overall measurement window of the assay was slightly reduced in the presence of the metal ions, their effect on the detection of interfering substances was negligible. Of the 29 substances tested, 12 showed significant interference with Bradford assay performance at one or more analyte concentrations. Among these compounds, six were detergents previously reported to affect Bradford assay performance, thus validating our experimental approach. In addition, our study identified several compounds with a substantial impact on the assay that, to our knowledge, have not been reported earlier. An amino acid, tryptophan, interfered with the assay, albeit only at millimolar concentrations. More strikingly, all three tested transfection reagents demonstrated measurable interference, which was especially pronounced when non-fat milk was used as the analyte. Furthermore, both tested oligoarginine peptides included in the panel also disrupted the assay, supporting earlier suggestions that CBBG can interact with smaller peptides, particularly those rich in positively charged residues.

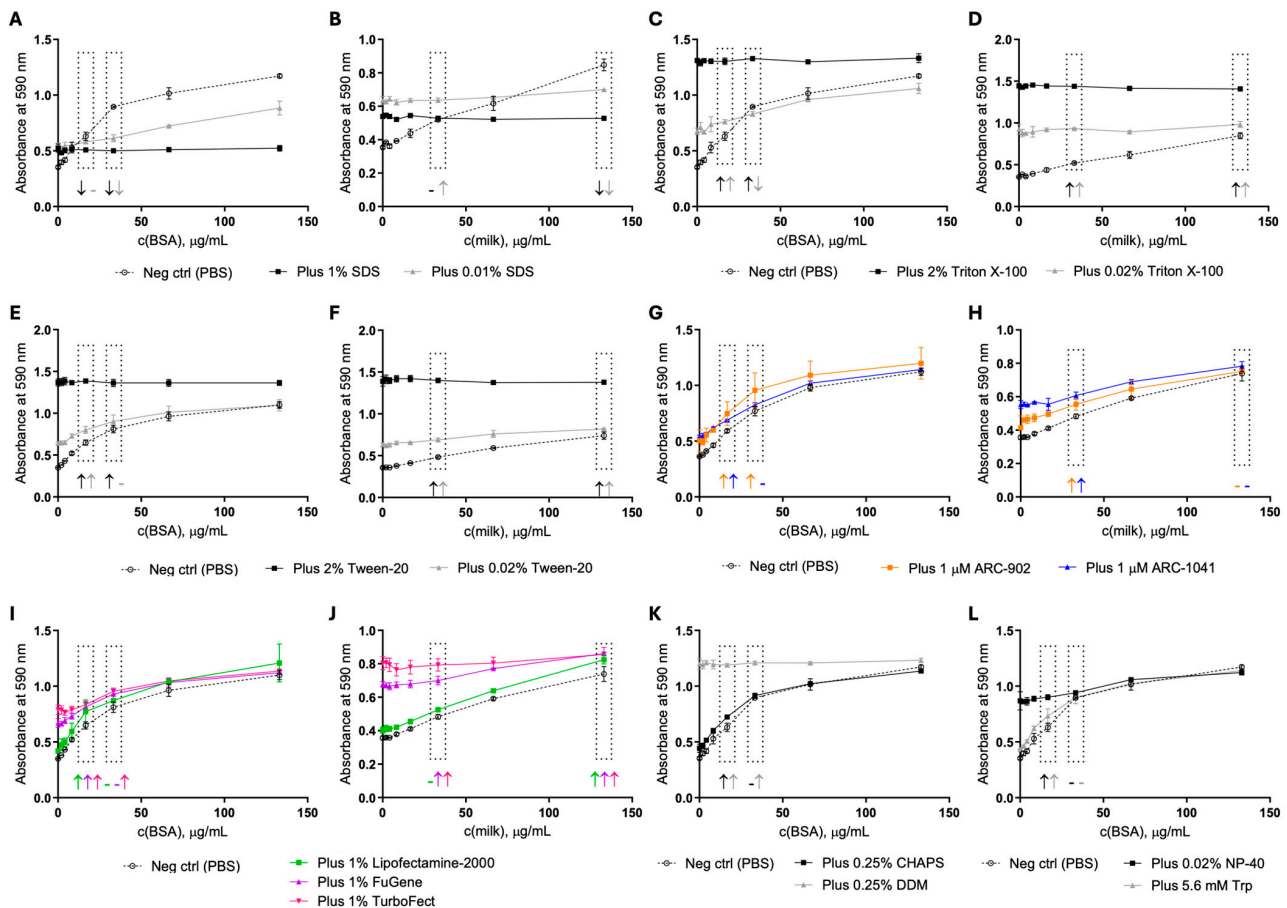


Figure 1. Examples of the analyte calibration curves measured by Bradford assay in the presence or absence of the interfering compounds from the validation set. Each panel represents data from a single independent experiment (mean and standard deviation of replicates); the data are shown only for compounds and concentrations which featured statistically significant interference with the analyte detection at least at one concentration of the analyte in all independent experiments ($N \geq 2$). Analytes: in panels (A,C,E,G,I,K,L) BSA; in panels (B,D,F,H,J) non-fat milk. The interfering compounds together with the final total concentrations used in the assay are listed below each panel (or a pair of panels if data for both BSA and milk are shown). The boxes indicate analyte concentrations at which the interfering effects of compounds were systematically assessed. The arrows under the boxes show statistically significant effects for the given experiment ($p < 0.05$); dashes indicate no statistical significance relative to the positive control. The color codes of the symbols are consistent with the color codes used for the interfering compound annotation; the direction of the arrow indicates whether, in the presence of the shown concentration of the interfering compound, the measured signal is higher ($\uparrow$) or lower ($\downarrow$) than for the analyte alone.

As a general trend, interference was more pronounced at lower protein concentrations—a predictable outcome, as higher analyte levels are more likely to outcompete the interfering compound for CBBG binding. For milk samples, interfering effects remained detectable even at the highest analyte concentrations, likely because the calibration curve had not yet reached a saturation plateau (in contrast to BSA, which exhibited a typical sigmoidal curve with a clear upper plateau). This difference is consistent with the previous reports [166,167] and can be explained by the fact that the reconstituted non-fat milk contained only 20% or protein by weight, and by the distinct amino-acid compositions of the analytes. BSA is relatively arginine-rich [168], and its strong positive charge density provides a large number of high-affinity binding sites for CBBG, which is known to preferentially interact with basic residues, particularly arginine. In contrast, the dominant proteins in non-fat milk, the caseins, are enriched in glutamate and aspartate residues and contain markedly fewer

basic amino acids [169,170]. When the effect of oligoarginine peptides and transfection reagents was explored with another widely used calibrant represented by BGG, statistically significant interference was still observed at 16.6 µg/mL calibrant protein (Supplementary Figure S8).

In line with the previous reports (Table 1), all 12 interfering compounds markedly increased the intercept of the calibration curve. However, the net effect on signal intensity in the presence of analyte could not be systematically classified as a false positive or false negative: the same compound, at the same concentration, could either enhance or suppress the signal depending on the analyte concentration and the specific region of the calibration curve (see, e.g., Figure 1B). This also implies that a commonly used simple correction—subtracting the difference between intercept values measured in the presence *versus* absence of an interfering compound—cannot reliably account for the complex nature of the interference and may not ensure adequate accuracy. A more robust approach should be preferred, testing the compound across a wide range of analyte concentrations (spanning several orders of magnitude) to fully capture its impact on assay behavior.

3.3. Assessment of the Compound Concentrations Interfering with the Assay in the Absence of Proteins

Although the interfering compounds varied in their specific effects, the substances consistently elevated the intercept of the calibration curve, functioning as false positives at analyte concentrations near the limit of detection. Based on this observation, we focused further analysis on this parameter to determine the lowest concentration at which each compound begins to interfere with the assay. To achieve this, we assessed the statistically significant differences in CBBG absorbance at 590 nm in the absence of analyte by pooling of data from all experiments, thereby isolating the compound-specific signal and eliminating variability due to differing protein concentrations across independent experiments. For experiments conducted in the absence of analyte, concentration ranges were extended (e.g., digitonin up to 100 µM; amino acids up to 20 mM; synthetic peptides up to 133 µM) to map the dose–response profile of each interfering compound. Together, these concentration choices ensured coverage of both physiologically relevant and mechanistically informative ranges, enabling a systematic and chemically grounded assessment of interference across all compound classes tested.

The results are summarized in Table 2 (results in the presence of calcium and magnesium ions) and Supplementary Figure S4A (results in the absence of divalent cations). Overall, the minimal interfering concentrations differed markedly even among chemically related compounds (the dose–response curves are shown in Supplementary Figure S5). For instance, despite both being mild non-reducing detergents, DDM and CHAPS exhibited substantially different interference thresholds. Less mild non-ionic detergents (Triton X-100, Tween-20, NP-40) generally showed tolerable concentrations below 0.007%, notably lower than concentrations typically used in protein extraction protocols (even accounting for the dilutions of the lysates). Among transfection reagents, Lipofectamine® 2000 had the least effect on CBBG absorbance, whereas TurboFect exhibited the strongest interference. Strikingly, for compounds ARC-902 and ARC-1041, a statistically significant increase in CBBG absorbance was observed at sub-micromolar concentrations of the synthetic peptides. This last observation is particularly noteworthy, as it underscores the potential for substantial bias in protein quantification when comparing treated and untreated lysates—especially in workflows involving cell membrane-penetrating peptides or transfection reagents. These compounds can become enriched within cells or extracellular vesicles relative to the surrounding medium [171–174], potentially distorting total protein concentration estimates obtained via the Bradford assay and leading to inaccurate interpretations of the downstream data.

Table 2. Interfering effects of tested compounds on CBBG absorbance at 595 nm in the absence of protein analyte.

Compound	Class of Compound	Lowest Tested Concentration Interfering with the CBBG Signal at 590 nm	Statistical Significance and Fold Change Relative to Free CBBG at 590 nm ^b	Highest Tested Concentration Not Interfering with the CBBG Signal at 590 nm ^c	Change in Spectral Characteristics of CBBG in the Presence of the Compound
SDS	Detergent	0.010% <i>v/v</i>	*** 1.66×	NA	Increase in absorbance at 650 nm
Triton X-100	Detergent, aromatic compound	0.013% <i>v/v</i>	*** 1.31×	0.0067% <i>v/v</i>	Increase in absorbance at 620 nm
Tween-20	Detergent, alcohol	0.0082% <i>v/v</i>	*** 1.27×	0.0027% <i>v/v</i>	Increase in absorbance at 625 nm
NP-40	Detergent, alcohol, aromatic compound	0.0083% <i>v/v</i>	*** 1.49×	0.0027% <i>v/v</i>	Increase in absorbance at 625 nm
CHAPS	Detergent, alcohol	0.25% <i>v/v</i>	*** 1.28×	0.11%	Increase in absorbance at 625 nm
DDM	Detergent, alcohol	0.012% <i>v/v</i>	*** 1.27×	0.0041% <i>v/v</i>	Increase in absorbance at 620 nm
Digitonin	Detergent, alcohol	NA	ns	100 µM	NM
Glycerol	Solvent, alcohol	NA	ns	5.0% <i>v/v</i>	NM
DMSO	Solvent	NA	ns	1.0% <i>v/v</i>	NM
DMF	Solvent	NA	ns	1.0% <i>v/v</i>	NM
Glycine	Amino acid	NA	ns	10 mM	NM
L-arginine	Amino acid	20 mM	* 1.20×	10 mM	Increase in absorbance at 595 nm
L-tryptophan	Amino acid	5.6 mM	** 1.23×	2.2 mM	Increase in absorbance at 595 nm
BPA	EDC, aromatic compound, phenol	NA	ns	5.0 µM	NM
BPF	EDC, aromatic compound, phenol	NA	ns	5.0 µM	NM
DDE	EDC, aromatic compound	NA	ns	5.0 µM	NM
HCB	EDC, aromatic compound	NA	ns	5.0 µM	NM
MEHP	EDC, aromatic compound	NA	ns	5.0 µM	NM
MEHHP	EDC, aromatic compound	NA	ns	5.0 µM	NM
PCB170	EDC, aromatic compound	NA	ns	5.0 µM	NM
PCB180	EDC, aromatic compound	NA	ns	5.0 µM	NM
PFOA	EDC, perfluorinated compound	NA	ns	5.0 µM	NM

Table 2. Cont.

Compound	Class of Compound	Lowest Tested Concentration Interfering with the CBBG Signal at 590 nm	Statistical Significance and Fold Change Relative to Free CBBG at 590 nm ^b	Highest Tested Concentration Not Interfering with the CBBG Signal at 590 nm ^c	Change in Spectral Characteristics of CBBG in the Presence of the Compound
PFOS	EDC, perfluorinated compound	NA	ns	5.0 μ M	NM
Fugene [®] 6 ^a	Transfection reagent	0.67% <i>v/v</i>	*** 1.48 $\times$	0.22% <i>v/v</i>	Increase in absorbance at 585 nm
Lipofectamine [®] 2000 ^a	Transfection reagent	1.0% <i>v/v</i>	** 1.17 $\times$	0.22% <i>v/v</i>	Increase in absorbance at 585 nm
TurboFect ^a	Transfection reagent	0.22% <i>v/v</i>	*** 1.44 $\times$	0.074% <i>v/v</i>	Increase in absorbance at 585 nm
ARC-902	D-arginine-rich peptide conjugate	370 nM	*** 1.06 $\times$	180 nM	Increase in absorbance at 585 nm
ARC-1041	D-arginine-rich peptide conjugate	550 nM	*** 1.18 $\times$	180 nM	Increase in absorbance at 585 nm
DTT	Reducing agent (thiol)	NA	ns	5.0 mM	NM

Abbreviations: NA, not achieved; NM, not measured; ns, not significant. ^a The percentage shown corresponds to the dilution of the stock solutions; the concentrations of the stock solutions were not specified by the producers. ^b The fold change shown corresponds to the ratio of absorbance values measured in the presence vs. absence of the lowest tested concentration of compound interfering with the CBBG signal; statistical significance (one-way ANOVA with Dunnett's test for multiple comparisons): *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. ^c The indicated concentrations can be considered safe if the same assay format is used as reported here.

3.4. Validation of the Interfering Effect of Synthetic Peptides and Transfection Reagents in Cancerous Cell Lysates

To extend our analyses into more physiologically relevant environments, we evaluated the effects of synthetic peptides and transfection reagents in lysates derived from the lung adenocarcinoma (HCC-44, A549) or bone osteosarcoma (U2OS) cell lines. The lysates were prepared in the presence of Triton X-100; the detergent concentration was adjusted such that it remained non-interfering at two of the three lysate dilution points, thereby allowing selective assessment of additional interfering agents. Synthetic peptides were tested at a final concentration of 200 nM, slightly exceeding the previously determined interference threshold (180 nM), whereas transfection reagents were tested at 0.3% (*v/v*), a concentration informed by earlier experiments showing interference at approximately 0.22% or lower. Two quantitative parameters were compared between conditions with and without interfering compounds: (i) the absorbance at 590 nm for the lowest lysate concentration (Figure 2A–C) and (ii) the slope of the linear regression across the lysate dilution series (Figure 2D–F).

Across all three lysate types, both synthetic peptides and two of the three transfection reagents (TurboFect and Fugene[®] 6) produced statistically significant effects on both metrics ($p < 0.05$), indicating perturbation of apparent protein concentration. Lipofectamine displayed the least pronounced interference profile, significantly affecting the 590 nm signal at the lowest lysate dilution in U2OS and A549 lysates, and altering the regression slope in HCC-44 and A549 lysates. Overall, these results confirmed that the interference patterns identified in simplified model systems remained detectable in complex biological matrices, thereby validating the robustness and practical relevance of the trends observed earlier.

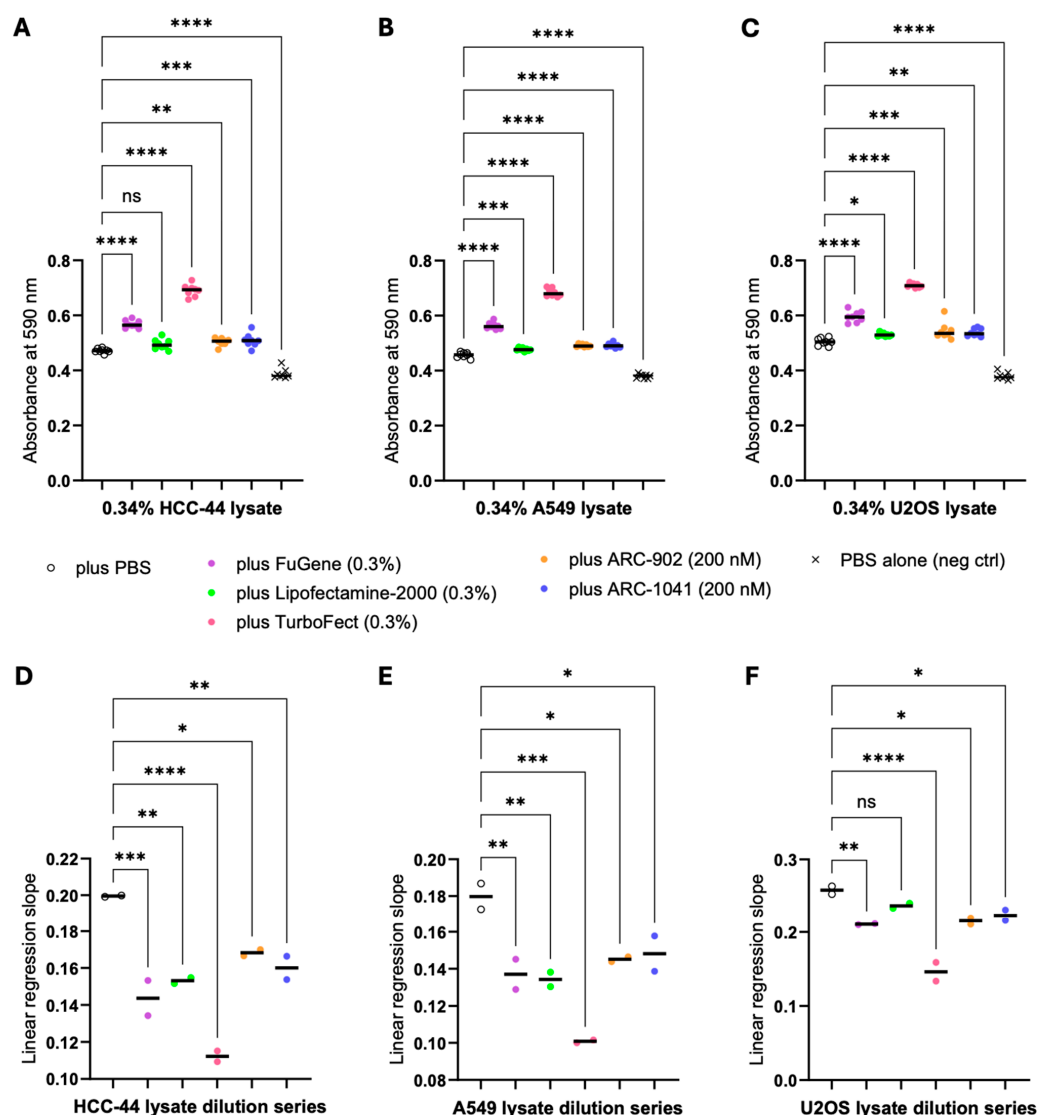


Figure 2. Interfering effects of transfection reagents and synthetic peptides spiked into lysates of the cancerous cell lines. (A–C) Pooled signals ($N = 2$ in quadruplicates) measured for the most dilute concentration of the lysate (0.34% v/v , cell line indicated at the bottom of each panel) in the presence or absence of the interfering compounds (listed in the middle section of the image); negative control signal shown for reference. (D–F) Slope of the linear regression plotted for three lysate dilutions ($N = 2$) in the presence or absence of the interfering compounds. Note that in (D,E), the y -axis does not start from zero. Statistical significance of grouped comparisons (one-way ANOVA with Dunnett's test for multiple comparisons): **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns—not significant.

3.5. Clustering of Interfering Compounds According to the Shift of CBBG Absorbance Spectrum

The distinct alterations observed in the shape of analyte response curves in the presence of various interfering compounds (Figure 1) indicated that some interfering compounds could affect the CBBG absorbance spectrum differently than true protein analytes (i.e., the changes are not limited to increase in absorbance at 590 nm). For several compounds, such spectral effects have been indeed previously reported (as indicated in Table 1). To investigate this phenomenon systematically, we measured full absorbance spectra of CBBG in the presence of compounds that significantly affected absorbance at 590 nm; the resulting spectra are presented in Supplementary Figures S6 and S7.

Our analysis revealed two major spectral patterns arising from addition of the interfering compounds. In the first case, represented by detergents, a marked increase in CBBG absorbance at higher wavelengths was evident—with the maxima in the range of

615–625 nm (and in the case of SDS, extending up to 650 nm). For other compounds of interest (including arginine, tryptophan, synthetic peptides, and transfection reagents), the spectral shift of CBBG resembled that observed in the presence of genuine protein analytes, with enhanced absorbance around 590 nm. In addition, certain compounds (e.g., tryptophan) exhibited characteristic intrinsic absorbance in the UV region. Mechanistically, CBBG binds proteins through a combination of electrostatic and charge-assisted hydrogen-bonding interactions with basic residues (most prominently arginine) and hydrophobic or van der Waals contacts with aromatic and nonpolar side chains [175–177]. Non-ionic detergents lack charged headgroups, and although SDS is an ionic surfactant, its hydrophobic tail contains no positively charged groups; consequently, these classes of detergents do not participate in direct electrostatic interactions with the sulfonated dye structure. Instead, they interfere primarily through hydrophobic or micelle-mediated effects, which can perturb the dye's spectral properties (particularly at higher wavelengths [147]) without mimicking true protein-binding modes. In contrast, cationic transfection reagents and oligoarginine-rich peptides can interact directly with the negatively charged sulfonate groups of CBBG, forming electrostatically stabilized complexes that closely resemble the spectral signature of the protein–dye complex, thereby producing strong false-positive responses.

To further systematize the spectral data and distinguish compound-specific spectral fingerprints, we performed clustering analysis based on the full absorbance spectra of CBBG in the presence of each interfering substance at its highest tested concentration. Normalized spectra from several independent experiments were included for each compound to account for variability. As reference, we included both negative controls (PBS with CBBG) and positive controls (133 µg/mL BSA with CBBG) in the analysis. Due to the limitations of the clustering algorithm, which could process only eight conditions per run, the set of interfering compounds was divided into two subsets according to the spectral patterns mentioned above. The clustering analysis results are presented in Figure 3, which includes both hierarchical clustering heatmaps (where absorbance at each wavelength is treated as an individual variable) and PCA plots derived from the same dataset.

For the compounds in subset A, along with the positive and negative controls, PCA revealed four distinct clusters (Figure 3C): (1) the negative control (PBS); (2) SDS; (3) positive control (BSA) and CHAPS; and (4) the remaining detergents. The separation between the negative and positive controls was clearly resolved along the PC1. Notably, CHAPS clustered closely with the BSA control, despite its spectrum not overtly resembling that of a typical analyte-bound CBBG profile. In contrast, the distinct clustering of SDS was consistent with its pronounced and atypical spectral shift toward higher wavelengths, as observed in the raw spectral data. The corresponding hierarchical clustering heatmap (Figure 3A) supported these observations. It confirmed that the normalized CBBG spectra in the presence of CHAPS were most similar to those observed in the presence of BSA, suggesting that complexation of CHAPS with CBBG mimics protein–dye interactions. Meanwhile, the remaining detergents clustered together, yet the algorithm reliably grouped replicate measurements of the same compound, indicating strong internal consistency within treatments.

In contrast to subset A, the clustering results for subset B revealed a more heterogeneous distribution of samples (Figure 3D). Along the PC1, samples containing tryptophan were clearly separated from the others, largely due to the intrinsic absorbance of this amino acid, which was particularly pronounced in the normalized spectra at lower wavelengths. Along the PC2, the negative and positive controls (PBS and BSA, respectively) formed distinct clusters. The remaining treatments clustered between the two controls, reflecting varying degrees of interference. Lipofectamine® 2000 was positioned closest to the negative control, consistent with earlier observations showing minimal spectral alteration. In con-

trast, the two other transfection reagents and the oligoarginine peptides (ARCs) clustered closer to the positive control, confirming their behavior as false analytes. The hierarchical clustering heatmap corroborated these trends, generally grouping replicates of the same compound together, thus demonstrating good reproducibility; minor inconsistencies were observed among the transfection reagents. Nevertheless, the overall clustering patterns aligned well with the spectral data, providing further support for the classification of interference types based on the full-spectrum profiling.

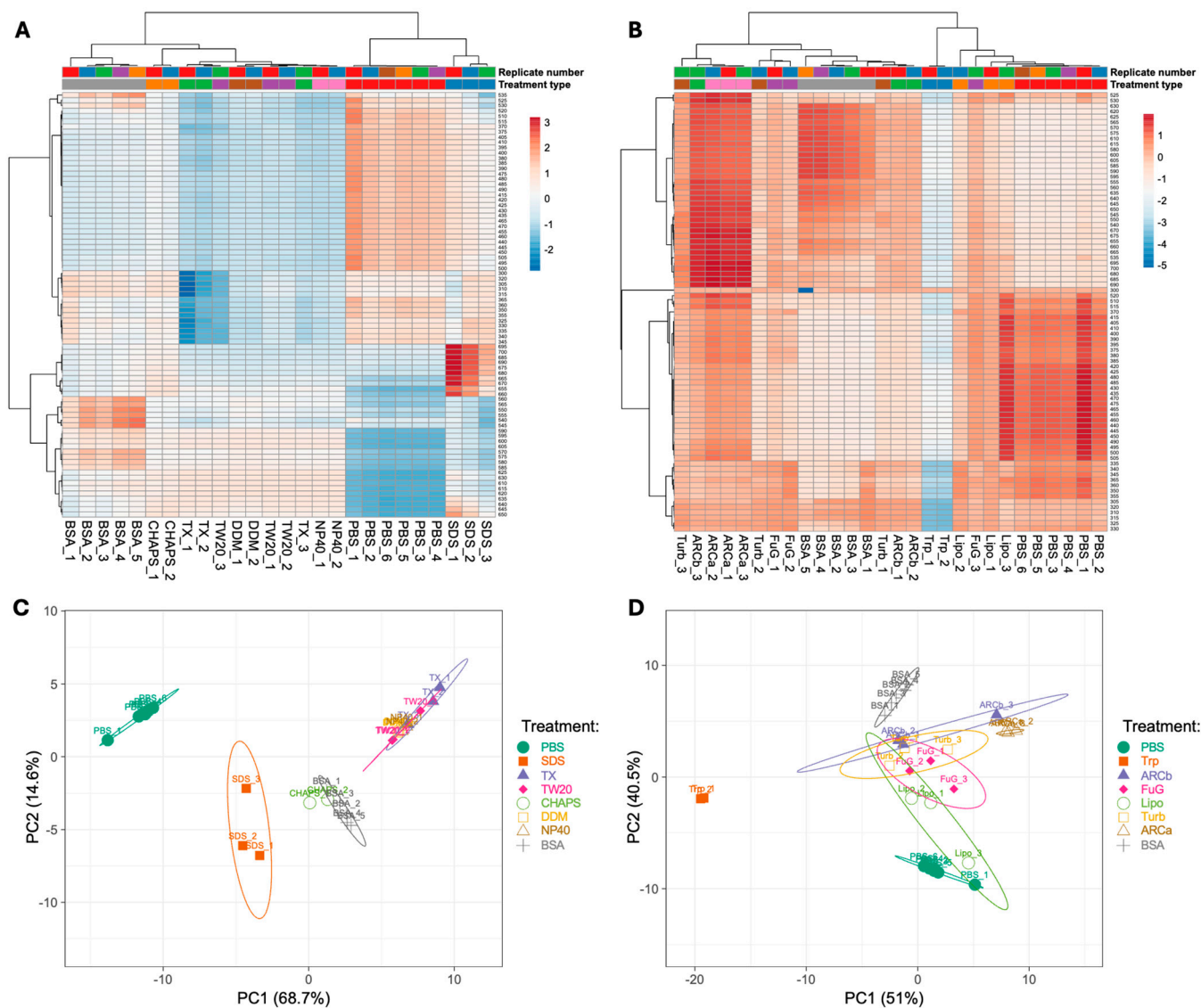


Figure 3. Clustering of interfering compounds based on the absorbance spectrum shift of CBBG dye in the absence of analytes. **(A,B)** Heatmaps based on the normalized absorbance spectra of CBBG dye in the presence of PBS only (negative control), BSA (positive control), or various interfering compounds at the highest concentration tested (abbreviations: TX, Triton X-100; TW20, Tween-20; ARCa, ARC-902; ARCb, ARC-1041; FuG, Fugene® 6; Lipo, Lipofectamine® 2000; Trp, L-tryptophan; Turb, TurboFect). Sample annotations are listed at the bottom (different numbers represent independent experiments), and wavelength annotations on the right side of each panel. **(C,D)** PCA plots based on the same data as in the case of heatmaps. Sample annotations are listed on the right side of each panel (different numbers represent independent experiments); the circles represent confidence intervals (95% probability).

3.6. Comparison with the Lowry Assay Reveals Assay-Specific Susceptibility to Interference

Finally, to compare the Bradford assay with a widely used alternative protein quantification method, we evaluated the effects of selected novel interfering compounds in the Lowry assay. The latter is based on the Biuret reaction of peptide bonds with copper ions under alkaline conditions, followed by reduction of the Folin–Ciocalteu reagent by copper-treated proteins and selected amino acid residues, resulting in the formation of a blue chromophore measurable at approximately 750 nm [178,179]. Oligoarginine peptides and transfection reagents were selected for comparison, as these compounds were identified among the most prominent novel interfering agents in the Bradford assay, whereas Tween-20 was used as a positive control, since detergent interference has been reported for the classical Lowry assay.

Calibration curves were generated using both BSA and BGG as protein standards in the presence or absence of the selected compounds. Representative calibration curves are shown in Supplementary Figure S9, while the corresponding slopes obtained by linear regression analysis are summarized in Figure 4A. Similarly to the Bradford assay, high concentrations of Tween-20 (0.02%), transfection reagents (1%), and oligoarginine peptides (1 μ M) altered the response of the Lowry assay. However, the direction and magnitude of these effects depended on the protein standard used. In some cases, the slope of the calibration curve increased, whereas in others, it decreased, highlighting the complex chemistry underlying the Lowry assay and complicating mechanistic interpretation of the resulting artefacts.

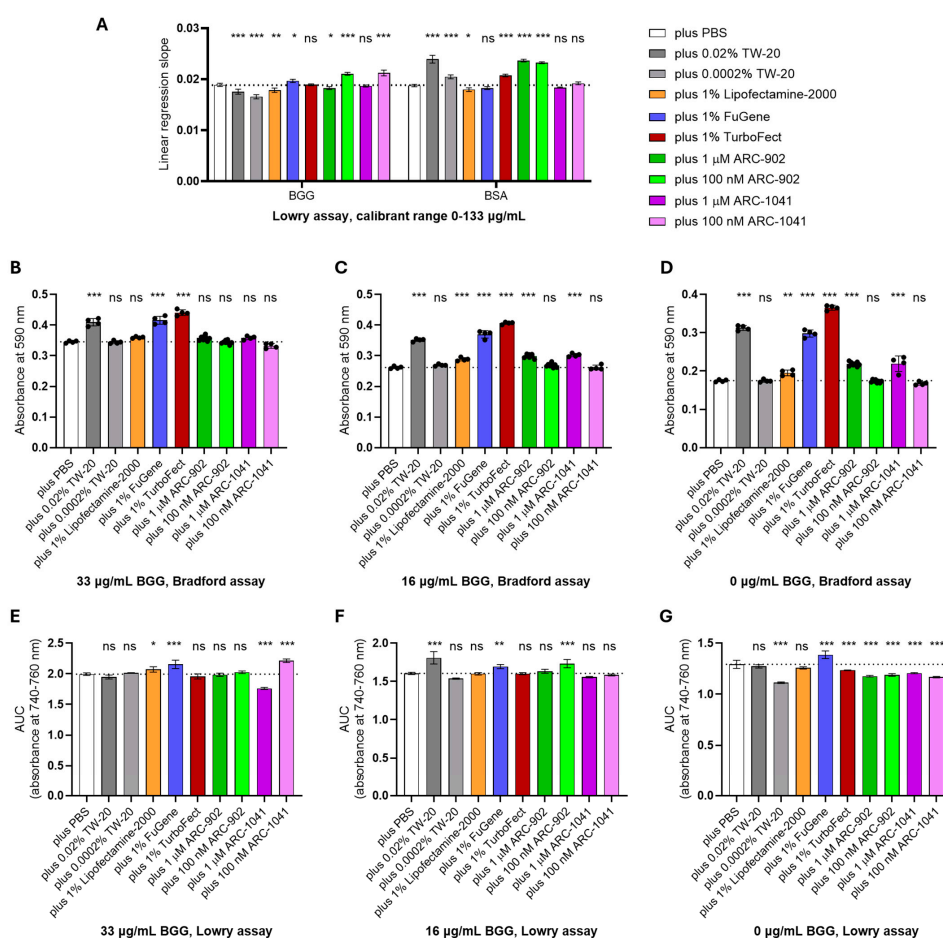


Figure 4. Comparison of interfering effects in Lowry vs. Bradford assays. (A) Lowry assay linear regression slope values for two different calibrants (left, BGG; right, BSA) in the absence or presence

of a set of interfering compounds. (B–D) Bradford assay results at different concentrations of the calibrant (listed at the bottom of each panel; results of individual measurements are shown as dark circles); (E–G) corresponding Lowry assay results. The names and final concentrations of the interfering compounds are listed to the right of panel (A), or below the X-axis in panels (B–G). All panels show mean and standard deviation of replicates for two independent experiments performed in quadruplicate (in the case of ARC-902) or duplicate (for all other compounds); dashed lines show the signal corresponding to the negative control. In all panels, asterisks indicate statistical significance of grouped comparisons related to the data measured in the absence of interfering compounds (one-way ANOVA with Dunnett's test for multiple comparisons): *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns stands for non-significant.

To enable a direct comparison between the Bradford and Lowry assays, signal changes were further evaluated at representative BGG concentrations of 0, 16.6, and 33.3 $\mu\text{g/mL}$ in the presence or absence of the interfering compounds (Figure 4B–G). Consistent with observations in the Bradford assay, the greatest relative errors were observed at lower protein concentrations, where the contribution of the interfering compound to the total signal was proportionally greater. Nevertheless, the magnitude of the artefacts was generally lower in the Lowry assay. While the Bradford assay consistently produced overestimations of protein concentration in the presence of oligoarginine peptides and transfection reagents, the Lowry assay exhibited a more variable response, with both increases and decreases in signal observed depending on the interfering compound and calibration standard employed. These findings suggest that the newly identified interfering compounds are not exclusive to the Bradford assay, although their impact appears substantially less pronounced in the Lowry method.

4. Conclusions

In conclusion, this study serves as a reminder that despite its technical simplicity, speed, and widespread use, the Bradford assay remains vulnerable to interference from a range of commonly used laboratory compounds. Our findings demonstrate that the nature of interference is not uniform; rather, it varies depending on the chemical properties of the interfering substance and, in some cases, mimics the spectral response of genuine protein analytes. At the same time, the large number of interference reports should not be interpreted as evidence that the Bradford assay lacks utility. Rather, its continued widespread adoption across diverse research fields has enabled researchers to identify and document assay-specific artefacts under many different experimental conditions. Our results underscore that interference effects cannot always be reliably corrected by simple adjustment of the calibration curve according to the intercept value. Spectral analysis provides valuable information for distinguishing between different modes of interference, yet comprehensive evaluation—particularly across a range of analyte concentrations—is essential for accurate interpretation. Notably, the newly identified interfering compounds also affected the Lowry assay, although their impact was generally less pronounced than in the Bradford assay. This observation suggests that assay substitution alone may not fully eliminate interference-related artefacts and highlights the importance of validating protein quantification methods within the context of the specific sample matrix and experimental workflow.

Among the limitations of this work is the fact that only 29 compounds were experimentally validated as potential interferents in the Bradford assay, and only classical formulations of the Bradford assay and Lowry assay were used. Still, we hope that this work contributes to renewed awareness within the proteomics and biochemical research communities about the limitations of the Bradford assay and encourages more informed and critical application of this widely used method. By highlighting common sources of interference and their underlying mechanisms, we aim not to discourage the use of the assay, but rather to support its more reliable implementation in contemporary research workflows.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/sci8070145/s1>, Figure S1: Structures of oligo-D-arginine conjugates ARC-902 and ARC-1041 used as the Bradford-interfering compounds in the current study; Figure S2: Examples of the analyte calibration curves measured by the Bradford assay in the absence or presence of Ca^{2+} and Mg^{2+} ions; Figure S3: Examples of the analyte calibration curves measured by the Bradford assay in the presence or absence of the compounds from the validation set that did not interfere with the assay; Figure S4: Assessment of the interfering effect of various compounds on the signal of CBBG in the absence (panel A) vs. presence (panel B) of Ca^{2+} and Mg^{2+} ions; Figure S5: Dose–response curves for the compounds of interest (COI) after addition of CBBG in the absence of analyte; Figure S6: Absorbance spectra of Coomassie Brilliant Blue G-250 (CBBG) in the presence of interfering compounds (subset A); Figure S7: Absorbance spectra of CBBG in the presence of interfering compounds (subset B) or BSA; Figure S8: Examples of the BGG calibration curves measured by the Bradford assay in the presence or absence of the interfering compounds; Figure S9: Examples of the analyte calibration curves measured by the Lowry assay in the presence or absence of the interfering compounds; Supplementary Methods—Suggested Bradford assay protocol.

Author Contributions: Conceptualization, D.L.; methodology, D.L.; validation, all authors; formal analysis, all authors; resources, D.L.; writing—original draft preparation, N.N., E.L. and D.L.; writing—review and editing, all authors; visualization, D.L.; supervision, D.L.; project administration, D.L.; funding acquisition, D.L. All authors have read and agreed to the published version of the manuscript.

Funding: D.L. was supported by internal financing from the Institute of Chemistry, University of Tartu, Estonia (PLTKTARENG21).

Data Availability Statement: The raw data corresponding to Table 2, Figures 1 and 2 and Supplementary Figures S2–S7 are available as GraphPad Prism 6 or GraphPad Prism 10 files from the free online repository FigShare (<https://doi.org/10.6084/m9.figshare.30749102>).

Acknowledgments: We thank Ago Rinken group members for the maintenance of the microplate reader platform.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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