

Mass Spectral Compatibility of Four Proteomics Stains

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Received June 27, 2007

With the recent introduction of new fluorescence stains to the proteomics market, there is now more choice available. SYPRO Ruby, LavaPurple, Flamingo, and Krypton total protein stains were compared for ease of use, image quality, and compatibility with protein identification by peptide mass fingerprinting (PMF) (MALDI-TOF). All four stains produced good images but with slightly different staining patterns. SYPRO was found to inhibit identification of cysteine and tryptophan containing peptides, which reduced protein identification.

Keywords: peptide mass fingerprinting • epicocconone • Deep Purple • LavaPurple • Krypton • SYPRO Ruby • Flamingo • fluorescence • total protein stain

Introduction

Two-dimensional gel electrophoresis is one of the most widely used techniques in proteomics today.¹ Recent advances in pre-fractionation techniques and sample partitioning have enabled thousands of spots to be separated on a single large format gel.^{2–4} In addition, advances in mass spectrometry (MS) now allow proteins to be accurately identified from as little as femtomole or attomole amounts of sample.⁵ Of the MS methods available for down-stream identification, the most popular is MALDI-TOF, either with or without subsequent tandem MS (MS/MS), as it lends itself to high-throughput analysis techniques.⁶ Although it is theoretically possible to identify any visualizable spot on a protein gel, this is rarely the case. A gel of over a thousand spots may produce as few as 300 significant matches.⁷ Sample processing is a major factor affecting how many peptides are recovered from each spot and, ultimately, whether a significant identification occurs.⁸

In the 20th century, the most common gel stains used were silver and Coomassie brilliant blue (CBB R-250 or G-250). Both of these stains are colorimetric in nature, relying on densitometry for image analysis. CBB, an organic dye (Scheme 1; **I**), was developed a number of decades ago and is believed to complex with arginine, lysine, tyrosine, and histidine residues in proteins.⁹ This stain is cheap and easy to use and has been found to be compatible with MS, though there have been reports that the long staining and/or destaining times in strong acid and methanol leads to random methylation of glutamic acid, aspartic acid, glutamine, and asparagines.¹⁰ However, CBB is still a popular choice for staining, prior to MS.¹¹ Several variations of protocols have been developed over the years to reduce background staining in the gel and increase sensitivity,

leading to a colloidal Coomassie (CCB G-250) procedure that allows for detection down to tens of nanograms per spot.^{12,13} These procedures do still lack the sensitivity of silver or fluorescent protocols and have a limited dynamic range (between 1 and 2 orders of magnitude), making the stain ill suited for quantification and identification of minor components of complex samples such as plasma, where there is a large range in protein concentration.⁷ Recently, CBB R-250 has been used to quantify proteins in gels and blots by infrared fluorescence;¹⁴ however, this technique requires specialized equipment and has sensitivity no better than other fluorescent stains.¹⁵

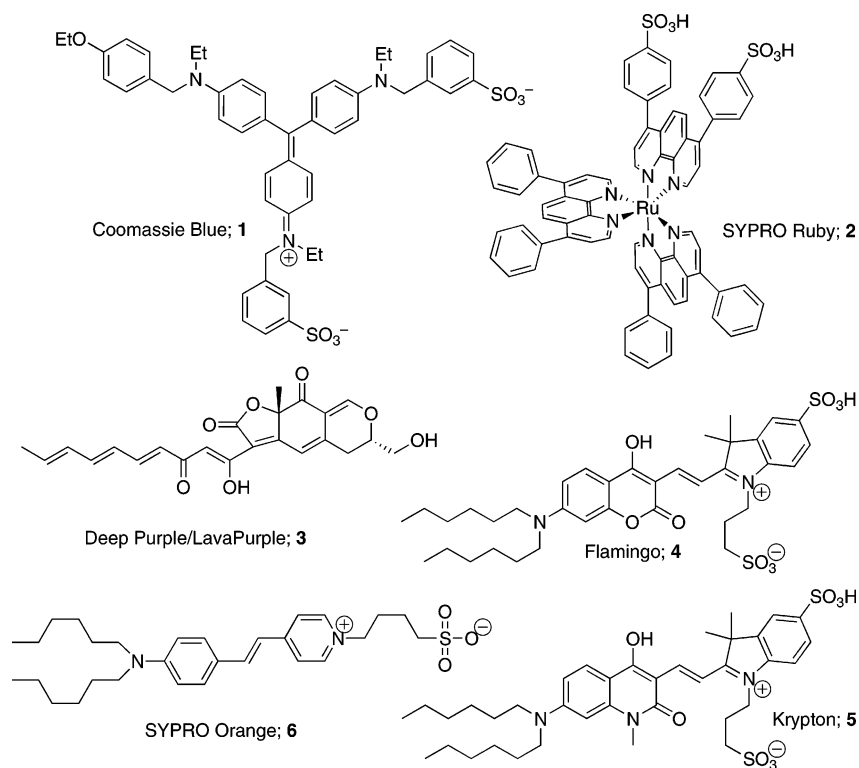
Silver staining protocols are still some of the most sensitive procedures available, involving the binding of silver ions to the peptide core of a protein followed by precipitation of metallic silver.¹⁶ The many variations of this procedure can produce femtomole sensitivity; however, results are highly variable, sensitive to small variations in timings of the many steps, and after all have a low dynamic range.^{17,18} Although advances have been made in the protocols, mass spectral compatibility is still an issue.^{19,20}

The limitations of traditional colorimetric stains have led to the development of a number of fluorescent stains that have sensitivities approaching or equaling silver and large dynamic ranges allowing accurate quantification over as many as 4 orders of magnitude of protein concentration in gels.²¹ Four such stains are SYPRO Ruby (Invitrogen), LavaPurple (FLUORotechnics, also known as Deep Purple from GE-Healthcare), Flamingo (BioRad), and Krypton (Pierce). SYPRO Ruby, the first and most commonly used fluorescent dye, is a formulation of a bathophenanthroline complex (Scheme 1; **2**) of ruthenium (II) that, like CBB (**I**), associates with cationic residues on the protein. The success of SYPRO Ruby has generated a number of SYPRO copies such as RUBEO (Geno-Tech) and Lumitein (Biotium) among others. The ruthenium complex is inherently fluorescent and needs to be washed from the gel to reduce nonspecific background staining.²² The stain

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Scheme 1. Structures of Common Fluorescent Stains for Gel Electrophoresis

has been shown to have significantly better quantitative characteristics than the colorimetric stains while achieving similar low nanogram sensitivity as silver.²³

LavaPurple is a fluorescence stain based on epicocconone (3)²⁴ that was introduced soon after SYPRO Ruby as Deep Purple total protein stain (GE Healthcare).²⁵ Epicocconone has low fluorescence in the green until it reacts with basic amino acids to form an internal charge transfer (ICT) complex that is highly fluorescent in the orange/red. The dye also associates with the SDS shell around proteins in gels, thereby increasing its quantum yield by an order of magnitude. This dual mechanism requires minimal destaining to produce low background fluorescence. A unique feature of epicocconone is that the covalent binding to proteins is reversible depending on the pH.²⁶ The stain has been shown to be sensitive in the picogram (femtomole) range for proteins in gel electrophoresis and to exhibit greater than 4 order of magnitude of linearity.²⁷ In addition, the reversible nature of the stain means that it is easily removed under the conditions used for tryptic digestion of proteins prior to MS analysis by peptide mass fingerprinting (PMF). Although the extinction coefficient for epicocconone is modest ($\epsilon = 20\,000$), it increases dramatically on formation of the ICT complex with proteins. Another convenient feature of LavaPurple is that the ICT complex has two excitation maxima (390 and 490 nm) and long Stokes' shift (>100 nm), allowing manual spot picking on a UVA transilluminator (365 nm) or black-light illumination (~ 400 nm). SYPRO Ruby also has a bimodal excitation profile (280 and 450 nm), which allows both imaging of gels with a CCD camera system or blue-laser scanners and manual spot picking on a UVB transilluminator.

Flamingo (4) and Krypton (5) are two recently introduced stains that show considerable promise. Both are coumarin–cyanine dye hybrids originating from Dyomics²⁸ that associate with the SDS shell around proteins in gels. Little literature is available for these stains as they have only recently been

introduced, though it is suggested that they provide sensitivities comparable to SYPRO Ruby and are compatible with down-stream processing by MS.^{15,29,30} One possible shortcoming is that even though the extinction coefficient for Krypton is high ($\epsilon = 105\,000$), the Stokes' shift is small in the presence of proteins (34 nm; $\lambda_{\text{ex}} = 518$ nm, $\lambda_{\text{em}} = 552$ nm). Similarly, Flamingo's Stokes' shift changes from ~ 60 to 30 nm ($\lambda_{\text{ex}} = 515$ nm, $\lambda_{\text{em}} = 545$ nm) in the presence of protein. Both stains have small excitation maxima around 270 and 320 nm, making manual spot cutting using a UV transilluminator difficult or impossible. There are three papers that compare fluorescent staining in gels that focus on spot detection and photostability.^{15,21,31} The aim of this paper is to look in detail at the mass spectral compatibility of four common fluorescent gel stains.

Experimental Section

Materials. All gels, focusing strips, Flamingo protein stain, and reagents other than those stated below were purchased from BioRad (Hercules, CA). LavaPurple (also known as Deep Purple; GE-Healthcare) was obtained from FLUORotechnics (Sydney Australia), Krypton protein stain was purchased from Pierce Biotechnology (Rockford, IL), and SYPRO Ruby was purchased from Invitrogen (Carlsbad, CA). Proteomics-grade trypsin, boric acid, and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO), and glycerol was purchased from BDH Chemicals (Poole, UK).

Gel Electrophoresis. Electrophoresis standards (BioRad 161–0320; 36 μL) that are prerduced were dissolved in isoelectric focusing buffer (3 mL; 7 M urea, 2 M thiourea, 65 mM DTT, 2% CHAPS, 2% sulfobetaine 3–10, 1% Biolyte 3–10, 40 mM Tris, 0.002% bromophenol blue). The sample was divided into 12 aliquots (240 μL each) and used to rehydrate 12 \times 11 cm pH 3–10 strips (BioRad, 163–2016) from a single batch. The strips were focused using an IpG-phore 3 unit (GE-Healthcare)

for a total of 65 000 Vh. The strips were washed in second dimension equilibration buffer containing the standard alkylation step (50 mL; 375 mM Tris-HCl containing 6 M urea, 2% w/v SDS, 5 mM tributylphosphine, 2.5% acrylamide, 20% glycerol) for 2×10 min then embedded onto the top of Criterion 8–16% Tris-HCl gels (BioRad, 345–0041) with 0.5% agarose containing 0.01% bromophenol blue as a tracking dye. The second dimension was run using a Dodeca unit (BioRad) with a constant voltage (200 V) for approximately 1.5 h (until the tracking dye had just run off the end of the gel). The gels were then randomized, and three gels were stained with each of the four dyes.

Gel Staining and Image Analysis. Gels were stained as per the manufacturers recommended protocol for the highest sensitivity results. Briefly, gels stained with LavaPurple were fixed overnight in 15% ethanol, 1% citric acid; stained for 1 h in a 1:200 dilution of LavaPurple concentrate in 100 mM sodium borate buffer pH 10.8; destained for 30 min in 15% ethanol; and acidified for 30 min in 15% ethanol, 1% citric acid before being imaged.

Gels stained with Flamingo were fixed in 40% ethanol, 10% acetic acid overnight, stained in 1:10 dilution of stain in water for 3 h, and rinsed in water for 5 min prior to imaging.

Gels stained with Krypton were fixed in 40% ethanol, 10% acetic acid for 2×30 min, washed with water for 5 min, stained in a 1:10 dilution of stain in water (overnight), washed in 5% acetic acid for 5 min and water for 2×15 min, and imaged.

Gels stained with SYPRO Ruby were fixed in 50% methanol, 7% acetic acid for 2×30 min, stained in fresh 1 \times stain overnight, washed in 10% methanol and 7% acetic acid for 2×15 min, and imaged.

All gels were imaged with a Typhoon 9200 (GE-Healthcare) equipped with a 532 nm excitation laser and either a 560 long-pass filter (Flamingo, LavaPurple) or 610–30 band-pass filter (Krypton, SYPRO Ruby) and 100 μ m resolution. The photomultiplier tube setting was altered for each stain to optimize sensitivity to background ratios. Imaging of SYPRO gels was performed suboptimally using a 532 nm laser.

Gel images were analyzed using the Analysis Toolbox application of ImageQuant-TL software (GE Healthcare). Spot boundaries were kept constant for each of the stains, whereas spot volumes were averaged and standard deviations calculated to determine the co-efficient of variation for each stain based upon the group average for that stain. Selecting a nonspot area of the gels and using the local average function for boxes of identical sizes determined the background for each stain. Dynamic range is defined here as the difference between the volume of the brightest and faintest spots. Signal to background is defined as the brightest spot volume divided by the average background.

Mass Spectrometry. The two best gels of each stain group were chosen for spot excision. Identical spots from duplicate gels of each stain were manually excised using either a traditional transilluminator (Flamingo and SYPRO Ruby), a transilluminator that has been modified with black-light globes (NEC FL-15 BL-B; LavaPurple), or by printing a 1:1 ratio image onto paper placing the image under the gel that is resting on a clean plastic sheet (Krypton). All gels were reimaged to confirm correct excision of spots. PMF analyses were performed commercially by the Australian Proteome Analysis Facility (APAF) under the following conditions. The gel plugs were placed into a low protein binding 96 well plate. Plugs were destained by sonication in 50% acetonitrile/25 mM ammonium

bicarbonate for 3×20 min. The plugs were then dehydrated and reswelled with 25 mM ammonium bicarbonate containing 15 ng/ μ L proteomics grade trypsin. The plugs were allowed to take up the buffer for 1 h at 4 °C, and then excess trypsin was removed and 20 μ L of 25 mM ammonium bicarbonate was added. The plate was then incubated at 37 °C for 16 h. The lysate was desalted and concentrated on ZipTip C18 pipet tips (Millipore) before being loaded onto a MALDI target in matrix (α -cyano-4-hydroxycinnamic acid, 4 mg/mL in 70% v/v acetonitrile, 0.1% v/v TFA) and allowed to air-dry. MALDI mass spectrometry was performed on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics in MS mode. A Nd:YAG laser (355 nm) was used to ablate the sample. The spectra were acquired in reflectron mode in the mass range 500–4000 amu after calibration to the trypsin autolysis peak at 2211.105 in every run.

The data was analyzed by APAF using standard search parameters³² with a mass accuracy of 50 ppm. Peaks were filtered to include only 500–4000 amu with a maximum of 200 peaks/sample. A minimum S/N of 20 and minimum area of 200 was required for each peak and no more than 30 peaks per 200 Da was allowed. Peptide matching allowed for up to 1 missed cleavage and the following fixed modifications; carbamidomethyl and propionamide of cysteine and variable modifications; oxidation of cysteine and/or methionine were allowed.

The data were exported in a format suitable for submission to the database search program MASCOT (Matrix Science Ltd, London, UK). High scores (Mowse score > 79) in the peptide database search that indicated a likely match were confirmed or qualified by operator inspection. Proteins that were found to have keratin contamination were removed from the calculations.

Statistical Analysis. The number of proteins that were identified by each stain was determined as a proportion of the total number of spots cut from the gel and the success rate of each stain compared using a proportion test of the two populations. Peptides that were recovered from the spots of each stain were analyzed for amino acid composition and compared to the theoretical composition of the proteins using the database tool ProtParam (Swiss-Prot). Any amino acid that was observed to vary by more than 25% from the theoretical was selected for further peptide analysis. Proteins were theoretically digested using PeptideMass (Swiss-Prot), and the resultant peptides were compared to those recovered from each of the stains. Any statistical variation in amino acid content was calculated using a pair-wise comparison (Supporting Information).

Results and Discussion

Staining and Image Analysis. Staining of gels according to the manufacturer's directions produced consistent gels that were quite similar (Figure 1). Spots were counted by eye, and all stains produced a similar number of identifiable spots (SYPRO Ruby 34; Flamingo 38; Krypton 39; and LavaPurple 41); however, all four of the stains produced images that varied from that reported by BioRad when stained with silver.³³ Even though there are only 7 proteins in the 2D-proteins standards, there are several isoforms, and all of the fluorescent stains were able to reveal a greater number of spots than silver staining.

Variability of the staining intensity of the same spots between the triplicates of each stain (coefficient of variation; CV), the dynamic range of staining (defined here as the difference

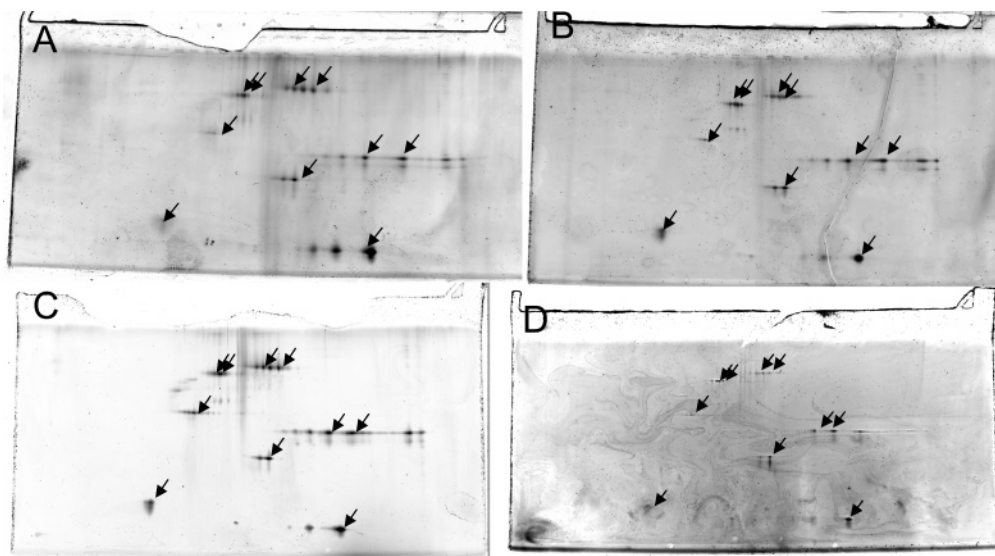


Figure 1. BioRad Criterion 8–16% gels that have been stained with (A) LavaPurple, (B) Flamingo, (C) Krypton, and (D) SYPRO Ruby. The gels were loaded with BioRad 2D standards. Gels were run in triplicate and analyzed with ImageQuant software, and spots were cut from duplicate gels. The spots were then digested and analyzed using a Applied Biosystems 4700 MALDI-ToF system. The arrows indicate the spots excised from each gel for PMF analysis.

Table 1. Analysis of Differences of Staining for the Four Stains Tested

protein	SYPRO Ruby		LavaPurple		Flamingo		Krypton	
	total (%) ^a	CV (%) ^b	total (%)	CV (%)	total (%)	CV (%)	total (%)	CV (%)
conalbumin	19.9	12.0	19.7	21.6	22.8	15.8	20.1	23.2
BSA	8.4	3.9	7.2	37.3	8.2	7.2	8.4	35.2
actin	6.3	5.4	2.0	23.1	6.9	55.6	4.3	18.4
GADPH	27.1	5.0	20.4	12.4	26.3	12.0	21.0	44.3
carbonic anhydrase	13.4	5.7	6.0	15.4	6.7	19.9	7.9	52.4
soybean trypsin inhibitor	9.6	3.6	1.9	15.7	4.2	4.6	5.8	36.2
myoglobin	15.4	4.1	42.9	15.7	25.0	14.6	32.5	23.9
Average CV ^c		5.7		20.2		18.5		33.4
Dynamic range ($\times 10^7$) ^d	0.4	3.6	4.6	12.4	1.8	13.2	2.3	14.2
Background (counts)	6491	2.4	6242	15.6	5230	31.7	767.3	10.5
Signal to background ^e	616:1	1.3	7530:1	6.6	3780:1	38.3	30145:1	22.2

^a Proportion of total fluorescent density accounted for by each protein, including all visible isoforms. ^b Variation observed between three duplicate gels. ^c Average of all the CV values for proteins in the sample. ^d Defined as the difference between the volume of the brightest and faintest spots. ^e Defined as the brightest spot volume divided by the average background.

between the faintest and darkest spot), and the background staining of each stain (measured by the average counts of a region of the gel with no protein spots) were all compared (Table 1). SYPRO Ruby exhibited the least variation between the three gels with an average CV of 5.7%. The variation in LavaPurple (20.2%) and Flamingo (18.5%) were similar whereas Krypton was higher (33%). The relative dynamic range of the stains was determined by subtracting the volume of the faintest spot of each stain from the brightest spot of the same gel. As no internal standard is used for normalization, this is only a relative measure. SYPRO Ruby-stained gels had the smallest dynamic range (4.0×10^6 counts), whereas the other three stains were similar and an order of magnitude better than SYPRO Ruby. The exceptionally low background exhibited by Krypton stained gels meant that it had by far the highest signal to background ratio (30135:1). The other three stains had similar backgrounds.

With the availability of several fluorescent stains, it is now possible to select from a range of products, all providing sensitivity in the range of silver but with expanded dynamic ranges. The stains studied here all provide high quality images

that allow quantification of proteins from 2D gels by volume analysis of spots. The staining protocols used, however, affect the ease with which the gels may be used for downstream processing (Table 4). SYPRO Ruby, Flamingo, and LavaPurple all have relatively simple protocols that require little hands-on time, and each stain could be used to excise the spots from the gels using readily available transilluminators. The use of methanol and high concentrations of acetic acid in the fixative and destain solutions make SYPRO Ruby and Flamingo somewhat more difficult to use than LavaPurple, especially on a large scale as would be required for high-throughput proteomics. Krypton gel stain requires a large amount of the workers time with 7 steps and seems to contain cyanide (smell of bitter almonds) so that gel handling had to be carried out in a fume hood. In addition, the stain did not fluoresce using any of the illuminators available to the researchers (UVA, UVB, UVC, or black-light illumination), making spot cutting difficult and time consuming.

All four stains tested produced images that were suitable for cutting of spots, though SYPRO Ruby had a relatively high background and thus a lower signal-to-noise ratio (Table 1).

Table 2. Results from the PMF Analysis of Spots Cut from 2D Gels

stain	% of total identified	average coverage	average number peptides	average Mowse score	average missed cleavage	number of false positives ^a
SYPRO Ruby	58	40	14.7	87.0	5.2	1
LavaPurple	89	59	24.3	129.5	9.3	0
Flamingo	63	58	24.5	145.6	11.2	3
Krypton	80	53	20.7	118.3	11.6	1

^a Number of identifications from MASCOT that are not in the standard mix.

This may be due, in part, to the suboptimal imaging of the SYPRO gels (532 nm excitation instead of 488 nm). Although producing similar results overall, each stain had distinct staining patterns for each of the protein spot groups. Comparative data of the staining pattern of each stain is presented in Figure 1. Flamingo was the only stain to identify a fifth isoform of GADPH and had a staining pattern roughly the same as Krypton, as would be expected. LavaPurple stained soybean trypsin inhibitor (STI) poorly, and SYPRO Ruby stained neither STI nor myoglobin efficiently.

Though there is significant literature describing the staining characteristics of SYPRO Ruby compared to traditional colorimetric stains¹¹ and Deep Purple,¹⁵ little has been published regarding the comparison of the two other fluorescent stains described here.

Protein Identification by PMF. The amount of protein loaded onto each gel, according to the manufacturer's instructions, is sufficient for CBB G-250 visualization. This amount was chosen to ensure that sufficient protein would be present for identification by MALDI-TOF PMF. Spots were selected from each protein group that had the highest staining density (Figure 1) and, where appropriate, multiple spots from each protein were used.

LavaPurple was able to identify 89% of the spots cut, followed by Krypton with 80%, Flamingo with 63%, and SYPRO Ruby with 58% (Table 2). Of the proteins that were identified, the average coverage was similar for LavaPurple, Flamingo, and Krypton (58.5, 58, and 53.1%, respectively), with SYPRO Ruby-stained proteins being slightly less well covered (40%). Thus, the average number of peptides recovered for Flamingo, LavaPurple, and Krypton were also similar (24.5, 24.3, and 20.7, respectively) whereas SYPRO Ruby-stained gel plugs returned on average 14.7 peptides. All stains returned MASCOT scores above the identification threshold,³⁴ Flamingo returned the highest average score (145.6), followed by LavaPurple (129.5), Krypton (118.3), and SYPRO Ruby (87). When analyses allowed 1 possible missed cleavage, SYPRO Ruby returned the least number of peptides that contain missed cleavages in the proteins identified (5.2 on average; 35%), LavaPurple produced the next lowest average count (9.3; 38%), and Flamingo and Krypton had similar numbers of peptides that contained missed cleavages recovered (11.2; 46% and 11.6; 56%, respectively). Though not widely reported, missed cleavages in tryptic digestion is a common phenomenon with >40% of peptides identified generally containing at least one missed cleavage.³⁵ The average number of missed cleavages with LavaPurple was taken as evidence that the reversible-covalent binding mechanism of epicocconone to lysine residues does not produce more missed cleavages in PMF analysis as has previously been postulated. Of note was the fact that SYPRO Ruby, Flamingo, and Krypton all returned at least one false positive, with MASCOT scores that were above the acceptance threshold set by MASCOT (79), significant coverage (38–46%), and number of peptides recovered (between 11 and 24). In all cases, the

Table 3. Comparison of Identical Spots Cut from Duplicate Gels of the Various Gel Stains

stain	number of identifications on both gels	average difference in coverage (%)	average difference in no. of peptides
SYPRO Ruby	4	7.5	2.8
LavaPurple	6	5.1	1.5
Flamingo	4	7.3	5.0
Krypton	7	9.9	6.1

Table 4. Comparison of the Staining Protocols for the Four Stains Tested

	SYPRO Ruby	LavaPurple	Flamingo	Krypton
Fix time	60 min	overnight	overnight	65 min
Stain time	overnight	60 min	180 min	overnight
De-stain time	60 min	60 min	10 min	35 min
No. of steps	5	4	3	7
Total time ^a	120 min	120 min	190 min	100 min
Noxious fumes	Yes	No	Yes	Yes

^a Excluding overnight step

protein involved was GADPH. Krypton and SYPRO Ruby both returned a single such result (identifying the source of GADPH as porcine or bovine, respectively) and Flamingo produced 3 such results (identifying 3 of 4 rabbit GADPH spots as bovine). Reducing the allowable mass accuracy to 20 ppm (data not shown) decreases the number of false positives for Krypton and Flamingo to zero and one, respectively.

The ability of each stain to reproducibly identify proteins was examined by comparing the 10 duplicate spots cut from gels (Table 3). Krypton identified the highest number of spots in both gels with 7 of 10 followed by LavaPurple with 6 and Flamingo and SYPRO Ruby both with 4. Spots that were identified in both gels were then compared for similarities in sequence coverage and number of peptides recovered. LavaPurple exhibited the least variability between identical spots identified (5.1% average difference in sequence coverage and an average difference of 1.5 peptides recovered). SYPRO Ruby and Flamingo had similar variation in the coverage (7.3% compared to 7.5%); however, SYPRO Ruby had less variation in the number of peptides recovered (an average difference of 2.8 compared to 5.0). Krypton had the most variation in coverage (an average difference between sequence coverage of 9.9%) and number of peptides recovered from each spot (6.1 peptide difference on average).

Downstream processing of protein spots is still predominantly performed using MALDI-TOF PMF as it is fast and cost-effective in time and resources.³⁶ Protein standards were used for this study because the sample covers a range of pIs and molecular weights without introducing the complexity of an unknown sample, thus making it possible to detect false positive results from the PMF analysis. It was thus possible to limit outside influences on the MS results and more accurately determine what effect each of the stains had on peptide

Table 5. Statistical Analysis of Peptides Recovered from the Four Stains Compared to Theoretical Values for All Proteins

	theoretical peptides		SYPRO Ruby	Lava Purple	Flamingo	Krypton
	no missed cleavages	including one missed cleavage				
Total peptides	200	487	166	419	433	433
Percentage of Pos+ charged amino acids	12.1%	12.1%	10.7%	10.4%	11.1%	10.1%
Percentage of Neg- charged amino acids	13.6%	13.6%	13.1%	14.0%	14.5%	14.3%
Cys peptides	29.0%	33.5%	9.0% $p \ll 0.001$	33.5%	33.5%	38.9%
His peptides	27.0%	31.4%	42.8% $p < 0.01$	35.1%	35.8%	43.6% $p < 0.001$
Met peptides	17.0%	20.3%	31.9% $p < 0.01$	26.0%	21.7%	25.6%
Trp peptides	13.0%	15.4%	4.8% $p < 0.01$	12.9%	12.5%	15.1%

recovery. In theory, it should be possible to recover all peptides from a protein digested with trypsin; however, in practice, it is rare for coverage to exceed 50% due to many of the peptides being too small to see by MS and loss of other peptides during processing.³⁷ Poor MS results have also been blamed on staining procedures particularly with SYPRO Ruby,^{38,39} though the mechanism of staining for SYPRO Ruby has been reported to be noncovalent and reversible.²¹ There is little in the literature regarding the effect of Flamingo or Krypton on MS, though both are claimed to be compatible with PMF.^{40,41} Certainly the mode of action as deduced from their structures would indicate both form noncovalent complexes with the SDS shell around proteins in the gel similar to SYPRO Orange (6)⁴² and should not affect PMF results.

Using a two-population test of proportions, LavaPurple was found to identify significantly more proteins than either Flamingo or SYPRO Ruby ($p < 0.05$; Table 5). Although LavaPurple also identified more proteins than Krypton, it was not found to be statistically significant ($p > 0.05$). There was no significant difference between the other stains. No significance was found in the difference of coverage, number of peptides, or missed cleavages. It has been suggested by Tannu et al.³⁸ that Deep Purple provides better coverage of proteins than SYPRO Ruby-stained proteins due to the fact that epicocconone binds to lysine residues, resulting in more missed cleavages and thus greater coverage. Our results show that this is not the case as LavaPurple did not show statistically any greater number of missed cleavages than the other stains (Table 2).

All of the protein stains (except LavaPurple) produced at least one false positive protein identification as determined by the selection criteria used (Supporting Information). In each case, the false identification was a related protein from another species, which can be filtered out in the database search parameters when the sample is from a known source. However, errors can occur intra-species; for example, an estrogen receptor was recently falsely identified as being present in a human mitochondrial extract using standard PMF selection criteria (at least four peptides recovered, with a sequence coverage of >25% and a Mowse score >79), suggesting that these criteria may need to be more stringent.^{43,44} The surprising result that SYPRO Ruby gel staining may be responsible for reduced mass spectrometric compatibility prompted us to investigate whether there was any particular characteristic associated with the peptides recovered from gel-spots when different stains are used (Table 5). The other stains also identified all peptides identified with SYPRO Ruby, and there was no statistical difference between the percentages of acidic or basic amino acid-containing peptides identified. A statistical analysis of the peptides identified in all the gels led to the conclusion that SYPRO Ruby had a reduced incidence of peptides containing

cysteine and tryptophan, compared to the theoretical recovery allowing for 0–1 missed cleavages (Table 5). This is consistent with the finding of Lanne et al.³⁷ who recovered no cysteine-containing peptides from SYPRO Ruby stained gels when alkylation was performed on a BSA standard in solution prior to gel running whereas 2–4 peptides were recovered from CBB stained gels. These data suggested that the ruthenium atom of the dye (2) may be reacting in a ligand exchange reaction. Whereas the Ru(II) pyridyl complexes are generally considered to be inert to substitution, under the conditions of tryptic digestion (pH 8.5), all cysteine residues can be expected to be cysteinyl anions, powerful nucleophiles, that have previously been reported to readily form complexes with ruthenium through ligand displacement.⁴⁵ Similarly, tryptophan derivatives are known to form η^6 -complexes with Ru(II) much more readily than phenylalanine or tyrosine.⁴⁶ The formation of any sort of complex between the dye and particular amino acids would be expected to interfere with mass spectral identification of the peptides affected. We also found a significant increase in methionine and histidine containing peptides in PMF results when SYPRO Ruby was used as a stain (Table 5). The other stains produced no significant reductions but Krypton identified a larger number of peptides containing histidine compared to the other stains.

Conclusions

All four stains produce high-quality images with good dynamic ranges and signal-to-background ratios, with Krypton having the best signal to background and SYPRO Ruby the lowest. LavaPurple had the best dynamic range of the four stains with SYPRO Ruby having the lowest. In terms of mass spectral compatibility, LavaPurple had clear advantages with the best coverage, largest number of positively identified proteins, and lowest number of false positives. SYPRO Ruby was found to reduce mass spectral compatibility by reducing the recovery of peptides containing cysteine and, to a lesser extent, tryptophan. Though Krypton and Flamingo are based on similar dyes, significant differences were observed in staining characteristics and peptide recovery, suggesting formulation and staining protocol may play a part in identification efficiency.

Acknowledgment. We thank Narelle Jay of the Australian Proteome Analysis Facility (APAF) for PMF analysis and Neil Hopkins (University of Western Sydney) for advice on statistical analyses and Prof. Duncan Veal for general support and supervision. This research has been facilitated by access to the Australian Proteome Analysis Facility established under the Australian Government's Major National Research Facilities Program.

Supporting Information Available: Statistical analysis of peptide mass fingerprinting data for all four stains studied. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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PR070398Z