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A comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels

A comparison between two fluorescent metal chelates for staining proteins separated by electrophoresis has been carried out. One of these chelates is ruthenium II tris (bathophenanthroline disulfonate) and the other is commercial Sypro Ruby. Both can be efficiently detected either with UV tables or with commercial laser fluorescence scanners. The sensitivity and homogeneity of the stains and the interference with mass spectrometry analysis have been investigated. It appears that both stains perform similarly for protein detection, while ruthenium II tris (bathophenanthroline disulfonate) performs better for mass spectrometry analyses and as cost-effectiveness ratio. However, Sypro Ruby is easier to use as a stain.

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1 Introduction

Proteomics analyses impart special constraints on the detection techniques used after 2-D gel electrophoresis. For example, in addition to the standard sensitivity issues, proteomics put special emphasis on the linearity and on the homogeneity of the staining techniques. Consequently, silver staining, which is still the most sensitive nonradioactive detection technique, is far from ideal in proteomics. The linearity is rather poor [1] and the protein-to-protein variation is known to be important. In addition to these pure detection issues, the detection methods used in proteomics must give minimal interference with the microcharacterization techniques used afterwards, *i.e.* in most cases mass spectrometry analysis by MALDI-TOF or MS/MS. Here again, silver staining is not optimal, and losses in peptide masses or problems in MS/MS have been documented [2].

Apart from silver staining, the most widely used detection technique relies on colloidal Coomassie Blue [3]. While this technique provides much better results in terms of linearity, homogeneity and interference with MS, its sensitivity is much too low. Thus, either high loads of proteins must be used, at the risk of protein losses by precipitation, or the analysis is restricted to major proteins only. There is thus a need for other detection techniques allying a sensitivity close to the one of silver staining to the good features of classical organic stains such as Coomassie Blue. With the development of imaging systems, fluorescence has come again in the field of protein detection for proteomics, with various approaches [4]. One of the approaches is covalent grafting of fluorescent probes onto the protein molecules, with the ability of multiplexing [5]. However, such methods are not trouble-free. As fluorescent probes decrease protein solubility, labelling must be carried out as a trace reaction, with subsequent losses in sensitivity. Even in this scheme, offset migrations of the labelled proteins compared to the bulk of the unlabelled ones are quite common, which induces problems for subsequent spot excision for analysis. Another approach is noncovalent binding of the probe to the proteins after gel electrophoresis. In this case, two different schemes can be used.

In the first scheme, the fluorescent probe has no special affinity for the proteins, but behaves differently in the gel (water-type environment) and on the proteins (more hydrophobic, especially in SDS electrophoresis). In this case, probes that do not fluoresce in water but only in hydrophobic environments are favored. This scheme has been first illustrated by naphtalene sulfonate derivatives [6, 7], and more recently by probes excitable with visible light such as Nile Red [8] or styryl dyes (sold as Sypro Orange and Sypro Red by Molecular Probes (Eugene, OR, USA)) [9]. However, these probes also lack sensitivity, for example compared to silver staining, as the hydrophobic environment imparted to proteins by SDS is highly labile. Any treatment removing SDS from the gel will also do so at the protein level, thereby decreasing sensitivity. Conversely, any replenishment of SDS at the protein level will increase background fluorescence, and thus decrease contrast. In both cases, detection of minor proteins is limited.

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Abbreviation: RuBPS, ruthenium II tris (bathophenanthroline disulfonate)

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In another noncovalent scheme, a fluorescent molecule having a true affinity for proteins is used. In this case, binding of the fluorophore to the proteins will build up a fluorescent image at the correct places. By analogy with the absorptive dyes used for protein detection, acidic molecules containing sulfonic acid groups are likely to bind efficiently to proteins. Unfortunately, sulfo derivatives of classical fluorophores (*e.g.* sulfo rhodamine) do not bind efficiently to proteins. However, metal chelates made with the chelator bathophenanthroline disulfonate bind strongly to proteins [10]. While the iron chelate is not fluorescent, a fluorescent europium chelate has been described with very good sensitivity [11]. This europium chelate is excitable only with UV light, which by far is not the best choice for a linear and space-resolved detection. These limits are broken by ruthenium chelates, first introduced for DNA detection [12]. These chelates can be easily excited with UV and visible light. The use of a pure ruthenium bathophenanthroline disulfonate (RuBPS) has been recently described for protein detection after electrophoresis [13]. In addition, a commercial fluorescent stain for protein detection on SDS gels, IEF gels and blots has been recently described [14–16] under the trade name Sypro Ruby. Although the formulations of these various commercial stains seem to vary for each application and are held secret, it is claimed that these formulations use a ruthenium chelate, and the spectra of Sypro Ruby and RuBPS are quite comparable [13]. We therefore decided to compare pure ruthenium bathophenanthroline disulfonate and Sypro Ruby for protein detection in gels.

2 Materials and methods

2.1 Fluorescent probe synthesis

Ruthenium II bathophenanthroline disulfonate chelate was prepared as follows: 0.2 g of potassium pentachloro aquo ruthenate ($K_2Cl_5Ru.H_2O$), purchased from Alfa Aesar (Karlsruhe, Germany) (26.9% Ru) were dissolved in 20 mL boiling water and kept under reflux. A deep red-brown solution resulted. Three molar equivalents of bathophenanthroline disulfonate, disodium salt, *i.e.* 0.9 g of the anhydrous compound, were added and the refluxing continued for 20 min. The solution turned to a deep greenish brown, and considerable foaming could occur. Meanwhile, a 500 mM sodium ascorbate solution was prepared (10–15 mL are sufficient). Five mL of this solution were then added to the refluxing mixture and refluxing was continued for another 20 min. Here again, considerable foaming occurred. The solution turned rapidly to a deep orange-brown. After cooling, the pH was adjusted to 7 with sodium hydroxide and the volume was adjusted to 26 mL with water. This gives a 20 mM stock solution, which can be stored in the fridge for several months. This protocol was found much more reliable than the ones using hypophosphite [13, 17].

2.2 Gel electrophoresis

Proteins were separated by SDS-PAGE, either in the standard Tris-glycine system, or in the Tris-taurine system [18]. Molecular weight markers (Bio-Rad, Hercules, CA, USA, broad range) were diluted 200 and 2000-fold in SDS sample buffer to reach a concentration range of 10 and 1 ng/μL for each band. The required volumes were loaded on top of a 10% gel to give the adequate concentrations, ranging from 10 to 400 ng *per* band. For 2-D separation, the first dimension was IEF with immobilized pH gradients and sample application by in-gel rehydration [18], using a urea-thiourea mixture as solubilizing agent [19].

2.3 Detection of proteins after electrophoresis

Proteins were detected after electrophoresis either with the pure ruthenium chelate (RuBPS) or with Sypro Ruby. In both cases, staining was performed in polypropylene food boxes, as recommended for Sypro Ruby. In all cases, the solution to gel volume ratio was between 5 and 10.

For Sypro Ruby detection, gels were fixed for 1 h in 20% ethanol 7% acetic acid. They were then placed in Sypro Ruby solution according to the manufacturers instructions [16]. Staining was carried out either for 3 h or overnight. For RuBPS staining [13] , gels were fixed overnight in 30% v/v ethanol 10% v/v acetic acid. The gels were then rinsed 4×30 min in 20% ethanol. Thorough removal of acetic acid is required, as acids strongly quench the fluorescence of the chelate. The gels were then stained for 3–6 h in 20% ethanol containing 100–200 nM of ruthenium chelate, *i.e.* 5 to 10 μL of stock solution *per* liter of staining solution. Finally, in both stains, the gels were reequilibrated in water (2×10 min) prior to imaging, either on a 302 nm UV table or with a laser scanner equipped with a 488 or 532 nm laser. We used a Molecular Dynamics Fluorimager (Sunnyvale, CA, USA) with a 488 nm laser.

2.4 MS analysis

Stained proteins spots or bands were excised (on a UV table for fluorescent detection), and shrunk in 1 mL of 50% ethanol for 2 h. Each gel slice was cut into small pieces with a scalpel, washed with 100 μL of 25 mM NH_4HCO_3, agitated for 8 min with a vortex mixer. After settling of the gel pieces, the supernatant was removed. Gel pieces were dehydrated with 100 μL of acetonitrile for 8 min. This operation was repeated twice. Gel pieces

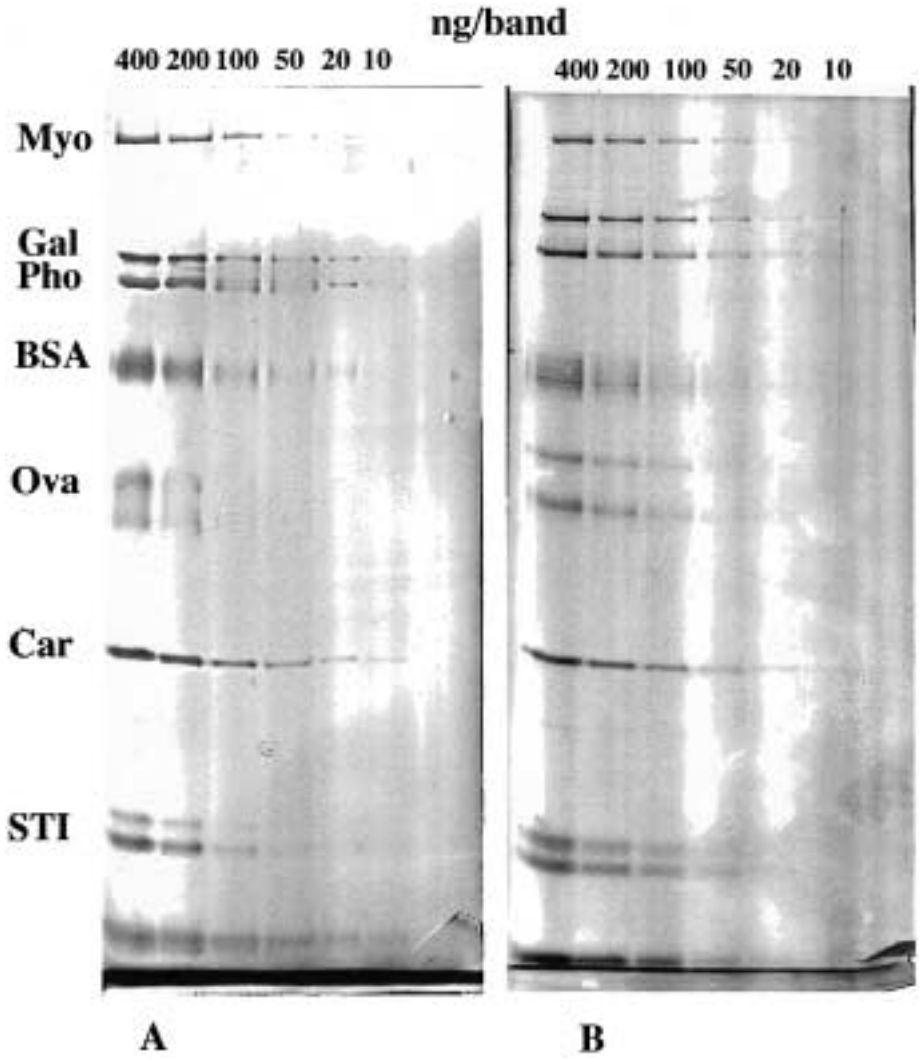

Figure 1. Sensitivity evaluation of RuBPS and Sypro Ruby. Serial dilutions of molecular weight markers (Bio-Rad, broad range) were separated by SDS PAGE (1.5 mm thick gels, 10% acrylamide). The gel was then stained with RuBPS (A) or Sypro Ruby (B). Amount of proteins *per* band are indicated on top of the gel. The protein markers are the following: Myo; myosin heavy chain; Gal: beta galactosidase; pho: phosphorylase A; BSA: bovine seum albumin; Ova: ovalbumin; Car: carbonic anhydrase; STI: soybean trypsin inhibitor

were completely dried with a Speed Vac (Fisher, Strasbourg, France) (15 min) before reduction-alkylation. Gel pieces were covered with 100 μL of 10 mM DTT in 25 mM NH_4HCO_4 and the reaction was left to proceed at 57°C for 1 h. The supernatant was removed, 100 μL of 55 mM iodoacetamide in 25 mM NH_4HCO_4 were added and reaction was left in the dark at room temperature for 1 h. The supernatant was removed and the washing procedure with 100 μL of NH_4HCO_4 and acetonitrile was repeated three times. Gel pieces were completely dried with a Speed Vac before tryptic digestion. The dried gel volume was evaluated and three volumes of trypsin (12.5 ng/μL) in 25 mM NH_4HCO_4 (freshly diluted) were added. The digestion was performed at 35°C overnight. The gel pieces were centrifuged and 5 μL of 25% H_2O/70% acetonitrile/5% HCOOH were added. The mixture was sonicated for 5 min and centrifuged. The supernatant was recovered and the operation was repeated once. The supernatant volume was reduced under nitrogen flow to 4 μL, 1 μL of H_2O/5% HCOOH were added and 0.5 μL of the mix were used for MALDI-TOF analysis.

Mass measurement were carried out on a Bruker (Karlsruhe, Germany) BIFLEX™ MALDI-TOF equipped with the SCOUT™ High Resolution Optics with X-Y multisample probe and gridless reflector. This instrument was used at a maximum accelerating potential of 20 kV and was operated in reflector mode. A saturated solution of α-cyano-4-hydroxycinnamic acid in acetone was used as a matrix. A first layer of fine matrix crystals was obtained by spreading and fast evaporation of 0.5 μL of matrix solution. On this fine layer of crystals, a droplet of 0.5 μL of aqueous HCOOH (5%) solution was deposited. Afterwards, 0.5 μL of sample solution was added and a second 0.2 μL droplet of saturated matrix solution (in 50% H_2O/50% acetonitrile) was added. The preparation was dried under

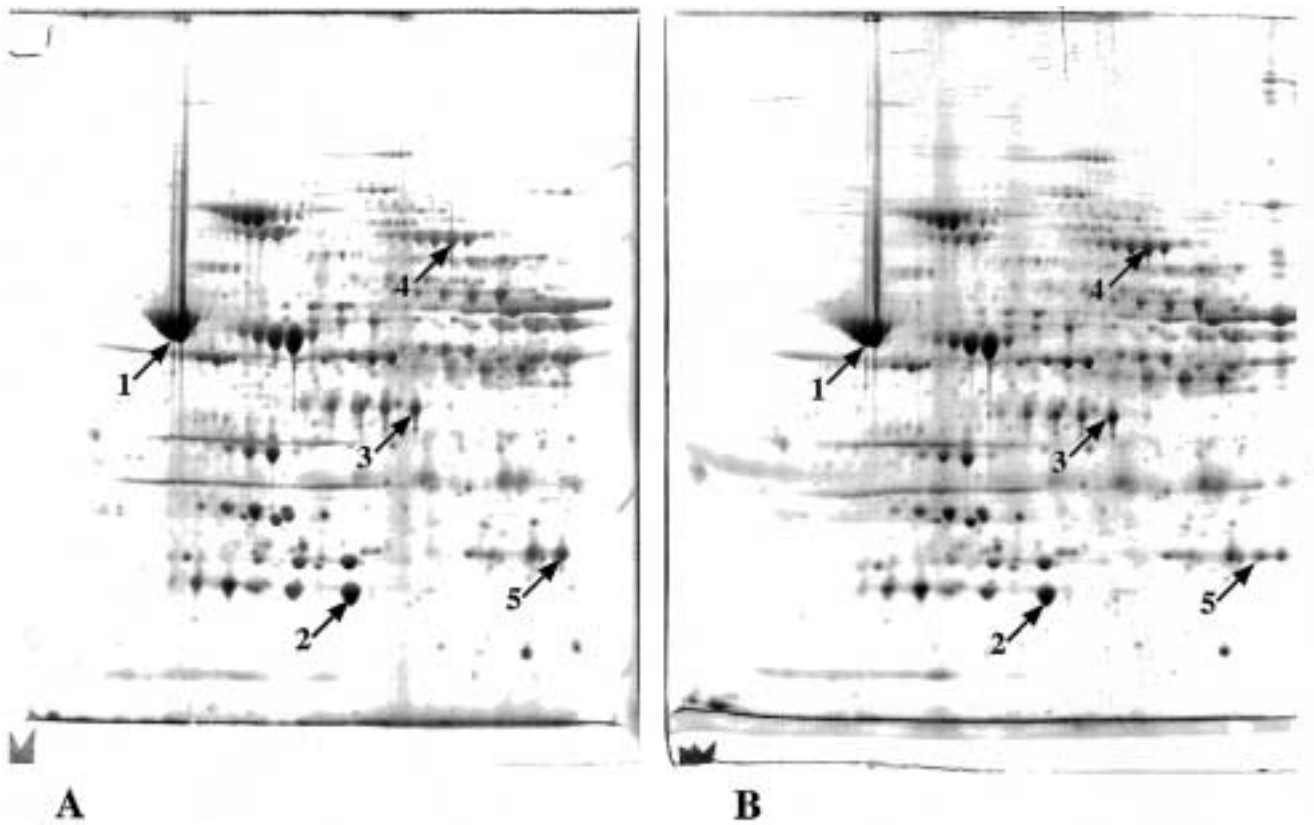

Figure 2. Comparison of detection by fluorescence with RuBPS and Sypro Ruby. A total extract prepared from bovine heart mitochondria was separated by 2-D gel electrophoresis. 300 μg were loaded on the first dimension gel (IPG, pH 4–8). The gels were then stained with RuBPS (A) or Sypro Ruby (B). Second dimension gels: 10% acrylamide. The numbered spots have been excised and tested by mass spectrometry analysis.

vacuum. The sample was washed one to three times by applying 1 μL of aqueous HCOOH (5%) solution on the target and then flushed after a few seconds. Internal calibration is performed with angiotensin 1046 542 Da, Substance P 134 7 736 Da, bombesin 1620 807 Da, and adrenocorticotropic hormone 2465 199 Da. Protein identification was performed using MS-FIT or Mascot softwares, using a mass accuracy of 50 ppm.

3 Results and discussion

3.1 Sensitivity evaluation

This test was carried out by staining serial dilutions of protein markers separated by SDS-PAGE. Typical results are shown in Fig. 1, and demonstrate that there is no obvious superiority from one stain to the other. This implies in turn that Sypro Ruby is less sensitive than silver staining, as RuBPS has been shown to be less sensitive than silver staining [13]. This is in contrast to former claims concerning Sypro Ruby [16] and can be explained either by a suboptimal silver staining protocol, or by the fact that Sypro Ruby evaluation has been carried out by camera-based systems. In contrast to scanners, cameras allow signal accumulation by integration over time (exposure) and thus higher sensitivities than scanners. It must be recalled however, that signal accumulation is obtained at the expense of linearity, as overexposition and pixel bleaching is experienced with saturated spots.

3.2 Homogeneity evaluation

This was tested by 2-D electrophoresis of complex protein extracts with several hundreds of various proteins. The results are shown in Fig. 2. Here again, both stains perform quite similarly. This similarity extends beyond the patterns obtained, as the peak intensities for the spots (in relative fluorescence units (rfu)) are comparable for both stains (2000 rfu for the brightest pixels of spot 1). The patterns are also similar both for scanner detection and on the UV table (not shown).

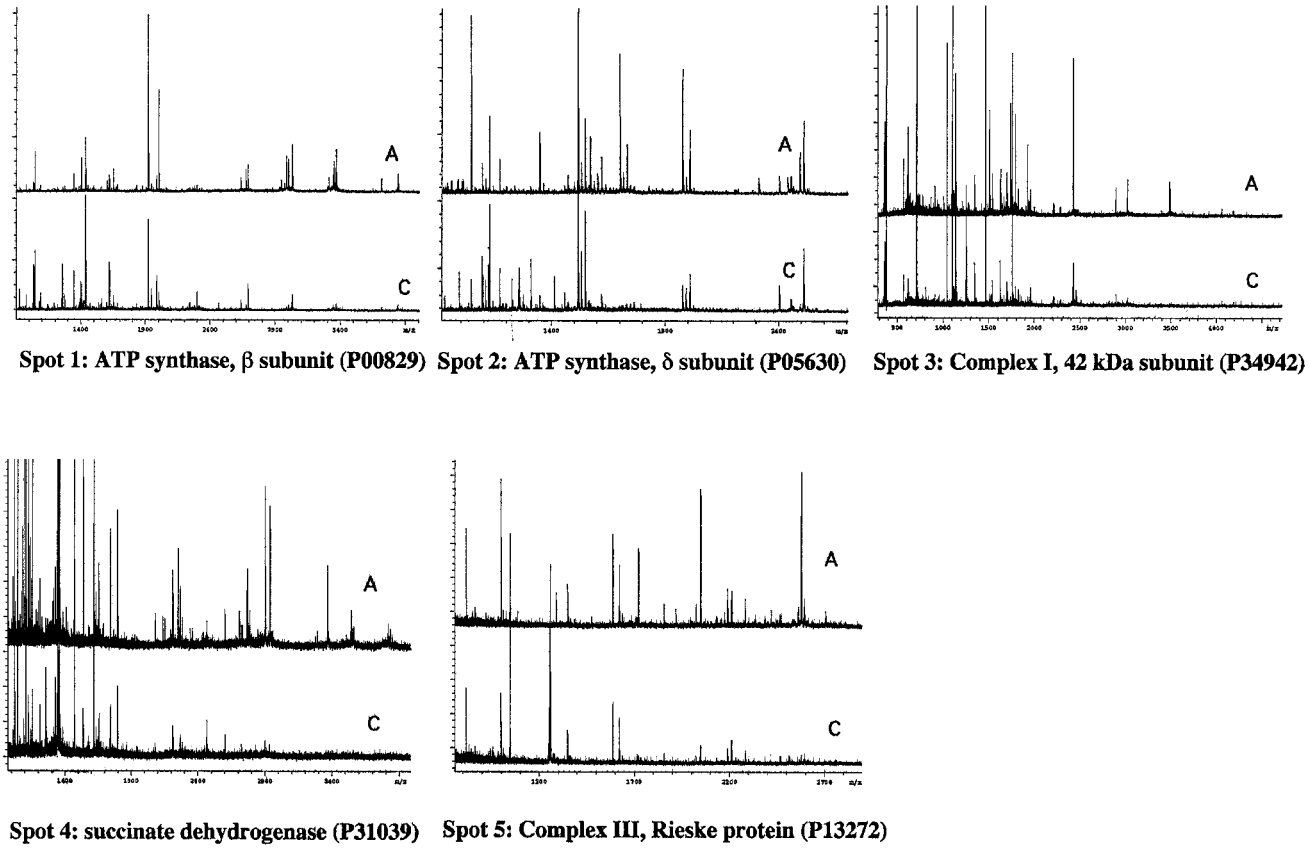

Figure 3. Comparison of mass spectra obtained from 2-D spots. Identical spots, excised from 2-D gels stained either with RuBPS or Sypro Ruby were analyzed by MALDI-TOF mass fingerprinting. The raw spectra are displayed, so that the signal-to-noise ratio can be easily evaluated. A, staining with RuBPS (ascorbate). C (control), staining with Sypro Ruby

3.3 Interference with MS

In order to test this feature, several equivalent spots were excised from 2-D gels stained with RuBPS or Sypro Ruby and submitted to MALDI-TOF analysis. Typical spectra are shown in Fig. 3. In this case, RuBPS performed better than Sypro Ruby, at least for peak intensities. In some cases, more peaks were detected with RuBPS (*e.g.* spot 5) but the reverse is also true (*e.g.* spot 1). The sequence coverage in each case is given in Table 1. It can be seen that the sequence coverage is as good or better with RuBPS than with Sypro Ruby.

Table 1. Sequence coverage of spots 1–5 analyzed by MALDI-TOF-MS after staining with RuBPS or Sypro Ruby

Protein	Sequence coverage with RuBPS	Sequence coverage with Sypro Ruby
ATP synthase, b subunit (P00829)	68%	42%
ATP synthase, d subunit (P05630)	57%	55%
Complex I, 42 kDa subunit (P34942)	41%	39%
Succinate dehydrogenase (P31039)	48%	25%
Complex III, Rieske protein (P13272)	37%	38%

4 Concluding remarks

The harmonious combination of good sensitivity, good linearity and minimal interference with MALDI-TOF analysis make both fluorescent stains an attractive choice, especially for analysis of 2-D gel-separated proteins [13, 16]. Both perform as well as far as pure detection performances are concerned. When it comes to interference with MS, RuBPS seems somewhat superior to Sypro Ruby, but the difference is much less important than with silver staining [13]. In addition, RuBPS is compatible with MS/MS analysis, provided that cleaning of the sample with reverse phase adsorption is carried out (Van Dorsselaer *et al.* unpublished data).

The difference between the two stains in mass spectrometry interference can arise from various causes. One could be the acidic pH of Sypro Ruby (pH 4.4) which may provide harsher fixation of the proteins and thus lower recoveries of peptides. Another cause could come from the additional chemicals present in Sypro Ruby. When gels are placed from Sypro Ruby into water, they swell enormously, which is indicative of the presence of additional chemicals in addition to the dye itself. These additional chemicals, however, provide a much simpler staining protocol for Sypro Ruby than with RuBPS. This simplicity has, however, a dramatic impact on the cost of the stain. Staining of a large 2-D gel with Sypro Ruby costs *ca.* 76 euros (70$), while the cost of the reagents used for preparing a 25 mL batch of concentrated RuBPS is 62 euros (*ca.* 55$). However, this 25 mL batch allows theoretically (*i.e.* without taking the shelf life into account) to stain 2500 large 2-D gels with chemical costs of 0.03 euros (0.025$) *per* gel. This may pay for some effort in making the stain and in using a slightly more complicated protocol.

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