

## Capabilities and limitations of gel electrophoresis for elemental speciation: A laboratory's experience\*

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**Abstract:** Gel electrophoresis is a fractionation/separation technique that yields valuable information in the field of metalloproteomics, often referred to as metallomics. This paper is based on four years of practical experience of the authors' lab in this domain and highlights the capabilities and limitations of gel electrophoresis. Pitfalls of the technique were recognized by identifying the origin of artefacts in the separation, species degradation being the most important. Gel electrophoresis can be accomplished under either native or denaturing conditions. The speciation of vanadium and selenium among serum and yeast proteins, respectively, is used to illustrate these two major modes. The most powerful approach is two-dimensional denaturing gel electrophoresis. This review of the methods used in our laboratory also describes the application of the two major detection techniques, autoradiography on the one hand and electrothermal vaporization—or laser ablation (LA)—inductively coupled plasma–mass spectrometry (ICP–MS) on the other.

**Keywords:** gel electrophoresis; electrothermal vaporization; laser ablation; autoradiography; vanadium; selenium; ICP–mass spectrometry; speciation.

### INTRODUCTION

Slab gel electrophoresis (SGE), especially its two-dimensional variant, is a widely used technique in the field of proteomics. One can speak of a real separation technique because of the high resolution achieved with two-dimensional gel electrophoresis (2DE). To better assess the position of gel electrophoresis amongst the fractionation techniques relevant for speciation, some characteristics of these methods have been summarized in Table 1. This table shows that only two-dimensional liquid chromatography can compete with gel electrophoresis. Moreover, the former still has to be further developed to be a serious alternative to 2DE in the field of proteomics.

The present article concentrates on the rationale of method development and the associated pitfalls and techniques available for detection of the trace elements in the gels. This article does not pretend to be an exhaustive review of gel electrophoresis for speciation nor a very detailed description of the work carried out at the authors' lab. For a more detailed description of the SGE techniques and methods available for speciation, the reader is referred to refs. [1,2] and the publications cited therein. For more comprehensive information on the research carried out at our laboratory and the validation of

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\*Paper based on a presentation at the 2<sup>nd</sup> International Symposium on Trace Elements in Food (TEF-2), Brussels, Belgium, 7–8 October 2004. Other presentations are published in this issue, pp. 65–143.

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the methods developed, the reader is referred to our research articles, published elsewhere and quoted in this overview.

**Table 1** Position of gel electrophoresis among the fractionation techniques relevant to speciation.

	Maximum theoretical plate number N and/or peak capacity C	Mechanisms available	Parameters driving the separation	Separation range	Ref.
HPLC	N > 5000; C = 200 In practice, C = 50–100	Reversed-phase Ion exchange	Hydrophobicity $K_a$		
SEC	N = 5000 In practice, C = 10	Exclusion	Hydrodynamic radius	$M_r > 10$ kDa	
FFF	~SEC	Sedimentation Flow Thermal, etc.	Hydrodynamic radius	$M_r > 1000$ kDa $M_r > 1000$ kDa $M_r > 10$ kDa	
UC	In practice, C = 10	Sedimentation Archibald's principle	Mass density $\rho$ , $M_r$	Subcellular fractions Mass distributions	
CE	N = 500 000	Electrophoretic mobility Sieving, pH gradient Isotachopheresis	$\mu$ $M_r$ , pI		[10]
SGE (IDE)	C = 100	pH gradient Sieving, with detergent Sieving, without detergent	pI $M_r$ Hydrodynamic radius		
LC <sup>2</sup>	N = 100 000; C = 200 <sup>2</sup>	i. IEC i. SEC i. RPC	See the		[11]
LCxCE on-line	In practice, C = 500–1000	ii. RPC ii. CZE ii. CZE	1D methods		[12]
2DE	C = 10 000 In practice, C = 500–1000	i. pH gradient ii. sieving	i. pI ii. $M_r$	3 < pI < 9 $M_r > 5$ kDa	[13]

Two examples of method development, covering the broadest possible spectrum of separation modes relevant to speciation, are given:

- Speciation of V, which forms labile complexes with proteins. This was done with one-dimensional, non-denaturing gel electrophoresis, combined with radioanalytical techniques for the detection of the trace element.
- Speciation of Se, which is covalently bound in proteins. Two-dimensional, denaturing gel electrophoresis could be used in this case, combined with mass spectrometric techniques for the detection of the trace element.

## METHOD DEVELOPMENT

The importance of the first steps of the method development can hardly be overestimated. If some precautions are not taken to guarantee the quality of the separation, a totally erroneous picture of the distribution of the trace element can be obtained.

### Contamination [3]

Although our study was not exhaustive and did not have the intention of being representative for all commercially available gels and ready-to-use buffers and strips, a gel submitted to a “blank” separation in a clean room, with all chemicals necessary for the separation (without the staining step), was subjected to panoramic trace analysis via pneumatic nebulization—ICP—MS. None of the elements monitored (Li, V, Cr, Mn, Ni, Cu, Zn, As, Se, Mo, Pd, Ag, Cd, Pt, Tl, Pb) were found to be present above the detection limit. Contamination was hence not considered a limiting factor if the separation is performed in a strictly monitored environment, as a clean room, and with chemicals of the highest purity. Yet, it seems obvious that if the separation is not performed according to these standards or if other more widely encountered elements, such as Ca or Fe, are considered, results should be scrutinized for contamination.

### Vanadium [4]

Native gel electrophoresis is a widely used technique, in which the tertiary structure of the proteins is preserved. Hence, it is often applied if an enzyme has to retain its activity after separation. This is why it is a possible separation technique for metal–protein complexes, which would be disturbed in the presence of denaturing chemicals. Unfortunately, the state-of-the-art 2DE protocols rely on the use of denaturing chemicals, which cannot be used for such complexes. For this reason, native gel electrophoresis is limited to one-dimensional separations.

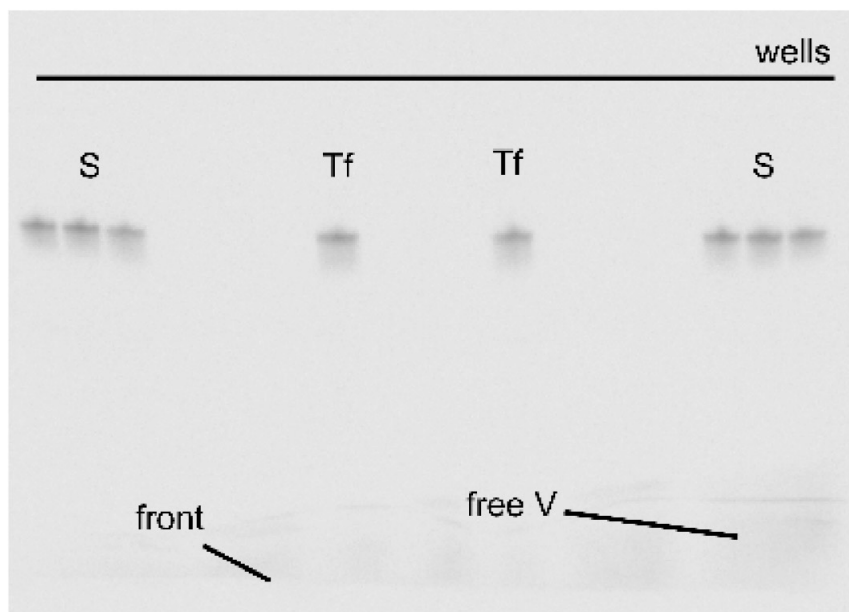
As proof-of-concept, the fractionation of serum proteins incubated with V was selected. At basal concentration, V is transported by transferrin, forming one of the most labile metal complexes of this protein. However, if transferrin, previously incubated with  $^{48}\text{V}$  as vanadate, was submitted to native gel electrophoresis, V was detected at the anode and was hence no longer bound to transferrin, as depicted in Fig. 1. To ensure this was not an artefact stemming from the sample preparation, the samples were submitted to ultrafiltration. As expected, the main V fraction was detected in the solution containing transferrin, and not as an unbound, low-molecular-mass species.

The complex can be further stabilized during the separation, using the blue native protocol. Blue native gel electrophoresis is a technique used for, for example, labile protein associations, reminiscent of the fragile association between metals and proteins. This was not yet sufficient to ensure a successful separation, since the other components of the buffers readily complexate V, disturbing the equilibrium between bound and unbound species. At last, if all the chemicals present in the buffers were carefully chosen for their low affinity for V-species, V was detected at the migration distance of transferrin, as shown in Fig. 2. The same distribution between bound and unbound V is found as with ultrafiltration, namely, a low amount of unbound V. When applied to serum, the same result was obtained.

The lesson to be learned from this kind of experiment is that a separation protocol for proteins alone cannot be directly extrapolated to metal–protein complexes: a careful assessment of the separation parameters and especially of the chemicals used, has to take place.



**Fig. 1** Autoradiogram of a gel obtained after the separation of a sample containing  $^{48}\text{V}$  as a transferrin-complex. Anode at the bottom. Although the expected species is a V-transferrin complex (marked Tf), nearly all V is detected as low-molecular-mass species at the front. Reproduced by permission of Wiley-VCH [4].

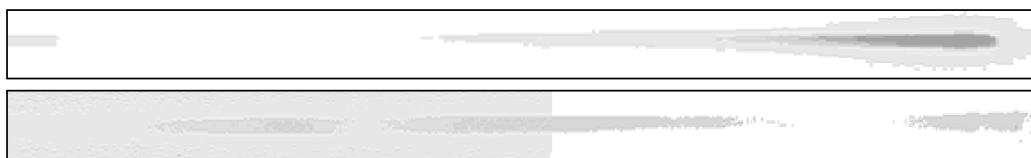


**Fig. 2** Autoradiogram of a gel obtained after blue native electrophoresis of serum (lanes S) and a sample only containing transferrin (lanes Tf). In both cases,  $^{48}\text{V}$  is detected in combination with transferrin. Anode at the bottom, samples applied in wells, the position of which is indicated by the bold line. The same gel was later silver-stained to confirm the identity of the protein bands. Reproduced by permission of Wiley-VCH [4].

## Selenium

Covalent bonding of the trace element to the protein is no assurance of stability during electrophoresis either. Let us examine the example of Se and 2DE. Yeast was grown in the presence of  $^{75}\text{Se}$ -containing selenate, in a sulfur-poor medium. It is expected that Se is incorporated instead of sulfur, in a nonselective manner, so that it is incorporated in proteins that normally contain methionine or cysteine, the two sulfur-containing amino acids. After lysis of the yeast cells and purification of the proteins thus obtained, the proteins were submitted to the first dimension of 2DE, isoelectric focusing (IEF), a separation according to pI. For the details of the experiment, the reader is referred to [5]. This separation was done in an immobilized pH gradient (IPG) strip, showing a linear pH gradient from pH 3 to 10.

A homogeneous distribution of proteins containing  $^{75}\text{Se}$  is expected, which was confirmed by filtration and size exclusion. Yet, after separation by IEF, all radioactivity was detected at the anode (by autoradiography, see further), as shown in Fig. 3. The long separation time is probably responsible for the acid decomposition of the side chain, leading to the formation of negatively charged inorganic Se, attracted to the anode.



**Fig. 3** Autoradiogram of IPG strips. Linear gradient, left pH 10, right pH 3. Above: without derivatization by iodoacetic acid, Se is detected at the anode. Underneath: after carboxymethylation using iodo-acetic acid, Se is distributed among all proteins.

In order to protect the Se side chain, a derivatization step with iodo-acetic acid was required prior to separation. This time, Se-containing proteins were detected at all pIs. The second dimension confirmed the existence of Se-containing proteins (see autoradiography).

## Conclusions on method development

The results obtained with a gel electrophoresis method should not be trusted if not previously validated. Confirmatory experiments are necessary to ensure that results yielded by gel electrophoresis are trustworthy. It should not be overlooked that, in comparison with high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE), such a separation is a lengthy process and this long duration can jeopardize the stability of the species. A further problem, which is hardly addressed in the literature, is contamination of the gels or chemicals. But this risk is often easy to avoid if precautionary measures are taken.

## DETECTION

Two methods of detection were implemented in our laboratory: autoradiography and solid sampling ICP-MS.

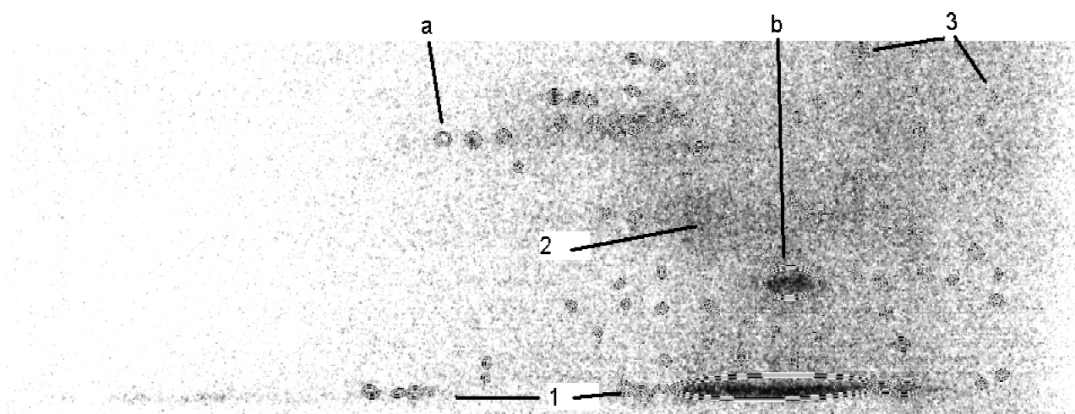
### Autoradiography

Although some users may be scared by the implementation of a radioanalytical method, some advantages of autoradiography are undeniable. First, contamination is surely not an issue, unless an excess of “cold” element is present, which could disturb the distribution of the element. Second, a whole picture

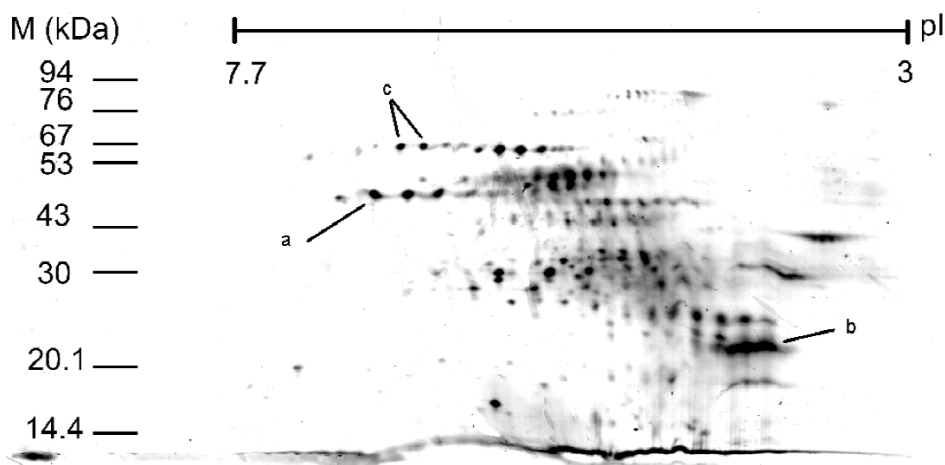
of the gel is obtained without further treatment or revelation. Further, autoradiography can be performed at low temperatures, allowing the detection of metals in a frozen gel. This can be particularly useful if the metal is not covalently bound to the protein and would not resist a chemical fixation (e.g., the first steps of silver- or coomassie blue-staining) of the proteins to prevent their diffusion after separation. The relatively high spatial resolution (a pixel typically has a size of  $0.5 \text{ mm}^2$ ) is also interesting.

The example of the distribution of Se amongst yeast proteins is given in Fig. 4. It is the same sample as formerly mentioned, stemming from yeast grown in  $^{75}\text{Se}$ -containing medium. After lysis, the proteins were chemically treated to prevent breakdown of the Se-containing side chains during separation. The radioactivity in the gel was located by autoradiography, and thus the Se-containing proteins could be identified (Fig. 4). This picture can be compared with the silver-stained gel, presented in Fig. 5, which shows nearly all proteins present in the sample. This comparison shows that Se is more or less homogeneously distributed among the proteins, as is expected from the nonspecific incorporation of this element in yeast. Please note that the Se sample had a low specific radioactivity in this experiment, which had a negative impact on the signal-to-background ratio and thus the autoradiography quality.

Yet, the use of radiotracers is not acceptable for in vivo human studies or not feasible if no radiotracer with an appropriate half-life and a sufficiently high specific activity can be produced. Solid sampling ICP-MS techniques are a valuable alternative to the use of radiotracers, if the problem of contamination is correctly addressed.



**Fig. 4** 2DE of Se-yeast proteins. Autoradiogram of the gel. a and b: Se-containing proteins. 2 is a region of the gel dense in proteins, the Se signals cannot be resolved here. The region noted 1, is an artefact from the IPG strip and consists of proteins which have not entered the separation gel. The specific activity of the sample was low compared to the natural radioactivity, which explains the high background in this picture (as in the artefacts noted 3).



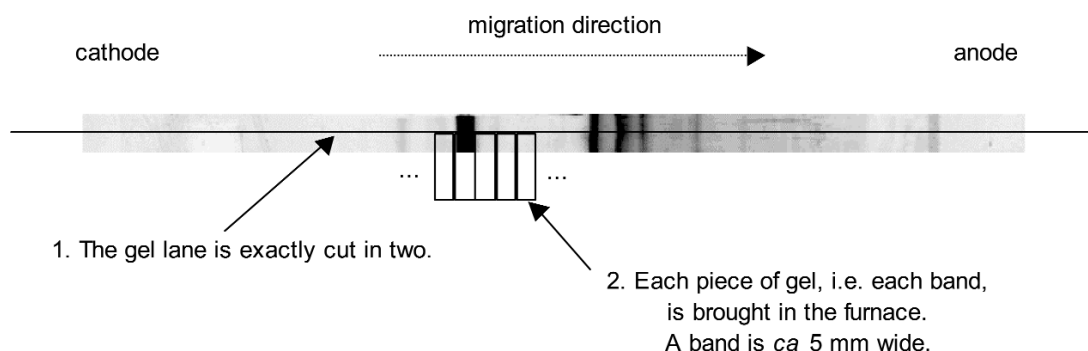
**Fig. 5** 2DE of Se-yeast proteins, protein map obtained upon silver-staining of the gel depicted in Fig. 4. Only the region of pH 3–7.7 is shown. Three protein spots were marked to facilitate comparison: a, b, and c. Proteins c are absent from the Se map (Fig. 4) and thus are not Se-containing proteins.

### Electrothermal vaporization–ICP–MS [6,7]

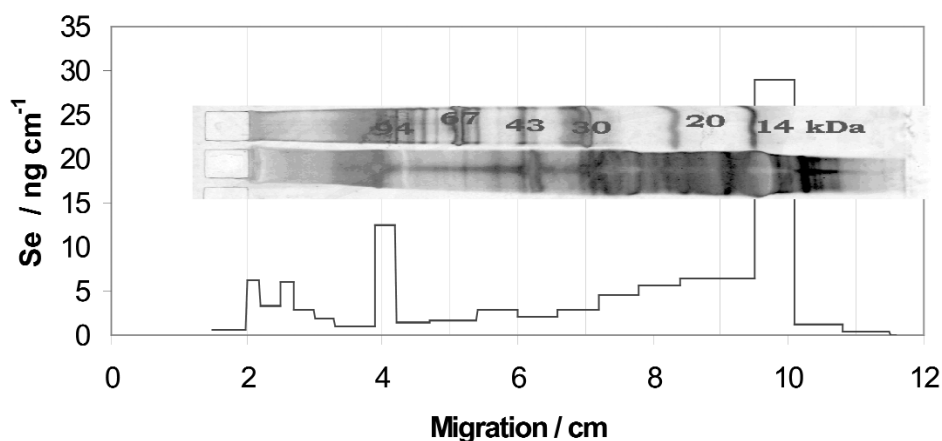
Electrothermal vaporization (ETV)–ICP–MS is the first technique that was explored in our laboratory because of its excellent limits of detection, but also because it offers truly quantitative results. The availability of a real blank, the gel itself, is an important advantage. With it, a real procedural limit of detection (LoD) can, for example, be determined. The blank model can even be further refined by using a piece of gel hydrated with a protein solution (e.g., albumin) and subsequently stained. All chemicals are then present, and perfectly matrix-matched standards can be used for external calibration.

The sampling strategy for ETV–ICP–MS is depicted in Fig. 6. A gel lane, in this case obtained after one-dimensional SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) of Se yeast, is first silver-stained to reveal the protein spots. A gel lane can then be cut in two halves, parallel to the migration direction: one half is used for elemental analysis, the other for protein analysis. The half kept for elemental analysis is then cut into subsamples in the direction of migration. This means that an ETV sample is one protein band, if the resolution of the SDS–PAGE is high enough. Each protein band is introduced into the graphite furnace of the ETV–ICP–MS set-up for the determination of its trace element content. The total profile can then be reconstructed from the various band measurements and, once again, compared with the protein profile obtained after staining. In the case of Se, it could be proved that the method was sensitive enough for Se yeast (see Fig. 7) or even human serum (LoD: 40 pg Se per band) and offered a quantitative recovery.

This approach is sensible for a gel lane, where 20 to 30 bands are detected. The analysis lasts 2 to 3 h in this case. Yet, it is not a solution for a two-dimensional gel, which should be divided into at least hundreds of spots. For this kind of gel, laser ablation (LA) was implemented. Nonetheless, it should be kept in mind that ETV–ICP–MS can offer quantitative results for a couple of spots even after 2DE.



**Fig. 6** Sampling strategy: Preparation of a gel after SDS-PAGE for solid sampling ETV-ICP-MS. Reproduced by permission of the Royal Society of Chemistry [6].



**Fig. 7** Detection of Se by ETV-ICP-MS after 1DE (SDS-PAGE) of the water-soluble fraction of Se-yeast proteins. The lanes after silver-staining of the yeast proteins (lower lane) and molecular mass markers (upper lane) are also given. Cathode and application point at the left. Reproduced by permission of the Royal Society of Chemistry [6].

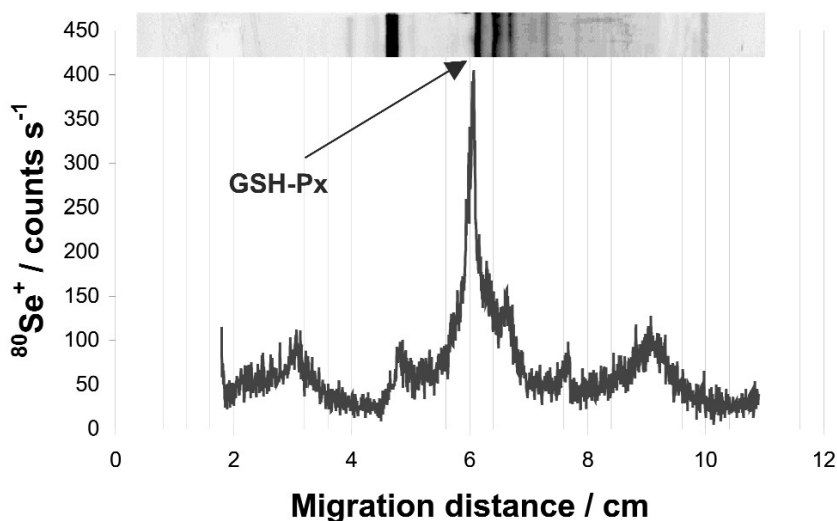
### Laser ablation-ICP-MS [3,8]

Compared to ETV-ICP-MS, LA-ICP-MS is a more versatile solid sampling technique. In combination with gel electrophoresis, two approaches can be distinguished.

- After one-dimensional gel electrophoresis (1DE), a gel lane can be scanned in the migration direction. The lane is translated under the laser beam, while the trace element is monitored and the corresponding signal intensity plotted as a function of the migration distance. In this way, an electropherogram is obtained for the trace element and the data obtained can be compared with the stained gel.
- For a two-dimensional gel, single spots can be investigated. This is no longer a systematic scanning, but each protein spot is selectively analyzed. The analysis time for one spot is no longer than 10 s, the translation to the following spot and the optical focusing on the spot takes less than 20 s, so that a typical analysis time per spot is 30 s. This allows the analysis of a whole two-dimensional gel in less than one day. A further advantage is that, if necessary, with the proper pattern recognition software automation is thinkable, as has been done with matrix-assisted laser desorption/ionization (MALDI)-MS in combination with 2DE.



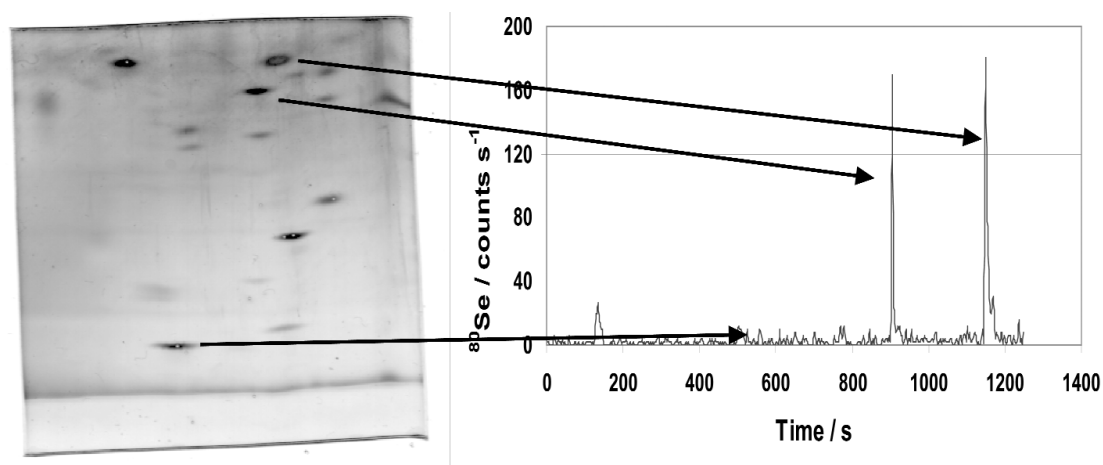
An example of the scanning for Se after gel electrophoresis is given in Fig. 8. It is a gel lane, similar to the one analyzed with ETV-ICP-MS. A similar profile is obtained, with a much higher resolution. The fact that the standard ablation cell cannot host a whole gel lane is, of course, a disadvantage. A lane has to be cut into two or three fractions, and the discontinuity in the signal (and background) stem from this fact. An improved cell design could solve this problem [3].



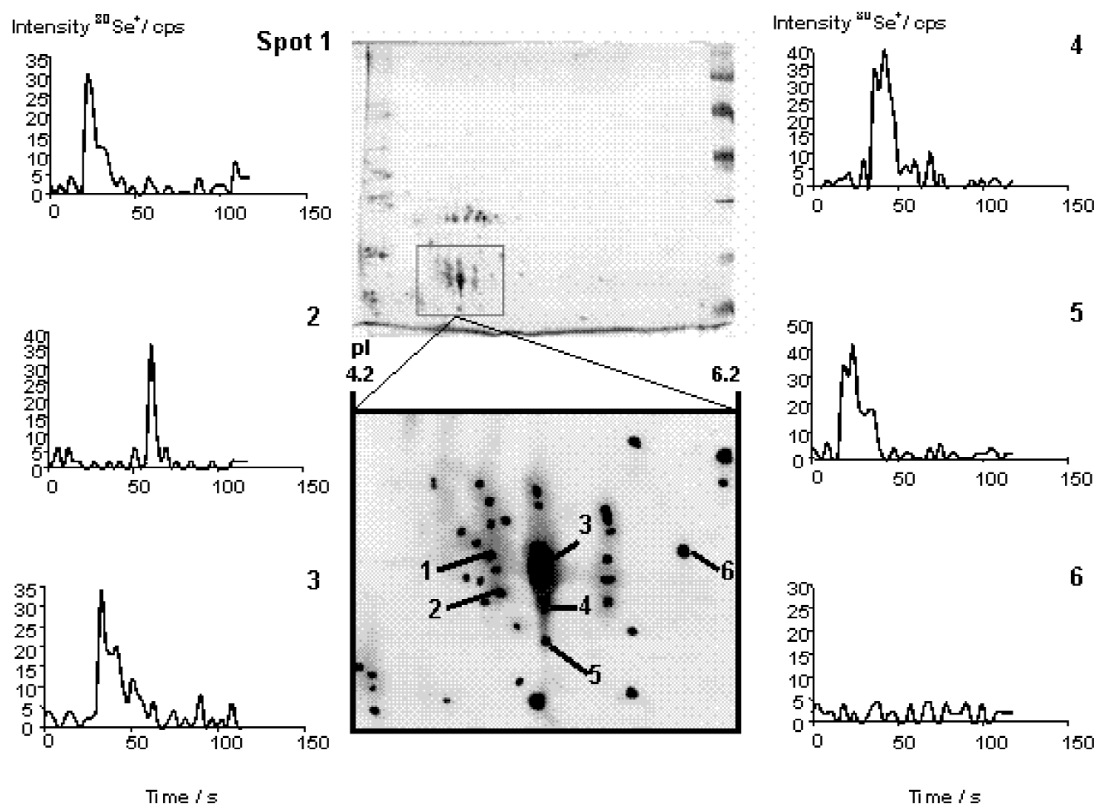
**Fig. 8** Detection of Se by LA-DRC-ICP-MS after 1DE (SDS-PAGE, anode at the right). Sample: Glutathione peroxidase (GSH-Px) extract from red blood cells, represents in total 6.75 pg Se. Reproduced by permission of Wiley-VCH [3].

Quantification is not mandatory for two-dimensional gels. The real question in this case is whether or not a metal-containing protein has been encountered, which can be translated in terms of “is the trace element present above the detection limit?”. In the case of Se, LA was combined with a dedicated ICP-MS method, based on the use of the dynamic reaction cell (DRC) to overcome the spectral overlap of the signals of  $^{80}\text{Se}^+$  and  $^{40}\text{Ar}_2^+$  by selective ion-molecule chemistry with CO as a reaction gas and hence, to improve the LoD. The LoD was then as low as 0.5 pg Se per spot. This is low enough to detect Se in a protein spot after silver-staining where only one atom of the metalloid is present per protein. Figure 9 gives the example of the analysis of a portion of a 2D gel obtained upon separation of the proteins from Se yeast [3]. Moreover, with the set-up used, a LA crater has a diameter of 120  $\mu\text{m}$  at maximum. The proteins spots are considerably larger, such that after LA-ICP-MS analysis, sufficient sample is left for other investigations.

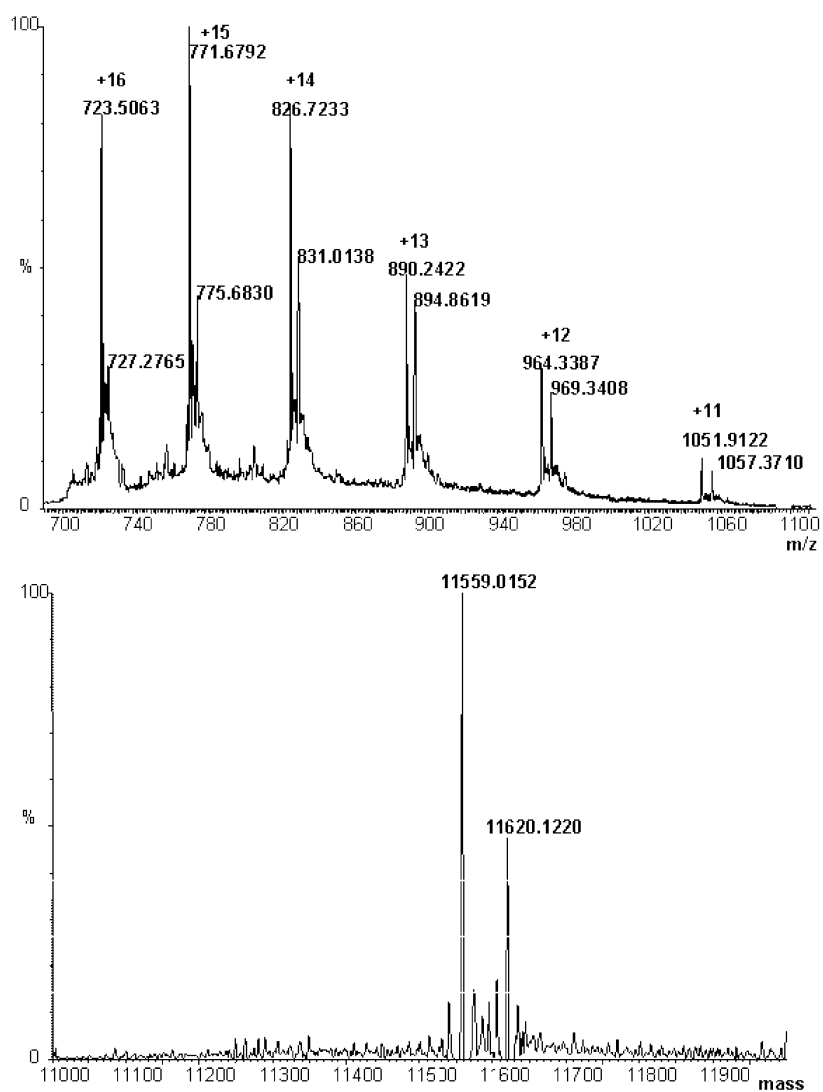
The example of the combination of LA-ICP-MS and electrospray (ES)-MS of a 2DE gel is given for the analysis of yeast proteins in, respectively, Figs. 10 and 11 [8]. Spot 5 from Fig. 10 was identified as a Se-containing protein by LA-ICP-MS and further analyzed by ES/time-of-flight/MS. The spectrum obtained with ES-MS (Fig. 11) showed the presence of two species in this spot, probably the Se-containing protein at mass 11 559 and its carboxymethylated form at mass 11 620. Although no attempt was made to further identify the protein, this showed that the two approaches, ICP-MS and ES-MS, are complementary in order to characterize the Se-containing proteins present in one sample after 2DE.



**Fig. 9** 2DE of Se yeast. Detection of Se by LA-DRC-ICP-MS. The actual part of the gel which was analyzed is shown ( $3 \times 3.5$  cm; pI ca. 5–7;  $M_r < 30$  kDa; (+,+) bottom right). (a) Beyond the front, no protein present; (b) protein containing no Se; (c,d) two Se-containing proteins. Reproduced by permission of Wiley-VCH [3].



**Fig. 10** 2DE of Se yeast proteins, water-soluble fraction. Detection of Se by LA-ICP-DRC-MS.  $^{80}\text{Se}^+$  signals for spots 1 to 6. Only spot 6 is not a Se-containing protein. Reproduced by permission of the Royal Society of Chemistry [8].



**Fig. 11** Electrospray/time-of-flight/MS of the protein from spot 5 of Fig. 10. Above: mass spectrum with the charges and mass-to-charge ratios of the protein. Underneath: deconvoluted signal. The peak at mass 11 620 is probably due to the carboxymethylation of the protein at mass 11 559. Reproduced by permission of the Royal Society of Chemistry [8].

### A THIRD DIMENSION? [9]

It should be noted that the material obtained after gel electrophoresis is useable for other types of analysis than elemental analysis or identification of the proteins. A further step can be added, like the identification of the seleno-amino acids in the proteins. Indeed, a condition *sine qua non* to classify a protein as selenoprotein, where Se is necessary for the enzymatic activity of the protein, compared to a Se-containing protein, where Se is simply incorporated instead of sulfur, is the presence of seleno-cysteine. In principle, the spots obtained by 2DE can be excised, lysed, and analyzed for their amino acid content with CE hyphenated with DRC-ICP-MS, as this approach offers a sufficiently low absolute detection limit to cope with the little material available.

### CONCLUSION

Although the existence of some pitfalls—such as contamination or species conversion—cannot be ignored, gel electrophoresis is a powerful tool for the fractionation or separation of high-molecular-mass compounds, such as metalloproteins. The two main separation modes, one- or two-dimensional, offer a real separation of the proteins. While non-denaturing 2DE still has to be further developed, denaturing 2DE is already a very powerful separation technique for covalently bound elements, such as Se or even phosphorus and sulfur. In combination with autoradiography or solid sampling-ICP-MS, gel electrophoresis plays an important role in metalloproteomics.

### ABBREVIATIONS

1DE	one-dimensional gel electrophoresis
2DE	two-dimensional gel electrophoresis
DRC	dynamic reaction cell
CE	capillary electrophoresis
ES	electrospray
ETV	electrothermal vaporization
FFF	field flow fractionation
GSH-Px	glutathione peroxidase
HPLC	high-performance liquid chromatography
ICP-MS	inductively coupled plasma-mass spectrometry
IEF	isoelectric focusing
LA	laser ablation
LoD	limit of detection
MALDI	matrix-assisted laser desorption ionization
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
SGE	slab gel electrophoresis
UC	ultracentrifugation

### ACKNOWLEDGMENT

CCC was Postdoctoral Fellow of the Fund for Scientific Research, Flanders (Belgium) (F.W.O., Vlaanderen) at the time this work was conducted. FV and RC acknowledge the F.W.O. for financial support (research project G.0037.01 and G.0026.01, respectively).

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