Review

Real and imaginary artefacts in proteome analysis via two-dimensional maps[☆]

Pier Giorgio Righetti *

Polytechnic of Milano, Department of Chemistry, Giulio Natta, Materials and Engineering Chemistry, Via Mancinelli 7, Milano 20131, Italy

Abstract

The present review touches on a long-lasting debate on possible artefacts (i.e. generation of spurious spots, not belonging to the biological sample under analysis) induced by the separation technique (in this case, two-dimensional mapping) per se. It is shown here that some of the biggest offenders, always blamed in the past (at least since 1970, i.e. since the inception of gel-base isoelectric focusing protocols), namely deamidation (of Asn and Gln residues) and carbamylation (due to cyanate produced in urea solution), simply do not occur in properly handled samples and have never indeed been demonstrated in real samples, except when forced in purpose. Conversely, two unexpected major artefacts have been recently shown to plague 2D mapping. One is formation of homo- and hetero-oligomers in samples that have been reduced but not alkylated prior to entering the electric field. The phenomenon is highly aggravated in alkaline pH regions and can lead to an impressive number of spurious spots not existing in the original sample. Thus, alkylation (best if performed with acrylamide or vinylpyridines) is a must for avoiding such spurious spots, as well as sample streaking and smearing in the alkaline gel region, and for maintaining sample integrity. In fact, the other unexpected artefact is desulfuration (β-elimination) by which, upon prolonged electrophoresis, the sample looses an −SH group fro Cys residues. This loss, in the long run, is accompanied by massive protein degradation due to lysis of a C−N bond along the polypeptide chain. Here too, alkylation of −SH groups of Cys almost completely prevents this noxious degradation phenomenon.
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Keywords: Two-dimensional maps; Artefacts; Carbamylation; Beta elimination

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"Let us not gird science to our loins as the warrior buckles on his sword. Let us raise science aloft as the olive branch of peace and the emblem of hope"
Speech by Brevet Lieutenant Colonel John Wesley Powell at a Darwin memorial meeting in Washington, 1882

1. Prelude

The loss of a friend is always a very sad event, but the loss of Zdenek Deyl is mourned by all of us in the field of Separation Science, since the work he undertook in this area was most valuable and will leave a long-lasting trace in our daily life. He was a humble, hard-working scientist, who must have lived according to the motto by J.W. Powell that opens this review. His contribution to the growth of the Journal of Chromatography was outstanding, since he painstakingly took care of collecting manuscripts at the numerous meetings on capillary zone electrophoresis, and since he was the editor of annual review volumes and special bibliography sections, that must have cost him a tremendous amount of work. I vividly remember him sitting at a small desk in the hall of the meeting grounds, patiently soliciting manuscripts from the attendees, always smiling at anyone approaching him, the eternal cigarette hanging from his lips, like his hero Humphrey Bogart in the movie Casablanca. Yes, because, as he told me, when, in the mid sixties (of last century) I was doing my post-doctoral work at MIT, he was also working at Harvard, way up to the very end of Massachusetts Avenue (I humbly stopped at No. 77, on the banks of the Charles river). We were taking some common classes with a professor of mathematics, a chap called Tom Lehrer, who was also an excellent pianist. Mathematics, we learned too little, I am afraid, but we spent plenty of nights at the Blue Parrot, a pub in Harvard Square, where Lehrer played piano at the lively rhythm of rag time. The walls of the pub were literally carpeted with giant posters of H. Bogart, of course. We did not know each other, in those days, but later on we became acquainted and we met innumerable times at meetings and also in Verona, where he often came collaborating with Dr. Tagliaro and other scientists at GSK.

Zdenek left important contributions in the Journal of Chromatography Library, where he edited quite a few books in the series [1–4]. He was also very active in assembling special volumes for the Journal of Chromatography B that had specialized in Biomedical Applications, to be distinguished from his twin J. Chromatogr. A, devoted to general Separation Science and to theoretical aspects in the field. It turned out that his enthusiasm was so contagious that I found myself collaborating with him in a couple of these special volumes [5,6]. It was a great experience, although, I confess, he took on his shoulders most of the tedious job in collecting and sending out manuscripts for refereeing. I think he fully deserved a space in the Pantheon, and I am glad that H.J. Issaq invited him to contribute to that monumental book "A Century of Separation Science", that should be mandatory reading in this field. A delightful account of his life as a scientist can be found in the chapter he wrote [7].

I would like to end this prologue to my review in his honour with an anecdote that I suspect very few know. One might think that his work and dedication in collecting all manuscripts at the various meetings should have been generously rewarded. Well, the twists of fortune are unpredictable. One day, as he was returning from a meeting in USA, with a briefcase full of manuscripts for the special issue, he ended up in the Centraal Station in Amsterdam, waiting for a train to bring him back to Prague. Having spent a sleepless night in the airplane, he was dozing on a bench on the platform, when his voluminous briefcase attracted the attention of a young guy desperate to get money for his daily dose of drug. In an instant, the chap was running out holding his precious material. Zdenek, suddenly awaken, was in a hot pursuit. The duo fled out of the station, running towards the canals. Zdenek was not so much worried for his portable computer, tucked in the briefcase among the manuscripts, but for the contributions that he had gathered with such a great care. Well the thief, seeing that he could not shake loose the pursuer, had a stroke of genius: he opened the purse, took out the computer and threw the briefcase in the canal. The most humble of the canals, mind you, the Singel, the canal of the poor workers, no princely or royal treatments in the Prinsengracht or Keizergracht. Poor Zdenek was left sorrowful, looking at the manuscripts spewing out of the bag and gently floating away on the muddy waters. That year the special issue of J. Chromatogr. A took quite an extra while to appear. Ironically, the sad reward had been written long before by Fjodor Dostojevskij in his famous novel *Crime and Punishment*!

2. Introduction

Proteomics demands high-resolution separation techniques to penetrate the complexity of even the simplest organisms [8]. The relatively small number of genes in the human genome (perhaps as few as 23–25,000) [9] has reinforced the major role of co- and post-translational modifications in generating complexity. Given this situation, one of the most fundamental requirements in protein separations is that the analytical technique does not further modify the sample during analysis. Two-dimensional electrophoresis (2D) remains the highest resolution separation method; however, it has been tarnished by suggestions of artefactual modifications induced during the process or during the sample treatment. Human serum has always been taken as an example of such artefacts due to extreme complexity of its 2D maps and due to the appearance of strings of spots that seem to be generated by a major parental species. Since most of these minor spots appear on the acidic side of the major one, deamidation has been claimed as the major villain for this high heterogeneity. Hydrolysis of asparagines and glutamine residues, generating negatively charged aspartic and glutamic acid residues, is a well-known biochemical event, although no proof has ever been put forward that it would or should occur during the IEF/IPG process per se, notwithstanding claims to the contrary. The only report hinting at such deamidation events came from Sariouglu et al. [10]. These authors generated 2D maps of human plasma and upon analysis of tryptic fragments of trains of spots, found indeed the 1 Da difference typical of Asn → Asp and Gln → Glu transitions. Yet, their conclusions were that such processes occurred in vivo during the lifetime of plasma proteins, not in vitro, during sample analysis. In addition, such extensive trains of spots could only be found in sera not in other human tissues (e.g., liver). There is ample evidence that their conclusions should be right: in an extensive investigation on the heterogeneity of maize zeins (storage proteins with a very high content of Gln and Asn residues, up to 25% of total amino acid residues), in order to extensively deamidate such residues,

Righetti et al. [11] reported incubation times of 3 weeks in borate buffer, pH 9.5, at 55 °C. Having thus cleared the grounds from the Fata Morgana of deamidation as a source of artefactual protein heterogeneity, let us now look at potential sources of true troubles.

3. Reducing agents

Thiol agents are typically used to break intramolecular and intermolecular disulphide bridges. Cyclic reducing agents such as dithiothreitol (DTT) or dithioerythritol (DTE) are the most common reagents admixed to solubilizing cocktails. These chemicals are used in large excess (e.g., 20–40 mM) so as to shift the equilibrium toward oxidation of the reducing agent with concomitant reduction of the protein disulphides. As this is an equilibrium reaction, loss of the reducing agent through migration of proteins away from the sample application zone can permit reoxidation of free Cys to disulphides in proteins, which would result not only in horizontal streaking but also, possibly, in formation of spurious extra bands due to scrambled −S−S− bridges and their cross-linking different polypeptide chains. Even if sample is directly reswollen in the dried IPG strip, as customary today (although other procedures exist, such as cup loading), the excess DTT or DTE will not remain in the gel at a constant concentration, since, due to their weakly acidic character, both compounds will migrate above pH 7 and be depleted from the alkaline gel region. Thus, this will aggravate focusing of alkaline proteins and be one of the multifactorial factors responsible for poor focusing in the alkaline pH scale and for generation of a multitude of artefactual spots. E.g., Righetti et al. [12], when reporting the focusing of recombinant pro-urokinase and urinary urokinase, two proteins with rather alkaline pI values, in IPG gel strips, detected a continuum of bands focusing in the pH 8–10 region, even for the recombinant protein, which exhibited a single, homogeneous band by SDS–PAGE. Since this protein has an incredible number of Cys residues (no less than 24!) this extraordinary heterogeneity was attributed to formation of scrambled disulphide bridges not only within a single polypeptide chain but also among different chains in solution (Fig. 1). Curiously this happened even if the protein was not subjected to reduction of −S−S− bridges prior to the IPG fractionation, but this could also have a logical explanation. According to Bordini et al. [13], when probing the alkylation by acrylamide of −SH groups in proteins by MALDI-TOF, the primary site of attack, even in proteins having both disulphide bridges and free −SH groups, was found not to be a free −SH residue, as it should, but it was systematically one of the −SH engaged in disulphide bridges! This could only be explained by assuming that, at alkaline pH values (pH ca. 10), disulphide bridges are weakened and probably constantly broken and reformed. The situation would be aggravated when using β-mercaptoethanol, since the latter compound has an even lower pK value, thus it is more depleted in the alkaline region and will form a concentration gradient towards pH 7, with a distribution in the gel following its degree of ionization at any given pH value along the IEF strip [14]. This is probably the reason for the dramatic loss of any pH gradient above pH 7.5, lamented by most users of conventional IEF in
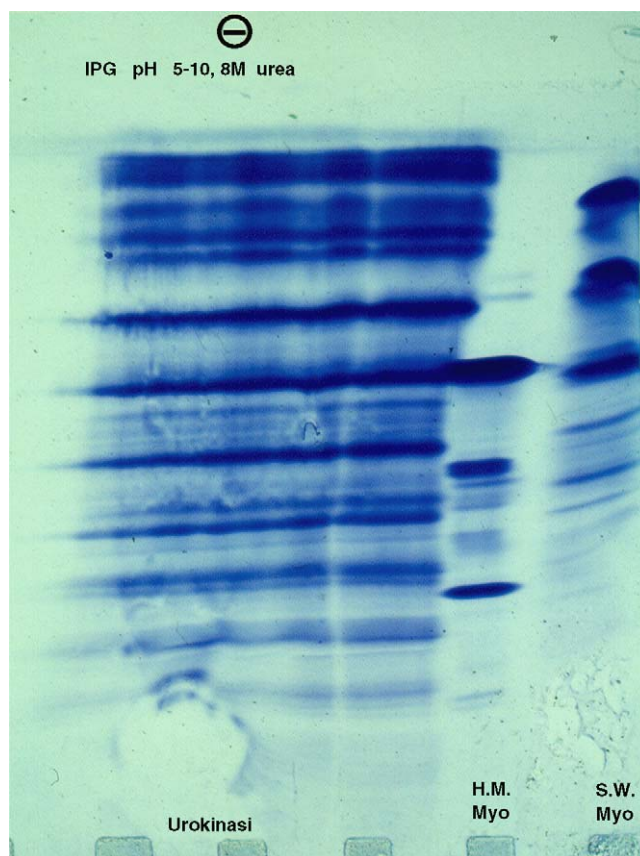

Fig. 1. Isoelectric focusing of recombinant pro-urokinase. Gel: IPG pH 5–10, reswollen in 8 M urea. Anodic sample application, Run continued for 5 h at 500 V/cm. Staining in micellar Coomassie Blue G-250. The first three samples to the right represent r-pro-urokinase. H.H. Myo: horse hearth myoglobin; S.W. Myo: sperm whale myoglobin (from Righetti et al. [12], by permission.

CAs, when generating 2-D maps. At least one lesson can be learned from these experiments: lack of blocking −SH groups of proteins (whether free or engaged in −S−S− bridges) is most deleterious and could bring about severe sample heterogeneity in the first IEF dimension, resulting in the appearance of false protein spots in a 2D map.

4. Reduction and alkylation prior to the 1st dimension or in between the 1st and 2nd dimensions?

This is also a very important aspect of sample solubilization and pre-treatment in 2D map analysis. Alkylation will prevent all the noxious phenomena reported above, like reformation of disulphide bridges, producing smears and even spurious bands due to inter-chain cross-linking, even among unrelated polypeptide chains. As clearly shown in Fig. 2, in the absence of alkylation (left panel) an impressive number of homo-oligomers (up to a dodecamer) of β-chains can be seen, together with homo- and hetero-dimers of α- and β-globin chains. All these spurious spots are abolished when reducing and alkylating prior to the IEF step (right panel). However, although reduction and alkylation is performed by anyone working with 2D maps, this last step is not done at the very beginning of the sample treatment, just prior to
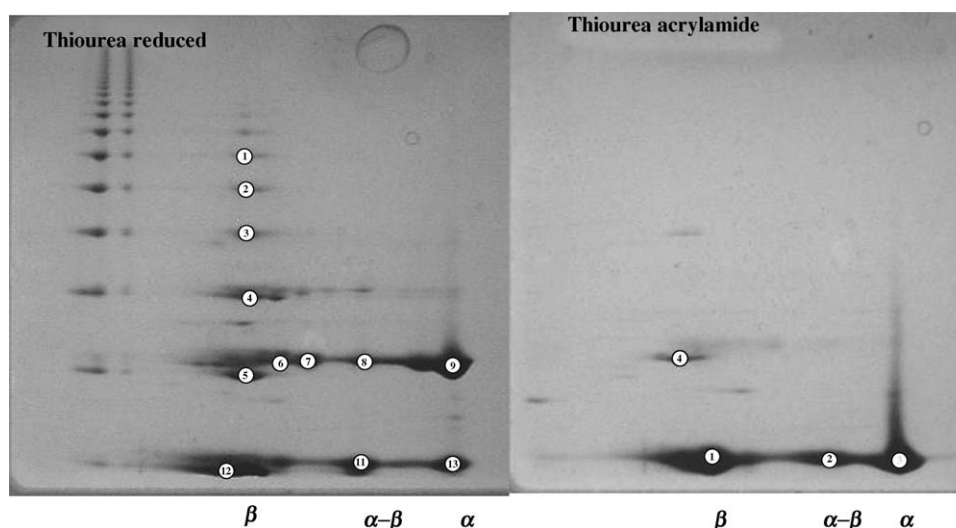
Fig. 2. Two-dimensional maps of a red blood cell lysate (98% globin chains). Left panel: sample reduced but not alkylated; right panel: sample reduced and alkylated with acrylamide prior to the IPG step. Note, in the first case, the presence of homo- and hetero-dimers of α- and β-globins, in addition to a series of oligomers (up to a dodecamer) of β-globin chains. These artefacts are fully abolished in the alkylated sample in the right panel (from Herbert et al. [33], by permission).

the 1st dimension IEF/IPG run, but in between the 1st and 2nd dimension, during the interfacing of the IEF/IPG strip with SDS-denaturing solution, in preparation for the SDS–PAGE final step. This probably stems from earlier reports by Goerg et al. [15,16], who recommended alkylation of proteins with iodoacetamide during the interfacing between the 1st and 2nd dimension on the grounds that this treatment would prevent point streaking and other silver-staining artefacts. Clearly, in the light of the above discussion, it appears to be a much smarter move to reduce and alkylate the sample just to start with, i.e. prior to even the 1st IEF/IPG dimension. The drawbacks of alkylation with iodoacetamide in between the two-dimension runs have also been highlighted by Yan et al. [17], although on different grounds than as discussed above. These authors noted that in most 2D protocols, the discontinuous Laemmli [18] buffer is used, which calls for a stacking and sample gels to be equilibrated in a pH 6.8 buffer. In order to prevent pH alterations, most people use a modified stacking buffer with a reducing agent (DTT or DTE) and alkylating agent (iodoacetamide) at pH 6.8 so that the strips can be loaded as such after these two treatments, avoiding any further pH manipulations [19]. However, at this low pH both reduction and alkylation are not so efficient, since the optimal pH for these reactions is usually at pH 8.5–8.9 [20]. As a result of this poor protocol, Yan et al. [17] have reported additional alkylation by free acrylamide during the SDS–PAGE run, with the same protein exhibiting a major peak of Cys-carboxyamidomethyl and a minor one of Cys-propionamide. It should be borne in mind, in fact, that whereas the risk of acrylamide adduct formation is much reduced in IPG gels (but not in unwashed IEF gels!), it is quite real in SDS–PAGE gels, due to the fact that these gels are not washed and that surfactants, in general, hamper incorporation of monomers into the growing polymer chain [21]. Different alkylating residues on a protein will complicate their recognition by MALDI-TOF analysis, a tool much in use today in proteomics. Here too, however, although Yan et al. [17]

clearly identified the problem, they did not give the solution we are proposing here, namely to reduce and alkylate the sample prior to any electrophoretic step. They suggest to still retain the original protocol of alkylation in between the two dimensions, but to increase the pH of the equilibration to pH 8.0, add a much large amount of alkylating agent (125 mM iodoacetamide) and increase the time of incubation to 15 min. We again stress that although this new protocol is an improvement over previous ones, it still does not cure the problems of the 1st IEF/IPG dimension, namely smears and formation of spurious bands, both due to reoxidation of reduced but non-alkylated Cys residues. As an alternative to alkylation, Olsson et al. [22] have proposed oxidation of thiol groups in proteins to mixed disulphides, by using, in the gel rehydration solution, an excess (100 mM) of hydroxyethyldisulphide (HO-CH_2-CH_2-S-S-CH_2-CH_2-OH; in their procedure, cup-loading at the anodal gel side is the preferred way of sample application. A simplified protocol was also recently published by the same group) [36]. Note that this is an equilibrium reaction; thus an excess HED has to be present at all times during the IEF/IPG run. In addition, this procedure has one drawback: during the interfacing between the first and second dimension, this reagent will be lost, which means that prior to SDS–PAGE, the IPG strip has to be treated with alkylating agents again.

As a brief conclusion to this section, we believe that reduction and alkylation of any sample that has to undergo an IEF/IPG separation has to be performed prior to entering the electric field, as amply documented in a series of papers we published on this topic [23–25].

5. Correct alkylation protocols. Is there a risk of over-alkylation?

Having established that reduction and alkylation of the protein sample prior to any focusing step is a must, if one wants
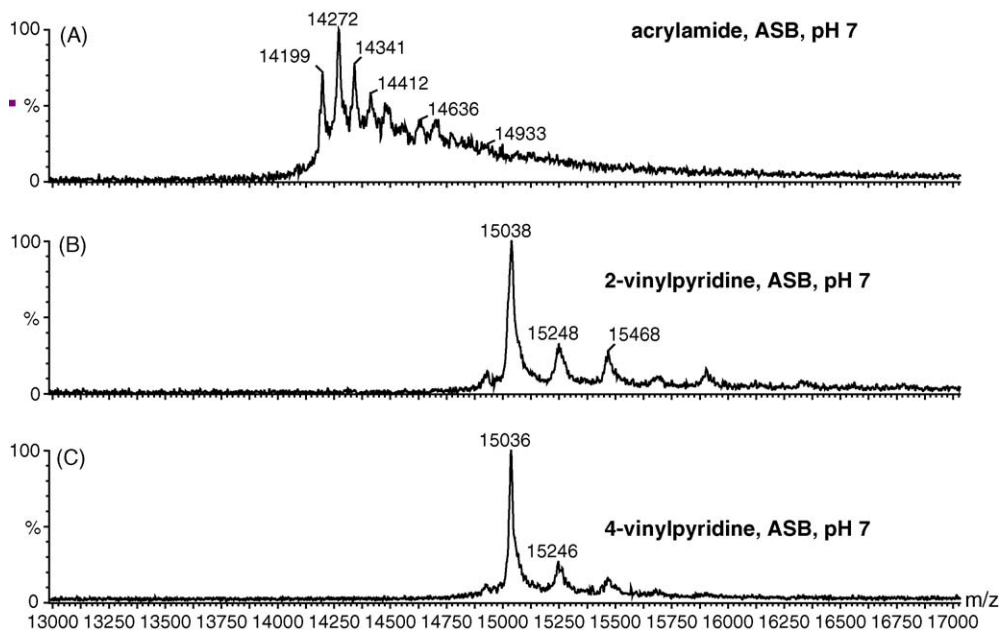
Fig. 3. Alkylation efficiency of lysozyme with either acrylamide (panel A), or 2-vinylpyridine (VP) (B) of 4-vinylpyridine (lowermost panel) in presence of a surfactant (sulphobetaine) at pH 7.0. Note the poor reactivity of acrylamide as compared to 2- or 4-VP. With the two last reagents, the two additional peaks at higher Mr values represent the mono- and bi-adducts with sinapinic acid (from Sebastiano et al. [27], by permission).

to avoid all sorts of spurious spots, there still remains the open question on the correct way to perform it. A note of caution is here expressed on the use of iodoacetamide, by far the most common −SH group alkylator. In most protocols for IEF/IPG in proteome analysis, a 7 M urea, 2 M thiourea mixture is used as universal solubilizing agent. A thorough investigation by ESI-MS [24] demonstrated that indeed thiourea was competing

with the free −SH groups of proteins for reaction and scavenging iodoacetamide at a fast rate (in *ca.* 5 min of incubation all iodoacetamide present in solution had fully disappeared). Iodoacetamide was found to add to the sulphur atom of thiourea; the reaction was also driven in this direction by the fact that once this adduct was formed, thiourea was deamidated and the reaction product generated a cyclic compound (a thiazolinone
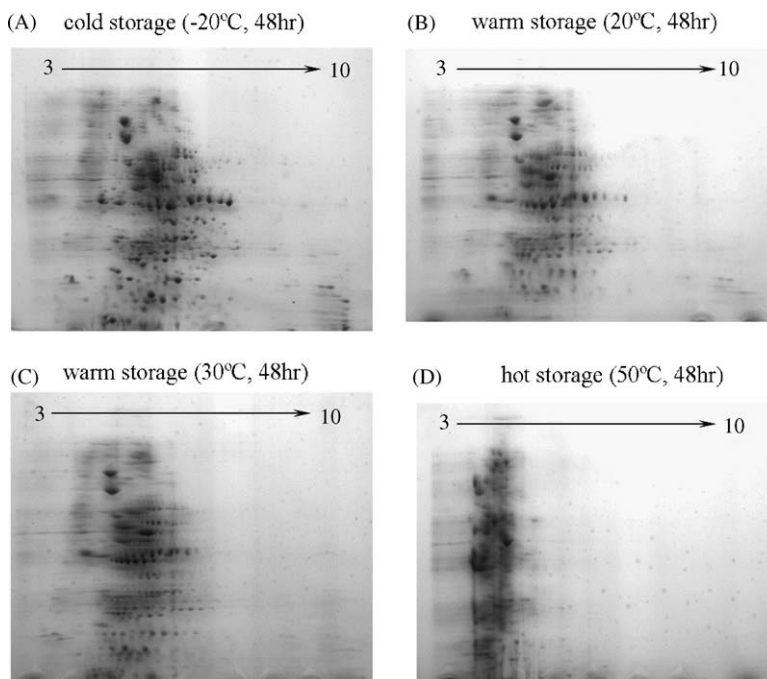

Fig. 4. Two-dimensional maps of an *E. coli* lysate stored for 48 h at progressively higher temperatures (form −20 up to 50 °C). Note how, as the temperature is increased, progressively higher sample carbamylation is experienced till, at 55 °C, all alkaline spots have completely disappeared and are reduced to a narrow range of acidic spots, indicating massive carbamylation of basic side chains (from McCarthy et al. [34], by permission).

derivative). Thus, those using urea/thiourea solutions should be aware of this side reaction and of the potential risk of not achieving full alkylation of the free, reduced −SH groups in Cys residues. As an alternative to alkylating with iodoacetamide, alkylation with acrylamide (producing a thioether derivative, Cys-S-β-propionamide) has been recommended [26]. Acrylamide is insensitive to the presence of thiourea, so that the alkylation reaction is not hampered. However, the reaction might be slow and might not reach completion. As a remedy to it, Sebastiano et al. [27] have proposed 2- and 4-vinylpyridine (VP) as efficient alkylators. These reagents appear to drive the alkylation reaction to completion, even in the most adverse cases of proteins very rich in cysteine groups such as albumin (35 Cys) and phosphorylase (36 Cys) and in presence of alkylation inhibitors such as surfactants and organic solvents (see Fig. 3). These two vinylpyridine compounds appear moreover, to be endowed with a few highly desirable properties:

(a) In the one hand, they seem to drive the alkylation reaction to 100%, a most desirable property, indeed, not achieved, up to the present, with any other agent.
(b) In the other hand, another most important property is the absolute specificity of the reaction: even up to 24 h of incubation, we are unable to find any other alkylation site than Cys, with all other potentially reacting groups (notably Lys) fully intact.
(c) As a third bonus, reaction of Cys residues with vinylpyridines appears to be very fast, since it is usually over within 1 h of incubation.
(d) Finally, the adduct formed on the −SH group appears to be highly stable towards hydrolysis [13], contrary to IAA in which at least the amido bond can be easily hydrolyzed, introducing a charge in an otherwise neutral compound.

The absolute specificity of the reaction could be linked to the fact that one can efficiently drive the reaction at neutral pH (7.0 or neighbouring values), i.e. at pH values about half a way between the pK values of the two reacting groups (5.4 for vinylpyridine, 8.3 for the −SH of Cys). At these pH values, both partners should still bear a fractional charge (ca. 10%), positive for vinylpyridine and negative for Cys. The two partners would still be able to seek each other out in solution by electrostatic attraction and drive the reaction at a fast speed. Conversely, when alkylating with normal agents, such as IAA or acrylamide, reaction kinetics slow down considerably if one lowers the pH from ca. 8.9 (where the −SH group is highly negatively charged, thus highly reactive) to pH 7.0, a pH guaranteeing only a modest fractional charge on Cys. Thus, in this last case, specificity of reaction would be obtained at the expense of incredibly long reaction times, unable in addition to offer 100% conversion.

There is a final question to be asked, namely, is there a risk of over-alkylation? In this case, alkylating proteins would be quite deleterious, since spurious spots would be created due to excess alkylation, which would go, in addition to −SH groups, also onto Lys residues, thus generating trains of spots with different pI values. Indeed our protocols have been recently challenged by Luche et al. [29] who reported incomplete and even spurious

alkylation on Lys residues, both phenomena producing marked artefacts in 2D maps. These authors, however, did not seem to be aware of the work of Sebastiano et al. [27] on VPs, neither of the work of Mineki et al. [26], who reported 97% alkylation of SH groups in BSA with high levels of acrylamide. I believe that Luche et al. [29] made the fundamental mistake of not removing the excess alkylant during the IPG run (were alkylation
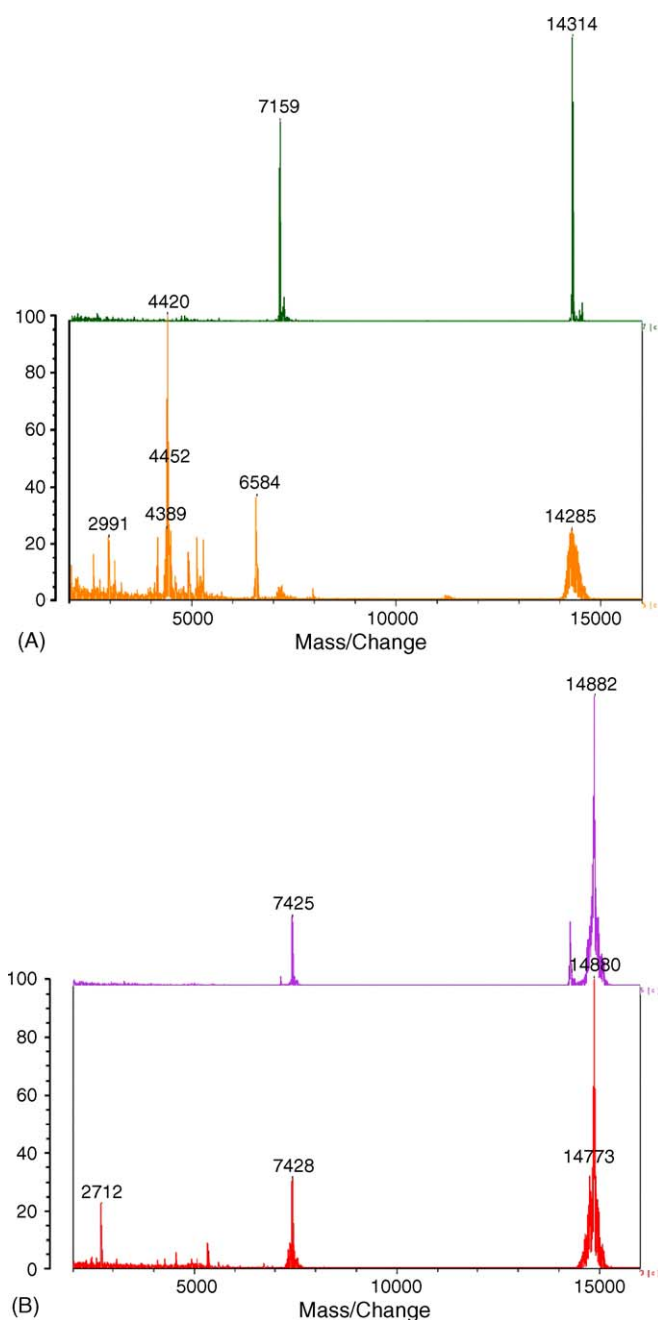

Fig. 5. Desulfuration (β-elimination) of lysozyme in an electric field. Panel A: mass spectra of control lysozyme (upper two peaks, representing the single and doubly charged species) and lysozyme exposed for 48 h to the electric field. Note, in this last case, the massive sample degradation into a series of fragments, with a massive disappearance of the intact lysozyme peak (Mr 14314). Panel B: same as A, but with the sample fully alkylated. *Note:* In this last case, the maintenance of sample integrity, with negligible formation of lysates at lower Mr values (from Herbert et al. [35], by permission).

will continue undisturbed!). In fact, in all alkylating protocols, the excess of alkylator should be removed prior to entering the electric field so as to prevent the reaction from proceeding and even being accelerated by the application of the voltage gradient.

6. Carbamylation

Carbamylation is widely quoted as being a problem in 2D gel analysis and the associated sample preparation steps [30]. This modification occurs when iso-cyanate, a urea break-down product, covalently modifies lysine residues, thus inducing a change in isoelectric point. Urea is used at up to 9 M concentrations in sample preparation and 2D gels because of its ability to disrupt protein structure and effect denaturation without the need for ionic surfactants such as SDS. In solution, urea is in equilibrium with ammonium cyanate. By using mixed-bed ion exchangers, urea solutions can be prepared that are initially cyanate free, but the concentration of ammonium cyanate will slowly increase over time until equilibrium is reached. However, if a cyanate scavenger, such as the ε-amino group of lysine, is present, the formation of cyanate will continue unabated until the scavenger is completely consumed. At temperatures below 37 °C, the degradation of urea proceeds slowly and concentrations of cyanate do not reach problematic levels within the time most sample preparation procedures take. Higher temperatures accelerate the rate at which ammonium cyanate is produced and thus should be avoided when preparing protein samples in urea. Cyanate has been shown to react with nucleophilic groups such as the protein amino terminus, the amino side chains of lysine and arginine residues and the sulfhydryl group of cysteine residues [31–33]. The reaction occurs more rapidly in alkaline conditions when the nucleophilic groups are deprotonated and

thus more reactive and the relative reactivity of the residues is dependent on their pK_a. The free base forms of aliphatic amines, such as the ε-amino group of lysine, are present at very low concentrations below pH 8. The carbamylation reaction of amines by isocyanic acid is strongly pH dependent and a pH of 8.5–9.5 is usually optimal for modifying lysine residues. In contrast, the α-amino group at a protein's amino terminus is neutral, with a pK_a of $\sim$7, and may be selectively modified by reaction at near neutral pH. McCarthy et al. [34] have studied carbamylation using 7 M urea and 2 M thiourea under a range of experimental temperatures to establish when, and if, it occurs and what can be done to minimise the modification. The actual time required for protein extraction from a tissue is usually short compared to the time required for procedures such as reduction and alkylation and IPG rehydration and focusing. Therefore, the temperature during these post-extraction procedures is the most critical factor. Their experiments have shown that carbamylation simply does not occur during electrophoresis in the presence of urea, even with prolonged run times. However, upon prolonged storage conditions (48 h) at high temperatures (50 °C) massive carbamylation occurs, as shown in Fig. 4. Thus, it can be safely concluded that under normal operating conditions of IEF/IPG, the sample is not at risk of any carbamylation event.

7. β-Elimination (desulfuration)

This is the only true noxious and unexpected artefact in proteome analysis [35]. It results on the loss of an H_2S group (34 Da) from Cys residues for proteins focusing in the alkaline pH region. Upon such an elimination event, a dehydro alanine residue is generated at the Cys site. In turn, the presence of a double bond in this position elicits lysis of the bond in C−N, generating a number of peptides of fairly large size from an intact protein. The first
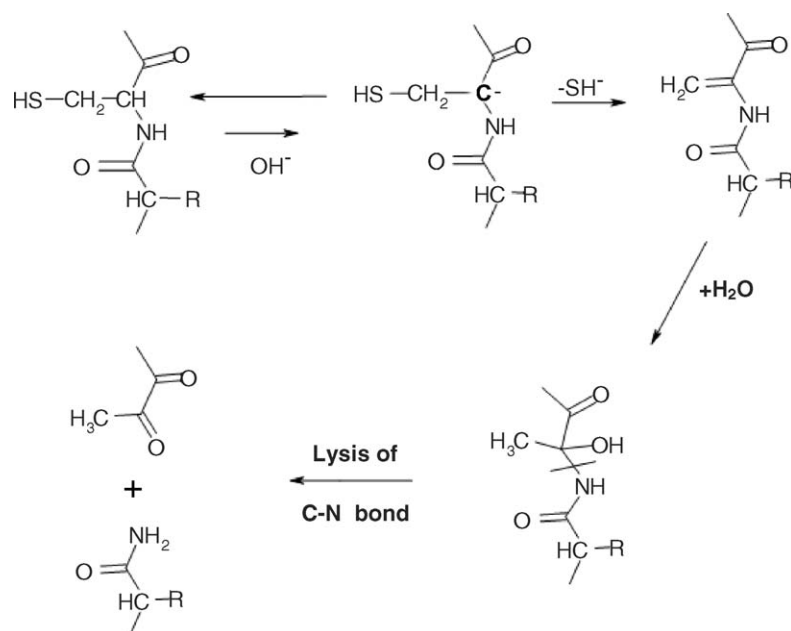

Fig. 6. Reaction scheme of a desulfuration event, followed by rupture of the polypeptide chain at the level of the dehydro alanine formed. Note that the polymer chain is not lysed at the level of the peptide bond, but along a C−N bond (from Herbert et al. [35], by permission).

process seems to be favoured by the electric field, probably due to the continuous harvesting of the SH$^-$ anion produced. In order to demonstrate this phenomenon, we have taken lysozyme (a basic protein) and subjected it to electrolysis in a multicompartment electrolyzer (MCE) at pH = pI = 9.5. In the control spectrum, only a single peak was visible corresponding to the Mr value of the intact protein (14313 Da). After 6 h in the electric field, the same spectrum revealed two additional compounds, one centred at *m/z* 14278 (corresponding to the loss of 34 Da) and one at *m/z* 14215 (loss of 98 Da). Such Mr decrements are consistent with the loss of one and three H$_2$S groups, respectively. Such a process is known as β-elimination from Cys residues, transforming them into dehydro alanine residues. If the process is continued for 24 h, the peak of the intact protein disappears, giving rise to an heterogeneous spectrum of peaks exhibiting progressively mass losses down to *m/z* 14152. This seems to be an electrically driven process, since the same lysozyme solution, if left standing on a test tube for the same time intervals at the same pH, does not show any degradation. The situation seems to be even more complex than the one just described. When the entire spectrum of *m/z* values is explored (see Fig. 5A, lower tracing), it appears as though, upon prolonged electrophoretic times (48 h), a number of low Mr peaks appear (in the Mr interval 2990–6583 Da), suggesting that after β-elimination, the protein is progressively broken down into small to large peptide fragments. This must occur at the expense of the intact protein species, since the peak of native lysozyme (*m/z* 14313) essentially disappears.

The only remedy found to this noxious degradation pathway is the reduction and alkylation of all Cys residues prior to their exposure to the electric field. Alkylation appears to substantially reduce both β-elimination and the subsequent amido bond lysis, as clearly shown in Fig. 5B. The series of events leading to desulfuration and subsequent lysis of the C–N bond along the polypeptide chain is illustrated in Fig. 6.

8. Conclusions

Artefacts such as deamidation and carbamylation, blamed since the very inception of 2D map analysis, in the late seventies of last century, and used as a weapon for rejecting unwanted manuscripts in journals, simply do not occur in any IEF/IPG protocol performed with a minimum of care. Definitely, the biggest offenders are maladroit procedures omitting proper sample reduction and alkylation, as, most unfortunately, performed up to at least 2002. It is clearly shown here that proper sample handling and well-implemented sample alkylation at the very start of any 2D fractionation have the following immediate advantages:

1. Considerably reduce of smears in the alkaline region, above pH 8;
2. Prevent formation of spurious bands due to mixed disulphide bridges;
3. Abolish formation of a mixed population of Cys-propionamide and Cys-carboxyamidomethyl species, due to alkylation with acrylamide and iodoacetamide, respectively

4. Prevent degradation of alkaline proteins into large peptides via a desulfuration event.

It is hoped that this brief survey will help scientists to adopt correct procedures and avoid pitfalls that could lead to disastrous results in proteome analysis.

9. Note added

In order to further clarify some points expounded here and to answer to some comments of the referee, I will summarize here the advantages and limitations of the present technique, especially in regard to 2VP alkylation, which I deem most suitable a procedure for handling proteins prior to 2D mapping.

1. *Alkylation with acrylamide*. In this case, high molarities of alkylant are required, typically of the order of 50 to 100 mM acrylamide. The reason is that even at pH 8.5 to 9.0, where the −SH group of Cys (pK 8.3) is highly ionised, and thus highly reactive, acrylamide is a slow reacting species. Thus, in order to shift the equilibrium towards the formation of reacted species, high levels of acrylamide are required.
2. *Alkylation with 2-VP*. Here the reaction conditions are quite different. First of all, due to the much higher reactivity of 2-VP, its concentration can be reduced to 20 mM. It should also be noted that the reaction here is conducted at neutral pH values (pH 7.0). This is optimal for the reactivity of both compounds (i.e. 2-VP and −SH groups of Cys), since it is about half a way between the pK values of the −SH groups (8.3) and the pK value of the tertiary amino group of 2-VP (pK 5.4): under these conditions, the reaction is driven by the partial negative and positive charges, respectively, of the two reacting species and continues at a fast rate till full alkylation of all Cys residues. As an additional bonus, this neutral pH too during reaction ensures its very high specificity, since at this pH value the ε-amino groups of Lys, being fully protonated, are unreactive. Thus, 2-VP appears to be among the best alkylating agents, since it couples 100% reactivity with 100% specificity, conditions rarely achievable with any other alkylating agent.
3. *Alkylation with acrylamide derivatives versus iodinated compounds*. It is well known that the typical alkylating agent for Cys residues, exploited in most protocols up to the present, is iodoacetamide. Indeed, our data have shown that alkylation with acrylamide and/or its derivatives is to be preferred, especially when dealing with 2D maps. The preferred solubilization cocktail for tissue and cell proteins, in use today, contains a mixture of 2 M thiourea and 7 M urea. We have demonstrated that thiourea is a scavenger of iodoacetamide, to the point at which all the amount added (typically of the order of 50 mM and higher) is consumed within 5 min of incubation [24,25]. Thus, under these conditions, alkylation with iodoacetamide is incomplete and hardly quantitative. This also is the reason why the classical ICAT reagent [37,38] cannot possibly be used in 2D mapping; besides the fact that this reagent is terribly expensive, since it has too a reacting iodinated tail, it will be immediately destroyed upon incuba-

tion of proteins dissolved in the solubilization cocktail, thus impeding proper tagging and proper quantitative analysis.

4. *Health hazards*. It should be remembered that both acrylamide and 2-VP are neurotoxins, as typical of all compounds containing a reactive acrylic double bond (as reviewed in [39]). Thus, great care should be taken in handling them. For acrylamide, which is less toxic than 2-VP, because it is a solid, one should nevertheless be careful about not accidentally breathing the powder when handling it for preparing a polyacrylamide gel or for alkylating purposes. In the case of 2-VP, although this compound is more toxic because of being more volatile (its physical state is a liquid), nevertheless it is more easily handled just because it can be transferred by pipetting. In both cases, though, these compounds should be handled only under a fume hood.

5. *2-VP or 4-VP*? In principle, one could use either of these compounds, since they have the same alkylating efficiency and very similar pK values. However, we much prefer using 2-VP due to its higher stability upon storage and under reacting conditions.

6. *Why 2-VP*. There is an additional advantage in using 2-VP alkylation, especially when the protein sample is further analysed by MS. In MS, all peptides labelled with 2-VP, due to the positive charge in this molecule, will give a much stronger signal, considering that most peptide analyses are performed in the positive ion mode.

7. *More on 2-VP and 4-VP*. Although S-pyridylethylation is scarcely used today for blocking −SH groups in proteins, this reaction has been known for more than 35 years [40,41] and it has been accepted as the best method for the modification of Cys residues in proteins for subsequent analysis and sequence determination. An extra bonus of this reaction is that whereas free cysteine and cystine residues are unstable under conditions used for acid hydrolysis of peptide bonds, their 2-VP or 4-VP derivatives are fully stable under the same harsh hydrolysis conditions, thus permitting quantitative recovery of these amino acid residues. This reaction has also been proposed for measuring D-Cys, homocysteine, glutathione, tryptophan, dehydroalanine and furanthiol in food flavours.

Acknowledgements

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