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Review

Determination of the isoelectric point of proteins by capillary isoelectric focusing

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Abstract

Different ways of determining isoelectric points (p*I*) of proteins in capillary isoelectric focusing are reviewed here. Due to the impossibility of direct pH measurements in the liquid phase, such assessments have to rely on the use of p*I* markers. Different types of p*I* markers have been described: dyes, fluorescently labelled peptides, sets of proteins of known p*I* values. It appears that, perhaps, the best system is a set of 16 synthetic peptides, trimers to hexamers, made to contain each a Trp residue for easy detection at 280 nm. By a careful blend of acidic (Asp, Glu), mildly basic, with p*K* around neutrality (His), and basic (Lys, Arg) amino acids, it is possible to obtain a series of p*I* markers with p*I* values quite evenly distributed along the pH scale, possessing good buffering capacity and conductivity around their p*I* values and thus focusing as sharp peaks. Another approach to p*I* determination is the monitoring of the current during mobilization: this allows, with the aid of known p*I* markers, to calibrate the system with a p*I*/current graph. Pitfalls and common errors in p*I* determinations are reviewed here and guidelines given for minimizing such errors in p*I* estimation.

Keywords: Reviews; Isoelectric points; Isoelectric focusing; Proteins; Amino acids

Contents

1. Introduction

Isoelectric focusing (IEF) is an electrophoretic technique by which amphoteric compounds are fractionated according to their isoelectric points (p*I*) along a continuous pH gradient. Contrary to zone electrophoresis, where the constant (buffered) pH of the separation medium establishes a constant charge density at the surface of the molecule and causes it to migrate with constant mobility (in the absence of molecular sieving), the surface charge of an amphoteric compound in IEF keeps changing, and decreasing, according to its titration curve, as it moves along a pH gradient until it reaches its equilibrium position, i.e. the region where the pH matches its p*I*. There, its mobility equals zero and the molecule comes to a stop.

There are basically two types of pH gradients. In one version, the gradient is created, and maintained, by the passage of an electric current through a solution of amphoteric compounds which have closely spaced p*I* values, encompassing a given pH range. The electrophoretic transport causes these carrier ampholytes (CAs) to stack according to their p*I*

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values, and a pH gradient, increasing from anode to cathode, is established. At the beginning of the run, the medium has a uniform pH, which equals the average p*I* of the CAs. Thus, most ampholytes have a net charge and a net mobility. The most acidic CA moves toward the anode, where it concentrates in a zone whose pH equals its p*I*, while the more basic CAs are driven toward the cathode. A less acidic ampholyte migrates adjacent and just cathodal to the previous one and so on, until all the components of the system reach a steady state. After this stacking process is completed, some CAs still enter zones of higher, or lower, pH by diffusion where they are not any longer in isoelectric equilibrium. But as soon as they enter these zones, the CAs become charged and the applied voltage forces them back to their equilibrium position. This pendulum movement, diffusion versus electrophoresis, is the primary cause of the residual current observed under isoelectric steady-state conditions. Finally, as time progresses, the sample protein molecules also reach their isoelectric point, a pH region where they merge (or focus, tout court) at the p*I* zone, having zero net charge [1,2].

In its improved version, immobilized pH gradients (IPGs), the major problems of CA-IEF (e.g. uneven conductivity, uneven buffering capacity, pH gradient instability, poor engineering of the pH gradient) seem to have been completely solved. IPGs are based on the principle that the pH gradient, which exists prior to the IEF run itself, is copolymerized, and thus insolubilized, within the fibres of a polyacrylamide matrix [4,5]. This is achieved by using, as buffers, a set of six non-amphoteric, weak acids and bases, having the following general chemical composition: CH_2=CH–CO–NH–R, where R denotes either two different weak carboxyl groups, with p*K* values 3.6 and 4.6 or four tertiary amino groups, with p*K* values 6.2, 7.0, 8.5 and 9.3 (available under the trade name Immobiline from Pharmacia-Upjohn). A more extensive set, comprising 10 chemicals (a p*K* 3.1 acidic buffer, a p*K* 10.3 basic buffer and two strong titrants, a p*K* 1 acid and a p$K > 12$ quaternary base) is available as 'p*I* select' from Fluka, Buchs, Switzerland.

IPGs represent perhaps the ultimate development in all focusing techniques, a big revolution in the field, in fact. Due to the possibility of engineering the pH gradient at whim, from the narrowest (which, for practical purposes, has been set at 0.1 pH units over a 10 cm distance) to the widest possible one (a pH 2.5–12 gradient), IPGs permit the highest possible resolving power, in the one hand, and the widest possible collection of spots (in two-dimensional maps) on the other hand. The chemistry is precise and amply developed; so are all algorithms for implementing any possible width and shape of the pH gradient. Due to its unique performance, IPGs represent now the best possible first dimension for two-dimensional maps and are increasingly adopted for this purpose.

Having stated that, let us clear the grounds from myths and fata morgana's which keep popping up from time to time in the scientific literature. There would appear to exist also a third approach to pH gradient engineering, namely the so called "thermally engendered pH gradients", which could be generated either by forming a temperature gradient (via outside thermostats) or by using tapered capillaries (the heating would come from inside, i.e. Joule heating!) [6–10]. Well, these approaches simply do not work, no matter how strong are the claims made by the "inventors". Had these scientists bothered to read earlier literature, they would have discovered that Lundahl and Hjertèn [11] had proposed (and simply dismissed) them already in 1973!

Another important statement is here due. Due to their nature and chemistry, IPGs have never been implemented in a capillary format, and probably never will. Thus, this review will only deal with CA-IEF (cIEF, where "c" stands for capillary), the only method largely adopted in most biochemistry laboratories. A large number of reviews have already appeared covering all aspects of cIEF, and are listed here for further readings [12–25]. A most recent and broad review, worth perusing, covers all aspects of cIEF and other capillary-based separations as applied to proteomics analysis [26]. A further review, although not connected at all with cIEF, gives a broad coverage on the use of IEF for investigating post-translational processing and chemical modification of proteins [27].

2. On the definition of isoelectric point and on the factors affecting its measurement

The p*I* is a singularity point in a titration curve, corresponding to the pH in solution at which the net surface charge, and thus the mobility, of a protein equals zero. If referred to a random coil, the p*I* only depends on a protein amino acid composition, and can thus be computed from analytical or sequence data. In contrast, in a native structure, where interactions among amino acid stretches can occur, the experimental p*I* may be subtly altered. As a rule, when moved to a hydrophobic environment, a group shifts its dissociation so as to favour the uncharged form (i.e. the p*K* of an acid increases and that of a base decreases). An example is the unique separation induced between Aγ and Gγ (two globin fetal chains differing by a Gly → Ala substitution at the residue 136, thus having identical surface charge) in presence of the surfactant Nonidet P-40, which suppresses one charge (probably of a Lys residue) in the Aγ chain [28].

Measuring a p*I* value with precision is not an easy task, especially, as we will see, in CA-IEF. A p*I* is a physico-chemical parameter and its precise assessment might be quite precious in establishing the identity of a protein. But a number of experimental parameters might alter its value. For instance, p*I* measurements should be performed at a strictly controlled temperature, since ionization constants (p*K* values) are temperature-dependent. The standard heat of ionization is rather modest for carboxyl groups, but can be quite high for amino groups [29], e.g. the p*K* of a carboxyl varies from 4.50 at 25 °C to 4.56 at 4 °C, thus with a Δp*K* of only 0.06. However, the ε-amino group

of Lys varies from 10.00 at 25 °C to 10.67 at 4 °C, with a much larger ΔpK (0.67, larger by one order of magnitude!). Also urea strongly affects p*I* determinations. In IEF experiments in presence of 6 M urea, Ui [30] had suggested an overall correction factor of 0.42 pH units, to be subtracted from the apparent p*I* of a given protein in this medium. However, Gianazza et al. [31] reported that the urea effect varies with the various Ampholine pH ranges, from 500th to 700th of a pH unit per unit of urea molarity in going from acidic to alkaline pH ranges, in agreement with a report by Gelsema and De Ligny [32], suggesting that the use of a unique correction term irrespective of the p*I* values of the ampholytes used is incorrect. If one were to use other types of hydro–organic solvents (e.g. mixtures of water–ethanol, water–2-propanol, water–methanol, water–acetonitrile or water–dimethyl sulphoxide, as often reported in capillary zone electrophoresis and sometimes in cIEF) a whole series of corrections for the acidity constants of analytes would have to be applied [33,34]. Were this not enough, a note of caution should be spent on p*I* measurements performed on open-face, gel stabilized systems, such as gel slabs. For a long time it had been known that p*I* values of alkaline proteins, as measured in thin-layer IEF, were consistently lower (by as much as 1–2 pH units) than those determined in density gradient IEF. Delincée and Radola [35] found this to be due to CO_2 absorption by the open gel surface in thin-layer gels, this interference being quite strong above pH 8.2–8.3.

The situation is much better in the case of IPGs. Bjellqvist et al. [36] have established a relevant pH scale for IPG runs in 8 M urea at 20 °C, while validating a correspondence between the focusing position on such a gradient and the protein p*I* as computed from its known sequence. At least in the pH 4–7.5 interval [37], the accordance between the experimental p*I* of a protein and the figure computed for a random-coiled structure from type and number of dissociating side-chains is so close (within one third to one half of a charge unit!) so as to allow the assignment of a polypeptide spot in a two-dimensional map to a known sequence (the second positional parameter, M_r, being immediately derived from amino acid composition). Any discrepancy from the expected focusing position, and exceeding the experimental error, may then be assumed to imply some structural modification in the species under investigation. By this approach, Bjellqvist et al. could identify either 4 [37] or 18–20 cases [38] of blocked NH_2 termini; for other proteins, they raised doubts about the polypeptide chain being glycosylated or even incorrectly sequenced [38]. This is truly extraordinary and one could ask how could they possibly do that. The fact is that the discrepancies they found between theoretical and experimental p*I* values [38] were of the order of a few hundredths of a pH unit, i.e. well above (about one order of magnitude) the current resolving power with narrow-range IPGs (in a side to side comparison of different isoforms, a few thousandths of a pH unit represent a sufficient ΔpI for unambiguous resolution) [39].

3. Isoelectric focusing in coated and uncoated capillaries

There are basically two ways for performing cIEF: the use of coated or uncoated capillaries. In the first case, due to suppression (in the best cases elimination) of electroendoosmotic flow (EOF), the process requires a two-step protocol: a focusing step, followed by mobilization, in order to force the focused (thus stationary) bands past the detector [40,41]. In the second case, a partial, dynamic coating (with soluble polymers, such as hydroxypropyl methyl cellulose or hydroxyl ethyl cellulose) is allowed, so as to partially quench the EOF, and focusing takes place while the entire pH gradient and train of bands is moving towards the capillary outlet, at the detection port [42,43]. We much prefer the first method, since it offers two advantages: (a) a much higher reproducibility (run-to-run and day-to-day); and (b) a much reduced chance of accidental protein adsorption to the silica wall, since said wall is well shielded by a covalently attached, highly hydrophilic layer of polymer; e.g. Huang and Richards [44], in commercially available neutral polymer coated capillaries, reported consecutive runs (>113) of several proteins with p*I* values 2.75–9.45 with good migration time reproducibility (<2% R.S.D.). Tang et al. [45] developed a method, for routine cIEF of recombinant immunoglobulins, in coated capillaries, offering an R.S.D. in peak area <2% intraday and <8% interday. The R.S.D. for mobilization times of the various IgG peaks was <1% intraday and <3% interday. With a highly hydrophilic, highly hydrolysis-stable acrylamide derivative (acryloyl amino ethoxy ethanol) [46,47], Talmadge et al. [48] have been able to perform several hundreds of analyses by cIEF with zwitterion mobilization. With yet another N-substituted acrylamide (*N*-acryloyl amino propanol), Gelfi et al. [49] reported highly reproducible separations and peak profiles for >350 runs.

4. Salts and solubilizers

Salts at high concentration in a sample could cause problems in cIEF separations, i.e. excessive current, which is destructive to both proteins and coatings, in the early phase of separation, and can cause distortion and constriction of the pH gradient, thus reducing peak capacity and reproducibility. An on-line desalting protocol was described by Clarke et al. [50] for effectively removing high salt levels from samples by voltage ramping. Conditions for cIEF for separating human cerebrospinal fluid (CSF) proteins were also examined by Manabe et al. [51]. Since the salt concentration of CSF is as high as that of plasma, desalting was deemed necessary for proper cIEF and was carried out with a miniaturized dialysis apparatus. In case salt removal might not be easily performed, Mao and Pawliszyn [52] reported a computational method for correcting the deformed separation patterns caused by presence of salt in the imaged

cIEF system. Another cause of concern is the potential corrosion of covalent coatings due to use of strong acids and bases and anode and cathode, respectively. We recommend to abolish all non-buffering ions in a cIEF system, which means using also weak electrolytes at the electrodic compartments; e.g. instead of phosphoric acid, one can adopt acetic acid, or zwitterions, such as free Asp or Glu or imino diacetic acids, as anolytes; conversely, as catholytes, one should use free Lys, Arg or Tris, ethanolamine, and the like, in lieu of the highly corrosive NaOH, unless one uses highly hydrolytically stable N-substituted acrylamides [53,54].

Another non-negligible hazard of cIEF is the possibility of protein precipitation at or near the p*I* value. Isoelectric proteins have a minimum of charge, thus a minimum of solvation and therefore poor solubility at the p*I*; this is worsened by the fact that, in a focusing process, the analyte peak is highly concentrated. Neutral or zwitterionic reagents can be added to cIEF for mitigating precipitation problems. A variety of additives, compatible with cIEF, were tested by Conti et al. [55] in cIEF of native proteins. Glycerol, ethylene glycol, propylene glycol, sulfobetaine, taurine, *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), saccharose and sorbitol were found to be effective. Synergistic effects were observed for combinations of sugars and taurine. In agreement with that, Tran et al. [56] reported good separations of an envelope glycoprotein of the human immunodeficiency virus (HIV) in a combination of 6% saccharose and 0.085 M CAPS.

5. Isoelectric point measurements in capillary isoelectric focusing

Finally, we enter the real argument of this review! Let us clear the grounds: in cIEF, there is no other way for charting the course of pH gradients than to use p*I* markers, contrary to CA-IEF in gel slabs, where direct pH measurements can be performed [3] or to IPGs, where the precise chemistry of pH engineering allows direct interpolation of p*I* values [5]. Fig. 1 gives an example of mapping pH gradients and extrapolating p*I* values in the cIEF of different RNases [57]. This is brought in as a negative example, for which the authors should be scolded: one should never use simply a few markers and make a linear interpolation in a wide (3–10) pH gradient! For sure, the pH course will not be linear, this is a chemical dogma. Due to the fact that, in all synthetic approaches, there are many more acidic CAs and fewer (and poorer too, in terms of buffering capacity and conductivity) basic ones, the plot will be largely non-linear. In fact, Shimura et al. [58] found that the relationship between detection time versus pH was not linear in most cases and thus concluded that the use of a linear calibration over the entire pH gradient would be erroneous. As luck goes, perhaps their p*I* measurements [57] were not so terrible, since their unknown proteins happened to focus rather close to some

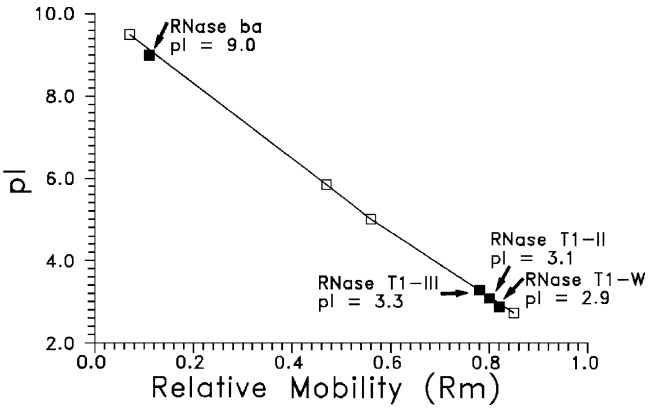

Fig. 1. Calibration graph for p*I* determination using a set of marker proteins. The markers (open squares) are: ribonuclease a (p*I* 9.45); carbonic anhydrase (p*I* 5.90); β-lactoglobulin B (p*I* 5.1); unsulphated cholecystikinin flanking peptide (p*I* 2.75). The four solid squares represent four unknown proteins, whose p*I* values have been determined by linear interpolation in the calibration graph (from [57] with permission).

of the p*I* markers; nevertheless, this is not an example to follow.

That non-linearity could be the case is also shown in the example of Fig. 2 [59]. This is another approach for p*I* assessments: monitoring the current during the mobilization step. If the peaks of the mobilized stack of proteins are monitored simultaneously with the rising current due to the passage of the salt front in the capillary (it should be recalled here that one of the most popular methods for protein elution after cIEF is to add salt, e.g. NaCl, to one of the electrode reservoirs), one can correlate a given p*I* value (which should already be known from the literature) with a given current associated with the transit of a peak at the detector port. The system can thus be standardized and used for constructing a calibration graph to be adopted in further work, without resorting to internal standards. It can be appreciated, from Fig. 2, that the p*I* (and thus pH) plot is not linear.

In another approach, Slais and Friedl [60] proposed the synthesis of 10 different dyes, of the aminomethylnitrophenol

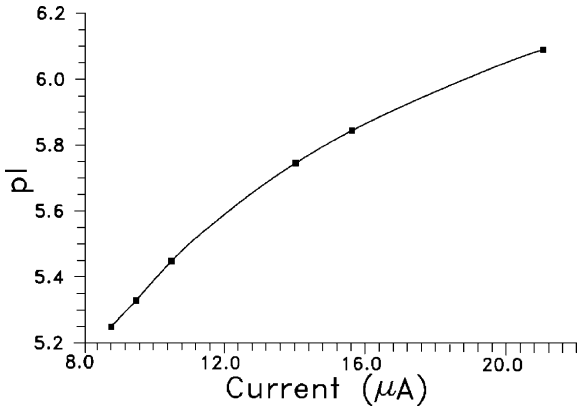

Fig. 2. Calibration graph for p*I* determination using the current, during the mobilization step, as a parameter in cIEF. The six experimental points represent six forms of transferrin, containing different amounts of sialic acid and of iron (from [59] with permission).

and of the aminomethylated sulphonaphthalene families. These dyes covered the grounds from pH 3.9 up to 10.3, and appeared to be quite evenly distributed along the pH scale. They had the following advantages: they would be definitely more stable than protein p*I* markers (know to be subjected to ageing, a process leading to deamidation and thus to strings of spots situated on the acidic side of the parental protein) and, in addition, they could be detected by both, UV absorption (in the near UV region) as well as fluorimetric detection, since they could be excited in the same UV region to fluoresce [61]. While this approach is certainly most interesting, there is a caveat, though: already at its inception, Molteni et al. [62], who had tested them prior to their official release, lamented that these dyes could be easily adsorbed by the silica wall, producing additional EOF and unstable drifting of the pH gradient. In another approach, Shimura and Kasai [63] suggested stable, fluorescence-labelled peptides for assessing protein p*I* values. This approach was problem-prone too, since, due to the fact that the markers could only be detected by fluorescence, the protein too, whose p*I* values had to be measured, had to be derivatized with fluorescence markers as well. This posed a serious problem, since it is known that the derivatization of proteins at all possible reacting sites is an impossible task, thus one would have to find conditions for minimal labelling, i.e. attaching the probe to a single reactive group. For that reason, Kobayashi et al. [64] proposed peptide digests from protamine (this relatively basic protein being selected for the specific purpose of obtaining also alkaline p*I* markers) and two additional synthetic peptides (Gly–Gly–Gly and Gly–Gly–His), all of them labelled with dansyl chloride and subsequently purified by preparative IEF in the Rotofor, as p*I* markers in cIEF. Since dansyl absorbs more close to 300 nm, with protein maxima of absorption being at 280 nm, they reasoned that measuring absorption at both wavelengths would allow to distinguish protein peaks from those of the p*I* markers. According to these authors, resolution (and precision on p*I* assessments) of 0.1 pH unit would be routinely achieved, whereas a p*I* 0.01 resolution could be possible only under certain conditions.

In yet another approach, Mohan and Lee [65] proposed a hybrid technique, consisting in using a set of 10, commercially available, p*I* marker proteins, combined with seven, UV-absorbing, tryptic peptides isolated from cytochrome *c* (see Table 1 for their list and p*I* values). By using this approach (allowing to map pH values high up in the alkaline pH region), combined with admixing to the pH 3–10 Pharmalytes the pH 9–11 Ampholine interval and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), they could resolve and measure the p*I* (pH 12) of even the very alkaline peptide bradykinin.

Perhaps the best approach, though, could be the one of Shimura et al. [66], who have proposed a set of 16 synthetic oligopeptides (trimers to hexamers) as isoelectric point markers for cIEF, fully compatible with UV absorption detection (see Table 2). The synthetic approach was quite smart, indeed. Each peptide was made to contain one Trp residue for detection by UV absorption and other residues having ionic side-chains, responsible for giving sharply-focusing peak during cIEF. In order to obtain this set of 16 p*I* markers, with a fairly even distribution along the pH scale, some rules were followed: the basic ones were made to contain mostly Lys and Arg residues; the neutral ones had to be made with His residues (remember, it is the only amino acid able to buffer along neutrality!) and the acidic ones were made to contain progressively higher level of Glu and, finally, Asp residues. The p*I* values of these peptides were determined by slab-gel IEF by using commercial carrier ampholytes. The focused peptides in the gel were detected at 280 nm and the pH gradient was determined with the aid of an oxidized metal membrane electrode. The p*I* values of the peptides range from as low as 3.38 up to 10.17. The measured values agreed well with the predicted ones, based on amino acid composition, with root mean square differences of 0.15 pH units. The sharp focusing, stability, high purity and high solubility of these synthetic p*I* markers should facilitate the profiling of a pH gradient in cIEF and the determination of p*I* values of proteins. Note the last column in Table 2, giving the slope of the charge over the pH axis at the p*I* value: the higher this value, the better is the ampholyte, since it will exhibit good conductivity and good buffering capacity in the proximity of its p*I*. These figures are very high when the p*I* is rather close to the p*K* values of the ionizable side-chains (accordingly, these values are above unity at the extremes of the pH scale, i.e. for the most acidic and most basic peptides and for number 36, whose p*I* falls in proximity of the p*K* value of the imidazole ring in His). According to this rule, the worst possible one is number 37, having a p*I* value (5.91) quite removed for the p*K* of the γ-carboxyl

Table 1
List of proteins/peptides for p*I* calibration[a]

Symbol	Protein/peptide	p*I*
P1	β-Lactoglobulin	5.20
P2	Carbonic anhydrase B (bovine)	5.85
P3	Carbonic anhydrase B (human)	6.55
P4	Myoglobin, acidic band	6.85
P5	Myoglobin, basic band	7.35
P6	Lentil lectin, acidic band	8.15
P7	Lentil lectin, middle band	8.45
P8	Lentil lectin, basic band	8.65
P9	Trypsinogen	9.30
P10	Cytochrome *c*	10.25
C1	EETLME**Y**LENPK	3.67
C2	EDLIA**Y**LK	4.37
C3	TGQAPGFT**Y**TDANK	5.50
C4	MI**F**AGIK	8.50
C5	**Y**IPGTK	8.59
C5	GIT**Y**K	8.59
C6	I**F**VQK	8.75
C7	TGPNLHGL**F**GR	9.44

[a] P: proteins; C: peptides from cytochrome *c*. Letters in bold: F, phenylalanine; Y, tyrosine.

Table 2
The 16 synthetic *p*I markers for cIEF

No.	Peptides	p*I* det.	S.D.	p*I* cal.	Δp*I*	dz/d(pH)
43	H–Trp–Asp–Asp–Asp–OH	3.38	0.041	3.38	0.00	1.70
42	H–Trp–Glu–Glu–H	3.78	0.038	3.82	−0.04	1.25
41	H–Trp–Asp–Asp–Arg–OH	4.05	0.038	4.16	−0.11	1.48
40	H–Trp–Glu–Glu–His–OH	4.28	0.035	4.54	−0.26	1.38
39	H–Trp–Asp–Asp–His–His–OH	5.31	0.022	5.24	0.07	0.53
38	H–Trp–Glu–His–OH	5.52	0.025	5.48	0.04	0.43
37	H–Trp–Glu–Arg–OH	5.91	0.078	6.06	−0.15	0.12
36	H–Trp–Glu–His–His–OH	6.66	0.024	6.42	0.24	1.31
35	H–Trp–Glu–His–Arg–OH	7.00	0.015	7.04	−0.04	0.77
34	H–Trp–GLu–His–His–His–Arg–OH	7.27	0.012	7.34	−0.07	1.23
33	H–Trp–Glu–Tyr–Tyr–Lys–Lys–OH	8.40	0.028	8.46	−0.06	0.52
32	H–Trp–Tyr–Lys–OH	8.40	0.028	8.62	−0.22	0.37
31	H–Trp–Tyr–Tyr–Tyr–Lys–Lys–OH	9.50	0.022	9.36	0.14	1.90
30	H–Trp–Tyr–Tyr–Lys–Lys–OH	9.68	0.029	9.52	0.16	1.73
29	H–Trp–Tyr–Lys–Lys–OH	9.99	0.025	9.76	0.23	1.49
28	H–Trp–Tyr–Lys–Arg–OH	10.17	0.019	10.02	0.15	1.11

Symbols and abbreviations: det., measured p*I*; cal.: calculated p*I*; S.D., standard deviation; Δp*I*, differences between measured and calculated p*I* values; dz/d(pH), variation of the charge over the pH axis in the proximity of the p*I* value.

of Glu and, of course, very far away from the p*K* of the guanidine moiety of Arg. Fig. 3A shows the remarkable mapping of the pH course in cIEF obtainable with this set of 16 markers. Fig. 3B is an enlargement of the time portion from 9 to 11 min, showing the still good resolution of the high p*I* markers, the fusion between the peptides numbers 28 and 29 being surely due to the lack of extension of the pH gradient towards more alkaline pH values. The authors correctly stress one important point, often overlooked by experimenters: p*I* values of samples should be estimated by assuming a linear relationship for pH against detection time only between two flanking marker peptides!

6. Pitfalls and fata morgana's

As a concluding remark, I would like to offer here some examples, proving what has been stated above (i.e. that

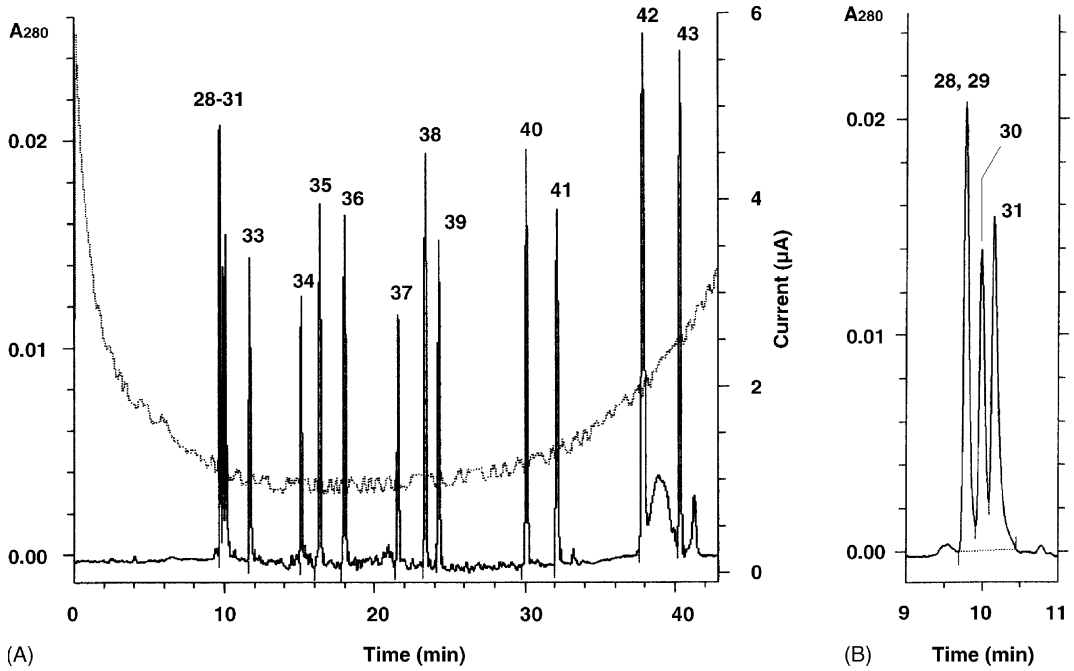

Fig. 3. (A) cIEF separation of the 16 p*I* markers. The capillary (27 cm × 50 mm i.d.) was filled with the ampholyte solution and the mixture of the peptide marker solution (0.25 mM of each peptide) was injected from the anodic side for 30 s. Focusing was carried out at 500 V/cm for 2 min at 25 °C. The focused peptide zones were mobilized by a low-pressure rinse mode, while maintaining a field strength of 500 V/cm. The peptides were detected via their absorption at 280 nm. The numbers above the peaks correspond to the code numbers of the peptides in Table 2. (B) Enlargement of the portion from 9 to 11 min of (A). The bow-shaped profile in (A) is the monitoring of the current (in μA) (from [66] with permission).

utmost care should be taken when assessing p*I* values, in general, not only in cIEF). Let us take Fig. 4 in Wu et al. [67]: these authors show ample baseline resolution between two myoglobins having p*I* values of 6.8 and 7.2 (thus $\Delta pI = 0.4$). This graph has been obtained by cIEF in a miniaturized instrument, with a 12 mm capillary, containing a pH 3–10 gradient. Thus, the slope of such a gradient, assuming a linear profile, is 0.58 pH/mm. How can they possibly obtain such an exquisite resolution, when the $\Delta pI = 0.4$ between these two peaks is substantially smaller that the slope of the pH gradient over the separation axis? In principle, they should barely see a single peak with a split apex or at best with a shoulder! Does this mean that my friend Pawliszyn is a follower of the teachings of Don Juan [68], in the famous book by Castaneda (i.e. a chewer of peyote buttons)? I have no way to tell that, but I suspect I know what is going on here: the pH gradient must be highly non-linear, and quite flat around the p*I* values of the two species. Judging from the resolution given in their figure, I would guess that the pH slope in the proximity of the two peaks is not 0.58 pH/mm but rather 0.2–0.3 pH/mm. Which brings about another subtle notion: not only pH gradients can be altered in a number of accidental ways (e.g. high salt in sample, severe EOF, etc.) but also in a quite unexpected way, by the proteins themselves. Proteins are, in the vast majority of cases, good carrier ampholytes, thus they dictate the pH in the region in which they focus. Due to the high concentrating power of IEF (typically zones in a focused peak are concentrated by a factor of 1000-fold as compared to when they are injected admixed to the whole solution filling the capillary), the high levels of a protein in a zone could easily alter the pH slope in the proximity of its p*I*. Thence the need of accepting pH courses mapped only between two p*I* markers flanking the protein of interest!

Let us take another interesting example: Hempe et al. [69] give an extended table of p*I* values of quite a number of haemoglobin (Hb) variants, all separated by cIEF and all exhibiting p*I* values accurate to the third decimal digit (e.g. Hb A, p*I* 6.972; F, p*I* 7.060; S, p*I* 7.210; C, 7.445). They in general use a pH 6–8 gradient, but admixed with pH 3–10 carrier ampholytes (thus probably more extended than 2 pH units) over a 20 cm separation distance. Thus, typically, the slope of their gradient is 0.1 pH/cm, or if you prefer, 0.01 pH/mm (but probably higher than that, considering that they have also 10% of broad-range CAs admixed to the narrow-range ones). How can they possibly resolve (and give p*I* values of) species which lie apart by only a few thousands of a pH unit? I live in a catholic country, close to the Vatican, yet, in >10 years of working with haemoglobins, I have never been able to perform this kind of miracles. They clearly state that they are unable to resolve Hb A_2 from Hb C which, according to their calculations, differ in p*I* values by only 0.003 of a pH unit. However, they seem to be able to resolve the same Hb A_2 from Hb E, such species differing by 0.007 of a pH unit. It just so happens that, in reality, we were able to obtain that kind of separation, but not by a miracle, but through hard working and unique pH engineering (see Fig. 6.2 on p. 320 of [5]) [70]. How did we do that? We prepared a 10 cm long (standard length) IPG slab, containing a pH 7.55–7.65 span over the entire gel length. This means that we had a pH slope of 0.001 pH/mm (i.e. at least one order of magnitude shallower than what Hempe et al. could possibly achieve). As there are 2–3 mm of clear gel in between the two bands, we calculated that the ΔpI between them would be of the order of 0.003–0.004 pH units. But, even though we know precisely that we have engineered a truly linear pH gradient between the two gel extremities, and we know all possible physico-chemical parameters of our gel and pH gradient (its buffering power, molarity of Immobilines in the gel, precise p*K* values of all Immobilines, both free in solution and grafted in the gel, temperature coefficients of all Immobiline p*K* values, well, you name it), we did not dare to give any precise p*I* value, not even to the second decimal digit, imagine to the third decimals! Unlike Muzio Scevola, the staunch defender of Rome, who sacrificed his arm in the fire to convince King Porsenna to go back home, we do not want to risk our limbs! So, what is the significance of giving p*I* values, in cIEF, accurate to the third decimal digit? I do not know, but let us go back to the above example. Hempe et al. [69] have given the following values: Hb A_2, p*I* 7.412; Hb E, p*I* 7.405. Looking at our figure on the separation of these two Hb species, and considering that both of them focus just about in the gel middle (pH span 7.55–7.65, remember), it would appear that the true p*I* values of these Hbs should be around pH 7.6. I let you draw your conclusions, but it would seem that never mind the second and third decimal digits, even the first decimal appears to be off the target! More modestly, Lupi et al. [71], in an extensive investigation on the polymorphism of α_1-antitrypsin in sera, have given the p*I* values of the various isoforms with only two decimal digits. Also, Cifuentes et al. [72], even though they reported exquisite resolution of seven isoforms of recombinant human erythropoietin (see their Fig. 5), did not dare to give any p*I* value, but simply stated that such species had p*I* values in the pH 3.78–4.69 range.

One more advice, though: although the pH course in cIEF might be accidentally altered by a number of factors, remember that there exists the possibility that you alter it in purpose for achieving resolution of species resilient to separation. A case in point is offered in Fig. 4, which is at the basis of the good separations of glycated Hbs, as well as umbilical cord Hbs reported in [53,54]. As one can see, a pH 6–8 interval (excellent for separation of just about all Hb species) is not quite linear, as assessed by experimentally monitoring the pH course. Such a gradient can be flattened, around the p*I* of the resilient species, by adding separators, i.e. fairly large amounts of poor carrier "ampholytes" focusing around pH 7.0, but as broad and large plateaus, thus able to markedly flatten the pH course in that region. A good mixture of separators is an equimolar amount of β-alanine and 6-amino caproic acid (0.33 M each); by this method, we could

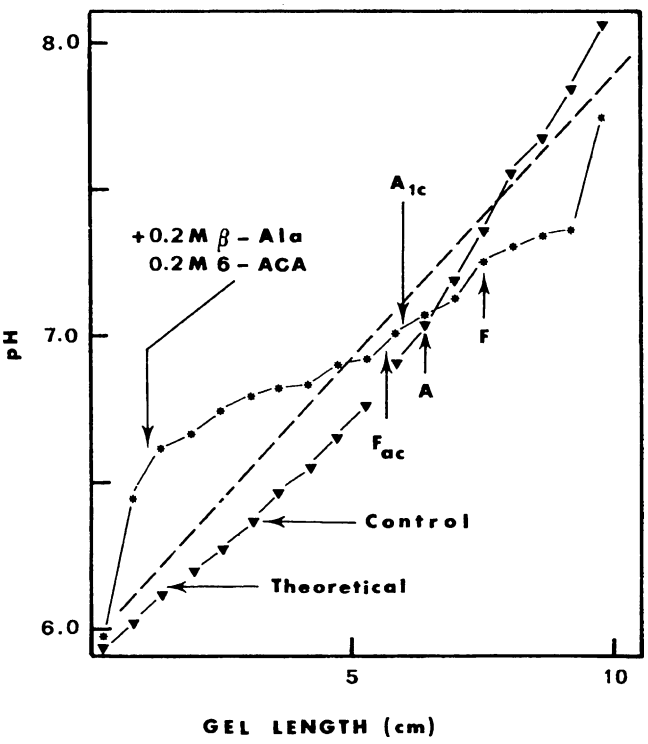

Fig. 4. Evaluation of the pH course in Ampholine gels. Broken line: theoretical pH gradient in a gel containing only pH 6–8 ampholytes, by assuming a linear pH course. Filled circles: pH gradient obtained in a gel containing 2% Ampholine pH 6–8 and a mixture of 0.2 M β-alanine and 0.2 M 6-amino caproic acid. Filled triangles: experimental pH gradient obtained with 2% Ampholine pH 6–8 in the absence of separators. In these last two cases, at the end of IEF, gel segments at 5 mm intervals were cut along the separation track, eluted with 300 μl of 10 mM KCl and read in a pH meter, under argon so as to avoid CO_2 adsorption in the alkaline part of the gradient. The arrows indicate the p*I* position, in the gradient with separators, of haemoglobins F, A, A_{1c} and F_{ac} (A_{1c}: glycated haemoglobin; F_{ac}: acetylated fetal Hb) (from [73] with permission).

improve the separation of the desired species by a factor of at least three times [73].

7. Tips and free psychiatric counselling

According to Woody Allen, life is not worth living, at least in New York, if you do not spend 50% of your time lying horizontal on the couch of your brain shrinker. Perhaps we scientists too need this treatment, subjected as we are to the insults of a daily avalanche of papers, continuous birth of new journals, unyielding experiments and the like. In order to minimize your trouble, and to save you from expensive sessions with a psychiatrist, I have borrowed these few guidelines from Woody's counsellor:

(1) Be modest, give your p*I* values to the first decimal digit. If you feel like Superman, give them to the second decimal, but at your own risk!
(2) If your analytes lie so close to each other, instead of giving absolute p*I* values, to the third decimal digit, give relative values, i.e. Δp*I* values. Basically, this is like using a differential pH meter [74].
(3) Expect to get different results from different brands of carrier ampholytes (Pharmalyte, Ampholine, Bio Lytes, Servalytes), since they might have different interacting properties with your proteins [75].
(4) Even when using the same brand of CAs, expect to obtain different results from batch to batch (remember, CAs synthesis is truly chaotic).
(5) If you use additives (surfactants, urea, organic solvents), assessing p*I* values might be quite hazardous and should be done with outmost care and proper correction factors.
(6) Do remember that p*I* (and p*K*) values are temperature sensitive, so the temperature of measurements should always be given (or correction factors adopted).
(7) Expect to have difficulties in p*I* measurements in the alkaline pH region: that is the region which is less populated in carrier ampholytes; moreover, such CAs have higher chances to be "poor", i.e. to exhibit poor buffering capacity and conductivity at the p*I* value. Were this not enough, remember that the more alkaline is your pH course, the more the solution will absorb CO_2. Of course, this problem is alleviated in closed systems, such as a capillary; however, the reservoirs are in general not protected from atmospheric CO_2.
(8) If all the above fails, start chewing peyote buttons (for instructions, see [68]). Your experiments might not improve, but you could not care less!

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References

[1] H. Svensson, Acta Chem. Scand. 15 (1961) 325.
[2] H. Svensson, Acta Chem. Scand. 16 (1962) 456.
[3] P.G. Righetti, Isoelectric Focusing: Theory, Methodology and Applications, Elsevier, Amsterdam, 1983.
[4] B. Bjellqvist, K. Ek, P.G. Righetti, E. Gianazza, A. Görg, W. Postel, R. Westermeier, J. Biochem. Biophys. Methods 6 (1982) 317.
[5] P.G. Righetti, Immobilized pH Gradients: Theory and Methodology, Elsevier, Amsterdam, 1990.
[6] C.H. Lochmüller, S.J. Breiner, C.S. Ronsick, J. Chromatogr. 480 (1989) 293.
[7] C.H. Lochmüller, C.S. Ronsick, Anal. Chim. Acta 249 (1991) 297.
[8] C.W. Whang, E.S. Yeung, Anal. Chem. 64 (1992) 502.
[9] K. Slais, J. Chromatogr. A 684 (1994) 149.
[10] T. Huang, J. Pawliszyn, Electrophoresis 23 (2002) 3504.
[11] P. Lundahl, S. Hjertèn, Ann. N. Y. Acad. Sci. 209 (1973) 94.
[12] P.G. Righetti, C. Gelfi, J. Capillary Electrophor. 1 (1994) 27.
[13] P.G. Righetti, C. Gelfi, M. Chiari, in: P.G. Righetti (Ed.), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996, pp. 509–538.
[14] P.G. Righetti, A. Bossi, Anal. Chim. Acta 372 (1998) 1.

[15] J.R. Mazzeo, I.S. Krull, in: A.N. Guzman (Ed.), Capillary Electrophoresis Technology, Marcel Dekker, New York, 1993, pp. 795–818.
[16] P.G. Righetti, M. Chiari, in: A.N. Guzman (Ed.), Capillary Electrophoresis Technology, Marcel Dekker, New York, 1993, pp. 89–116.
[17] S. Hjertèn, in: P.D. Grossman, J.C. Colburn (Eds.), Capillary Electrophoresis: Theory and Practice, Academic Press, San Diego, CA, 1992, pp. 191–214.
[18] T.J. Pritchett, Electrophoresis 17 (1996) 1195.
[19] F. Kilàr, in: J.P. Landers (Ed.), Handbook of Capillary Electrophoresis, CRC Press, Boca Raton, FL, 1994, pp. 95–109.
[20] T. Wehr, M.D. Zhu, R. Rodriguez-Diaz, Methods Enzymol. 270 (1996) 358.
[21] R. Rodriguez-Diaz, M.D. Zhu, T. Wehr, J. Chromatogr. A 772 (1997) 145.
[22] R. Rodriguez-Diaz, M.D. Zhu, T. Wehr, Electrophoresis 18 (1997) 2134.
[23] P.G. Righetti, C. Gelfi, M. Conti, J. Chromatogr. B 699 (1997) 91.
[24] M. Taverna, N.T. Tran, T. Merry, E. Horvath, D. Ferrier, Electrophoresis 19 (1998) 2527.
[25] K. Shimura, Electrophoresis 23 (2002) 3847.
[26] Y. Shen, R.D. Smith, Electrophoresis 23 (2002) 3106.
[27] E. Gianazza, J. Chromatogr. A 705 (1995) 67.
[28] P. Comi, B. Giglioni, S. Ottolenghi, M.A. Gianni, G. Ricco, U. Mazza, G. Saglio, C. Camaschella, P.G. Pich, E. Gianazza, P.G. Righetti, Biochem. Biophys. Res. Commun. 87 (1979) 1.
[29] S. Fredriksson, J. Chromatogr. 135 (1977) 441.
[30] N. Ui, Biochim. Biophys. Acta 229 (1971) 567.
[31] E. Gianazza, P.G. Righetti, S. Bordi, G. Papeschi, in: B.J. Radola, D. Graesslin (Eds.), Electrofocusing and Isotachophoresis, Walter de Gruyter, Berlin, 1977, pp. 173–179.
[32] J.W. Gelsema, C.L. De Ligny, J. Chromatogr. 130 (1977) 41.
[33] K. Sarmini, E. Kenndler, J. Chromatogr A 806 (1998) 325; 811 (1998) 201; 818 (1998) 209; 833 (1999) 245.
[34] K.I. Roy, C.A. Lucy, J. Chromatogr. A 964 (2002) 213.
[35] H. Delincée, B.J. Radola, Anal. Biochem. 90 (1978) 609.
[36] B. Bjellqvist, C. Pasquali, F. Ravier, J.C. Sanchez, D. Hochstrasser, Electrophoresis 14 (1993) 1357.
[37] B. Bjellqvist, G.J. Hughes, C. Pasquali, N. Paquet, F. Ravier, J.C. Sanchez, S. Frutiger, D. Hochstrasser, Electrophoresis 14 (1993) 1023.
[38] B. Bjellqvist, B. Sasse, E. Olsen, J.E. Celis, Electrophoresis 15 (1994) 529.
[39] G. Cossu, P.G. Righetti, J. Chromatogr. 398 (1987) 211.
[40] S. Hjertèn, M.D. Zhu, J. Chromatogr. 347 (1985) 265.
[41] S. Hjertèn, J.L. Liao, K. Yao, J. Chromatogr. 387 (1987) 127.
[42] J.R. Mazzeo, I.S. Krull, Anal. Chem. 63 (1991) 2852.
[43] W. Thormann, J. Caslavska, S. Molteni, J. Chmelik, J. Chromatogr. 589 (1992) 321.
[44] T.L. Huang, M. Richards, J. Chromatogr. A 757 (1997) 247.
[45] S. Tang, D.P. Nesta, L.R. Maneri, K.R. Anumula, J. Pharm. Biomed. Anal. 19 (1999) 569.
[46] E. Simò-Alfonso, C. Gelfi, R. Sebastiano, A. Citterio, P.G. Righetti, Electrophoresis 17 (1996) 723.
[47] E. Simò-Alfonso, C. Gelfi, R. Sebastiano, A. Citterio, P.G. Righetti, Electrophoresis 17 (1996) 732.
[48] K.W. Talmadge, M.D. Zhu, L. Olech, C. Siebert, J. Chromatogr. A 744 (1996) 347.
[49] C. Gelfi, M. Curcio, P.G. Righetti, R. Sebastiano, A. Citterio, H. Ahmadzadeh, N. Dovichi, Electrophoresis 19 (1998) 1677.
[50] N.J. Clarke, A.J. Tomlinson, G. Schomburg, S. Naylor, Anal. Chem. 69 (1997) 2786.
[51] T. Manabe, H. Miyamoto, K. Inoue, M. Nakatsu, M. Arai, Electrophoresis 20 (1999) 3677.
[52] Q. Mao, J. Pawliszyn, J. Chromatogr. B 729 (1999) 355.
[53] M. Conti, C. Gelfi, P.G. Righetti, Electrophoresis 16 (1995) 1485.
[54] M. Conti, C. Gelfi, A. Bianchi-Bosisio, P.G. Righetti, Electrophoresis 17 (1996) 1590.
[55] M. Conti, M. Galassi, A. Bossi, P.G. Righetti, J. Chromatogr. A 757 (1997) 237.
[56] N.T. Tran, M. Taverna, M. Chevalier, D. Ferrier, J. Chromatogr. A 866 (2000) 121.
[57] S.M. Chen, J.E. Wiktorowicz, Anal. Biochem. 20 (1992) 84.
[58] K. Shimura, Z. Wang, H. Matsumoto, K. Kasai, Anal. Chem. 72 (2000) 4747.
[59] F. Kilàr, J. Chromatogr. 545 (1991) 403.
[60] K. Slais, Z. Friedl, J. Chromatogr. A 695 (1995) 113.
[61] M. Horkà, T. Willimann, M. Blum, P. Nording, Z. Friedl, K. Slais, J. Chromatogr. A 916 (2001) 65.
[62] S. Molteni, H. Frischknecht, W. Thormann, Electrophoresis 15 (1994) 23.
[63] K. Shimura, K. Kasai, Electrophoresis 16 (1995) 1479.
[64] H. Kobayashi, M. Aoki, M. Suzuki, A. Yanagisawa, E. Arai, J. Chromatogr. A 772 (1997) 137.
[65] D. Mohan, D.S. Lee, J. Chromatogr. A 979 (2002) 271.
[66] K. Shimura, Z. Wang, H. Matsumoto, K.I. Kasai, Electrophoresis 21 (2000) 603.
[67] X.Z. Wu, N.S.K. Sze, J. Pawliszyn, Electrophoresis 22 (2001) 3968.
[68] C. Castaneda, The Teachings of Don Juan: A Yaqui Way of Knowledge, Pocket Books, New York, 1974.
[69] J.M. Hempe, J.N. Granger, R.D. Craver, Electrophoresis 18 (1997) 1785.
[70] A. Bianchi-Bosisio, J. Rochette, H. Wajcman, E. Gianazza, P.G. Righetti, J. Chromatogr. 330 (1985) 299.
[71] A. Lupi, S. Viglio, M. Luisetti, M. Gorrini, P. Coni, G. Faa, G. Cetta, P. Idarola, Electrophoresis 21 (2000) 3318.
[72] A. Cifuentes, M.V. Moreno-Arribas, M. De Frutos, J.C. Diez-Masa, J. Chromatogr. A 830 (1999) 453.
[73] G. Cossu, M. Manca, M. Gavina Pirastru, R. Bulletta, A. Bianchi-Bosisio, P.G. Righetti, J. Chromatogr. 307 (1984) 103.
[74] E. Rovida, C. Gelfi, A. Morelli, P.G. Righetti, J. Chromatogr. 363 (1986) 159.
[75] A.B. Chen, C.A. Nickel, A. Flanigan, G. Hunt, K.G. Moorhouse, J. Chromatogr. A 744 (1996) 279.