The Epos of Isoelectric focusing, the Alpha Centaury of Electrokinetic Methodologies

I was born in 1968. The year that rocked the world, according to Mark Kurlansky (Vintage books, Random House, London, 2005) and quite a few others. There has never been a year like 1968, and it is unlikely that there will ever be one again; a year in which a spontaneous combustion of rebellious spirits occurred around the world. Universities were leading the protest, that started at Berkeley with Jerry Rubin, spread to NYC with the civil right movement leader Abbie Hoffman, and continued in Paris with Cohn-Bendit and his army equipped with *sanpietrino's* (the porphyry cubes thrown at the police, named after the pavement of S. Peter's square). Quite a few world leaders were swept ashore by the big surf of 1968: L.B. Johnson did not run for re-election, Robert Kennedy and Martin Luther King were assassinated, De Gaulle was soon to retire. For the irony of it, during the NYC major strike, also the sidewalks of the town were swept by LeRoy Jones and his Motherfuckers: the big piles of garbage were hauled by subway from the NYC streets and composed in a huge pyramid in front of the newly opened Lincoln Centre. Also Allen Ginsberg, the poet and guru of LSD, did not stay idle: in 1968 he visited Castro in Cuba and proposed to him not to execute the opponents of the regime, but rather feed them for the rest of their lives with hallucinogenic mushrooms and then give them jobs operating the elevators at the Havana Riviera (the luxury hotel he was staying in). He did not stay long in that hotel, though, since, upon hearing this request, Castro put him on a plane and deported him to Czechoslovakia, as this happened to be the first outbound plane leaving after he was seized by the guards in his hotel room. As luck goes, he was soon expelled also from this country!

More humbly, in 1968, I was doing my time (not as convict, though) at MIT (Cambridge, Mass) as a post doctoral fellow at the Department of Nutrition and Food Science. As luck goes, we were visited one day by a Japanese scientist who delivered a lecture on isoelectric focusing (IEF) of ferritins. Japanese truly amaze me, they are always on the forefront: nobody had ever heard of IEF and no one could quite figure out how it worked. It was instant love, a passion that devoured my life and my energies, like the Moloch swallowing the poor underground workers in Metropolis, the classic movie of Fritz Lang (1927). Upon returning to the university of Milano in 1970, I spend the next decade working out every aspect of it, synthesizing novel types of carrier ampholytes, fractionating them in narrow ranges with a continuous flow IEF device, exploring ways and means of counteracting the decay of the steady-state (the infamous cathodic drift), applying it to all sorts of clinical chemistry problems (by intensive screening of foetal haemoglobins in newborns, together with G.F. Cossu, we practically eradicated thalassemia from Sardinia; see P.G. Righetti, *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier, Amsterdam, 1983). Yes, IEF was just a big revolution in Separation Science as the student movements of 1968 were!

By the end of the seventies, though, it was apparent that IEF in soluble carrier ampholytes was beginning to show the crippling disease of age. So, we teamed up with scientists at LKB Produkter (later to be incorporated into Pharmacia) to work out a totally new concept, immobilized pH gradients (IPGs), that seemed to iron out all the wrinkles of conventional IEF. This new work of art was unveiled in April 22, 1982, at the electrophoresis meeting organized by D. Stathakos in Athens and acclaimed with standing ovations. At least that is what we thought. In reality, we presented these data to an almost

empty room, since most of the delegates had never been to Athens before and surely enjoyed lovely spring weather on the Acropolis, on the Licabetto, on the Plaka, strolling just about around any corner in the capital except at the Hellenic Academy of Science, where the meeting was held. Discouraging as this may be, it gave us a leeway over the competition and set us working for another decade on all aspects of IPG development: synthesis of all possible Immobilines (from barely six to sixteen, from vacuum to plenum chemistry, as we wrote in a review), theoretical simulations for developing extended pH gradients (in those day we thought one could use them only for generating 1-pH-unit wide gradients), preparative aspects, including the idea of multicompartment electrolyzers with Immobiline membranes, that allowed harvesting ultra-clean protein species in a liquid vein. A concept that was abandoned in the nineties, but resurrected recently as a pre-fractionation tool in proteome analysis. With our work, we brought democracy into the realm of electrokinetic methodologies: when running IPGs in a pH 3-10 gradient, for analysing a total cell lysate, as a first dimension of a 2D map (another great discovery of the seventies, O'Farrell *docet*), we realized that a linear pH profile would not be "democratic", but aristocratic, since it would give plenty of extra space to fewer alkaline proteins and confine the much more abundant acidic proteins into a shantytown. So, we worked out a non-linear IPG pH 3-10, the only "democratic" gradient giving equal rights to the population at large. It was the biggest mistake of our life, since we did not think of patenting it: today 99% of the scientists engaged in proteome analysis have adopted it, but not only we do not see a penny, but are not even given credit for it (that is why I still move around on a red bicycle instead of a Ferrari Testa Rossa)! See P.G. Righetti, *Immobilized pH Gradients: Theory and Methodology*, Elsevier, Amsterdam, 1990). Yes, IPGs turned out too to be a major innovative step in Separation Science.

What would the next decade (the nineties) bring to us was largely determined by a meeting organized in Boston (April 10-12, 1989) by Barry Karger: the First International Symposium on High Performance Capillary Electrophoresis (CZE). To all those who thought that this First would also be the Last, the following years would bring a great disappointment, since there followed a deluge of meeting on CZE, still much in vogue today, although, all in all, CZE did not live up to the expectations, namely of surpassing HPLC: there seems to be no way for us electrophoreticists to prevail over chromatographers. And that is precisely what we found out by spending a decade working with CZE. As long as we applied the technique to DNA separations, we always got plenty of good results, but when we approached proteins… well, matters were not so smooth. No matter how hard we worked in developing good coating for the silica wall, novel acrylamide derivatives endowed with high hydrophilicity and very high resistance to hydrolysis, including some recent chemicals (quaternarized piperazines) able to spontaneously react with the silica surface and alkylate silanols, proteins had a love affair with the silica (naked or dressed up, mind you) and kept being adsorbed by this surface. Do not trust street peddlers selling you this technology for protein separations (peptides do much better, though): it hardly works and that is why, for protein analysis, we will never be able to compete with HPLC. Notwithstanding that, I did not overcome my obsession with IEF and reported plenty of IEF variants applied to a capillary format. See P.G. Righetti, *Capillary Electrophoresis in Analytical Biotechnology*, CRC Press, Boca Raton, 1996.

What did the third millennium bring about? Proteomics, of course, and I am now living in a lab with the walls pasted with dry, large 2D maps filled with thousands of tiny blue spots… am I in heaven? Funny, people think that the third millennium was the birth of proteomics; they should better dig deeper into the eighties. This is precisely what electrophoreticists started doing in the eighties: with the extensive use of 2-D maps they colonized bit by bit the entire earth, by pouring hundredths of thousands of 2-D gels, small, large and very large, enough to cover the earth surface with a patchwork blanket

(punctuated by millions of tiny stars, all the proteins spots in a rainbow of colours, blue, silver, gold, iridescent silvers). Additionally, the Anderson's concocted a grandiose scheme, by which their lab was transformed into a "Molecular Anatomy Program", bringing a renaissance aura into conventional anatomy. Enough with classifying bones and other anatomical details: they would create a Human Protein Index (HPI), a formidable new diagnostic tool. Physicians would approach the patient bed with huge tomes containing the entire HPI and confront the 2-D map of the patient with the standards in the book: any deviation from, say, that peculiar spot close to the Pleiades, or that nebula close to Orion, would be a clear indication of a given pathology. Meanwhile, the intrepid paladins had started a program of computerized processing of gel images and, aptly so, their first software was named Tycho, in honour of the Danish astronomer Tycho Brae: after all, looking at the thousands of tiny spots in a 2-D map was like observing a minute portion of a starry sky in a clear night. How comes, then, that 2-D map analysis had to wait the third millennium to become well-ingrained in the bioanalytical field? What scientists lacked in the eighties, to transform 2-D map analysis into a smashing success, was a valid support not so much for pattern recognition (which was progressing at a brisk pace via computer modelling) but for protein identification. The latter was truly a painful job: the lucky ones could identify a few proteins via antibodies, a few attempted (with scarce success) sequencing from the NH_2-termini; but the vast majority of spots resisted identification. This task was the rate-limiting step; success came >10 years later, when some brilliant scientists managed to analyse proteins directly with mass spectrometers. The other big impediment was the erratic spot profile obtained by performing the first dimension in conventional IEF with soluble carrier ampholytes (CA), a la Svensson-Vesterberg, if you like. There were no fixed stars in the firmament of 2-D maps: the apparent pI values kept changing, from batch to batch of CAs and, of course, from brand to brand, as manufactured by different companies (a chaotic synthesis, as you might remember). To that we finally posed a remedy with the introduction of IPGs, aptly considered by far the best first dimension in modern 2D map analysis. So, what am I doing at present? Again, trying to bring democracy in the field of protein analysis. You are surely aware that all proteomes are not democratic; oligarchic, at best. Take the human serum proteome, for example: the first 50 proteins represents about 99% of the total amount of protein mass but only less than 0.1% in number. They swamp and obliterate the "silent majority". So, we are presently working with "equalizer beads", a library of hexameric peptide ligands, made with the 20 natural amino acids (thus containing, in principle, some 64 million different ligands), able to capture all components of a mixture, sharply cutting the concentration of the abundant species and enriching the rare and very rare ones. Digging deep down in the mine to bring to light the "hidden proteome". Watch the current literature for the latest news.

Projections for the future? Just a major one: I want to give up and let the young generation carry the Olympic torch. It is just about time!

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