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1 **Review Article**

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3 High resolution separation methods for the determination of intact human erythropoiesis

4 stimulating agents. A review

5

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21

22 **Keywords:**

23 Erythropoietin (EPO)

24 capillary electrophoresis

25 high performance liquid chromatography

26 mass spectrometry

27 doping control

28 EPO biosimilars

29

30

31 **Abbreviations:**

32 AGP, Alfa 1-acid glycoprotein

33 BGE, Background electrolyte.

34 BRP, Biological reference preparation.

35 BSA, Bovine serum albumin.

36 CE, Capillary electrophoresis

37 CERA, Continuous erythropoiesis receptor activator.

38 CHO, Chinese hamster ovary

39 CIEF, Capillary isoelectric focusing.

40 CMC, Carboxymethyl chitosan.

41 CZE, Capillary zone electrophoresis.

42 DEAE-agarose, Diethylaminoethyl-agarose.

43 DPO, Darbepoetin alfa.

44 DTT, Dithiothreitol.

45 eEPO, Equine erythropoietin.

46 ELISA, Enzyme linked immunosorbent assay.

47 EOF, Electroosmotic flow.

48 EP, European pharmacopoeia.

49 EPO, Erythropoietin.

50 ESA, Erythropoiesis stimulating agent.

51 ESI, Electrospray ionization.

52 ESP, Erythropoiesis stimulating protein.

53 FOBs, Follow-on biologics.

54 FTICR, Fourier transform ion cyclotron resonance.

55 HMW, High molecular weight.

56 hEPO, Human erythropoietin.

57 HILIC, Hydrophilic interaction chromatography

58 HPLC, High performance liquid chromatography.

59 HRP, Horseradish peroxidase

60 HSA, Human serum albumin.

61 IAC, Immunoaffinity chromatography.

62 IEF, Isoelectric focusing.

63 IEF-PAGE, Isoelectric focusing - Polyacrylamide gel electrophoresis

64 IEX, Ion exchange.

65 IT, Ion trap.

66 LacNAc, *N*-Acetyllactosamine.

67 LC, Liquid chromatography.

68 LIF, Laser induced fluorescence

69 MS, Mass spectrometry.

70 NESP, Novel erythropoiesis stimulating protein.

71 NeuNAc, *N*-Acetylneuraminic acid.

72 NeuNGc, *N*-Glycolylneuraminic acid.

73 PBS, Phosphate buffer saline

74 PEG, Polyethyleneglycol.

75 PTM, Post-translational modification.

76 PVP, Polyvinylpyrrolidone.

77 QC, Quality control.

78 rhEPO, recombinant human erythropoietin.

79 RMT, Relative migration time

80 RP, Reversed phase.

81 SDS, Sodium dodecyl sulphate.

82 SDS-PAGE, Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis.

83 SE, Size exclusion.

84 SEBs, Subsequent entry biologics.

85 SPS, Sodium Pentasulfonate.

86 TOF, Time of flight.

87 uhEPO, Urinary human erythropoietin.

88 WHO, World health organization

89 WADA, World antidoping agency.

90

91 **Abstract**

92

93 Human erythropoietin (hEPO), a hormone involved in the formation of red blood cells, is
94 a 30 kDa glycoprotein with a high carbohydrate content. The production of recombinant hEPO
95 has made possible its widespread therapeutic use and its banned use in competition sports.
96 Methods to analyze EPO and other erythropoiesis stimulating agents (ESAs) are necessary for
97 the characterization and quality control of these biopharmaceuticals and also for doping control.
98 In this paper, high resolution separation methods, namely high performance liquid
99 chromatography (HPLC) and capillary electrophoresis (CE), with special attention to CE-
100 coupled mass spectrometry, are reviewed. The usefulness of these techniques when applied in
101 different modes to separate the glycoprotein isoforms, aggregates or excipients are detailed. In
102 addition, sample preparation methods that have been applied to ESA samples for subsequent
103 determination by HPLC or CE, as well as the potential compatibility of other preparation
104 methods, are discussed. Applications of the HPLC and CE methods regarding regulatory
105 considerations for biopharmaceuticals analysis, with emphasis on biosimilars, and doping control
106 are also included. Finally, limitations of the present methods and their possible solutions are
107 considered.

108

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119

120 **1. Introduction**

121

122 Human erythropoietin (hEPO) is a glycoprotein hormone produced mainly in the kidneys
123 that regulates the erythropoiesis process, that is, the formation of red blood cells [1]. The large-
124 scale production of a recombinant version of human erythropoietin (rhEPO) by biotechnological
125 methods has made possible its widespread therapeutic use as well as its misuse in sport
126 competitions. It has a molecular mass of about 30 kDa, 40% of which corresponds to three N-
127 linked and one O-linked carbohydrate chains attached to the polypeptide chain. A representation
128 can be observed in Figure 1 [2]. Glycosylation depends on several factors such as the organism
129 or cell line in which EPO is produced or the culture conditions. This has given rise to the
130 production of several EPO products featuring variations in their glycosylation pattern. In
131 addition, several modifications have been carried out in recombinant products, giving rise to
132 different erythropoiesis stimulating agents (ESAs).

133 The nomenclature and main characteristics of EPO and EPO-related substances have
134 been summarized by Jelkmann [3] and by Reichel and Gmeiner [4]. For EPO-type substances,
135 the stem “-poetin” is used according to the International Nonproprietary Names system of the
136 World Health Organization (WHO). The term “epoetin” corresponds to eukaryotic cell-derived
137 rhEPO, with a peptide sequence identical to that of urinary human EPO (uhEPO). A prefix is
138 used to indicate changes in the peptidic sequence, as in the case of darbepoetin alfa (DPO, also
139 known as novel erythropoiesis stimulating protein or NESP). Greek letters after the name are
140 employed to indicate analogues of a given EPO type substance with an altered glycosylation, as
141 in the case of epoetin-α and epoetin-ω. For other EPO related substances, pegylated epoetin-β is
142 known as CERA (continous erythropoiesis receptor activator), and the name Hematide accounts

143 for a synthetic dimeric EPO mimetic peptide linked to polyethyleneglycol (PEG). Schemes of
144 EPO, DPO, and CERA are shown in Figure 2 [3].
145 Analysis methods are aimed at the control of the drug for its therapeutic use as well as in
146 situations of doping control. In particular, the increase in biosimilars and uncontrolled drugs
147 claimed to be rhEPO is enlarging the need for these analysis methods. In addition, ESAs are
148 now produced in many countries around the world as exemplified in Table 1, which shows a
149 non-comprehensive list of ESAs available worldwide; further information about ESAs in the
150 world can be found in [5-8]. Depending on the source of information, mainly the web pages of
151 the manufacturers, contradictory data are sometimes found. Efforts are also being made for the
152 in-depth characterization of recombinant EPO [9-12].
153 When performing EPO determination, it is important to consider that variations in
154 glycosylation result in alternate versions of the same protein called glycoforms. These
155 glycoforms can be separated using different techniques and appear as different peaks in capillary
156 electrophoresis or liquid chromatography, and as different bands or spots in gel electrophoresis,
157 these peaks or bands being known as isoforms.
158 Different strategies can be followed to perform the determination of glycoproteins. Some
159 of them include the controlled hydrolysis of the glycoprotein, resulting in the study of glycans
160 and/or glycopeptides. In other instances, hydrolysis is performed to solely release the glycans,
161 leaving the peptide sequence intact, thus enabling the determination of glycans and/or of the
162 peptide sequence. Yet in other cases, hydrolysis is not carried out and the whole intact
163 glycoprotein is analyzed. This review will focus on the determination of intact EPO and other
164 ESAs, namely, erythropoiesis stimulating proteins (ESPs). The determination of glycoforms of
165 intact glycoproteins presents several advantages in comparison to hydrolyzed glycoproteins, such

166 as saving time, less sample manipulation (less influence of the operator and the sample treatment
167 on the results) and obtaining a more holistic information about the glycoprotein.
168 A number of analytical methods for EPO and related substances have been recently
169 summarized [4]. In that book chapter chromatography, mass spectrometry, immunoassay, and
170 electrophoresis are considered. Special attention is paid to this last technique by including
171 different methods performed using zone electrophoresis in agarose suspension, isoelectric
172 focusing both in solution and in polyacrylamide gels, sodium dodecyl sulphate-polyacrylamide
173 gel electrophoresis (SDS-PAGE) and bidimensional electrophoresis. In the present review, high
174 resolution separation techniques, namely high performance liquid chromatography (HPLC) and
175 capillary electrophoresis (CE), will be considered. Each of the analytical steps, from sample
176 preparation to detection, with special attention paid to mass spectrometry (MS) coupled to CE,
177 will be discussed. Finally, applications of these techniques for the determination of biosimilars
178 and for doping control will be covered. The review will address the methods published and also
179 potential approaches to solve current limitations. In this regard, methods employed to analyze
180 other glycoproteins or other aims will be discussed focusing on their potential applicability for
181 HPLC or CE determination of ESAs.
182
183
184 **2. Sample preparation**
185
186 The process by which a sample is made ready for analysis by HPLC or by CE depends on
187 the type of sample, the aim of the analysis, the HPLC or CE separation mode as well as the

188 detection technique to be employed. In this context, two main groups of samples can be
189 considered: pharmaceutical preparations and biological fluids.
190 2.1 Sample preparation methods carried out for HPLC or CE determination of ESAs
191 For pharmaceutical preparations, the ESA concentration is usually high enough to allow
192 its detection by most of the usual methods (e.g., UV and MS). The sample preparation step is
193 aimed at eliminating substances that may interfere during analysis. The need to remove these
194 substances, as can be the case for formulation excipients, in CE analysis depends on the capillary
195 coating and the background electrolyte (BGE) employed. For example, EPO products formulated
196 with human serum albumin (HSA) as excipient can be directly analyzed using a BGE containing
197 nickel chloride and amine-coated capillaries [13]. Alternatively, analysis using widely available
198 and inexpensive bare fused silica capillaries requires prior removal of HSA, which can be
199 efficiently carried out with commercial anti-HSA cartridges. In this case special attention must
200 be paid to avoid the presence of phosphate in the purified rhEPO sample as this prevents its
201 determination by capillary zone electrophoresis (CZE) [14]. Low-molecular weight sample
202 components can be readily removed using centrifugal ultrafiltration devices [15-17]. ESA
203 samples containing several excipients, including polysorbate 80, were prepared using these
204 devices and analyzed by CZE without problem [18]. However, some authors [19] reported,
205 without presenting results that these devices are unable to remove polysorbate 80 and that they
206 preferred the use of a commercial non-ionic detergent trap cartridge prior to the centrifugal
207 filtration to prepare ESA samples containing this excipient. In order to increase EPO recovery
208 passivation of the filtration devices with 5% BRIJ 35 was recommended [14,20]. Similar
209 recoveries were achieved for devices with 10 kDa or 30 kDa cut-off membranes [14].When the
210 analysis was carried out by capillary isoelectric focusing (CIEF), better results were obtained if

211 excipients were not completely removed or, for a better control of the salt content, known
212 amounts of salts were added after excipients were eliminated [15,21]. For characterization by
213 CE-MS, a high rhEPO concentration (about 3 mg mL^{-1}) is desirable. To increase the EPO
214 concentration, ultrafiltration through a 10 kDa cut-off membrane device was used, followed by
215 freeze-drying and subsequent dissolution in a small volume of water [22].

216 Sample preparation for the determination of EPO and other ESAs in culture supernatants
217 and biological fluids is far more complex than in pharmaceutical preparations due to the low
218 analyte concentration and the complexity of the sample matrix. In these samples the preparation
219 step is aimed not only to purify the ESA but also to concentrate it. The determination of ESAs in
220 these complex samples can be of interest from a therapeutic point of view (e.g., culture
221 supernatant control during production, pharmacokinetics studies, etc) as well as for doping
222 control considerations.

223 Purification of rhEPO from culture supernatants for subsequent CZE analysis has been
224 carried out through a series of chromatographic steps including ion-exchange (IEX), reversed-
225 phase (RP), and gel filtration or size-exclusion (SE) modes [23]. The protocol followed for the
226 EPO purification is referred to other papers [24-25] and it appears to be based on the seven-step
227 procedure developed by Miyake et al. [26] for urinary EPO. It includes ethanol precipitation,
228 diethylaminoethyl-agarose (DEAE-agarose) fractionation, sulfopropyl-Sephadex
229 chromatography, gel filtration, and hydroxylapatite chromatography. Samples purified from cell
230 cultures in this way were of high purity, thus making CE analysis possible [23].Although the
231 length of the purification procedure is not indicated, the large number of steps involved implies
232 that it is a laborious and time-consuming method that would not be adequate for doping or drug
233 production control.

234 The on-line coupling of immunoaffinity with CZE separation has been attempted using
235 rhEPO [27]. Polyclonal anti-EPO antibodies were attached to glutaraldehyde-treated glass beads
236 and packed between two frits in a small Polybrene-coated capillary column, which was then
237 linked to a separation capillary of the same diameter and type. The authors indicated that the
238 capture and release of EPO were observed but the resolution of isoforms was completely lost.

239 2.2 Suitability of other methods for sample preparation prior to HPLC or CE determination of
240 ESAs

241 As seen above, only a handful of sample preparation methods have been specifically
242 described for the isolation of intact ESAs for analysis by CE or HPLC. In the following
243 paragraphs, other methods described to purify EPO for determination by other techniques will be
244 considered and their suitability for subsequent determination by CE and HPLC analysis
245 discussed.

246 Immunoaffinity using anti-EPO antibodies has been performed on different platforms
247 such as magnetic beads, ELISA plates, monolith disks and, mainly, chromatographic columns.
248 Monoclonal antibodies covalently linked to magnetic beads were used by Wognum et al. to
249 purify EPO from serum and plasma [28]. The elution buffer included PEG 8000. Polyclonal anti-
250 EPO antibodies also attached to magnetic beads were used to purify different ESAs from equine
251 plasma [29-30]. In this case, desorption was carried out using PEG 6000 in phosphate buffer
252 saline (PBS). In both cases, PEG should be eliminated if CE or SE-HPLC is used to analyze
253 intact EPO isoforms. A modification of the method by Wognum et al. that includes elimination
254 of PEG, has proved useful to isolate rhEPO and ESA from equine plasma [31] and DPO from
255 human plasma [32]. An alternative method for EPO elution from magnetic beads derivatized
256 with polyclonal antibody, consisting of 1% sodium dodecyl sulphate (SDS) in PBS under

257 vigorous shaking and subsequent removal of SDS by dialysis, was effective to isolate EPO from
258 human serum [33].

259 The optimization of EPO purification using monoclonal anti-EPO 9C21D11 on
260 immunoaffinity well plates (similar to the ones used in Enzyme Linked Immunosorbent Assays -
261 ELISA) led to around 56% recovery of hEPO in plasma without isoform discrimination [34].
262 Although the total protein content had been reduced 3000 times, the remaining concentration of
263 interfering proteins from plasma was still too high and would not be compatible with the direct
264 CZE determination on uncoated capillaries or with the SE-HPLC determination of EPO. Figure 3
265 shows the total concentration of proteins (black line, right scale) and that of hEPO (bars, left
266 scale).

267 Gupta et al. [35] prepared three immunocolumns using three different commercial
268 antibodies from different sources to purify EPO and analogues in the development of a method
269 aimed at detecting horse doping. No indication about the nature of the packing material or the
270 antibodies characteristics was provided. By using an antibody/antigen molar ratio of about 1
271 million (50 mg antibody for each column), incubating the sample in the column for 4 h, and
272 employing 100 mM glycine pH 2.8 as eluent, equine EPO (eEPO), rhEPO, and DPO were
273 purified with about 87% recovery. Analyses of the intact ESAs in the purified samples were
274 performed by ELISA and gel electrophoresis with either Coomasie or immunological staining.
275 The desorption agent could be easily eliminated with centrifugal filtration devices; however, the
276 amount of EPO purified in this way (about 20 ng) would not be sufficient for determination by
277 CE or SE-HPLC with the currently available detection methods. When this immunoaffinity
278 chromatography (IAC) method was slightly modified by using 0.2 M glycine buffer pH 2.5 as
279 desorption agent and used to isolate rhEPO and darbepoietin from horse plasma, it was observed

280 that albumin co-eluted with the ESPs [36]. Among the different strategies assayed to eliminate
281 co-elution, the combination of lectin (concanavalin A) affinity with IAC provided the best results
282 with 70% recovery of rhEPO or darbepoietin; however, the samples were still contaminated with
283 albumin and other proteins. This procedure would most probably be useful in the determination
284 of epoetins by RP-HPLC or IEX-HPLC but not by CZE on uncoated capillaries, or by SE-HPLC.
285 Another IAC procedure, potentially compatible with further CE or SE-HPLC
286 determination of the purified EPO, was developed by Mi et al. using anti-EPO polyclonal
287 antibodies bound to Sepharose [37]. Recoveries in the range 78-86% were achieved using Gly-
288 HCl pH 2.5 as elution buffer. This buffer could be easily removed after the immunopurification
289 step. The binding capacity (2 μg) should be slightly increased to obtain enough purified EPO to
290 be detected by the usual procedures.
291 IAC with columns made of the 9C21D11 anti-EPO antibody bound to a chromatographic
292 support with hydrazide groups was demonstrated to be useful to isolate the glycoprotein from
293 serum and urine prior to Western blot isoelectric focusing analysis [38]. Although the use of
294 bovine serum albumin (BSA) as a component of the elution buffer would make the method
295 inappropriate for the subsequent determination of EPO by either CZE on uncoated capillaries or
296 by SE-HPLC, it could probably be compatible with CZE analysis using the Bietlot and Girard
297 method [13] and with HPLC in the RP and IEX modes.
298 IAC was also demonstrated to be adequate for eliminating interferences caused by
299 unfractionated heparin on IEF-PAGE of EPO [39]. This anticoagulant, used by some athletes
300 during recombinant EPO therapies, forms a heparin gradient on the IEF gel that modifies or even
301 destroys the IEF EPO pattern. Saturation of samples with urea was also shown to eliminate the
302 heparin interferences on IEF gels [39]. Although the effect of heparin on EPO determination by

303 CE has not been studied, it could also affect focusing of EPO in CIEF and potentially modify its
304 charge, which would affect its electrophoretic mobility in CZE. Among the two methods to
305 remove heparin, IAC seems to be more appropriate for CE analysis because it eliminates heparin
306 at the same time as the rest of the interfering compounds from the sample matrix. It should also
307 be noted that a BGE frequently used for CZE of EPO contains 7M urea [15,18,40] and that such
308 a concentration of urea is usually included in the sample mixture for CIEF [15,21], a situation
309 that could help to avoid interactions between heparin and EPO.

310 A commercial kit based on anti-EPO IAC and made of disposable monoliths was shown
311 to be useful to purify different recombinant EPOs from several matrices without isoform
312 discrimination [41-42]. A picture of the device is shown in Figure 4.The usefulness of this
313 technique for subsequent analysis by CZE on uncoated capillaries or SE-HPLC may be limited
314 due to the presence of BSA in the pH-adjustment buffer provided with the kit. The elimination of
315 BSA would be required and the impact of such a modification studied. The same antibody (anti-
316 EPO 3F6) had been previously employed in larger columns using a pH 2.2 solution
317 supplemented with an inhibitor of aspartyl proteases (pepstatin) as desorption agent [43]. In that
318 case, nothing was mentioned about the need to use a pH adjustment buffer including BSA. In
319 principle, this IAC process, if followed by removal of the desorption agent, could be compatible
320 with CE or HPLC analysis.

321 As discussed, both polyclonal and monoclonal antibodies have been used as affinity
322 ligands. When considering their use, they should be carefully chosen to allow EPO capture and
323 elution without crossreaction with other proteins, as would be the case for the AE7A5 clone,
324 which can crossreact with other proteins such as eukaryotic or bacterial, and namely with the

325 urinary proteins Tamm Horsfall glycoprotein, alfa- antichymotrypsin, alfa 2-thiol proteinase
326 inhibitor, and alfa 2-HS glycoprotein [44-45].
327 Besides antibodies, other affinity ligands have also been used for EPO purification.
328 Among them, the use of synthetic peptides developed by a combinatorial strategy has recently
329 been shown to be effective for isolating rhEPO from Chinese Hamster Ovary (CHO) cell culture
330 supernatant spiked with the recombinant glycoprotein [46]. The selected peptides were
331 immobilized on Sepharose and EPO was eluted with 90% recovery and around 96% purity using
332 100 mM sodium acetate buffer, pH 3.0, 0.25 M NaCl. These conditions should be compatible for
333 further CE or HPLC analysis provided that the sample is first cleaned of the elution buffer.
334 Lectins have also been used to purify EPO [47]. However, these affinity ligands, if not combined
335 with another isolation step, may not be appropriate for preparing EPO samples to be analyzed by
336 CE or HPLC as lectins show affinity for a wide range of glycoproteins.
337 Purification of rhEPO from human plasma was also performed by depletion of the seven
338 major plasma proteins followed by reversed phase desalting and purification [48]. This method,
339 which has been optimized for tandem mass spectrometry analysis of tryptic peptides, leads to
340 samples containing proteins that would make it unsuitable for CZE analysis of intact EPO on
341 uncoated capillaries or by SE-HPLC.
342 An alternative sample preparation method for EPO doping control in blood samples has
343 been recently published [49]. The method is based on EPO solubility and stability at acidic pH
344 and consists of sample (serum or plasma) precipitation with perchloric acid, followed by
345 neutralization of the supernatant and further elimination of the precipitate formed during
346 neutralization. Although this method was shown to be valid for performing IEF in gel followed
347 by blotting assay, it may not be adequate for CZE analysis on uncoated capillaries as it was

348 observed when performing sample preparation of another glycoprotein (alfa-1 acid glycoprotein,
349 AGP) from serum using perchloric precipitation. AGP is also a very acidic glycoprotein; and
350 although most of the interferences from the sample were eliminated by acidic precipitation, the
351 remaining ones markedly decreased the electroosmotic flow (EOF). This is most probably due to
352 adsorption to the capillary wall [50]. In all likelihood, for EPO the remaining interferences would
353 also make this preparation method incompatible with SE-HPLC. This isolation method could
354 potentially be appropriate for samples to be analyzed by CE using coated capillaries.

355 2.3 General concerns for sample preparation for ESAs determination

356 Independent of the specific purification method chosen, EPO-containing samples should
357 generally be subject to pre-treatment steps. For example the modification, reduction and even
358 disappearance of the EPO bands in the IEF analysis of urine samples have been related to the
359 enzymatic activity from micro-organisms. Besides the activity from normal flora or other
360 microbial contaminants, concerns arose recently that purposely-added proteolytic enzymes, to
361 avoid doping control, can distort EPO determination results. Physical and chemical methods to
362 stabilize human urine samples for doping control have been summarized elsewhere [51]. The
363 addition of a general protease inhibition cocktail was the method of choice in some protocols,
364 and was considered more efficient in preventing EPO degradation by proteases than was heating
365 or the addition of pepstatin [45]. Coating plastic urine collection containers with a chemical
366 mixture of antibiotics, antimycotics and protease inhibitors was also advised [52]. In addition,
367 when analyzing urine samples, the need to solubilize precipitates prior to the purification step is
368 considered mandatory because the precipitates can contain up to 85% of the total amount of EPO
369 in the sample [53]. This is particularly relevant when analyzing frozen samples.

370

3. High performance liquid chromatography for the determination of intact EPO and other ESAs

High performance liquid chromatography (HPLC) methods have been reported for the determination of intact EPO and other ESP preparations in a variety of situations that included identification, purity assessment and assay of the active ingredient. Of the several HPLC modes SE-HPLC, IEX-HPLC and RP-HPLC have been successfully applied; a summary can be observed in Table 2. As its name implies SE-HPLC separates compounds according to their size (or hydrodynamic volume), which can, in turn, be approximated to the molecular weight. SE-HPLC columns are made of porous packing materials that allow compounds to interact with the pores according to their size. Thus, in the absence of non-specific interactions, larger molecules interact less with the stationary phase and elute ahead of smaller molecules. Depending on the column porosity different ranges of molecular size can be adequately separated. For RP-HPLC, separation is obtained based on differences in hydrophobicity between the compounds, with the more hydrophobic species showing greater retention. RP-HPLC columns vary in their hydrophobicity depending on the bonded phase used. Typically, C4 and C8 bonded phase columns with wide pores (300 Å) have been used for protein separations. In IEX-HPLC, separation involves coulombic (also called electrostatic or ionic) interactions between the components and ionic functional groups on the stationary phase. Both cationic and anionic groups have been used for the separation of proteins.

The formation of dimers and high molecular weight (HMW) aggregates constitutes one of the main degradation pathways of EPO. Their presence in pharmaceutical proteins has been a

394 major concern to manufacturers and regulatory authorities alike due to their immunogenic
395 potential. As such, the monitoring and control of these species in EPO and other ESAs have been
396 the subject of several studies using chromatographic techniques.

397 Early reports have shown that SE-HPLC conditions could be used to study dimer and
398 HMW aggregate formation in EPO samples placed under stress conditions [54-55]. Silica-based
399 columns providing separation in the molecular weight range of 10– 500 kDa (medium-range)
400 were used. The detection of dimers and oligomers was found to be adequate by monitoring with
401 UV at either 214 nm or 280 nm. However, the use of a low angle laser light scattering detector
402 provided increased sensitivity for the detection of very large aggregates [54]. These two studies
403 showed that heat-stressed EPO samples led mainly to the formation of HMW aggregates.
404 Dimerization was favored when samples were placed under denaturing and reducing conditions
405 in the presence of dithiothreitol (DTT), presumably through the cleavage of disulfide bridges and
406 the subsequent random re-oxidation of the free thiols [55]. Similar SE-HPLC conditions were
407 later used to monitor dimers and HMW aggregates in formulation studies of EPO [56] and DPO
408 [57]. RP-HPLC conditions have also been shown to separate EPO dimers and HMW aggregates
409 [55]. In this case, both dimers and HMW aggregates showed increased hydrophobicity compared
410 to monomeric EPO.

411 A quantitative test for dimers and HMW aggregates has been prescribed in the European
412 Pharmacopoeia (EP, also abbreviated as Ph.Eur.) monograph for EPO concentrated solution
413 since the first publication of the monograph [58]. It is based on SE-HPLC with UV detection at
414 214 nm and applies to unformulated bulk preparations. A limit for total aggregates, including
415 dimers and HMW aggregates, of less than 2% is specified. The method has been successfully

416 applied in collaborative studies for the establishment of the EP biological reference preparations
417 for EPO [59-60].
418 The applicability of SE-HPLC for monitoring dimers and HMW aggregates in final
419 products has been complicated by the presence of excipients. For instance, formulations
420 containing HSA as a stabilizer are not amenable to determination by SE-HPLC due to the
421 interference of HSA with the detection of the EPO monomer. In most formulations the amount of
422 HSA is much larger than the amount of EPO (2500 µg mL^{-1} of HSA vs 30-100 µg mL^{-1} of EPO).
423 In formulations where polysorbate 80 is present instead of HSA, there have been reports of peaks
424 due to the polysorbate interfering with the detection of EPO dimers and oligomers [61-63]. To
425 circumvent this problem detection by fluorescence was used [63-64]. Interestingly, other studies
426 have not reported the interference of polysorbate peaks on the detection of dimers and aggregates
427 [65-66] despite using separation conditions that do not appear too dissimilar, that is, phosphate-
428 containing mobile phases and silica-based gel columns. It is not clear at this time on what basis
429 the latter conditions enabled to differentiate the polysorbate 80 peaks from the EPO dimers and
430 aggregates without further evaluation.
431 Methods based on RP-HPLC have also been used for the analysis of HSA-containing and
432 polysorbate-containing EPO formulations. RP-HPLC conditions using wide-pore, mildly
433 hydrophobic, C4 bonded (as opposed to the strongly hydrophobic, C18 bonded phase) reversed-
434 phase columns were first reported by De Paolis et al. [55] and later used to provide adequate
435 separation between HSA and EPO [67]. This was also the case with strongly hydrophobic, C18-
436 bonded phase columns that enabled the separation of HSA, EPO and aggregates [68]. Notably,
437 the latter conditions enabled the partial separation of the Ser-125 unglycosylated variant that was
438 detected in EPO-omega formulations. Similarly, underglycosylated EPO obtained from

439 transgenic pigs was also separated from CHO-derived EPO by RP-HPLC conditions [69]. In
440 both cases, the underglycosylated EPO was found to elute after EPO as a consequence of its
441 increased hydrophobicity.

442 A method based on anion-exchange HPLC with fluorescence detection was reported for
443 the analysis of HSA-containing formulations [70]. Fluorescence detection was found to be more
444 sensitive than UV at either 214 or 280 nm. In addition, the method provided selectivity between
445 EPO-alfa and EPO-beta. IEX-HPLC conditions were also reported for the fractionation of EPO
446 glycoforms. As expected, the gradient elution of EPO on anion-exchange columns using
447 increasing salt concentrations resulted in fractions containing isoforms with decreasing average
448 pI [71-72].

449 The EPO content measured by SE-HPLC and RP-HPLC has been correlated to the
450 biological activity in pharmaceutical finished products using the EP biological reference
451 preparation (BRP) for EPO as reference [66-67]. The average potency determined by RP-HPLC
452 was 11% higher than by the bioassay, whereas it was 2.5% higher by SE-HPLC. The authors
453 suggested that the latter method could be used as an alternative to the bioassay for the potency
454 determination of EPO preparations.

455 An attempt at the identification of intact EPO-alfa, EPO-beta and DPO by RP-HPLC
456 coupled on-line to electro-spray ionization mass spectrometry (ESI-MS) has been described [73].
457 While the chromatographic step enabled the three components from a mixture to be resolved, the
458 mass spectra of each of the three ESAs were of poor quality in terms of their mass resolution and
459 signal intensity. The authors hypothesized that this was likely due to the presence of clusters of
460 multicharged species deriving from the various isoforms.

461

462

463 **4. Separation and detection of intact EPO using capillary electrophoresis methodology**

464

465 Capillary electrophoresis (CE) modes of free zone (CZE) and isoelectric focusing (CIEF)
466 are well suited for the isoform separation of glycoproteins [74-75]. In particular, both
467 pharmaceutical preparations and natural samples of EPO have been analyzed using CE
468 [15,40,76]. It should be noted that the EP prescribes CZE as an identification method in its
469 monograph for Erythropoietin Concentrated Solution [58,77]. Most of the work on CE analysis
470 has been carried out using UV detection (at 214 nm) which has a limited sensitivity (in the range
471 of 10^{-6} M). Other detection techniques such as MS have been used and will be reviewed in this
472 paper.

473 In this section a general overview of the different CE methodologies for the
474 determination of intact EPO will be presented with a particular emphasis on papers published
475 during the period from 2006 until January 2011, which have been summarized in Table 2. Works
476 performed earlier have been already reviewed by us [74]. Considerations regarding different
477 possibilities to improve detection will also be discussed. The reader may also refer to review
478 papers of protein separation using CE that contain a summary of EPO (intact and digested)
479 studies [75,78].

480 4.1 Separation of intact EPO using CZE

481 Most of the recent work carried out by CE for the determination of isoforms of intact
482 EPO has been based on the method prescribed by the EP [58,77]. This method uses CZE for
483 identification of EPO (assay B). It uses uncoated fused silica capillaries and a separation buffer
484 composed of 0.01 M tricine, 0.01 M NaCl, 7 M urea and 2.5 mM putrescine. This diamine is

485 employed to avoid adsorption of the protein on the capillary surface and to control the EOF.
486 Separation is carried out at 35ºC and UV detection at 214 nm is used. A critical point in the use
487 of this CE method is the capillary preconditioning. The monograph recommends rinsing new
488 capillaries with 0.1 M NaOH (60 min) and with the separation buffer (60 min), the latter applied
489 using 20 kV (for 12 h). Between runs, the monograph recommends rinsing the capillary
490 successively with water (10 min), 0.1 M NaOH (5 min) and separation buffer (10 min). With
491 these precautions the method provides the separation of 8 isoforms in 60 min with good
492 repeatability for the EP BRP standard.

493 However, some researchers have claimed a lack of reproducibility with the EP method.
494 For instance, Zhang et al. [19] have found that the causes of the poor reproducibility of the
495 method are: i) inefficient elimination of the excipient (e.g., polysorbate 80 in EPO-alfa) in the
496 sample and ii) inadequate capillary conditioning procedure. As mentioned in Section 2, removal
497 of polysorbate 80 from formulated EPO-alfa can be achieved using a commercial non-ionic,
498 detergent trap cartridge. The EPO recovered from the cartridge is then desalted using the method
499 proposed in the EP monograph prior to analysis by CE. For the capillary conditioning, the
500 authors highlighted the critical role of putrescine in the separation method. According to them, a
501 portion of the protein anionic isoforms is associated via electrostatic interactions with the
502 putrescine anchored on the silica surface through hydrogen bonding, ionic, and/or hydrophobic
503 interactions. The putrescine-isoform association reduces the net electrophoretic migration of the
504 isoforms, resulting in a modification of the migration velocity. The authors suggest that the rinse
505 of the capillary is a critical point for obtaining good repeatability of the migration time for the
506 EPO isoforms. This step is necessary to completely remove the putrescine and re-establish the
507 pH on the silica surface. Consequently, the authors proposed a rinsing procedure consisting of a

508 combination of base, acid, and water rinses over an extended period of time (more than 260
509 min). Although the recommended rinsing procedure to be done after every 10 injections is long,
510 its efficiency was demonstrated by the good reproducibility of the absolute migration time (for
511 peak number 2) of the EPO samples (RSD 1.8%, for 3 different samples with n=3 for each
512 sample) and even better for the relative migration time to the water peak (RMT) (RSD 0.2%,
513 under the same conditions). For peak areas, the inter-run RSD values (for 4 runs with a total
514 n=10) ranged from 0.2 to 0.7% (for the more abundant ones) and from 3.3 to 6.8 % for isoforms
515 3 and 8 (those being the less abundant isoforms). As expected, better RSD values (ranging from
516 0.0 to 6.8 %) for areas were obtained in these experiments when intra-run values were
517 considered. The use of relative migration time to the EOF as (indicated by the water peak)
518 instead of the absolute migration time has been also advised by other authors. This is even in
519 cases where good repeatability of the absolute migration time (RSD < 0.6%, n= 6) has been
520 achieved for the separation of EPO isoforms [18]. The experience of our group is also that an
521 appropriate rinsing of the capillary is crucial for obtaining good repeatability among injections,
522 when using a procedure for sample preparation and a CE buffer similar to that of the EP [18].
523 With a shorter capillary conditioning step (35 min between consecutive analysis) than the one
524 proposed by Zhang et al. (20 min between consecutive analyses and 260 min every 10 analysis)
525 [19], the repeatability of the migration time for the peaks of the isoforms of the EP-BRP EPO
526 sample was in the range of 0.29-0.56% (RSD, n=6) for analyses in the same day and in the range
527 0.98-1.72% (RSD, n=18) for analyses carried out in three different days with two different
528 instruments.
529 Other authors proposed the use of commercial polymeric coatings, such as hexadimetrine
530 bromide (Polybrene) or Ultratrol LN [79] to avoid the lack of reproducibility that had previously

531 been observed for uncoated capillaries [80]. Using volatile buffers compatible with MS detection
532 6 isoforms were detected for the EP BRP standard when using the Polybrene coating, and more
533 than 7 peaks were observed when Ultratrol LN was used. However, a lower number of peaks was
534 obtained in the CE separations in comparison to those obtained using non volatile BGEs
535 containing diamine additive, which could complicate the interpretation of the MS spectra (vide
536 infra).

537 Another commercial polymeric cationic coating, carboxymethyl chitosan (CMC), has
538 shown promising results for rhEPO isoforms separation [81]. New capillaries were rinsed with 1
539 M NaOH (30 min) followed by 0.1 M NaOH (10 min) and water (10 min). Then, capillaries were
540 dynamically coated by rinsing them with 0.6% (w/v) of commercial CMC (MW 29.000) solution
541 in 0.1M HCl (30 min). Finally, the capillaries were left to stand (40 min) to allow CMC to
542 adsorb on the fused silica wall and followed by a 10 min purge with air. Between runs the
543 capillaries were flushed with the separation buffer (2 min). Using a volatile buffer of 100 mM
544 acetate (pH 4.5) and -30 kV for separation voltage, 9 isoforms of EPO (NCPC Gene Tech
545 Biotechnology Development, China) were separated in 9 min. Reproducibility of the migration
546 times for the isoforms ranged from 0.75 to 1.29 (RSD (%), n=5). Although, base-line resolution
547 was not achieved for the 9 isoforms, the CMC coating showed good potential for rhEPO
548 determination using both CZE and CZE-MS.

549 Amine-coated capillaries (eCAP) and a separation buffer containing 8 M urea and 300
550 mM phosphate were used for QC of rhEPO produced by several companies from Korea, China,
551 and India and compared to the one produced by Amgen (Epogen) in terms of the isoform
552 distribution [82], as mentioned in Section 6.1 of this paper. Information about capillary
553 conditioning and repeatability of the separation method was not provided.

554 A similar, but not identical, CE method to the one prescribed by the EP has been
555 developed for quantification of the 8 isoforms in samples of Epoetin beta [83]. The method used
556 70 mM MES buffer instead of 0.01 M tricine and 0.01 M sodium acetate employed by the EP
557 method. Forty production batches of this pharmaceutical protein were selected to cover adequate
558 range of precisely established potency values. The percentage of each isoform in the set of
559 samples was determined using CE analysis. The sialylation degree of each batch was also
560 measured. Several relationships between biological activity and chemical parameters were
561 statistically evaluated. As a consequence, a suitable model for correlating physico-chemical
562 parameters (percentage of each isoform and sialic acid content) and biological activity of the
563 EPO samples was established. The results obtained using the model were equivalent to those
564 obtained using the established *in vivo* bioassay in terms of accuracy, but they were superior in
565 terms of precision. The CE method was also proposed as an alternative to animal
566 experimentation in Quality Control (QC) of EPO.

567 Recently [23] the CZE method of the EP was used for determination of the sialic acid
568 content per molecule of different rhEPO subfractions; these subfractions were obtained using
569 chromatography from a conditioned medium of CHO cells expressing rhEPO. The sialic acid per
570 molecule for each subfraction was calculated using a first order polynomial equation of the
571 relative proportion (expressed as %) of each of the isoforms separated by CZE. The sialic acid
572 per molecule content from each subfraction was correlated to the *in vivo* bioactivity of the
573 fraction determined on the basis of increased number of reticulocytes following each EPO
574 fraction injection into a murine model. The authors observed a low correlation coefficient (0.76)
575 among these two parameters, suggesting the possibility that other glycan moieties structures
576 besides sialic acid residues may have a significant impact on *in vivo* bioactivity of rhEPO

577 subfractions, a suggestion in accordance with the known influence of branching of the N-linked
578 glycans on the EPO *in vivo* activity [25].

579 4.2 Separation of intact EPO using CIEF

580 Since EPO isoforms can be separated based on differences in their respective isoelectric
581 point [4], CIEF is also well suited for isoform determination. However, due to difficulties in
582 achieving reproducible separations by CIEF only one report has been published during the period
583 reviewed [84]. In this article, CIEF was carried out with a commercial instrument (iCE 280 from
584 Convergent Bioscience) that used a separation cartridge which contains an internally
585 fluorocarbon-coated fused silica capillary (i.d. 100 μm) with integrated buffer reservoirs. The
586 detection was carried out by whole column (50 mm) imaging using UV light at 280 nm and a
587 CCD camera that takes images every 30 sec during the separation process. The sample was
588 prepared by mixing an aliquot of the EPO stock solution (typically 1 mg mL^{-1} after desalting)
589 with 2% (v/v) ampholytes (Fluka pH 3-10), 1% (v/v) polyvinylpyrrolidone (PVP) and 4 M urea.
590 The anolyte was 100 mM phosphoric acid with 1% (w/v) PVP and the catholyte was 100 mM
591 NaOH also containing the same amount of PVP. The polymer was used to increase the viscosity
592 of the sample and to decrease the effect of the EOF in the separation. When the electric field (1.5
593 kV) was applied, the focalization of the EPO isoforms and the detection took place
594 simultaneously. With this procedure, isoforms 1-9 were separated in different EPO preparations
595 in 5 min (see Figure 5) with good reproducibility (RSD values for migration time relative to the
596 pI standard of 0.3% and 0.9% for run-to-run and day-to-day, respectively). The sensitivity of the
597 technique with UV detection was estimated to be in the range of 10^{-6} M; at such a level, it is of
598 interest for pharmaceutical preparations analysis, but it would be of limited value for some
599 applications such as doping control.

600 4.3 Improving detection sensitivity in CE for intact EPO

601 Due to its favorable resolution, the potential of CZE for doping control is good [85].
602 CIEF and gel CE could potentially be valid for this purpose taking into consideration the
603 experience acquired using IEF in gel [86] and SDS-PAGE [87-88]. However, in our opinion, the
604 major limitation for the development of a doping control method based on CE techniques is
605 detection. It is tempting to speculate that the use of laser induced fluorescence (LIF) detection,
606 which is around 1000 times more sensitive than UV, could help for these applications.
607 Nonetheless, some difficulties have to be overcome before achieving the monitoring of EPO
608 isoforms using LIF detection. These problems are analyzed and potential solutions based on the
609 literature are presented in the next few paragraphs.

610 LIF detection is one of the most sensitive techniques for CE [89]. However, few analytes
611 have native fluorescence when excited by visible laser light. For this reason, covalent
612 derivatization and laser with emission in the visible region of the spectrum must be used to get
613 detection sensitivity in the low nM range. For LIF detection of proteins non-covalent and
614 covalent derivatization procedures have been proposed [90]. Non-covalent derivatization relies
615 on the adsorption of a dye, which is dissolved in the separation buffer, on the hydrophobic
616 patches of the proteins of the sample. The adsorption takes place inside the column once the
617 sample has been injected and the dye wavelength and emission intensity increase with respect to
618 unbound dye. In this way the protein-dye complex emits fluorescence when it passes through the
619 laser beam at the detection point. However, hydrophobic interaction of such substances with
620 glycoproteins is generally very small or prevented. In fact, to our knowledge, no substance has
621 been reported to be useful for the non-covalent derivatization of EPO isoforms. Although several
622 methods for covalent derivatization through amino group of Lys residues have been proposed for

623 protein derivatization, multiple derivatization products (leading to multiple peaks or broad peaks
624 in CE) and slow reaction (when the protein is at low concentration (< 10^{-9} M) in the sample)
625 limit covalent derivatization of proteins. The use of on-column derivatization using submicellar
626 concentration of detergent (SDS or sodium pentasulfonate - SPS) in the separation buffer have
627 been used successfully in many cases [91]. However, the use of an ionic detergent in the
628 separation buffer may also prevent the separation of isoforms of glycoproteins [92]. Preliminary
629 results recently obtained by our group for another glycoprotein (prostate-specific antigen) have
630 shown the feasibility of separating isoforms by CZE of on-column fluorescently-labeled
631 glycoprotein [93].

632 Covalent derivatization through thiol group offers better perspective for avoiding
633 multiple derivatization products since Cys amino acids are less abundant than Lys in proteins.
634 This strategy has proved effective for separation of isoforms of some glycoproteins [94] and its
635 applicability to the determination of EPO isoforms is currently under study in our group.

636 It should be mentioned that native fluorescence of proteins has been probed [95] using
637 laser light at 275 nm that is able to excite the fluorescence of the tryptophan residues of the
638 proteins. However, this approach could be difficult to carry out for proteins in most natural
639 samples due to impurities that are always present in natural samples which fluoresce at the same
640 wavelength. This can lead to a strong fluorescence background and a poor sensitivity for LIF
641 detection in this type of samples.

642

643

644 5. Determination of intact EPO and other ESAs by capillary electrophoresis-mass 645 spectrometry

646

647 Owing to the structural information that MS detectors can provide, this technique has
648 been coupled online to CE (CE-MS) for the determination of different compounds including
649 glycoproteins [74]. In particular, several papers were published in 2005-2006 on the
650 determination and characterization of intact EPO and DPO by CE-MS [79,96-98]. Intact standard
651 EPOs (BRP EPO, epoetin alfa, and epoetin beta) were fairly well, though not completely,
652 characterized by CE-MS. After a thorough analysis of the deconvoluted mass spectra for each
653 electrophoretic peak, about 64 glycoforms of EPO differing in their glycan moieties were
654 characterized by CE-time of flight (TOF)-MS [79]. Minor modifications of these 64 glycoforms,
655 such as acetylation and exchange of *N*-acetylneuraminic acid (NeuNAc) with *N*-
656 glycolylneuraminic acid (NeuNGc), were also observed. Detection of NeuNGc in the glycan
657 moiety of EPO is especially relevant as an indicator of misuse of EPO (doping) because glycans
658 containing NeuNGc are produced by CHO cells [99] but not by human cells [10,100-101]. In
659 spite of the extensive work carried out, some post-translational modifications (PTMs) involving
660 shifts of 1 or 2 Da such as deamidation (Δ = 1 Da), addition of 2 fucose molecules *vs* addition of
661 1 molecule of sialic acid to the glycan (Δ = 1 Da), cleavage of disulfide bridge (Δ = 2 Da),
662 hydroxylation *vs* methylation (Δ= 2 Da), and combinations of oxidation and water loss (Δ = 2
663 Da), in a 30 kDa (approx.) protein could not be assessed due to performance limitations of the
664 MS equipment employed [96]. From 2007 to the present, only a few papers dealing with the CE-
665 MS determination of intact EPO have been published [22,102].

666 When CE-MS determination of proteins or glycoproteins is carried out, several practical
667 problems should be considered. From a separation point of view, as it is known, proteins can
668 undergo non-specific adsorption to the capillary wall, and/or lack of reproducible EOF can

669 occur. Highly alkaline BGE (to ensure the electrostatic repulsion of analytes from the capillary
670 wall) or coated capillaries (covalent or non-covalent – either dynamic or semi-permanent –
671 coatings) have been used to prevent adsorption. Between these different options, the most
672 common approach for CE-MS of proteins is the use of capillaries with non-covalent, semi-
673 permanent coatings. For instance, polybrene [79,96,98], different ionene polymers [97], and N-
674 substituted acrylamide copolymers (UltraTrol™) with high reversed EOF (UltraTrol™ HR)
675 [102] or almost suppressed EOF (UltraTrol™ LN) [22,79,102], have been used for separation of
676 glycoforms of EPO by CE-MS. The efficiency of the CE-MS determination of glycoprotein
677 glycoforms depends on the partial separation obtained in the CE analysis, the ionization
678 efficiency and the performance of the MS employed for the detection; the best results were
679 obtained using UltraTrol™ LN as coating. When comparing the two UltraTrol coatings [102],
680 UltraTrol™ LN provided better results in terms of the determination of the glycoforms of EPO,
681 but UltraTrol™ HR showed higher stability and less leakage, which, from the MS detection
682 point of view, is a positive characteristic. With UltraTrol™ LN better glycoform resolution was
683 achieved for EPO than for DPO due to the higher microheterogeneity of the latter glycoprotein
684 [102]. In addition, better separation of EPO glycoforms was obtained with UltraTrol™ LN in
685 comparison to the polybrene coating [79] and with either of these two coatings than with ionene
686 polymers [97]. In Figure 6, the electropherogram of EPO BRP analyzed by CE-MS using an
687 UltraTrol™ LN coated capillary is shown.

688 In all of these papers dealing with CE-MS of EPO, which are collected in Table 2, the use
689 of a volatile BGE was compulsory due to the MS detection. Acetic acid – ammonium acetate
690 buffers [97], or acetic acid (1M or 2M) [22,79,96,98,102], with [79,98] or without [22,79,96-
691 97,102] methanol, were used as BGE for the CE separation.

692 In the above-mentioned papers, the coupling of the CE instrument to the MS detector,
693 operating in electrospray ionization (ESI) mode, was through a sheathflow interface. In most
694 instances [22,79,96,98,102], the composition of the make-up liquid was a mixture of water and
695 isopropanol (1:1, v/v) containing 1% (v/v) acetic acid at a flow rate in the range of 3.3 – 4 µL
696 min^{-1} operating in the positive ion detection mode. For the negative ion detection mode [97] a
697 completely different make-up liquid composition was required and consisted of 5 mM
698 ammonium acetate in 50 % v/v water/acetonitrile at a flow rate of 10 µL min^{-1}. However, in the
699 latter case the performance of the analysis of EPO glycoforms was poor. Although some peaks
700 assigned to EPO glycoforms by the authors were resolved by CE-MS, their MS spectra was very
701 weak and without the typical MS peaks pattern of multiply-charged glycoproteins. This is likely
702 due to a combination of the separation conditions, the ionization settings and the detector
703 employed. For instance, the negative ionization mode was used, though positive ionization mode
704 tends to be the best option for the determination of proteins as ammonia seems to give rise to
705 inefficient ionization of proteins. In fact, Neususs et al. [96] were unsuccessful in detecting EPO
706 glycoforms in the negative ionization mode, while, as mentioned above, succeeded using
707 positive ionization mode. Besides,the dilution of the eluent from the CE column was larger as
708 they used a higher make-up liquid flowrate (10 µL min^{-1} *vs* 3.3-4 µL min^{-1}).
709 Different MS instruments with different MS analyzers such as ion trap (IT) [97,102],
710 time of flight (TOF) [79,96,98] and extra-long flight path TOF [22] have been used for the
711 determination of EPO glycoforms. Two equipments with IT analyzer (Agilent [97] and Bruker
712 [102]) have been used. The two instruments led to different results in terms of identification of
713 EPO glycoforms, probably because different separation conditions and ionization settings were
714 used. The Bruker MicrOTOF™ has been the only "normal" TOF analyzer used for the CE-MS

715 determination of EPO glycoforms and provided better results than IT analyzers. This was
716 expected as, in general, TOF analyzers show better performance than IT analyzers when there is
717 a high number of partially co-migrating glycoforms over a broad mass/charge range. Reasons
718 include better suitability for larger m/z values (quadrupole based instruments including ITs have
719 limited mass range), better de-clustering due to higher vacuum and shorter or no storage time in
720 TOF instruments. For similar separation conditions (same BGE and capillary coating) and
721 ionization settings (sheathflow composition) 64 glycoforms of EPO were detected with a TOF
722 analyzer [79] while only 17 were detected with an IT analyzer [102]. Recently, a new study [22]
723 was published dealing with improvement of the determination of EPO by CE-MS where an
724 extralong ion flight path TOF (5 m flight path, maXis quadrupole time-of-flight mass
725 spectrometer also called maxis 4G UHR-TOF from Bruker) was used as opposed to the "normal"
726 TOF analyzer (2 m flight path, Bruker MicrOTOF™), also called benchtop TOF-MS. In this
727 work EPO was presented as a proof-of-concept for resolution improvement in MS spectra
728 achieved for large proteins (Figure 6). With the extralong flight path TOF analyzer, isotopically
729 resolved MS spectra can be obtained leading to the accurate determination of the empirical
730 formula of the protein and, as a consequence, to the determination of the accurate structure of the
731 protein having PTMs involving mass shifts as small as 1 Da (such as deamidation). The authors
732 determined the accurate empirical formula of the protein by comparison between the isotopically
733 resolved, deconvoluted MS spectra and the theoretical isotopic MS spectra of the protein.
734 However, the authors recognize that even with the very high resolution provided by the extra-
735 long flight path TOF analyzer, the unambiguous identification of PTMs involving very small
736 mass shift (1 Da) is not always accomplished due to the comigration of glycoforms and the
737 overlap of the isotopic pattern. However, in view of the technical improvement provided by the

738 extra-long flight path TOF, the publication of the CE-MS determination of the glycoforms of
739 EPO with this new instrumentation in the near future can be expected.
740 There are other MS instruments with high mass resolution and accuracy such as orbitrap MS and
741 Fourier Transform Ion Cyclotron Resonance (FTICR)-MS, but their features –resolution and
742 accuracy- depend on the measuring time. As a consequence, in order to obtain the best
743 performance when FTICR-MS is coupled to CE separation, the measured mass range is reduced
744 in order to devote the required time to measure each m/z ion. Although CE-FTICR-MS has been
745 used to analyze protein glycoforms [103], it has not been applied yet to the characterization of
746 EPO.
747 As mentioned in the Introduction section, the determination of glycoforms of intact
748 glycoproteins presents several advantages in comparison to hydrolyzed glycoproteins. This
749 approach permits obtaining the whole glycoform profile without further "reconstruction" of the
750 glycoprotein structure. However, the observed microheterogeneity cannot always be
751 unequivocally assigned to either the glycan structure or the protein backbone. For instance, a 16
752 Da mass shift can be assigned to several pathways: hydroxylation, exchange of NeuNAc with
753 NeuNGc or oxidation at either methionine, histidine (to oxo-histidine), cysteine (to sulfenic acid)
754 or proline. Although it may be possible to ascertain the nature of such a PTM from the MS
755 results and the electrophoretic behavior of the glycoform (as the PTM may have an effect on the
756 migration of the glycoprotein), a combination of the CE-MS determination of the intact
757 glycoprotein with other techniques is very useful to characterize the structure of glycoproteins.
758 Such approaches could involve selective digestion processes (e.g., deglycosylation, proteolytic
759 digestion) and the determination of the different fractions (glycans, glycopeptides, and peptides)
760 by different techniques such as HPLC (IEX, RP, or HILIC), CE, MS, and hyphenations CE-MS

761 and HPLC-MS. CE-MS analysis of an intact glycoprotein can provide the glycoforms profile of
762 the target glycoprotein and, as a consequence, the global composition of its glycan moiety, but
763 the exact structure of the glycans cannot be completely determined. In addition, when different
764 glycosylation sites are present in the glycoprotein, as it is the case for EPO, it is not possible to
765 determine the glycan composition of each glycosylation site individually. Besides, there are
766 several possible isomeric structures associated with a given monosaccharide composition that
767 cannot be distinguished by simple mass spectrometric measurements without fragmentation. For
768 instance, a tetra-antennary structure is equivalent to one tri-antennary glycan containing one *N*-
769 acetyllactosamine (LacNAc) repeat. Moreover, information relative to the interglycosidic
770 linkages cannot be obtained using this methodology [79]. Thus, in general, the above-mentioned
771 approaches complementary to CE-MS of the intact analyte are useful for a deeper
772 characterization of the glycoprotein.

773 Finally, one of the problems of the CE-MS determination of glycoforms of glycoproteins
774 is its lack of sensitivity. For instance, the concentration of the standard EPO analyzed in the
775 articles cited above was in the range of 1.5 – 15 mg mL^{-1}. While these concentrations may be
776 appropriate for characterizing pharmaceutical preparations they would not be suitable for EPO
777 concentration expected in human biological fluids. To our best knowledge, there is only one
778 published article [97] where intact standard urinary EPO was analyzed by CE-MS and the MS
779 results were not very useful for the comparison of the glycoform profile between recombinant
780 and urinary EPO due to lack of quality in the MS spectra as it was mentioned before.
781 Improvements in the purification and concentration methods for EPO determination should be
782 developed, as discussed in Section 2, enabling future comparisons of the glycoform pattern of
783 recombinant and endogenous EPO. The knowledge of these differences could be of interest both

784 in pharmaceutical drug development, in order to decrease chances of immunological reactions,
785 and for doping control.
786
787
788 **6. Applications**
789
790 **6.1 Regulatory considerations and Biosimilars**.
791
792 EPO was one of the first recombinant protein pharmaceuticals to reach the market at the
793 end of the 1980s and, just as for any other therapeutic entity, market authorization was granted
794 on the demonstration of safety, quality and efficacy. These are the three pillars on which the
795 regulatory approval process is based in most jurisdictions and they have been the focus of
796 extensive harmonization efforts that culminated in the development of the International
797 Conference on Harmonization (ICH) guidelines that are now widely accepted by regulatory
798 agencies and manufacturers [104]. In addition, in several jurisdictions manufacturers must
799 comply with pharmacopoeial requirements for the establishment of quality attributes for the drug
800 substance. Altogether, these regulatory considerations have led to the development and
801 widespread adoption of high resolution separation techniques and methods for the
802 characterization of biopharmaceuticals due in large part to their increased precision and
803 quantitative nature. This has been particularly the case for EPO.
804 As mentioned in Section 4, capillary electrophoresis has been prescribed as an
805 identification test since the publication of the first EPO monograph of the European
806 Pharmacopoeia (EP) [58,77]. The test is based on the separation of intact EPO isoforms by CZE

807 and applies to the analysis of the drug substance. As such, a sample treatment is required to
808 eliminate excipients that may be present for stability purposes. The isoform profile of the test
809 sample is compared to that of the EPO BRP for the identification of the specific isoforms. The
810 percentage content of each isoform in the test sample is then calculated and must be within a
811 specified range.

812 The quantitative determination of dimers and other high molecular mass aggregates by
813 SE-HPLC has also been a prescribed test in the EP monograph for EPO since its initial
814 publication [58,77] (see section 3).

815 As indicated above, EPO was one of the first recombinant protein pharmaceuticals to
816 reach the market and it has been one of the most successful, with worldwide annual sales
817 exceeding 10 billion US dollars in 2010. Soon after the patent expiry of the two innovator
818 products, EPO-alfa (Eprex and Epogen) and EPO-beta (Neorecormon), in Europe and the USA
819 many biopharmaceutical companies indicated their intention to bring their own EPO version to
820 the market. This led regulatory authorities to look into the concept of biosimilars, which was
821 formally adopted in Europe in 2005 [105]. It differs significantly from the concept of generics
822 used for small molecule pharmaceuticals. In the standard generic approach the demonstration of
823 bioequivalence is made with a reference product through appropriate bioavailability studies. This
824 approach is not scientifically feasible for biopharmaceuticals due to their complex nature.
825 Instead, a comparability exercise is recommended and it is the degree of similarity that
826 ultimately is evaluated. In Canada, the terminology "subsequent entry biologics" (SEBs) has
827 been adopted and a pathway similar to that of Europe has been recommended [106]. In the
828 United States, where the terminology of follow-on biologics (FOBs) is used, no guidelines have
829 been issued as of the beginning of 2011.

830 The first EPO biosimilars were approved in 2007 in Europe (under the names Binocrit,
831 Epoetin alfa Hexal and Abseamed) using the EPO-alfa innovator product, Eprex, as comparator.
832 Since then a few others have been marketed. Among the contentious issues to surface in the
833 debate between innovators and biosimilar manufacturers was the lack of availability of the
834 comparator's bulk drug substance for biophysical and chemical comparability testing. In such a
835 case, a biosimilar manufacturer must rely on obtaining it through purification from the
836 comparator's finished drug product. This has led to the publication of studies on the biophysical
837 comparability of EPO purified from different formulations [107-108].

838 Using a wide range of techniques that included CE and HPLC, Deechongkit et al. [107]
839 were the first to compare EPO-alfa isolated from two commercially-available formulations,
840 namely Eprex and Epogen. EPO was purified from formulations by IEX –HPLC. They used CZE
841 to examine the isoform profiles of the two purified EPO products and found them to be similar.
842 On the other hand, determination of the dimers and HMW aggregates by SE-HPLC showed that
843 the purification procedure gave rise to a slight increase in the aggregate content for one of the
844 two products. Ultimately, significant differences were observed in the tertiary structure and they
845 concluded that the two proteins were not structurally identical. Heavner et al [108] conducted a
846 follow-up study and made a counter-argument to the approach of Deechongkit et al. by showing
847 that the purification procedure used irreversibly altered the physical structure of the purified
848 EPO.

849 EPO products obtained from several countries around the world, although not strictly
850 considered biosimilars, have been compared to innovator products such as Eprex [109] and
851 Epogen [82]. In the latter study, the determination of EPO isoforms was carried out by CZE and
852 showed differences in isoform profiles between several EPO products obtained from Asia. Both

853 studies showed wide variability in terms of glycoform profiles, content and biological activity,
854 and even batch-to-batch consistency. Isoform profile determination by CZE was also used in the
855 comparative assessment of the approved biosimilar, Binocrit, to the innovator product, Eprex.
856 Binocrit was found to be comparable, of high quality, safe and efficacious [110]. A similar
857 conclusion was reached by another group on the quality assessment of two biosimilars, Binocrit
858 and Retacrit, and two innovator products, Eprex and Dynepo [111]. Again, CZE determination of
859 isoform profiles was used along with SE-HPLC.

860 A recent article featuring the ESA product, Aranesp, has brought forward into the public
861 domain another contentious issue hotly debated for some time between innovators and biosimilar
862 manufacturers. It relates to the degree with which differences in quality attributes are acceptable
863 [17]. Among several other techniques, they used CZE to demonstrate significant differences in
864 the isoform distribution profile between different batches of the product and concluded that these
865 variations had been deemed acceptable by health authorities since the batches had been marketed
866 without any label alterations.

867

868 **6.2 Doping control**

869

870 EPO and the rest of the ESAs are included in the List of Prohibited substances by the
871 World Anti-Doping Agency (WADA); they are part of the substances classified as prohibited at
872 all times (in- and out-of-competition) [112].

873 For epoetin alfa, epoetin beta (both produced in CHO cell lines) and epoetin omega
874 (produced in hamster kidney cells) the basis for detecting rhEPO doping relies on the different
875 glycosylation of the protein as a function of the organism or cell line in which it is produced. As

876 a result of glycosylation changes, the isoelectric points and molecular weights of recombinant
877 EPOs are higher than in uhEPO. The isoforms in darbepoetin are more acidic than those in
878 uhEPO due to its increased number of sialic acid residues from the higher number of
879 glycosylation positions. CERA can be distinguished from endogenous EPO due to its higher
880 molecular weight. Epoetin delta (produced in human fibrosarcoma cell line) in comparison to the
881 endogenous EPO shows more acidic isoforms on IEF, but these differences are not sufficient to
882 differentiate them according to the WADA's criteria; doping with epoetin delta is detected by
883 differences observed by SDS-PAGE. A summary of ESA doping strategies and their abuse
884 detection methods was published by Jelkmann [113].

885 None of the methods approved for EPO-related doping control is based on HPLC or CE
886 techniques. The method presently used for EPO and other ESAs doping control is based on
887 Western blot after IEF and, if additional scientific evidence is needed, SDS-PAGE or an
888 equivalent procedure can be used [114]. IEF allows partial discriminatiion of isoforms of
889 endogenous (either urinary, plasma or serum) EPO from those of different recombinant epoetins.
890 Identification windows for uhEPO and ESAs are established by WADA [114]. Detection is
891 based on a double blotting procedure followed by chemiluminescent detection, which provides
892 high selectivity and sensitivity [86]. In addition to these gel electrophoretic methods, ELISA-
893 based methods (to measure EPO and CERA) are among the techniques used at present by
894 WADA-accredited laboratories to detect ESAs [115-116].

895 A CE competitive immunoassay which, according to the authors, could be potentially
896 useful for EPO doping control has been developed using silica dioxide nanoparticles as pseudo
897 stationary phase and chemiluminescence detection. The assay is based on separation of EPO
898 labeled with horseradish peroxidase (EPO-HRP) from the immunocomplex-HRP. High

899 sensitivity (0.9 ng mL^{-1} = 3 x 10^{-11} M) and high correlation with commercial immunoassay kit
900 are achieved. However, the assay does not separate EPO isoforms and cannot differentiate
901 endogenous from exogenous EPO; thus doping detection should be based on total EPO
902 concentration [117].

903 Taking into account the separation mechanisms involved in differentiation of exogenous
904 from endogenous EPO by IEF or SDS electrophoresis performed in conventional gels, EPO
905 doping control by CIEF, CZE, or gel CE should potentially allow distinguishing EPO from both
906 origins. However, doping control based on separation of isoforms of intact EPO (or related
907 molecules) either by HPLC or by CE has not been carried out. Similarly, gel CE has not been
908 applied for EPO doping control. The main limitations are the low concentration of endogenous
909 and exogenous EPO or EPO-related substances in biological fluids (in the range of mIU mL^{-1},
910 this is about pg mL^{-1}) and the complexity of the matrices which include a high number of
911 proteins in a broad concentration range. To solve these limitations, sample preparation methods
912 aimed to isolate and concentrate EPO substances, and detection methods providing enhanced
913 sensitivity while preserving isoform resolution, are needed.

914 The CE or HPLC published papers dealing with differentiation of endogenous from
915 exogenous EPO substances based on isoform separation have focused on the development of
916 methods using standard glycoproteins of both origins. In our laboratory, a preliminary study
917 performed by CZE with UV detection showed different isoform profiles for human EPO
918 standards from recombinant and urinary origin. uhEPO showed some isoforms migrating slower
919 than the isoforms in rhEPO (see Figure 7) [85]. If an almost constant size is assumed for all
920 isoforms, the slower isoforms in uhEPO would correspond to more acidic forms than in rhEPO,
921 a result that is in accordance with those obtained by conventional electrophoresis by Wide et al.

922 [118] and by Lasne and de Ceaurriz [86]. Although this result was encouraging, the sensitivity
923 obtained was not enough to analyze endogenous and exogenous EPO at the levels existing in
924 natural samples.

925 Differentiation of urinary and recombinant human EPO standards has been attempted by
926 coupling CE-ESI-MS [97],as mentioned above. Although the authors indicated that a unique m/z
927 value was observed for rhEPO and a different one for uhEPO, identification of those ions was
928 not performed and the low sensitivity of this CE-MS method prevented its use for doping
929 control.

930 An attempt to increase sensitivity while maintaining CZE resolution of EPO isoforms has
931 been performed using standard rhEPO. Combination of large volume sample stacking and
932 reversed pH junction allowed about 50 times on-line sample preconcentration at the cost of
933 reduced resolution [119]. However, this increase in sensitivity was insufficient for doping
934 control.

935

936

937 **7. Concluding remarks and future perspectives**

938

939 At the present time HPLC and CE methods developed for the determination of EPO and
940 other ESAs enable most of the challenges presented by such complex proteins to be addressed.
941 Sufficient isoform resolution can be achieved using CE methods, particularly in CZE and CIEF
942 modes, although some isoforms can also be separated by RP-HPLC and IEX-HPLC. These two
943 HPLC modes have also been shown to be adequate for the separation of ESAs from excipients.

944 In addition, monitoring of the formation of dimers and aggregates can be achieved by RP-HPLC
945 and especially by SE-HPLC.
946 HPLC and CE separation techniques are very useful for the analysis of pharmaceutical
947 products. In fact, SE-HPLC and CE tests are prescribed by the European Pharmacopoeia for
948 quantitation of EPO dimers and aggregates and for EPO identification through the separation of
949 isoforms, respectively. The importance of ESA determination is increasing enormously with the
950 continuing expansion of biosimilars.
951 The main limitation of HPLC and CE for the determination of ESAs is the insufficient
952 sensitivity provided by UV or MS detection. This fact, together with the difficulty in analyzing
953 ESAs in samples of high complexity, limits their application for pharmacokinetics studies and
954 doping control. Two approaches can be followed to overcome these difficulties. Purification and
955 concentration can be achieved by applying sample preparation methods aimed at isolating the
956 target ESA from its complex matrix. Different sample preparation methods based on
957 immunorecognition have been successfully applied for performing doping control following the
958 IEF WADA accepted method. These results could be adapted to prepare ESA samples to be
959 analyzed by CE or HPLC. Alternative ligands, such as aptamers, whose affinity for EPO has
960 been already demonstrated [120-121] could be tested for purification and concentration
961 applications. The aptamer approach would require more sensitive detection compatible with the
962 separation technique. In this regard, LIF is known to provide higher sensitivity than UV
963 detection and, together with fluorescent derivatization through the amino or thiol groups, could
964 still allow CZE separation of isoforms, as was recently developed [93-94]. Another promising
965 strategy has been developed for LIF detection in CIEF [122]. LOD in the high femtomolar range
966 has been achieved by employing photo-bleached ampholytes, derivatization through the thiol

967 groups using Chromeo P450 as fluorescent derivatization reagent, and a 532 nm laser for
968 fluorescent excitation. These methods or similar ones could be assayed for CE-LIF of EPO
969 isoforms.

970 In order to automate and simplify the analysis, sample preparation, CE or HPLC
971 separation, derivatization, and detection could be applied on-line either by coupling HPLC
972 columns or by performing all the steps in the same CE column. Furthermore, miniaturization via
973 the analysis in microchip format would decrease the analysis time, sample volumes and reagent
974 consumption, and provide disposable platforms for analysis, thus avoiding cross-contamination
975 between samples.

976

977 Figure legends.

978

979 Figure 1. Molecular model of erythropoietin with complex N-linked glycans at sites N24, N38
980 and N83. O-linked glycans are not represented. The structure shown is just one possible
981 conformation. Reprinted permission from Macmillan Publishers Ltd. (C. Sheridan, Nature
982 Biotech., 25 145-146) copyright (2007) [2]. The picture was a courtesy of M.R. Wormald, R.A.
983 Dwek and P.M. Rudd.

984

985 Figure 2. Schemes of epoetin, darbepoetin, and CERA: glycosylation and pegylation sites (for
986 CERA). Reprinted from W. Jelkmann, "Recombinant EPO production-points the nephrologist
987 should know" Nephrol. Dial. Transplant. (2007) 22, 2749-2753 by permission of Oxford
988 University Press [3].

989

990 Figure 3. Total protein content (black line and right scale) and hEPO (bars and left scale) present
991 in each fraction through the immunopurification procedure using immunoaffinity well plates:
992 initial plasma sample, unbound fraction, first wash, second wash, and elution fraction.
993 Reprinted from J. Chromatogr. B 878, J. Mallorqui, E. Llop, C. de Bolos, R. Gutierrez-Gallego,
994 J. Segura, J.A. Pascual "Purification of erythropoietin from human plasma samples using an
995 immunoaffinity well plate" 2117-2122, Copyright (2010) with permission from Elsevier [34].

996

997 Figure 4. Immunoaffinity purification set-up. The anti-EPO antibody is covalently bound to the
998 monolith disk which is held inside the plastic housing. The device is used in a multi-channel
999 vacuum manifold. Reprinted from J. Chromatogr. A 1217, M. Lonnberg, Y. Dehnes, M. Drevin,

1000 M. Garle, S. Lamon, N. Leuenberger, T. Quach, J. Carlsson, "Rapid affinity purification of
1001 erythropietin from biological samples using disposable monoliths" 7031-7037, Copyright (2010)
1002 with permission from Elsevier [41].
1003
1004 Figure 5. CIEF comparison of different EPO samples using a microchip-like separation device
1005 with internally fluorocarbon-coated fused silica microchannel. EPO samples (1 mg mL^{-1}
1006 containcontaining 4 M urea, 1% PVP and 2 % ampholytes pH 3-10). Reprinted from J. Chrom. A
1007 1190, P. Dou, Z. Liu, J.G. He, J.J. Xu, H.Y. Chen, "Rapid and high-resolution glycoform
1008 profiling of recombinant human erythropoietin by capillary isoelectric focusing with whole
1009 column imaging detection" 372-376, Copyright (2008) with permission from Elsevier [84].
1010
1011 Figure 6. Electropherogram of an EPO BRP PE sample (ca. 120 ng injected): CE-MS analysis
1012 using a soluble linear polyacrylamide coated capillary and 1 M acetic acid as BGE. All the traces
1013 are the extracted ion electropherograms (EIEs) of the average mass of the most abundant charge
1014 state. Traces of same color represent EIEs of glycoforms showing the same number of sialic acid
1015 (SA) residues. Within these groups the glycoforms are separated by the number of hexose-N-
1016 acetyl-hexosamine (HexHexNAc) units. Reprinted from J. Proteom. 74, A. Taichrib, M. Pelzing,
1017 C. Pellegrino, M. Rossi, C. Neususs, " High resolution TOF MS coupled to CE for the analysis
1018 of isotopically resolved intact proteins" 958-966, Copyright (2011) with permission from
1019 Elsevier [22].
1020
1021 Figure 7. CZE electropherograms of A) uhEPO, B) rEPO to which mannitol had been added and
1022 then removed, and C) rhEPO. Electropherograms are aligned according to the retention time of

1023 the EOF (Negative peak not shown). Modified from M. de Frutos, A. Cifuentes, J.C. Diez-Masa,

1024 "Differences in capillary electrophoresis profiles of urinary and recombinant erythropoietin",

1025 Electrophoresis with permission from John Wiley and Sons [85].

1026

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1031

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Revised Table 1

Table 1. **Non – comprehensive list of Erythropoiesis Stimulating Agents (ESAs) around the world and the geographical areas of production ***

ESA	Manufacturer	Geographical area of origin
Abseamed	Medice Arzneimittel Pütter	Germany
Aranesp	Amgen	USA
Binocrit	Sandoz	Germany
Bioetin	Nanogenpharma	Vietnam
Biopoin	CT Arzneimittel	Germany
Bioyetin	Probiomed	Mexico
Ceriton	Rambaxy	India
Dynepo	Shire	Ireland
EPIAO	3SBio(Shenyang Sunshine Pharmaceuticals)	China
Epocim	CIMEQ	Cuba
Epocrin	Sotex	Russia
Epoetal	Pliva	Croatia
Epoetin Alfa Hexal	Hexal Biotech Forschungs	Germany
Epofer	Emcure	India
Epofit	INTAS Pharmaceuticals	India
Epoform	Eipico	Egypt
Epogen	Amgen	USA
Epogin	Chugai Pharmaceutical Company	Japan
Epokine	CJ Corporation	South Korea
Eporatio	Ratiopharm	Germany
Eporise	Zuventus	India
Eporon	Dong-A Pharmaceutical Company	South Korea
Eposino	Shandong Kexing	China
Epostim	Reliance Genemedix	UK
Epotin	Claris Lifesciences	India
Epotrex-NP	Novell Pharmaceutical	Indonesia
Epoyet / Epoimmun / Hemax / Hypercrit / Zyrop	Bio Sidus	Argentina
Eprex	Janssen – Ortho Biotech	USA
Eritina	Chalver Laboratories	Columbia
Eritrogen	Bioprofarma	Argentina
Eritromax	Blausiegel	Brazil
Erlan	Landsteiner Scientific	Mexico
Erykine	INTAS Pharmaceuticals	India
Erypo	Janssen-Cilag	Europe
Erypoietin	Amoun Pharmaceuticals	Egypt
Erypro	Biocon Pharmaceuticals	India
Erytrex	Cinnagen / Zhavari	India / Iran
Espo	Kirin Brewery Company, Pharmaceutical Division	Japan
Espogen	LG Life Sciences	South Korea
Exetin	Pisa Farmaceutica	Mexico
Gerepo	North China Pharmaceutical Group Corporation	China
Hemapo	Innogene Kalbiotech	Singapore
Hemoprex	Bergamo	Brazil

Hemotin	Itaca Laboratorios	Brazil
Huan’er Bo	Beijing Four Rings Pharmaceuticals	China
JR-013	Kissei / JCR Pharmaceuticals	Japan
Mircera	Roche	Switzerland
Negortire	Armstrong Laboratorios de Mexico	Mexico
Nephrodil	Feron	India
NeoRecormon	Roche	Switzerland
Nespo	Dompe Biotec (withdrawn 2008)	Italy
PDpoetin	Pooyesh Darou Pharmaceuticals	Iran
Procrit	Amgen (for Centocor Ortho Biotech)	USA
Pronivel	Elea	Argentina
Relipoietin	Reliance Life Sciences	India
Renogen	Biomedics	Philippines
Repotin	BioClones	South Africa
Retacrit	Hospira/STADA	Germany
Shanpoietin	Shanta Biotechnics	India
Silapo	Bioceuticals Arzneimittel AG	Germany
Tinax	Eurofarma	Brazil
Vintor	Emcure	India
Wepox	Wockhardt	India
Zyrop (see Epoyet)	Zydus Biogen	India

* Adapted from [5,7-8].

Table 2

Table 2. **Determination of intact ESAs by high resolution separation methods***

ANALYTE	**ANALYSIS TECHNIQUE AND MODE / DETECTION**	**SPECIES DETECTED**	**REFERENCE**
rhEPO	SE-HPLC / UV	Monomer, dimers and HMW aggregates	[54] [55] [56] [58] [59] [60] [61] [62] [65] [66] [107] [111]
rhEPO	SE-HPLC / Fluorescence	Monomer, dimers and HMW aggregates	[63] [64] [111]
rhEPO	RP-HPLC / UV	Monomer, aggregates and HSA	[55] [67]
rhEPO	RP-HPLC / UV	Monomer, aggregates, HSA and underglycosylated EPO	[68]
rhEPO	RP-HPLC / UV	EPO and underglycosylated EPO	[69]
rhEPO	RP-HPLC / TOF-MS	EPO	[73]
rhEPO	IEX-HPLC / UV	Isoforms	[108]

rhEPO	IEX-HPLC / Fluorescence	Isoforms	[70] [71] [72]
rhEPO	CZE / UV	Isoforms	[19] [23] [81] [82] [83] [107] [110] [111] [119]
rhEPO	CIEF / UV	Isoforms	[84]
rhEPO	CZE / TOF-MS	Glycoforms	[22] [79] [96] [98]
rhEPO	CZE / IT-MS	Glycoforms	[102]
rhEPO	CZE / IT-MS	Isoforms. MS spectra did not allow glycoforms characterization	[97]
rhEPO	CE immunoassay / Chemiluminescence	EPO concentration	[117]
DPO	SE-HPLC / UV	Monomer, dimers and HMW aggregates	[57]
DPO	RP-HPLC / TOF-MS	DPO	[73]
DPO	CZE / UV	Isoforms	[17]

DPO	CZE / IT-MS	Isoforms. Glycoforms were not characterized.	[102]
uhEPO	CZE / IT-MS	Isoforms. None of them was characterized based on MS spectra.	[97]
Endogenous EPO in human sera	CE immunoassay / Chemiluminescence	EPO concentration	[117]

* CE methods published before 2006 were reviewed on [74].

Figure 1

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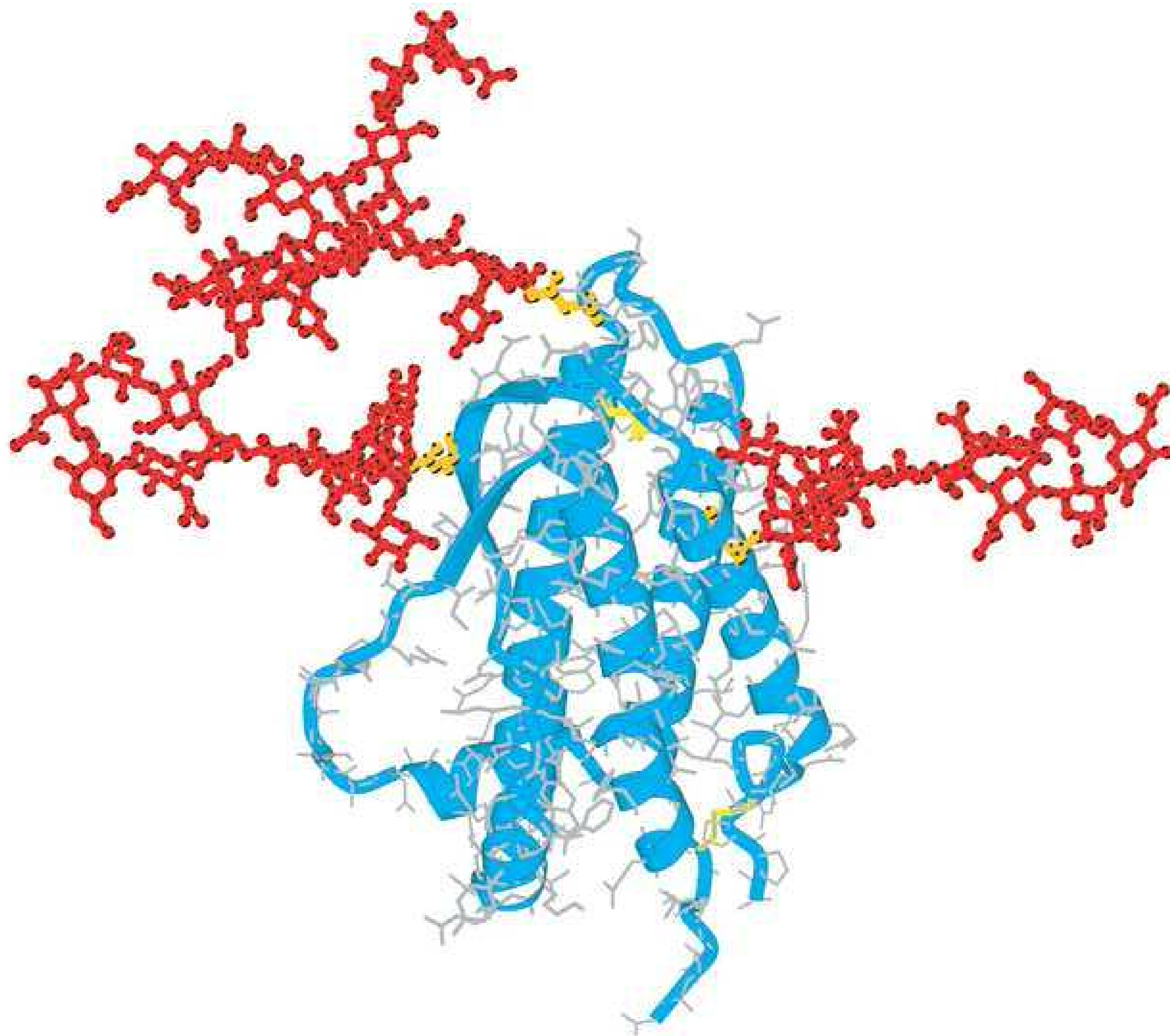

Figure 2

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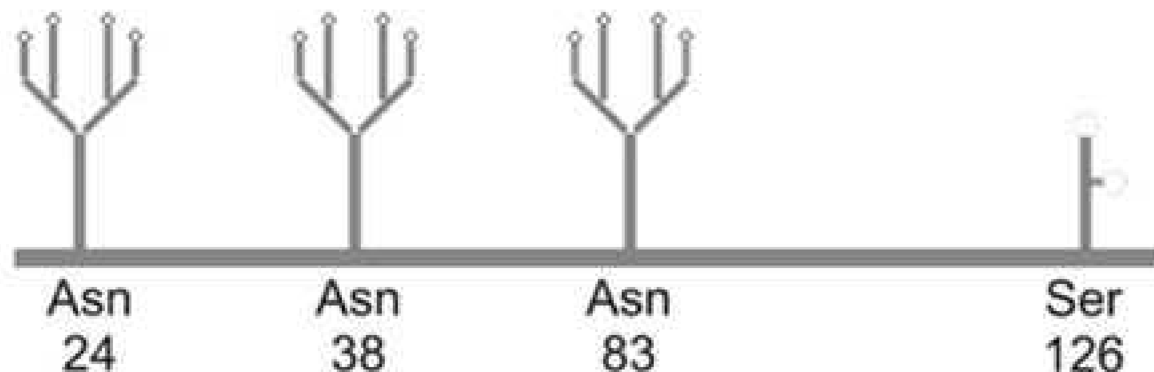
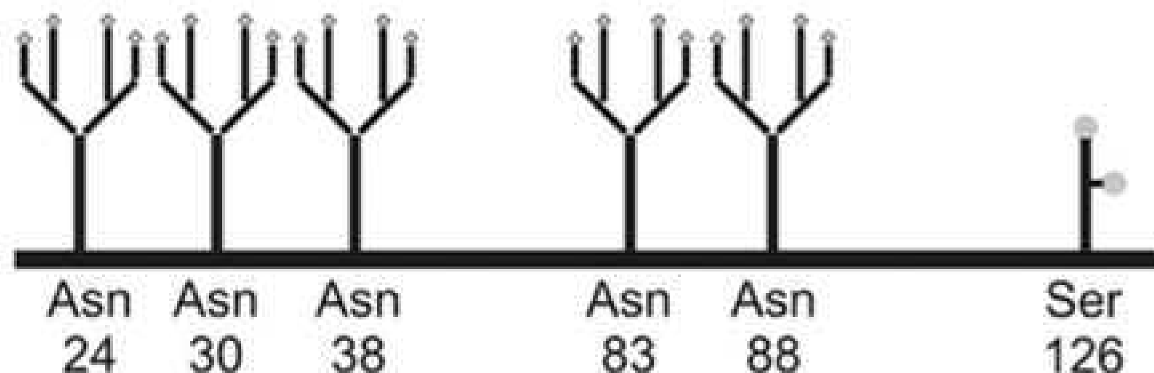
Epoetin (–α, –β, etc.)

same amino acid as human EPO



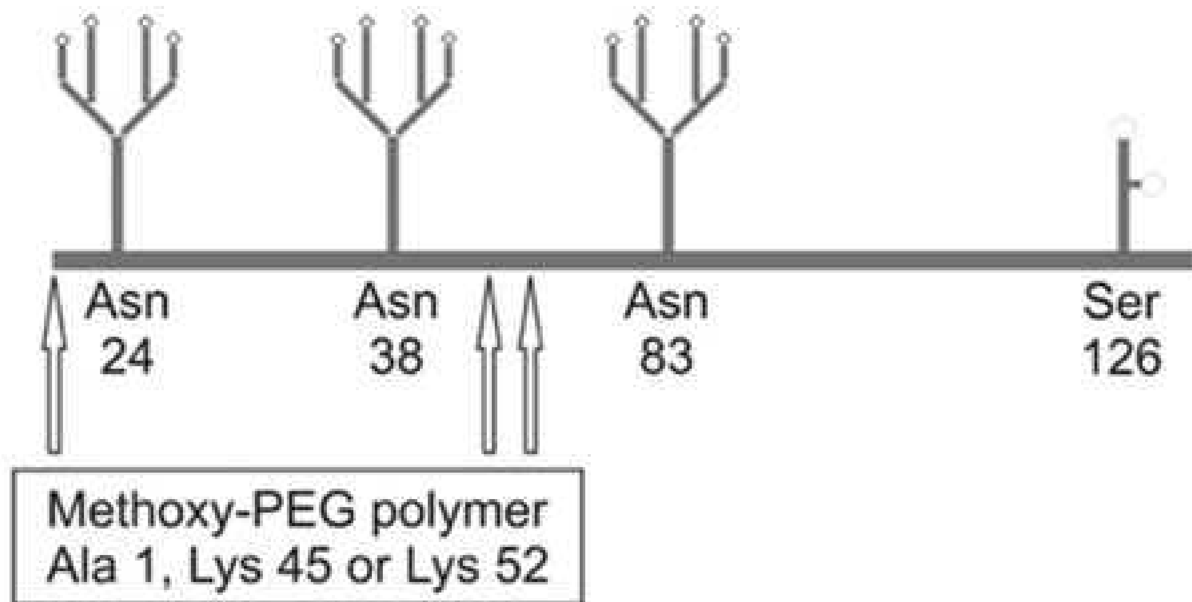
Darbepoetin

altered amino acid sequence



CERA

posttranslational pharmaceutical modification



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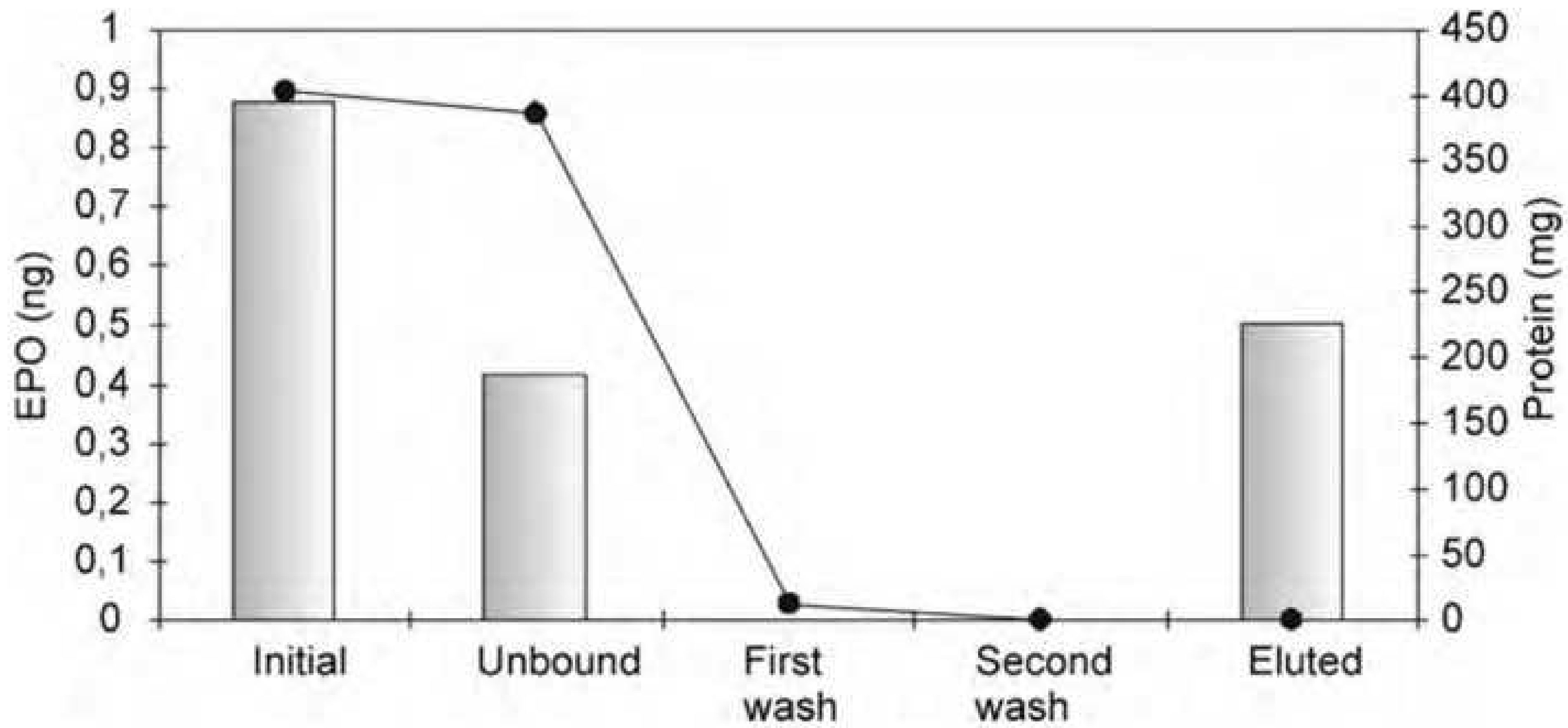

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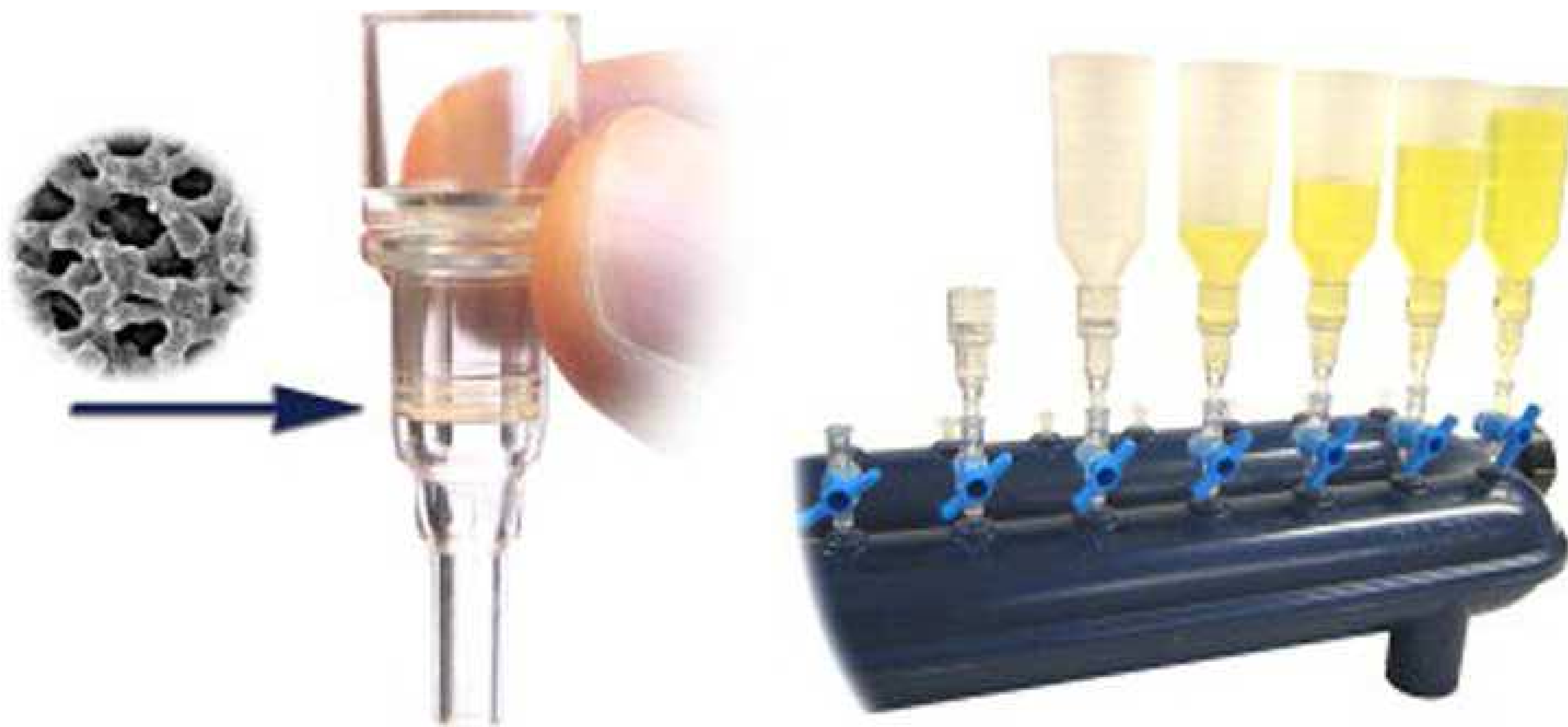

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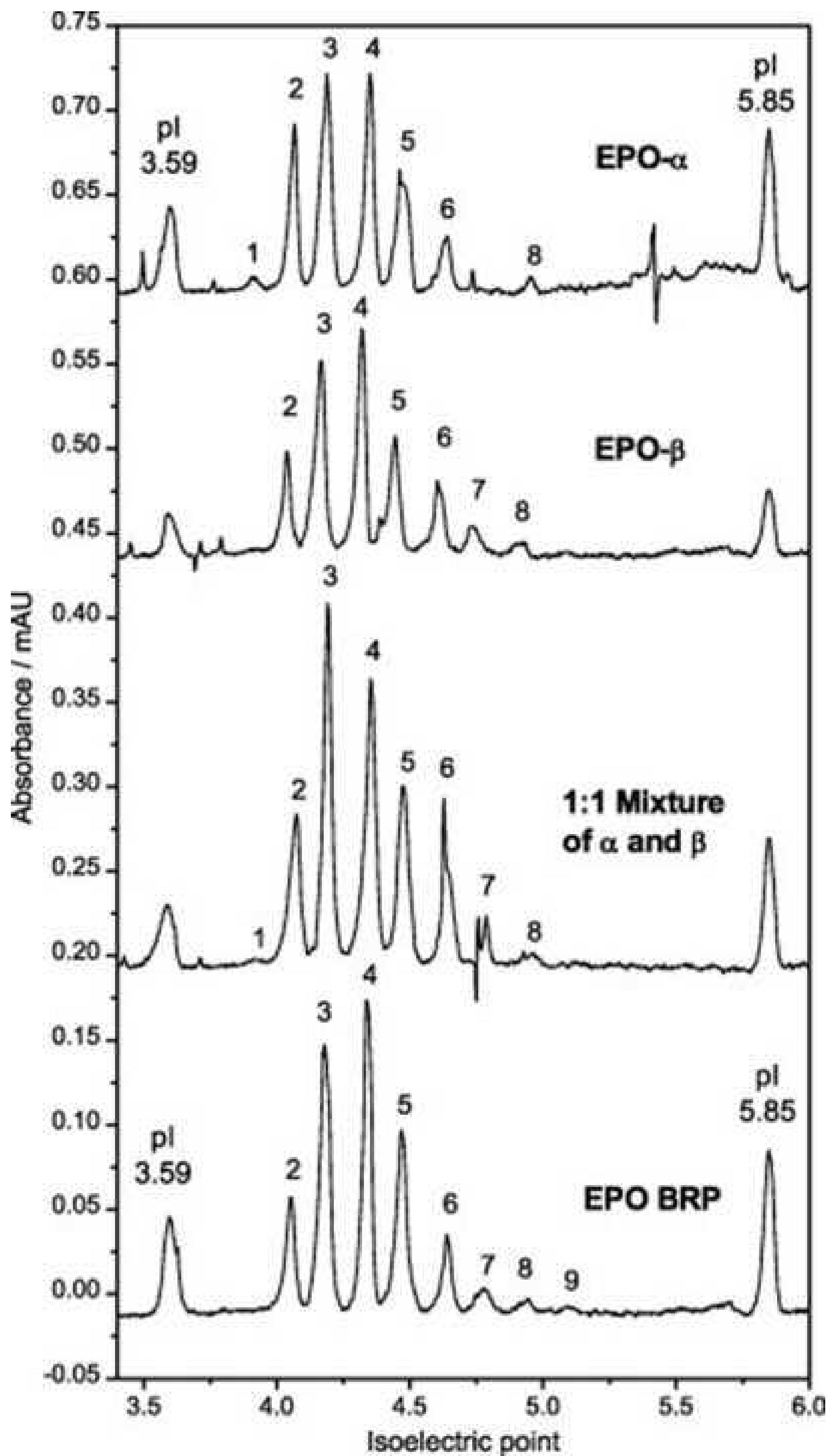

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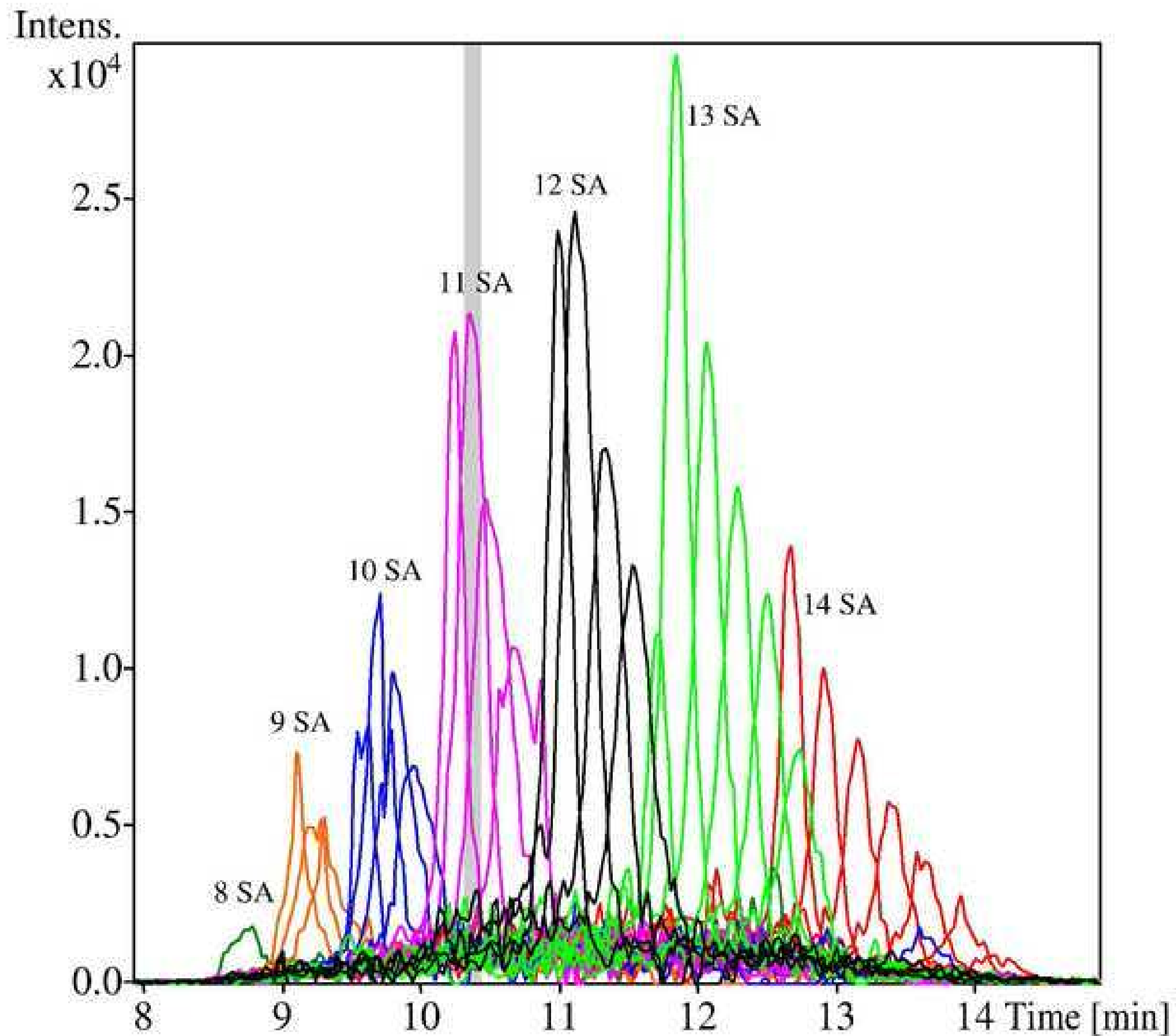

Figure 7
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