B02 CAPILLARY ELECTROPHORESIS

GENERAL PRINCIPLES

Capillary electrophoresis is a physical technique of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field.

The migration velocity of an analyte under an electric field of intensity E, is determined by the electrophoretic mobility of the analyte and the electro-osmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electric charge, molecular size and shape) and those of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity and additives). The electrophoretic velocity (ν_{ep}) of a solute, assuming a spherical shape, is given by the equation:

$$\nu_{ep} = \mu_{ep} E = (q / (6\pi\eta r)) \times (V / L)$$

q = effective charge of the solute,
η = viscosity of the electrolyte solution,
r = Stoke's radius of the solute,
V = applied voltage,
L = total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent is generated inside the capillary, called electro-osmotic flow (EOF). The velocity of the EOF depends on the electro-osmotic mobility (μ_{eo}) which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electro-osmotic velocity (ν_{eo}) is given by the equation:

$$\nu_{eo} = \mu_{eo} E = ((\varepsilon\zeta) / \eta) \times (V / L)$$

ε = dielectric constant of the buffer,
ζ = zeta potential of the capillary surface.

The velocity of the solute (ν) is given by:

$$\nu = \nu_{ep} + \nu_{eo}$$

The electrophoretic mobility of the analyte and the electro-osmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the EOF and their velocities will be smaller than the electro-osmotic velocity. Cations will migrate in the same direction as the EOF and their velocities will be greater than the electro-osmotic velocity.

Under conditions in which there is a fast electro-osmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run.

The time (t) taken by the solute to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is given by the expression:

$$t = l \,/\, (\nu_{ep} + \nu_{eo}) = (l \times L) \,/\, ((\mu_{ep} + \mu_{eo})\ V)$$

In general, uncoated fused silica capillaries above pH 3 have negative charge due to ionized silanol groups in the inner wall. Consequently, the EOF is from anode to cathode. It is recommended that the EOF is maintained constant from run to run if good reproducibility is to be obtained in the migration velocity of the solutes. For some applications, it may be necessary to reduce or suppress the EOF by modifying the inner wall of the capillary or by changing the concentration, composition, and/or pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone, according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion).
In this ideal case the efficiency of the zone, expressed as the parameter N corresponding to the number of theoretical plates, is given by:

$$N = ((\mu_{ep} + \mu_{eo}) \times V \times l) \,/\, (2 \times D \times L)$$

D = molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size and unlevelled buffer reservoirs can also significantly contribute to band dispersion.

Separation between two bands (expressed as the resolution, Rs) can be obtained by modifying the electrophoretic mobility of the analytes, the electro-osmotic mobility induced in the capillary and by increasing the efficiency for the band of each analyte, according to the equation:

$$Rs = (\sqrt{N}\ (\mu_{epb} - \mu_{epa})) \,/\, (4(\bar{\mu}_{ep} + \mu_{eo}))$$

μ_{epa} and μ_{epb} = electrophoretic mobilities of the two analytes separated,
$\bar{\mu}_{ep}$ = mean electrophoretic mobility of the two analytes.

$$\bar{\mu}_{ep} = \tfrac{1}{2}\ (\mu_{epb} + \mu_{epa})$$

92 APPARATUS
93
94 An apparatus for capillary electrophoresis is composed of :
95 — a high-voltage, controllable direct-current power supply,
96 — two buffer reservoirs, held at the same level, containing the prescribed anodic and
97 cathodic solutions,
98 — two electrode assemblies (the cathode and the anode), immersed in the buffer
99 reservoirs and connected to the power supply,
100 — a separation capillary (usually made of fused silica) which, when used with some
101 specific types of detectors, has an optical viewing window aligned with the detector. The
102 ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the
103 solution prescribed in the monograph,
104 — a suitable injection system,
105 — a detector able to monitor the amount of substances of interest passing through a
106 segment of the separation capillary at a given time. It is usually based on absorption
107 spectrophotometry (UV and visible) or fluorimetry, but conductimetric, amperometric as
108 well as mass spectrometric detection can be useful for specific applications. Indirect
109 detection is an alternative method used to detect non-UV-absorbing and non-fluorescent
110 compounds,
111 — a thermostatic system able to maintain a constant temperature inside the capillary is
112 recommended to obtain a good separation reproducibility,
113 — a data acquisition system.
114
115 The definition of the injection process and its automation are critical for precise
116 quantitative analysis. Modes of injection include hydrodynamic injection and
117 electrokinetic injection. The amount of each sample component introduced
118 electrokinetically depends on its electrophoretic mobility, leading to possible
119 discrimination using this injection mode.
120
121 **Hydrodynamic injection**
122 The sample is injected into the capillary by applying a pressure difference (Δp) between
123 the ends of the capillary. The volume of sample injected can be calculated by the
124 Hagen-Poiseuille equation:
125
126 $V_{inj} = (\Delta p\, d_i^4\, \pi\, t_{inj})/(128\, \eta L)$
127
128 t_{inj} = injection time period,
129 η = dynamic viscosity of the buffer,
130 d_i = internal diameter of the capillary,
131 L = total length of the capillary.
132
133 According to the equation, under constant pressure, the injection volume decreases when
134 the length of the capillary is increased. However, the internal diameter of the capillary (d_i)
135 has a dominant influence on the injection volume, since doubling the capillary internal
136 diameter allows the injection of 4 times as much sample while keeping the plug length (l_{inj})
137 constant:
138

139 $l_{inj} = (\Delta p\ d_i^2 t_{inj})/(32\ \eta L)$
140
141 **Electrokinetic injection**
142 In this mode of injection, analytes are injected into the capillary by applying an electrical
143 field (E) across the capillary. Neutral analytes migrate into the capillary with the EOF
144 whereas the charged analytes move according to their own electrophoretic mobilities and
145 to the EOF ($\mu_{ep} + \mu_{eo}$). The amount of each analyte injected depends on its apparent
146 mobility. In contrast to the hydrodynamic mode of injection, this is therefore discriminating
147 mode and consequently, the analyte sample vial may only be used for one injection, since
148 the concentration of the analytes will change after the injection.
149
150 The injection amount (Q_{inj}) of each analyte can be calculated from the following equation:
151
152 $Q_{inj} = (E\ k_b\ \mu_{app}\ t_{inj}\ \pi\ d_i^2\ c_s) / 4\ k_s$
153
154 μ_{app} and t_{inj} = apparent electrophoretic mobility ($=\mu_{ep}+\mu_{eo}$) and injection time period,
155 respectively,
156 k_b/k_s = ratio of conductivities of the background electrolyte and sample,
157 c_s = concentration of the analyte in the sample.
158
159 The plug length (l_{inj}) is not dependent on the capillary internal diameter and can be
160 estimated from the following equation:
161
162 $l_{inj} = E\ (k_b/k_s)\ \mu_{app} t_{inj}$
163
164 This injection mode is mainly used for capillary gel electrophoresis using viscous gel or
165 polymer solution.
166
167 An internal diameter tolerance of ±2 μm, ±3 μm, and ±3 μm is recommended for 25 μm, 50
168 μm, and 75 μm fused silica capillaries, respectively.
169
170 Use the capillary, the buffer solutions, the preconditioning method, the sample solution
171 and the migration conditions prescribed in the monograph of the considered substance.
172 The electrolyte solution employed is filtered to remove particles and degassed to avoid
173 bubble formation that could interfere with the detection system or interrupt the electrical
174 contact in the capillary during the separation run. A rigorous rinsing procedure should be
175 developed for each analytical method to achieve reproducible migration times of the
176 solutes.
177
178 CAPILLARY ZONE ELECTROPHORESIS
179
180 **PRINCIPLE**
181
182 In capillary zone electrophoresis, analytes are separated in a capillary containing only
183 buffer without any anticonvective medium. With this technique, separation takes place
184 because the different components of the sample migrate as discrete bands with different
185 velocities. The velocity of each band depends on the electrophoretic mobility of the solute

and the EOF in the capillary (see General Principles).
Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused silica surfaces.
Using this mode of capillary electrophoresis, the analysis of both small (M_r < 2000) and large molecules (2000 < M_r < 100 000) can be accomplished. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-size ratio can be effected; size refers to hydrodynamic size or hydrodynamic volume. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

OPTIMISATION

Optimisation of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of separations are instrumental and electrolyte solution parameters.

Instrumental parameters

Voltage. A Joule heating plot is useful in optimizing the applied voltage and capillary temperature. Separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result thereof, viscosity gradients in the buffer inside the capillary. This effect causes band broadening and decreases resolution.

Polarity. Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the EOF will move toward the cathode. If the electrode polarity is reversed, the EOF is away from the outlet and only charged analytes with electrophoretic mobilities greater than the EOF will pass to the outlet.

Temperature. The main effect of temperature is observed on buffer viscosity and electrical conductivity, and therefore on migration velocity. In some cases, an increase in capillary temperature can cause a conformational change in proteins, modifying their migration time and the efficiency of the separation.

Capillary. The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing both effective length and total length decreases the electric field (working at constant voltage) which increases migration time. For a given buffer and electric field, heat dissipation, and hence sample band-broadening, depends on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected and the detection system employed.

Since the adsorption of the sample components on the capillary wall limits efficiency, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer

233 composition to prevent protein adsorption. In other strategies, the internal wall of the
234 capillary is coated with a polymer, covalently bonded to the silica, that prevents interaction
235 between the proteins and the negatively charged silica surface. For this purpose,
236 ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and
237 anionic polymers are available.
238
239 **Electrolyte solution parameters**
240
241 *Buffer type and concentration*. Suitable buffers for capillary electrophoresis have an
242 appropriate buffer capacity in the pH range of choice and low mobility to minimise current
243 generation.
244
245 Matching buffer-ion mobility to solute mobility, whenever possible, is important for
246 minimising band distortion. The type of sample solvent used is also important to achieve
247 on-column sample focusing, which increases separation efficiency and improves
248 detection.
249
250 An increase in buffer concentration (for a given pH) decreases EOF and solute velocity.
251
252 *Buffer pH*. The pH of the buffer can affect separation by modifying the charge of the
253 analyte or additives, and by changing the EOF. In protein and peptide separation,
254 changing the pH of the buffer from above to below the isoelectric point (pI) changes the
255 net charge of the solute from negative to positive. An increase in the buffer pH generally
256 increases the EOF.
257
258 *Organic solvents*. Organic modifiers (methanol, acetonitrile, etc.) may be added to the
259 aqueous buffer to increase the solubility of the solute or other additives and/or to affect the
260 degree of ionisation of the sample components. The addition of these organic modifiers to
261 the buffer generally causes a decrease in the EOF.
262
263 *Additives for chiral separations*. For the separation of enantiomers, a chiral selector is
264 added to the separation buffer. The most commonly used chiral selectors are
265 cyclodextrins, but crown ethers, polysaccharides and proteins may also be used. Since
266 chiral recognition is governed by the different interactions between the chiral selector and
267 each of the enantiomers, the resolution achieved for the chiral compounds depends
268 largely on the type of chiral selector used. Other factors controlling the resolution in chiral
269 separations are concentration of chiral selector, composition and pH of the buffer and
270 temperature. The use of organic additives, such as methanol or urea can also modify the
271 resolution achieved. For the development of a given separation it may be useful to test
272 cyclodextrins having a different cavity size (α-, β-, or γ-cyclodextrin) or modified
273 cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionic/ionisable
274 (aminomethyl, carboxymethyl, sulfobutyl ether, etc.) groups. When using modified
275 cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins
276 need to be taken into account since it will influence the selectivity.
277
278
279 CAPILLARY GEL ELECTROPHORESIS

PRINCIPLE

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve, under conditions where the EOF is suppressed. Molecules with similar charge-to-size ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, DNA fragments and SDS-treated proteins), which often have similar charge-to-size ratios, can thus be separated according to their molecular hydrodynamic size by capillary gel electrophoresis.

CHARACTERISTICS OF GELS

Two types of gel are used in capillary electrophoresis: cross-linked gels and entangled linear polymer solutions.

Cross-linked gels are prepared inside the capillary by polymerisation of the monomers. They are usually bonded to the fused silica wall and cannot be removed without destroying the capillary. If the gels are used for protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate and the samples are denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When non-reducing conditions are used (for example analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Separation in cross-linked gels can be optimised by modifying the separation buffer (as indicated in the capillary zone electrophoresis section) and controlling the gel pore size during the gel preparation. For cross-linked polyacrylamide gels, the pore size can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the pore size of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of these gels, only electrokinetic injection can be used.

Entangled linear polymer solutions contain hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a capillary. Replacing the gel before every injection generally improves the separation reproducibility. The dynamic pore size of the gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A reduction in the gel dynamic pore size leads to a decrease in the mobility of the solute for the same buffer. Since the dissolution of these polymers in the buffer gives low viscosity solutions, both hydrodynamic and electrokinetic injection techniques can be used.

CAPILLARY ISOELECTRIC FOCUSING

327
328 **PRINCIPLE**
329
330 In isoelectric focusing, the molecules migrate under the influence of the electric field until
331 they reach their isoelectric point, in a pH gradient generated by ampholytes having pI
332 values in a wide range (poly-aminocarboxylic acids), dissolved in the separation buffer.
333
334 The three basic steps of isoelectric focusing are loading, focusing and, if needed,
335 mobilisation.
336
337 **Loading step**. Two methods may be employed:
338
339 — loading in one step: the sample is mixed with ampholytes and introduced into the
340 capillary either by pressure or vacuum;
341 — sequential loading: a leading buffer, then the ampholytes, then the sample mixed with
342 ampholytes, again ampholytes alone and finally the terminating buffer are introduced into
343 the capillary. The volume of the sample is kept small enough not to modify the pH
344 gradient.
345
346 **Focusing step**. When the voltage is applied, ampholytes migrate toward the cathode or
347 the anode, according to their net charge, thus creating a pH gradient from anode (lower
348 pH) to cathode (higher pH). During this step the components to be separated migrate until
349 they reach a pH corresponding to their isoelectric point (pI) and the current drops to very
350 low values.
351
352 **Mobilisation step**. Unless imaging is used, mobilisation is required for detection, using
353 one of the following methods.
354 Three methods are available:
355
356 — in the first method, mobilisation is accomplished during the focusing step under the
357 effect of the EOF; the EOF needs to be small enough to allow the focusing of the
358 components ;
359 — in the second method, mobilisation is accomplished by applying positive pressure after
360 the focusing step;
361 — in the third method, mobilisation is achieved after the focusing step by adding salts to
362 the cathode reservoir or the anode reservoir (depending on the direction chosen for
363 mobilisation) in order to alter the pH in the capillary when the voltage is applied. As the pH
364 changes, the proteins and ampholytes are mobilised in the direction of the reservoir which
365 contains the added salts and pass the detector.
366
367 The separation achieved, expressed as ΔpI, depends on the pH gradient ($\mathrm{d}pH / \mathrm{d}x$), the
368 number of ampholytes having different pI values, the molecular diffusion coefficient (D),
369 the intensity of the electric field (E) and the variation of the electrophoretic mobility of the
370 analyte with the pH ($-\mathrm{d}\mu / \mathrm{d}pH$):
371
372

$$\Delta pI = 3 \times \sqrt{((D (\mathrm{d}pH / \mathrm{d}x)) / (E (-\mathrm{d}\mu / \mathrm{d}pH)))}$$

373 **OPTIMISATION**
374
375 The main factors to be considered in the development of separations are:
376
377 **Voltage**. Capillary isoelectric focusing uses very high electric fields, 300 V/cm to 1000
378 V/cm in the focusing step.
379
380 **Capillary**. The EOF needs to be reduced or suppressed depending on the mobilisation
381 strategy (see above). Coated capillaries tend to reduce the EOF.
382
383 **Solutions**. The anode buffer reservoir is filled with a solution with a pH lower than the pI of
384 the most acidic ampholyte and the cathode reservoir is filled with a solution with a pH
385 higher than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium
386 hydroxide for the cathode are frequently used.
387
388 Addition of a polymer, such as methylcellulose, in the ampholyte solution tends to
389 suppress convective forces (if any) and EOF by increasing the viscosity. Commercial
390 ampholytes are available covering many pH ranges and may be mixed if necessary to
391 obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point
392 whereas narrower ranges are employed to improve accuracy. Calibration can be done by
393 correlating migration time with isoelectric point for a series of protein markers.
394
395 During the focusing step precipitation of proteins at their isoelectric point can be prevented,
396 if necessary, using buffer additives such as glycerol, surfactants, urea or zwitterionic
397 buffers. However, depending on the concentration, urea denatures proteins.
398
399 MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)
400
401 **PRINCIPLE**
402
403 In micellar electrokinetic chromatography, separation takes place in an electrolyte solution
404 which contains a surfactant at a concentration above the critical micellar concentration
405 (*cmc*). The solute molecules are distributed between the aqueous buffer and the
406 pseudostationary phase composed of micelles, according to the partition coefficient of the
407 solute. The technique can therefore be considered as a hybrid of electrophoresis and
408 chromatography. It is a technique that can be used for the separation of both neutral and
409 charged solutes, maintaining the efficiency, speed and instrumental suitability of capillary
410 electrophoresis. One of the most widely used surfactants in MEKC is the anionic
411 surfactant sodium dodecyl sulfate, although other surfactants, for example cationic
412 surfactants such as cetyltrimethylammonium salts, are also used.
413
414 The separation mechanism is as follows. At neutral and alkaline pH, a strong EOF is
415 generated and moves the separation buffer ions in the direction of the cathode. If sodium
416 dodecyl sulfate is employed as the surfactant, the electrophoretic migration of the anionic
417 micelle is in the opposite direction, towards the anode. As a result, the overall micelle
418 migration velocity is slowed down compared to the bulk flow of the electrolyte solution. In
419 the case of neutral solutes, since the analyte can partition between the micelle and the

420 aqueous buffer, and has no electrophoretic mobility, the analyte migration velocity will
421 depend only on the partition coefficient between the micelle and the aqueous buffer. In the
422 electropherogram, the peaks corresponding to each uncharged solute are always
423 between that of the EOF marker and that of the micelle (the time elapsed between these
424 two peaks is called the separation window). For electrically charged solutes, the migration
425 velocity depends on both the partition coefficient of the solute between the micelle and the
426 aqueous buffer, and on the electrophoretic mobility of the solute in the absence of micelle.
427
428 Since the separation mechanism in MEKC of neutral and weakly ionised solutes is
429 essentially chromatographic, migration of the solute and resolution can be rationalised in
430 terms of the retention factor of the solute (k), also referred to as mass distribution ratio
431 (Dm), which is the ratio of the number of moles of solute in the micelle to those in the
432 mobile phase. For a neutral compound, k is given by:
433
434 $$k = (t - t_0) / (t_0 \times (1 - (t / t_{mc}))) = K (V_S / V_M)$$
435
436 t = migration time of the solute,
437 t_0 = analysis time of an unretained solute (determined by injecting an EOF marker which
438 does not enter the micelle, for instance methanol),
439 t_{mc} = micelle migration time (measured by injecting a micelle marker, such as Sudan III,
440 which migrates while continuously associated in the micelle),
441 K = partition coefficient of the solute,
442 V_S = volume of the micellar phase,
443 V_M = volume of the mobile phase.
444
445 Likewise, the resolution between two closely-migrating solutes (Rs) is given by:
446
447 $$R_S = (\sqrt{N} / 4) \times ((\alpha-1) / \alpha) \times (k_b / (k_b + 1)) \times ((1-(t_0 / t_{mc})) / (1+(t_0 / t_{mc}) \times k_a))$$
448
449 N = number of theoretical plates for one of the solutes,
450 α = selectivity,
451 k_a and k_b = retention factors for both solutes, respectively ($k_b > k_a$).
452
453 Similar, but not identical, equations give k and Rs values for electrically charged solutes.
454
455
456 **OPTIMISATION**
457
458 The main factors to be considered in the development of separations by MEKC are
459 instrumental and electrolyte solution parameters.
460
461 **Instrumental parameters**
462
463 *Voltage*. Separation time is inversely proportional to applied voltage. However, an
464 increase in voltage can cause excessive heat production that gives rise to temperature
465 gradients and viscosity gradients of the buffer in the cross-section of the capillary. This

466 effect can be significant with high conductivity buffers such as those containing micelles.
467 Poor heat dissipation causes band broadening and decreases resolution.
468
469 *Temperature*. Variations in capillary temperature affect the partition coefficient of the
470 solute between the buffer and the micelles, the critical micellar concentration and the
471 viscosity of the buffer. These parameters contribute to the migration time of the solutes.
472 The use of a good cooling system improves the reproducibility of the migration time for the
473 solutes.
474
475 *Capillary*. As in capillary zone electrophoresis, the dimensions of the capillary (length and
476 internal diameter) contribute to analysis time and efficiency of separations.
477 Increasing both effective length and total length decreases the electric field (working at
478 constant voltage), increases migration time and can improve the separation efficiency.
479 The internal diameter controls heat dissipation (for a given buffer and electric field) and
480 consequently the sample band broadening.
481
482 **Electrolyte solution parameters**
483
484 *Surfactant type and concentration*. The type of surfactant, in the same way as the
485 stationary phase in chromatography, affects the resolution since it modifies separation
486 selectivity. Also, the log k of a neutral compound increases linearly with the concentration
487 of surfactant in the mobile phase. Since resolution in MEKC reaches a maximum when k
488 approaches the value of $\sqrt{(t_{mc} / t_0)}$, modifying the concentration of surfactant in the mobile
489 phase changes the resolution obtained.
490
491 *Buffer pH*. Although pH does not modify the partition coefficient of non-ionised solutes, it
492 can modify the EOF in uncoated capillaries. A decrease in the buffer pH decreases the
493 EOF and therefore increases the resolution of the neutral solutes in MEKC, resulting in a
494 longer analysis time.
495
496 *Organic solvents*. To improve MEKC separation of hydrophobic compounds, organic
497 modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolyte solution.
498 The addition of these modifiers can influence the migration time and the selectivity of the
499 separation. Since the addition of organic modifiers affects the critical micellar
500 concentration, a given surfactant concentration can be used only within a certain
501 percentage of organic modifier before the micellisation is inhibited or adversely affected,
502 resulting in the absence of micelles and, therefore, in the absence of partition. The
503 dissociation of micelles in the presence of a high content of organic solvent does not
504 always mean that the separation will no longer be possible; in some cases the
505 hydrophobic interaction between the ionic surfactant monomer and the neutral solutes
506 forms solvophobic complexes that can be separated electrophoretically.
507
508 *Additives for chiral separations*. For the separation of enantiomers using MEKC, a chiral
509 selector is included in the micellar system, either covalently bound to the surfactant or
510 added to the micellar separation electrolyte. Micelles that have a moiety with chiral
511 discrimination properties include salts of *N*-dodecanoyl-L-amino acids, bile salts, etc.
512 Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins,

513 added to the electrolyte solutions which contain micellised achiral surfactants.
514
515 *Other additives*. Several strategies can be carried out to modify selectivity, by adding
516 chemicals to the buffer. The addition of several types of cyclodextrins to the buffer can
517 also be used to reduce the interaction of hydrophobic solutes with the micelle, thus
518 increasing the selectivity for this type of compound.
519
520 The addition of substances able to modify solute-micelle interactions by adsorption on the
521 latter, is used to improve the selectivity of the separations in MEKC. These additives may
522 be a second surfactant (ionic or non-ionic) which gives rise to mixed micelles or metallic
523 cations which dissolve in the micelle and form co-ordination complexes with the solutes.
524
525 QUANTITATION
526
527 With the exception of capillary isoelectric focusing, peak areas are typically divided by the
528 corresponding migration time to give the corrected area in order to:
529
530 — compensate for the shift in migration time from run to run, thus reducing the variation of
531 the response,
532 — compensate for the different responses of sample constituents with different migration
533 times.
534
535 Where an internal standard is used, verify that no peak of the substance to be examined is
536 masked by that of the internal standard.
537
538 **CALCULATIONS**
539
540 From the values obtained, calculate the content of the component or components being
541 examined. When prescribed, the percentage content of one or more components of the
542 sample to be examined is calculated by determining the corrected area(s) of the peak(s)
543 as a percentage of the total of the corrected areas of all peaks, excluding those due to
544 solvents or any added reagents (normalisation procedure). The use of an automatic
545 integration system (integrator or data acquisition and processing system) is
546 recommended.
547
548
549 SYSTEM SUITABILITY
550
551 In order to check the behaviour of the capillary electrophoresis system, system suitability
552 parameters are used. The choice of these parameters depends on the mode of capillary
553 electrophoresis used. They can include: retention factor (k) (only for micellar electrokinetic
554 chromatography), apparent number of theoretical plates (N), symmetry factor (A_s) and
555 resolution (R_s). In previous sections, the theoretical expressions for N and R_s have been
556 described, but more practical equations that allow these parameters to be calculated from
557 the electropherograms are given below.
558
559 **APPARENT NUMBER OF THEORETICAL PLATES**

560
561 The apparent number of theoretical plates (N) may be calculated using the expression:
562
563 $$N = 5{,}54\ (t\ /\ \omega_h)^2$$
564
565 t = migration time or distance along the baseline from the point of injection to the
566 perpendicular dropped from the maximum of the peak corresponding to the component,
567 ω_h = width of the peak at half-height.
568
569 **RESOLUTION**
570
571 The resolution (Rs) between peaks of similar height of two components may be calculated
572 using the expression:
573
574 $$R_S = ((1{,}18\ (t_2 - t_1))\ /\ (\omega_{h1} + \omega_{h2}))$$
575
576 $$t_2 > t_1$$
577
578 t_1 and t_2 = migration times or distances along the baseline from the point of injection to the
579 perpendiculars dropped from the maxima of two adjacent peaks,
580 ω_{h1} and ω_{h2} = peak widths at half-height.
581
582 When appropriate, the resolution may be calculated by measuring the height of the valley
583 (Hv) between two partly resolved peaks in a standard preparation and the height of the
584 smaller peak (Hp) and calculating the peak-to-valley ratio:
585
586 $$p\ /\ \upsilon = H_p\ /\ H_\upsilon$$
587
588 **SYMMETRY FACTOR**
589
590 The symmetry factor (A_s) of a peak may be calculated using the expression:
591
592 $$A_s = \omega_{0,05}\ /\ 2d$$
593
594 $\omega_{0,05}$ = width of the peak at one-twentieth of the peak height,
595 d = distance between the perpendicular dropped from the peak maximum and the leading
596 edge of the peak at one-twentieth of the peak height.
597
598 **REPEATABILITY**
599
600 Tests for area repeatability (standard deviation of areas or of the area/migration-time
601 ratio) and for migration time repeatability (standard deviation of migration time) are
602 introduced as suitability parameters. Migration time repeatability provides a test for the
603 suitability of the capillary washing procedures. An alternative practice to avoid the lack of
604 repeatability of the migration time is to use migration time relative to an internal standard.
605

606
607
608 **SIGNAL-TO-NOISE RATIO**
609
610 The detection limit and quantitation limit can be estimated using signal-to-noise ratios of
611 3 and 10 respectively. The signal-to-noise ratio (*S*/*N*) is calculated using the expression:
612
613 $$S / N = 2H / h$$
614
615 H = height of the peak corresponding to the component concerned, in the
616 electropherogram obtained with the prescribed reference solution, measured from the
617 maximum of the peak to the extrapolated baseline of the signal observed over a distance
618 equal to twenty times the width at half-height,
619 h = range of the background in an electropherogram obtained after injection of a blank,
620 observed over a distance equal to twenty times the width at the half-height of the peak in
621 the electropherogram obtained with the prescribed reference solution and, if possible,
622 situated equally around the place where this peak would be found.
623
624 A test for the verification of the signal-to-noise ratio for a standard preparation (or the
625 determination of the limit of quantitation) may also be useful for the determination of
626 related substances.
627
628
629
630 ADJUSTMENTS OF OPERATING CONDITIONS FOR CAPILLARY
631 ELECTROPHORESIS
632
633 The operating conditions described in the pharmacopoeial procedures were validated
634 during the elaboration of the monograph. Compliance with the system suitability criteria is
635 required to verify that conditions for satisfactory performance of the test or assay are
636 achieved.
637 Capillary length may be adjusted to suit the individual dimensions of the capillary
638 electrophoresis instrument used. If a capillary with a different length is used, the suitability
639 of use should be verified.
640 If the intended analytical performance is not met either due to the instrument or to different
641 capillary lengths, then:
642 - the voltage,
643 - rinsing conditions,
644 - temperature settings,
645 - refreshment frequency of the electrolyte solution at the inlet and outlet,
646 - and injection conditions
647 may be adjusted to satisfy the analytical performance, provided these adjustments are
648 properly evaluated by the users and do not modify fundamentally the pharmacopoeial
649 procedures. Additional verification tests and/or revalidation for any adjustments made
650 may be required.
651 No further changes are authorised, unless otherwise stated in the individual monograph.