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Review

On-line multidimensional liquid chromatography and capillary electrophoresis systems for peptides and proteins

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Abstract

Peptides and proteins are gaining increasing attention in biosciences and, consequently, in analysis. This overview highlights the different approaches to couple on-line various separation techniques for the determination of proteins and peptides. The first section discusses the liquid chromatography (LC)–LC coupling, the second one reviews the on-line LC–capillary electrophoresis (CE) coupled systems and the third section summarizes the strategies for on-line CE–CE. The advantages, disadvantages, most relevant difficulties and particular systems for on-line coupling are discussed. Special attention is paid to the interface between the two dimensions. Applications are summarized in tables and a few typical examples are discussed. Many multidimensional separation methods are available, and it is demonstrated that peptide and protein mapping, or quantitation of proteins or peptides in various samples (aqueous solutions, cells, plasma) require different coupled systems. For mapping a semi-quantitative detection is often sufficient, while comprehensiveness is very important. For quantitation of a certain peptide or protein at a low concentration level a validated method should be used, while a heart-cut transport of the first dimension to the second one can offer sufficient selectivity. The combination with mass spectrometry as part of the total system is stressed and illustrated.

Keywords: Capillary electrophoresis; Comprehensive; Heart-cut; Liquid chromatography; Multidimensional separations; On-line coupling; Peptides; Proteins

Contents

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1. Introduction

For the bioanalysis of peptides and proteins in biological samples, one-dimensional separation methods often do not have sufficient selectivity. In these cases, multidimensional methods may offer higher selectivity [1–7]. An increase in selectivity of a multidimensional system can only be obtained when the dimensions are based on different separation mechanisms. The second dimension of a multidimensional system should not destroy the resolution achieved by the previous one [4]. Adequate interfacing is needed to transport the various fractions from the first to the second dimension.

The interfacing for multidimensional column separation systems can be performed in various ways. The effluent of the first separation system can be transported to the next system manually (off-line) or automatically, e.g. with the aid of a robot (at-line), or via connecting tubing and/or a valve that directly transports a stream of liquid to the next system (on-line). In general, the on-line combination of separation systems enables a significantly faster analysis of a complex matrix in comparison with an off-line or at-line combination.

As an on-line interface in a coupled system, a loop or a column is generally used to trap a fraction of interest, where a switching valve is employed to inject this fraction into the next separation step [8]. If the separation of the complete sample in all dimensions is achieved, the system is considered to be completely multidimensional or comprehensive [9]. In this case the time required for performing a separation in the second dimension must be the same as for e.g. filling a loop with effluent from the first dimension. Such a comprehensive on-line system can only be designed by making adjustments in the analytical dimensions (flow rates, column dimensions) of the system components, because the second dimension separations have to be performed much faster. An important reason for the development of comprehensive multidimensional separation methods is the challenge to establish protein profiles of cells and other biological samples. These profiles are very important for research in the field of proteomics. It should be noted that in proteomic research multidimensional column systems are mainly used for the separation of peptide mixtures after digestion of proteins. Recently, Wang and Hanash [10] published a review on multidimensional LC-based separations in proteomics, including off-line techniques as well.

If a coupled system separates only a fraction, retrieved from the first dimension, in the second dimension it is called a heart-cut system [8]. These systems are not comprehensive; however such separations are important for the determination of structurally closely related peptides and proteins in biological samples such as plasma and tissues, which require analytical methods with high selectivity and sensitivity. Multidimensional heart-cut systems often offer sufficient selectivity and sensitivity for the assay of these compounds in a relatively short time [11–17].

Two-dimensional gel electrophoresis is widely used for protein profiling [18–21]. It usually uses isoelectric focusing (IEF) followed by sodium dodecyl sulfate polyacrylamide gel sieving electrophoresis (2D IEF-SDSPAGE), which two are orthogonal separation modes. For this reason the system provides high resolution power for the separation of complex samples containing proteins [3,4]. However, 2D gel electrophoresis has some limitations: extensive sample handling, time consuming, difficult to automate, decreased resolving power for proteins with a molecular mass of <15 kDa as a result of their high mobility in the gel and the gel cannot be coupled on-line to a mass spectrometer (MS). Moreover, quantitation in 2D gel electrophoresis is also a problem. After staining the spots can be detected, but a direct (semi)quantitative detection method is not available [18–21]. When using an off-line multidimensional column system, direct quantitative detection with UV absorbance, laser-induced fluorescence (LIF) or MS detection is possible. Therefore, off-line multidimensional LC systems have often been applied for profiling in proteomics research [22–25]. If the multidimensional column system is on-line coupled there are additional advantages: limited loss of analyte due to adsorption to the walls of vials or pipettes, which often occurs in analysis of peptides or proteins in the pmol range [26]. Moreover, increased reproducibility of the method can be achieved due to the lack of manual sample treatment necessary for the transfer of the analytes from the first to the second dimension [8]. On-line coupling is also easy to automate [8] and operator involvement is reduced [26]. Disadvantages of on-line coupling are the increased complexity of the system which may result in a higher risk of peak broadening due to dead volumes [8], and the necessity for relatively long retention or migration times in the first dimension to enable the (comprehensive) coupling [4].

The present review focuses on the development of various on-line multidimensional separation systems for the determination of peptides and proteins. The use of on-line solid-phase extraction (SPE) for the preconcentration and sample clean-up prior to a LC separation might be advantageous in peptide analysis [27,28], however these systems are not included in this review. SPE is only mentioned in this review if it is applied as an interface between two separation systems. A review about preconcentration techniques coupled on-line and in-line to CE was published earlier [29] and will

not be discussed in this review either. The majority of the multidimensional systems for proteins and peptides has been applied in the field of proteomics for profiling including MS detection. Some systems have been applied for quantitation of some peptides or proteins in biological samples. This review will discuss the differences between these two types of approaches. Emphasis will be placed on the design of the systems, and their applicability will be demonstrated with some typical examples. A coupled system can be composed of various separation techniques. Especially LC and CE are widely used. Gas chromatographic methods are not discussed in this paper because of the decomposition of peptides and proteins at high temperatures. As some recent reviews on 2D-LC systems for proteins and peptides are available [10,30,31], in this review special attention will be paid to CE-containing systems. The first section will discuss on-line LC–LC, the second one will overview on-line LC–CE and the third section will summarize on-line CE–CE. The role of MS detection for an increase of selectivity and for providing structure information will also be shown.

2. LC–LC systems

2.1. General aspects

Because LC can be employed in various separation modes, for example normal-phase (NP), reversed-phase (RPLC), size exclusion (SEC), ion exchange (IEX) or affinity chromatography, it is a very powerful tool and offers many possibilities for multidimensional systems. The difference in operation between an on-line coupled column system and a conventional one-dimensional chromatographic system is the direct transfer of analytes from the first column to the second column. This transfer may be the source of three types of problems:

(1) Solvent incompatibility between the mobile phases of the first and the second system.
(2) Excessive band broadening between columns during passage of valves, loops or detector.
(3) The need of a much faster separation in the second dimension compared to the first separation, if a comprehensive system is chosen.

According to (1), the transfer solvent from the first column must allow the focusing of the analytes on the second column and must be miscible with the solvent used in the second dimension. According to (2), the second column preferably must have focusing. This is the reason that, for instance, a SEC-RPLC coupling is very popular. The mobile phases are miscible and the RPLC allows preconcentration at the top of the column of the aqueous SEC fractions. Coupling of LC with CE is complicated because there is generally a large difference in peak volume of the LC and the injection volume of the CE. However, according to (3), the comprehensive mode of a LC–LC system often is more difficult to develop compared to a LC–CE or CE–CE system, because, in general, CE can be performed more easily in a fast mode.

The interfacing in multidimensional LC systems can be divided into two classes: trapping via a loop or direct introduction on the top of the column of the second dimension, both in combination with one or more valves. Trapping in a loop is always possible, while trapping on the column has limitations. Trapping with an additional trapping column between the two separation columns is also possible. The possibility of trapping on a column depends on the choice of the second retention mechanism and the mobile phase of the first column. A loop has the disadvantage of a possible additional peak broadening.

Most separation modes can easily be combined if their mobile phases are compatible. However, combination is not always possible. The interfacing of NP and RP systems is particularly difficult, due to mobile phase immiscibilities. To overcome this problem, Sonnefeld et al. [32] used a system in which the fraction of interest was transferred from the first (NP) column to a packed precolumn and the NP eluent was removed by passage of an inert gas and vacuum. Once the solvent was removed, the analytes were desorbed from the precolumn using a RP eluent and transferred to the second (RP) column. Another method to overcome solvent immiscibilities has been developed by Takeuchi et al. [33]. They used a micro column (0.35 mm i.d.) in the first dimension and a conventionally sized column (4.6 mm i.d.) in the second dimension. Due to the reduced peak volume generated by the use of the micro column, solvent removal was not necessary. The resulting 2D separation system was evaluated by the separation of several aromatic hydrocarbons in a fuel for body warmers. Consequently, the use of micro columns in multidimensional separations has allowed coupling of seemingly incompatible separation modes [32,33]. Micro columns are also better suited than conventionally sized columns for analysis of small sample volumes. Other advantages of miniaturization of a LC system are the low consumption of mobile phases, increased mass sensitivity, higher separation efficiency and the more efficient combination with a MS detector.

A summary of 2D-LC systems is shown in Table 1. Some aspects are important: the target proteins and/or peptides, the type of first and second dimension, the type of interface (valves with a loop or valves with a column), the type of detection and whether it is qualitative or (semi-) quantitative analysis. These aspects and some particular remarks are included in Table 1.

2.2. Typical examples of 2D-LC systems

A particular example of the analytical power of a 2D system for intact proteins is the coupling of IEC with RPLC, developed by Opiteck et al. [34] for the separation of proteins in an *Escherichia coli* cell lysate. This is the first comprehensive LC–LC system utilizing on-line mass spectrometry for detection, which adds, in essence, a third dimension to this 2D system, because the mass spectrometer can identify the presence

Table 1
An overview of 2D-LC systems for proteins and peptides

Compounds	First dimension	Second dimension	Interface	Detection	Remarks	Reference
Standard serum proteins	IEC	SEC	Eight-port valve with two loops	UV	Comprehensive; semi-quantitative; time of analysis: 3 h	[9]
Small peptides from porcine adrenal gland	IEC	RPLC	Two valves with trapping on RPLC	LIF	Comprehensive; semi-quantitative; time of analysis: 32 h; number of spots: 150 effluent from the IEC is concentrated, quantity of a single resolved peptide is around 16 pmol	[83]
Proteins from *E. coli* lysate	IEC	RPLC	Eight-port valve	UV and on-line MS (electro spray)	Comprehensive; semi-quantitative; total peak capacity of over 2500; sensitivity of around 3 pmol	[24]
Digest from human lung fibroblasts	IEC	RPLC	Two six-port valves with trapping on RPLC	On-line MS	Comprehensive; semi-quantitative	[84]
Proteins from *E. coli* lysate	IEC	RPLC	Eight-port valve with trapping on RPLC	Off-line MS	Comprehensive; semi-quantitative	[85–87]
Digested proteins from *S. cerevisiae*	IEC	RPLC	MudPIT technology	On-line MS	Comprehensive; semi-quantitative; time of analysis: 24 h; number of spots: 23,000 (theoretically); the IEC/RPLC combination is not multidimensional, the MS addition is	[39]
In vivo derived amyloid polypeptides	SEC	RPLC	Six-port valve with trapping on RPLC	On-line MS	Heart-cut; semi-quantitative with known recoveries; time of analysis: 30 min	[13]
Peptides from ovalbumine and serum albumin	SEC	RPLC	Four-port valve with two trapping columns	On-line MS	Comprehensive; semi-quantitative; time of analysis: 2.5 h; number of spots: 50	[26]
Tryptic digest of bovine serum albumin	SEC	RPLC	Two four-port valves with two trapping columns	On-line MS	Comprehensive; semi-quantitative; time of analysis: 5 h; number of spots: 520	[88]
Enkephalins in cerebrospinal fluid	SEC	RPLC	Two six-port valves with two loops	UV	Heart-cut; quantitative; time of analysis: 30 min; CLOQ: 2 μg/mL	[14,15]
Tyrosine-kinase, β-lactamase	SEC	RPLC	Two four-port valves with two trapping columns	UV, off line MS	Comprehensive; semi-quantitative; time of analysis: 2.5 h; number of spots: 800 (theoretically)	[89]
Enkephalins in plasma	SEC, IEC (strong cation exchange) and RP-SPE as sample clean-up	RPLC	Three six-port valves	UV, on-line MS	Heart-cut; quantitative; time of analysis: <1 h; LOD: 200 nmol/L (UV), 10 nmol/L (MS)	[16]
Heparine	SEC	IEC	Six-port valve with a loop	Refraction, conductivity	Heart-cut; quantitative; time of analysis: 1 h LOD < 100 ng	[17]
Model protein mixtures, digested and labeled	IEC (strong cation exchange)	RPLC	10-port valve	MS; acid-labile isotope-coded extractants (ALICE)	Comprehensive; quantitative	[40]
Human lung fibroblasts	IEC	RPLC	Four 10-port valve with one loop and four trapping columns	UV with off-line MS	Comprehensive; semi-quantitative; time of analysis: 96 min; number of spots: 1000	[37]
Digested milk proteins	Affinity chromatography	RPLC	Three 10-port valves with sample loops	On-line MS	Comprehensive; semi-quantitative	[90]

of coeluting peaks when they are not resolved by chromatography. The two LC systems are coupled by an eight-port valve equipped with two storage loops which is under computer control. The RPLC column samples the first IEC dimension two or three times per peak. The RPLC effluent is sampled by both a UV detector and an electrospray mass spectrometer. In this way, complex mixtures of large biomolecules can be rapidly separated, desalted, and analyzed for molecular weight in less than 2 h. The 2D chromatogram of *E. coli* lysate is shown in Fig. 1. This figure clearly demonstrates that the IEC dimension only (see the horizontal line at 37.5 min) does not have sufficient separation power. The extracted UV absorption data from RPLC and the corresponding total ion current traces from the MS at 37.5 min are presented in Fig. 2.

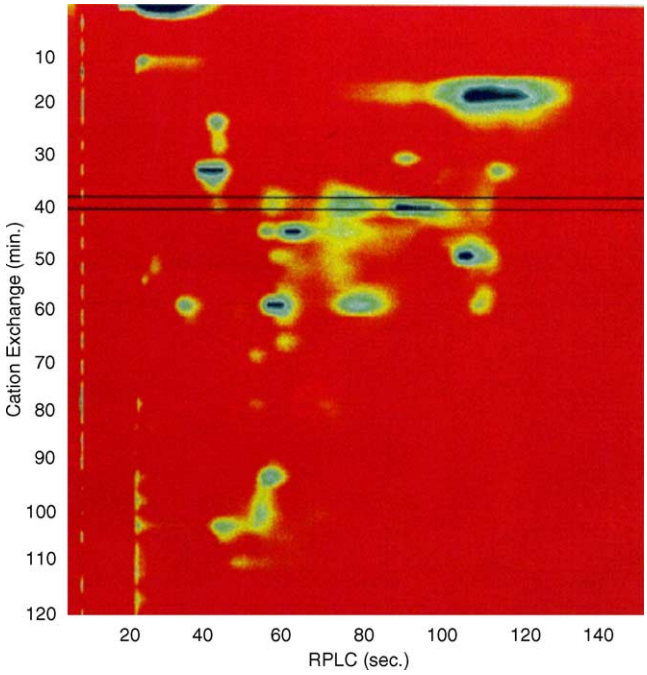

Fig. 1. The 2D chromatogram of *Escherichia coli* lysate [34].

This figure demonstrates that even the separation power of the 2D system is not sufficient. However, the MS provides additional separation power. The peak at 110 s in the RPLC run (Fig. 2a) has the mass spectrum shown in Fig. 3a, with its reconstructed molecular weight shown as 40,702 in Fig. 3b. A search of the Swiss-Prot database shows 38 proteins from *E. coli* within 2% of this molecular weight but only two proteins within 0.2%, GCPE protein (P27433) and hydrogenase-1 small-chain precursor (P19928). The total number of peaks counted in the chromatogram of Fig. 2 is roughly 2500. The mass spectrometer can be presented with as little as 3.2 pmol of analyte and still obtain an accurate molecular weight [34]. This example demonstrates the resolving power of the system and is a typical example of peptide mapping with a sensitive on-line MS detection. Most on-line MS detections are based on nanoelectrospray-MS/MS. MALDI-MS/MS is relatively sensitive and also important in the field of proteomics [35], but it can not be coupled on-line to LC. Using off-line MALDI-MS/MS or on-line nanoelectrospray-MS/MS a retrieved peptide mass fingerprint (PMF) is generally used to screen a protein sequence database. A match between the PMF and the patterns computed from the sequence database, employing rules for digest chemistry, are scored and used for protein identification. The success of such an identification depends on the size of the database and its error rate, the number of the matching peptides and their molecular weight (MW), the mass accuracy and the control over the digest chemistry [35].

A typical illustration of comprehensive LC–LC is the high resolution 2D-LC system based on high speed RP chromatography [36]. This system is developed for separations of peptides and proteins using non-porous 1.5 μm silica beads and conventional low-void-volume chromatographic equipment to prevent additional peak broadening. The 2D system consists of either an anion or a cation exchanger in the first dimension and two equivalent non-porous 14 mm × 4.6 mm

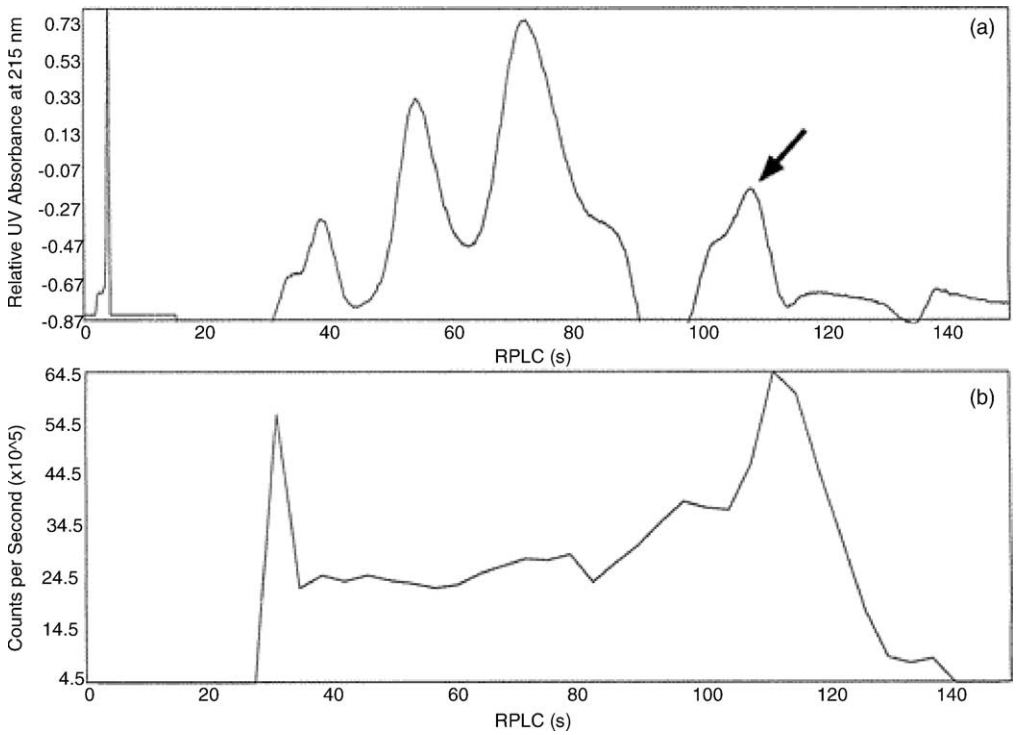

Fig. 2. (a) UV absorption data from RPLC chromatogram extracted from 37.5 min of Fig. 1. (b) Corresponding total ion current chromatogram. [34].

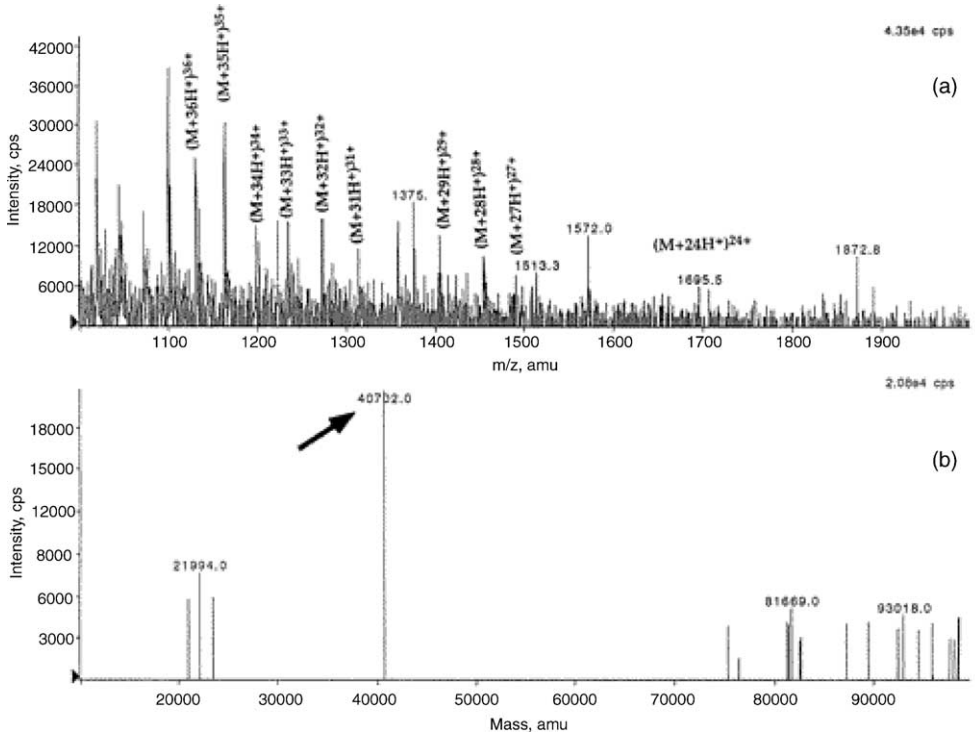

Fig. 3. (a) Mass spectrum from peak at 110 s of Fig. 2. (b) Corresponding Hypermass reconstruction of charge envelope [34].

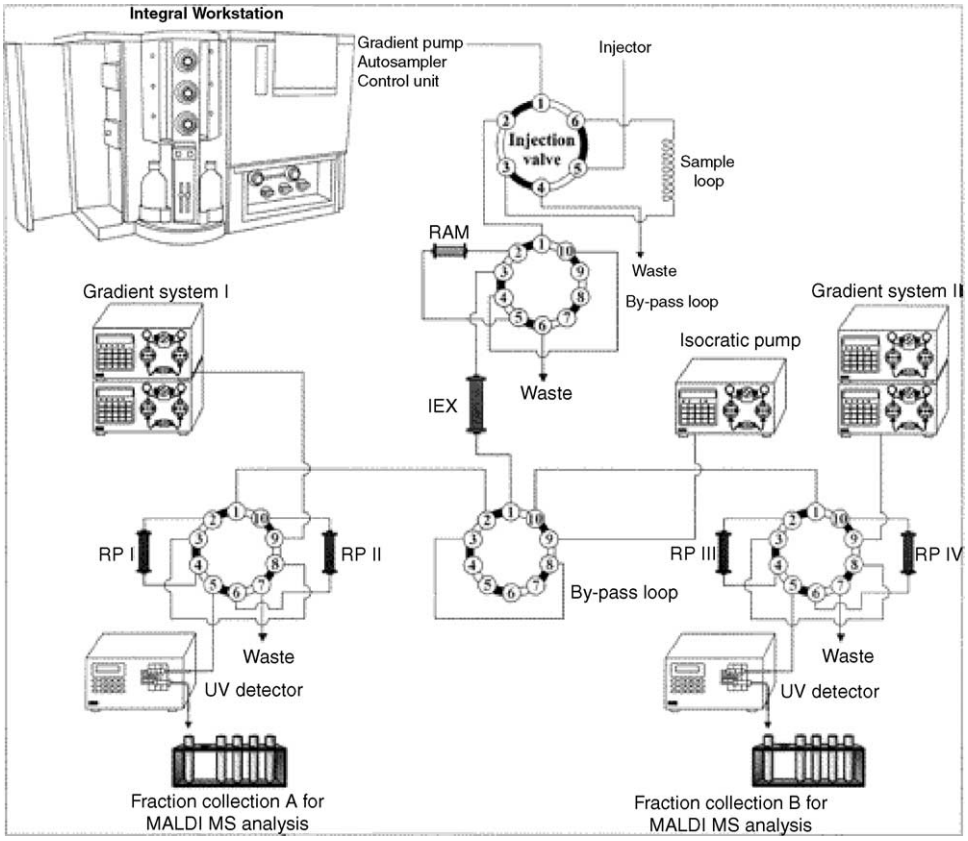

Fig. 4. Schematic representation of the on-line comprehensive 2D IEC-RPLC system, including an integrated sample preparation step [37]. The restricted access material is indicated with RAM.

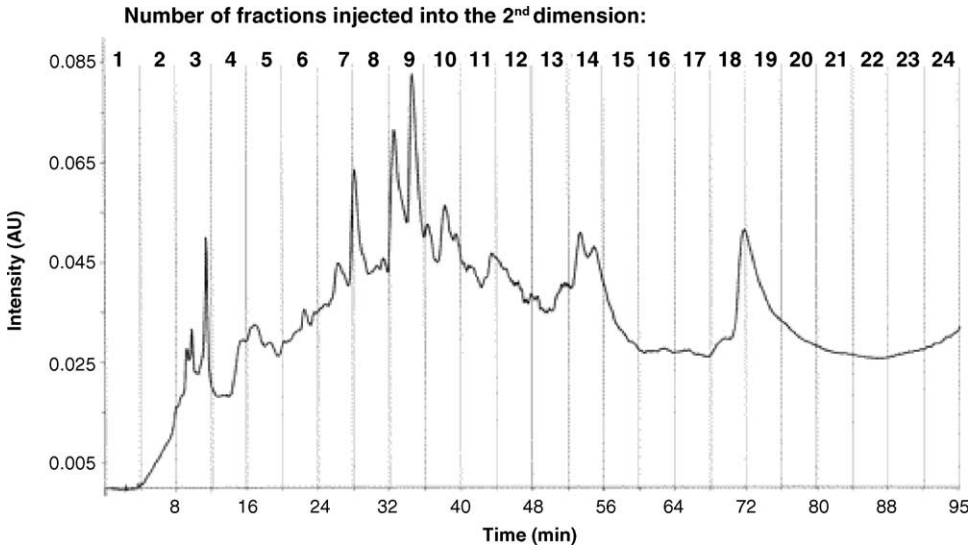

Fig. 5. The separation of human hemofiltrate on the analytical IEC column in the first dimension after first being subjected to selective enrichment on a RAM. Fractions (24 in total) were continuously transferred to the second dimension in 4 min intervals for subsequent analysis by reversed-phase chromatography [37].

i.d. RPLC columns in the second dimension which operate faster compared to other systems described in the literature [34,26]. While the first RP column is loaded with a fraction of the effluent from the ion exchanger, the analytes on the second column are eluted. Subsequently, after switching the valves, trapping is performed onto the second column while analytes are eluted from the first column. Consequently, the interface is the switching valve combined with trapping capabilities of the RP columns. An improvement of this fast 2D-LC system has recently been developed [37] for the analysis of peptides and small proteins. The system reproducibly resolves ∼1000 peaks within a total analysis time of 96 min. The design of the fully integrated platform for peptide and protein mapping is illustrated in Fig. 4. It consists of a sample fractionation column with silica based restricted access materials (RAM) based on the SEC principle coupled on-line with a comprehensive 2D-LC system. Peptides and small proteins are being enriched, whereas higher-molecular-weight matrix components can be flushed directly to waste. This size selective sample fractionation step is followed by anion or cation exchange chromatography as the first dimension. A new column switching technique, including four paral-

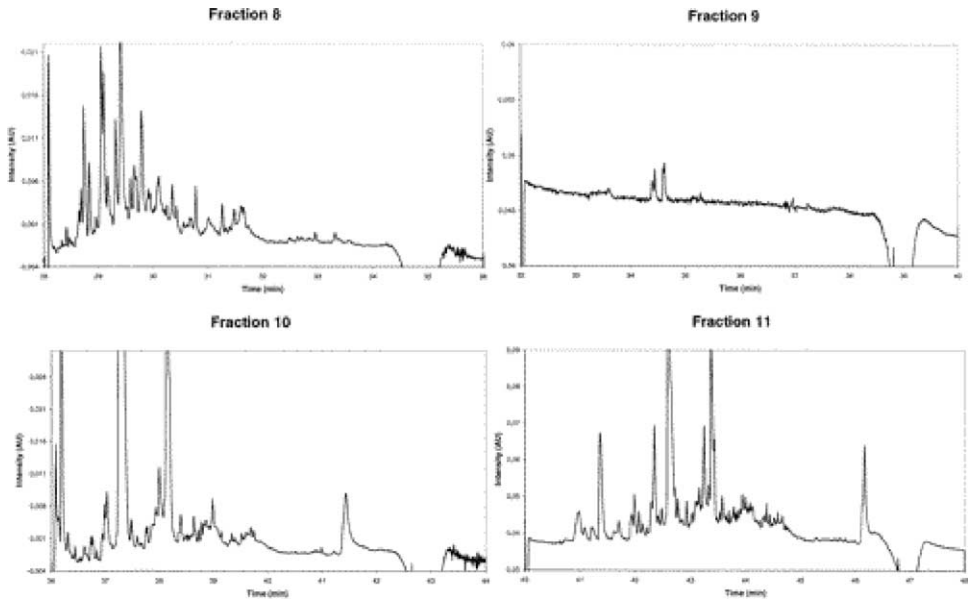

Fig. 6. Selected reversed-phase chromatograms corresponding to a complete two-dimensional RAM/IEC/reversed-phase run exemplifying the high resolving power within 8 min of analysis time [37]. The fraction number mentioned on the cation exchange chromatogram corresponds to the fraction indication on the reversed-phase chromatograms in Fig. 5.

lel RP columns, is employed in the second dimension for further separation. The system has been applied to protein mapping of human hemofiltrate as well as cell lysates originating from a human fetal fibroblast cell line. Fig. 5 shows the separation of human hemofiltrate on the analytical IEC column after first being subjected to selective enrichment on the RAM, while Fig. 6 shows a couple of reversed-phase chromatograms from selected fractions of the IEC, exemplifying the high resolving power within 8 min of analysis time. The 3D RAM/IEC/reversed-phase separation of human hemofiltrate is shown in Fig. 7. [37]. Selected peaks are collected and analyzed off-line by MALDI-TOF MS. Fig. 8 is an illustration of this application. It shows a MALDI-TOF spectrum corresponding to the peak (arrow) in the chromatogram, which originates from a human hemofiltrate sample. In this case, two masses were observed from the single UV peak [37]. On-line MS detection might be possible if other modes of MS detection, e.g. electrospray MS, are used.

The previously discussed separation systems are on-line. Vissers et al. [38] developed an automated at-line 2D capillary LC method which is based on micro fractionating, automated reinjection, and rechromatography using an automated micro column switching set-up to separate complex peptide mixtures from different origins. Although this procedure is not on-line, it is advantageous that the parts necessary for the sample transfer from the first dimension to the fraction collector are commercially available. The first-dimension separations were carried out by either RPLC or strong anion exchange chromatography. The second dimension separation was in all cases RPLC. Because of the large robustness of this system it can possibly be applied for the quantitation of peptides or proteins as well.

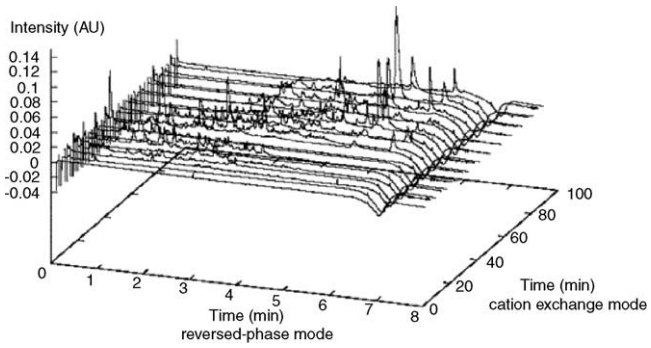

Fig. 7. The 3D RAM/IEC/reversed-phase separation of human hemofiltrate [37].

Another particular example is the powerful and well-known system described by Wolters et al. [39]. It is an automated method for so-called shotgun proteomics, named multidimensional protein identification technology (MudPIT). MudPIT works fast and is focused on the identification of proteins and peptides. The MudPIT-system itself integrates a strong cation exchange resin (SCX) and RP resin in one column. The RP material is positioned directly after the SCX material. Consequently, the combination of the SCX and the RP resin can be imagined as two different on-line coupled columns without an interface between them. After loading the peptides onto the SCX, the MudPIT experiment consists of a couple of identical cycles. Each cycle integrates several steps: equilibrating with buffer of a specific pH, elution of a peptide fraction from the SCX column onto the RP column using a salt gradient and subsequent elution with a RP gradient to a mass spectrometer. Depending on the pH, peptides with a particular p*I* elute off the SCX and are further sepa-

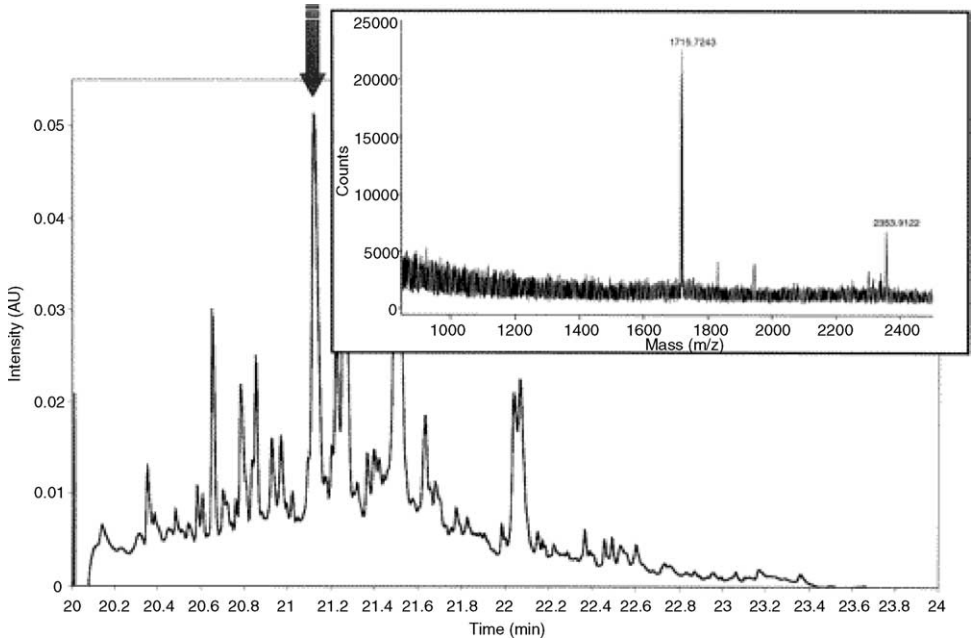

Fig. 8. Typical MALDI-TOF spectrum corresponding to the peak (arrow) in the chromatogram, which originates from human hemofiltrate sample. In this case, two masses were observed from the single UV peak [37].

rated on the RP material in relation to their hydrophobicities. Consequently, multiple 1D chromatograms are retrieved. The theoretical peak capacity of the total MudPIT system is ∼23,000 [39]. MudPIT is a powerful tool for proteomics. The system is capable of sensitive detection of peptides, due to the at-line concentration procedure prior to peptide loading onto the SCX and the MS detection, however it has not been developed for quantitative analysis. It can be questioned whether this integrated combination of SCX/RP is really multidimensional. Using the described set-up the whole effluent from the SCX dimension is directly transported to the RP dimension.

From Table 1 some conclusions can be drawn. The analytes of interest are often peptides retrieved from digested proteins. In case of the digested proteins, off-line sample handling is performed before injection onto the coupled system. This introduces at one hand the possibility of analyte concentration, at the other hand it is time consuming. Most of the systems use RPLC for the second dimension. This has two possible advantages: concentration on top of the RP column and the use of a loop with additional band-broadening is not necessary. In the systems which do not use RPLC in the second dimension, a loop is employed for interfacing. Another aspect is the low number of heart-cut systems, while all the described heart-cut systems have been validated for performing quantitation of proteins or peptides, contrarily to the comprehensive systems. Two examples of mapping systems are reported which have also been validated for quantitations of peptides [40,41].

3. LC–CE systems

3.1. General aspects

LC–CE coupling instead of LC–LC combination is generally more orthogonal. Also, the high separation speed of CE is advantageous, especially in the development of comprehensive multidimensional systems. In addition CE has a large separation efficiency, however, especially when trace-level concentrations have to be determined, the analyte detect ability in units of concentration is often insufficient.

Next to the interfacing problems discussed in the section on LC–LC, the on-line coupling of LC and CE may be the source of two additional problems: the large difference in peak volume of the LC and the injection volume of the CE and the way the electrode is used at the coupling side of the CE and the voltage is applied.

The transfer solvent from the first dimension preferably must allow stacking of the peptides or proteins in the CE capillary. Consequently, the ionic strength of the LC effluent has to be as low as possible. While most LC–CE systems are comprehensive because of the use of a slowly eluting LC system, fast CE (FCE) [42,43] can be necessary to enable comprehensiveness without this requirement for the LC system, although normal CE is often relatively fast in contrast to LC. It is important for the CE injection system to sample the first column as frequent as possible.

The differences in peak volumes of the LC (μL range) and the injection volume of the CE (nL range) are large in LC–CE coupling. Generally a T-piece can reduce these differences. However, in this case, only a part of the effluent from the LC will be injected into the CE. The major part is transported via the T-piece to waste. If this approach is chosen, focusing in the CE capillary is even more important to diminish loss in sensitivity. Another solution may be the use of a nano LC dimension with effluent peaks comparable to the injection volume of the subsequent CE dimension [44]. However, a disadvantage of using nano LC in the first dimension is the relatively small amount of sample that can be injected. Consequently, using nano LC, the sensitivity of the total system will not always be improved.

The stainless steel tubing of the LC dimension in most cases serves as the inlet electrode for the CE [9,11, 12, 37,43–46]. In this case, the inlet electrode of the CE is grounded, while the outlet electrode has a positive or a negative potential [9,11,12,37,43–46]. This is simple, however, it complicates the addition of on-line MS detection compared to a LC–LC system. However, in principle the positioning of a separate electrode is also possible.

In Table 2 an overview is given of the available multidimensional LC–CE systems. Some aspects are important: the type and dimension of the LC dimension, the speed of the CE dimension, whether the coupling is heart-cut or comprehensive, the type of interface, the detection mode and whether the system has been developed to quantify peptides or proteins or is more suitable for profiling purposes.

3.2. Typical examples of LC–CE systems

The combination of very short capillaries with normal high voltages (5–25 kV) will give very fast CE analyses [37,47]. However, if the speed of these analyses becomes very fast, a fast injection method will be necessary as well. In this case a valve with a loop cannot be used for the coupling. A unique optical-gating injection and detection system is developed by Larmann et al. [42] to enable injections that are fast in comparison with the CE analysis time. This system is based on a laser. The samples to be analyzed are tagged with fluorescein isothiocyanate (FITC), thus becoming sensitive for the 488 nm laser. The initial laser beam is split into a gating beam, focused near the injection end of the capillary and containing 95% of the laser power, and a probe beam, focused near the exit end of the capillary and containing the remaining 5% of the laser power. The optical-gating injection can be considered as an “inverse” injection method. As long as the gating beam is focused on the capillary, the analyte in the sample passing through the beam is photodegraded by the intense light of the gating beam. To inject a plug, the gating beam is temporarily blocked (usually for 5–50 ms) with a computer-controlled shutter. This allows the passage of a small plug of unaffected material through the capillary between the beams,

Table 2
An overview of LC–CE systems for peptides and proteins

Compounds	First dimension	Second dimension	Interface	Detection	Remarks	Reference
Tryptic digests of ovalbumin, fluorescently labelled	RPLC	CZE	Two six-port valves with two loops	Fluorescence	Comprehensive; semi-quantitative; time of analysis: 3 h; number of spots: 420 (theoretically)	[48]
Proteins and peptide fragments of horse heart cytochrome *c* and bovine heart cytochrome *c*	RPLC	CZE	Six-port valve	Fluorescence	Comprehensive; semi-quantitative; higher electric field strengths might be possible; influence of hydrodynamic flow reduced by using 15 μm i.d. capillaries	[49]
Tryptic peptides of horse heart cytochrome *c*	RPLC	CZE	Eight-port valve	LIF	Comprehensive; semi-quantitative	[42]
Tryptic peptides of horse heart cytochrome *c*	RPLC	FCE	Optical gating injection system	Fluorescence	Comprehensive; semi-quantitative; effective CZE capillary: 1.2 cm	[42]
Peptides of methoxy-arginine horse heart cytochrome *c* and trypsin	RPLC	FCE	Optical gating injection system	Fluorescence	Comprehensive; semi-quantitative; time of analysis: 7 min; number of spots: 25; separation length CZE capillary: 2 cm	[43]
Standard proteins thyroglobulin, bovine serum albumin, chicken egg albumin, horse heart myoglobulin	μSEC	CZE	Four-port valve	UV	Comprehensive; semi-quantitative; SEC flow rate 180 nL/min	[42]
Proteins (human, horse)	μSEC	CZE	Six-port valve with T-piece	UV	Comprehensive; semi-quantitative; time of analysis: 2 h; SEC flow rate 360 nL/min	[45]
Standard proteins thyroglobulin, bovine serum albumin, chicken egg albumin, horse heart myoglobulin	μSEC	CZE	Optical gating injection system	UV	Comprehensive; semi-quantitative; time of analysis: 2 h; two SEC columns: 100 and 250 μm i.d.; SEC flow rate is 235 nL/min (column dimension: 250 μm i.d.) and 20 nL/min (column dimension: 100 μm i.d.)	[44]
Proteins and amino acid standards in urine	μRPLC	CZE	Optical gating injection system	LIF	Comprehensive; semi-quantitative; time of analysis: 1 h; number of spots: 400; direct observation and routine manipulation of the micro-HPLC and CZE capillaries	[46]
Enkephalins in cerebrospinal fluid	SEC	CZE	Various valves, transverse flow and T-piece	UV	Heart-cut; quantitative; LOQ: 2.5 μg/mL; positioning of a trapping column between the SEC and the CZE	[11,12]
Bradykinin, neurotensin, lecine enkephalin	RPLC	CZE	Transverse buffer flow	UV, MS	Semi-quantitative; RPLC-CZE coupling comprehensive; time of analysis: 15 min	[91]
Bovine albumine, myoglobin	Gel filtration	CIEF	Eight-port valve with internal sample loop and dialysis membrane	UV	Comprehensive; semi-quantitative; time of analysis: 30 min; desalting with a dialysis membrane after the gel filtration column	[92]
Proteins in several cell lysates	CGE	RPLC	Eight-port valve with two loops	UV	Comprehensive; semi-quantitative; time of analysis: 2 h	[50]
Peptides from the salivary glands from animals	CIEF	RPLC	A valve with a loop as microinjector	UV	Comprehensive; semi-quantitative	[51]

where the components of the plug are separated and detected by their fluorescence when they pass the probe beam. No interface valve is needed because CE injections are controlled by the gating beam. The FCE system enables complete CE analysis in as little as 3 s. Because of the high speed, there is more freedom in monitoring the first separation dimension. Fig. 9 is a schematic diagram of the capillary mound of the FCE instrument. The capillary is only 10 μm in internal diameter (i.d.) and a low-concentration CE buffer (10 mM) is used. The effective separation capillary (distance between the gating beam and probe beam) is 1.2 cm. Consequently, with such a short capillary, the CE efficiency is limited [43]. One possible application of this method is the rapid fingerprinting of proteins: tryptic digests of known and unknown proteins

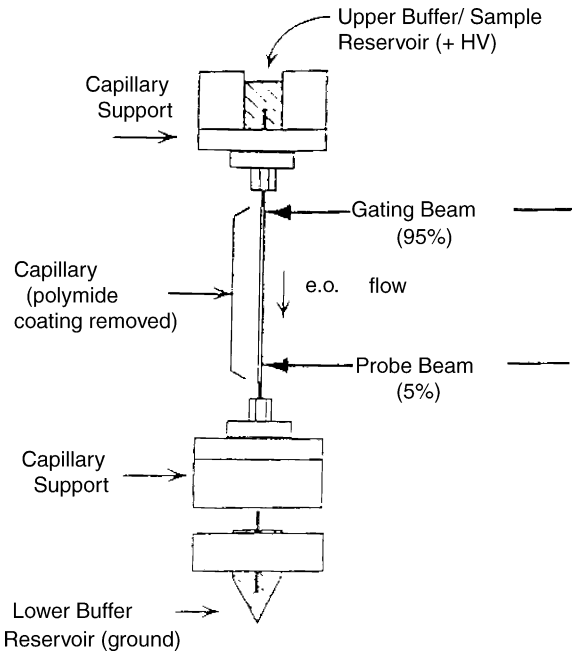

Fig. 9. Diagram of the capillary mount of the FCE instrument [42].

can be fluorescently tagged and analyzed semi-quantitatively by this method [42]. An application of FCE with another injection system is discussed and shown later.

In the development of the comprehensive coupling of LC with CE, Bushey and Jorgenson [48,49] initially used conventional LC columns and a sample loop as interface for the analysis of peptides samples. The use of LC micro columns in a comprehensive system is attractive, as mentioned earlier. However, the use of micro columns with small eluent volumes makes the collection of sample in loops impractical because of peak broadening. The coupling of separation techniques that operate with low flow rates requires the use of an interface design capable of transferring small volumes from the LC to the CE, while minimizing extra peak broadening. Therefore, an interface was designed, allowing micro-LC and CE to be combined in a comprehensive 2D system [44]. Fig. 10 represents the instrumental set-up. In the interface a cross flow of buffer has been used. The cross flow is the buffer flow from the CE buffer reservoir via the "flush buffer in" to the waste via "flush buffer out" as indicated in Fig. 10. The cross flow controls the injections of eluted LC mobile phase onto the CE capillary and the system is called the Transverse Flow Gating Interface.

Since the position of the capillaries with respect to each other in the interface is critical for a successful sample transfer from LC to CE, it was necessary to construct the interface from a transparent material (polycarbonate polymer, Lexan) [44]. The chemical resistance to acids, bases, and certain organic solvents of Lexan makes it an attractive material. Problems, such as air bubbles, trapped between the two capillaries, can easily be diagnosed with the design (Transparent Flow Gating Interface). The design also allows the positioning of

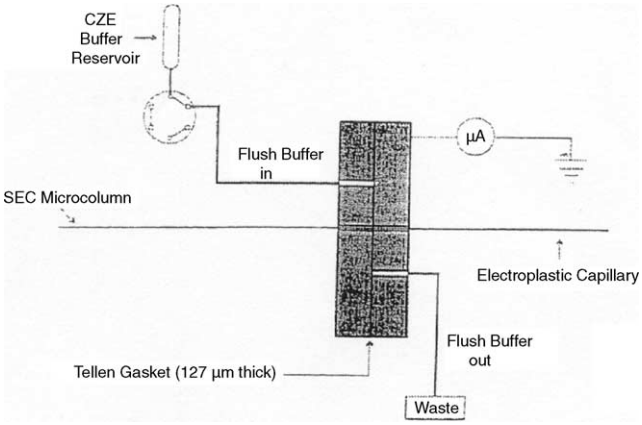

Fig. 10. Schematic of instrumental setup for 2D SEC-CE using a Transverse Flow Gating Interface [44].

the fritted outlet of the LC micro column directly in the interface and in the transverse buffer flow. This interface offers a large amount of flexibility in its operation. Adjustable parameters include the distance between the capillaries, the transverse buffer flow rate, injection time and voltage. Fig. 11 gives an indication of the resolving power of such a system. Moreover, it is an illustration of FCE as well. A SEC-CE system, using an interface with T-piece, a flush stream of buffer and also based on a type of flow gating, has recently been developed [11,12]. The flush stream is not positioned across, but it is applied in the interface using a switching valve. The SEC column is of normal size (4.6 mm × 30 mm), however, a preconcentration column is positioned between the SEC and the CE for trapping of a SEC fraction. Although this system is not comprehensive, a group of structurally related enkephalins could be quantified with a LOQ of 20 ng in cerebrospinal fluid.

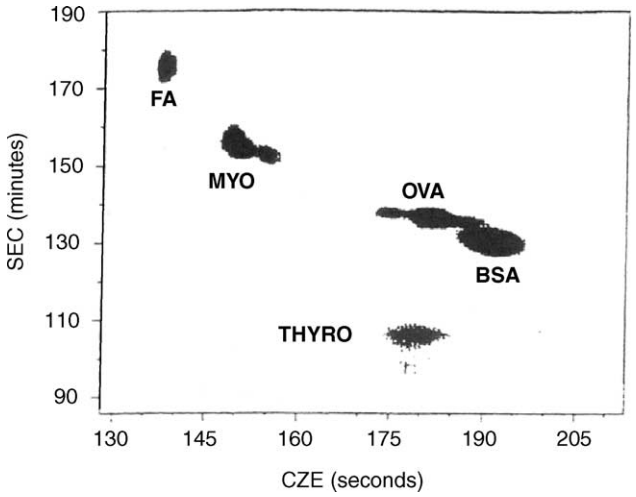

Fig. 11. Separation of protein standards by 2D SEC-CE with the flow gating interface and a 250 μm i.d. micro column. Each protein was present at 0.5% (w/v) with 2.5% (w/v) formamide. FA: formamide; MYO: horse heart myoglobulin; OVA: chicken egg albumin; BSO: bovine serum albumin; THYRO: thyroglobulin [44].

Beside the most important LC–CE couplings, there are two CE–LC system included in Table 2. These are a relatively old gel electrophoresis–liquid chromatographic system developed by Rose and Opiteck [50] and a relatively new CIEF-RPLC design [51]. The system of Rose was not further employed because it has one large disadvantage: the use of the polyacrylamide gel columns was not very reproducible, due to degradation of the polyacrylamide matrix with time and irreproducibility of the polymerization reaction. The advantage of CIEF in the first dimension of the system of Chen et al. [51] is its large preparative capability compared to most of the capillary-based electrokinetic separation techniques since the entire capillary is initially filled with a solution containing proteins/peptides and carrier ampholytes for the creation of a pH gradient in the capillary. The focused peptide fractions, which have a similar p*I*, are coinjected into the second RPLC dimension and further resolved by their differences in hydrophobicity [51].

From Table 2, a number of general conclusions emerge. Using LC–CE, the analytes of interest are peptides from digested proteins as well as intact proteins. Many systems are developed for detection of proteins. Most of the systems use RPLC or SEC as the first dimension. The eluent of RPLC allows stacking in the CE system because it contains relatively small amounts of ions. The effluent of SEC can allow stacking if only small amounts of salts are used in the mobile phase of the SEC. The transfer of all analytes in an effluent plug from the LC dimension into the CE capillary is for all systems impossible. Always a fraction of the analytes in an effluent plug is injected into the CE. Only a few heart-cut systems for quantitation of proteins or peptides have been described. The comprehensive systems have not been developed for the quantitation of proteins or peptides, however quantitative analysis may be possible.

4. CE–CE systems

4.1. General aspects

Electrophoretic separations can be based on various mechanisms: charge to mass ratio (capillary zone electrophoresis: CZE, isotachophoresis: ITP) isoelectric point (capillary isoelectric focusing: CIEF), with size (capillary gel electrophoresis: CGE) and even differences in hydrophobicity (micellar electrokinetic chromatography: MEKC). Consequently, when combining different electrophoretic principles for the development of a CE–CE system it is possible to develop orthogonal 2D systems. Compared to LC–CE combinations a CE–CE coupling may be easier to realize: the effluent peak of the first capillary will generally have a volume similar to the injection amount on the second capillary, however the transfer of this small volume may be difficult. Another advantage is the remarkable separation power [52,53].

Additional problems in CE–CE coupling compared to LC–CE couplings are: the samples must contain only low concentrations of salt and the low injection amount of the first dimension. Especially for sample components with low concentrations, large sample volumes have to be injected in order to obtain detectable amounts of components. A possibility is concentration of the sample to obtain an acceptable sensitivity. For this purpose, field amplification [29,54,55] or sample stacking [29,56,57] is often applied; however, these techniques can result in a disastrous decrease in separation power [58], especially for large sample volumes. Another solution is to improve the detection, by use of detectors which are more sensitive than UV, such as LIF [59,60], or by altering the detection cell configuration, such as in multireflection detection cells [61], Z-cell and bubble cell [62,63]. The injection of certain biological samples, e.g. urine or cerebrospinal fluid is generally possible; desalting is often needed. Desalting is also necessary to enable stacking.

A summary of CE–CE systems is shown in Table 3.

4.2. Typical examples of CE–CE systems

4.2.1. Coupling of CITP with CZE

The coupling of capillary ITP (CITP) with CZE in an online comprehensive arrangement is a promising technique for combined trace enrichment and analysis of samples with low limits of detection (LOD) [64]. The ability of ITP to introduce sample volumes in the μL range in combination with the 10- to 1000-fold preconcentration factor, compared to nL-size injections in normal CZE [65] is attractive for the analysis of low-concentration analytes in complex matrices. However, although these methods are 2D, in most studies CITP is only used as a concentration step prior to normal CZE. Recently, one comprehensive CITP-CE system is introduced for the determination of angiotensin [66]. This system uses a second UV–vis detector which is positioned after the ITP dimension to improve injection timing. The interfacing is performed by using a counterflow in a principle similar to the Transverse Flow Gating Interface.

4.2.2. Coupling of CIEF with CGE

If CIEF is coupled to CGE, a system can be designed based on the same principles as 2D gel electrophoresis. Consequently, it may have be similar separation power. Using CIEF, amphoteric compounds such as proteins are being focused in a pH gradient, established by carrier ampholytes, under a high voltage. A solution of phosphoric acid is usually used as the anolyte and a solution of sodium hydroxide as the catholyte. These solvents both differ from the buffers used in CZE. As a result, it is difficult to combine CIEF with CZE. The presence of high-density gel in a second CE dimension changes the separation mechanism to a size-based separation, minimizes solute diffusion [67–69] and prevents analyte adsorption to the capillary walls [70]. These factors all can improve separation efficiency for the proteins.

One system consisting of CIEF and CGE has been reported [71] for the analysis of hemoglobin (Hb), bovine carbonic

Table 3
An overview of CE–CE systems for peptides and proteins

Compounds	First dimension	Second dimension	Interface	Detection	Remarks	Reference
Angiotensin	ITP	CZE	Counterflow	UV	Comprehensive; semi-quantitative	[66]
Peptides (tryptic digest from *Aplysia california*	CZE	CGE	Moving capillary	Fluorescence	Comprehensive; semi-quantitative; time of analysis: 30 min; number of spots: 780; use of polyacrylamide gel	[70]
Cytochrome *c*, ribonuclease A, carbonic anhydrase II	CIEF	ITP	Microdialysis junction	UV	Comprehensive; semi-quantitative; time of analysis: 40 min; number of spots: 150	[93]
Bovine carbonic anhydrase, rabbit actin, bovine serum albumin and human hemoglobin	CIEF	CGE	Dialysis interface	UV	Not multidimensional; semi-quantitative	[71]
Ribonuclease	CIEF	CZE	Dialysis interface	UV	Not multidimensional; semi-quantitative	[94]
Proteins extracted from HT29 cancer cells	Submicellar CE	CZE	Resembling the optical gating injection system	LIF	Multiple heart-cut with comprehensive reconstruction; semi-quantitative; time of analysis: 8 h; number of plates: 100,000; sensitivity in the low zepto moles	[72]
Fluorescently labeled products from tryptic digests of cytochrome *c* (from bovine heart)	MEKC	CZE	Chip technology	Fluorescence	Comprehensive; semi-quantitative; time of analysis: 15 min; estimated number of spots: 500–1000	[80]
Fluorescently labeled products from tryptic digests of β-casein	MEKC	CZE	Chip technology	LIF	Comprehensive; semi-quantitative; time of analysis: 15 min; estimated number of spots: 150	[81]
Fluorescently labeled products from tryptic digests of ovalbumin, human hemoglobin and bovine hemoglobin	MEKC	CZE	Chip technology	LIF	Comprehensive; semi-quantitative; time of analysis: 15 min; estimated number of spots: 4200	[82]
Green fluorescent protein, ovalbumin, low-density lipoprotein and trypsin inhibitor	CIEF	CGE	Chip technology with four valves on chip	Fluorescence	Comprehensive; semi-quantitative; time of analysis: <10 min	[95]

anhydrase, rabbit actin and bovine serum albumin. A dialysis interface with a hollow fiber was developed to integrate CIEF with CGE into an on-line system. With the dialysis interface, small molecules can be introduced to or removed from the separation channel to change the ionic strength in the capillary and, consequently, the zeta-potential and the migration of the analytes. This system is easy to operate with only one high voltage source and three electrodes. Fig. 12 is a schematic diagram of this 2D-CE/CE system. The focusing is started when a voltage is placed over the CIEF capillary. After the focusing and catholyte buffer replacement, the anode (Fig. 12) was moved into the anolyte compartment of the second dimension capillary. A hemoglobin (Hb) sample containing various molecular weight markers was separated to check the effectiveness of this system. Fig. 13A shows the CIEF electropherogram of a Hb sample, without CGE separation. The four peaks indicate the four Hb variants A, F, S, and C. They have p*I* values of 7.10, 7.15, 7.25, and 7.50, respectively. The 1D CGE electropherogram of the Hb sample is shown in Fig. 13B, while the 2D separation is shown in Fig. 13C. This is not a real multidimensional system, since the CIEF buffer is transported continuously into the CGE. It could have been multidimensional if every fraction with the same p*I* had been separated individually in the CGE. The elution order of A, F, S and C is the same in the 1D CIEF electropherogram as in the 2D electropherogram, because their size is approximately the same. If this was not the case, their

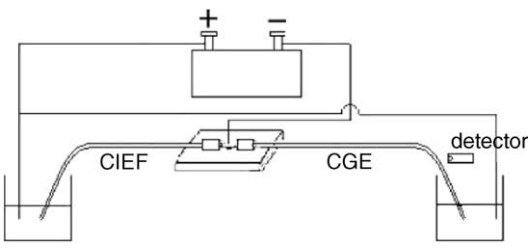

Fig. 12. Set-up of a 2D-CIEF/CGE system [71].

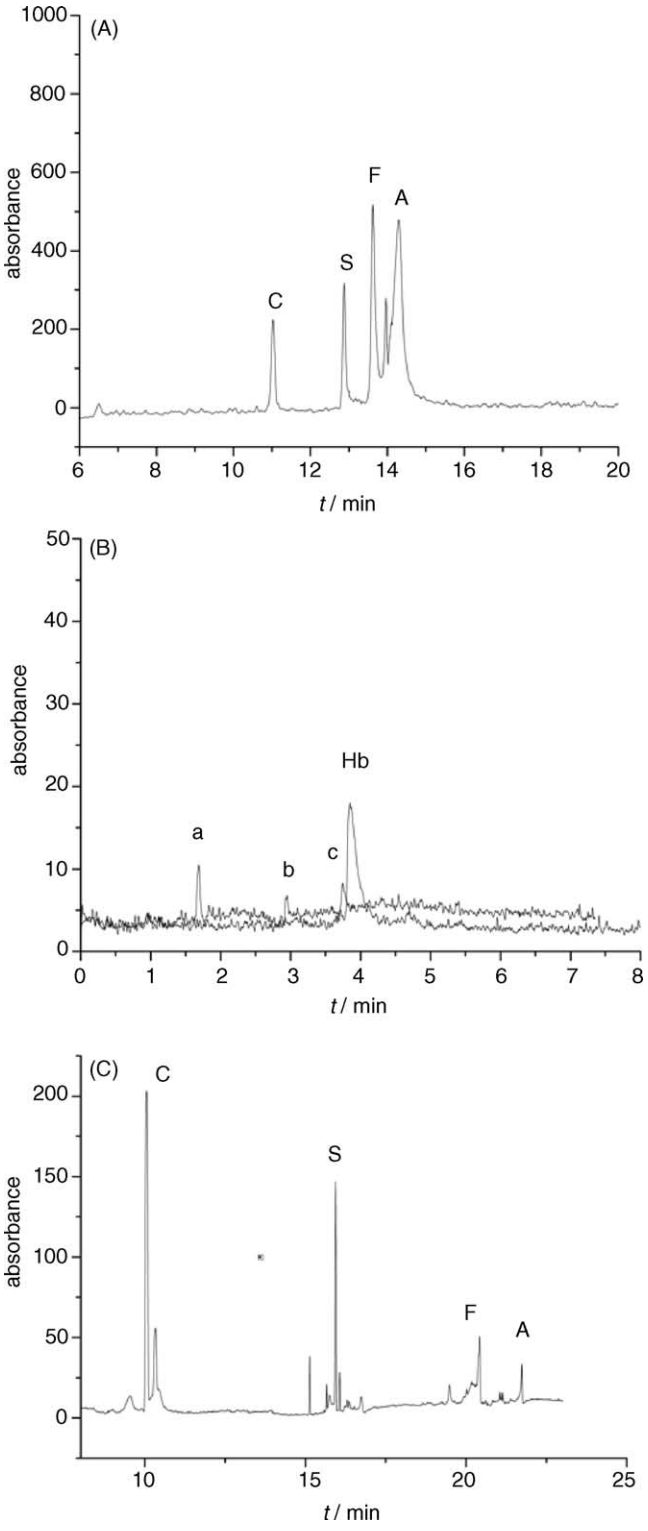

Fig. 13. (A) CIEF of hemoglobin. Hb (0.2%, w/v) was dissolved in 50 mM Tris–HCl containing pharmalyte (2%, v/v) and TEMED (0.1%, v/v) [71]. A, F, S and C stand for the four variants of hemoglobin with different p*I*. (B) CGE of proteins: (a) carbonic anhydrase (bovine, 31,000), (b) actin (rabbit, 43,000), and (c) albumin (bovine serum, 66,200) and hemoglobin (Hb), all 0.1% (w/v) in CGE buffer. The concentration of Hb is 0.2% (w/v) SDS was used as both the buffer and the electrolytes [71]. (C) The 2D electropherogram of Hb. A, F, S and C stand for the four variants of hemoglobin with different p*I* [71].

elution order in the CGE could be changed. It should be noted that compounds **a**–**c** in Fig. 13B have not been identified in Fig. 13C. The elution order of these substances could not be determined in the 2D system. Consequently in principle, using this set-up the CGE dimension can reverse the resolution achieved by CIEF.

4.2.3. Coupling of CZE with CGE

As previously stated, in gel electrophoresis analyte migration is hindered by the structure of the high-concentration and cross-linked polyacrylamide gels (sieving and/or interaction with the polymer matrix). One system, based on the combination of CZE and high-density gel electrophoresis. was reported for the analysis of some target peptides, a tryptic digest of trypsinogen and an individual B2 neuron from the marine mollusk *Aplysia californica* [70]. The second dimension consists of a number of channels. These channels are filled with *N*,*N*′-methylenebis(acrylamide) cross-linked polyacrylamide gel, chemically bound to the inner wall of the glass channel. Reproducible, bubble-free and stable gels are easily obtained in the thin channels. Injection into the second dimension is performed by moving the outlet end of the first capillary dimension across the entrances of the channels. Consequently, the second dimension can be considered as a couple of parallel positioned separation capillaries. An important advantage of this approach is that it is completely comprehensive in contrast to the previously reported system. Fig. 14 presents a schematic diagram of this 2D-CZE/CGE system. Fig. 15 shows the optimization results of a series of separations of a sample containing model peptides using different channel fillings: buffer and a series of gel-fillings with concentration varying from 5 to 50%*T* (the total concentration of monomer in the gel) at a constant degree of cross-linkage of 3.3%. The sample mixture consists of a number of peptides: Leu-enkephalin, Met-enkephalin, angiotensin II and some smaller peptides consisting of two or three amino acids. These peptides are not separated in the CZE dimension. In free solution channel electrophoresis, this peptide mixture is separated into two poorly shaped bands. No improvement was found for a variety of buffer solutions in different pH ranges. For the peptides, only the channels with a high concentration of gel show significant separation efficiencies especially for the small peptides.

4.2.4. Coupling of MEKC with CZE

The coupling of MEKC with CZE is favorable because of its orthogonality [72]. It resembles the orthogonality of a RPLC-CZE system. Michels et al. developed a system consisting of submicellar capillary electrophoresis coupled to CZE for the detection of proteins, labeled with a fluorogenic reagent. The transverse flow-gated principle of Lemmo and Jorgenson [44] was used. The developed system differs from that of Jorgenson in three important respects. First, a CE in both dimensions was used, which eliminates the use of chromatography, with its associated pumps and large sample vol-

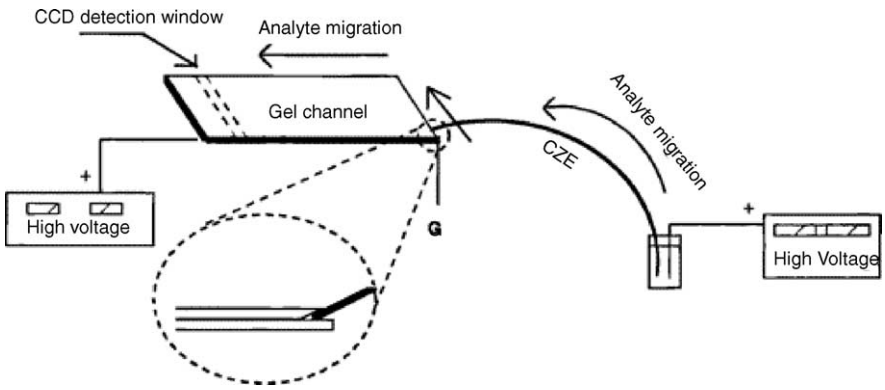

Fig. 14. Schematic diagram of the CZE-CGE separation system showing the CZE capillary, gel channel, interface and the power supply arrangement [70]. G is the ground.

umes. Second, the entire fraction is transferred from the first dimension to the second, eliminating the loss of sample in the flow-gated interface. Third, an extremely high sensitive fluorescence detection is used, which allowed the detection of zeptomoles of proteins [72], however the amount of sample which can be injected is smaller.

4.2.5. Microfluidic devices

The number of reports concerning microfluidic devices (chips, micro total analysis system or μTAS) has increased dramatically since the initial concept was introduced some years ago [73,74]. In particular, microchip CE devices have been shown to offer many advantages over conventional CE systems: parallel channels in one device, reduced amounts of reagents and waste, increased precision and accuracy and the potential for disposable devices. In addition, lower voltages are required with microchip CE due to the short channels that are employed for the separation. This enables the use of smaller power supplies, leading to portable analysis systems [73]. Recently several reviews were published concerning various aspects of chip technology. One deals with sample pretreatment on microchips [75], others review sample introduction [76], cyclic electrophoretic and chromatographic separation methods [74] and theory and technology of μTAS [77]. Two reviews report various applications of chips [78,79], including peptide and protein analysis. Although the chip technique is promising, there are only three reports, from Ramsey and co-workers [80–82], about 2D electrophoretic applications for the determination of peptides or proteins so far. A microfabricated fluidic device was developed that combines micellar electrokinetic chromatography and high-speed electrophoresis for the 2D analysis of peptides. Second-dimension analyses were performed every few seconds, with total analysis times of around 15 min for tryptic peptides. The peak capacity of the two-dimensional separations was estimated to be 500–1000. The orthogonality of the separation techniques, an important factor for maximizing peak capacity and resolution, was verified by examining each technique independently for peptide separations. The 2D separation strategy was found to greatly increase the resolving power over that obtained for either dimension alone [80]. This system was further improved [81,82]. The chip described in ref. [82] enables MEKC separations in a 19.6 cm long serpentine channel. Subsequently, the peptides were rapidly sampled into a 1.3 cm long second-dimension channel, where they were separated by CZE. The contribution to band broadening in the channels was further minimized. Analysis of rhodamine B routinely produced plate numbers of 230,000 and 40,000 in the first (MEKC) and second (CE) dimensions, respectively, corresponding to plate heights of 0.9 and 0.3 μm. The electric field strengths were 200 V/cm for MEKC and a relatively high 2400 V/cm for CE. In less than 15 min, two-dimensional separation of bovine serum albumin tryptic digest produced a peak capacity of 4200 (110 in the first dimension and 38 in the second dimension). Fig. 16 shows the 2D separation of a bovine serum albumin tryptic digest. The theoretical projections of the 2D data onto each axis are also shown. The system was used to identify a peptide from a tryptic digest of ovalbumin using standard addition and to distinguish between tryptic digests of human and bovine hemoglobin [82].

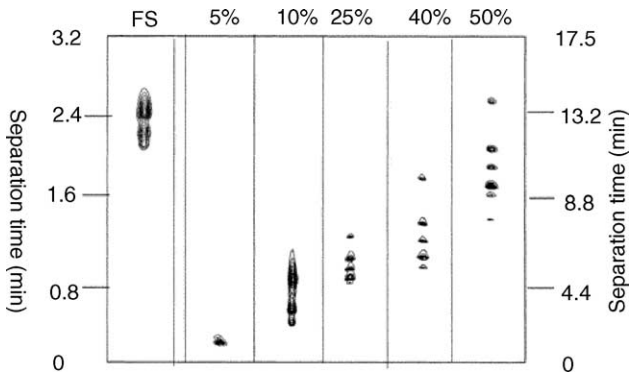

Fig. 15. Comparison of free solution (FS) and gel-filled channels for the separation of a series of five labeled peptides of between two and eight residues. The total concentration of monomer in the gel (%*T*) is shown in each electropherogram. The concentration of cross-linking agent (%*C*) is 3.3% in all cases [70].

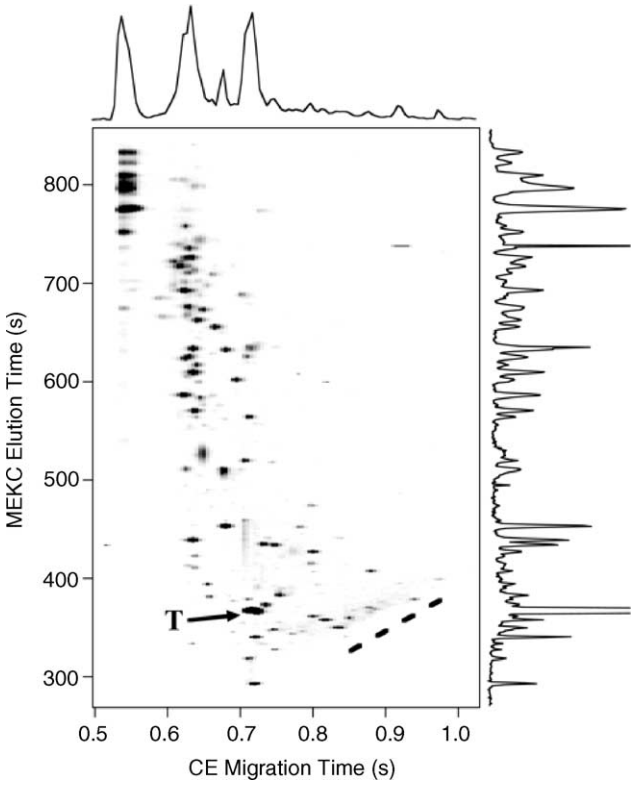

Fig. 16. The 2D separation of a bovine serum albumin tryptic digest. The projections of the 2D data onto each axis are shown. Spots marked with a "T" represent unreacted 5-TAMRA dye. The dotted line illustrates correlation in the separation mechanisms [82].

From Table 3, a number of conclusions can be drawn. So far, no CE–CE system has been used for quantitation of peptides or proteins. It should be noted that sample pretreatment consisting of a desalting procedure, is nearly always necessary before using these systems, in contrast to the systems starting with a LC mode. Chip technology is still further improved, resulting in increased separation power in very short time. Using chip technology, the transfer of effluent from the first dimension to the second dimension can be performed with limited band broadening.

5. Conclusions

For protein and peptide mapping a semi-quantitative detection is often used, while comprehensiveness and selectivity of the separation system is very important. For quantitation of a specific peptide or protein it is essential to develop a validated sensitive method, while a heart-cut system can then have sufficient selectivity. Generally, the more or less direct injection of biological samples and the speed of the separation system are important. For the development of an on-line multidimensional system to determine peptides and proteins some general strategies can be given. If samples containing considerable amounts of salts have to be injected, SEC or RPLC separation in the first dimension is favorable, while either chromatographic or electrophoretic separation in the second dimension can be used, depending on the nature of the peptides or proteins (lipophilicity, size, charge, etc.). If direct injection of biological samples is required, SEC is a very good choice as the first separation dimension. Combinations with RPLC, IEC or electrophoretic techniques result in systems with large selectivity.

For quantitation purposes LC–LC, LC–CE and CE–CE combinations can be used, with on-line coupling of suitable detection systems (LIF, MS). If target analysis of low-concentrated peptides or proteins is the aim, a (pre)concentration step has to be included in the system. Affinity chromatography seems first choice when low concentrations of specific peptides or proteins must be determined. However, for such a system antibodies should be available for immobilization Trapping on a micro column or concentration on the top of a chromatographic column in the second dimension and stacking in the electrophoretis (for charged compounds is often used.

For high-throughput analysis, for instance in proteomics, it is necessary to separate the compounds at a high speed and combination with tandem MS is mandatory if the system must be employed for profiling purposes. Although, electrophoretic separation techniques in many cases give fast separations, (nano-)LC–CE (FCE) coupling should be further developed. In such a system both dimensions enable separations within minutes and seconds, respectively, enabling the analysis of large numbers of samples within a short period of time. Coupling of CE with MS including different ionization techniques is now becoming routine. For a universal application of fluorescence detection derivatization of the analytes may be necessary, which is a limitation.

Relatively new approaches, such as CE–CE, may have the potential to supersede the conventional LC–LC techniques in some cases, However, the LC–LC systems are still much easier to develop, in particular if only a heart-cut system is required. The on-line addition of a MS detector to a LC–LC system can be achieved more easily as well, compared to a LC–CE or a CE–CE system. However, a LC–CE system often seems to be more orthogonal and selective than LC–LC, but generally the sensitivity is less because only a part of a LC fraction can be introduced into the CE. If a comprehensive system is required, a LC–CE system may also have the advantage of a rapid CE step but such a system is still less robust. In CE–CE, which can have a large orthogonality as well, direct injection of biological samples is generally not possible unless a simple desalting procedure is applied. Using LC–LC or LC–CE direct sample injection is sometimes possible. By use of chip-technology very fast separations can be achieved. Chip-technology is now becoming available with on-line MS detection. If chips will be developed in such a way that direct injection of biological sample is possible with the addition of an on-line desalting step, e.g. with a hollow fiber, a very powerful system will be obtained.

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