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Review

Determination of enzymatic activity by capillary electrophoresis ☆

Zdeněk Glatz *

Department of Biochemistry, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic

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Abstract

Enzymes are biological catalysts that play an important role in biochemical reactions necessary for normal growth, maturation and reproduction through whole live world. Their accurate quantitation in biological samples is important in many fields of biochemistry, not only in routine biochemistry and in fundamental research, but also in clinical and pharmacological research and diagnosis. Since the direct measurement of enzymes by masses is impossible, they must be quantified by their catalytic activities. Many different methods have been applied for this purpose so far. Although photometric methods are undoubtedly the most frequently used, separation methods will further gain their position in this field. The article reviews different possibilities for the assay of enzymatic activity by means of capillary electrophoresis (CE). Both the off-line and on-line enzyme assays based on CE are discussed.

Keywords: Biocatalysts; Enzymes; Ribozymes; Enzymatic activity; Pre-capillary assay; On-capillary assay; Post-capillary assay; EMMA

Contents

1. Introduction

Enzymes together with ribozymes (RNA molecules with catalytic function) are biocatalysts necessary for every living organism. They catalyze all chemical reactions required for its survival and reproduction rapidly, selectively and efficiently [1]. What is more, the isolated enzymes can also catalyze these reactions, so the excellent properties of enzymes can be used in many fields – in catalysis in industrial processes, as well as, in a wide range of analytical systems [2]. The determination of enzymes is probably one of the most frequently encountered procedures in biochemistry and molecular biology. As the enzymes are presented in living cells in complex mixture with other proteins, it is impossible to measure directly their concentrations. For example about 3000 proteins were identified in such simple cell as bacteria *Escherichia coli*, in human cell this amount increases up to 20,000. But anyway the most relevant property of particular enzyme in the biochemical context is its catalytic activity, so it can be simply quantified by the measurement of the rate of given reaction.

The range of techniques used to measure the enzymatic activity is vast and depends on the nature of the chemical change. Application of particular technique will depend on the ground of convenience, cost, the availability of appropriate equipment and

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* Tel.: +420 549493957; fax: +420 541211214.
E-mail address: glatz@chemi.muni.cz.

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reagents and the level of the sensitivity required. Within these limits a wide scope of methodology is now available including photometric, electrochemical, radiochemical and separation techniques like HPLC a capillary electrophoresis (CE) [3–5].

In the last two decades, CE has become powerful technique, which can provide highly efficient separations and large peak capacities [6–10]. Today the availability of sophisticated automated equipment for CE makes this technique suitable to be implemented in routine laboratories for separation of a variety of molecules including organic and inorganic ions, neutral molecules, biopolymers such as peptides, proteins, DNA and RNA molecules or their fragments, even viruses and whole bacterial cells. Compared to the other methods, the CE based methods are rapid, can be automated, and require only small amounts of samples. All these parameters predict CE as one of the method of choice for the assay of enzymatic activities.

Monitoring of enzymatic reaction by CE, which has been under active investigation in recent years, could be divided in two fundamental groups on the basis of reaction location. In *heterogeneous enzyme assay*, one of the reactants, mostly the enzyme is immobilized on the surface of a suitable carrier material forming microreactor, which can be directly integrated in given CE apparatus. As a result a mixing and a reaction occur between the immobilized enzyme and the substrate(s) presented in the background electrolyte. The resulting product(s) is (are) then directly transported and on-capillary detected. Application of enzyme microreactor brings not only the improvement in the storage stability in contrast with the enzymes presented in the solution, but also increasing of reaction rate due to the much higher enzyme/substrate(s) ratio. In contrast, all reactants are presented in solution during *homogeneous enzyme assay*. This variant includes (i) enzyme reaction prior CE separatiom – pre-capillary assay; (ii) enzyme reaction during CE separation – on-capillary assay, also known as electrophoretically mediated microanalysis (EMMA) and (iii) enzyme reaction post CE separation – post-capillary assay. However, it is necessary to emphasize that the objectives of *heterogeneous and homogeneous enzyme assays* are totally different. While the *heterogeneous enzyme assay* is primarily oriented on the determination of substrates and inhibitors or for the reaction kinetic monitoring, the *homogeneous enzyme assay* is mainly utilized for the determination of enzymatic activity in given samples.

Since the field of immobilized enzymatic reactors has been recently exhaustively reviewed by Křenková and Foret [11], this paper is oriented on the area of homogeneous enzyme assays. The last review dedicated to the similar subject was already published by Bao et al. in 1997 [12].

2. Homogeneous enzyme assay

The classic enzyme assay commonly includes a number of operations: (i) the mixing of reactants and the initiation of the reaction, (ii) the incubation of the reaction mixture, and finally (iii) the detection of the reaction product(s) or substrate(s). In the pre-capillary assay, only the determination of product(s) or substrate(s) is performed by means of this efficient separation technique, so this variant can be classified as an off-line assay. It is also the most frequently used enzyme assay based on CE. On the contrary, the on-capillary as well as post-capillary assays utilize the migration in the electric field as the basic principle of CE for the other operations, therefore these variants can be classified as on-line assays.

2.1. Pre-capillary assay

In a typical pre-capillary assay, all but one of the components of the reaction mixture is added to the reaction vessel. It can be also directly the sample vial situated in the heated autosampler of given CE instrument, its thermostating thus insures that the enzymatic reaction will be performed under strictly defined conditions. After a short period of pre-incubation, the reaction is started by adding the missing component, which can either be the enzyme or the substrate. Two approaches are typically used for the preparation of samples for the analyses. The first possibility is to withdraw the aliquots from the reaction mixture at predetermined intervals and to stop the enzymatic reaction by boiling or by addition of various denaturants (Fig. 1). The centrifugation of precipitated proteins is usually performed to prevent the capillary clogging. The second possibility is a monitoring of the reaction rate by the repeated injections from the same vial in combination with the sequential CE runs (Fig. 2). This arrangement brings also particular automation of the assay. Although both hydrodynamic and electrokinetic sample injections are used in such analyses, the application of hydrodynamic injection by pressure prevails. So-called “short end injection” procedure has been also applied in several studies to get the rapid analyses [14–16]. It can be achieved by placing the sample vial at the outlet, the buffer vial at the inlet and then applying vacuum. The CE separation is then performed with reverse polarity on the part of the capillary nearest to the detector. It brought not only substantial reduction of time of analyses form tens of minutes to tens of seconds, but also approximately twice increase in detection sensitivity (Fig. 2B). It is especially suitable for the enzymatic reaction with short linear phase and for the kinetic studies.

The formation of the product(s) or depletion of the substrate(s) can be monitored by one of the CE modes in the dependence on the physicochemical nature of given compounds. The separations are performed both in the classic capillary as well chip formats. As in other CE application fields, capillary zone electrophoresis (CZE) is probable the most frequently used mode for this purpose, following by micellar electrokinetic capillary chromatography (MEKC). Chiral modifications of CZE and MEKC have been also used in several studies [17,18]. In addition, capillary gel electrophoresis (CGE) has been applied for the analyses collagen fragments for determination of collagenase activity [19] and capillary isoelectric focusing for the separation of peptides in trypsin activity assay [20]. Recently even capillary electrochromatography (CEC) in monolithic polymer columns has been tested for the study of glutathione S-transferase [21].

Because almost all detection techniques can be combined with CE, the choice of certain one is dependent on the required sensitivity and detector availability in given laboratory.

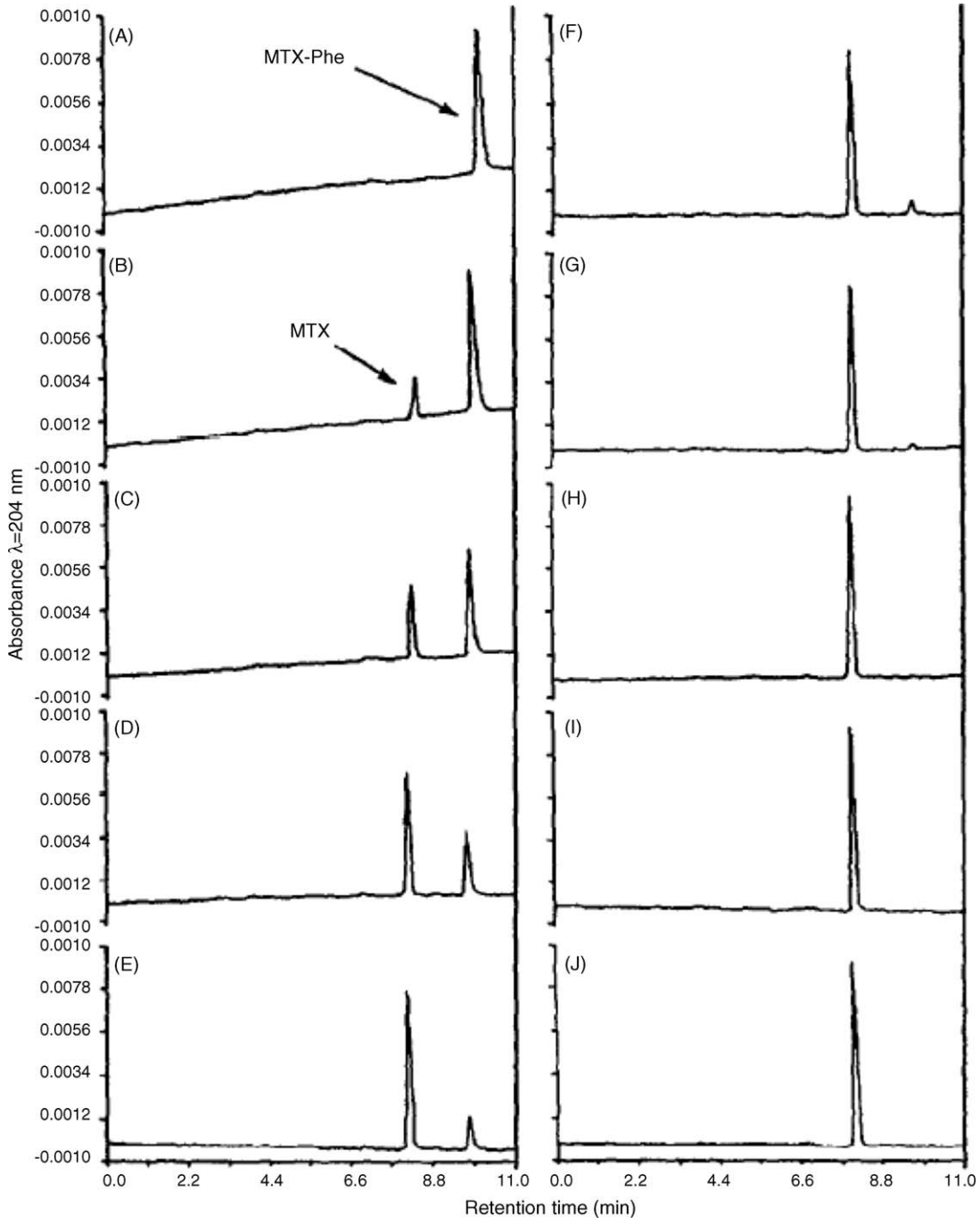

Fig. 1. Monitoring of the carboxypeptidase reaction by CE analyses of the samples withdrawn from the reaction mixture after (B) 0 min, (C) 1 min, (D) 2 min, (E) 3 min, (F) (4) min, (G) 5 min, (H) 7 min, (I) 10 min and (J) 15 min incubation; (A) control without enzyme. Reprint from [13] with permission.

Although UV–VIS detection is most common mainly for the reason that the commercial CE apparatus are normally equipped with this type of detector, the application of LIF detection increases especially in clinical or analogously oriented studies. Since the requirement on high throughput analysis systems increases also in the field of enzyme assays especially with respect to drug discovery, two approaches were recently proposed for this purpose. Xu and Ewing developed a multichannel microchip with optically gated sample introduction [22]. Multiple assays of β-galactosidase were demonstrated in four parallel channels (Fig. 3). In addition, three unique enzyme assays were simultaneously performed in different channels for inhibition study of β-galactosidase by various inhibitors. Ma et al. performed similar study on LDH by means of 96-capillary array electrophoresis coupled with a multiplexed absorption detector (Fig. 4), whereas the reactions were accomplished in a combinatorial array of 96 reaction microvilas [23].

From the time when Krueger et al. used CE for the first time for assay of enzymatic activity of endopeptidase Arg-C [24], CE based methods have been developed for many enzymes

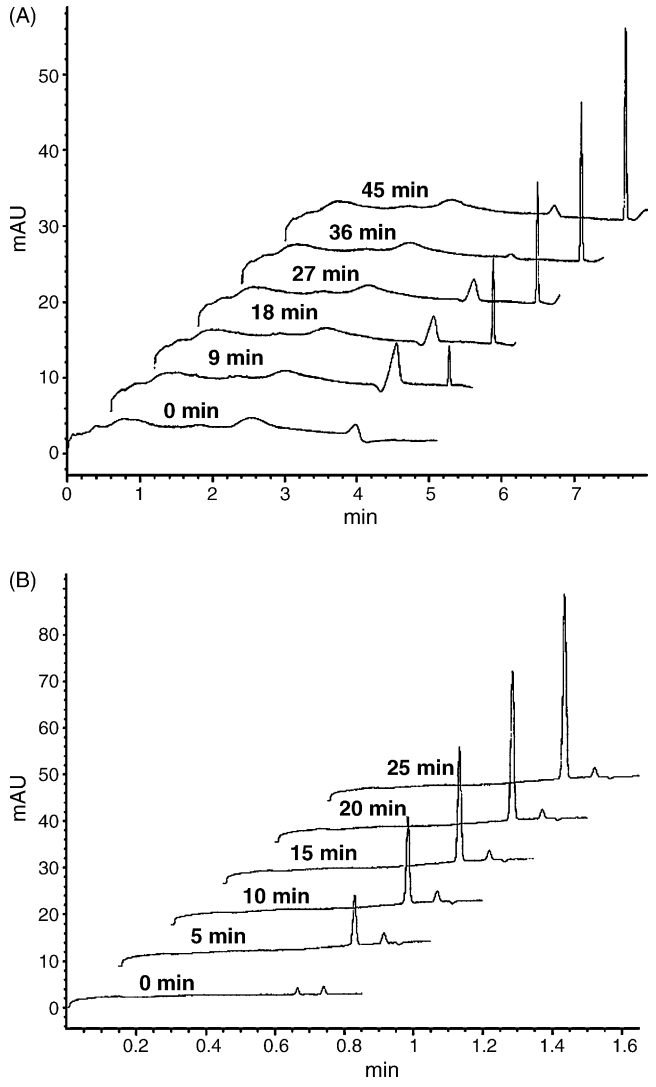

Fig. 2. Monitoring of the production of bromide by means of haloalkane dehalogenase enzyme reaction by CE with the repeated injections from the same vial using classic long-end (A) and short-end (B) injection procedures; reprint from [14] with permission.

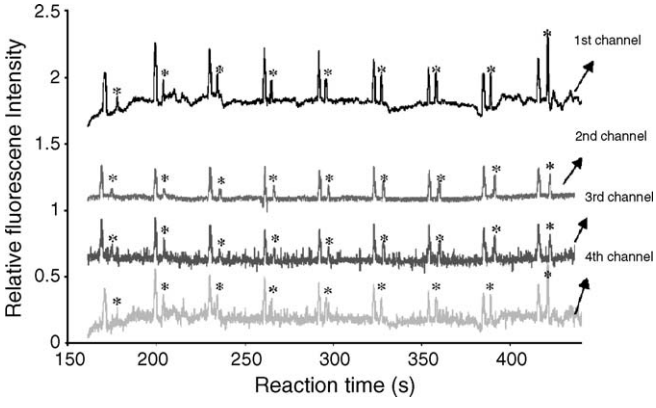

Fig. 3. Parallel monitoring of the enzymatic reaction of β-galaktosidase in a four-channel multichannel microchip; reprint from [22] with permission.

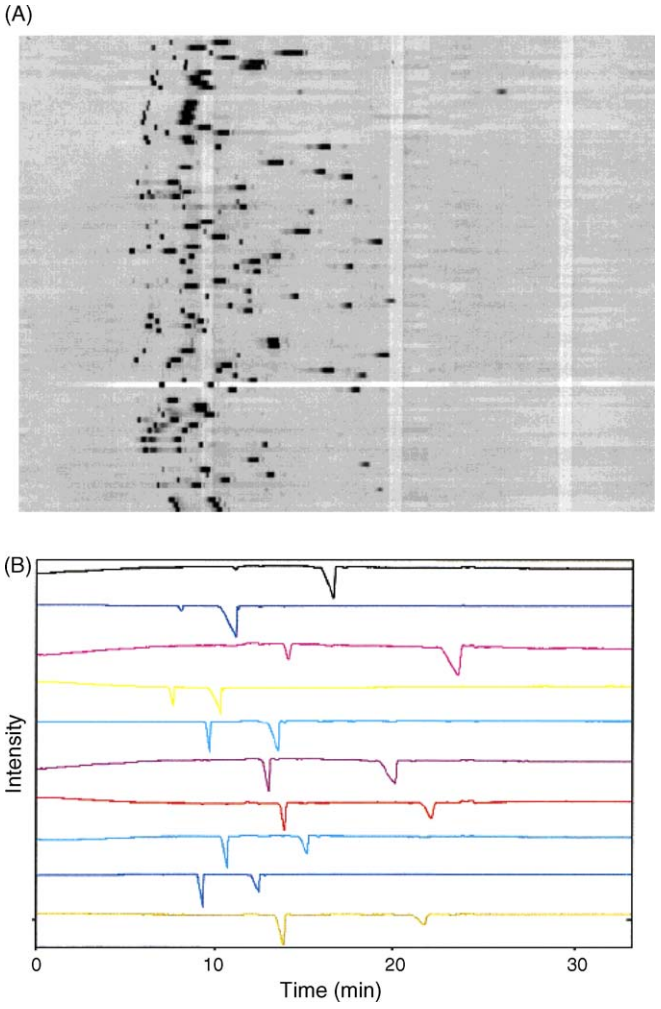

Fig. 4. Reconstructed absorption image of combinatorial screening of LDH enzyme activity in a 96-capillary array (A) and the electropherograms of products after a 180-min reaction for different LDH concentrations (B); reprint from [23] with permission.

of diverse action and origin. Saevels et al. even monitored the ribozyme activity by means of CE [25]. The chronological summary of pre-capillary methods is given in Table 1.

2.2. *On-capillary assay*

As mentioned above, the enzyme assay requires a number of operations. The EMMA methodology introduced by Bao and Regnier [123] couples all these operations in one integrated technique. It utilizes different electrophoretic mobilities of enzyme and substrate(s) to initiate reaction inside the capillary and to separate the components of the reaction mixture from each other for the final on-capillary quantification. The resulting automation of all assay steps both minimises the possible errors and also saves the experimental time.

Upon now, two main approaches have been proposed for loading and consequential mixing of reaction compounds in the capillary during the EMMA methodology. In the continuous mode of EMMA (long contact mode), the capillary is initially completely filled with one of the reactants while the second

Table 1
Summary of pre-capillary assays

Enzyme	CE mode	Detection	Note	Ref.
Endoproteinase Arg-C	CZE	UV/200 nm		[24]
Glutathione-peroxidase	MEKC	UV/214 nm		[26]
Chloramphenicol acetyl transferase	CZE	UV/247 nm		[27]
Tripeptidase	CZE	UV/200 nm		[28]
Carboxypeptidase Y	CZE	UV/200 nm	Optimization of transpeptidation reaction	[29]
Protein kinase	CZE	UV/200 nm		[30]
Protein phosphatase				
Carboxypeptidase A	CZE	UV/204 nm		[13]
β-Galactosidase	CZE	LIF	LOD 1.6 molecules	[31]
Dipeptidyl peptidase IV	CZE with lin. polymer	UV/200 nm	Coated capillary, assay and inhibition study	[32]
ADP glucose pyrophosphorylase	CZE	UV/254 nm	BGE contained 1 M trimethylammoniumpropane sulfonate	[33]
Adenylosuccinase	CZE	UV/254 nm		[34]
ADP ribosyltransferase	CZE	UV/254 nm	BGE contained 2.5 mM 1-hexanesulfonate	[35]
Polyphenol oxidase	CZE	UV/325 nm	More then one substrate in reaction mixture	[36]
Cathepsin D	CZE	UV/214 nm		[37]
5′-Nucleotidase	CZE	UV/254 nm		[38]
γ-Glutamyl hydrolase	MEKC	UV/300 nm		[39]
2,3-Sialyltransferase	CZE	LIF		[40]
Hyaluronidase	CZE	UV/200 nm		[41]
Endoglycanases	CZE	Fluorescence	In vitro and in vivo reaction	[42]
5-Aminolaevulinic acid hydratase	MEKC	UV/220 nm		[43]
Hyaluronate lyase	CZE	Fluorescence	Kinetic study of hyaluronic acid depolymerization	[44]
Oligopeptidase	CZE	UV/220 nm	Coated capillary	[45]
γ-Glutamyl hydrolase	MEKC	UV/300 nm		[46]
Ubiquitin carboxyl-terminal hydrolases	CZE	UV/200 nm		[47]
N-Acetyl-β-glucosaminidase	CZE	UV/214 nm or fluorescence		[48]
UDP glucuronyl transferase, β-glucuronidase	CZE	UV/320 nm		[49]
Aminopeptidase X	CZE	UV/185 nm		[50]
N-Acetyltransferase 1	CZE	UV/305 nm		[51]
Phospholipase C	MEKC	UV/196 nm		[52]
β-Galactosidase	CZE	UV/306 nm	Extended lightpath capillary, sugar derivatization	[53]
Purine nucleoside phosphorylase	CZE	UV/200 nm		[54]
Prolidase	MEKC	UV/200 nm	Kinetic study	[55]
Protease	CZE	UV/185 nm	Minor protease from *Pseudomonas aeruginosa*	[56]
β-Glucuronidase	MEKC	UV/200 nm		[57]
Creatine kinase	CZE	UV/256 nm	Off-line and on-line assays	[58]
VanX enzyme	CZE	UV/200 nm	Dipeptidase involved in vancomycin resistance	[59]
Proteases	CZE	LIF	General assay for proteases, coupling SPE and CE	[60]
β-Glucuronidase	CZE	Fluorescence	Urine samples	[61]
Src kinase, protein kinase C	CZE	LIF		[62]
Galactosyltransferase	CZE	UV/214 nm	Kinetic study	[63]
Purine nucleoside phosphorylase	CZE	UV/210 nm	Erythrocyte samples	[64]
Adenine phosphoribosyl transferase		UV/254 nm		
Adenosine deaminase		UV/254 nm		
Hypoxanthine guanine phosphoribosyl transferase		UV/260 nm		
Trypsin	cIEF	LIF		[20]
Elastase	MEKC	UV/200 nm	Kinetic study	[65]
Glutamate synthase	CZE	Fluorescence		[66]
Cathepsin D	MEKC	LIF	Bovine skeletal muscle samples	[67]
Bilirubin oxidase	MEKC	VIS/450 nm		[68]
Adenosine deaminase	cITP-CZE	UV/254 nm	Erythrocyte samples	[69]
Ornithine transcarbamylase	MEKC	UV/200 nm	Kinetic study	[70]
Galactosyltransferase	CZE	LIF		[71]

Table 1 (*Continued*)

Enzyme	CE mode	Detection	Note	Ref.
Sulfoesterase	CZE	Indirect UV/254 nm	BGE contained chromate	[72]
Angiotension converting enzyme	CZE	UV/254 nm		[73]
Phospholipase A_2	MEKC	UV/196 nm		[74]
Rhodanese	CZE	UV/200 nm	Short end injection	[15]
β-Galactosidase	CZE	LIF	10 μm capillary, LOD 600 molecules	[75]
β-Glucuronidase	CZE	UV/250 nm	Kinetic study	[76]
Phospholipide hydroperoxide Glutathione peroxidase	CZE	UV/232 nm		[77]
Haloalkane dehalogenase	CZE	UV/200 nm Indirect UV/315 nm/375 nm	Short end injection	[14]
GTPase	CZE	UV/254 nm	Inhibition study	[78]
Angiotension converting enzyme	CZE	UV/228 nm		[79]
LDH	Multiplex CZE	UV		[23]
Proteases	MEKC	UV/200 nm	Identification of particular protease by addition of specific inhibitor	[80]
3′,5′-Cyclic nucleotide-phosphodiesterase	CZE	UV/260 nm	Kinetic study, short end injection, EOF reversion by hexadimethrin	[16]
Angiotension converting enzyme	CZE	UV/214 nm	Inhibition study	[81]
μ- and *m*-calpain	MEKC	LIF		[82]
β-Glucuronidase	CZE	LIF	Microchip CE, kinetic study	[83]
Extracellular signal-regulated kinase	CZE	UV/185 nm		[84]
Src-kinase, IR-kinase, PKA-kinase	CZE	LIF	Microchip CE, multiplexed enzyme assay	[85]
Flavanone 3-hydroxytransferase Casein kinase	CZE	UV/220 nm		[86]
Catechol-*O*-methyltransferase	CZE	UV/200 nm	Off-line and on-line assays, BGE contains 12 mM hydroxypropyl-β-cyclodextrin	[87]
Alanine:glyoxylate aminotransferase	CZE	Indirect UV/350 nm/200 nm	Capillary internally coated with polyimide	[88]
Farnesyltransferase	CZE, MEKC	LIF	Kinetic study	[89]
Proteinases	CZE	LIF	Using fluorescence-quenched protein-dye conjugates as a substrate	[90]
UDP-*N*-acetylglucosamine enoylpyruvyl transferase	CZE	UV/200 nm	Kinetic study	[91]
CYP 3A isoenzyme	CZE	LIF, UV	Kinetic study	[92]
Transglutaminase	CZE, MEKC	LIF	Kinetic study	[93]
Extracellular signal-regulated kinase	CZE	UV/185 nm		[94]
Protease MCP-01	CZE	UV/202 nm	Autolysis study	[95]
Angiotension converting enzyme	MEKC	UV/230 nm	Inhibition study	[96]
Phospholipase A_2	MEKC	LIF	Microchip CE	[97]
Cathepsin D	CZE	UV/214 nm	On-capillary sample stacking	[98]
cAMP dependent protein kinase	MEKC	LIF	Inhibition study	[99]
Acyl-CoA hydrolase	CZE	UV/260 nm	Kinetic study	[100]
Extracellular signal-regulated kinase	CZE	Fluorescence	Microchip CE	[101]
Extracellular signal-regulated kinase	CZE	LIF	Multiplex capillary assay	[102]
Alkaline phosphatase	CZE	LIF	Combination of capillary array electrophoresis with laser microchip detection	[103]
CYP450 isoenzymes	Achiral and chiral CZE and MEKC	UV/195–360 nm	CYP2C19, CYP2D6*1, CYP2C9*1, CYP1A2, CYP3A4,	[17]
Adenosine deaminase Purine nucleoside phosphorylase S-adenosyl-L-homocysteine-hydrolase	CZE MEKC	UV/254 nm		[104]
LDH	CZE	EC	Carbon fiber micro-disk bundle electrode	[105]
Ribonuclease H	CZE	LIF	BGE contains 20% acetonitrile	[106]

Table 1 (*Continued*)

Enzyme	CE mode	Detection	Note	Ref.
Alkaline phosphatase	CZE	EC	Yoctomole sensitivity, single carbon fiber microcylinder electrode	[107]
Oxalyl CoA decarboxylase	CZE	UV/200 nm		[108]
Collagenase	CGE	LIF		[19]
Protein farnesyltransferase Caax-specific prenyl-protein endoprotease Isoprenylcysteine carboxyl methyltransferase	CZE	LIF	Monitoring of RAS protein posttranslation modifications	[109]
Pyrimidine 5′-nucleotidase	MEKC	UV/254 nm		[110]
ATPase	MEKC	UV/254 nm	In situ monitoring on muscle fibres	[111]
Flavin-containing monooxygenase FMO3 and FMO1	Chiral CZE	UV/200 nm		[18]
β-Galactosidase	CZE	LIF	Multichannel microchip CE	[22]
UDP galacturonic acid C4-epimerase	CZE	UV/254 nm		[112]
Theanine synthetase	MEKC	Indirect UV/360 nm		[113]
GTPase	CZE	LIF	Flow gated injection for real time detection	[114]
α-Glucosidase α-1,3-*N*-Acetylgalactosaminyltransferase	MEKC	LIF	Multiple sampling form single cell	[115]
Thymidylate kinase Thymidine kinase	CZE	UV/267 nm		[116]
Protein kinase B	CZE	UV/190 nm		[117]
α-1,3-Galactosyltransferases	CZE	UV/214 nm UV/224 nm UV/270 nm	Post-reaction derivatization	[118]
GTPase	CZE	UV/254 nm	PVA coated and uncoated capillaries	[119]
CYP3A4 (CYP450 isoenzyme)	CZE	Fluorescence	Warfarine, kinetic study Verapamil, kinetic study	[120] [121]
Glutathione S-transferase	MEKC, CEC	UV/200 nm	Reaction with styrene oxide	[21]
Alkaline phosphatase	CZE	EC	Amperometric PMMA-microchip with integrated gold working electrode	[122]

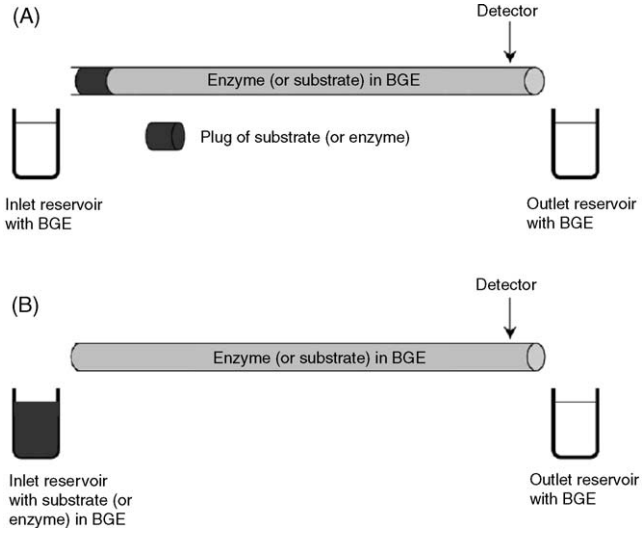

Fig. 5. Schematic illustration of the continuous mode of EMMA: zonal sample introduction (A); moving boundary sample introduction (B); reprint from [124] with permission.

reactant is introduced as a plug or continuously from the inlet vial (Fig. 5). In contrast, in plug–plug mode of EMMA (short contact mode), enzyme and substrate(s) are introduced in the capillary as distinct plugs, the first reactant injected being the one with the lower electrophoretic mobility (Fig. 6). The enzymatic reaction is then initiated in both modes by the application of an electric field since the zones interpenetrate due to the differences in their electrophoretic mobilities.

It is necessary to emphasize that plug–plug mode has several advantages. What is generally the most important for enzymatic reactions, less amount of reactants is needed because only a plug is required as opposed to filling of the capillary and buffer reservoirs. For example the whole kinetic study on rhodanese by means of plug–plug mode of EMMA [125] required only 20 μl of the enzyme preparation (this amount was given be CE apparatus used), whereas approximately 50-times larger amount would be necessary for identical study by means of traditional, for example spectrophotometric assay. It addition, the electropherogram evaluation is simpler that is especially evident from the Fig. 7 where the same the enzymatic system – the conversion of NAD(P) to NAD(P)H in the oxidation of glucose-6-phosphate (G-6-P) to 6-phosphogluconate by glucose-6-phosphate dehydrogenase (G-6-PDH) – was analysed. In their pioneering work,

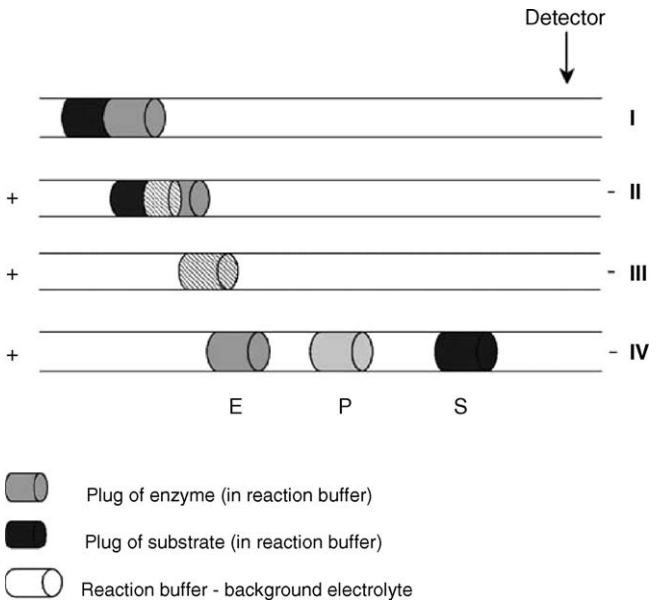

Fig. 6. Schematic illustration of the classical plug–plug mode of EMMA; reprint from [124] with permission.

Bao and Regnier studied this enzymatic reaction by means of the continuous EMMA mode [123]. They added both the substrate (G-6-P) and coenzyme (NADP) required for the catalyzed reaction to the background electrolyte, whereas the enzyme (G-6-PDH) was injected into the capillary. Kwak et al. used for the same purpose the plug–plug EMMA mode loading the reaction compounds as separate plugs into the capillary [126]. As a first plug (a plug with lower electrophoretic mobility) the enzyme solution was loaded, as a second plug (a plug with higher electrophoretic mobility) the coenzyme (NAD) solution was loaded, both solution were prepared using the reaction buffer containing the substrate (G-6-P).

Although the plug–plug variant brought substantial improvements in EMMA methodology, one limitation still left – the necessity to have the electrophoretic conditions compatible with both the separation of substrate(s) and product(s) of the enzymatic reaction and the enzymatic reaction itself. However, sometimes it was impossible to use the classical EMMA arrangement since the conditions required for the enzymatic reaction and for the electrophoretic separation were totally different. This problem was elegantly solved by Van Dyck et al., who introduced the integration of partial filling technique in the EMMA methodology [127]. In this EMMA modification, the part of the capillary is filled with the buffer best possible for the enzymatic reaction whereas the rest of the capillary with the background electrolyte optimal for separation of substrate(s) and product(s). From the time when Van Dyck et al. combined the EMMA methodology with MEKC for study of bovine plasma amine oxidase activity, several other background electrolytes have been applied in this innovative EMMA modification – low pH background electrolyte in combination with direct detection for absorbing inorganic anions with high mobilities [125,128], 10 mM chromate – 0.1 mM cetyltrimethylammonium bromide (CTAB) in combination with indirect detection for non-absorbing inorganic anions with high mobilities [129,130] and 30 mM sorbic acid – 0.1 mM CTAB in combination with indirect detection for non-absorbing organic anions with moderate mobilities [131].

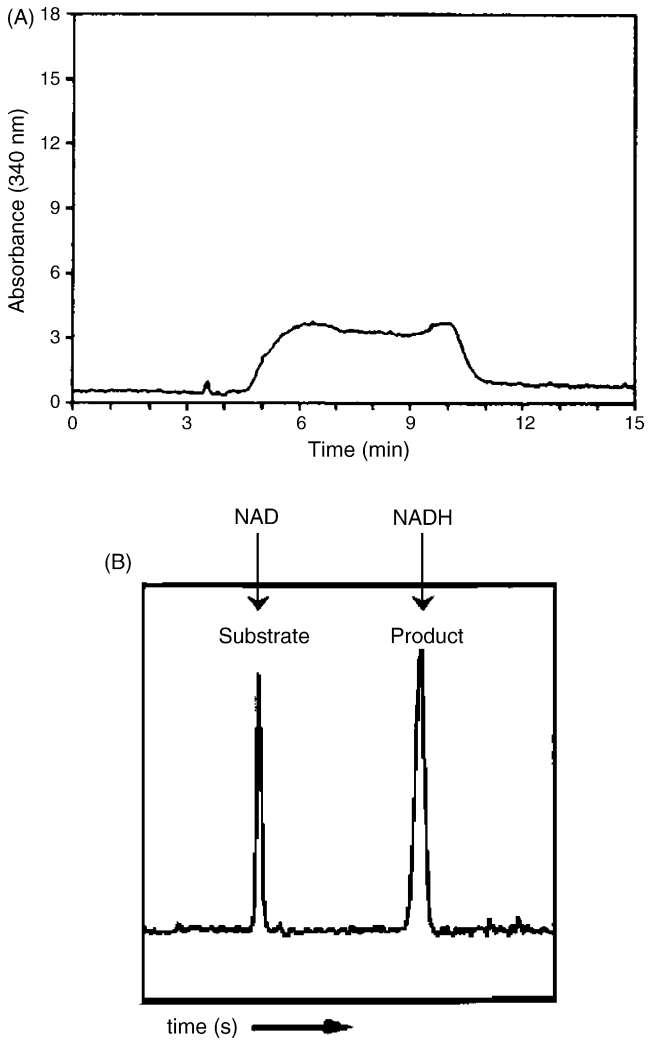

Fig. 7. Typical electropherogram, after zonal sample introduction, showing the formation of NADPH during the process of G-6-PDH migration through the capillary (A); reprint form [123] with permission. Typical electropherogram of the classical plug–plug EMMA assay of G-6-PDH (B); reprint form [126] with permission.

The EMMA methodology particularly its plug–plug mode can be classified as an initial rate enzyme assay, since the reaction proceeds only during the short period of the zones interpenetration. Knowing the mobility as well the length and distance of the reactants zones the reaction time thus can be simply calculated. Similarly, the time of the complete zones interpenetration can be calculated [132]. It can be useful for the prolongation of the reaction and consequent accumulation of more product(s) since turning off the voltage at this time point allows the reaction to continue in the absence of an electric field. This EMMA modification called "zero potential amplification" brings the substantial improvement of the detection sensitivity.

Since 1992 when Bao and Regnier came with this methodology, it has been applied on many enzymatic systems not only for assays of enzymatic activities, but also for determination of substrates, Michaelis constants, inhibitors and inhibition constants

Table 2
Summary of on-capillary (EMMA) assays

Enzyme	EMMA mode	CE mode	Detection	Note	Ref.
Glucose-6-phosphate dehydrogenase	Continuous	CZE	UV/340 nm	Epoxy-based coating	[123]
Leucine aminopeptidase	Continuous	CZE	LIF		[133]
Alkaline phosphatase β-Galactosidase	Continuous	CGE	VIS/405 nm	Gel filled capillaries	[134]
Glucose-6-phosphate dehydrogenase Alcohol dehydrogenase	Continuous and plug–plug	CZE	UV/260 nm UV/340 nm	Enzymatic CE microreactor	[135]
Alcohol dehydrogenase Leucine aminopeptidase Alkaline phosphatase β-Galactosidase	Continuous	CZE	UV/340 nm VIS/405 nm VIS/405 nm VIS/405 nm	Selectivity in EMMA by control of product detection time	[136]
Alkaline phosphatase	Continuous	CZE	EC VIS/405 nm	Carbon electrode	[137]
Lactate dehydrogenase	Continuous	CZE	LIF	Detection limit: 1.3×10^{-21} mol of LDH	[138]
Leucine aminopeptidase	Continuous	CZE	VIS/405 nm		[139]
Adenosine deaminase	Plug–plug	CZE	UV/254 nm		[140]
Adenosine deaminase	Plug–plug	CZE	UV/254 nm	Inhibition study	[141]
Glucose oxidase Galactose oxidase Catalase	Continuous	CZE	Chemiluminescent		[142]
Lactate dehydrogenase	Continuous	CZE	UV/280 nm UV/340 nm		[143]
Phosphodiesterase I	Plug–plug	CGE	UV/260 nm	Degradation of oligonucleotides with	[144]
Glucose-6-phosphate dehydrogense Acid phosphatase	Plug–plug	CZE	UV/200 nm		[145]
β-Galactosidase	Continuous		LIF	EMMA on microchip-inhibition study	[146]
Hexokinase/apyrase Lactate dehydrogense/glucose-6-phosphate dehydogenase	Continuous	CZE	UV/260 nm	Double enzyme-catalyzed CE microreactors	[147]
Alkaline phosphatase	Plug–plug	CZE	UV/230 nm	Kinetic study, coated capillary	[148]
Creatine kinase	Plug–plug	CZE	UV/256 nm		[58]
Glucoamylase	Plug–plug	MEKC	UV/280 nm	Measurement of glucoamylase activities in sake rice koji	[149]
Glucose-6-phosphate dehydrogenase	Plug–plug	CZE	UV/260 nm		[126]
Protein kinase	Continuous	CZE	Fluorescence	EMMA on microchip-inhibition study	[150]
Acetylcholinesterase	Continuous	CZE	LIF	EMMA on microchip-inhibition study	[151]
Lactate dehydrogenase	Continuous	CZE	EC	Carbon electrode	[152]
Leucine aminopeptidase	Plug–plug	CZE	Two-photon excited fluorescence detection	EMMA on microchip	[153]
Fructose-biphosphate aldolase Fructose-1,6-diphosphatase	Plug–plug (partial filling)	CZE	Indirect UV/254 nm	30 mM sorbic acid–0.1 mM CTAB	[131]

Table 2 (*Continued*)

Enzyme	EMMA mode	CE mode	Detection	Note	Ref.
Alkaline phosphatase	Combination	CZE	LIF	Inhibition study of ALP by theophyline	[154]
Alkaline phosphatase	Continuous	CZE	LIF	EMMA on microchip	[155]
β-Galactosidase	Plug–plug	CZE	Fluorescence	EMMA on microchip	[156]
Amine oxidase	Plug–plug (partial filling)	MEKC	UV/254 nm	EMMA combined with MEKC	[127]
α-Glucosidase β-Galactosidase, β-*N*-Acetylglucosamidase	Plug–plug	CZE	UV/214 nm VIS/405 nm		[157]
Catechol-*O*-methyltransferase	Plug–plug	CZE	UV/200 nm	12 mM Hydroxypropyl-β-cyclodextrin	[87]
γ-Glutamyltransferase	Plug–plug	MEKC	UV/380 nm	EMMA combined with MEKC	[158]
Rhodanese	Plug–plug (partial filling)	CZE	UV/200 nm		[125]
Alkaline phosphatase	Combination	CZE	LIF	Inhibition study	[159]
β-Glucosidase	Plug–plug	CZE	UV/214 nm	EMMA in coated capillary with linear polyacrylamide	[160]
Rhodanese	Plug–plug (partial filling)	CZE	UV/200 nm	Inhibition study	[128]
Haloalkane dehalogenase	Plug–plug (partial filling)	CZE	Indirect UV/315 nm/375 nm	10 mM chromate–0.1 mM CTAB	[129]
Angiotensin converting enzyme	Plug–plug	CZE	UV/230 nm	Kinetic study, at-inlet reaction	[161]
Haloalkane dehalogenase	Plug–plug (partial filling)	CZE	Indirect UV/315 nm/375 nm	10 mM chromate–0.1 mM CTAB, inhibition study	[130]
Angiotensin converting enzyme	Plug–plug	CZE	UV/230 nm	Inhibition study, at-inlet reaction	[162]
Glucose-6-phosphate dehydrogense	Continuous	CZE	EC		[163]
Sulfotransferase A1	Plug–plug	MEKC	UV/260 nm UV/274 nm	Inhibitor preseparation Kinetic study	[164]
Alkaline phosphatase	Continuous	CZE	EC		[165]

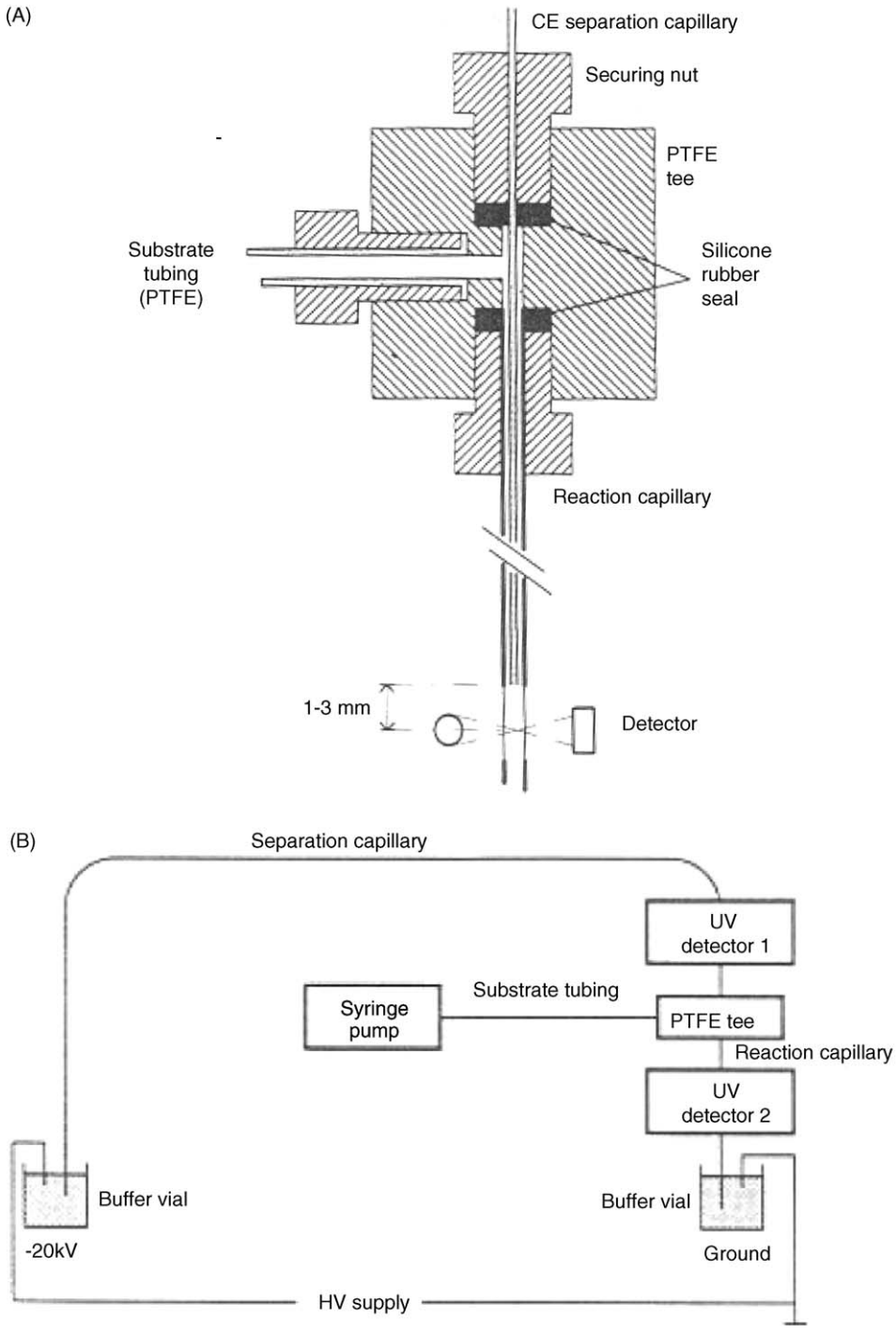

Fig. 8. Schematic of a three-way PTFE tee post-capillary reactor (A). CE system with this type of post-capillary reactor (B); reprint form [168] with permission.

(Table 2.). As it can be seen from the Table 2, different CE modes have been combined with various detection techniques, while separation has been performed in uncoated or coated fused silica capillaries, in gel filled capillaries, even in chip format. More detailed information about the EMMA methodology could be found in three recently published reviews. The first review is oriented mainly on the basic principles and modes of EMMA [124], whereas the other two cover the application scope of EMMA methodology on enzymatic as well as non-enzymatic systems [166,167].

2.3. Post-capillary assay

A technique combining CE with a micro post-capillary reactor for on-line enzyme assays has been developed by Emmer and Roeraade. The home made CE system equipped with two UV detectors was used in their studies oriented on two NADP dependent dehydrogenases – G-6-PDH and 6-phosphogluconate dehydrogenase (6-PGDH). While the first detector monitored the classic CE separation of given enzyme(s), the second one detected NADPH as the product of enzymatic reaction formed

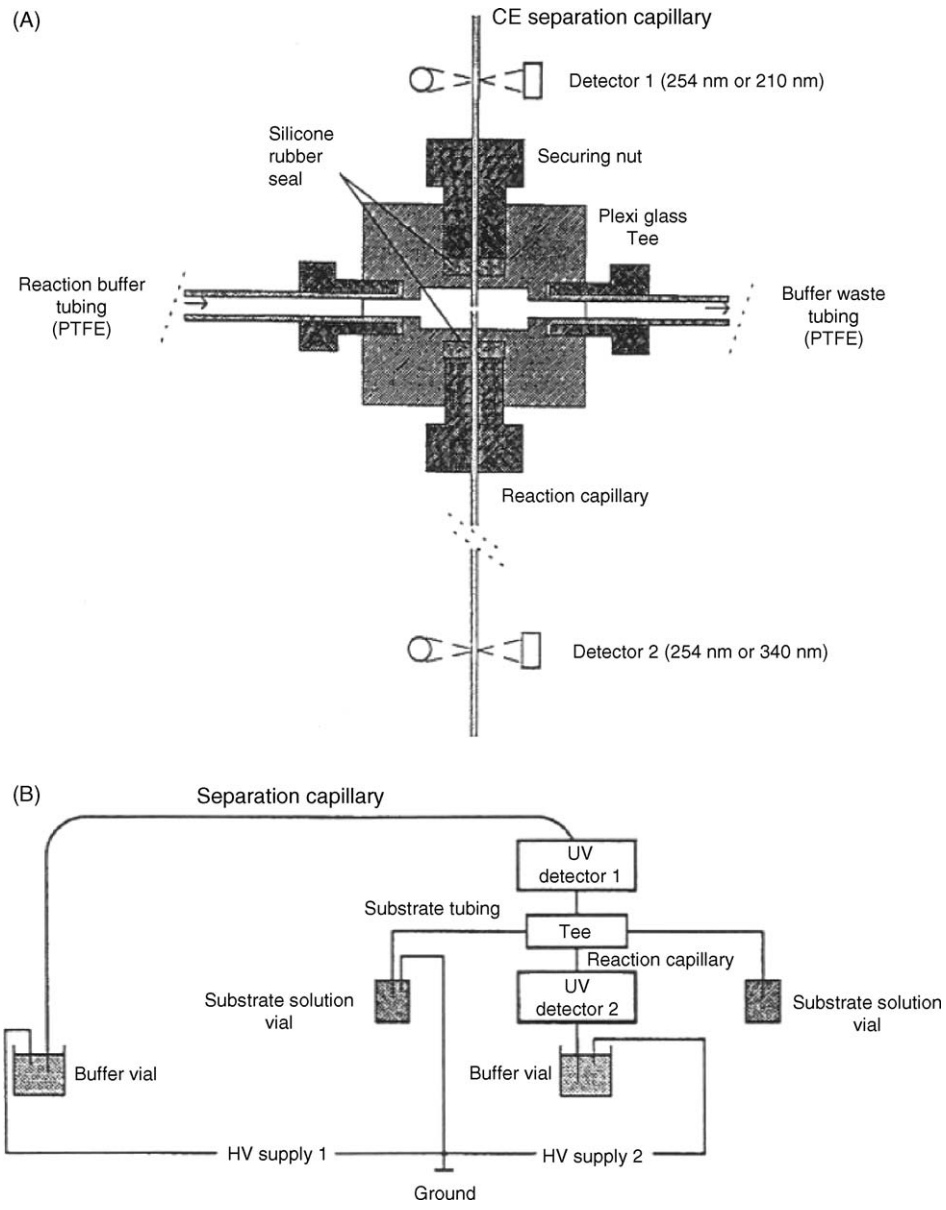

Fig. 9. Schematic of four-way Plexiglas tee post-capillary reactor (A). CE system with this type of post-capillary reactor (B); reprint form [169] with permission.

in the post-capillary section. Fluorosurfactant with negligible effect on the enzymatic reactions was added to the background electrolyte to avoid the enzyme sorption on the wall of separation capillary.

In the first work, Emmer and Roeraade used the post-capillary reactor based on the pressurised sheath flow of the substrate [168] (Fig. 8A). It consisted from a three-way PTFE tee in which both the separation and reaction capillaries were mounted. The separation capillary of 50 μm I.D. was inserted into the reaction capillary of 530 μm I.D. being positioned 1–3 mm above the detection window of the reaction capillary. Substrate solution was introduced into the tee by a PTFE tube by means of syringe pump (Fig. 8B). Since the possibility of sensitivity enhancement was exhausted in this setup, the post-capillary reactor based on the liquid junction was used in their second work [169] (Fig. 9A). The dilution of the reactant is thus minimised since the capillaries for the CE separation, as well as post-capillary reaction were of the same I.D (50 μm). It resulted in enhancement of the detection sensitivity. What is more, the flow could be easily stopped for accumulation of more reaction product as in the case of zero potential amplification in EMMA methodology also resulting in improvement of the sensitivity. The junction between the separation and reaction capillaries was formed inside a four-way Plexiglas tee. Their distance was adjusted to be about 50 μm. The other two way of tee were used as inlet, respectively outlet of the substrate solution. Several possibilities of the introducing of substrate solution into the reaction capillary were utilized in this work. Even though the differences in pH or ionic strength between substrate solution and BGE were tested, the better results were obtained using second HV power supply for generation of the flow (Fig. 9B). Both the conditions for the CE separation and enzymatic reac-

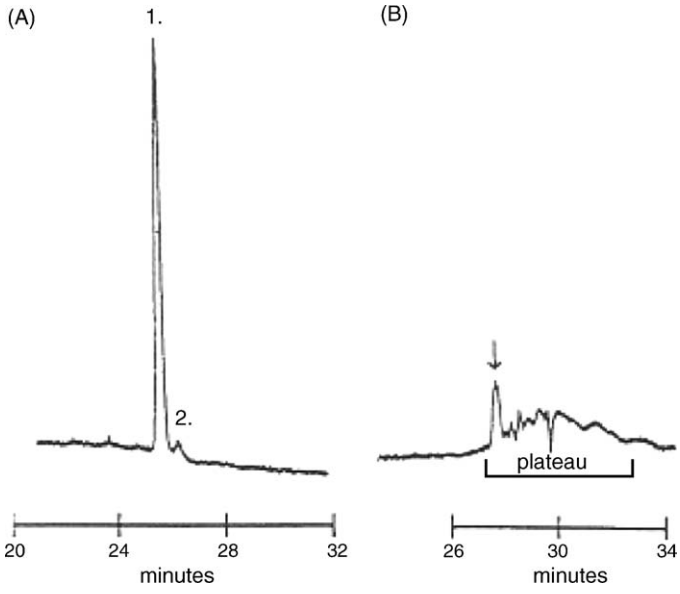

Fig. 10. CE separation and post-capillary enzyme assay of G-6PDH and 6-PGDH. Signal from the first detector (A), signal from the second detector (B); reprint form [169] with permission.

tion thus could be more easily optimised. As example the CE separation and post-capillary enzyme assay of G-6-PDH and 6-PGDH mixture are given in the Fig. 10. The first and second peaks on the electropherogram are 6-PGDH, respectively G-6-PDH (Fig. 10A). NADPH on the record of the enzyme assay (Fig. 10B) was formed in the G-6-PDH reaction since G-6-P was used as a substrate.

Although the main limitation of such technique is probably in incompatibility of post-capillary reactor with most commercial CE instruments; this is also the explanation why the concept did not find larger implementation; it still has some advantages. It is especially the possibility to obtain both classic electropherogram and the corresponding profile of enzymatic activity simultaneously, what should be useful particularly in isoenzyme analysis. In addition, the small sample volumes are required as in the case of EMMA methodology and the analyses are also fully automated. As a result, the other development can be expected by integrating of post-capillary reactors in microchip CE systems.

3. Conclusion

Due to the large diversity in the chemical and physical characteristics of substrate(s) and product(s) of enzymatic reaction, many different methods have been applied for the assay of enzymatic activities. Although photometric methods are undoubtedly the most frequently used, CE based methods will further gain their position in this field. It is for the most part given by the large application diversity of CE including the separation of ionic compounds and neutral molecules, small molecules as well as biomacromolecules; moreover, such separations are characterized by high efficiencies and resolution. The application versatility is further filled out by availability of suitable detection technique from common UV–VIS spectrophotometry through highly sensitive LIF up to mass spectrometry as an absolute detection technique. Besides these methods are rapid, require only small amounts of samples and their on-line variants can be even fully automated. So the development new CE based method above all oriented on the separations in multi-array capillaries or in the chip format can be expected. While the bioanalytical systems based on microfluidics, also called "lab-on-chips" or "micro total analysis systems" (microTAS), are still not very common, they represent a very challenging and fast-developing area of research. They bear the promise of developing in the near future low cost, powerful and high throughput systems for biological and medical research, in strong synergy with the post-genomic revolution. A high throughput and companion informatics systems have also great potential in the biotechnology and pharmaceutical development.

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