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Review

Analysis of metallothionein by capillary electrophoresis

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ABSTRACT

Metallothioneins (MTs) belong to proteins playing a key role in metal ion homeostasis, maintenance of the redox pool and free radical scavenging in both prokaryotic and eukaryotic cells. Strong interactions of the MTs with essential and non-essential metal ions as well as unique MT structure and behavior under various conditions are subjects of numerous studies. Among other analytical techniques, capillary electrophoresis (CE) has been proven to be an effective tool not only for determination of MT in biological samples, but also for the identification of its isoforms and sub-isoforms in various types of samples. Moreover, CE has a great potential to investigate MT–metal and MT–protein interactions, which has not been fully utilized yet. Thus, it is not surprising that numerous studies devoted to the optimization of CE conditions such as background electrolyte composition, electrolyte modifiers and/or capillary surface modifications have been carried out since MT's discovery in 1957. From the MTs' detection point of view, optical detectors including absorbance, laser-induced fluorescence have been employed. Also mass spectrometric detection coupled to the various ionization techniques including inductively coupled plasma (ICP) and electrospray ionization (ESI) has been utilized for detail MT characterization and sensitive determination. In this paper, articles published from eighties to 2011 are reviewed, presenting both optimization of key parameters of CE method for MT determination as well as utilization of CE as a routine analytical technique for further investigation of complex biological and biochemical processes where MT is a key component.

Contents

1. Introduction

Since the discovery of metallothioneins (MTs) [1], these low-molecular mass proteins have been attracting attention of

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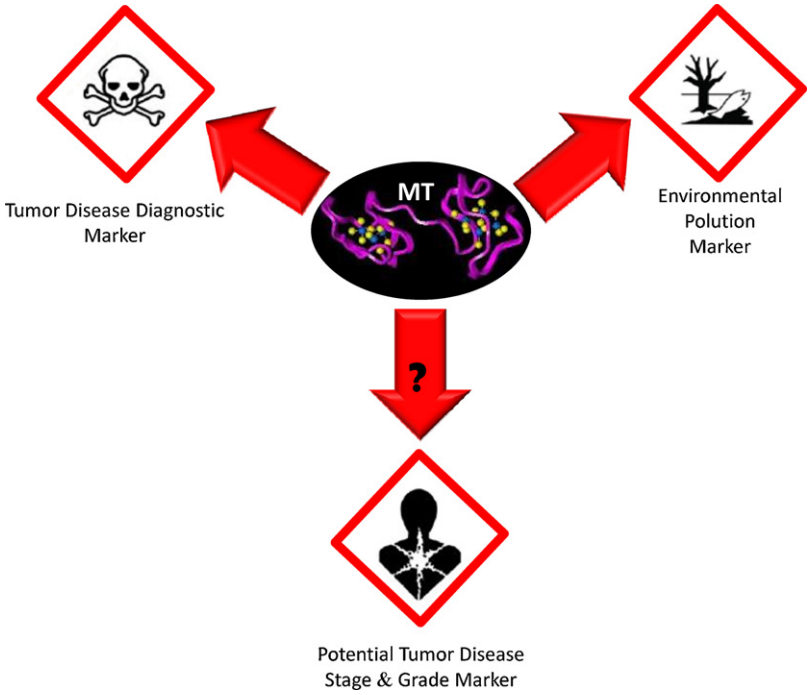

Fig. 1. MTs can be used as an environmental pollution marker and tumor diagnostic marker, however potentially it can be used as a marker of the tumor disease progression.

numerous scientists due to their unique properties and key functions in organisms. MTs are consisting of two domains: β-domain which is able to bind 3 divalent or 6 monovalent metal ions, and α-domain binding 4 divalent or 6 monovalent metal ions. There were 4 mammalian MT isoforms (MT-1, MT-2, MT-3, and MT-4) discovered up to now and 13 MT-like human proteins identified [2]. Despite the physical–chemical similarity of the forms, their roles and occurrence in tissues vary significantly. MT-1 and MT-2 and their sub-isoforms are present almost in all types of soft tissues; MT-3 is expressed mostly in brain tissue but also in the heart, kidneys and reproductive organs, and MT-4 gene was detected in epithelial cells [3]. MTs without metal ion, so-called apo-MTs, are present in zinc deficient cells. These exceptional metal binding abilities are enabled by highly abundant cysteine, which can comprise up to one-third of all amino acids. Despite the extensive research the proper functions of MTs are still not completely

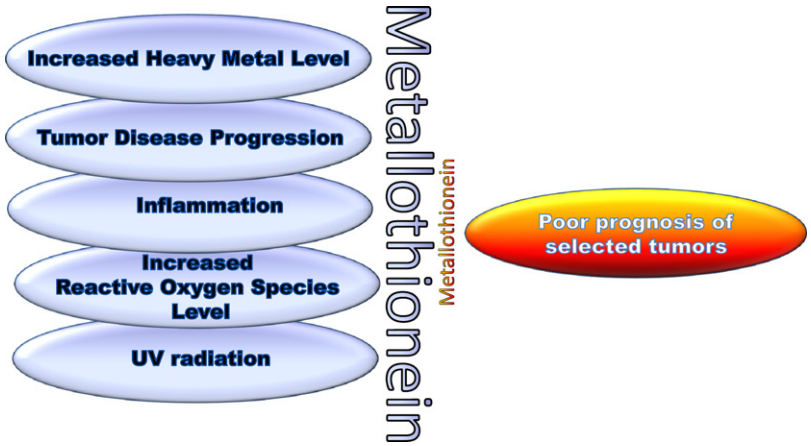

Fig. 2. Factors enhancing the MT expression include UV radiation, inflammation, increased level of reactive oxygen species, and increased level of heavy metals as well as tumor disease progression. The down-expression of MT is connected to poor prognosis in case of selected tumors.

elucidated. Nevertheless, MT has been implicated in a wide range of roles including toxic metal detoxification, homeostasis maintaining of both zinc and copper [4], prevention of DNA oxidative stress damage [5], and/or the redox pool maintenance [6]. It is not surprising that MTs are involved in many pathways and cycles dependent on metal ions or scavenging of reactive oxygen radicals [7,8]. *Metal ions dependent involvement*: The anti-apoptotic properties of MTs are discussed in the relation to the uncontrolled malignant cell proliferation (Fig. 1). This feature can be used for diagnostics of various cancer types [7–10]. The relation of MTs with transcription processes has been described also under physiological conditions in normal proliferating cells [11–14]. Due to the metal-induced MT's expression [15,16], this multifunctional protein is used as a marker of environmental pollution [17–20]. Furthermore, the overexpression of metal-binding biomolecules including MTs, glutathione and phytochelatins for phytoremediation of environment polluted with metal ions has been applied [21–24]. *Redox pool maintaining*: Free radical scavenging function of MT has been suggested as an explanation of the ability of MT to protect the organism against UV radiation-induced damages followed by melanoma occurrence [25]. Besides this, a review considering the role of MT in aging process was published by Swindell [26]. Moreover, it was found that the MT participates in the processes induced by acute phase cytokines that mediate inflammation. The inflammatory response may be accompanied by a number of systemic changes referred to collectively as the acute phase response. The organs participating in the response include liver, which synthesizes increased amounts of metallothionein and antioxidants [27–31]. Factors influencing the MT expression are shown in Fig. 2.

2. Analytical techniques for MT detection

Based on the abovementioned fact, metallothioneins represent a group of highly important analytes extensively studied due to their unique properties and essential biological functions. Little information about MTs is found in general reviews focused on proteomics [32,33] as well as metallomics and/or metalloproteomics [34–36]. Also, general analytically oriented reviews are mentioning MTs as analytes even though these papers are usually primarily oriented on the advances in particular analytical technique rather than on MT itself [37–40]. On the other hand, articles devoted to the MTs only, their properties, structure and behavior have been published, too [41–43]. In 2002, the review providing an interesting summary of the knowledge about MT's primary, secondary and tertiary structures, reactivity and mechanism of metal binding was published by Romero-Isart and Vasak [41]. In 2010, Blindauer and Leszczyszyn published a comprehensive review on MT's structural and functional diversity, covering also the certain aspects of MT's analytical chemistry particularly mass spectrometric and nuclear magnetic resonance (NMR) analysis [43].

Another group of reviews includes articles summarizing the whole range of various analytical methods used for MTs analysis [44]. An example is a paper by Vodickova et al. reviewing analytical methods for the determination of plant metallothioneins. Special attention is given to chromatographic and electrophoretic methods and their combination with selected detection techniques (AAS, MS, and ICP MS). In 2010, Adam et al. presented a review article on trends in analytical techniques for MT determination including the isolation procedures from various samples [45]. Similar paper summarizing methods applied for MT detection has been published by Ryvolova et al. [3]. This article is paying attention not only to the structural analysis of MTs but also to the molecular biology methods, separation techniques combined with various detectors as well as electrochemical detection including Brdicka reaction and H-peak. Studies of metal binding reactions in metallothioneins by spectroscopic, molecular biology, and molecular modeling techniques were reviewed in 2002 by Chan et al. [46]. This article highlights the contribution of ^{113}Cd, ^{1}H NMR and X-ray diffraction method to determine MT structure and to observe tetrahedral coordination of metals in two isolated domains. Moreover, the role of optical spectroscopy particularly circular dichroism and luminescence in understanding of complicated metal binding chemistry is described. More recently, a review on determination of MTs in aquatic organisms has been published by Shariati and Shariati [47]. This article covers electrochemical methods such as differential pulse and cathodic stripping voltammetry, saturation analysis methods based on Cd, Ag and Hg, spectrophotometric methods as well as chromatographic and electrophoretic techniques. Also, MT-mRNA and immunological techniques followed by PCR are summarized in this article. Even though this review is limited to aquatic organisms, it demonstrates clearly that the range of suitable analytical methods is broad.

The other group of reviews deals with particular analytical techniques used primarily for MT determination. The work by Maret showed that various chemical modifications of MT's cysteine thiol groups with fluorescent probes allowed determination of three states of the protein of interest: metallothionein (zinc-bound thiolate), thionein (free thiols), and thionin (disulfides). The examination of MT's zinc-binding properties with fluorescent chelating agents revealed that the affinities vary significantly for the seven zinc ions. Finally, fluorescence resonance energy transfer (FRET) sensors for investigating MT's structure and function were created [48]. Torreggiani describes Raman spectroscopy as a fitting method for MT analysis due to its ability to define the metal-binding properties. He found that the oxidation state of cysteine residues and their participation in the metal complexation can be clearly defined, as well as the potential participation of histidine residues. Based on the presented results it can be concluded that Raman spectroscopy can be also useful tool for providing information on the favorite sites of the radical attack and radical-induced modification in protein folding [49]. The review by Liu and Wang focused on the metal release and sequestration by MTs monitored by electrochemistry and surface plasmon resonance (SPR) [50]. Also the group of electrochemical methods was used for MTs determination as it is summarized in the following reviews [3,45,51]. The electrochemical determination of MTs is based on catalytic processes, which proceed at very negative potentials on mercury electrodes. These processes accompanied by evolution of hydrogen from supporting electrolyte components, include the presodium wave and/or the Brdicka reaction. It was found that SH groups present in MTs are responsible for catalytic processes. The catalytic signal of a MT at nanomolar concentrations can be detected on mercury electrodes using potentiostatic electrochemical methods. However, as the determination of various isoforms is concerned, there must be a very sophisticated detection system connected with separation one. CE as one of the analytical methods commonly used for MT analysis, especially due to its ability to separate MT isoforms and to capture the metal–protein interaction, is of great potential. Therefore, this review primarily focuses on the using of this promising separation technique in MT analysis. The summary of scientific papers containing various types of information on MT is shown in Fig. 3.

3. CE of MT

Since the introduction of CE by Jorgenson and Lukacs in 1981 [52], the technique has rapidly developed into a versatile analytical tool. Generally, the separation mechanism in CE is based on the electrophoretic mobility of the analyte in the electric field as well as on its size. The electrophoretic mobility is directly

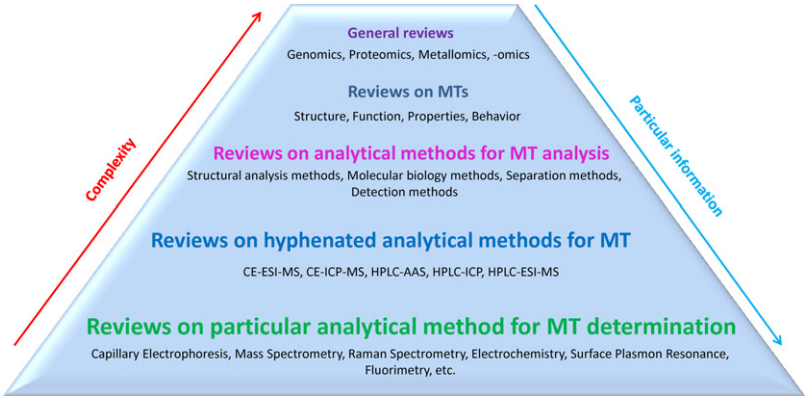

Fig. 3. Scientific papers containing various types of information on MT. Reviews focused mainly on genomics, proteomics, metallomics and other – omics contain general information about MT only. Then, reviews on structure, function, properties and behavior of these proteins follow with more detailed information on MT but still of limited interest for analytical chemists. Reviews on analytical methods for MT analysis and reviews on hyphenated analytical methods for MT are of great importance for analytical chemists because these types of reviews summarize current state of art of all analytical methods used for MT detection. On the very base of the pyramid, reviews on particular analytical method for MT determination are located, because these reviews highlight a single method only but comprehensively.

proportionate to the charge and indirectly proportionate to the electrolyte viscosity and Stokes' radius of the analyte [53–56]. As the MT is concerned, separation of its isoforms and metal affinity studies are the main areas of CE application. Already in 1998, the strategies for the qualitative and quantitative analysis of metallothionein isoforms by capillary electrophoresis were summarized by Beattie [57]. At the same time, coupled techniques for MT analyses were reviewed by Lobinski et al. [58]. Later on, CE applied for MT determination was a subject of review by Minami et al. [59]. Also hyphenated techniques for MT characterization and quantification were reviewed by Prange and Schaumlöffel [60]. Developments in quantification methods for metallothionein were summarized by Dabrio et al. [61]. Generally, most papers focused on optimization of CE analysis of MTs have been published at the end of the last and at the beginning of this century. Several researchers, such as Richards and Beattie [62–74], Minami and coworkers [59,75–87], Virtanen [88–95], and Wilhelmsen [96–99] studied systematically CE of MT. Their results as well as the results from other groups are discussed in the following subchapters.

3.1. MT sample pre-treatment

The extraction of peptides/proteins from biological matrices can be performed using various sample-preparation techniques such as homogenization, centrifugation, precipitation, solvent extraction, liquid–liquid extraction, solid-phase extraction, (micro)dialysis and ultrafiltration. Using a sample preparation technique or a combination of several sample-preparation techniques is frequently insufficient to selectively extract the target analyte from a biological matrix. In such cases, combination with a separation technique such as liquid chromatography or capillary electrophoresis can be used to separate the target analyte from interfering co-extracted matrix components [100]. The sample pre-treatment improves the practicability of the CE and simplifies the interpretation of the obtained signals. However, it cannot prevent the variation of the migration times of different electrophoretical runs, which thus remains the main problem of the speciation analysis via CE. Generally, the MTs have been determined in a variety of animal and plant matrices. In combination with CE, mostly animal tissues are in the center of interest. Size exclusion chromatography and anion exchange chromatography are the most common techniques used for sample preparation followed by CE separation [60,101,102]. The pre-treatment procedures are summarized below. Their using for a sample preparation for CE is uncommon, but it could be very convenient for some future applications.

3.1.1. Blood, blood serum and cells

Isolation and consequent detection of MT in blood and/or blood serum samples is not so frequently carried out in comparison with tissues analysis. Heat treatment of a sample (approx. 100 °C for more than 5 min) to denature and to remove high molecular mass proteins from samples proposed by Erk et al. [103] is nowadays successfully applied to blood and blood serum samples [104,105]. Moreover, Petrlova et al. showed that using of tris(2-carboxyethyl)phosphine as a reducing agent could be beneficial for quantification of MT. The modified method was utilized for the preparation of blood and blood serum samples of patients with various tumor diseases [106,107] or fish sperm preparation [108]; in such samples these proteins have not been quantified before. Caulfield et al. used heat treatment for the preparation of human red blood cells [109]. Cells were disrupted by repeated freeze–thawing cycles. The lysates obtained were heat treated and analyzed. The authors had drawn blood from patients by venipuncture into tubes containing heparin. The presence of heparin or other compounds such as ethylenediaminetetraacetic acid (EDTA) can seriously affect quantification of MT in blood, blood serum or blood fractions, when electrochemical methods are used. Adam et al. showed that the presence of EDTA influenced voltammetric signals markedly [110].

3.1.2. Animal tissues

To isolate MT from animal tissues, a preparation of crude extract from a tissue and purification of such extract by using gel filtration is one of the most commonly used protocols [34]. Tissue extract is prepared in the presence of Tris–HCl with added sucrose [64], glucose and antioxidant species (mercaptoethanol, dithiothreitol and/or TCEP) [44]. This extract is centrifuged or heat treated with subsequent centrifugation. Erk et al. reported on comparison of different procedures to purify MT from the digestive glands of mussels (*Mytilus galloprovincialis*) exposed to cadmium: heat treatment (at 70 and 85 °C), solvent precipitation, and gel-filtration [103]. They found that the most convenient approach was using heat treatment for the preparation of both heavy metal stressed and non-stressed tissues with consequent voltammetric detection. Moreover, Beattie et al. successfully utilized solid phase extractors for MT isolation [65].

3.1.3. Plant tissues

Preparation of plant tissues, cells and parts to isolate phytochelatins (included into MT Class III) have been shown in many papers and reviewed repeatedly [111]. MT Class I and II cannot

be found in plant tissues without genetic modification of a plant genome. Macek et al. inserted MT yeast and human genes into tobacco to enhance their ability to accumulate metal ions [112]. To detect MT, Diopan et al. prepared crude extracts from these plants and heat treated the extracts. Results on content of MT were similar to those detecting expression of mRNA [113].

3.2. Optimization of CE method for MT determination

In this section, an overview of articles focused on key CE parameters optimization for MT determination is presented. Three main factors such as electrolyte, capillary and detection method have to be optimized in order to reach required resolution and sensitivity within a reasonable time scale. The general impact of these parameters on the CE separation was summarized elsewhere [114–118]. Briefly, the fundamental constituent of CE is electroosmotic flow (EOF), a bulkflow of the liquid in the capillary, resulting from the surface charge on the inner wall of capillary as well as the electric field applied on the solution. The EOF controls the amount of time solutes remain in the capillary by the superposition of flow on the solute mobility. The modification of the EOF results in the separation performance variations. The main means influencing the EOF include electric field, electrolyte pH, ionic strength and composition as well as electrolyte additives, capillary wall coating and separation temperature. EOF changes proportionally with the electrical field, however high electrical field generates Joule heating in the capillary. On the other hand, the decrease of the electric field reduces the efficiency and resolution. High ionic strength of the background electrolyte generates high current causing the Joule heating. However, low ionic strength and low buffer capacity causes distortion in peak shapes (if the conductivity of the electrolyte differs from the conductivity of the analyte) as well as increased adsorption on the capillary wall. The ionic strength also affects the zone width. Additives to the electrolyte are responsible for changes in magnitude and direction of the EOF (anionic surfactants can increase the EOF, cationic surfactants can decrease the EOF) influencing the resolution as well as the efficiency. Organic modifiers change the ζ-potential and electrolyte viscosity thus causing complex changes of EOF.

3.2.1. Electrolyte composition and electrolyte modifiers

Background electrolyte (BGE) as a key component of CE method can be changed easily according to the type of analyte and its properties. Optimal BGE composition, concentration and pH are the main parameters to be determined to obtain stable, reproducible and valuable results. Even though the majority of CE experiments are carried out in aqueous solutions such as borate, phosphate, acetate and others, also non-aqueous capillary electrophoresis methods have been developed [119–121]. Moreover, addition of certain modifiers to the electrolyte solutions can significantly influence the separation by minimizing wall interactions, improving selectivity, resolution and controlling the electroosmotic flow [122]. Except the electrolyte composition, the pH value is a main parameter. The pH regulation is an important purpose of the BGE in order to keep the migration velocity of weak electrolyte components and the velocity of the electroosmotic flow (EOF) constant. The pH is of key importance for all electromigration phenomena in systems with weak electrolytes [123]. During the years of MT research, a variety of CE conditions have been tested, optimized and verified. Short summary of selected CE conditions is listed in Table 1.

Already in 1993, Beattie et al. [64] introduced a CE method for MT isoforms separation employing 10 mM Tris–HCl buffer with pH 9.1. Purified and semi-purified MT samples were analyzed for qualitative assessment of purity, relative isoform abundance and separation characteristics of MT from different species. Numerous methods for MT separation have been developed employing BGEs of various composition and pH value. Also, separation of MT isoforms from horse, rabbit and rat at low pH was demonstrated [99].

In 1996, Virtanen et al. presented a systematic study of the impact of buffer composition, concentration and pH as well as temperature and voltage on separation of MT isoforms [94]. Later, the same author introduced CE method using Tris–tricine buffer containing methanol as a BGE [91]. Tricine buffer as well as Tris-borate buffer was employed in following studies by Virtanen et al. [90,92].

CE and reversed phase high performance liquid chromatography (RP-HPLC) methods were compared by separation of rabbit liver MT isoforms [70]. CE was performed in phosphate buffer (10 mM) at pH 2.5, 7.0 and 11.0. CE at pH 2.5 resolved three distinct peaks of rabbit liver MT, which were incompletely resolved at pH 7.0 or 11.0. RP-HPLC at pH 2.5 gave two peaks and the resolution was not as good as with CE at the same pH. At pH 7.0 and 11.0, RP-HPLC was not able to resolve MT-1 and MT-2 at all.

The application of electrolyte modifiers and additives is a common method for influencing the separation performance. Generally, the resolution, analysis time, sensitivity and analyte detectability can be significantly improved by the addition of either organic solvent – methanol, ethanol, acetonitrile, etc., inorganic molecules – EDTA and/or $CuSO_4$ as well as organic compounds including SDS and/or cyclodextrines into the BGE. For MT determination numerous additives have been tested for separation improvement.

Electrolyte modifiers such as methanol, ethanol, propanol, acetonitrile, and acetone were tested by Virtanen et al. in 1998 [95]. It was shown that the general trend was the same for all organic solvents, but the rate of the effect was different. A 40% content of the protic solvents – methanol, ethanol, and 2-propanol – in the buffer led to a decrease in electroosmotic mobility and current in the ranges of 75–90% and 47–67%, respectively. Whereas for acetonitrile and acetone the decrease in electroosmotic mobility was almost linear, for all the protic solvents used the decrease was steeper on addition of the first 15–20% of solvent.

Modification of BGE by cyclodextrines for MT isoform analysis was presented by Wilhelmsen et al. in 2004 [96]. Using phosphate buffers of 500 and 100 mM, at pH 1.5 and 7.4, respectively, modified by various types of cyclodextrines, better results with respect to resolution, peak heights and baseline stability for the various MT forms were obtained. EDTA was also tested as an electrolyte additive and it was found to be useful for identification of MT isoforms [78].

Depending on the concentration of the electrolyte modifier, principally different separation modes called micellar electrokinetic chromatography can be introduced. In this approach, the ionic surfactant is added to the BGE in the concentration exceeding the critical micellar concentration leading to the micelle formation. A fraction of the analyte is incorporated into the micelle and migrates at the micelle's velocity. Upon the interaction between the analyte and the micelle, known as micellar solubilization, the reaction quickly reaches an equilibrium. Therefore, the migration velocity of the neutral analyte depends on the fraction of the analyte incorporated by the micelle [124]. Systematic optimization of micellar electrokinetic chromatography (MEKC) method in terms of capillary length, pH, buffer and SDS concentration used as a buffer modifier was carried out for MT determination [63]. The authors found that optimal conditions depend on the MT sample. In case of sheep MT, the pH should be around 8.4 and SDS concentration in the range of 80–90 mM. In contrast, the best separations of rabbit MT were obtained at pH 10.4 and SDS concentration of 100 mM. The comparison of numerous conditions was also investigated in other paper by this author [71].

Table 1
Summary of basic parameters (capillary length and internal diameter, BGE composition and pH, type of modifiers, voltage and detection method) applied for MT analysis.

Capillary length (internal diameter)	BGE composition	BGE pH	Additives	Voltage (kV)	Detection	LOD	Ref.
110 cm (50 μm)	50 mM Tris	9.1	NO	25	ICP-MS	^{114}Cd 0.21 mg/L MT 2.36 mg/L	[183]
100 cm (75 μm)	50 mM Tris–HCl	9.1	NO	30	Absorbance (185 or 214 nm)	MT 1–10 μg/ml	[64]
100 cm	5 mM acetate or 10 mM acetate	6	NO	20	ICP-MS or ESI-MS	–	[150]
100 cm (75 μm)	100 mM acetic acid–100 mM formic acid	2.3	NO	30	Absorbance (200 nm) ESI-MS	–	[157]
100 cm (50 μm)	50 mM Tris	9	NO	25	ICP-MS	^{114}Cd 0.01 mg/L	[144]
100 cm	12 mM Tris	7.5	NO	20	ICP-MS	0.01 mg/L ^{114}Cd	[146]
(75 μm)	30 mM acetate	7.2			ESI-MS Absorbance		
100 cm (75 μm)	12 mM Tris–HCl 25 mM ammonium-acetate	7.5 6.8	NO	20	ICP-MS ES-MS	–	[149]
75 cm	20 mM Tris–HNO_3	7.4	NO	30	ICP-MS	^{114}Cd	[149]
100 cm	25 mM ammonium acetate	6.8		20	ESI-MS	0.0008 μg/ml ^{64}Zn 0.0021 μg/ml	
70–120 cm (75 μm)	20 mM Tris–HNO_3	7.4	NO	30	ICP-MS	–	[129]
81 cm (75 μm)	70 mM Tris	7.5	5% MeOH	20	Absorbance (200 nm) ICP-MS	MT 3–4 μg/ml	[174]
79 cm (75 μm)	70 mM Tris	7.4	NO	20	Absorbance (254, 220 and 280 nm)	0.31 μg/ml	[102]
77 cm (50 μm)	20 mM Tris	7.8	NO	25	ICP-MS Absorbance (200 nm)	MT-1 1.52 mg/L ^{114}Cd 0.11 mg/L	[143]
61 cm (75 μm) 74–79 cm (75 μm)	70 mM Tris	7.4	NO	20	Absorbance (200, 220, 254 nm) ICP-MS	MT 182–263 ng/ml	[142]
74 and 79 cm (75 μm)	70 mM Tris	7.4	5% MeOH	20	ICP-MS	6 ng/ml	[151]
70 cm (75 μm)	100 mM Tricine–NH_3	7.2	NO	30	ICP-MS	–	[179]
70 cm (75 μm)	20 mM Tris	7.4	NO	30	ICP-MS Absorbance (250 nm)	MT-1 0.011 mg/L MT-2 0.023 mg/L	[184]
70 cm (75 μm)	20 mM Tris	7.4	NO	30	ICP-MS	MT-1 72.6 mg/L	[148]
70 cm (75 μm)	20 mM Tris	7.4	NO	30	ICP-MS ESI-MS	MT 80 mg/L ^{114}Cd 3.5 mg/L	[156]
70 cm (75 μm)	20 mM Tris–HNO_3	7.4	NO	30	ICP-MS	–	[101]
70 cm (75 μm)	20 mM Tris–HNO_3	7.4	NO	30	ICP-MS	–	[185]
57 cm (50 and 184 μm)	200 mM Formic acid	2.2	NO	−25 to −30	ESI-MS	–	[140]
57 (50 μm)	110 mM Tris–110 mM borate	6.9	NO	11	Absorbance (200 nm)	–	[90]
57 cm (75 μm)	110 mM Tris–110 mM borate	6.9	NO	11	Absorbance (200 nm)	–	[94]
57 cm (75 μm)	100–500 mM Borate	8.4–10.4	SDS (75–100 mM)	10	Absorbance (190–340 nm)	–	[63]
57 cm (75 μm)	20 mM phosphate	7.0	NO	20	Absorbance (200 nm)	28 ng/ml	[136]
57 cm (25 μm)	100 mM sodium borate	8.4	75 mM SDS	20	ESI-MS	–	[182]
57 cm (50 or 75 μm)	110 mM Tris–110 mM borate	6.9	MeOH (10–40%)	11–22	Absorbance (200 nm)	–	[95]
50 cm (75 μm)	100 mM borate	8.4	75 mM SDS	10	Absorbance (200, 214, 254 and 280 nm)	–	[62]
37 cm (50 μm)	150 mM phosphate	3.5	NO	−12	Absorbance (214 nm)	–	[186]
37 cm (75 μm)	20 mM H_3PO_4, 50 mM acetic acid–50 mM formic acid, 100 mM acetic acid	2 2.5–5 5–6	NO	25	Absorbance (200 nm)	–	[165]
24 cm (25 μm)	Sodium phosphate (25–800 mM) and borate buffers (25–500 mM)	1.5–8.0 and 8.0–10.0	NO	8–20	Absorbance (200, 254 and 280 nm)	–	[97]
24 cm (25 μm)	Phosphate 500 and 100 mM	1.5 and 7.4	Cyclodextrines	10 and 16	Absorbance (200, 254 and 280 nm)	–	[96]
17 cm (25 μm)	200 mM sodium phosphate	2.0	NO	10 or 12	Absorbance (200 nm)	5 μg/ml	[99]

3.2.2. Capillary surface modifications

The surface of fused silica capillaries is covered by silanol (SiOH) groups at a density of ~4.5 nm^{-2} [125,126]. The p*I* of silica but also the pK_a of SiOH both depend on the quality of the silica material, but are also affected by the nature and composition of the solvent within the capillary. The p*I* of silica varies between ~2 and 3. Since the extent of deprotonation and thus the surface charge of silica is highly related to the pH of the adjacent solution, pK_a of SiOH groups has been referred with 5.3–6.3; however, other authors even assigned a broader pH range between 3.5 and 8.2 [127]. The protein interaction with capillary surface is a complex process which was in detail described by Stutz [127]. To avoid or diminish the undesirable interactions of the analyte with the capillary wall, numerous approaches have been taken including capillary wall coating [128]. The main purpose of the coating is a suppression of protein adsorption onto the capillary wall ensuring high efficiency and reproducibility for separation. Generally, there are two common methods of deactivation the capillary wall: (a) dynamic and (b) permanent coatings. In case of dynamic coating, depending on the nature of additive, interactions are based on columbic attractions, hydrogen bonding or van der Waals forces. The main advantage of dynamic coating is the simplicity of its preparation. Regeneration of the surface between runs can be performed effectively by using strong basic solutions [128]. Dynamic coating additives can be either present in the electrolyte during the run or it can be used during conditioning prior to the separation. Permanent coatings are pre-attached to the surface of the silica wall and therefore excluded from the buffer electrolyte system during runs. It can be distinguished between the substances that are covalently attached to the capillary surface and the coatings that are not covalently attached but adsorbed to the surface by physical or ionic forces. Comparatively, adsorbed coatings are simple to prepare whereas covalent bonded coatings require elaborate chemical reactions [128].

Even though numerous CE analyses of MTs have been performed in uncoated silica capillaries [59,94], several articles describing utilization of surface-modified capillaries have been published. One of the most widely used surface coatings for MT separations is a linear polyacrylamide [66,76–80,84–86]. This coating eliminates the EOF and enables to separate MT isoforms in a buffer of near physiological pH [59]. Polyamine-based surface modifications were also utilized for MT separation [66,71]. Paper by Wang and Prange [129] presents the comparison of different surface coatings (permanent polyAAMPS coating, dynamic PEI coating and dynamic Qpip coating) for MT analysis. Among these three types of coated capillaries, anionic polymer (polyAAMPS)-coated columns offered unique improvements in the separation of MT isoforms compared to the uncoated ones. Based on the comparison of three tested coating it follows that (polyAAMPS)-coated columns provided the best separation efficiency.

3.2.3. Detection techniques

3.2.3.1. CE with optical detection. Two main modes of optical detection in CE are photometric (absorbance) and fluorimetric detection. These types of detection have certain advantages as well as disadvantages. Even though the absorbance detection is highly universal especially in the deep UV range of spectra, its sensitivity is dependent on the optical pathlength, which is given by the capillary diameter. Thus, strategies to compensate and improve analyte detectability in CE have been attempted, including on-line preconcentration methods [130–132]. On the other hand, fluorimetric detection exceeds due to the extremely high sensitivity, however the applicability is limited for the fluorescent or fluorescently labeled compounds. In case of MT analysis, fluorimetric detection coupled to CE is not very common method and to author's best knowledge it was used only for the separation of MT-mRNA using SYBR I green as a fluorescent label [133] and in studies by Krizkova et al. [134,135].

MT absorbance detection is mostly carried out at 200 nm employing the light absorption by the peptide bond due to the absence of aromatic amino acids in MTs' primary structure. However, it was shown that the metal binding process leads to the increase of absorbance in the range from 220 to 280 nm. The absorption maximum of the MT-metal complex is between 240–255 nm. Therefore studies employing the wavelength in this range were published [96,97]. In order to obtain additional spectral information spectrophotometric detection using diode array detector was used to monitor the metal–protein interaction [88–90]. The diode array detector also allows the assignment of a peak to an isoform complexing a metal, and the evaluation of the degree of complexation. The UV spectra of the main peaks are typical for metallothioneins, containing shoulders for Zn-thiol and Cd-thiol bondage at 225 nm and 250 nm, respectively.

As noted previously, the sensitivity of absorbance detection is limited and therefore large volume sample stacking (LVSS) technique was applied for MT analysis in the paper by Alvarez-Llamas et al. [102]. They achieved a 10-time improvement in concentration-based LODs. The other way how to use photometric detection was shown by Knudsen Beattie [136], who described an in-house made on-line solid-phase extractor for the analysis of metallothionein isoforms in sheep fetal liver. It was shown that the precision was <10% and detection limits were at least 28 ng/ml for standards and 272 ng/ml for biological samples. It can be considered as a versatile way how to use CE-UV for real sample analysis.

3.2.3.2. CE with mass spectrometric detection. Mass spectrometric (MS) detection has developed into one of the most popular and useful detection techniques in separation science. MS is well suited for protein analysis due to its sensitivity and selectivity, and due to its ability to characterize proteins. MS can considerably enhance the utility of CE by providing information about the identity of the separated compounds. Therefore, coupling of CE to MS creates a powerful analytical tool for the characterization of intact proteins. For CE–MS of proteins, electrospray ionization (ESI), matrix assisted laser desorption/ionization (MALDI), and inductively coupled plasma (ICP) ionization have been used.

3.2.3.3. ICP-MS. ICP-MS has several unique characteristics such as high analytical throughput, excellent detection limits for most elements, minimal matrix effects, broad dynamic range (up to 8 orders of magnitude) and information of isotope ratio [137]. In proteomics, ICP-MS is especially valuable due to the additional element-specific information about the metal–protein complexes. Using different types of sample introduction systems, ICP-MS can be easily combined with various separation methods, including gas chromatography (GC), high-performance liquid chromatography (HPLC), and CE. Recently, on-line coupling of gel electrophoresis and ICP-MS was also achieved [138,139].

The application of CE–ICP-MS has made significant progress in MT analysis in last few decades. The metal complexes of two major MT isoforms, i.e. MT-1 and MT-2, were separated and elements contained in the isoforms selectively detected by CE–ICP-MS coupled via various interface designs [140]. Even a home-made system hyphenating CE with ICP-MS for cadmium speciation of protein-binding and free cadmium ions in solution was reported by Lavorante et al. [141]. The feasibility to isolate metallothionein compounds extracted from the cyanobacterium *Synechococcus* PCC7942 was demonstrated in this paper. Two commercially available interfaces based on microflow nebulizers were evaluated by Alvarez-Llamas et al. using two ICP-MS detectors (quadrupole and double focusing) [142]. In the other paper [143], new interface was constructed using a commercial microconcentric nebulizer

and home-built cyclonic spray chamber. MT isoforms were separated and the bound metals detected to characterize the interface. It was found that the separation resolution was improved compared to that achieved by on-capillary UV detection. Comparison of a cross-flow and microconcentric nebulizer for chemical speciation by CE–ICP-MS was performed using MT standard samples [144]. In addition, other instrumental designs of CE–ICP-MS coupling were characterized using MTs [145–147].

A new approach for the quantification of metallothionein isoforms used by Schaumlöffel et al. via sulfur measurements was possible due to the fixed number of sulfur atoms (twenty one) per one MT molecule [148]. Further measurements of Cu, Zn, and Cd followed by determination of the sulfur-to-metal ratio in MT enabled characterization of the stoichiometric composition of the metalloprotein complexes. The introduction of the on-line isotope dilution technique into CE–ICP-MS permitted the quantification of separated MT isoforms and the characterization of their metal compositions. The complementarity of ICP sector-field double-focusing MS and ESI-MS in CE was applied for the determination and identification of metal complexes with MT induced in the liver of rats exposed to the intravenously administered Cd^{2+} [149]. Isotope dilution analysis was developed for the accurate quantification and the determination of the stoichiometry of metal complexes. The characterization of MT sample was completed by ESI-MS allowing the determination of molecular masses of the resulting complexes and identification of the ligands as MT-1 and MT-2 isoforms. Moreover, the complementarity of CE–ICP-MS and CE–ESI-MS couplings was evaluated for the characterization of metal complexes with MT isoforms by Mounicou et al. [150]. The study highlights the potential of CE–ICP-MS and CE–ESI-MS when used in parallel for characterization of metal complexes with biomolecular ligands. Similarly to UV-absorbance detection, the sensitivity of MS-ICP is limited because it depends on the amount (mass) of analyte that enters the detector per second. Extremely low amount of the effluent provided by CE may be problematic. For this reason, LVSS method is advantageous not only for absorption but also for MS detection of MTs [151].

3.2.3.4. ESI-MS. The analysis of biogenic ions has advanced significantly due to the sensitivity, accuracy and precision of ESI-MS. On the other hand, efficient coupling of CE to ESI-MS is challenging. Earlier CE–ESI-MS interfaces typically employed a liquid junction or a coaxial sheath liquid flow to maintain electrical connection between CE and ES introduction [152–156]. The band broadening as well as contamination issues introduced by the presence of the extra liquid diluting the CE effluent has to be addressed. Therefore, the use of small i.d. capillaries and/or sprayers, amine modification of the inner surface, and gold coating of the exterior as well as the tip of the sprayer have enabled direct, sheathless interfacing of CE to ESI-MS [140]. Special attention must be paid to the choice of background electrolyte composition, its concentration and pH. Widely used buffers (borate, phosphate, etc.) cause many problems in ESI. Ammonium acetate buffer [150] or formic acid [140,157] based buffers were employed as suitable buffers for electrospray ionization of MTs.

Because CE–ICP-MS is unsuitable for obtaining molecular mass information from the different apothionein metalloforms (forms of MT without any metal ion), CE–ESI-MS is preferred for this purpose [158]. However, quantification by ESI-MS can only be accepted for standards with a high similarity to the analytes. Unfortunately, this is usually not applicable for MTs [159]. Due to the high diversity among MT species the absence of suitable reference material precludes their quantification by molecular MS. There is widely assumed that the intensities of ESI-MS peaks of the similar species are directly correlated with their relative concentration in the sample. This fact has been extended to the determination of different MT proteins coexisting in a sample. Perez-Rafael et al. [159] assumed that every MT protein shows an individual and particular behavior when ionized at certain conditions. Consequently, the intensities of the ESI-MS signals of distinct MT proteins, even though they are closely related isoforms of the same organism, cannot be directly related with their relative abundances in a sample. This is correct even when these peptides or metal–MT complexes are analyzed under the same conditions.

CE–ESI-MS in combination with circular dichroism and UV–vis spectrometry was demonstrated as a powerful tool for a detailed analysis of the Zn/Ag(I) replacement mechanism in mammalian Zn_7–MT-1 and constitutive Zn_4–αMT and Zn_3–βMT fragments, as well as into the Ag(I) loading to the corresponding apoforms [160]. Monitoring of metal displacement from the recombinant mouse liver MT Zn_7-complex by CE–ESI-MS during titration studies offers complementary information to that obtained from circular dichroism, UV–vis or NMR. The titration of the Zn_7–MT complex with Cd(II) showed the sequential displacement of the Zn(II) by Cd(II). Moreover, these experiments revealed an unusually high stability of the Cd_6Zn_1–MT suggesting a structural role for the remaining Zn(II) ion [161]. Detection techniques used in CE on MT with their key advantages are summarized in Fig. 4.

3.3. Mathematics in CE of MTs

Mathematics can be utilized in CE generally in two ways: (a) prediction and modeling of the behavior of certain analyte, (b) improvement of obtained data. Modeling of electrophoretic behavior in CE is an effective way how to optimize separation conditions. Several papers suggest semiempirical relationships between the electrophoretic mobility of the analytes and their structural parameters, including molecular mass, charge or number of amino acid residues [162,163]. A general equation relating electrophoretic mobilities to the pH of the running electrolytes was used for selecting the optimum pH for separation of mixtures of ionizable compounds such as peptides [164] or flavonoids [163]. Rabbit liver apothioneins are relatively large molecules with up to 34 ionizable groups. The electrophoretic behavior of their subisoforms can be investigated in the acidic pH range, due to the fact that bound metal ions of the metallated subisoforms are released from the amino acidic structures. Benavente et al. introduced general equation that can be used to model the electrophoretic mobility of rabbit liver apothioneins as a function of the pH of the separation electrolyte [165]. The ability of these relationships to explain the migration behavior of these relatively complex polyprotic proteins in the pH range between 2 and 6 was discussed in this paper.

The second type of mathematical approach (chemometrics) can be used when physical separation of sample components is not fully accomplished and quantification difficulties stemming from poorly resolved peaks. Chemometrics methods are used in separation techniques to optimize the separation conditions, improve the resolution and quantification, and check peak purity. The performance of CE–ESI-MS is limited and resolution problems could arise when handling complex mixtures of protein isoforms [166]. Traditionally used methods for the MS data analysis may be excessively time consuming and therefore chemometrics-assisted multiway data analysis is an alternative for handling such complex data sets [158,167,168]. Mathematical approach for MT data processing was carried out by Benavente et al. [158]. Parallel factor analysis and multivariate curve resolution-alternating least squares were applied to the data sets. Using both methods it was possible to discriminate a characteristic fingerprint for MT samples, based on the electrophoretic profiles and the pure mass spectra of the three model components, which contributed in a different way to explaining the variance of each protein type.

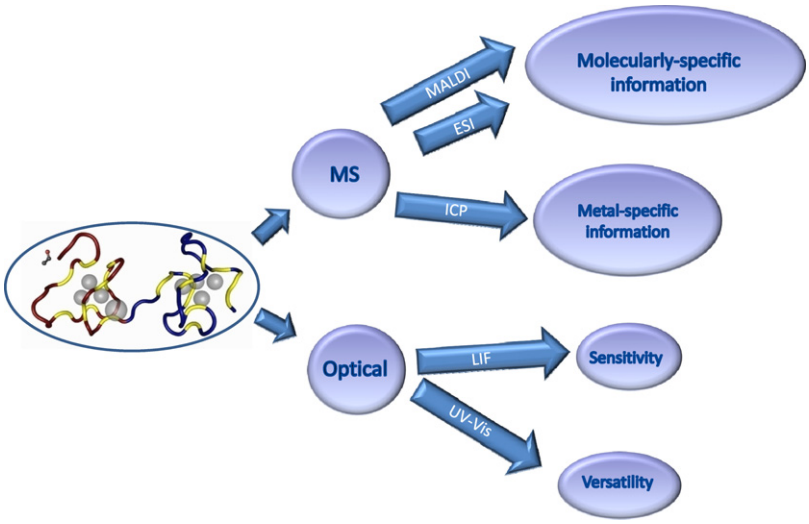

Fig. 4. Detection methods used in CE of MTs and their key advantages. Mass spectrometry and optical detectors belong to the most commonly used detectors coupled with CE. Mass spectrometers can be used to investigate molecular specific information and metal-specific data. On the other hand, optical detectors are versatile and, under specific conditions, sensitive to the presence of MT.

3.4. Chip-based CE

Miniaturized integrated analytical systems have matured greatly since the initial lab-on-a-chip concept was proposed [169–172]. The advantages of miniaturized systems include speed of analysis, increased precision and accuracy, portability, increased throughput, reduced cost and waste as well as disposability. Due to the instrumental simplicity CE represents one of the most suitable analytical methods for miniaturization. The majority of chip-based CE instruments are in-house made. However, also several commercially available platforms have been introduced. One of them was used for MT analysis by Krizkova et al. [134,135]. In the first paper, chip-based CE was used for quantitative study of MT oxidation by H_2O_2 resulting in significant decrease in peaks heights and shift of peaks positions to higher molecular mass, corresponding with the time of oxidation. Moreover, it was observed that the proportion of high-molecular forms of MT was markedly increased. The oxidative changes were successfully reversed by using reducing agent prior to electrophoresis. This method can reveal the stability MT aggregates, of which biological role still remains unclear [134].

In the second study, the chip-CE with fluorescence detection was used to determine structural changes of MT with increasing concentration of zinc(II) ions and under various redox conditions. Formation of MT aggregates with increasing zinc concentration was observed by spectrophotometry, chip-CE, and SDS-PAGE. It was found that reduced MT forms aggregate more readily compared to oxidized MT. Using the chip-CE allowed relative quantification of MT aggregation as a decrease in the area of the signal corresponding to the monomer form of MT [135].

4. CE as a routine method for MT detection in real samples

CE methods optimized for MT analysis during past years have been applied to the wide range of real samples including rabbit liver [62,64,90,91,99], horse [90,96,99] and human kidney [62], rat liver [99,149] as well as sheep liver [63], yeast [173] and mussels [174]. Currently CE is already generally accepted as a suitable analytical tool for determination of MTs in both standard and real samples. Therefore the following section aims to overview works that employ CE as a routine analytical technique for further investigation of complex biological and biochemical processes involving MTs. The chapter is divided according to field of application.

4.1. Environmental analysis

Due to the fact that expression and translation of MT is enhanced by heavy metals, these proteins have been widely used as specific biomarkers to detect exposure to Cu, Hg, Cd, Ag, and Zn. Speciation of different MT-metal isoforms in organisms of interest requires both high efficiency separation technique as well as specific and highly sensitive detector to cope with naturally occurring low metal-MTs levels in biological systems. Both are fulfilled by coupling of CE to the ICP-MS detection as mentioned in the following examples of real samples analysis. It was found that brown cells (found in the red glands of *Mercenaria mercenaria*) accumulate, detoxify and excrete cadmium. Reduced glutathione provided the initial defense against Cd^{2+} toxicity prior to MT induction. Then, triggered MT expression fully protected the cells treated with these adverse ions [175].

The expression of mammalian and fish MTs in *Escherichia coli* as a strategy to enhance metal biosorption efficiency of bacterial biosorbents for lead(II), copper(II), cadmium(II), and zinc(II) was suggested. MT was expressed in either the cytoplasmic or periplasmic compartment of host cells to explore the localization effect on metal biosorption. The results showed that MT expression led to a significant increase (5–210%) of biosorption efficiency especially for biosorption of Cd(II). Periplasmic expression of MTs appeared to be more effective in facilitating the metal-binding ability than the cytoplasmic MT expression. Notably, disparity of the impacts on biosorption ability was observed for the origin of MT, as human MT (MT1A) was the most effective biosorption stimulator compared to MTs originating from mouse (MT1) and fish (OmMT) [176].

4.2. Clinical analysis

Clinical application of MT as a potential cancer marker is frequently discussed [7,8,10]. Determination of MT isoform ratio by CE–ICP-MS was demonstrated as beneficial for improvement of the diagnostic method for chronic hepatic disorder. Together with metal content in the liver tissue of patients suffering with hepatocellular carcinoma, MT isoform characterization is a promising tool for increasing the accuracy of the diagnosis [75]. Resistance on the presence of specific drugs is one of the most important issues in cancer chemotherapy. One of the mechanisms suggested to contribute to this phenomenon is the sequestration of alkylating

agents by metallothionein (MT) *in vivo*. Cadmium-induced human bladder tumor T24 cells were exposed to two therapeutic agents – chlorambucil and melphalan. MT-2a was determined by capillary electrophoresis. It was found that 56% of the MT isoforms in induced cells represented acid drug adducts of MT-2a. One to four equivalents of drug were found to be covalently adducted. Major binding sites on metallothionein were located in the C-terminal domain by peptide mapping, consistent with previous studies *in vitro* [177]. In order to further characterize mechanisms of MT induced resistance, the study was performed on urothelial cancers. The authors established several cisplatin-resistant sublines of the bladder carcinoma cell line RT112, examined the cross-resistance profiles to different cytotoxic drugs and measured the expression of MT-1 and MT-2 with capillary electrophoresis [178].

Moreover, Wolf et al. used capillary zone electrophoresis for the analysis of human brain cytosols. Samples were separated after different pre-treatment steps and were compared, with special emphasis on the detection of the isoform metallothionein-3 [101]. MT-3 mostly occurs in brain but other isoforms can also be present in this tissue. The induction of metallothionein in mouse cerebellum and cerebrum with low-dose thimerosal injection was investigated [82]. It can be concluded that low-dose thimerosal affects the cerebellum as MTs, acute-phase protein, were induced in the cerebellum but only slightly in the cerebrum. MT-1, but not MT-2, may act as a neuroprotective protein against thimerosal in the cerebellum. The function of MT-3 induced by thimerosal in the cerebellum is still not fully elucidated.

4.3. Others

Species analysis of metallothionein-like proteins (MLPs) in liver tissues from Elbe-Bream (*Abramis brama* L.) and Roe Deer (*Capreolus capreolus* L.) using CE combined with ICP-MS detection was reported. In order to allow systematic development of the method, commercially available MT preparations of rabbit liver were used. For commercial MT preparations, the relative standard deviations (RSDs) in the retention times were 0.9% for MT-1 and 1.9% for MT-2; the RSD's in the peak areas were less than 6% for MT-1 and 16% for MT-2, respectively. Under optimized conditions, the MLPs in the real samples could be separated efficiently in less than 10 min. By comparison with the migration times of commercially available MT preparations, two of the observed peaks could be assigned to MT-1 and MT-2 [179]. Besides these samples, complexes of MT can be investigated in other types of tissues such as rat and rabbit ones [146,180,181]. Based on such studies, it was found that mammalian MTs are characteristically N^{α}-acetylated. However CE-ESI-MS of hepatic MT-2 from rats treated with zinc(II) revealed two isoforms differing by a mass equivalent to that of a single acetyl group [182].

5. Conclusion

The brightest point of the CE history was probably its application for human genome sequencing project. Nowadays its significance is again increasing due to the miniaturization and introduction of microchip CE to the analytical praxis. The portability, being the main requirement for miniaturized devices, enables to analyze biological samples out of the laboratory and decrease significantly the costs of analysis. MTs are considered as biomarkers for environmental pollution as well as for medical biomarkers and therefore their effective and rapid determination may impact significantly the actions taken in such cases. Moreover, due to the extreme complexity of MT functions, regulation processes and induction mechanisms in wide range of organisms, the detailed studies of this metalloprotein family are still required. CE has already proved to be the suitable tool for it.

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