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Nanotechnology in bio/clinical analysis

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Abstract

Nanotechnology is being exploited now in different fields of analytical chemistry: Single cell analysis; in chip/micro machined devices; hyphenated technology and sampling techniques. Secretory vesicles can be chemically and individually analyzed with a combination of optical trapping, capillary electrophoresis separation, and laser induced fluorescence detection. Attoliters (10^{-18} l) can be introduced into the tapered inlets of separation capillaries. Chip technology has come of age in the field of genomics, allowing faster analyses, and will fulfil an important role in RNA and peptide/protein analysis. The introduction of nanotechnology in LC–MS and CE–MS has resulted in new findings in the study of DNA adduct formation caused by carcinogenic substances, including anticancer drugs. Sample handling and introduction also can benefit from nanotechnology: The downscaling of sample volumes to the picoliter level has resulted in zeptomole (10^{-21}) detection limits in the single-shot mass spectrum of proteins.

Keywords: Nanotechnology; Chips; Miniaturization

1. Introduction

The potential of nanotechnology has been highlighted recently by its impact on scientific research budgets [1] and the need for separation and identification devices at the nanoscale level [2]. Harvard will spend $70 million on a Center for genomics and proteomics, and a Center for imaging and mesoscale structures. In both a wide range of new technologies should be developed, including DNA chips and devices to ''perturb'' proteins and estimate their functions and activities [1]. Lithographic methodology is at the heart of nanotechnology, and the recent introduction of ''Dip-Pen'' nanolithography may be a crucial step forward in a technique more than 4000 years old. An atomic force microscope (AFM) tip was used to construct alkanethiols with 30 nm linewidth resolution on a gold thin film in a manner analogous to that of a dip pen. Molecules of interest are positioned by the AFM tip on a solid substrate through capillary transport. The technique is a potentially useful tool for the generation of nanoscale devices [2].

Nanotechnology – which utilizes nanovolumes and/or nanoflows or less in separation sciences – is not limited to the production of micromachined devices. In this paper we summarize various applica-

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tions of nanotechnology in bio/clinical research in the areas of (1) single cell analysis, (2) DNA, RNA and protein/peptide analysis on micromachined devices, (3) hyphenated technology such as capillary liquid chromatography–electrospray ionization mass spectrometry (LC–ESI–MS), and (4) sampling techniques.

2. Single cell analysis

Biosensors detect chemical species with high selectivity on the basis of molecular recognition rather than the physical properties of analytes. Zare et al. demonstrated a biosensor system, based on the response of living cells, that can detect specific components of a complex structure fractionated by a microcolumn separation technique [3]. The system applied ligand–receptor binding and signal-transduction pathways, upon separation of a specific agent by capillary electrophoresis (CE).

The single cell biosensor (SCB) for CE was based on monitoring Ca^{2+} changes within one or more PC-12 cells with the use of the Ca^{2+}-sensitive dye fluo-3 (A). Separation of the analytes was performed by CE, and the capillary effluent was directed to PC-12 cells cultured on a microscope cover slip ~20 to 40 μm from the channel outlet. Substances that evoked changes in $[Ca^{2+}]$, were monitored with an epi-illuminated fluorescence microscope, high voltage supply (Fig. 1A).

The use of ligand–receptor binding and signal-transduction pathways to biochemically amplify the presence of a substance upon electrophoretic separation was also demonstrated by measurements of the transmembrane current in a Xenopus leavis oocyte microinjected with messenger RNA encoding a specific receptor (Fig. 1B). Here the CE–SCB systems were utilized as qualitative analytical tools. The presence or absence of species of interest above the detection limit was achieved rapidly, with high sensitivity and selectivity. Quantitation can be obtained by coupling the effluent from a CE capillary to a sensor comprised of various cells (Fig. 2A and B), although a series of problems, such as cell-to-cell variability of response and differential reduced sensitization, have to be overcome [3,4].

Coupling of chemical separations with single-cell biosensors has led to the isolation of novel ligands, the screening of mRNA expression, and the analysis of very small volumes associated with single cells. Progress in the quantitative capabilities of the system

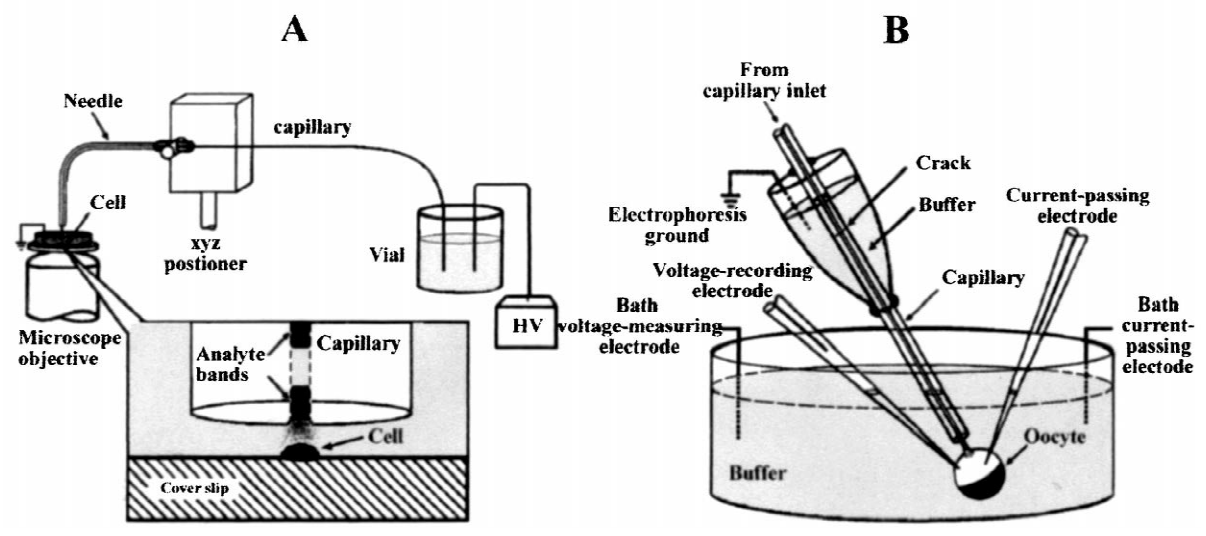

Fig. 1. Single-cell biosensor (SCB) system for CE (From Ref. [3] p. 75, Fig. 1). (A) ACE–SCB device based on monitoring Ca^{2+} changes within one or more PC-12 cells with the use of the Ca^{2+}-sensitive dye fuo-3. Analyte were separated by capillary electrophoresis. (B) ACE–SCB system based on membrane current measurements on a Xenopus laevis oocyte expressing a cloned membrane receptor. (From Ref. [3] p. 75, Fig. 1).

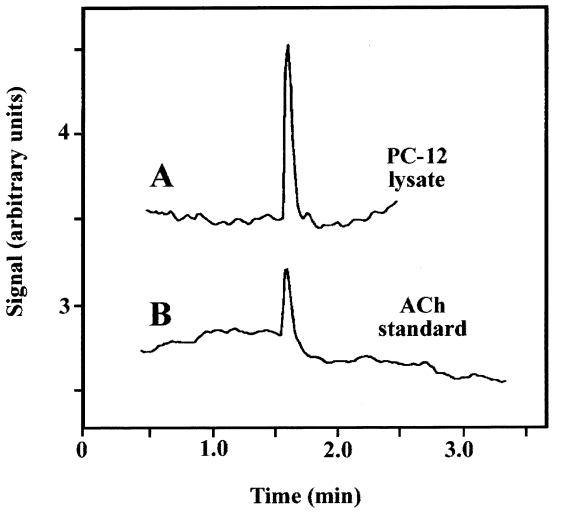

Fig. 2. Identification of a single component present in a complex biological matrix with the use of the CE–SCB fluorescence system. In (A), a sample derived from the lysate of PC-12 cells was electrophoretically separated, producing a single peak in the first 150 s of separation. This peak was identified as Ach in (B) by electrophoretic separation of a standard solution and comparison of the migration times of the unknown and the standard. Experimental conditions were as follows: 10 s gravity injections (+10 cm above the outlet) of lysate and Ach standard (0.8 m*M*) were separated with a field of ~400 V/cm in a 25 μm I.D. capillary. Between four and five PC-12 cells were used as the sensor. The electropherograms are offset on the vertical axis. Fluo-3 AM ester was included in the measurement buffer. (From Ref. [3], p. 76, Fig. 4).

has been made with the rat mast cell line RBL-2H3. These cells contain adenosine receptors, and the potassium current invoked by ADP and ATP is reversible, the latter being essential for quantitation. The system does not desensitize readily and allows the construction of a calibration dose–response curve. An unknown concentration can be measured and quantified because of the reversible nature of the response. When cells transfected with custom receptors are used, identification of the ligands becomes possible, and a detector can be designed for given substances with improved selectivity of this system [5–7].

2.1. Chips/micromachined devices

Chip technology, developed in the past five years, is an incredible technical achievement in the rapid and automated performance of genetic identification. By a series of photolithographic and chemical steps on the solid-phase of a small surface, relatively dense arrays can be generated, e.g. oligonucleotide arrays for DNA chip technology. The oligonucleotides produced by computer-directed in-situ microfabrication may bind complementary fluorescent-labeled DNA fragments from an unknown sample. Subsequent scanning registers the position of positive signals, which in turn provides information concerning the identity of DNA fragments. As well as DNA sequencing, genetic errors in known genes can be detected. The presence of infective organisms can be determined, and genetic markers such as HLA, important in transplantation and forensic medicine, can be typed. DNA, RNA and protein/peptide chips are revolutionary new milestones in genetics and proteomics.

2.1.1. DNA analysis

The use of CE in a polymer network to detect point mutations in DNA, instead of traditional polyacrylamide gel electrophoresis (PAGE), is a step towards nanoscale DNA analysis [8]. CE in a polymer network has been investigated in single-strand confirmation polymorphism (SSCP). SSCP is a method to detect point mutations, essential in the diagnosis of several diseases. Point mutations give rise to conformational changes in single stranded DNA, and lead to a mobility shift of the DNA fragments on neutral polyacrylamide gels. This SSCP technique is a convenient and efficient method for the detection of point mutations [9]. Mutations in the p53 gene, located on the short arm of chromosome 17, have been investigated using this new technique. The gene is known to be frequently mutated in malignancies, and can therefore be used as a molecular marker for (pre-) malignant cells [10]. The p53 gene contains five evolutionary conserved regions, and the mutations are clustered in regions II–V; one region has recently been shown to be susceptible to damage by products present in tobacco smoke. Two single-strand DNA fragments of 372 bp length, differing in only one nucleotide, have been separated.

Manz et al. pioneered planar chip technology for capillary electrophoresis in the analysis of ''antisense'' oligonucleotides [11]. A micromachined

chemical analysis device based on capillary gel electrophoresis (CGE) has been used for fast size separation of a synthetic mixture of fluorescent phosphorothioate oligonucleotides. The device consists of a channel system formed on the surface of a glass plate by a standard photolithographical process. The channel system is completely filled with a non-cross linked polyacrylamide gel solution with 10% monomer concentration. The use of electric field strengths of up to 2300 V/cm results in very fast analysis times with plate numbers of up to 200 000 without degradation of the gel matrix. An integrated volume-defined sample injector allows unbiased electrophoretic introduction of sample plugs of 150 μm length. Fast automated repetitive sample injection and analysis can be demonstrated with excellent reproducibilities for both migration time and peak height.

Further development of such a device resulted in a micromachined chemical amplifier which was used successfully to perform the polymerase chain reaction (PCR) in continuous flow at high speed: ''PCR on a roller coaster'' [12,13]. The device is similar to an electronic amplifier, and relies on the movement of a sample through thermostated temperature zones on a glass microchip. Substance (e.g. DNA) input and output is continuous, and amplification is independent of input concentration. Reaction times varied between 1.5 and 20 min (Fig. 3).

It has been questioned whether ''DNA-chip'' technology will in fact speed up the genome initiative [14] but developments in the past five years have been positive [15–20]. In the mid 1990s the challenge of the human genome project was to increase the rate of DNA sequence acquisition by two orders of magnitude, and to complete sequencing by the year 2000. Stimpson introduced a rapid detection method using a two-dimensional optical wave guide that allows measurement of real-time binding, or melting, of a light-scattering label on a DNA-array. A particular label on the target DNA acts as a light-scattering source when illuminated by the evanescent wave of the wave guide, and only the label bound to the surface generates a signal [19]. Imaging/visual examination of the scattered light permits interrogation of the entire array simultaneously. The

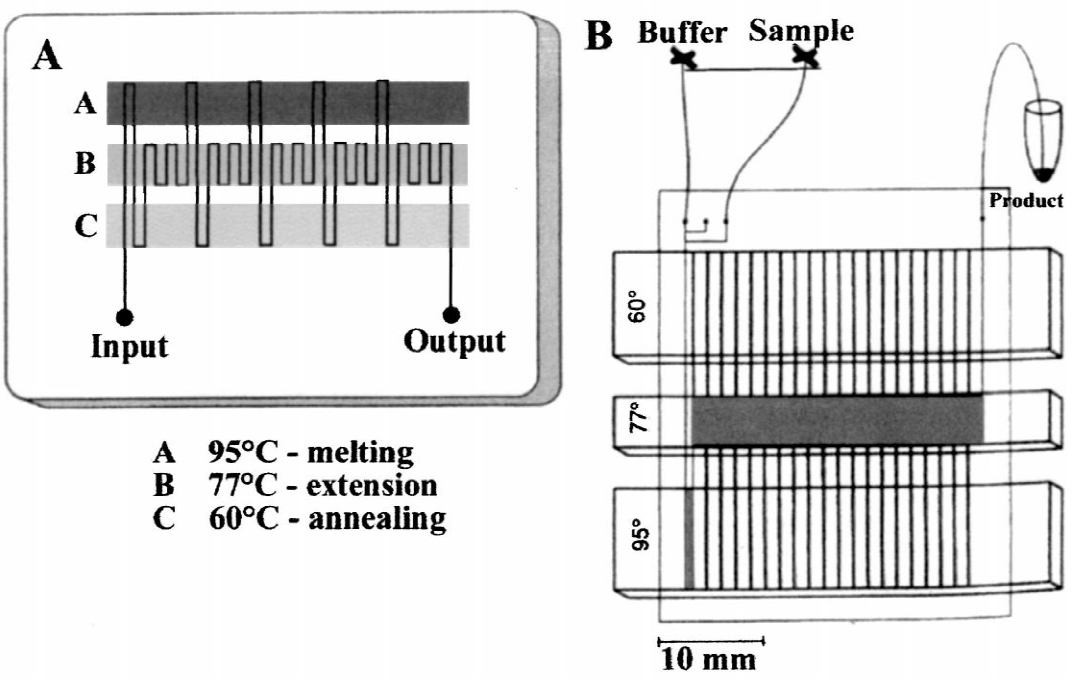

Fig. 3. Chip layout. (A) Schematic of a chip for flow through PCR (Ref. [13], p. 1046, Fig. 1). (B) Layout of the device used in the study of Ref. [13].

methodology is a powerful tool in diagnostic techniques that require rapid cost-effective detection of variations in known sequences. Pastinen et al. described minisequencing as a specific tool for DNA analysis and diagnostics on oligonucleotide arrays [17]. Mutations are detected by extending immobilized primers that anneal to their template sequences, immediately adjacent to the mutant nucleotide positions, with single labeled dideoxynucleoside triphosphates using a DNA polymerase. The arrays were prepared by coupling one primer per mutation on a small glass area. Genomic fragments spanning nine disease mutations, which were selected as targets for the assay, were amplified in multiplex PCR reactions on the primer array. The genotypes of homozygous and heterzygous genomic DNA samples were unequivocally defined at each analyzed nucleotide position by the highly specific primer extension reaction. Compared to hybridization with immobilized allele-specific probes in the same assay format, the power of discrimination between homozygous and heterozygous genotypes was one order of magnitude higher using the minisequencing method [17]. Single-nucleotide primer extension is, therefore, a promising possibility for future high-throughput mutation detection and genotyping using high density DNA-chip technology.

2.1.2. RNA analysis

The quality of RNA is crucial for RNA-related diagnostic tests, and disposable plastic chips can be useful in the quality assessment of RNA [21]. Two major rRNA components (18S and 28Sr RNA) have been separated by electrophoresis in injection-modeled acrylic chips with a microchannel 100 μm in width, 40 μm in depth, and with 1 cm of separation. Microchannels were filled with 4 g/l hydroxypropylmethyl cellulose as a sieving polymer, and 5 mg/l ethidium bromide for RNA staining. The fluorescent signals were detected by a fluorescent microscope equipped with a photometer and a 590 nm emission filter. The assay is rapid (<3 min), reproducible, RNase-free, and requires only 1–2 μl of sample. The detection limit is approximately 10 mg/l, 100-fold lower than that for agarose gel electrophoresis. As only 0.1 nl of the loaded sample was introduced for electrophoresis, the detectable peaks of rRNA in the separation were derived from less RNA than is present in one single cell.

2.1.3. Protein/Peptide analysis

New developments and applications relating to pyrrole copolymerization have resulted in the investigation of pyrrole-modified biomolecules on microelectrode arrays. Livache et al. recently described two important developments in this area [22]. Firstly, the development of multiplexed silicon chips bearing 128 microelectrodes instead of the usual 48 for passive chips. Secondly new applications of the grafting process concerning not only DNA chips, but also peptide chips. Copolymerization of pyrrole peptides in the chips and their immunological detection appears possible, and the technology exhibits a high dimensional resolution and real versatility [22].

3. Hyphenated technology

Probably the most impressive results of nanotechnology thus far are in the coupling of (capillary) separation techniques to powerful detectors such as MS and NMR. A wide variety of publications on this topic are available with important implications in the field of bio/clinical sciences.

3.1. On-line nanoscale liquid chromatography–mass spectrometry

MS has the potential to become a standard analytical tool for detecting and identifying biological and clinical substances. Several mass spectral techniques have been used in this area, and satisfactory results have been obtained even without on-line coupling of separation techniques with MS [23]. Nanoscale LC–MS has fulfilled a striking role in biological sciences very recently, i.e. in the detection and identification of DNA adducts. Humans are exposed to a wide array of chemical compounds which can interact with biological macromolecules such as proteins, RNA and DNA. Interactions with DNA can lead to a new covalent bond between the carcinogen and DNA, leading to damaged DNA and so-called DNA-adducts [24]. This damage can cause mutations and possibly lead to induced carcinogen-

esis. Detection and identification of these DNA adducts is an important research topic in cancer etiology, and insights into adduct formation and removal may yield tools in cancer prevention. DNA adduct formation also plays an important role in cancer treatment; agents such as *cis*-platin form DNA adducts preventing cell division.

An analytical method for carcinogen DNA adducts must be capable of detecting in the pg to fg range and elucidating adduct structure. ^{32}P-Postlabeling is an analytical assay sufficiently sensitive to detect adducts isolated from in vivo sources [25], but it can not provide structural information [26]. Analysis of DNA adducts from in vivo and in vitro sources with MS is usually accomplished by GC–MS [27]. Combined LC–ESI–MS can detect adducts at the nucleotide level without prior derivatization. This allows the study of sugar-phosphate modifications which is not possible with GC–MS. ES–/MS–MS has been exploited to characterize 2′-deoxynucleotide adducts [28].

ESI–MS is a concentration-sensitive device compatible with miniaturized chromatographic techniques [29]. Various groups have coupled CE (vide infra) and LC techniques with ESI–MS to increase sensitivity, and coupling of capillary LC to ESI–MS improves mass sensitivity (the amount injected on-column) down to the low-picogram range [30–32]. The introduction of electrospray interfaces operating at nanoflows has enabled the coupling of liquid nanochromatography (nano-LC columns 75 μm I.D.) with ESI–MS [33–35]. The use of a NanoFlow LC–ESI–MS system to analyse DNA adducts has been reported by the groups of Esmans and Van den Eeckhoudt [36]. An in vitro reaction mixture, resulting from the interaction of 2′-deoxyguanosine 5′-monophosphate with bisphenol A diglycidyl ether and the injection of 2′-deoxyadenosine, was analyzed (Fig. 4).

Mass sensitivity can be improved by a factor of 3300 using NanoFlow LC–ESI–MS. The sensitivity of three injection methods were compared: i.e., split, large-volume, and column-switching injections (Fig. 5).

NanoFlow LC–ESI–MS has been used to analyse 2′-deoxynucleotide adducts isolated from an in vitro mixture of calf thymus DNA and bisphenol A diglycidyl ether. Column-switching techniques in combination with nano-LC allowed on-line sample clean-up, removing the unmodified 2′-deoxynucleotides.

Using this type of injection, new components such as cross-linked adducts have been identified, undetectable by capillary or conventional LC–ESI–MS techniques [36]. The coupling of nano-LC to ESI–MS with several commercially available interfaces may be associated with a sudden drop in the electrospray total ion current depending on the percentage of organic modifier in the mobile phase. Vanhoutte et al. evaluated the performance of their nano LC–MS system with different mobile phase compositions [37]. Standard uncoated, non-tapered fused-silica tips (20 μm I.D.), and several other electrospray capillaries were evaluated using different mobile phase compositions. Tapered but uncoated fused-silica tips increased the performance of the nano-electrospray system, but the best results were obtained with gold coated tapered tips [37].

3.2. On line capillary electrophoresis–mass spectrometry

Capillary zone electrophoresis has been coupled to ESI–MS to detect DNA adducts [38–40], and offers intriguing possibilities for monitoring DNA adduct formation and removal following exposure to substances such as anticancer agents. The CE separation of the DNA adducts of *cis*-platin and carboplatin has already been elegantly exploited using laser-induced fluorescence detection [41]. In studies of the formation of DNA-adducts by PGE, Deforce et al. applied ‘‘sample stacking’’ to load more sample on the capillary, with negative sample ions concentrated on the capillary [42]. A 100-fold increase of sensitivity can be obtained with ‘‘sample stacking’’; for DNA-adducts of phenyl glycidylether the sensitivity is 2.9×10^{-7} *M*, without loss of resolution or separation efficiency. Coupling of the CE technique with MS has been established using an ESI interface, using a triaxial CE probe [43,44]. A coaxial sheath liquid was delivered at the capillary exit to enhance the process (Fig. 6).

Optimal sensitivity was accomplished using 80/20 isopropanol/2 m*M*, and ammonium carbonate as sheath liquid. A major difficulty in on-line coupling of CE to ESI–MS is the minute amount of sample

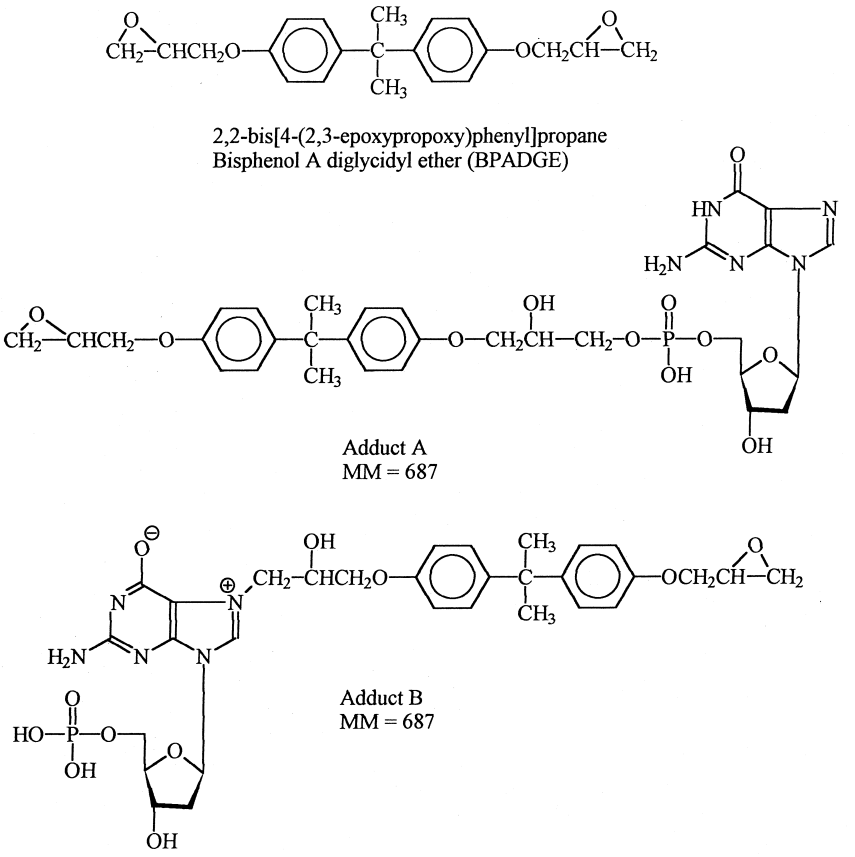

Fig. 4. Structures of BPADGE, phosphate-alkylated alfa-GMP/BPADGE (adduct A) and base-alkylated alfa-GMP/BPADGE (adduct B). (From Ref. [36], p. 3162, Fig. 1).

injected on the capillary leading to sensitivity problems in MS-detection. The use of the ''sample stacking'' technique in the on-line configuration [42], results in a detection limit in the SIM mode of 3.63×10^{-9} *M*, constituting a 200-fold gain in sensitivity [43,44]. With this technique, adducts formed between Phenyl Glycidyl Ether and the four 5′-monophosphate nucleotides were monitored, the latter being selected as lead components for the study of adduct formation using calf thymus DNA. CE–ESI–QTOF–MS offers a similar sensitivity to CE–ESI–MS under SIM conditions, but yields more information on interactions between platinum analogues and oligonucleotides [42]. CE–ESI–QTOF–MS and LC–ESI–QTOF–MS will fulfil an important role in proteomics. The advances in DNA-sequencing and the rapidly increasing amount of genome sequence data becoming available have changed the scope of protein analysis. Databases now provide the sequence of more than 300 000 proteins. The sequencing of complete genomes gives rise to a number of questions. ''Which of the genes are expressed in the organism?'', and in higher eucaryotes, ''which genes are expressed in which cell types?''. The complete 2D-PAGE map of the proteins expressed in a given cell type has recently been termed the proteome [45]. Once a protein is identified, the questions are: ''Is this protein known?'' ''Does this protein contain secondary modifications ?''. ''What are these modifications, where are they located, and what are their functions ?''. These questions can now be answered by MS, and various examples have been described such as the dissection of multi-protein complexes. The

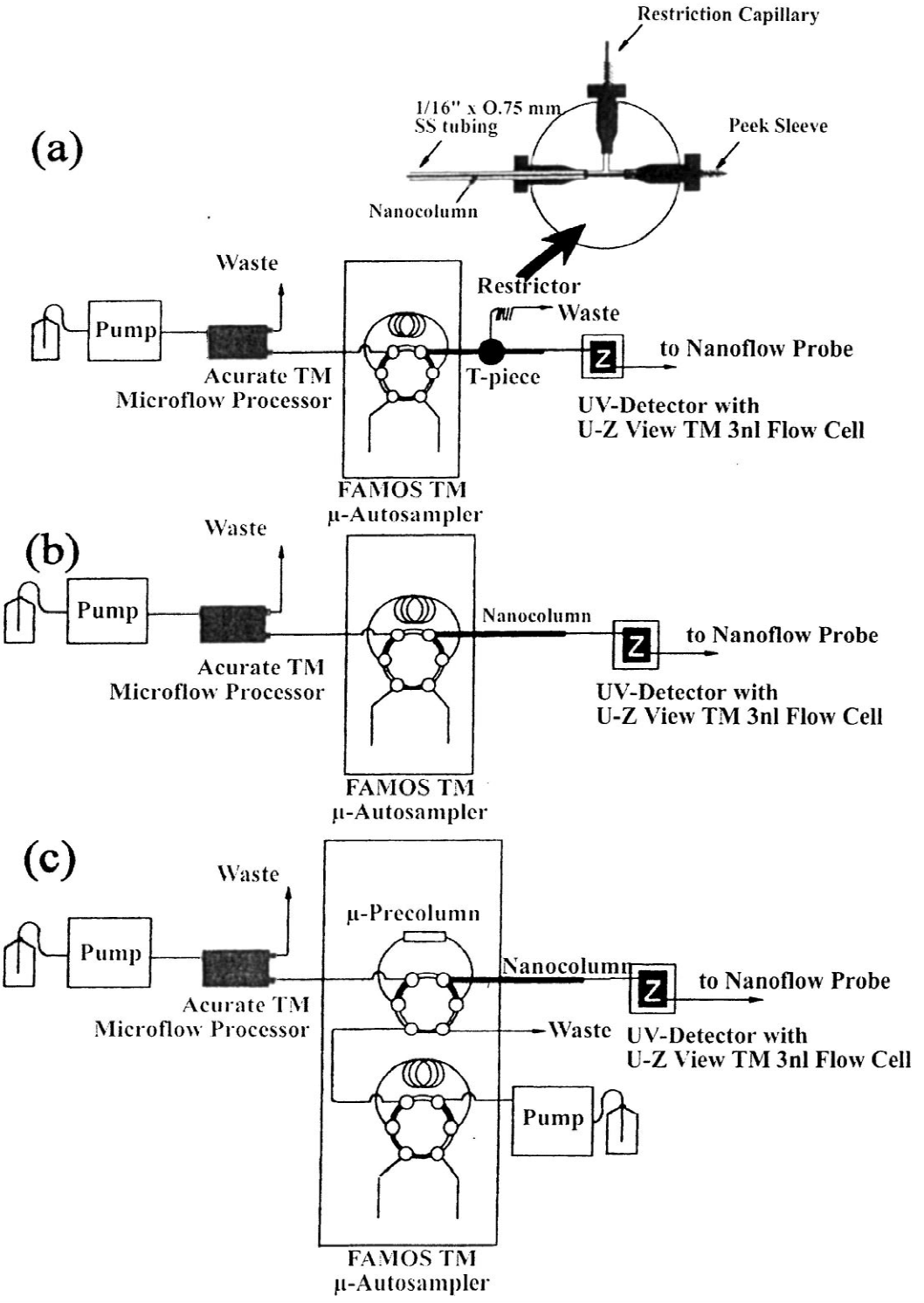

Fig. 5. Schematic diagrams of the three injection methods used in Nano flow LC/ESI/MS: (a) Split injections; (b) large-volume injections, and (c) column-switching injections. (From Ref. [36], p. 3165, Fig. 5).

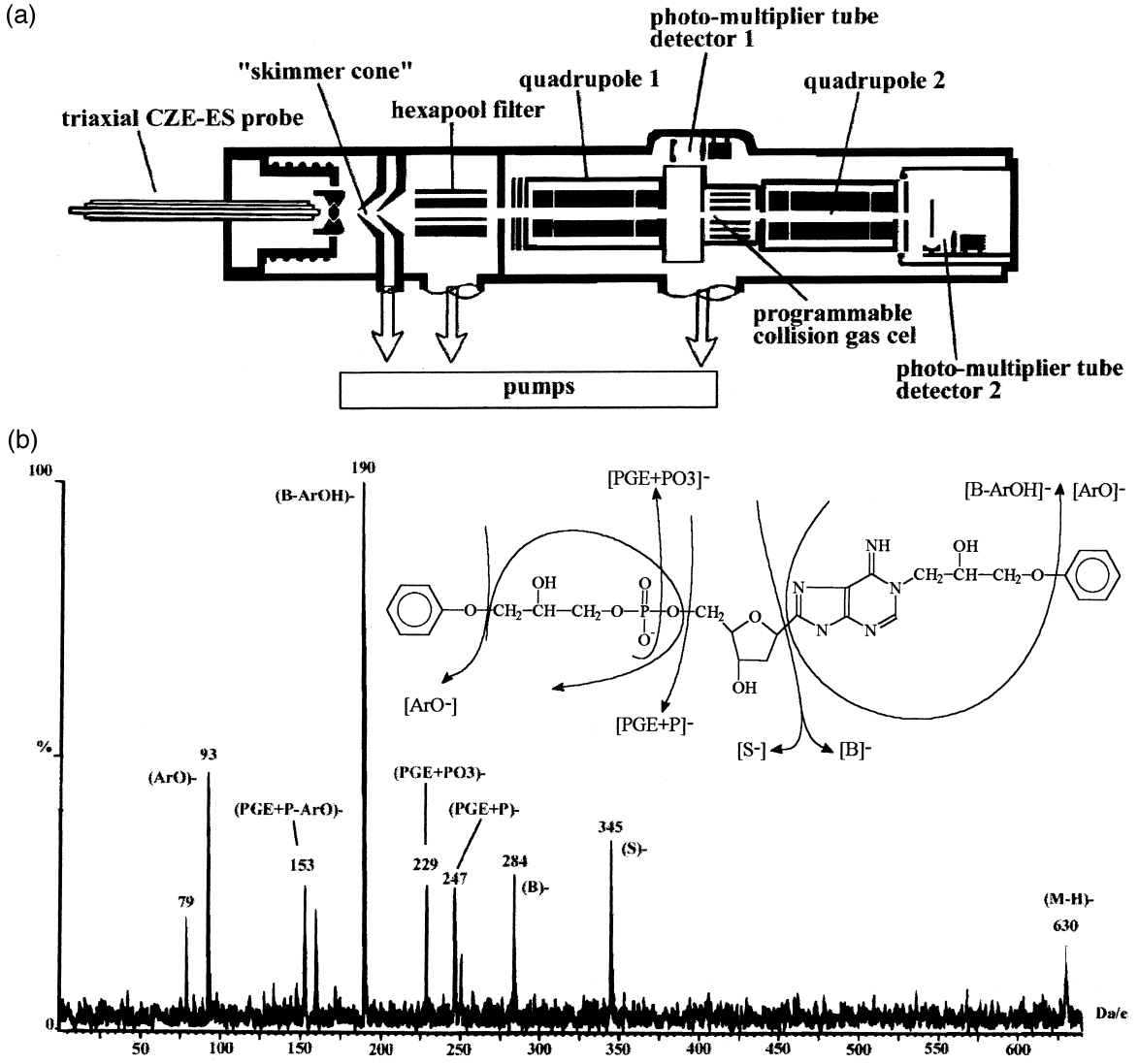

Fig. 6. (A) CE/ESI/MS with the triaxial probe (Thesis, Deforce [42], p. 82, Fig. 3); (B) CZE–ES–MS–MS spectrum of the daughters of monoalkylated dAMP (mother ion mass $(M-H)^- = 480$), present in the hydrolysate of the reaction of DNA with PGE (Ref. [39], p. 3583, Fig. 10). Here the sample stacking technique was used following an injection of 100 mbar for 30 s. The collision energy was set at 25 eV.

combination of nanoflow electrospray coupled with the time-of-flight mass analyser enables the stability of large multi-protein complexes to be assessed. The interpretation of the data, and its relationship to solution data, is a critical problem in this type of analysis. Robinson et al. used the protein tetramer transthyretin to demonstrate that under pre-defined MS conditions it is possible to match solution conditions to MS data [46]. The conditions were then applied to study protein variants which form fibrils under the solution conditions required for their measurement, therefore preventing the generation of solution data. The transthyretin tetramer interacts with a second protein, retinol binding protein, to form a six-protein complex that binds and transports vitamin A. ESI–QTOF–MS analysis of the complex

reveals not only the intact multi-protein complex, but also high energy collisions enabling the study of subunit interactions [47].

As well as MS, the on-line coupling of separation techniques to NMR offers a powerful and time saving method for the separation and structural elucidation of unknown compounds and molecular compositions of mixtures [48]. With on-line capillary LC–NMR, coupling only a few ml of deuterated solvents are necessary to perform separations on the nanoliter scale. On the basis of stopped-flow 2D-NMR spectra, a so far unknown retinyl acetate dimer was identified in one capillary LC–NMR experiment [49].

In addition to capillary LC–NMR separations, CE–NMR and capillary electrochromatography–NMR experiments have been exploited on the nanoscale level in biological studies [50]. It is to be expected that hyphenation of capillary separation methods with NMR spectroscopy and MS will become increasingly important in structure elucidation in science and industry.

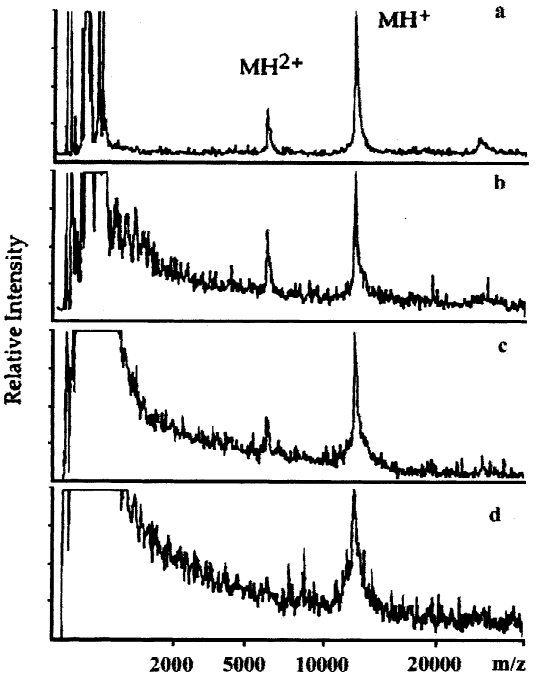

Fig. 8. MALDI spectra of horse cytochrome *c* obtained from the 250 picoliter vials containing absolute amount of (a) 1 fmol, (b) 100 amol, (c) 50 amol, and (d) 25 amol (From Ref. [51], p. 59).

4. Sampling techniques, limitations and conclusions

Nanotechnology offers interesting possibilities in a key phase of substance analysis, i.e. sample introduction. The sample stacking technique discussed above is one such example. Jespersen et al. described significant improvements in the absolute detection limits for proteins in matrix-assisted laser desorption–ionization mass spectrometry (MALDI–MS) using picoliter vials (Fig. 7) [51]. By the reduction of the sample volume from a few μl down to 250 pl, and concomitant reduction of the sample spot area from a few mm^2 down to 0.01 mm^2, low attomole detection limits were obtained for bradykinin and cytochrome *C*.

The detection limit in a single-shot mass spectrum of bradykinin was as low as 250 zeptomole (10–21 mol) (Fig. 8).

Limitations of nanotechnology exist in sampling,

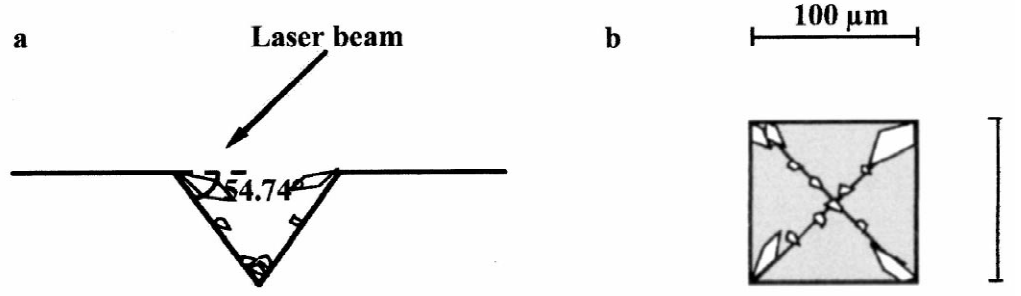

Fig. 7. Schematic drawing of a 250 picoliter vial, showing the position of the micro-crystal; (a) cross sectional view of the vial, and (b) top view of the vial. (From Ref. [51], p. 56).

sample pretreatment and separations, i.e. chromatography and electrophoresis, and in detection and signal processing. With single cell analysis tissue integrity has been lost, and with it some information on cell–cell communication, tissue architecture and flow conditions. With CE, sampling still remains a problem with respect to reproducibility, especially when compounds of interest originate in a biological matrix such as plasma. Single cell analysis is limited by the threshold level of substance for the cellular system used, e.g. a receptor.

In quantitative analysis, the limit of quantitation is the most important performance characteristic of the analytical method. Despite this fact, such limits are rarely published in contrast to the limit of detection. The general approaches to the limit of quantitation were developed for classical, spectral and electroanalytical methods and must be modified if used in electrophoresis and chromatography. The reason for this is the two dimensional nature of the electrophoretic and chromatographic signal in contrast to the one-dimensional signal usually considered in limit of quantitation concepts. The higher dimensionality of the signal needs to be used if trace levels of analytes are to be quantified, and exact methods for the limit of quantitation defined. Particular aspects of the calculation, estimation and lowering of the limit of quantitation which are specific for electrophoresis and chromatography are the model of the signal and the influence of sampling frequency, column efficiency, and signal processing.

Microarray devices such as DNA, RNA and protein/peptide chips, and picoliter vials are mostly fabricated by UV photolitography. Here the optimal UV wavelength is a clear limitation. With picoliter vials, limitations at the molecular level may well be encountered. The zeptomole level has been attained and improvements in sample preparation may lead to further lowering of detection limits. For instance, increasing the number of micro-crystals will lead to increased absorption of analyte ions from the vials. The potential to transfer single cells to the picoliter vials, followed by the addition of the matrix solution, raises the possibility of following the cell-specific processing of proteins, post-translational modifications of peptide sorting, and targeting to different intracellular sites. In addition, the ability to investigate very small sample spots, could provide a convenient means of coupling capillary electrophoresis separation systems to MALDI–MS. Diatoms are potentially an area beyond micromachining [52]. Diatoms are microscopic, single-celled algae possessing rigid cell walls (frustules) composed of amorphous silica. Depending on the species of diatom and growth conditions, these frustules can display a wide range of different morphologies. It is possible to design and produce specific frustule morphologies that have potential applications in nanotechnology, operating at detection levels 10 to 1000 times below the limits achieved with micromachining devices.

The next millennium will see technical and methodological changes in chromatographic analysis that are broader, more pervasive, and more advanced than at any time during the last five decades. Detergent removal, extraction and preconcentration of substances such as peptides and proteins from gel-separated samples prior to nanoscale LC–ESI–MS, and automated fraction into nanoelectrospray needles are already possible today, e.g. in the analysis of MHC-class peptides. Micromachined devices have generated impressive results in DNA, RNA and peptide/protein analysis. Nanoscale technology has been exploited in single cell analysis as well as in LC–ESI–MS and CE–ESI–MS. By reducing the sample volume from a few μl to 250 pl, and simultaneously reducing the sample spot area from a few mm^2 to 0.01 mm^2, low attomole detection limits have been obtained for bradykinin and cytochrome *c*. Here one can already speak of picoscale technology. The most profound results of nanoscale technology may be expected in the field of proteomics and a direct insight into substances determining the phenotype of organisms is already available.

References

[1] D. Malakoff, Science 283 (1999) 610.
[2] R.D. Piner, J. Zhu, F. Xu, S. Hong, C.A. Mirkin, Science 283 (1999) 661.
[3] J.B. Shear, H.A. Fishman, N.L. Allbriton, D. Garigan, R.N. Zare, R.H. Scheller, Science 267 (1995) 74.
[4] L.A. Greene, M.M. Sobeih, K.K. Teng, in: G. Banker, K. Goslin (Eds.), Culturing Nerve Cells, MIT Press, Cambridge, MA, 1991, p. 207.
[5] Q. Omar, K. Jardemark, I. Jacobson, A. Moscho, H.A. Fishman, R.H. Scheller, Science 272 (1996) 1779.

[6] K. Jardemark, O. Orwar, I. Jacobson, A. Moscho, R.N. Zare, Anal. Chem. 69 (1997) 3427.
[7] K. Jardemark, C. Farre, I. Jacobson, R.N. Zare, O. Orwar, Anal. Chem. 70 (1998) 2468.
[8] A.W.H.M. Kuypers, P.M.W. Willems, M.J. Van der Schans, P.C.M. Linssen, H.M.C. Wessels, J. Chromatogr. 621 (1993) 149.
[9] M. Orita, H. Iwahana, H. Kanazana, K. Hayashi, T. Sekiya, Proc. Natl. Acad. Sci. USA 86 (1989) 2766.
[10] J.M. Nigor, S.J. Baker, A.C. Preisinger, J.M. Jessup, R. Hostetter, K. Cleary, S.H. Binger, N. Davidson, S. Baylin, P. Devilee, T. Gloveri, F.S. Collins, A. Weston, R. Modali, C.C. Harris, B. Vogelstein, Nature 342 (1989) 705.
[11] A. Manz, E. Verpoorte, C.S. Effenhauser, N. Burggraf, D.E. Raymond, H.M. Widmer, Fres. J. Anal. Chem. 348 (1994) 567.
[12] R. Peters, R. Sikorski, Science 280 (1998) 1956.
[13] M.U. Kopp, A.J. de Mello, A. Manz, Science 280 (1998) 1046.
[14] M. Barinaga, Science 253 (1991) 1489.
[15] T. Livache, B. Fouque, A. Roget, J. Marchand, G. Bidar, R. Teoule, G. Mathis, Anal. Biochem. 255 (1998) 188.
[16] J.G. Hacia, W. Makalowski, K. Edgemon, M.R. Erdos, C.M. Robbins, S.P. Fodor, L.C. Brody, F.S. Collins, Nat-Genet. 18 (1998) 155.
[17] T. Pastinen, A. Kurg, A. Metspalu, L. Peltonen, A.C. Syvanen, Genome Res. 7 (1997) 606.
[18] J.G. Hacia, L.C. Brody, M.S. Chee, S.P. Fodor, F.S. Collins, Nat-Genet. 14 (1996) 441.
[19] D.I. Stimpson, J.V. Hoijer, W.T. Hsieh, C. Jou, J. Gordon, T. Theriault, R. Gamble, J.O. Baldeschwieler, Proc. Natl. Acad. Sci. USA 92 (1995) 6379.
[20] C. Moon, G.M. Preston, C.A. Griffin, E.W. Jabs, P. Agre, J. Biol. Chem. 268 (1993) 15772.
[21] M. Ogura, Y. Agata, K. Watanabe, R.M. MC Cormick, Y. Hamaguchi, Y. Aso, M. Mitsuhashi, Clin. Chem. 44 (1998) 2249.
[22] T. Livache, H. Bazin, P. Caillat, A. Roget, Biosens-Bioelectron 13 (1998) 629.
[23] S. Jespersen, Thesis, University of Leiden, Leiden, 1999.
[24] K. Hemminki, Arch. Toxicol. 52 (1983) 249.
[25] K. Randerath, E. Randerath, Drug Met. Rev. 26 (1994) 67.
[26] M.P. Chiarelli, J.O. Lay, Mass Spectrom. Rev. 11 (1992) 447.
[27] M. Saha, A. Abushamaa, R.W. Giese, J. Chromatogr. A 712 (1995) 345.
[28] K. Vanhoutte, W. Van Dongen, E.L. Esmans, E. Van den Eeckhout, H. Van Onckelen, Eur. Mass Spectrom. 2 (1996) 181.
[29] G. Hopfgartner, K. Bean, J. Henion, R. Henry, J. Chromatogr. 647 (1993) 51.
[30] K. Vanhoutte, P. Joos, F. Lemière, W. Van Dongen, E.L. Esmans, J. Mass Spectrom., Rapid Commun. Mass Spectrom. S (1995) 143.
[31] T.Y. Yen, N.I. Christova-Gucoguieva, N. Scheller, S. Holt, J.A. Swenberg, M.J. Charles, J. Mass Spectrom. 31 (1996) 1271.
[32] E. Rindgen, R.J. Tureskey, P. Vouros, Chem. Res. Toxicol. 8 (1995) 1005.
[33] M.S. Wilm, M. Mann, Int. J. Mass Spectrom. Ion Processes 136 (1994) 167.
[34] M.S. Wilm, M. Mann, Anal. Chem. 68 (1996) 1.
[35] M.T. Davis, D.C. Stahl, S.A. Hefta, T.D. Lee, Anal. Chem. 67 (1995) 4549.
[36] K. Vanhoutte, W. Van Dongen, I. Hoes, F. Lemière, E.L. Esmans, H. Van Onckelen, E. Van den Eeckhout, R.E.F. van Soest, A.J. Hudson, Anal. Chem. 69 (1997) 3161.
[37] K. Vanhoutte, W. Van Dongen, E.L. Esmans, Rapid Commun. Mass Spectrom. 12 (1998) 15.
[38] J.P. Barry, C. Norwood, P. Vouros, Anal. Chem. 68 (1996) 1432.
[39] D.L.D. Deforce, F.P.K. Ryniers, E.G. Van den Eeckhout, F. Lemière, E.L. Esmans, Anal. Chem. 68 (1996) 3575.
[40] P. Jenning, W. Schrader, M. Linscheid, Rapid Commun. Mass Spectrom. 8 (1994) 1035.
[41] M. Sharma, R. Jain, E. Jonescu, H.K. Slocum, Anal. Biochem. 228 (1995) 307.
[42] D. Deforce, Thesis, University Gent, Gent, 1999.
[43] D. Deforce, F. Ryniers, F. Lemière, E. Esmans, E. Van den Eeckhout, Anal. Chem. 68 (1996) 3575.
[44] D. Deforce, F. Lemière, E. Esmans, A. de Leenheer, E. Van den Eeckhout, Anal. Biochem. 258 (1998) 331.
[45] M.R. Wilkins, Biotechnol. 14 (1996) 61.
[46] E. Nettleton, M. Sunde, V. Lai, J. Kelly, C. Dobson, C. Robinson, J. Mol. Biol. 281 (1998) 553.
[47] A.A. Rostom, M. Sunde, S.J. Richardson, G. Schreiber, S. Jarvis, R. Bateman, C.M. Dobson, C.V. Robinson, Proteins 52 (1998) 3.
[48] K. Albert, M. Dachtler, T. Glaser, H. Händel, T. Lacker, G. Schlotterbeck, S. Strohshein, L.H. Tseng, U. Braumann, HRC/J. High Res. Chromatogr. 22 (1999) 135.
[49] G. Schlotterbeck, L.H. Tseng, H. Händel, U. Braumann, K. Albert, Anal. Chem. 69 (1997) 1421.
[50] K. Pusecker, J. Schewitz, P. Gförer, L.H. Tseng, K. Albert, E. Bayer, Anal. Chem. 70 (1998) 3280.
[51] S. Jespersen, W.M.A. Niessen, U.R. Tjaden, J. van der Greef, E. Litborn, U. Lindberg, J. Roeraade, Rapid Commun. Mass Spectrom. (1994) 581.
[52] J. Parkinson, R. Gordon, Trends Biotechnol. 17 (1999) 190.