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Journal of Chromatography A, 1000 (2003) 567–581
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Review

Contributions of liquid chromatography–mass spectrometry to ''highlights'' of biomedical research

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Abstract

Combined chromatographic and mass spectrometric techniques and in particular liquid chromatography–mass spectrometry (LC–MS) have been contributing in a decisive way to the progress of life sciences in general. Thus, the number of document entries in the US National Library of Medicine (MEDLINE) for articles dealing with LC–MS was 738 in 1991 and 2285 in 2001, with a total of 13 147 for the whole 10-year period, an increase of 310%. From these figures, we can ascertain that the total usage of combined LC–MS techniques is of the order of 40% relative to all of the MS publications collected in MEDLINE for the same period. However, from the perspective of real advances in medicine, it becomes difficult to identify what is outstanding in this field. The aim of this review was not to provide another LC–MS review, but an overview of the current status of the presence, visibility and impact of combined LC–MS techniques in biomedical research. The idea being to spot ''highlight'' literature contributions with the potential to become in the short or medium term real assets in a doctor's daily medical practice. In other words, after several truly remarkable technical achievements reported within the past decade, are we any closer to making LC–MS a useful and practical diagnostic tool for molecular diagnostics and personalized medicine? To approach this question, a literature survey was carried out to define: (i) the presence of LC–MS in the biomedical literature (MEDLINE) and its weight relative to the whole field of biological and biomedical mass spectrometry; (ii) the role of LC–MS in recent milestone biomedical contributions; and (iii) the present and future role of new LC–MS technology in medical diagnosis.

Keywords: Reviews; Liquid chromatography–mass spectrometry

Contents

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0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00601-0

1. Introduction

The present contribution is to be taken as a personal experience on a top-down approach to the generation of a scientific report in commemoration of the ''100 Years of Chromatography''. During these years, chromatography in its various modes of operation has contributed in a decisive, and in some cases, totally indispensable way to the advance of our knowledge in multiple fields of science. The technique permeates all disciplines and the biomedical field has become very relevant in this respect, especially when we consider the on-line or off-line coupling of powerful chromatographic separation means with highly selective and sensitive analytical tools, such as the mass spectrometer. The number of documents in international databases is staggering and difficult to digest. All of them have contributed, either in a very modest or significant manner, to significantly improve our current knowledge of human diseases, and their prevention and treatment. However, if we ask ourselves what is outstanding in this field from the perspective of real advances in medical practice, the answer is not obvious and whatever it is, it is likely to be highly subjective. This is why the present contribution is an experiment! Thus, it is not intended as a review article on liquid chromatography–mass spectrometry (LC–MS) in biomedicine, but as a commemorative 100th anniversary assay to expose the value of the technique from a medical perspective. In other words, once the pertinent question was defined, a medical literature search was undertaken to ascertain to what extent could such a question be answered with existing data. The goal herein thus focuses in providing an overview of the current status of presence, visibility and impact of combined LC–MS techniques in biomedical research. For this purpose, a focused literature survey was carried out to define: (i) the presence of LC–MS in the biomedical literature (US National Library of Medicine, MEDLINE) and its weight relative to the whole field of biological and biomedical mass spectrometry; (ii) the role of LC–MS in recent milestone biomedical contributions (Chemistry Highlights 2002 [1] and Top 10 medical/health stories 2002 [2]); and (iii) the present and future role of new LC–MS technology in medical diagnosis.

The choice of MEDLINE as the search database provides a clear picture of the spread and number of biomedical applications of combined chromatography and mass spectrometric techniques, including literature on the description of new methods or techniques in the field as well (see below). On the other hand, whereas the analysis of the data resulting from this search can certainly be considered as objective and truly representative of the selected search parameters, published descriptions of literature ''highlights'' [1,2] were specifically ''selected'' in order to prevent predesigned and/or subjective personal choices, as indicated above.

2. Presence of LC–MS in the biomedical literature and its weight relative to the whole field of biological and biomedical MS

At present it is evident that combined LC–MS is providing a wealth of information on critical fundamental and basic research issues in biology, biochemistry, structural biology or biomedicine. As an indication of the increasing relative weight of this modern analytical technique in the field of life sciences in general, the number of document entries in the PubMed data base of MEDLINE for the term ''mass spectrometry'' collectively went from 1771 in 1991 to 6294 in 2001, 35 192 being the total number of MS related documents that can be found in the database within this 10-year period. Of these, 738 entries correspond to articles dealing with LC–MS in 1991 and 2285 in 2001, with a total of 13 147 for the whole period. Thus, it could be argued that in the

past 10 years, the number of mass spectrometry items in the MEDLINE has increased over 350% and that the number of publications on LC–MS applications have kept pace, increasing their number by 310% (Fig. 1). From these figures, we can ascertain that the total usage of combined LC–MS techniques in 1991 was of the order of 42% relative to all MS publications collected in MEDLINE for that year and that 10 years later this percentage, while a bit lower, is still a sizeable 36% of the total. The latter moderate decrease may also reflect the increasing use of new off-line sample introduction methods, such as direct infusion techniques in electrospray ionization systems [3] or sample deposition on MALDI targets [4], as opposed to truly on-line LC–MS systems [5].

The continuous growth experienced within this time period in the accumulative number of LC–MS related documents on a yearly basis is clearly illustrated in Fig. 2. The light grey bars represent the number of items retrieved per year when searching for the terms ''LC–MS or LCMS or liquid chromatography mass spectrometry'' appearing at any place in the document. As shown, the other two bars show that LC–MS related terminology does not appear with the same frequency in the titles and abstracts of papers published in many biomedical oriented journals. This is to be expected, since in medical and clinical papers the technology used is often simply a means to reach an objective, as opposed to the situation in analytical journals where the development and/or application of a technique can be the prime concern in itself. This does not imply, however, that analytical developments in LC–MS biomedical methods or techniques are missed on MEDLINE since many of the so-called keynote contributions can be retrieved from this database. Thus, a combined search for the terms above ''AND methods OR techniques'' returns 5213 items. If the search is further refined to include the term ''new'', the turnout is then 895 documents for the 1991–2001 period, or about 7% of the total of 13 147 LC–MS documents found for this period.

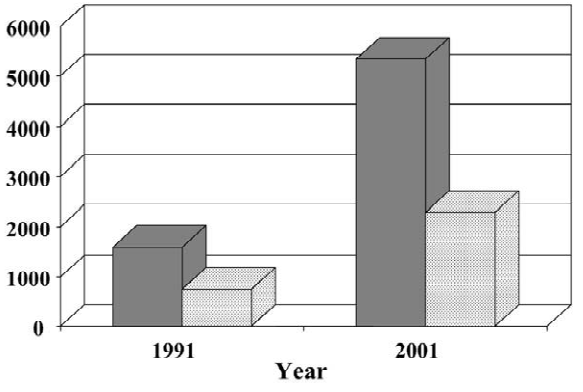

Fig. 1. Grey bars, total number of MS related documents in MEDLINE corresponding to the years 1991 and 2001. Dotted bars, total number of LC–MS related documents in MEDLINE corresponding to the years 1991 and 2001.

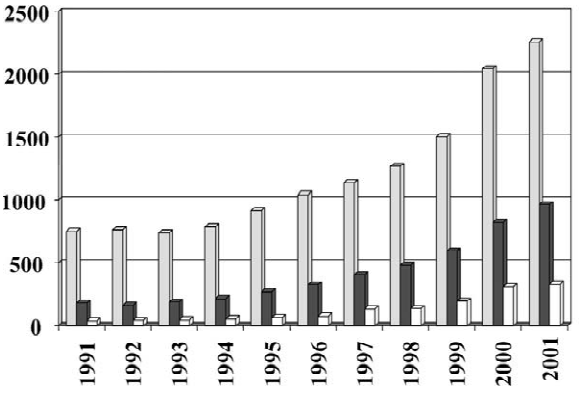

Fig. 2. Accumulative number of documents for LC–MS in MEDLINE database from 1991 to 2001. Light grey bars, LC–MS technique mentioned in document text; dark grey bars, in title/abstract; and white bars, in title only.

2.1. New ionization and instrumentation techniques in LC–MS

Within this 10-year time period significant events have taken place in the field of ionization techniques for mass spectrometry, which are evident in the information contained in the plot of Fig. 3. Whereas the 1980s was dominated by the use of thermospray [6,7], a mild ionization technique that notably expanded the limits of high molecular weight mass spectrometry in biological and biomedical applications, it has now been replaced by the new atmospheric pressure ionization methods [8]. Thus, the total number of thermospray based applications found via this PubMed search is only of 216 for the entire 10-year period. This contrasts with a total of 2707 articles found for the same period describing

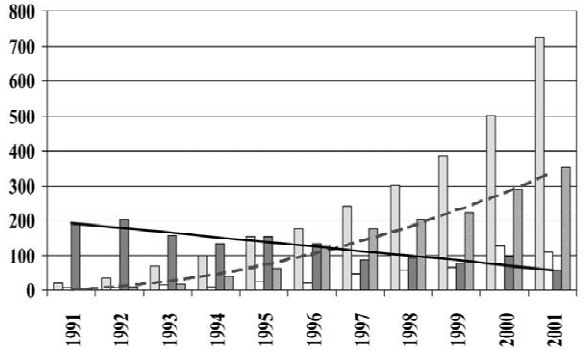

Fig. 3. Frequency of use of various MS ionization techniques for the years indicated. Total number of items found for each year. Dotted bars, electrospray (ESI); white bars, atmospheric pressure ionization (APCI); grey bars, fast atom bombardment (FAB); hashed bars, matrix assisted laser desorption ionization (MALDI).

the use of electrospray ionization (ESI) [9] or other atmospheric pressure ionization methods [10] like APCI with 496 articles, or ion spray [8] (261 articles in the data base). The yearly distribution trends of all of these documents is shown in the figure (except for ion spray), confirming the trend already observed in a 1995 review on ‘‘Biomedical and biochemical applications of liquid chromatography–mass spectrometry’’ [11]. From these data it becomes evident that API methods presently dominate the field of biomedical applications of mass spectrometry. Thus, the combined entries for electrospray, APCI and ion spray represent 54.5% of the total 10-year figure for all of the ionization techniques included in Fig. 3 (ESI, APCI, FAB, MALDI). On the other hand, it is clear that the use of fast atom bombardment (FAB) [12] for the study of high molecular mass biological material has been steadily decreasing with time, especially since 1997 and that it has been replaced by the more recent matrix assisted laser desorption ionization (MALDI) techniques, with up to 351 articles found for 2001. This is reflected in the tendency lines drawn over the bars in Fig. 3. The hashed line underlines the increasing frequency of use of MALDI vs. the decreasing usage of FAB methods (black). Disregarding FAB, both ESI and MALDI are today indispensable and powerful tools in all aspects of modern protein chemistry [13]. The coupling of all of these techniques to liquid separation methods, including capillary electrophoresis, has recently been reviewed [5,14].

As regards new instrumentation, database analysis also shows that tandem mass spectrometry (MS–MS), is clearly the dominant mass spectrometric technique in most publications. This could have an explanation in the increasing introduction and use of triple quadrupole and ion trap instruments [15,16] in biomedical oriented laboratories, which provide the means for tandem MS work. Also, the consolidated renaissance of time-of-flight (TOF) mass spectrometers [17] is evident and it is expected to grow even more over the immediate future, including their coupling to quadrupole systems (hybrid QTOF instruments).

2.2. New LC separation techniques in LC–MS

The information in the MEDLINE database also shows that most of the current applications of LC–MS techniques to date are still run on standard bore reversed-phase columns, whereas the use of capillary liquid chromatography (about 13%) is gaining popularity. A further search of the database reveals that relative to standard bore reversed-phase columns, entries for normal and bonded phases account for 21 and 4.5%, respectively. Nano-LC–MS is still sparsely used in the biomedical literature (0.22%) but applications of microbore columns are more popular (about 9% of the total for normal bore LC). On the other hand, the use of ion-exchange and ion pair methods in the biomedical LC–MS literature account for 70% of the total for standard reversed-phase applications. This figure needs to be taken with caution since documents retrieved in the search also turn out many off-line applications in the case of ion-exchange. Likewise, size exclusion methods represent up to 14% relative to the latter. On the other hand, although not strictly a liquid chromatography technique but a liquid phase separation method, capillary electrophoresis coupled to mass spectrometry represents a 5.13% of the total entries retrieved for LC–MS and it is clearly gaining a foothold in this field, as are the micro and nano-LC–MS methods [18].

3. LC–MS role in recent milestone biomedical contributions

Once we have obtained an overview picture of the position of combined LC–MS techniques in the biomedical literature (2.1 and 2.2 above), we could raise a number of pertinent questions regarding the actual practical value of combined LC–MS in everyday medical practice. In other words, questions that could be asked would be, how visible is LC–MS in medical and clinical research?, is it a real diagnostic tool?, and could it become a useful diagnostic tool in the doctor's office?

The field of mass spectrometry has been recently distinguished by a well deserved Nobel prize to John Fenn and Koichi Tanaka, proving the importance of fundamental work behind the spectacular advances of mass spectrometry. Mathias Mann, in a *Nature* article last year [19] was explaining that John Fenn wanted to develop a mass spectrometer that could be used in the doctor's office. Instead he invented electrospray, which immediately became a milestone technique in mass spectrometry and nobody would doubt today that this development has touched biomedical research at large. However, from a practical point of view, the question is whether mass spectrometry is just contributing to the collection of lasting observations and investigations (some of which fade rapidly) that create a continuum of events eventually leading to real progress in any given field or, whether in certain cases it is contributing to real medical milestones as well. In other words, can we infer from the literature the impact, if any, that combined LC–MS techniques are having on medical progress in disease diagnosis, treatment development and therapeutical monitoring.

However, although true scientific milestones are hard to pinpoint, this is the main goal of this exercise. To accomplish this task, a search strategy was outlined to identify the most recent medical highlights or breakthroughs where LC–MS played an unequivocal role. This became a complex task for what is a medical highlight and who defines it? To avoid clearly subjective personal definitions and/or selections, two useful sources of information were first identified in the literature. One of them is the *Chemical and Engineering News* (C&En) year-end review article on Chemistry Highlights 2002 [1] and the other the Top 10 Medical/Health stories 2002 from Medscape General Medicine [2].

3.1. Chemistry highlights 2002

In the first case, the C&EN article describes the chemical advances for the year in a range of areas—from organic chemistry to molecular medicine or attosecond spectroscopy. From these, the following areas were selected for an evaluation: medicinal and combinatorial chemistry, chemistry-based medicine, genomics and proteomics, carbohydrate chemistry and biochemistry. In total, these areas summed 68 highlighted articles selected as true milestones for the year 2002. A search through these articles showed that approximately 25% of them described the use of MS techniques. However, in some of these the role of MS was of high impact for the development of the results reported but of very low visibility. For instance, in one of these articles entitled ''Molecular properties that influence the oral bioavailability of drug candidates'' [20], all that could be found about the use of MS was the following sentence: ''Circulating concentrations of test compounds were determined using LC–MS–MS methods with demonstrated specificity and error over a concentration range of 10.0 ng/ml (LLQ) to 2500 ng/ml'' whereas in another entitled ''Structural basis for the activation of anthrax adenylyl cyclase exotoxin by calmodulin'' [21], all there is is a thank you note in the Acknowledgments to ''B. Bernat for help with mass spectrometry''. This is indeed an extreme case showing how much the technique can be downplayed in some cases, especially when there are medical teams behind the research reported.

Amongst the rest of the C&En highlighted articles, some were clearly dependent on the use of LC–MS analytical methodologies for the results obtained. For instance, one of them describes a new pharmacological approach to treating human amyloid diseases. These disorders range from type II diabetes to Alzheimer's disease, and involve the abnormal folding of usually soluble proteins into an insoluble, tightly packed shape known as a β-sheet. The proteins are then deposited outside cells and form aggregates (amyloid deposits that lead to tissue

damage). In this instance, Pepys and co-workers from the University College Medical School in London [22] describe the ''first example of a low molecular mass drug that profoundly depletes serum amyloid protein (SAP) from the circulation and the tissues''. SAP in amyloid deposits derives entirely from circulating SAP, which is produced, cleared and catabolized exclusively in the liver. The plasma and amyloid pools of SAP are in free dynamic equilibrium, and the new drug removes SAP from amyloid deposits in vivo by inhibiting its binding to amyloid fibrils and dissociating it from amyloid deposits. The drug, which is a dimeric derivative of proline, crosslinks pairs of native pentameric human SAP molecules, creating a decameric assembly that is cleared from the circulation by the liver. The significant depletion of plasma SAP thus achieved shifts the distribution of SAP between the plasma and amyloid pools. The SAP released from the tissues into the circulation is immediately targeted by the drug for clearance in the liver. Coupled with direct inhibition of SAP binding, this leads to rapid and extensive removal of SAP from the amyloid deposits. Plasma levels of SAP were monitored by multiple reaction monitoring with plasma samples subjected to protein precipitation, extraction, diazomethane methylation, reversed-phase HPLC separation and MS determination on an MS Quatro II with a deuterated internal standard. Clinical studies with this drug are under way.

Another highlighted article described how a group led by James Duan at Bristol Mayers [23] have designed a new class of substituted γ-lactams capable of inhibiting the metalloprotease that produces tumor necrosis factor. This factor is present in abnormally high amounts in cases of rheumatoid arthritis so that it could be argued that the inhibition of the converting enzyme that releases it from its membrane bound precursor may lead to improved therapeutics. In this work, a suitable lactam analogue, presently undergoing clinical trials, was synthesized in accordance with a rational drug design program and its pharmacokinetics in dog and rat plasma were established using a previously described LC–MS–MS procedure for direct plasma sample injection in multicomponent LC–MS–MS assays for high throughput [24].

Finally, the third selected ''highlight article'' is a collaboration from groups at the Rockfeller University and the Aaron Diamond AIDS Research Center [25] that has also been highlighted in *Nature Immunology* [26] (see Section 4.3).

3.2. Top 10 Medical/Health stories 2002

The rationale for the selection of these stories is summarized by the authors in the following paragraph form Medscape General Medicine [2]. ''In any one modern calendar year, much is observed and reported; some likely to be lasting, and some not''. It may be difficult to separate out those observations that are transient from those that might have more permanence. At best, scientific truth is a moving target, a work in progress. Nonetheless, the editors at *MedGenMed* decided to have a go at choosing what they believe to be the most important experimental results, medical observations, public health occurrences, or policy reports that appeared during 2002 to share with our readers, in part to inform, in part to stimulate their thoughts and memories, and perhaps to invite a bit of controversy''.

Thus, under this line of thought, the following stories were selected by the editors:

- The demise of postmenopausal *hormone replacement therapy*.
- *Molecular targeted therapies* come of age in oncology.
- Breast conserving surgery: final results.
- The narrowing gap between coronary stents and cardiac surgery.
- Intensive *insulin therapy* in critically ill patients.
- The *mouse genome sequenced*.
- Complete *genome sequence for the malaria mosquito*.
- HPV vaccination: a first step towards infection control.
- More nurses mean better patient care.
- Another malpractice crisis in the USA.

The major difference between this approach and the one taken by *Chemical & Engineering News* is that here only medical stories are highlighted and not specific literature reports. Thus, in this case one is forced to search the literature for representative MS articles with a direct bearing on the story selected.

Many contributions of combined LC–MS can be found for the stories highlighted above. The rest of

the stories are evidently not associated with the application of LC–MS techniques of any sort. Searching through those topics a continuum of scientific contributions, eventually leading to the 2002 stories above underlined, can be identified over the years for the different fields considered herein. Thus, various LC–MS approaches as well as other analytical techniques, have in cases markedly contributed to build up the foundations on which these top stories rest today.

3.2.1. The demise of postmenopausal hormone replacement therapy

To take up the first story, the termination on July 9 of the NIH Women's Health Initiative program effectively ended the period of enthusiasm about hormone replacement therapy, at least in the USA. Combined estrogen–progestin therapy was used there by 35% of postmenopausal women. The WHI program did prove that out of 161.809 women, 26% had higher risks of developing breast cancer, coronary heart disease, stroke or pulmonary embolism.

A lot of work has been carried out over time on the study of steroid biochemistry by mass spectrometry, as illustrated by the corresponding MEDLINE searches (1991–to date) for breast cancer (261 items retrieved), or breast cancer and hormone replacement therapy (78 items retrieved). Table 1 illustrates the number of items retrieved for a search for mass spectrometry and the indicated key words both in human/animal studies or exclusively in human studies, from 1991 to date. One thing that becomes clear from this table is that, although a good number of studies have been carried out by LC–MS techniques, this particular field is still dominated by classical GC–MS techniques.

Table 1
Contributions of MS to the study of steroid biochemistry and therapy

	Human/animal	Human
Steroid	2058	1015
Estrogen	384	192
Progestin	133	72
Steroid and ESI	152	66
Steroid and APCI	32	13
Steroid and API	45	20
Steroid and CG–MS	977	559
Estrogen and ESI	50	19
Estrogen and APCI	10	1
Estrogen and API	12	2
Estrogen and CG–MS	169	99

There is a vast array of scientific papers on these and related topics but no major contribution could be pinpointed with regard to the above highlighted story. Nevertheless, this is an active area that illustrates that not everything of significance in mass spectrometry occurs in structural biology or proteomics. Thus, standard MS techniques such as GC–MS are still very much active in the study of human diseases.

3.2.2. Molecular targeted therapies come of age in oncology

Regarding this topic, selective approaches and novel targeted drugs are presently considered the future of oncology therapy. Along these lines, the inhibition of the tyrosine kinase pathway has emerged as an important therapeutic target for cancer. Thus, treatment with Imatinib mesylate, a signal transduction inhibitor (STI), in cases of chronic myelogenous leukemia (CML) has been a remarkable therapeutical success, as illustrated by the high rate of success of a trial carried out on 553 CML patients, where 94.4% showed complete hematological responses and 97.2% experienced progression free survival after a 14-month follow-up. A recent article from Bakhtiar and co-workers at Novartis describes the pharmacokinetic study of this new antileukemia drug in human plasma by LC–MS–MS [27].

In this article its authors explain how it is known that a reciprocal translocation between chromosomes 9 and 22 yields a fusion oncoprotein known as Bcr-Abl (approx. 210 kDa). High catalytic activity of Bcr-Abl results in resistance to apoptosis, cell transformation, and malignancy. Such a high activity of the Bcr-Abl tyrosine kinase is a cytogenetic hallmark of CML and none of the therapeutic options is known to target the underlying cause of the disease.

Recently, Imatinib mesylate was approved by the FDA in a record time as an effective signal transduction inhibitor (STI571 or Gleevec™) for the treatment of patients at any of the three stages of CML: myeloid blast crisis, accelerated phase, and chronic phase, after failure of interferon-alpha therapy. Gleevec has been referred to as a milestone for drug

development in cancer and an ideal targeted drug at the molecular level. With high specificity, it competitively inhibits the binding of ATP to the kinase activation domain of Bcr-Abl. As a result, the activation loop of Bcr-Abl that modulates the catalytic activity of this enzyme is blocked from interaction with cellular ATP molecules. Fig. 4 shows the LC–APCI-MS–MS ion chromatograms for STI571

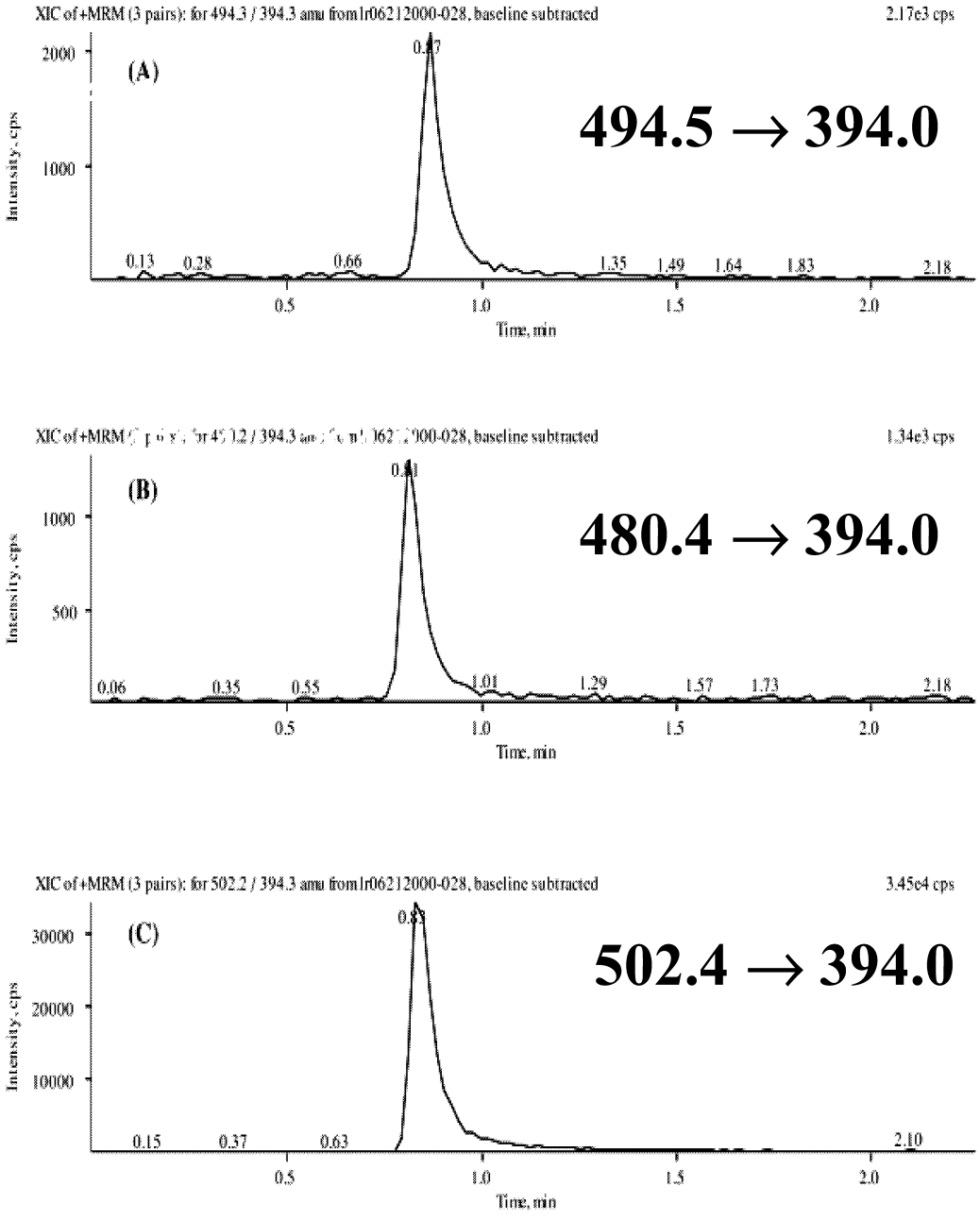

Fig. 4. LC–APCI-MS–MS ion-chromatograms; 4.00 ng/ml (LLOQ) of STI571 and CGP 74588 spiked with the internal standard (27 ng/sample) (adapted and reproduced from Ref. [27]).

(MW 494.5), its demethylated metabolite (MW 480) and deuterated internal standard (MW 502.4). These ion chromatograms illustrate the monitoring of the transition to the common fragment ion at m/z 394 arising from the cleavage of the piperazinyl group in the molecule.

3.2.3. Complete genome sequence for the malaria mosquito

A MEDLINE search for Malaria and mass spectrometry returned a total of 59 documents. One of these, recently published, represents a large-scale, high-accuracy (average deviation less than 0.02 Da at 1000 Da) mass spectrometric proteome analysis of selected stages of the human malaria parasite *Plasmodium falciparum*. The work entitled ''Analysis of the *Plasmodium falciparum* proteome by high-accuracy mass spectrometry'' is a collaboration of various groups in Denmark and The Netherlands [28] and describes the methodology used to define the proteome of the asexual (trophozoites and schizonts) and sexual (gametocytes) blood stages of the malaria parasite. Parasites were extracted by freeze–thawing and centrifugation, yielding soluble and insoluble (pellet) fractions. These complex protein mixtures were then analyzed ''gel free'' (differentially extracted membrane fractions) or separated into 10 molecular mass fractions by one-dimensional gel electrophoresis followed by excision of equally spaced bands after precisely removing hemoglobin and globin and subjecting them to tryptic digestion. The tryptic peptides were separated by reversed-phase liquid chromatography coupled to quadrupole time-of-flight mass spectrometry for peptide sequencing (nanoLC–MS–MS). Iterative calibration algorithms were used to achieve a final, average absolute mass accuracy of better than 20 ppm in both the precursor and fragment ions. As reported by the authors ''The analysis revealed 1289 proteins of which 714 proteins were identified in asexual blood stages, 931 in gametocytes and 645 in gametes. The last two groups provide insights into the biology of the sexual stages of the parasite, and include conserved, stage-specific, secreted and membrane-associated proteins. A subset of these proteins contain domains that indicate a role in cell–cell interactions, and therefore can be evaluated as potential components of a malaria vaccine formulation''.

4. Present and future role of new LC–MS technology in medical diagnosis

A detailed and combined Internet (various updating and news alert services: eurekalert, cancerpage and docguide) and MEDLINE search for new technologies or breakthroughs in mass spectrometry presenting a clear disease diagnostic potential points to the following scientific contributions as true milestone candidates: (a) imaging mass spectrometry; (b) high pressure LC–FTICR and AMT tag databases; and (c) protein chip-SELDI-TOF.

4.1. Imaging mass spectrometry

On March 30 2001 the Eureka Alert service of the AAAS (http://www.eurekalert.org) provided a public release note with the title ''New technology expands 'molecular photography' options''. The note explained that Kodak-Vanderbilt University Medical Center investigators led by Richard Caprioli had developed a new method of taking pictures of molecules in a slice of tissue. It went on saying ''The technique, called 'imaging mass spectrometry', offers scientists a new tool for visualizing where proteins are located in cells and tissues''. This kind of information is important to understanding how proteins work and how they change in disease states. The Vanderbilt team applied the new technology, described in the April issue of *Nature Medicine*, to taking ''molecular photographs'' of normal and malignant brain tissue slices''.

The new technology [29] uses a MALDI mass spectrometer with properly modified electronics and software to image tissue slices. The slices to be studied were coated with a suitable matrix material and subjected to laser shots that release molecules for analysis in the mass spectrometer. Each spot hit by the laser beam becomes a ''pixel'' in the final image, with each pixel containing a record of the molecules in that spot (Fig. 5). Separation science in the form of protein HPLC again plays a role in the identification of the mapped proteins. For this purpose, the authors made an extract of the appropriate portion of the tumor tissue, and then fractionated the proteins by HPLC. A representative UV chromatogram of such an extract is shown in Fig. 6. The on-line ion trap mass spectrometric analysis per-

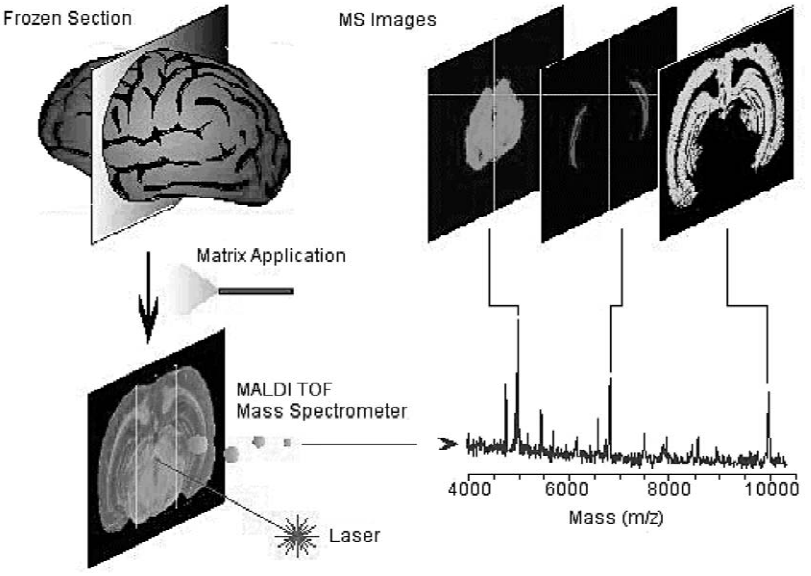

Fig. 5. Spatial tissue analysis by MALDI MS. Frozen tissue sections are mounted on a metal plate, coated with a UV-absorbing matrix and placed in the mass spectrometer. Ionized analytes desorbed from the tissue are determined in a TOF system. Mass spectrometric images are generated at specific molecular mass values (reproduced from Ref. [29]).

formed using ESI-MS permitted localization of the fraction containing the proteins of interest. For example, one of the proteins of molecular mass 4964 was eluted at 28.35 min in the chromatogram as illustrated in the figure. A sample of this fraction was spotted onto a MALDI target plate and subjected to on-target trypsin digestion. The analysis of the digest by MALDI MS identified thymosin β4 (Tβ4) as the protein matching the digest data precisely. The sequence analysis of the amino-terminal peptide (inset of Fig. 6) confirmed the identification of the protein.

The great diagnostic potential of the new technique has been highlighted by Suzanne Rostler in the news section of the Cancer Page at http://www.cancerpage.com/cancernews. This news release stated ‘‘A new technology that produces digital computer images of cells could improve the diagnosis and treatment of cancer. The investigators used imaging mass spectrometry to detect cells producing high levels of a specific protein that helps tumors to grow, called thymosin β-4. The discovery may be important when deciding exactly where to surgically remove tumors, since these proteins are potentially the best targets for diagnostic and therapeutic purposes’’, Dr Richard Caprioli, one of the study authors, told Reuters Health in an interview. This type of search can help scientists understand how proteins work inside of cells, and how they change as a result of disease.

Caprioli, from Vanderbilt University in Nashville, Tennessee, said in a prepared statement, ‘‘The presumption is that molecules which are distinct for this invasive tumor region are related to the proliferation process and might serve as good diagnostic markers or molecular drug targets’’. He said that using imaging mass spectrometry to develop new drugs ‘‘would be a major advancement in the drug discovery process’’.

4.2. High pressure LC–FTICR and AMT tag databases

Another public release, dated September 3 2002, from the Eurekalert service highlights high pressure LC–FTICR as a breakthrough mass spectrometry technology as it reduces the time needed to analyze a proteome from years to days. ‘‘The new high-throughput instrumentation uses very high-pressure capillary liquid chromatography combined with a

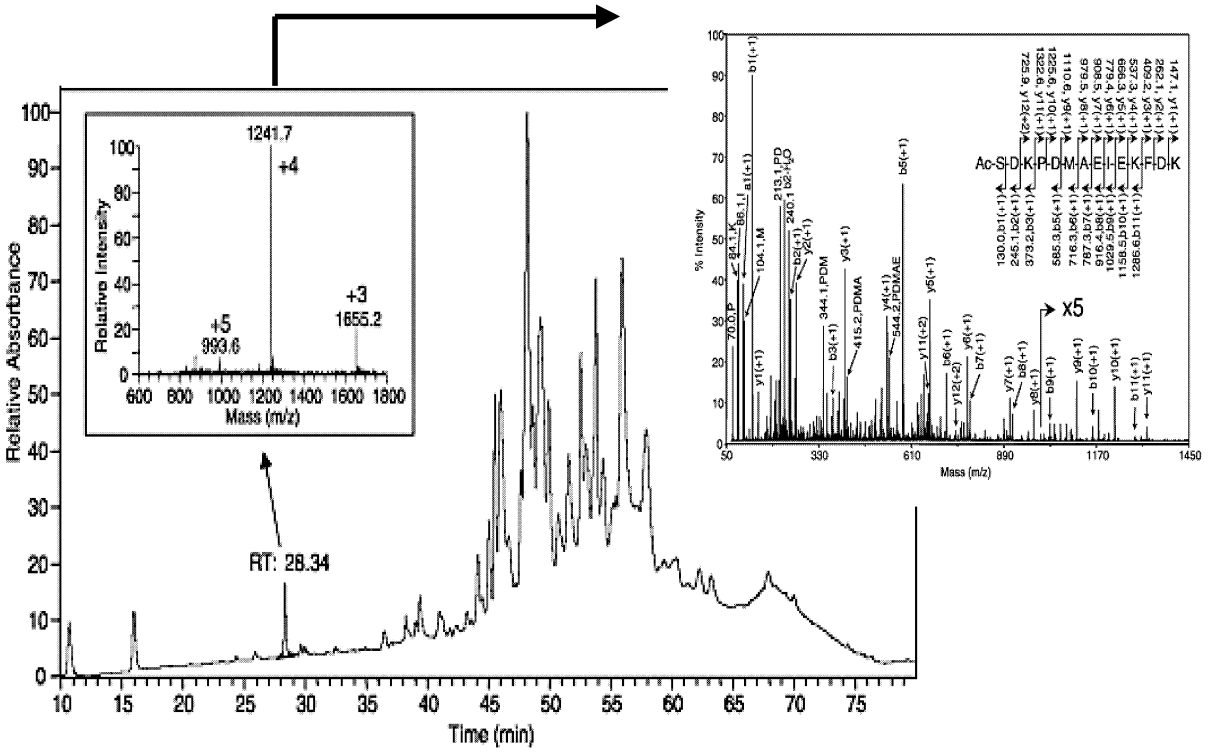

Fig. 6. UV chromatogram of an LC separation on a glioblastoma xenograft extract. The analyte of molecular mass 4964 was detected by online ESI-MS at 28.3 min. The mass spectrometric analysis by electrospray MS–MS of the N-terminal tryptic fragment of T.β4 (adapted and reproduced from Ref. [29]).

unique form of Fourier transform ion cyclotron resonance (FTICR) mass spectrometry, conceived by Battelle Fellow and Chief Scientist Dick Smith. This breakthrough technology enabled Smith's research team, collaborating with *Deinococcus radiodurans* experts from Louisiana State University, and the Uniformed Services University of the Health Sciences, Bethesda, to identify more than 61% of the predicted proteome (more than 1900 of the almost 3200 proteins predicted) of *D. radiodurans*, a radiation-resistant bacterium. These results represent the broadest coverage of any organism to date''. Called PROMS for protein mass spectrometer, the instrument is a 9.4-tesla FTICR system that researchers extensively modified with hardware and software tools that enable identification of an extremely wide range of proteins. The system has an exceptional capability for identifying proteins that exist in small quantities—with sensitivities up to 100 times greater than other methods.

PROMS is faster than tandem mass spectrometry (MS–MS). This is because while MS–MS requires the generation of one spectrum, or dataset, for each and every identified protein, with each and every sample analyzed, the PROMS approach can identify many proteins from one spectrum after initial validation using MS–MS. After validation takes place, the system uses biomarker tags to avoid the tedious MS–MS step. The time required for analysis with PROMS is thus shortened considerably with results produced 10–100 times faster.

A complete description of the technique has been published in the *Journal of Mass Spectrometry* [30]. In that article authors explain the basis of the experimental steps involved in accurate mass time (AMT tags) based proteomics, as shown in the scheme of Fig. 7. As shown in Fig. 7a, a proteome sample is first analyzed by LC–MS–MS using a conventional ion-trap mass spectrometer and peptides are identified by the resulting sequence in-

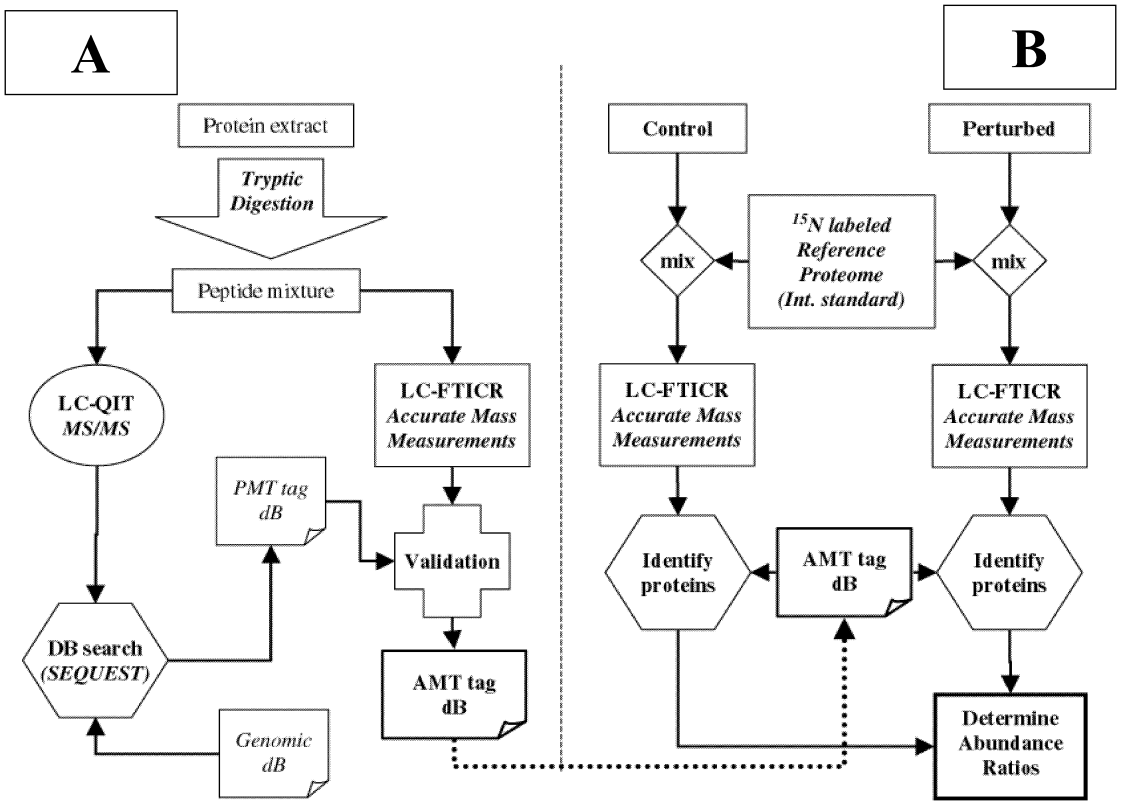

Fig. 7. Experimental steps involved in AMT tags based proteomics. (A) A proteome sample is analyzed by LC–MS–MS using a conventional ion-trap mass spectrometer and peptides are identified by the resulting sequence information as potential mass and time (PMT) tags. The same proteome sample is then analyzed under the same LC–MS conditions using an FTICR mass spectrometer. An AMT tag is established when a peptide eluting at the same time and corresponding to the calculated mass (within 1 ppm) of the PMT identified initially is observed. By analyzing a plethora of samples, an AMT tag database is constructed. (B) These peptides then function as markers to identify their corresponding proteins in all subsequent experiments analyzing a proteome sample from a specific organism, such as protein level gene expression profiling illustrated here (reproduced from Ref. [30]).

formation as potential mass and time (PMT) tags. The same proteome sample is then analyzed under the same LC–MS conditions using an FTICR mass spectrometer. An AMT tag is established when a peptide eluting at the same time and corresponding to the calculated mass (within 1 ppm) of the PMT identified initially is observed. By analyzing a plethora of samples, an AMT tag database is constructed. These peptides then function as markers to identify their corresponding proteins in all subsequent experiments analyzing a proteome sample from a specific organism, such as protein level gene expression profiling illustrated here (Fig. 7B).

4.3. Protein chip-surface enhanced laser desorption ionization

This is a relatively new technique known as Protein chip® (Ciphergen Biosystems, Inc., Fremont, CA) surface enhanced laser desorption ionization (SELDI)-time of flight (TOF) for which a total of 45 items can be found in MEDLINE starting in 1998. SELDI combined with liquid phase chromatography chip surfaces allows the selective capture of proteins on the chips. These are then analyzed by laser desorption in a time of flight mass spectrometer. The SELDI process captures one or more proteins of

interest on either the chemically or biologically modified surfaces of a protein chip array. The capture is a direct affinity chemistry process that does not require sample preparation. ‘‘Chemical or biomolecular noise’’ is reduced by washing away undesired non-retained materials. Next, the proteins retained on the chip are read by means of the SELDI system obtaining direct information on their molecular mass and if needed the target proteins are processed in situ by application of specific reactions and/or digestion processes. The surface chemistry of the arrays is based on classical chromatographic surfaces including normal and reverse capture phases, cation and anion exchange surfaces and immobilized metal affinity capture surfaces. Special affinity capture surfaces based on the use of specific antibodies, enzymes or receptors are also used for very specific capture processes.

This technique has been recently used for instance for the work described in one of the articles mentioned in *Chemistry Highlights 2002* of C&En ([25] see above and under IIa) also specially cited by the journal *Nature Immunology* [26]. According to the highlight cited from this journal ‘‘In *Science*, Ho and colleagues describe how they used mass spectroscopy and protein-chip technology to isolate three proteins that are secreted in response to stimulation of $CD8^+$ T cells from HIV-infected long-term non-progressors (LTPNs). These proteins were identified as α-defensins-1, -2 and -3. Blocking antibody for α-defensins abolished anti-HIV activity not attributable to β-chemokines. Furthermore, synthetic and purified human α-defensins inhibited HIV replication in vitro. Thus, α-defensins contribute to the anti-HIV activity of CAF’’.

It has been known since 1986 that CD8 T lymphocytes from certain HIV-1-infected individuals who are immunologically stable secrete a soluble factor, termed CAF, that suppresses HIV-1 replication. However, the identity of CAF had remained elusive despite an extensive search. A scheme of the analytical procedure is shown in Fig. 8. Supernatant fluids were harvested from stimulated and unstimulated CD8 T lymphocyte cultures derived from three LTNPs, as well as from four progressors and 15 normal controls. Each sample was analyzed on a protein chip system, based on the integration of chemically modified array surfaces with surface-enhanced laser desorption/ionization (SELDI) time-of-flight (TOF) mass-spectrometry (MS) detection. This allowed the identification of a cluster of small proteins secreted by stimulated CD8 T cells (Fig. 9).

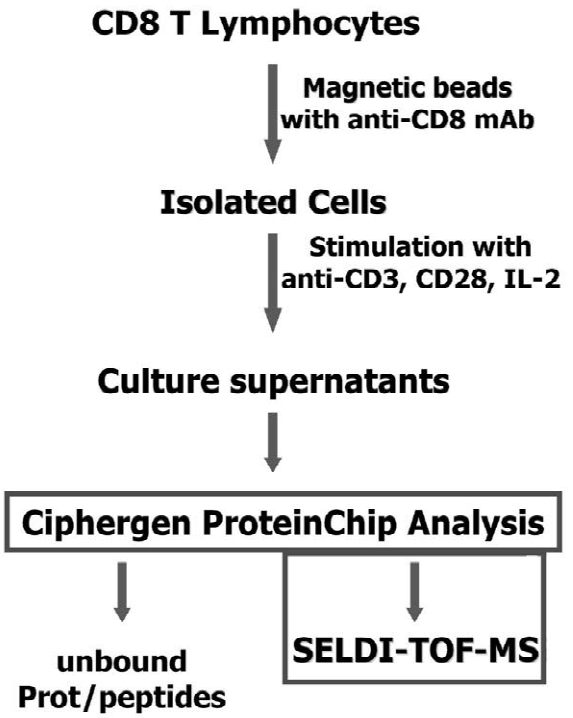

Fig. 8. Supernatant fluids were harvested from stimulated and unstimulated CD8 T lymphocyte cultures derived from three LTNPs, as well as from four progressors and 15 normal controls. Each sample was analyzed on the protein chip system (Ciphergen Biosystems, Fremont, CA), which is based on the integration of chemically modified array surfaces with surface-enhanced laser desorption/ionization (SELDI) time-of-flight (TOF) mass-spectrometry (MS) detection (from Ref. [25]).

The great potential of this technique as a diagnostic tool has been further highlighted in the Doctor’s Guide at www.docguide.com/news. One of the recent DG dispatches calls attention to how protein chip mass spectrometry may be able to identify cancer-associated protein profile panels, which can lead to a simple blood test for cancer detection. Lori L. Wilson, MD, Eastern Virginia Medical School, presented study results at the 25th Annual Meeting of the San Antonio Breast Cancer Symposium. ‘‘Currently, there are no single protein markers

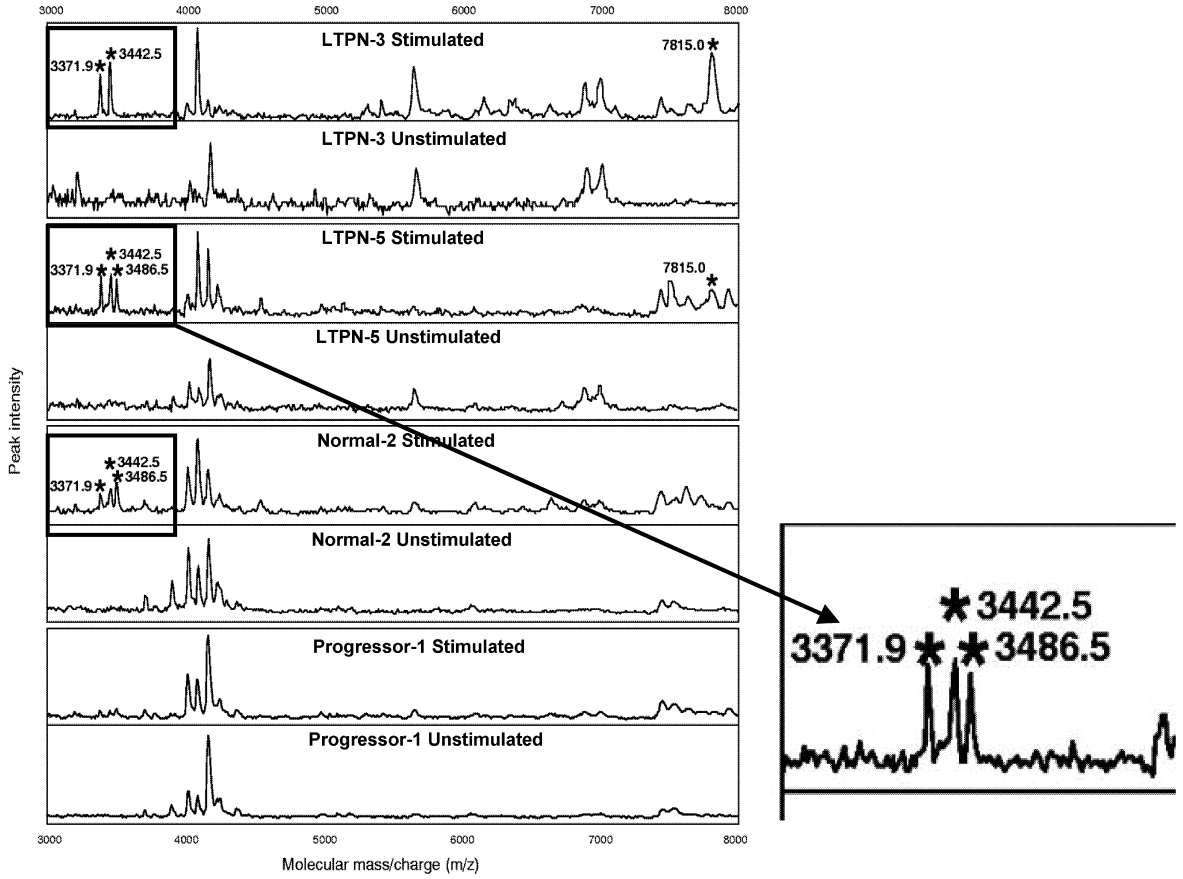

Fig. 9. Protein mass spectra of culture supernatants from stimulated and unstimulated CD8 T cells from two LTNPs, one normal individual, and one progressor. Protein peaks up-regulated after stimulation are highlighted (see inset) (adapted from Ref. [25]).

available for breast cancer screening,'' Dr Wilson said. ''Identification and analysis of a protein panel profile appears to have a greater potential for overcoming this limitation''. Dr Wilson and colleagues have used the procedure combining SELDI-TOF protein chip mass spectrometry technology along with a classification algorithm to identify protein profiles for detection of breast cancer. This innovative technology searches for multiple differentially expressed proteins and is able to create a protein profile panel.

Along these lines a public release from Eurekalert dated July 29 2002 and entitled ''A laser-based spectrometry screening tool may provide early and efficient detection of breast cancer'' points out that identification of disease-associated proteins could be a solution for early detection of breast cancers. The study carried out using the same technology by a team of John Hopkins researchers [31] gave evidence that SELDI mass spectrometry could lead researchers to the discovery of additional biomarkers that would indicate early stage breast carcinoma, and that this technology could fill a gap that now exists in breast cancer prevention.

5. Conclusions

It is clear from all of the above that the combined technologies of liquid phase separations and mass spectrometry, either on- or off-line, are providing the biomedical community with the knowledge foundation necessary to advance in the right direction in the control of human health it its widest sense. This is

obtained for the most part, however, with classical or standard state-of-the-art LC techniques as opposed to the popularization of novel MS techniques. In other words, most of the experimental work described in the articles selected as LC–MS highlights was carried out with standard or microbore LC columns whereas capillary and nano-columns or capillary electrophoresis columns are still in the development stages regarding biomedical applications. This is somewhat surprising as miniaturization and column on a chip technologies are gaining ground on account of the advantages they afford in terms of lower sample consumption and increased detection limits. However, these technologies, while very popular in a handful of specialized laboratories, still have to be introduced into the realm of medical schools. The dream of Nobel Laureate, Professor John Fenn has yet to be fulfilled, as there is no such a thing as a small desktop diagnostic mass spectrometer to be found in doctors' offices. Nevertheless, the technological breakthroughs presented above for the identification of disease biomarker proteins will probably contribute to moving this idea forward in the near future, especially as regards protein chip SELDI-TOF approaches where a full diagnostic profile of a patient could be obtained with a minimum of sample. If anything, technology is getting closer to the desktop diagnostic mass spectrometer and will probably come close to the identification of disease biomarkers on specific chromatographic chips coupled to miniaturized mass spectrometer systems.

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