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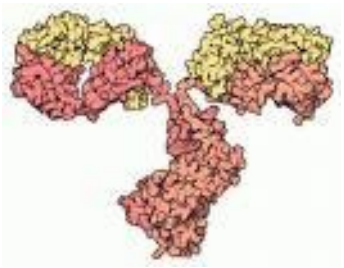
Immunoassay and Mass Spectrometry Analytical Methods for Targeted Proteomic Analysis



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To my family

Index

<u>Chapter 4:</u> The Role of ICP-MS in Metallodrugs-Proteins Interaction Monitoring

Introduction

Nothing should be done
if the consequences might not be serious.
George Bernard Shaw

Since the rediscovery of Gregor Mendel's findings in the early twentieth century we have known that the observable traits of an organism are largely controlled by inherited functional units. In the years that have followed, much effort and progress have been made in genetics and functional genomics to understand the molecular basis of inheritance and how it translates into a particular phenotype. However, the simple hope that genomes can be viewed as blueprints from which phenotypes can be inferred has not been fulfilled. The term phenotype is generally used to describe the observable traits of an organism (for example, morphology, size, physiology and behaviour), that emerge from its genotype and the specific environmental conditions. As the sensitivity of biological and biochemical methods has increased, the term has been extended to various aspects of cellular and molecular organization. Taking into account the complexity of cellular systems, techniques have been developed over the years that allow the comprehensive analysis of molecular components. Unfortunately, the global analysis of proteins was exceedingly difficult in the past. The classical biochemical analysis of proteins has been a daunting and time-consuming task in the past, and has yielded incomplete data sets that provided little quantitative information. In addition, changes in protein abundance cannot be simply inferred from DNA microarray data as mRNA abundance poorly correlates with protein abundance. Recently, and partly as a consequence of the advances in genome sequencing, enormous progress has been achieved in mass spectrometry based proteomics.

In the past decade mass spectrometry (MS) has experienced consistent instrumental improvements that enable biological research applications unheard of before. Due to such important developments and their associated creative applications the current role and potential of MS is enormous for proteomics based investigations. In fact, MS is today not just an established tool for structural proteomics research, it is more than ever at the forefront of functional proteomics. So far, however, most of the efforts devoted to proteomics have focused on developing methods that enable efficient identification (and where possible structural characterization) of large numbers of proteins in the samples, while protein quantification studies carried out so far are comparatively scarce. This situation is mainly derived from the great difficulties in obtaining the accurate and reliable determination of the

amount of a protein “buried” in a complex real protein mixture. Main challenges to be tackled when accurate protein quantitative analysis is pursued are i) the system to be analysed may be very small (e.g. a single cell, with 0.5 pL volume and ca. 50 pg total protein content); ii) the possible proteins expression dynamic range is very large (e.g. from 1 to 106 copies/cell) with proteins to be determined “buried” in a complex matrix; iii) the number of possible different proteins, e.g. in human samples, is huge (i.e. possibly 10–20 times the number of genes which codify the proteins) and iv) proteins show very large variations in their physicochemical properties.

In the following Ph.D. work, the line of research dealing with the developed of multiplex analytical methods for proteomic analysis is presented. The use of the term “targeted proteomic” refers to investigation in complex mixtures of specific proteins or peptides which are representatives for the definition of a particular state of the system analyze. Different applications were investigated, ranging from analysis of foods to human fluids or tissue samples.

The manuscript is divided into four chapters which define in principle the theme enclosed, which is further explained in detail with proper introduction, experimental section, results and discussion and conclusions by the collection of different subchapters.

In Chapter 1, the application of a classic shotgun proteomic approach for the determination of hidden peanut allergenic proteins in food samples at the trace levels is developed. As general observation, working with molecular mass spectrometry on this subject highlighted one of the most common limitation relates to this technique: the MS signal intensity coming from such “soft” ionization source is strongly affected by the species considered like protein, peptide, sample matrix and also solvents. Therefore, the aim to develope methods for multiplex quantification inserts an additional level of difficult since the targeted analytes might be of very different physical-chemical properties. During this former part of the work, in order to obtain a more sensitive method a step of selective enrichment of the sample was applied with the introduction of analytes (proteins) purification with magnetic beads opportunely functionalized with monoclonal antibodies raised against the target proteins. Use of antibodies as specific “hunters” of targeted proteins in complex samples, suggested the possibility to develope a special immunoassay which can be coupled with a final detection of analytes by ICP-MS.

Then, in Chapter 2 the first application for analysis in food samples by an immunoassay in ELISA format based on the use of a commercially available antibody labelled with lanthanide (Europium) ion is presented. The promising results obtained, pushed towards a direct comparison with the method prior developed in LC-ESI-MS for the same analytes. The best

sensitivity, precision and particularly the indipendence of ionization process from the sample analyzed by ICP-MS analysis, underline its role as technique of choice for quantification purpose. Furthermore, one of the principal feature of ICP-MS is its ability to perform multi-element detection in quasi-simultaneous way, fitting for purpose of multiplexed targeted proteomic. However, since ICP-MS detector information is elemental, only useful heteroatoms are measured (i.e. metals and non-metals) and it should be note that all structural molecular information are lost as consequence of atomization process in ICP source. A wide part of proteome does not contain useful elements for a direct ICP-MS detection and therefore it still invisible to the analysis. To overcome this problem recently two different strategies were recently introduced in literature: i) the direct labeling of protein to be determined and ii) labelling of an antibody followed by a specific antibody-antigen (antibody-protein) reaction. The direct labelling of a metal or elemental tag to the target protein can be performed in two different ways, as well: either the metal, mostly lanthanides, is introduced in the form of a coordination complex, e.g. with DOTA, or the heteroelement is bound directly to a specific amino acid via a covalent bond. For the introduction of a lanthanide into a protein, its complexing reagents based on DOTA (1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid) or DTPA (diethylentriamine-N, N,N',N'',N''-pentaacetic acid) are developed to target specific amino acid functions *via* thiol- or amine-reactive groups. Once covalently bound to the protein, the macrocycle ligand can be loaded by lanthanide ions with the result of thermodynamically highly stable complexes. These complexes, attached to desired proteins, are shown to be stable during a typical proteomic workflow, therefore the corresponding peptides could be characterised by MALDI- and ESI-MS and furthermore quantified by ICP-MS. The second strategy follows the principle of immunoassays and makes use of the same labelling reactions described in the above paragraph. Here, antibodies are labelled with complexes of different lanthanides, which serve as sensitive elemental tags in ICP-MS detection. Based on the pioneering work of Zhang et al. several concepts and applications have been developed for the multiplexing protein determinations. Different antibodies were labelled with different lanthanides, and thus, different proteins could be detected by ICP-MS simultaneously. In order to increase the detection sensitivity for these elemental-tagged immunoassays, gold nanoparticles were attached to antibodies as well. However, the possibility of a multiplexed detection solely based on Au nanoparticle labelling is limited. Thus, a new concept was recently presented, in which different lanthanides were incorporated into antibodies in the form of multiple metal-chelating ligand. This approach combines accordingly the multiplexing (simultaneous multi-element detection) and highly sensitive (low detection limits) capabilities of ICP-MS for its emerging use in protein analysis. For our

purpose, the second strategy involving the immunoassay was chosen, because the former approach posses a weak point in the needs of an additional post-labelling passage of characterization of the proteins prior to ICP-MS quantification.

Chapter 3 represents the core of this thesis, and it is dedicated to the development of labelling procedure for monoclonal antibodies with lanthanides complexation based on DOTA conjugation to biomolecule. For the first time, elemental labelled monoclonal antibodies were used in a liquid phase immunoassay with a virtual absence of sample manipulation, and the coupling of size exclusion chromatography to separate bound/unbound forms of antibodies was found successful. In order to provide additional information regarding the control of labelling procedure, the stoichiometry metal/antibody was determined by a new method which exploite the High Resolution-ICP-MS capability to perform measure of sulphur content into antibody at low levels. Then, knowing the sulphur content into protein backbone prior of lanyhanides loading, the stoichiometry of labelling can be asses by the ratio tag/sulphur, where this element is a direct measure of antibody concentration. In addition, an approach aiming to the increase in sensitivity by the introduction of a high number of tags into antibody is presented with the use of dendrimers as useful carriers of multiple copies of DOTA ligands.

Finally, Chapter 4 is an external subject of research respect to former three chapters. The idea of this study rely in a previous research regarding the study of interaction between metallodrugs with model proteins, at that time performed by UV-VIS spectroscopy and NMR techniques. The acquisition of competences and knowledge in proteomic techniques, especially with ICP-MS, suggested the idea of study the non-covalent interactions between proteins (albumin and transferrin) and metallodrugs by more sensitive and accurate methods. Also if ICP-MS was already used for these purpose, the separation techniques employed normally involves the coupling of capillary zone electrophoresys or ion exchange chromatography. Nevertheless, the robustness of these methods is still scarce and the harsh conditions necessar for separation of bound/unbound metal species does not reflect a natural environment in which interaction (i.e. human serum) take place. Then a new set up for this kind of studies involving size exclusion chromatography and ICP-MS is presented and results are further investigate by ESI-MS both on the whole tranferrin and on a model peptide mimicking the iron site.

E 'l duca lui: "Caron, non ti crucciare:
vuolsi così colà dove si puote
ciò che si vuole, e più non dimandare".
Dante Aligieri,
Inferno, Canto III

Chapter 1

Molecular (ESI) Mass Spectrometry for Targeted Proteomic in Foods

1.1. Use of Specific Peptide Biomarkers for Quantitative Confirmation of Hidden Allergenic Peanut Proteins Ara h 2 and Ara h 3/4 for Food Control by Liquid Chromatography–Tandem Mass Spectrometry.

Abstract. A liquid chromatography–electrospray-tandem mass spectrometry (LC–ESI-MS–MS) method based on the detection of biomarker peptides from allergenic proteins was devised for confirming and quantifying peanut allergens in foods. Peptides obtained from tryptic digestion of Ara h 2 and Ara h 3/4 proteins were identified and characterized by LC–MS and LC–MS–MS with a quadrupole-time of flight mass analyzer. Four peptides were chosen and investigated as biomarkers taking into account their selectivity, the absence of missed cleavages, the uniform distribution in the Ara h 2 and Ara h 3/4 protein isoforms together with their spectral features under ESIMS–MS conditions, and good repeatability of LC retention time. Because of the different expression levels, the selection of two different allergenic proteins was proved to be useful in the identification and univocal confirmation of the presence of peanuts in foodstuffs. Using rice crispy
and chocolate-based snacks as model food matrix, an LC–MS–MS method with triple quadrupole mass analyzer allowed good detection limits to be obtained for Ara h2 (5 μg protein g^{-1} matrix) and Ara h3/4 (1 μg protein g^{-1}matrix). Linearity of the method was established in the 10–200 μg g^{-1} range of peanut proteins in the food matrix investigated. Method selectivity was demonstrated by analyzing tree nuts (almonds, pecan nuts, hazelnuts, walnuts) and food ingredients such as milk, soy beans, chocolate, cornflakes, and rice crispy.

INTRODUCTION

In recent years peanut allergy seems to be on the rise in western countries, with a prevalence of 1% to 1.5% [1]. Together with tree nuts, peanuts account for the majority of food allergic reactions with fatal outcome both in children and in adults [2]. Since spontaneous resolution of peanut allergy is rare [3] and curative treatment is not yet available [4], strict elimination of peanuts is the rule, because traces of peanut can cause a severe allergic reaction in sensitive subjects [5, 6]. However, strict elimination is difficult, especially because food labeling practice can be still inadequate [7, 8] and contamination of nonallergenic foodstuffs with allergenic ingredients can easily occur during processing and transport. Recent developments intending to improve regulatory aspects of food labeling have led to increased advisory labeling [9–11].

Up to now several methods have been developed for the determination of peanut allergens in foods; these are mainly based on immunoassay (sandwich and competitive enzyme linked immunosorbent assay (ELISA) [12, 13], polymerase chain reaction (PCR), and real-time-PCR [14]. These approaches represent valid screening tools allowing simple analysis of a high number of samples. However, in such methods problems related to DNA–protein expression level, cross-reactivity, accuracy, and selectivity are still present. In a research program dealing with the development of new analytical strategies for the determination of hidden allergens in foods, very recently [15] we proposed a non-competitive sandwich immunoassay with inductively coupled plasma-mass spectrometric (ICP–MS) detection for the analysis of peanut proteins in different food matrices by using metal-tag antibodies. The method was demonstrated to be suitable for the detection of peanut allergens down to 1.5 mg peanuts kg^{-1} matrix with good precision. The ICP–MS system presents several advantages, including high sensitivity and the capability of performing multi-tag detection. However, this technique does not overcome the limitations of immunoassay methods in terms of accuracy and selectivity. In such a context, the high selectivity of mass spectrometry in protein analysis can overcome some of the difficulties associated with the correct identification of peanut proteins in complex food matrices. In this work we propose a proteomic-based approach for quantitative confirmation of peanut allergens in foods. In particular, we devised a liquid chromatography–electrospray-tandem mass spectrometry (LC–ESI-MS–MS) method with triple quadrupole (QqQ) analyzer for identification and determination of two major peanut allergens, i.e. Ara h 2 and Ara h 3-4[17], by using characteristic peptide biomarkers. Recently, LC–ESI-MS–MS methods for the detection of Ara h 1 in ice-cream and dark chocolate have been published [18, 19]. In those papers, the approach proposed was based on the detection of biomarker peptides

of one protein. i.e. Ara h 1, to directly identify peanut allergens at low parts per million level in food matrices. When complex matrices are analyzed, the selection of adequate peptide markers derived from different proteins for detection of hidden allergens is of great importance. In this context, very recently a characterization of potential peptide biomarkers of Ara h 1, A h 2, and Ara 3/4 using capillary LC-nanoESI-quadrupole-time-of-flight (QTOF)-MS–MS has been proposed [20]. In that work, attention was paid to the establishment of a list of peptides that remain stable after peanut processing.

The key feature of the research study proposed here is the development of a LC–ESI-QqQ-MS–MS method for two different proteins of the same allergenic source with the goal of combining the selectivity of the approach based on the detection of peptide biomarkers for different allergens with the capability of the method of performing quantitative analysis. In addition, attention was paid to assessment of method performance for both qualitative and quantitative purposes. The choice of two targets can offer the advantages of increasing the selectivity of allergen identification and better assessment of electrospray sensitivity for different peptides and thus the detection limits of the method.

EXPERIMENTAL SECTION

Chemicals. Ara h2 was purified from raw peanuts following a procedure reported in the literature [21], whereas Ara h3/4 purified from raw peanuts was kindly provided by Dr W. Becker from the Leibniz Center for Medicine and Biosciences at the Research Center Borstel (Borstel, Germany). Native protein solutions were prepared and diluted in deionized water (18 MΩ cm resistivity) which was obtained from a Milli-Q Element water-purification system (Millipore, Bedford, MA, USA). Acetonitrile (HPLC purity), trifluoroacetic acid, and formic acid (analytical reagent grade) were purchased from Carlo Erba (Milan, Italy). Trypsin from bovine pancreas, iodoacetamide, and dithiothreitol were from Sigma–Aldrich (St Louis, Missouri, USA).

Liquid chromatography–quadrupole/time of flight-mass spectrometry. Characterization of Ara h 2 and Ara h 3/4 peptides was carried out using a microLC–ESI-Q-TOF Micro (Waters,Milford, MA, USA) mass spectrometer. LC elution was performed on a Symmetry C18 capillary column (0.32 mm×150 mm, 5 μm) (Waters) using a gradient solvent system [(A) 0.1% (v/v) formic acid in water; (B) 0.08% (v/v) formic acid in acetonitrile]. Gradient elution was as follows: solvent B was set at 5% for 6 min, then delivered by a linear gradient from 5% to 90% in 28 min. Solvent B was maintained at 90% for 2 min before column re-equilibration (10 min). Flow-rate was 6 μL min^{-1}. Mobile phase was delivered by a CapLC

pump (Waters) equipped with a 48-vial capacity sample-management system. Standard or sample solution (10 μL) was loaded on a Symmetry 300 C_{18} (Waters) precolumn (0.32 mm×10 mm, 5 μm) and eluted with the mobile phase on to the chromatographic column for on-column analyte enrichment and to avoid column contamination. The nebulizing gas (nitrogen, 99.999% purity) and the desolvation gas (nitrogen, 99.998% purity) were delivered at flow-rates of 10 and 600 L h^{-1} respectively. Q-TOF external calibration was performed using a peptide mixture solution and a fifth-order nonlinear calibration curve was usually adopted. The interface parameters were: ESI voltage 3.0 kV, cone voltage 30 V, rf lens 0.5 V, source temperature 50 °C, desolvation temperature 100 °C. Identification with the Q-TOF instrument was performed using accurate MS and MS–MS measurements of the selected target peptides. Continuum mode TOF mass spectra were acquired over the m/z range 400–2000 using an acquisition time of 1 s and an interscan delay of 0.1 s. When operating in production mode, collision energy and the m/z range were optimized as a function of peptide charge and mass.

Liquid chromatography–triple quadrupole mass spectrometry. LC elution was carried out on a Poroshell C_{18} 300 Å (75 mm×2.1 mm, 5 μm particles) (Agilent Technologies, Santa Clara, CA, USA) using a gradient solvent system [(A) aqueous formic acid (0.1% v/v)/(B) 0.08% (v/v) formic acid in acetonitrile]. Gradient elution was the same as reported above. The flow-rate was 200 μL min^{-1}. The mobile phase was delivered by a Waters 2690 series Alliance quaternary pump equipped with a 120-vial capacity sample-management system. Injection volume was 30 μL. A Quattro LC triple-quadrupole instrument (Micromass, Manchester, UK) equipped with a pneumatically assisted ESI interface was used. The system was controlled by Masslynx software version 4.0 (Micromass). The nebulizing gas (nitrogen, 99.999% purity) and the desolvation gas (nitrogen, 99.998% purity) were delivered at flow-rates of 55 and 500 L h^{-1}, respectively. Full-scan, selected ion monitoring (SIM), product-ion, and selected-reaction monitoring (SRM) experiments were performed operating the mass spectrometer in PI mode. Optimized conditions of the interface were as follows: ESI voltage 3.5 kV, cone voltage 35 V, rf lens 0.3 V, source temperature 130 °C, desolvation temperature 250 °C. Fullscan mass spectra were acquired over the scan range m/z 200-2000 using a step size of 0.1 Da. SIM experiments were carried out on the doubly charged ions of selected peptides. For MS–MS experiments collision-induced dissociation (CID) was performed with a collision gas pressure of 2.3×10^{-3} mbar in the collision cell. The collision energies (CE) were optimized for the mass and charge state of each peptide in order to obtain selective and intense fragmentation. The SRM transitions monitored were as follows: m/z 807/1050 CE 35 eV (CCNELNEFENNQR, Ara h 2), m/z 950/1120 CE 35 eV (CMCEALQQIMENQSDR,

Ara h 2), m/z 649/1089 CE 30 eV (AHYQVVDSNGDR, Ara h 3/4), m/z 695/700 CE 35 eV (SPDIYNPQAGSLK, Ara h3/4). Leucine–enkephalin was used as internal standard (m/z 557/397; CE 20 eV). A ±0.5 mass resolution was set in Q1 and Q3.

Samples. Raw peanut samples (Arachis hypogaea) of the variety red skin from five different producers were purchased in commercial stores.

Sample treatment.

Method 1. Peanut extracts and peanut-containing food extracts were prepared by adding 10 mL Tris HCl 0.2 mol L^{-1} (pH 8.2) to 1 g ground sample. Proteins were extracted by shaking for 2 h at 60 °C, then the extract was centrifuged (3000 g, 5 min) and filtered through a 0.2-μm Nylon filter. Sample concentration was performed on a 5 kDa MW cut-off cellulose filter (Millipore Billerica, MA, USA) by adding 5 mL protein extract and reducing the volume to 0.5 mL. The sample was then digested with trypsin (5 μL, 0.6 mmol L^{-1} in tris HCl, pH 8.2). Different digestion conditions in terms of time (12 and 24 h) and temperature (37 °C, 50 °C) were tested. The final digestion conditions were: 50 °C for 24 h and reaction blocking with 1 μL trifluoroacetic acid. Milk, cacao powder, soy beans, tree nuts, rice crispy, cornflakes (all purchased from a major store chain) were extracted by applying the same procedure. Peptide extracts were stored at −20 °C until analysis.

Method 2. The same procedure as for method 1 except for a further liquid–liquid extraction step with hexane before sample concentration on the cut-off filter.

Method 3. Sample (1 g) was incubated overnight in 10 mL 0.2 mol L^{-1} Tris HCl (pH 8.2) and 100 μL trypsin 0.6 mmol L−1 at 50 °C with vigorous shaking. The resulting mixture was centrifuged at 14000 rpm for 5 min. The supernatant was filtered through a 0.45-μm centrifugal filter (Millipore). The extract was further purified using Strata-X polymeric multimode solid-phase cartridges (Phenomenex, Torrance, CA, USA). The cartridge was washed with 2 mL 95:5 (v/v) water–acetonitrile and peptides were eluted with 1 mL acetonitrile. The eluate was dried under a nitrogen stream and re-suspended in 200 μL 60:40 (v/v) water–acetonitrile containing 0.1% formic acid.

LC–QqQ-MS–MS method performance. Method performance was assessed in terms of limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, and selectivity. For this purpose a rice crispy/cacao-based snack was chosen as the matrix. LOD and LOQ values were calculated for standard solution and for matrix as amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively. Instrumental linearity was calculated by spiking aqueous solutions with different amounts (0.1–20% w/v) peanut extract. A matrix matched calibration curve was built-up by spiking matrix extract with different amounts of peanut extract (1–20% w/v). Method precision was assessed by performing two extractions of matrix

fortified at two peanut concentration levels (1.25 and 5% w/w peanuts/matrix) and performing five LC injection replicates for each extract. Method selectivity was evaluated by analyzing tryptic digests of tree nuts, soybeans, milk, cacao powder, and rice crispy samples.

RESULTS AND DISCUSSION

*LC–ESI-QTOF MS and MS–MS identification of Ara h 2 and Ara h 3/4 peptide biomarkers.*Ara h 2 and Ara h 3/4 whole proteins were initially characterized by ESI-QTOF MS analysis. No protein interferences were found in the mass spectra, suggesting an excellent degree of purification of both proteins. In particular, Ara h 2, which was purified from peanut seeds in our laboratory, exhibited the typical doublet in the 16–18 kDa range, as reported in the literature [21]. In a further step, the tryptic digests of purified Ara h 2 and Ara h 3/4 were individually analyzed in LC–QTOF-MS in order to identify putative peptide biomarkers. Proteins were digested both in the native and reduced/alkylated (using dithiothreitol and iodoacetamide) forms. The results did not show significant differences among the most abundant peptides observed for both proteins digested under the two different conditions. Since no analytical advantages were obtained by performing digestion of reduced/alkylated proteins, the work was performed on the native forms. Several doubly charged ions of the tryptic digest peptides were identified for both proteins allowing a good degree of sequence coverage to be obtained (74% for Ara h 2 and 60% for Ara h 3/4). Peptides were selected on the basis of the following criteria: signal intensity, retention time repeatability, lack of missed cleavages, specificity, and presence in the different Ara h 2 and Ara h 3/4 isoforms. In the case of Ara h 2 the peptides at m/z 807.2451 (CCNELNEFENNQR; doubly charged ion) and m/z 949.8894 (CMCEALQQIMENQSDR); doubly charged ion of the cysteine oxidized form were selected. Accurate mass measurements and product-ion MS–MS experiments were carried out to identify the peptide sequences and data collected for Ara h 2 protein are presented in Figure 1.

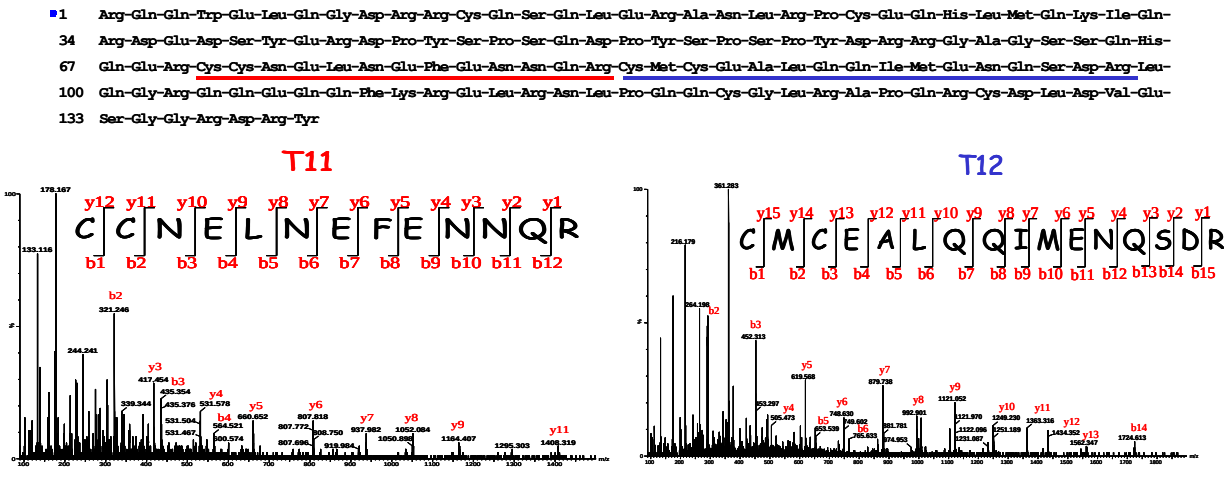

Figure 1. Aminoacid sequence of Ara h2 protein and the selected biomarker peptides T11 (red line) and T12 (blue line). Below: ESI-QTof mass spectra obtained by CID experiments for the accurate identification of the selected peptides T11 and T12.

No peptides covering the IgE-binding epitope giving the allergic reaction (two epitopes in the region aa57–74 contain the amino acid sequence DPYSP necessary for IgE binding, [22]) were found in the ESI mass spectrum of the tryptic digest of Ara h 2. Concerning Ara h 3/4, the peptides at m/z 648.8109 (AHYQVVDSNGDR, doubly charged ion), m/z 695.3468 (SPDIYNPQAGSLK, doubly charged ion), and at m/z 732.8384 (AQSENYEYLAFK) were found to be the most abundant. However, considering the protein sequences reported in databases it was possible to observe that the sequence at m/z 732 is not present in all isoforms thus suggesting that this peptide can not be a useful biomarker.

To demonstrate this, peanut extracts from different providers were analyzed and the presence of the peptide at m/z 732 was observed only in two of the five samples analyzed. In addition, taking into account recovery of the extraction procedure and that the concentration of the protein extract is determined and made uniform before digestion, the peptide was detected with different abundances in the two varieties in which it was present. These findings suggest that the peptides useful as biomarkers must be present in all the different isoforms of the investigated proteins in order to detect them when different peanut cultivars are used in the food industry.

Further, the analysis of more than one allergenic protein for confirmation purposes allows reduction of the problem of a different level of expression of a protein in the vegetable system, which can significantly affect the detection limit of the peptides. In the case of Ara h 2, the peptides at m/z 807 and 950 were found to be present with comparable signal intensity in all the varieties selected. In fact, the sequence coverage we obtained for Ara h 2 was predominantly on the conserved sequences and not on the sequences that differ in the various isoforms. On the basis of these results, the LC tandem mass spectrometry method using a triple-quadrupole was developed.

LC–ESI-QqQ-MS–MS method. The chromatographic behavior of the four selected peptides (Fig. 2) exhibited excellent stability of the retention times (RSD<1.5%, n=10).

In a preliminary step ESI ionization efficiency of the selected peptides was evaluated to study analytical sensitivity for quantitative purposes. In fact, since different ESI sensitivity is expected, it should be assessed to verify which peptides can be useful for obtaining the lowest detection limits. Calibration curves were calculated in SIM mode by performing several dilutions of the tryptic digests of Ara h 2 and Ara h 3/4 standard solutions. From the slope values obtained it was possible to observe comparable sensitivity for the peptides at m/z 807 [y = 3593 (±260)x] and m/z 950 (y = 2517 (±320)x] (Ara h 2). Instead, in the case of the Ara h 3/4 peptide m/z 695 [y = 22110 (±1140)x], remarkably, the highest sensitivity was observed. The peptide at m/z 649 provided a sensitivity comparable to that observed or the Ara h 2 peptides [y = 3720 (±167)x]. These findings suggest that the peptide at m/z 649 can be a good marker to improve method detectability.

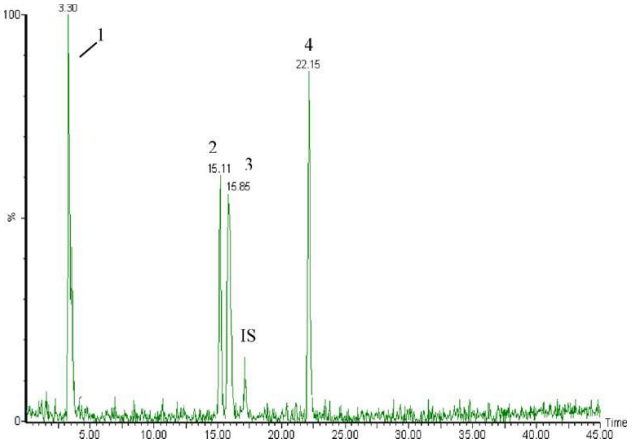

Figure 2. LC–ESI-QqQ-MS–MS SRM chromatograms of a tryptic digest of Ara h2 and Ara h3/4 standard solutions (20 μg mL^{-1}). Peaks: 1, m/z 649/1089 (Ara h3/4); 2, m/z 695/700 (Ara h3/4); 3, m/z 807/1050 (Ara h2); 4, m/z 950/1120 (Ara h2).

Using a low-resolution mass analyzer it is necessary to perform SRM experiments to obtain good selectivity in peptide identification in a complex matrix. For this reason, the product-ion mass spectra of the target peptides were acquired by optimizing the collision energy in order to obtain selective fragment ions with high intensity. The MS–MS spectra of peptides at m/z 807 (Ara h2), 950 (Ara h2), 695 (Ara h3/4), and 649 (Ara h3/4) showed fragment ions distributed under and above each selected precursor ion mass yielding useful analytical data for the quantitative method.

Sample treatment procedure. In the development of a sensitive method for the detection of hidden allergens in food products, sample preparation should be optimized to obtain the best sensitivity improving peptide detection limits. For this purpose, three different extraction methods were applied to peanuts. By using the method involving extraction of whole proteins followed by enzymatic digestion (method 1), the signals of all selected peanut peptides were observed with excellent signal-to-noise ratio in the LC–MS–MS SRM chromatogram.

However, it was possible to observe an increase of column back-pressure and a signal intensity decrease as a consequence of ESI source dirt when a number of subsequent analyses were run (i.e. more than 20 injections). In order to reduce sample impurities, an additional extraction step with hexane was introduced to eliminate the lipid fraction of the sample (method 2). However, even though the problems related to unclean extracts were solved, a significant decrease (between 20 and 50%) in the peak area of the investigated peptides was observed with respect to method 1. To improve sample treatment the simultaneous extraction and digestion of peanut proteins was performed followed by an SPE clean-up step (method 3). This resulted in a decrease of peptide signal intensity (between 50 and 90%) with respect to method 1. This behavior could be the consequence of incomplete recovery of the peptides during digestion and purification or of a different matrix effect that can affect ESI ionization efficiency. On the basis of these findings, method 1 was chosen. Different digestion conditions were then assessed, as reported in the experimental section. The best result in terms of recovery of the peptides from peanut-containing foods was observed when operating at 50 °C for 24 h. In particular, these conditions led to an increase of the peak area of the four peptides monitored of between 20 and 120% compared with the other milder digestion conditions.

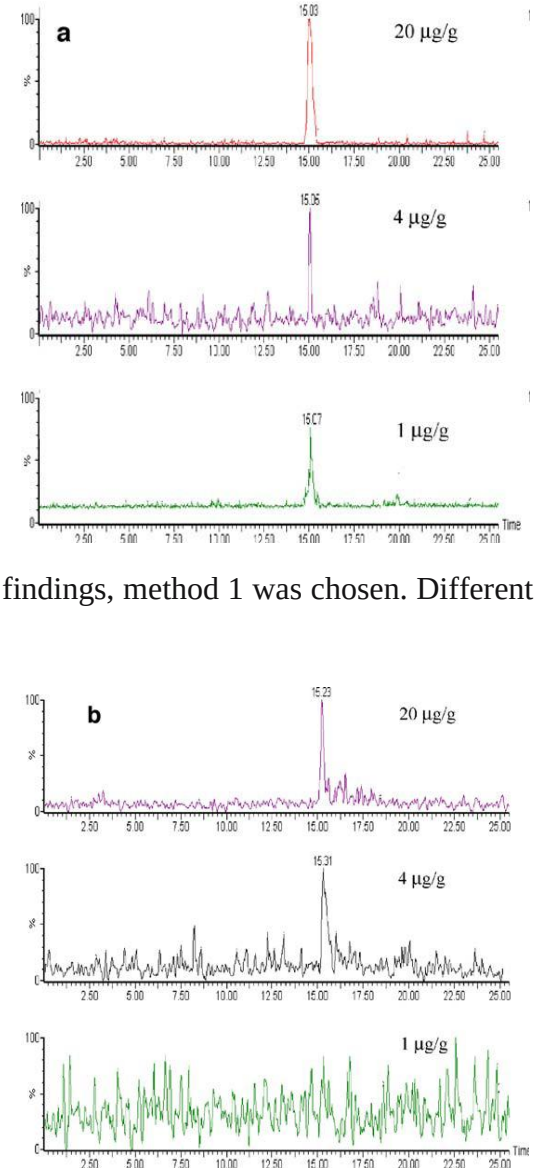

Figure 3. LC–ESI-QqQ-MS–MS SRM chromatograms of peptides (a) m/z 695/700 [SPDIYNPQAGSLK, Ara h3/4] and (b) m/z 807/1050 [CCNELNEFENNQR, Ara h2] in chocolate/rice crispy-based matrix fortified with Ara h2 and Ara h3/4 protein solutions at different concentrations.

Assessment of LC–ESI-QqQ-MS–MS method performance with foods. To assess the applicability of the LC–ESI-MS–MS SRM method for detection of peanut allergens in food, rice crispy/cacao-based snacks were fortified with different amounts of raw peanuts. Instrumental linearity calculated by using aqueous solutions spiked with percentages from 0.1 to 20% of a peanut peptide extract in general

demonstrated better sensitivity for the peptides selected for Ara h3/4 (y = 10.48 (±0.23)x and y = 3.78 (± 0.12)x for the peptides at m/z 695 and m/z 649 respectively) with respect to those selected for Ara h2 (y = 4.23 (±0.22)x and y = 2.68 (±0.18)x for the peptides at m/z 807 and m/z 950 respectively). The peptide m/z 695 provided the lowest detection limit (1.5 μg mL^{-1}) and quantitation limit (4.8 μg mL^{-1}). The LOD and LOQ values for matrix were calculated for all the peptides investigated. The best result was obtained for the peptide at m/z 695 (Ara h3/4); the LOD and LOQ were 1 and 3.7 μg protein g^{-1} matrix, respectively (Fig. 3a). In the case of Ara h2, the peptide exhibiting the lowest LOD and LOQ values was that at m/z 807 (LOD and LOQ values were 5 and 14 μg g^{-1}, respectively; Fig. 3b). Method precision, expressed as RSD, was lower than 10% when calculated for 1.25 and 5% w/w peanuts/matrix concentration levels for all the peptides investigated. Trueness was evaluated as matrix effect and the results exhibited the presence of a significant suppression effect (from 30 to 50%) on the signals of the peptides of Ara h2, whereas in the case of Ara h3/4 peptides the suppression effect was lower than 10%. In general, the use of the internal standard selected did not completely overcome the matrix suppression effect but reduced it to within 5 and 30% for Ara h3/4 and Ara h2 peptides, respectively.

Further, specificity of the peptide biomarkers was assessed for the matrix. In fact, Ara h 2 is a glycoprotein having sequence homologies with conglutin, the 2S albumin seed protein families, and the α-amylase from wheat. Ara h 3/4 is a protein belonging to the glycinin protein family and some of the IgE-binding epitopes (the C-terminal fragment appeared to bind more IgE) are conserved regions present in legumes (e.g. soy beans). Such IgE-binding sites provide a molecular explanation for the IgE cross-reactivity observed between soybean and peanut proteins [23]. For this purpose, research on protein databases was performed and the sequences of the selected peptides were found to be unique for peanuts. In fact, by analyzing peptide extracts from different food sources (tree nuts, soybeans, milk, cacao powder, rice crispy) no interfering signals were observed at the SRM transitions monitored for the peanut peptides. It should be noted that such selectivity was obtained by keeping a very narrow mass window (±0.5 Th) on the first quadrupole, thus reducing the number of ions transmitted to the collision cell in spite of method sensitivity. In fact, using a larger mass window (±1 Th), several signals were observed in the matrices tested. Such findings suggest that when a low-resolution mass analyzer is used, optimum selectivity can be obtained by selecting specific transitions and keeping a narrow m/z window on the first quadrupole for the MS–MS experiments.

CONCLUSIONS

The approach based on the simultaneous identification of biomarker peptides derived from two different proteins makes the proposed method highly selective towards peanut residues in foods. The capability of identification of both Ara h 2 and Ara h 3/4 at concentrations as low as 1 μg g−1 was demonstrated in a complex matrix such as rice crispy/chocolate-based snacks. Since the method combines highly selective identification with the capability of performing quantitative analysis at low concentration levels, it can be proposed as an effective quantitative confirmatory method for the detection and determination of hidden allergens in food. Further developments will concern the strategy of sample treatment with the aim of obtaining simultaneous extraction, purification, and enrichment of the investigated peanut proteins. The final goal is to reduce the time required for sample preparation and to make detection and quantification limits less prone to the effects of the complexity of the matrices.

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1.2. Selective and Rapid Immunomagnetic Bead-Based Sample Treatment for Liquid Chromatography–Electrospray Ion-Trap Mass Spectrometry Detection of Ara h3/4 Peanut Protein in Foods.

Abstract. Complex matrices commonly affect the sensitivity and selectivity of liquid chromatography–mass spectrometry (LC–MS) analysis. Thus, selective sample enrichment strategies are useful particularly to analyze organic biomarkers present in low abundance in samples. A selective immunomagnetic extraction procedure to isolate trace peanut allergen protein Ara h3/4 from breakfast cereals combined with microwave-assisted tryptic digestion and liquid chromatography–electrospray ion-trap tandem mass spectrometry (LC–ESI-IT-MS/MS) measurement was developed. Using protein A-coated magnetic bead (MB) support, anti-Ara h3/4 monoclonal antibodies (Abs) were used as selective capture molecules. The results obtained by LC–ESI-IT-MS/MS in terms of limit of detection (3mg peanuts/kg matrix) and a significantly reduced matrix effect demonstrated that the Ab-coated magnetic bead was very effective to selectively trap Ara h3/4 protein in breakfast cereals. The magnetic bead-based sample treatment followed by LC–IT-MS/MS method here developed can be proposed as very rapid and powerful confirmatory analytical method to verify the reliability of enzyme-linked immunosorbent assay (ELISA) screening methods, since the magnetic bead-LC–IT-MS/MS method combines good sensitivity to the identification capabilities of mass spectrometry.

INTRODUCTION

Hidden allergens in foods represent a major health problem in the food safety issue, since they are recognized to induce a wide variety of hypersensitivity reactions [1]. A substance is a hidden allergen when it is unrecognized or not declared on the product label. This omission is not always intentional and there are many ways for allergens to be hidden in food, for example, through misleading labels, allergenic foods that can contaminate other safe foods, food that is listed by an uncommon term, and ingredient switching. The USA and EU legislations [2,3] on food labelling only refer to the allergenic foods added as known ingredients and do not prevent the unintentional presence of trace allergens.

The most sensitive method used to screen the presence of hidden allergens in food rely on enzyme-linked immunosorbent assay (ELISA) [4–6]. ELISA allows to obtain detection limits between 0.1 and 10 mg peanuts/kg matrix and is easy to use. However, the presence of false-positive and false-negative results demands the availability of confirmatory methods. In addition, as well documented, the ELISA quantitative data are strongly dependent on the matrix tested leading to lack of accuracy, that still remains the main discussed analytical topic [7]. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been proposed as confirmation technique to identify and also quantify proteins, but its major limitation still remains the low sensitivity achieved in complex matrices with respect to immunoassays [8–11]. In a research program dealing with the development of new analytical strategies for the determination of hidden allergens in foods, we developed an ELISA-inductively coupled plasma mass spectrometry (ICP-MS) method [12] and a LC–electrospray (ESI) MS/MS method [8] for screening and confirmation purposes, respectively.

In particular,we demonstrated the potential of LC–ESI-MS/MS with triple quadrupole (QqQ) analyzer for the detection of peanut allergens (Ara h2 and Ara h3/4) in foods [8]. A proteomic bottom up-based approach based on the direct identification of Ara h2 and Ara h3/4, the biomarker peptides were used as a strategy to identify peanuts in complex food matrices. Selected reaction monitoring (SRM) analysis allowed to obtain very good selectivity in the biomarker peptide detection even with a low-resolution mass spectrometer by keeping a narrow mass window during precursor and product-ion transmission. This restriction allows to analyze complex digest extracts, even if it limits method sensitivity (0.5% (w/w) peanuts/matrix). In addition, besides ESI and LC column dirtying, a significant matrix effect reducing biomarker signal intensity was observed, affecting accuracy of the quantitative analysis.

To achieve better sensitivity and accuracy of the LC–MS/MS method together with an improvement of column life-time, an efficient sample treatment step involving a purification and concentration of allergen proteins from complex matrices is needed. The procedures routinely used for the extraction of compounds from liquid or solid matrices (i.e. solid-phase extraction, solid–liquid extraction, etc.), despite their attractive features of simplicity and availability of supports, lack selectivity leading to a partial co-extraction of interfering substances. In addition, a multi-step treatment can lead to analyte loss. Recently, to enhance selectivity of the extraction procedure and improve accuracy of the LC–MS results, immunosorbent (IS) supports have been proposed for the different applications [13–16]. Based on the high affinity and selectivity of antigen–antibody (Ag–Ab) interactions, the IS materials allow a selective extraction of the target compounds from complex mixtures. A strategy based on immuno-recognition with materials combined with LC–ESI-MS/MS can therefore represent one of the most specific and sensitive analytical approaches to reach the analytical challenge of hidden allergen detection in foods.

Herein we propose an antibody magnetic bead (MB)-based method for the selective enrichment of Ara h3/4 protein in food extracts. Protein A-magnetic beads bound to monoclonal anti-Ara h3/4 antibodies were used as affinity probes to isolate the specific target protein. Further, we discuss a LC–ESI-MS/MS method with a linear ion-trap (IT) mass analyzer for the identification and determination of the Ara h3/4 peptide biomarkers in breakfast cereals. As a result, the combination of the use of magnetic affinity probes with LC–ESI-IT-MS/MS analysis allows us to obtain an excellent sensitivity and selectivity for the identification and quantification of peanut as contaminant in this kind of food matrix.

EXPERIMENTAL SECTION

Chemicals. Anti-Ara h3/4 (Pn-b) monoclonal antibodies were obtained by using peanuts, which were roasted in shell to prevent molding and kindly provided by the Leibniz-Center for Medicine and Biosciences at the Research Center Borstel (Borstel, Germany). Antibody solutions were diluted with deionized water (Millipore, Bedford, MA, USA). Phosphate buffer (pH 8.0), acetonitrile (HPLC purity), and formic acid (analytical reagent grade) were purchased from Carlo Erba (Milan, Italy). Trypsin from bovine pancreas, leukine–enkefaline acetate hydrate, Trisma–HCl, Bradford reagent, bovine serum albumin (BSA), triethanolamine and DMP (dimethylprimelinidated·2HCl) were from Sigma–Aldrich (St. Louis, MO, USA). EDC *{*1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide*}*was from Pierce (Bonn, Germany). A commercial ELISA kit (Veratox quantitative kit) specific for peanut allergens waspurchased from Neogen (Lansing, MI, USA) and used following the instructions given by the producers.

Samples. Roasted peanuts and different commercial breakfast cereals samples (cornflakes, breakfast cereals based on cereal flakes and dried fruit, cereal mix with fruit and chocolate, cereal mix with rice crispy and powder milk) were obtained at a local food store. The sample treatment and the LC–MS/MSmethods were developed and validated by using cornflakes free of peanuts. In all the other products used to test the method peanuts were not present as an ingredient and the labeling "may contain trace of nuts and soy"was reported.

Preparation of functional magnetic beads. Magnetizable polystyrene beads (diameter 2.8±0.2 μm, surface area 3–9 $m^2/10^9$ beads) (Dynabeads, Invitrogen Dynal, Oslo, Norway) covalently coupled with recombinant protein A were used to capture anti-Ara h3/4 monoclonal immunoglobulins G (IgG) for the small-scale purification of Ara h3/4 protein in food extracts. The antibody solution (100 μL of a 1 mg/mL anti-Ara h3/4-IgG in sodium phosphate solution, 100 mM) was added to an Eppendorf containing 50 μL of pre-washed protein A-magnetic beads. Incubation was performed at room temperature for 4 h to allow the binding of the Fc portion of the immunoglobulin to protein A. The protein A-anti-Ara h3/4 IgG complex was isolated using the Dynal MPC (Invitrogen Dynal) magnet and washed twice with 500 μL of 200 mM triethanolamine, pH 8.2. To ensure an efficient binding of the IgG to protein A, a crosslinking reaction was added to the procedure. Protein A-IgG complex was then incubated with 500 μL of freshly made 20 mM DMP (dimethylprimelinidated·2HCl) in 200 mM triethanolamine solution for 30min. Cross-linking reaction was quenched by isolating the magnetic beads and incubating with 500 μL of 50 mM Tris–HCl pH 7.5 for 15

min. The beads were thus rinsed three times with 500 μL of 100 mM phosphate buffer with 0.02% Tween 20 before use.

Sample treatment. Peanut-containing food extractswere prepared by adding 10 mL of 100 mM phosphate buffer to 1 g of ground sample. Proteins were extracted by shaking for 2 h at 37°C, then the extract was centrifuged (3500×*g*, 5 min) and filtered on 0.2 μm nylon filter before treatment with the magnetic beads. Sample extract (800 μL) was incubated at different times (15min, 30 min, 1 h, 3 h and overnight) under slight agitation at room temperature with the protein A-IgG magnetic bead complex prepared as reported above. The beads were then washed three times with 500 μL of 100 mM phosphate buffer, pH 8.0. After discharging the phosphate buffer, the beads-conjugated sample was then re-suspended in 60 μL and digested with trypsin (5 μL, 0.6 mM in 100 mM phosphate buffer) by using an accelerated microwave-assisted digestion procedure (1 min, 700W) as reported in the literature [17]. The internal standard (5 μL of 130 mM of leukine–enkefaline) was added to the solution just before LC analysis.

Liquid chromatography–ion-trap mass spectrometry. LC separation was carried out on a Poroshell C_{18} 300 Å (75mm×2.1mm, 5 μm particles) (Agilent Technologies, Santa Clara, CA, USA) using a gradient solvent elution system [(A) 0.1% (v/v) formic acid inwater/(B) 0.08% (v/v) formic acid in acetonitrile] as previously described [8]. The flow-rate was 200 μL/min. The mobile phase was delivered by the Surveyor chromatographic system (ThermoElectron Corporation, San José, CA, USA) equipped with a 200-vial capacity sample tray. Injection volume was 50 μL. A LTQ XL linear ion-trap instrument (ThermoElectron Corporation) equipped with a pneumatically assisted ESI interface was used. The system was controlled by the Xcalibur software. The sheath gas (nitrogen, 99.999% purity) and the auxiliary gas (nitrogen, 99.998% purity) were delivered at flow-rates of 45 and 5 arbitrary units, respectively. Optimized conditions of the interface were as follows: ESI voltage 3.5 kV, capillary voltage 20 V, capillary temperature 200°C. MS/MS experiments were performed under both product-ion and SRM conditions with a collision gas (He) pressure of 2.3×10^{-3} mbar in the collision cell. In the product-ion scan mode the 200–1400 *m*/*z* range was monitored. The SRM transitions monitored were as follows: *m*/*z* 649/761 (AHYQVVDSNGDR, Ara h3/4), *m*/*z* 695/977 (SPDIYNPQAGSLK, Ara h3/4) both operating with collision energy (CE) 35 eV. Leukine–enkefaline (*m*/*z* 557/397; CE 20 eV) was used as the internal standard.

Magnetic bead-LC–MS/MS method validation. Validation of the whole analytical procedure was performed on the fortified samples of breakfast cereals (mix of cereal flakes).

For this purpose, breakfast cereal samples were fortified with different peanut amounts and measurements were carried out by monitoring the most abundant MS/MS transition for each peptide as above reported. The detection limits (LODs) and the quantitation limits (LOQs) were calculated as the concentration of the two peptides corresponding to a signal-to-noise ratio (S/N) 3 and 10, respectively. Linearity was assessed in the 2.5–2500 mg peanuts/kg matrix range. Precision was evaluated by performing five independent extractions of cornflake samples fortified with 1 mg peanuts/kg matrix using the functionalized magnetic beads and by performing two LC–MS/MS injections for each extract. The matrix effect was assessed by comparing the slopes of an instrumental and a matrix-matched calibration curve applying a *t*-test. The matrix-matched calibration curve was obtained by analyzing the breakfast cereal sample extracts fortified with different peanut amounts and treated applying the whole analytical procedure.

RESULTS AND DISCUSSION

Liquid chromatography–ion-trap-tandem mass spectrometry method. This study started from the previously published results dealing with the ESI-MS behaviour of the biomarker peptides selected for Ara h2 and Ara h3/4 [8]. The biomarker peptides were previously chosen following different criteria (i.e. absence of missed cleavages, good ESI sensitivity, no post-translational modification sites and detectability in both rawand roasted peanuts, sequence specificity) [8,11]. By analyzing a peanut protein aqueous solution, the LC–ITMS/MS profile of the peptides resulted to match that observed by LC–QqQ-MS/MS in terms of chromatographic behaviour and relative intensity. Also using the ESI-IT-MS instrument, the peptides chosen for Ara h3/4 exhibited a higher ionization efficiency with respect to those selected for Ara h2. On the basis of these results, in this work the immuno-strategy extraction method was developed targeting Ara h3/4 protein. The chromatographic profile of the two Ara h3/4 peptides showed both good peak shape and retention time stability [peptide of *m*/*z* 649 (t_R 1.50±0.03 min); peptide of *m*/*z* 695 (t_R 8.76±0.17 min)]. The identity of these two peptides was confirmed by both the doubly charge state detection, obtained by tuning the mass analyzer to higher resolution (UltraZoom scan) and by the ITMS/MS product-ion profiles. The MS/MS spectra exhibited several fragments of the y- and b-series to cover and confirm the peptide sequences (Fig. 1).

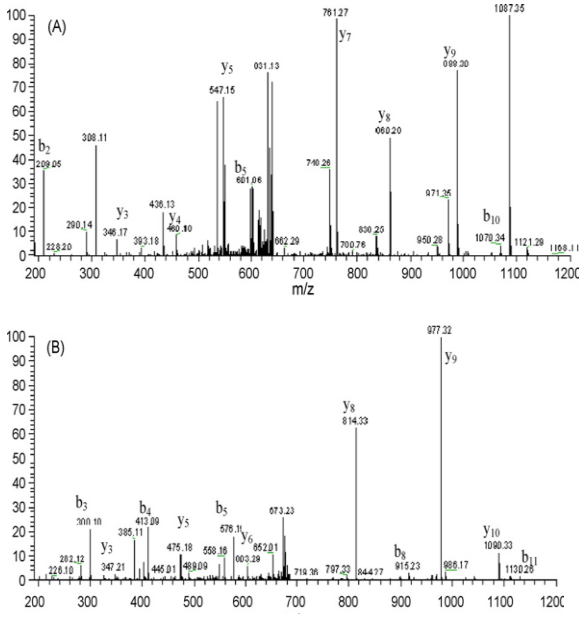

Figure 1. LC–IT–MS/MS product-ion mass spectra of the doubly charged tryptic peptides of Ara h3/4: (A) *m*/*z* 649/761 (AHYQVVDSNGDR) and (B) *m*/*z* 695/977 (SPDIYNPQAGSLK).

By using the IT mass analyzer, product-ion and SRM acquisition modes were performed at the same time allowing simultaneously the unambiguous identification of the two peptides together with the quantitative analysis without loss of sensitivity. By contrast, when using the QqQ analyzer, as in the previous work, the production mode has lower sensitivity with respect to SRM acquisition mode due to the different duty cycles and did not allow to obtain qualitative information at low concentration levels [8]. These findings highlight the great appeal of the linear IT mass spectrometer for this application.

Development of the immunoaffinity-based Ara h3/4 extraction method. Isolation of target low-abundant proteins in foods containing a number of protein rich-ingredients (i.e. milk, soy, rice, eggs, etc.) represents a great analytical challenge. The use of cut-off filters or other traditional approaches (i.e. ultracentrifugation) does not allow to obtain target-enriched purified samples and in most cases loss of analytes can occur. The magnetic bead support functionalized with anti-Ara h3/4 monoclonal antibodies could represent an easy-to-use, fast and selective approach for extraction, purification and concentration of the target protein from complex mixtures.

In this work, different Ab-protein A-magnetic bead immobilization conditions were tested. Further, the optimization of the Ara h3/4 protein extraction from breakfast cereal samples free of peanuts fortified with different peanut amounts was performed. Selectivity of the monoclonal Abs used in this work has been previously tested in term of cross-reactivity with other food ingredients by ELISA-ICP-MS [12]. The results obtained evidenced an excellent Ag–Ab selectivity, thus proving the suitability of these monoclonal Abs for magnetic bead coating in this application.

The efficiency of the protein A IgG binding reaction was initially tested by incubating the beads with the antibody solution (100 μg Ab) at different times (1–5 h) with and without the cross-linking reaction. The antibody-coated beads thus obtained were incubated with a peanut protein aqueous solution (4 h) and the extract digested with trypsin by microwave-assisted digestion (1 min, 700W). The LC–MS/MS analysis of the peptides at *m*/*z* 649 and *m*/*z* 695 exhibited a significant increase (>40%) of their peak area in all the cases in which the IgG was covalently bound to the magnetic beads by the cross-linker. Further, the increase of the antibody incubation time from 1 to 4 h in the presence of the cross-linker allowed to obtain magnetic beads with the maximum extraction efficiency. No significant increases were observed from 4 to 5 h binding reaction (Fig. 2).

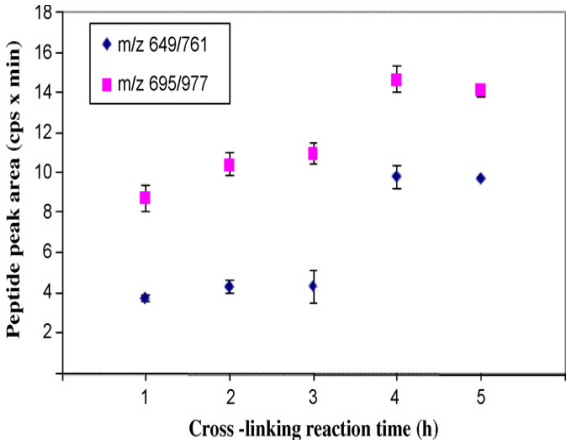

Figure 2. Plot of the Ara h3/4 LC–MS/MS peptide peak areas as a function of the antibody incubation time in the presence of the cross-linker.

In a further step of thework, the efficiency of the protein A-anti-Ara h3/4 IgG complex was studied by varying the amount of Abs (50, 70 and 100 μg) while keeping the incubation time constant at 4 h. The best LC–MS/MS resultswere obtained using 100 μg of antibody (data not shown). Confirmation of these findings was obtained by quantifying the antibody amount

after the binding reaction with the beads using a Bradford assay with a standard IgG. In fact, we found the amount of anti-Ara h3/4 bound to be 15.1±0.7 μg for 50 μL bead solution. This result is in good agreement with the typical amount of antibody bound capacity of magnetic beads declared by the manufacturer (i.e. 50 μL Dynabeads protein A are reported to isolate approximately 12–15 μg antibodies).

In the past, the use of EDC reagent to activate terminal carboxyl groups was proposed to improve the antibody coating on protein A-magnetic beads [18]. On this basis, we tested the efficiency of EDC-activated magnetic beads following the procedure reported in the literature [16]. By analyzing a peanut protein aqueous solution (50 μg peanuts/mL solution) no significant differences were observed between the magnetic beads prepared with and without EDC. These findings could be explained taking into account the results reported above about the typical amount of Ab bound to the beads that already reach an excellent value.

By keeping the coating reaction time at 4 h, the same amount of magnetic beads (50 μL of a 1.3 g/mL product density correspond to approximately 65 mg of beads) was used for the extraction of different volumes (200, 400, 600, 800, 1200 and 1800 μL) of peanut aqueous extract in order to evaluate both the trapping capacity of the functionalized support and the effect of different concentration factors. The sample was then incubated overnight. By keeping the sample concentration constant, itwas possible to observe a significant increase in the amount of trapped Ara h3/4 protein when sample volume increased from 200 to 800 μL, in particular for the peptide of *m/z* 695 (Fig. 3). Instead, by increasing the sample volume from 800 to 1800 μL, no signal increase was observed. The peanut protein extract was quantified using a Bradford assay with BSA as the standard and the total protein concentration resulted to be around 7 mg/mL. This finding suggests that the magnetic beads prepared following a 4 h-Ab and cross-linker incubation protocol reach the saturation when 800 μL of a 7 mg/mL peanut protein extract are used as incubation sample.

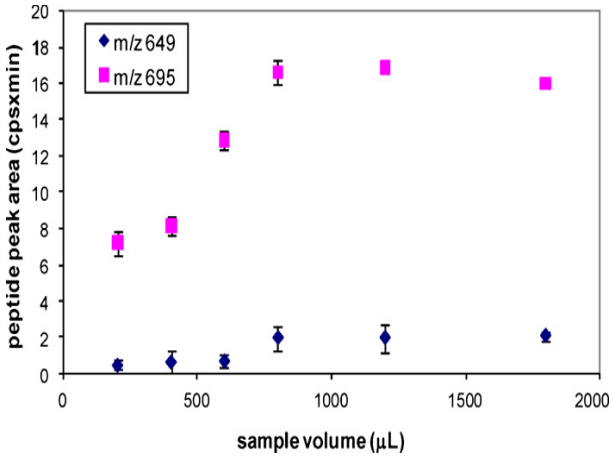

Figure 3. Plot of the Ara h3/4 LC–MS/MS peptide peak areas as a function of the sample volume (magnetic beads prepared by incubating the antibodies and the cross-linker for 4 h, sample incubation overnight, elution volume 70μL). Data corrected by the dilution factor.

Further, different sample incubation times were investigated (15min, 30 min, 1 h and 3 h and overnight) using 800 μL of aqueous peanut protein extract. The best results in terms of signal intensity and repeatability (RSD < 8%) were obtained by performing sample incubation for 3 h. No further signal increase was observed after the overnight incubation. As for selectivity, the overnight incubation of the magnetic beads without the Abs in a peanut aqueous extract resulted in a positive LC–MS/MS signal at the transitions monitored for the chosen peptides, suggesting the occurrence of non-specific binding. However, even if the results evidenced the capability of protein A to bind Ara h3/4, the MS signal was significantly lower (<10%) with respect to that obtained for the Ab-Ara h3/4 demonstrating that the Abs are required to reach an higher enrichment factor in the real samples. By keeping the sample incubation time at 3 h, a decrease of non specific interactions giving a signal comparable with that of the noise was observed. No decrease in capture efficiency was observed within 3 weeks when the antibody-coated magnetic beads were kept at 4°C. The final scheme of the extraction procedure and sample analysis is reported in Fig. 4.

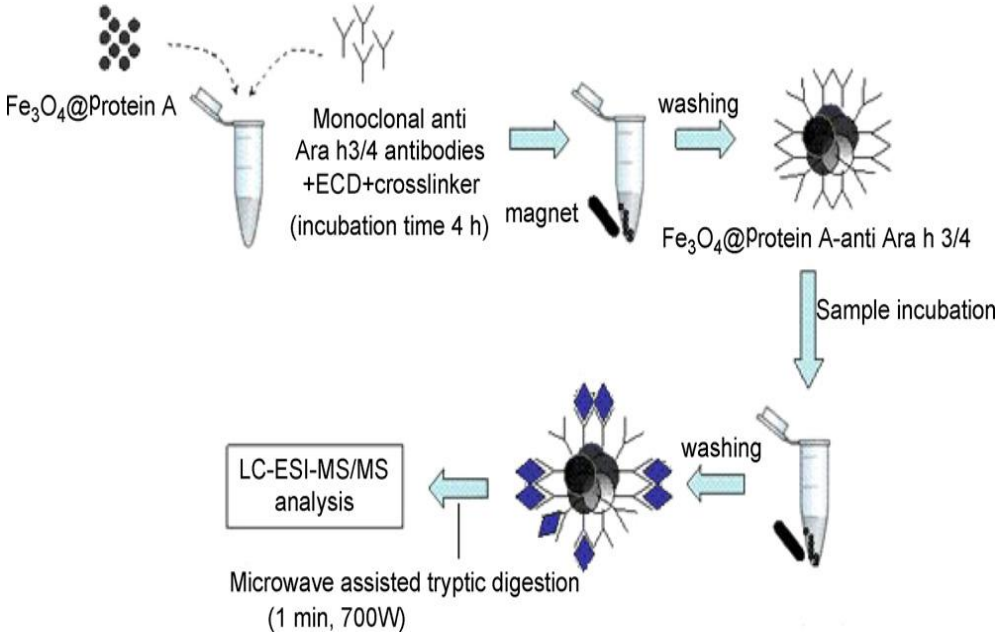

Figure 4. Scheme of the analytical protocol: Fe3O4-protein A-anti-Ara h3/4 IgG as affinity probes for selective enrichment and determination of Ara h3/4 protein from food sample using LC–ESI-MS/MS methods.

Method validation and application to the analysis of Ara h3/4 in breakfast cereal fortified with peanut. A good linearity with different sensitivity was established by using an aqueous peanut extract at different dilutions (percentages from 2.5 to 2500 μg peanut/mL solution) for the two Ara h3/4 peptides (y = 29.2(±0.7)x, r^2 0.988, n = 12 and y = 1.6(±0.2)x, r^2 0.986, n = 12 for the peptides at m/z 695 and m/z 649, respectively). The instrumental LOD and LOQ obtained for the peptide at m/z 695 exhibiting higher sensitivity resulted to be 0.8 and 2.5 μg

peanut aqueous extract/mL solution, respectively. Matrix-matched calibration curve calculated by using breakfast cereal fortified with different peanut amounts (percentages from 0.001 to 0.25% w/w peanut/matrix corresponding to 10–2500 mg peanuts/kg matrix range) and treated with the magnetic beads exhibited an excellent linearity (y = 69.1(±0.9)x, r^2 0.998, n = 12 and y = 3.4(±0.3)x, r^2 0.997, n = 12 for the peptides at m/z 695 and m/z 649, respectively).

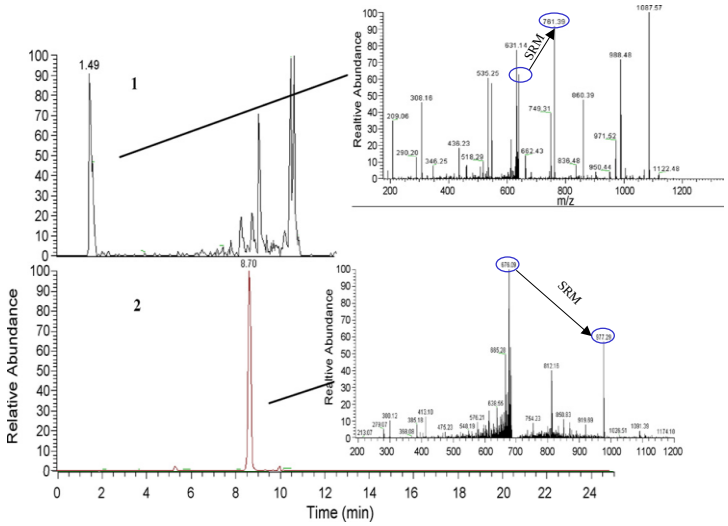

Figure 5. LC–MS/MS profile of a breakfast cereal (mix of cereal flakes) sample containing peanuts, treated with magnetic beads: Peaks: 1, peptide at m/z 649/761 and relative MS/MS product-ion mass spectrum (inset); 2, peptide at m/z 695/977 and MS/MS product-ion mass spectrum (inset).

Trueness assessment allowed us to evidence a significant signal enhancement by comparing with a t-test the slope of the curve calculated analyzing the aqueous peanut extract and that of the matrix matched calibration curve (peptide at m/z 695 $t$calculated = 188, peptide at m/z 649 $t_{calculated}$ =18, $t_{tabulated,0.05,11}$ = 2.2). The slope of the matrix-matched calibration curve increased more than two times for both the peptides. This result can be explained on the basis of the effects of purification and enrichment of the samples when Ab-coated beads are used. The LOD and LOQ obtained on matrix were 3 and 10 mg peanuts/kg matrix, respectively. The method precision expressed as RSD was lower than 8% when calculated at 10 and 100 mg peanuts/kg matrix concentration levels for all the peptides investigated. The method here developed was then applied for the detection of hidden peanut allergen Ara h3/4 in different commercially available breakfast cereal samples. In two out of seven samples (i.e. the cereal mix and the cereal mix with dried fruits, declared to "may contain traces of nuts") the presence of Ara h3/4 was confirmed, and the other samples were found to be negative. The production mass spectra and the LC

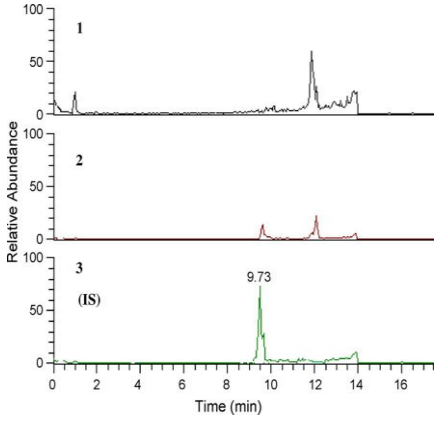

Fig. 6. LC–MS/MS profile of a breakfast cereal (mix of cereal flakes) sample containing peanuts without magnetic bead treatment. No signals corresponding to the two peptides investigated were detected. Chromatograms: 1, peptide at m/z 649/761; 2, peptide at m/z 695/977; 3, IS.

retention times were used to confirm the presence of peanut in samples (Fig. 5). In the positive samples, the peanut concentration was approximately 9.8±0.4 and 12.3±1.3mg/kg, respectively. The positive samples were analyzed by performing the same extraction procedure without the use of magnetic beads. No signals corresponding to the two peptide investigated were observed at their corresponding retention times (Fig. 6). In a final step, the LC–MS/MS quantitative results were verified by testing the same samples with a commercially available ELISA kit to detect peanut hidden allergens. The ELISA datawere in agreement with those obtained by LC–MS/MS for positive and negative samples. In the case of the two positive samples, the ELISA screening data were 14.4±0.9 and 17±2mg/kg, respectively. Taking into account that the Veratox kit could over estimate samples [19], the ELISA data were consistent with those obtained by LC–MS/MS to indicate that thismethodwas successful in confirming the presence of hidden peanut allergens in breakfast cereals.

CONCLUSIONS

A strategy to coat protein A-magnetic beads with monoclonal anti-Ara h3/4 antibodies was devised. To our knowledge so far this is the first time that a selective antibody magnetic support is developed for enrichment of hidden peanut allergens. In conclusion, our methodology demonstrated numerous advantages. The application of immunomagnetic isolation of Ara h3/4 from complex matrices coupled with a microwave-assisted digestion resulted in the most fast and reliable method to purify, concentrate and digest sample extracts before LC–MS/MS analysis. The method works with a single monoclonal antibody with the linear ion-trap mass spectrometer as high selective detector. Taken together, the sensitivity and selectivity reached by the sample treatment method coupled with the LC–IT-MS/MS analysis developed (LOD 3mg peanuts/kg matrix) allow to propose a powerful method suitable to confirm the results of the ELISA screening procedures.

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Chapter 2

The ICP-MS Immunoassay Approach for Targeted Proteomic Analysis

2.1. ICP-MS as A Novel Detection System for Quantitative Element-Tagged Immunoassay of Hidden Peanut Allergens in Foods.

Abstract. A novel ICP-MS-based ELISA immunoassay via element-tagged determination was devised for quantitative analysis of hidden allergens in food. The method was able to detect low amounts of peanuts (down to approximately 2 mg peanuts kg^{-1} cereal-based matrix) by using a europium-tagged antibody. Selectivity was proved by the lack of detectable cross-reaction with a number of protein-rich raw materials.

INTRODUCTION

Nowadays fluorescence-based enzyme-linked immunosorbent assay (ELISA) is the “gold standard” for general screening of food samples for allergen detection [1–3]. Recently, complementarydetection techniques have been developed for immunoassays [2]. Among these, inductively coupled plasma–mass spectrometry (ICP-MS) has been proposed for sensitive and quantitative element-tagged immunoassay for protein analysis in biological samples [4, 5]. Gold-cluster antibody and lanthanide (Eu, Tb, Dy,and Sm)-chelate antibody conjugates have been used to develop both direct competitive and non-competitive immunoassays, demonstrating that target human proteins can be detected at levels as low as 0.1–0.5 ng/mL and that a linear response to protein concentration over 3 orders of magnitude can be obtained [4].

ICP-MS detection offers several advantages with respect to more conventional fluorescence such as lower matrix effects from other components of the biological sample, low detection limits, excellent linearity of the response, long-term sample storage, and capability of multiple analyte detection via different element tags. However, despite its potential up to now, metal-tagged ICP-MS immunoassay has not been applied for detecting and measuring food allergens and hidden allergens, i.e., those deriving from unintentional contamination during food manufacturing. In order to assist in the control of allergen levels in foods to acceptable levels, analysts require test methods designed to produce reliable analytical information using high-throughput instrumentation.

In this context the method developed in this work combines the advantages of the non-competitive sandwich ELISA methods with the sensitivity and precision of ICPMS detection in quantitative protein analysis via elementtagged determination. The immediate result is an innovative and accurate method for the quantitative analysis of peanut proteins as hidden allergens in foods, having the ultimate goal of multiple allergen detection. Attention was focused on the determination of the Ara h1 and Ara h3/4 peanut proteins in cereal products.

EXPERIMENTAL SECTION

Reagents. Phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.4), 0.01% Tween 20 in PBS, 7M nitric acid (Carlo Erba, Milan), Eu-labeled anti-mouse affinity purified rabbit polyclonal Abs (Delfia, Perkin Elmer Life Science, MA, USA) were used. The stoichiometry of the europium/antibody system was directly calculated in a range of Eu-labeled antibody concentrations (0.01–600 nM) by using an external calibration method with NIST-traceable europium standard solutions (High-Purity Standards, Charleston, SC, USA). The stoichiometry (10±2 Eu atoms) was found to be independent of the Ab concentration and in good agreement with that declared by the manufacturer (8 atoms of europium/antibody). Ara h1 and Ara h3/4 purified peanut proteins and anti-Ara h1 and anti-Arah3/4 monoclonal antibodies were kindly provided by the Leibniz-Center for Medicine and Biosciences at the Research Center Borstel (Borstel,Germany). All antibody solutions were diluted with deionized water (Millipore, Bedford, MA, USA).

Instrumentation. Experimental measurements were performed on the ICPMS X SeriesII (Thermo Electron Corporation, Waltham, MA, USA) operating under Xt interface standard conditions. The instrument optimization was performed daily to assure a response of at least 50,000 cps/ppb for indium and 80,000 cps/ppb for uranium in the high mass range. A GS50 chromatographic pump (Dionex Corporation, Sunnyvale, CA, USA) was used to perform flow-injection analysis (FIA) with a 2% (m/v) nitric acid aqueous solution as a mobile phase delivered at a flow rate of 0.5 mL min^{-1}.

Sample treatment. Peanut and peanut-containing food extracts were prepared by adding 25 mL PBS to 1 g of ground sample. Proteins were extracted by shaking for 15 min at 60 °C, then centrifuged (14,000 rpm, 5 min) and filtered on 0.2-μm nylon filter. Milk, cacao powder, soy beans, tree nuts, rice crispies, cornflakes (all purchased in a big store), and cornflakes fortified with different amounts of peanuts were extracted by applying the same procedure. Using the BCA test the crude extracts were analyzed for protein content that was adjusted to 1 mg mL^{-1} using PBS.Protein extracts were stored at 20 °C until analysis.

Immunoassay procedure. The assay format was based on an indirect ELISA using well-plates coated with polyclonal antibodies targeting total soluble peanut proteins. Protein standard solutions and sample extracts diluted in PBS (100 μL) were incubated for 15 min at room temperature. After washing six times with 0.01% Tween 20 in PBS, two incubation steps were performed: the first with a mixture of the mouse anti-Ara h3/4 and anti-Ara h1 antibodies and the second with the Eu-labeled anti-mouse antibodies. Each incubation step was carried out as for the sample. After digestion with 7M HNO3 (100 μL, 1 h, room

temperature), a 25-µL sample was injected into the FIA-ICP-MS system. To test cross-reactivity, protein extracts of protein-rich raw materials (almonds, hazelnuts, walnuts, milk, soy beans) and other food products (cacao powder, cornflakes, rice crispies) commonly used in the food industry were analyzed.

RESULTS AND DISCUSSION

The non-competitive sandwich ELISA ICP-MS assay is based on the determination of the element used to tag the antibody. The assay was constructed using polyclonal immunopurified antibodies as capture reagents and anti-Ara h1 and anti-Ara h3/4 monoclonal antibodies (Ab I) for identification. Detection was performed with europium labeled rabbit anti-mouse polyclonal antibodies (Ab II). In the assay development, various parameters, such as Ab concentration, incubation time, and temperature, were shown to have a significant effect on method sensitivity.

Table 1: different concentration tested for optimization of Ab I and Ab II using a 25 ng mL^{-1} of peanut proteins extract.

Ab I ($\mu g\ mL^{-1}$)	Ab II ($\mu g\ mL^{-1}$)	^{153}Eu response(cps x s)
200	10	326145(±85924)
200	5	367631(±52313)
100	5	363801(±27765)
100	2	118065(±15729)
100	1	76434(±7932)
20	1	58062(±8982)
20	0,5	36039(±11324)

Ab I and Ab II concentrations were optimized as a function of the assay, since the results showed that the dynamic range of this method at low concentrations of antigen is limited by non-specific binding. Results for the optimization of Ab I and Ab II concentration are reported in Table 1 and Figure 1.

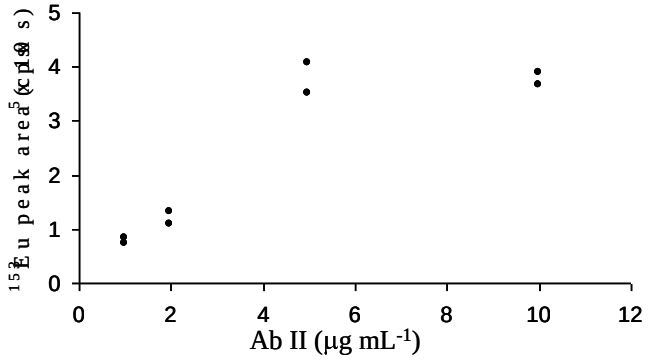

Figure 1: optimization of Ab II concentration with a fixed amount 100 µg mL^{-1} of Ab I. Non-competitive ELISA was always performed with a 25 ng mL^{-1} of peanut proteins extract.

In fact, by increasing both reagent concentrations (Ab I from 20 to 200 µg mL^{-1}; Ab II from 0.5 to 10 µg mL^{-1}) and incubation times from 15 min up to 1 h, Eu signal increased significantly both when positive and negative controls were analyzed. Should be note that increase in signal for negative control at higher concentration of Ab II derives from the presence of the immobilized antibody onto ELISA plate. Infact Ab II reacts with all the mouse IgG, and antibody immobilized on the plate surface fall in this category. Then, when a lower antigen concentration is used or a negative control, the number of possible free site for

antibody-Ab II interaction is more favourite. So, a kinetic control must be applied to counter balance this undesiderate effect. Changes in the intensities of europium observed for the increase in the incubation times are shown in Fig.2. Incubation was carried out at room temperature and at 37 °C, but not significant differences were observed in terms of Eu signal intensity. These findings suggested that in order to detect very low amounts of antigen it is necessary to maintain a low background of Eu and a low amount of proteins that can form non-specific binding to the anti-Ara h1 and Ara h3/4 antibodies. Hence, optimal conditions were those obtained using the highest amount of antibodies tested and the lowest incubation time at room temperature. To determine method performance, peanut extracts containing known protein concentrations in the 3–25 ng mL^{-1} range were preliminarily tested. The results showed an excellent linearity (Fig. 3A) and the detection limit of the immunoassay method (determined as 3 times the blank standard deviation/slope of the calibration curve) calculated for raw peanut protein extracts was determined to be 1.5 ng mL^{-1}. It is to be noticed that this value is directly affected by the binding affinity of the antibodies and by the non-specific background, since the ICP-MS instrumental detection limit for Eu-labeled Ab was as low as 0.1 ng mL^{-1}. The ELISA ICP-MS method developed was then evaluated in matrix for linearity, detection limit, and selectivity using known concentrations of peanut in cornflakes. Peanuts could be detected in foods down to approximately 2 mg peanuts kg^{-1} matrix. Good linearity was demonstrated by ùusing matrix matching samples obtained by fortifying cornflakes with peanut in the range of 10–100 mg peanuts kg^{-1} matrix (r^2 =0.998, n=10) (inset Fig. 3B). By exploring the linearity up to 700 mg peanuts kg^{-1} matrix, a saturation of the europium signal (corresponding to an Eu-labeled Ab concentration of 90 μg mL^{-1}) was observed (Fig. 3B). Such findings indicate the limit of the

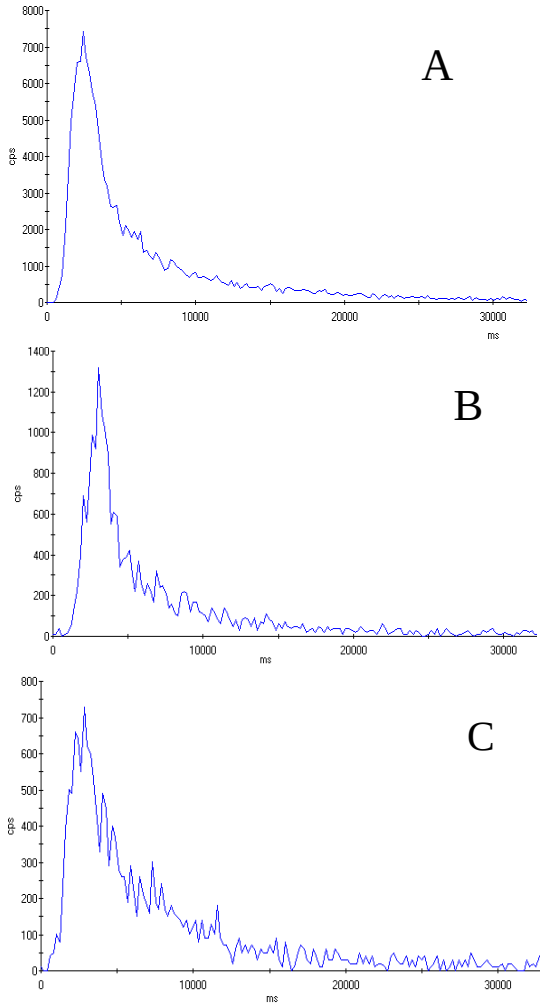

Figure 2. Effect of incubation time on the incubation step with Ab II in the ELISA test for a negative control. Incubation time was A) 1 hour, B) 30 minutes and C) 15 minutes.

plate immobilized antibody binding capability, since the response of the free in-solution Eu-labeled Ab was proved to be linear over the 2 ng mL^{-1} to 100 μg mL^{-1} range. As for selectivity, the results obtained did not exhibit a detectable cross-reaction, demonstrating that the monoclonal antibodies used are very specific toward peanut proteins considering the food products reported in the "Experimental Section". Further investigation on the cross-reactivity will be carried out on other tree nuts and cereals. Taking into account that commercially available tests for peanut allergens have detection limits ranging from 0.1 to 2.5 mg peanuts kg^{-1} matrix and that generally linearity is established in a narrower range [6], the ICP-MS-based method proposed is very promising, since it could guarantee adequate detection limits coupled with an improved linearity and reduced matrix effects.

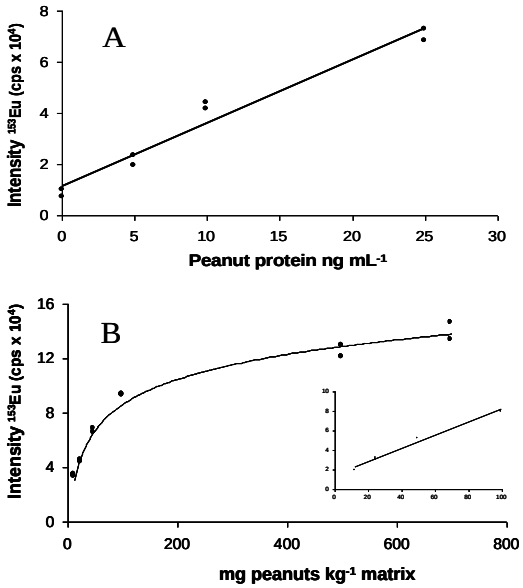

Figure 3: A) ELISA test for an extract of peanuts protein in the 3–25 ng mL^{-1} range; B) for a matrix of cornflakes up to 700 mg peanuts kg^{-1} matrix.

CONCLUSIONS

The capability of ICP-MS as a detection system for ELISA immunoassay in food analysis was proved. In particular, a selective and sensitive immunoassay able to detect low amounts of peanuts by using an Eu-tagged antibody was devised. The described immunoassay can be used as a quantitative tool to detect peanut proteins as hidden allergens in foods. In agreement with literature data [4, 5], the bottleneck of the resulting application was the immu- noassay protocol due to the non-specific reactivity of the reagents and lower detection limits could be reached by improving reagent quality. ICP-MS represent a powerful detection system from several points of view and namely in terms of sensitivity and precision of the quantitative data. Future perspectives are to exploit the capabilities of ICP-MS to perform multielement analysis by tagging directly the primary antibodies with different lanthanides and to develop a multi-tag ELISA assay able to simultaneously detect different allergens in food samples.

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2.2. Metal-Tag ICP-MS Immunoassay and Liquid Chromatography – Eectrospray Ionization Tandem Mass Spectrometry: A Comparison

Abstract. A comparison of two methods for the identification and determination of peanut allergens based on europium (Eu)-tagged inductively coupled plasma mass spectrometry (ICP-MS) immunoassay and on liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) with a triple quadrupole mass analyzer was carried out on a complex food matrix like a chocolate rice crispy-based snack. The LC/MS/MS method was based on the determination of four different peptide biomarkers selective for the Ara h2 and Ara h3/4 peanut proteins. The performance of this method was compared with that of a non-competitive sandwich enzyme-linked immunosorbent assay (ELISA) method with ICP-MS detection of the metal used to tag the antibody for the quantitative peanut protein analysis in food. The limit of detection (LOD) and quantitation of the ICP-MS immunoassay were 2.2 and 5 μg peanuts g^{-1} matrix, respectively, the recovery ranged from 86 ± 18% to 110 ± 4% and linearity was proved in the 5–50 μg g^{-1} range. The LC/MS/MS method allowed us to obtain LODs of 1 and 5 μg protein g^{-1} matrix for Ara h3/4 and Ara h2, respectively, thus obtaining significantly higher values with respect to the ELISA ICP-MS method, taking into account the different expression for concentrations. Linearity was established in the 10–200 μg g^{-1} range of peanut proteins in the food matrix investigated and good precision (RSD <10%) was demonstrated. Both the two approaches, used for screening or confirmative purposes, showed the power of mass spectrometry when used as a very selective detector in difficult matrices even if some limitations still exist, i.e. matrix suppression in the LC/ESI-MS/MS procedure and the change of the Ag/Ab binding with matrix in the ICP-MS method.

INTRODUCTION

Food allergies and related syndromes are one of the arising health issues in western countries:[1] recent estimations show that the affected population can be up to 1.5%.[2] Nuts, and namely peanuts,[3] are the major players in the food allergy scenario, even resulting in fatalities both in infants and adults.[4] Unfortunately, no effective clinical treatment is known, only relief of adverse symptoms. For this reason, prevention, i.e. a strict dietary control, is the only effective measure to be taken in the cases of severe allergy and therefore an accurate declaration of ingredients on food labels is essential, as stated in recent legislative changes aimed at improving consumer protection such as the European Union (EU) legislation on food labeling that came into force in November 2005 (EU Directive 2003/89/EC amending Directive 2000/13/EC).

Taking in account that accidental contamination with allergens can occur during food manufacturing or handling of the raw materials,[5] fast and reliable analytical methods are required to asses allergen levels in the final products. Although the fluorescence-based enzyme-linked immunosorbent assay (ELISA) is regarded as the 'gold standard' for general screening of food samples for allergen detection,[6,7] and it is presently used as the official screening method, several problems such as selectivity, accuracy and cross-reactivity lead to severe limitations in the applicability of this screening technique. Several approaches have been proposed to overcome these issues,[8] such as complementary detection techniques,[9] real-time polymerase chain reaction or proteomics-based ones.

Recently, inductively coupled plasma mass spectrometry (ICP-MS) via element-tagged immunoassay has been proposed as a selective and sensitive detection technique for immunoassays.[10,11] ICP-MS detection have several advantages if compared with conventional fluorescence, such as lower matrix effects caused by the other food components, low detection limits, excellent response linearity, long-term sample storage, and the capability of a multi-analyte detection using different elemental tags. However, few applications of the metal tagged ICP-MS immunoassay have been reported,[10–16] and the potential of the technique has not been fully assessed up to now.

To overcome some limitations of immunoassay techniques in terms of false positive or false negative results, confirmatory methods are needed. In this case the selectivity of mass spectrometry could be of great benefit in identifying peanut proteins even in complex matrices and could be used for confirmation purposes. Very recently, the advantages of liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) methods for the detection of peanut allergens in various food matrices have been

demonstrated.[17–19] A proteomics-based approach based on the direct identification of Ara h1 was used as a new strategy to identify peanuts in food matrices.[17,18] To improve the selectivity and thus the confirmation criteria in the allergen identification more than one target could be selected.[19] For this reason our research group developed a LC/ESI-MS/MS method with a triple quadrupole analyzer for the identification and determination of two of the major peanut allergens in food: Ara h2 and Ara h3/4.[20] In this paper we discuss a comparison of the two methods recently developed by our group, based respectively on Eu-tagged ICP-MS immunoassay[11] and on LC/ESI-MS/MS identification, and determination of Ara h2 and Ara h3/4 [20] applied to a complex food matrix like a chocolate rice crispy-based snack.

EXPERIMENTAL SECTION

Materials. Phosphate-buffered saline (1x PBS; 150mM NaCl, 20mM sodium phosphate, pH 7.4), 0.01% Tween 20 in 1x PBS, 7M nitric acid (Carlo Erba, Milan), Eu-labeled anti-mouse affinity purified rabbit polyclonal Abs (DELFIA1, Perkin Elmer Life Sciences, MA, USA) were used. The holmium standard solution (1000 mg L^{-1}) used for ICP-MS was purchased from High-Purity Standards (Charleston, SC, USA). Ara h2 was purified from toasted peanuts following a procedure reported in the literature.[21] Ara h1 and Ara h3/4 purified peanut proteins and anti-Ara h1 (Pn-t) and anti-Arah3/4 (Pn-b) monoclonal antibodies raised against proteins purified from toasted peanuts were kindly provided by the Leibniz Center for Medicine and Biosciences at the Research Center Borstel (Borstel, Germany). All antibody solutions were diluted with deionized water (Millipore, Bedford, MA, USA). Acetonitrile (HPLC purity), trifluoroacetic acid and formic acid (analytical reagent grade) were purchased from Carlo Erba (Milan, Italy). Trypsin from bovine pancreas was from Sigma-Aldrich (St. Louis, Missouri, USA). Toasted peanuts and food products (chocolate/rice crispy-based snacks) used in this work were obtained at a local food store.

Apparatus and procedures.

ELISA ICP-MS method. The assay format was based on indirect ELISA using well-plates coated with polyclonal immunopurified antibodies targeting total soluble peanut proteins. The immunoassay and flow-injection analysis (FIA)-ICP-MS procedures are described in detail in a previous paper.[11] Briefly, the sample solutions were incubated for 15 min at room temperature in to well-plates coated with polyclonal immunopurified antibodies targeting total soluble peanut proteins. After washing, two incubation steps were performed: the first with a mixture of the mouse anti-Ara h3/4 and anti-Ara h1 monoclonal antibodies (Ab I) and the

second with the Eu-labeled anti-mouse polyclonal antibodies (Ab II) for detection. After digestion with 7M HNO_3 (100 μL, 1 h, room temperature), 25 μL samples were injected into the FIA-ICP-MS system. Limits of detection and quantitation were calculated as three and ten times the standard deviation of blank solution on the slope of the calibration curve, respectively. Linearity was tested in the 2.5–500 μg peanuts g^{-1} matrix range. Accuracy was evaluated both in terms of matrix effect and recovery. In particularly, a t-test was used to compare the slopes of calibration curves built up in the range 2.5–50 (n = 15) μg peanuts g^{-1} matrix and by using aqueous peanut protein solutions. The recovery experiments were carried out both on unspiked food matrix and on the same matrix fortified with peanuts at three different concentration levels (5, 20 and 50 μg peanuts g^{-1} matrix). Recovery was expressed as the ratio percentage of the determined total peanut concentration to the added peanut concentration. Precision expressed as percentage coefficient of variance (CV%) was calculated by carrying out the extraction procedure on three samples and performing three FIA-ICP-MS analyses for each sample. Experimental measurements were made on an ICP-MS X SeriesII (Thermo Electron Corporation, Waltham, MA, USA) equipped with the trigger card and operating under Xt interface standard conditions. The instrument optimization was performed daily in the high mass range to assure a response of at least 50 000 counts per second (cps)/mgL^{-1} for indium and 80 000 cps/mgL^{-1} for uranium in the high mass range. A GS50 chromatographic pump (Dionex Corporation, Sunnyvale, CA, USA) and an EV750-100 inert injection valve (Rheodyne, Rohnert Park, CA, USA) were used to perform fully automated FIA with a 2% (m/v) nitric acid aqueous solution as the mobile phase delivered at a flow rate of 0.5 mL min^{-1}. Both isotopes of ^{151}Eu and ^{153}Eu together with the ^{165}Ho signal used as internal standard (10 μg L^{-1}) were monitored in the experiment. For qualitative and quantitative purposes the signal of ^{153}Eu was considered. The operating parameters of the ICP-MS instrument were as follows: RF power 1400 W; coolant gas flow 15.5 L min^{-1}; auxiliary gas flow 0.98 L min^{-1}; nebulizer gas flow 0.87 L min^{-1}; transient; nickel standard Xt cones; TRA data acquisition mode: dwell time 100 ms; duration time 60 s, standard resolution.

Liquid chromatography/triple quadrupole tandem mass spectrometry. LC elution was carried out on a Poroshell C-18 with pore size of 300 Å column (75 x 2.1 mm, 5 μm particles) (Agilent Technologies, Santa Clara, CA, USA) using a gradient solvent system [(A) aqueous formic acid 0.1% (v/v)/(B) 0.08% (v/v) formic acid in acetonitrile] as previously described.[20] The flow rate was 200 μL min^{-1}. The mobile phase was delivered by a 2690 series Alliance chromatographic system (Waters, Milford, MA, USA) equipped with a 120 vial capacity sample tray. Injection volume was 30 μL. A Quattro LC triple quadrupole instrument

(Micromass, Manchester, UK) equipped with a pneumatically assisted ESI interface was used. The system was controlled by MassLynx software version 4.0. The nebulizing gas (nitrogen, 99.999% purity) and the desolvation gas (nitrogen, 99.999% purity) were delivered at flow rates of 55 and 500 L h^{-1} respectively. Optimized conditions of the interface were as follows: ESI voltage 3.5 kV, cone voltage 35 V, RF lens 0.3 V, source temperature 130 °C, desolvation temperature 250 °C. MS/MS experiments were performed underm single reaction monitoring (SRM) conditions with a collision gas pressure of 2.3 x 10^{-3} mbar in the collision cell. The SRM transitions monitored were as follows: m/z 807/1050 collision energy (CE) 35 eV (CCNELNEFENNQR, Ara h2), m/z 950/1120 CE 35 eV (CMCEALQQIMENQSDR, Ara h2), m/z 649/1089 CE 30 eV (AHYQVVDSNGDR, Ara h3/4), m/z 695/700 CE 35 eV (SPDIYNPQAGSLK, Ara h3/4). Leucine-enkephalin was used as internal standard (m/z 557/397; CE 20 eV).

Sample treatment. Peanut-containing food extracts were prepared by adding 25 mL of 1 x PBS to 1 g of ground sample. Proteins were extracted by shaking for 15 min at 60 °C, then centrifuged (14000 rpm, 5 min) and filtered on 0.2 μm nylon filter. The whole sample treatment procedure for the subsequent ELISA-ICP-MS method is described fully by Careri et al.[11] In the case of the LC/ESI-MS/MS method[20] peanut containing food extracts were prepared by adding 10 mL of 0.2M Tris HCl (pH 8.2) to 1 g of ground sample. Proteins were extracted by shaking for 2 h at 60°C; then the extract was centrifuged (14000 rpm, 5min) and filtered on a 0.2-μm nylon filter. Sample concentration was performed on a 5 kDa molecular weight (MW) cut-off filter (Millipore, Billerica, MA, USA) by adding 5 mL protein extract and reducing the volume to 0.5 mL. The sample was then digested with trypsin (5μL, 0.6mM in Tris HCl, pH 8.2) for 24 h at 50°C before injection into the LC system.

Results and Discussion

Performance of the ELISA-ICP-MS based method. A non-competitive sandwich ELISA with ICP-MS detection was previously developed for screening purposes to detect hidden peanut allergens in food.[11] The assay, which is based on the determination of the element used to tag the antibody, exhibited good performances in terms of detection limits, linearity and selectivity on cornflakes. In addition, ICP-MS analysis conditions were carefully evaluated. Due to the small sample volume available and the nature of the samples, the FIA mode was chosen. The FIA mode offers several advantages in the sample analysis since it easy to use, there is no sample loss and it is a high-throughput analysis. With this configuration, it was observed that the introduction of digest samples into the ICP source toward a LC mobile phase flow did not cause any significant clogging in the nebulization system and in the sample cone, usually seen in the normal analysis mode: this could more likely due to the high nitric acid residual concentration. However, a significant memory effect on the europium signal coming from the six-port injection valve was observed, probably due to the sample sticking on the peek parts: it was necessary to inject 7 M nitric acid twice before introduction of the next sample to decrease the background noise to the level usually observed (700 cps). Such a configuration allows the analyses of a high number of samples to be performed making ICP-MS suitable for a rapid screening detection.

The method performance was further investigated considering a more complex matrix based on chocolate, milk and rice. The detection and the quantitation limits were 2.2 and 5 μg peanuts g^{-1} matrix, respectively. In contrast to ELISA-UV reading, by injecting 25 μL aliquots into the ICP source, the signal reading for each sample is performed on three different aliquots of the each well-plate sample to account for the variability in the FIA-ICP-MS method. Assessment of the dynamic response range in the 5–500 μg peanuts g^{-1} matrix range evidenced linearity between 5 and 40 μg g^{-1}, since a signal saturation starts to occur at the 50 μg g^{-1} level as shown in Figure 1.

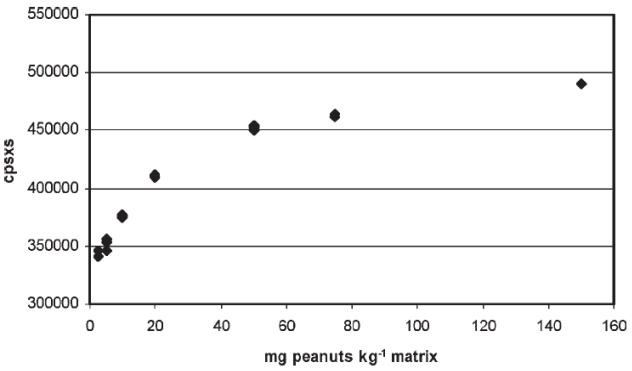

Figure 1: ICP-MS ELISA calibration curve obtained by using chocolate/rice crispy-based matrix fortified with different amounts of peanuts (2.5–160mg peanuts g^{-1} matrix range).

Trueness of the measurements was found to be affected by the presence of matrix effect on the antigen/antibody (Ag/Ab) binding and thus on the ICP-MS response of Eu-labeled Abs. By performing a t-test on the slope values of the matrix-matched calibration curve and the slope of the curve obtained by aqueous dilution of peanuts extracts, a significant difference at the 95% confidence level (t-calculated = 48, $t\text{-tabulated}_{12,\ 0.05}$ = 1.78) was highlighted. Namely, regression equations evidenced that in the presence of matrix ($Y = 3913 \pm 140X$) a ≈ 20% signal suppression was observed with respect to that obtained analyzing aqueous peanut extracts ($Y = 4874 \pm 160X$) to indicate a lower Ag/Ab binding in the presence of matrix interferences. The lowering in the Ab/Ag binding in such a complex extract compared to aqueous peanut solutions was also observed by analyzing the same matrix with a commercially available ELISA kit (Veratox quantitative peanut allergen test; Neogen Corporation, Lansing, MI, USA) based on colorimetric UV detection. This result strongly supports the critical role of the matrix on immunoassay performance and the lack of accuracy of quantitative analysis. For this reason the recovery values were calculated by using the standard addition method. Excellent recovery values were obtained on the three concentration levels tested (5 μg peanuts g^{-1} matrix: 86 ± 18%; 20 μg peanuts g^{-1} matrix: 110 ± 4%; 50 μg peanuts g^{-1} matrix: 89 ± 2%). From these results it should be noticed that there is a significant increase in recovery precision as the peanut concentration increases, suggesting a better repeatability of data at higher fortification levels.

As far as the cross-reactivity is concerned, the analysis of several food components (hazelnuts, Brazil nuts, almonds, walnuts, pecan nuts, macadamia, milk, soybeans, cacao powder) showed no significant effects and demonstrating that the monoclonal Abs used are very specific toward peanut proteins.

Performance of the LC/ESI-MS/MS method. The availability of triple quadupole mass spectrometers has significantly increased in the last decade and the selectivity of operating acquisition modes can allow identification of peptides in complex matrices even without high-resolution data. The capability of the LC/MS/MS method as a confirmatory tool is strongly related to its sensitivity. As for the immunoassay the sensitivity depends on the Ag/Ab binding affinity and thus on the quality of reagents used, in the case of the LC/ESI-MS/MS method this is strongly related to the ionization efficiency of selected peptides and to the presence of matrix suppression effects in the ESI source. In this work, a sudden degradation of performance in terms of MS/MS analyte response was observed after a few LC runs of the food sample extracts dependent on the source rapidly becoming dirty. In order to make

detection and quantification limits less dependent on the effects of the matrix complexity, it clearly appears that a more selective sample enrichment treatment is required.

However, by operating under SRM conditions, LOD and LOQ values for the peptide of Ara h3/4 at m/z 695 [SPDIYNPQAGSLK] were 1 and 3.7 μg protein g^{-1} matrix, respectively. Figure 2 shows the SRM chromatograms of peptide m/z 695/700 [SPDIYNPQAGSLK, Ara h3/4] in a chocolate/rice crispy-based matrix fortified with different amounts of peanuts.

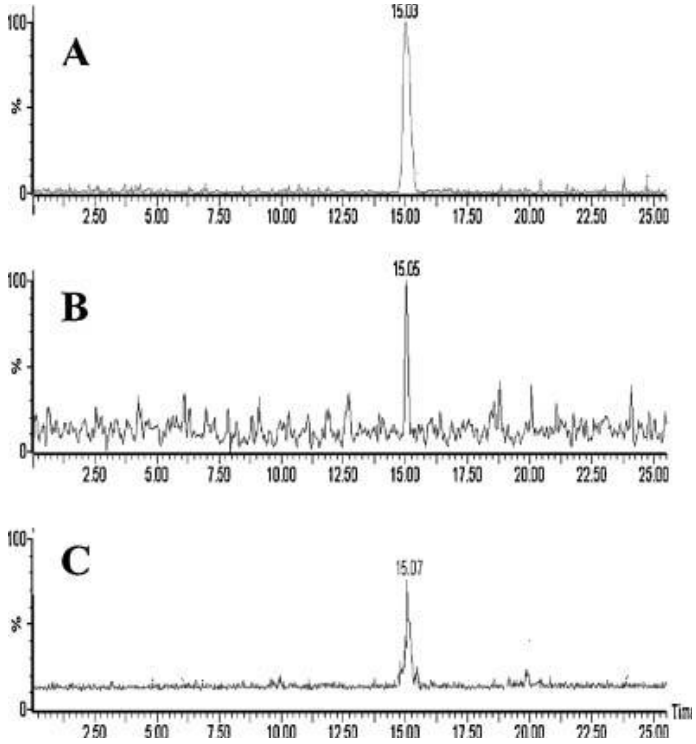

Figure 2. LC/ESI-QqQ-MS/MS SRM chromatograms of the peptide m/z 695/700 [SPDIYNPQAGSLK, Ara h3/4] in a chocolate/rice crispy-based matrix fortified with different amounts of peanuts: (A) 20 μg g^{-1}; (B) 4 μg g^{-1}; and (C) 1 μg g^{-1}.

Detection and quantitation of the peptide biomarker of Ara h2 at m/z 807 [CCNELNEFENNQR] led to poor sensitivity; LOD and LOQ values being 5 and 14 μg g^{-1}, respectively. Accordingly, better sensitivity was observed for the peptides selected for Ara h3/4 ($y = 10.48 \pm 0.23x$ and $y = 3.78 \pm 0.12$ x; m/z 695 [SPDIYNPQAGSLK] and m/z 649 [AHYQVVDSNGDR], respectively) with respect to those selected for Ara h2 ($y = 4.23 \pm 0.22x$ and $y = 2.68 \pm 0.18x$; m/z 807 and m/z 950 [CMCEALQQIMENQSDR], respectively). Excellent linearity was demonstrated for all the peptides selected by spiking the matrix extract with 1–20% peanut extract. Good method precision for all the peptide biomarkers investigated was obtained, the relative standard deviation (RSD) being lower than 10% (n = 10) when measurements were performed in the model food extract fortified with 1.25 and 5% w/w peanuts/matrix. The signals for the Ara h2 peptides were found to be strongly affected by ion suppression (from 30–50%), whereas this drawback was more negligible in the case of the detection of Ara h3/4 peptides (lower than 10%). The use of an internal standard allowed a reduction in the matrix effect of between 5% and 30% for the measurement of Ara h3/4 and Ara h2 peptides, respectively. The method was proved highly specific in the matrix investigated as a consequence of the specificity of the peptide biomarkers selected. Performing a search in protein databases, the sequences of the selected peptides were found to be unique for peanuts.

Comparison of the ELISA-ICP-MS and LC/ESI-MS/MS methods and conclusions. In this work mass spectrometry is presented as a detection technique for either screening and confirmation purposes. Findings highlight that this technique has versatility in different approaches for the detection and determination of hidden peanut allergens. Even though the methods used have two different purposes, they share some major limitations. In fact, their application to a complex matrix pointed out that in both cases trueness of the quantitative analysis is strongly influenced by the food under investigation and the complexity of the matrix still remains the main determining factor to improve detectability. In particular, as discussed before in the case of the LC/MS/MS method, the whole food matrix extract is injected into the LC system causing a significant signal suppression effect on the analyte signals at the ESI interface with respect to the signal obtained by analyzing standard solutions. In the case of the ELISA the matrix is able to influence the Ag/Ab binding by reducing the amount of bound protein.

It should be remarked that the reasons for the lack of accuracy demonstrated for the two methods have different origin. In the case of the LC/MS/MS method the main limitation is related to the working principle of the ESI source; by contrast, in the ICP-MS-based method, no significant instrumental drawbacks were observed apart from the memory effect on the injection valve, and the bottle-necks were found to be related to the immunoassay. As for the LOD values, very good detection was obtained with ICP-MS detection even by using an Ab not optimized for this kind of detection, whereas, in the case of the LC/MS/MS method, poorer sensitivity was observed. These findings suggest that actually the immunoassay still represents the most powerful technique for the evaluation at trace level of hidden allergens in foods. On the other hand the development of a sample treatment able to enrich Ara h2 and Ara h3/4 extract content could allow a significant improvement in the LOD values of the LC method and fully take advantage of the highly selective MRM analysis.

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Chapter 3

Elemental Labeling of Monoclonal Antibodies for Multiplexed ICP-MS Targeted Proteomic

3.1. Multiplexed Determination of Protein Biomarkers Using Metal-Tagged Antibodies and Size-Exclusion Chromatography – Inductively Coupled Plasma mass Spectrometry (SEC-ICP-MS)

Abstract. A liquid-phase immunoassay was developed for the simultaneous determination of five cancer biomarker proteins: alpha-fetoprotein (AFP), human corionic gonadotropin (hCG), carcino-embryonic antigen (CEA), ovarian tumour antigen (CA125/MUC16) and gastrointestinal tumor antigen (CA19-9). The method was based on the incubation of a serum (or tissue cytosol) with 5 antibodies, each labelled with a different lanthanide (Pr^{3+}, Eu^{3+}, Gd^{3+}, Ho^{3+} and Tb^{3+}, respectively) followed by the specific determination of the immunocomplex formed by size-exclusion chromatography with inductively coupled plasma mass spectrometric detection (SEC-ICP-MS). The sensitivity of the method was comparable with those attainable by ELISA or radioimmunoassay with the advantage of multiplexed analysis capacity, virtually no sample preparation and sample amount consumption ca. 3-times lower than an ELISA test. The method was validated for the analysis of the proteins in human serum and proved to be able to discriminate ovary and uterus tumour tissue samples from those of healthy subjects.

INTRODUCTION

The needs for the detection of low-level molecular biomarkers of disease and the monitoring of the efficiency of drug treatment are the driving force for the development of methods allowing absolute quantification of proteins (ideally several at the same time) in clinical samples, such as, e.g. human serum and biopsy tissues (*1-2*). The two principal classes of methods include immunoassays, based on the use of antibodies, using fluorescence (ELISA) or radioactivity (RIA) detection (*3*), or methods based on the use of isotopically labelled tags (ICAT or ITRAQ) followed by electrospray or MALDI mass spectrometry measurement (*4*). The procedures are relatively complex and suffer from the difficulties with multiplexing (immunoassays) or insufficient sensitivity or specificity for low level protein determination in biological matrices (molecular MS methods).

Elemental MS, in particular inductively coupled plasma mass spectrometry (ICP-MS), associated with the appropriate tagging strategies offers a number of features of interest for quantitative proteomics, including high sensitivity even in complex samples with reduced or lacking matrix effect. In addition, the availability of having up to one hundred elemental isotopes which can be simultaneously discriminated by ICP-MS allows multiplexing detection (*5-6*). The ICP-MS sensitivity and the possibility to produce a quantitative signal have been used in the concept of Metal Coded Affinity Tag (MeCAT) (*7-8*). The reagent, bearing a lanthanide incorporated as a DOTA complex, reacted to form covalent complexes with –SH containing proteins which were enzymatically degraded to produce specific peptides. The latter could be quantified *via* the lanthanide signal but a parallel identification step by molecular MS was necessary which reduced the field of practical applications.

Considerable attention has been paid to the tagging of antibodies with an ICP-MS detectable element, absent in biological matrix (*9-11*). Antibodies react with antigens with an extremely high degree of specificity even in complex matrices. The activity of the metal-tagged antibodies was shown to be preserved on the condition of a careful optimization of the labelling procedure. The tagging was usually achieved with a rare earth element introduced as a stable DOTA chelate (*12-13*) but other elements, such as Au and Ag, and more recently I and Hg were also used (*14-17*). Metal-tagged antibodies have been successfully used for the detection of two allergenic proteins of peanuts in food (*18-19*) in ELISA or for detection of specific proteins in Western Blot applications (*20*). Tissue imaging was successfully obtained combining laser ablation ICP-MS and immunohystochemistry techniques with labelled antibodies on tissue slides as well tissue microarray for cancer biomarkers (*21-22*). A unique attractiveness of ICP-MS is the possibility of straightforward multivariate (multiplex) analysis

owing to the simultaneous use of different tags for the different proteins and no overlap of the tags of different antibodies as developed for a massive multivariate single-cell bio-assay for detection of surface antigens in leukemia stem cells (*23-29*).

The above methods are based on a heterogeneous reaction (usually between an immobilized antigen and antibody in solution) and require tedious and error-prone preparation procedure including washing steps. During the development of the immunoassay the washing steps deserve particular attention, since in optimized conditions of antibody concentration and incubation time, they affect accuracy of the analysis as a consequence of possible high values of background with consequent poor sensitivity.

In this study, for the first time, size-exclusion chromatography with inductively coupled plasma mass spectrometric detection (SEC-ICP-MS) was proposed for the multiplexed determination of protein biomarkers using metal-tagged antibodies. The objective of this research was to find conditions in which a number of target proteins could be simultaneously determined as their complexes with antibodies directly in the sample matrix without any sample pre-treatment, such as e.g. solid-phase extraction or washing steps. For this purpose, we have made use of, on one hand, the selectivity of ICP-MS allowing the simultaneous determination of several rare-earth elements (each tagging a different antibody), and, on the other hand, of the selectivity of high-resolution size-exclusion LC allowing the on-line separation of the protein-antibody complex from the unreacted antibody and degradation products of the labelling reagent. Size-exclusion LC has been extensively used in combination with classical detection systems like UV-VIS or fluorescence spectroscopy, to study antigen-antibody reactivity (*30-35*). Those studies showed the ability of size exclusion chromatography to perform qualitative and quantitative analysis in complex matrix. However, they were limited to a single antibody-antigen interaction at a time because of the co-elution of the immunocomplexes of different proteins, as well of the free antibodies, and the inability of detection systems to discriminate signals from different analytes. Here, we are proposing a generic procedure for a straightforward liquid-phase immunoassay allowing the determination of five established cancer biomarkers (AFP, hCG, CEA, CA125 and CA19-9) based on the reaction with a cocktail of antibodies, each tagged with a different lanthanide, and size-exclusion ICP-MS.

EXPERIMENTAL SECTION

Samples, Reagents and Salts. *Proteins, serum and tissue lysates*. Full length alpha-fetoprotein (AFP) purified from human cord serum was from Abcam (Cambridge, UK). Ovarian tumor antigen (CA125/MUC6), gastrointestinal tumor antigen (CA19-9), carcinoembryonic antigen (CEA), human chorionic gonadotropin (hCG) were obtained from Sigma-Aldrich (St. Quentin-Fallavier, France). The proteins are known as established cancer biomarkers (36-37). The proteins were divided into single work aliquots immediately upon reception and stored at –20°C. Control human sera with a declared amount of tumour biomarkers were obtained from Data Medical Service (Milan, Italy). Samples of water-soluble fraction of lysates from ovaric and uterus tissues, both from tumoral (74 and 72 years old women with ovary carcinoma at unknown stage and uterus adenocarcinoma of 2nd degree, respectively) and healthy subjects were from Imgenex (San Diego, CA) and were purchased from Histoline (Milan, Italy). They were received in lysis buffer (phosphate buffer saline pH 7.4 containing 0.25% sodium deoxycholate, 0.1% SDS, 1 mM of EDTA, Na_3VO_4, NaF and PMSF, and 1 μg mL^{-1} of aprotin, pepstatin-A and leupeptin). The declared total protein concentration was 1 mg mL^{-1}. Tissue lysates, received frozen, were thawed, divided into single work aliquots and stored at –20°C.

Antibodies. Rabbit polyclonal anti-mouse (whole molecule) IgG Europium labelled (DELFIA) was from Perkin Elmer Life Science (Waltham, MA). Mouse ascitic fluid containing a declared 2 mg mL^{-1} of IgG was from Sigma-Aldrich (Schelldorf, Germany). Mouse IgG anti-human monoclonal antibodies (mAb) for α-CA19-9 (clone M39026), α-CA125 (clone X325), α-AFP (clone AFP-01), α-hCG (clone HCG1) and α-CEA (clone 1C11) were purchased from Abcam (Cambridge, UK). All mAbs were obtained in liquid form as IgG or protein A purified fractions. Upon reception, mAbs were divided into single working aliquots and stored at –20°C.

Labelling reagents. 1,4,7,10-tetraazacyclododecane-1,4,7-tris-aceticacid-10-maleimido-ethylacetamide (DOTA) was purchased from Macrocyclics (Dallas, TX). Lanthanide chlorides ($LuCl_3$, $HoCl_3$, $PrCl_3$, $EuCl_3$, $TbCl_3$, $GdCl_3$) with natural isotopic abundances were from Aldrich (Schelldorf, Germany). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and *N*-succinimidyl-*S*-acetylthioacetate (SATA) were from Pierce (Rockford, IL). Hydroxylamine (>99%) was from Fluka.

Salts and buffers. Analytical grade ammonium acetate, sodium phosphate monobasic (NaH_2PO_4), tris-(hydroxymethyl) aminomethane hydrochloride, sodium chloride, ethylene-diaminotetraacetic acid disodium salt (EDTA) were purchased from Sigma-Aldrich (St.

Quentin-Fallavier, France). Buffers used were: ammonium acetate buffer (100 mM, pH 6.8 as elution buffer and 20 mM, pH 6.0 for metal complexation), phosphate buffer (100 mM, pH 7.2, 2.5 mM EDTA), Tris buffer saline (20 mM Tris-HCl, 0,45% NaCl, pH 7.0), phosphate buffer saline (10 mM in phoshate, 150 mM in NaCl pH 7.4, 2.5 mM EDTA), deacetylation buffer (100 mM phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4). Ultrapure water 18 MΩ cm from a Milli-Q system (Millipore, Bedford, MA) was used throughout the work.

Instrumentation. An Agilent model 1200 binary pump, equipped with an autosampler, automatic injection system with a loop of 900 μL and a UV-VIS detector was used as solvent delivery system. The system was controlled by Agilent ChemStation software. The ICP mass spectrometer was an Agilent 7500 CE fitted with a Meinhardt nebulizer. The exit of the column was connected by means of PEEK tubing to the ICP-MS nebulizer. Nanosep centrifugation tubes with low protein membrane and a 30 kDa cut-off (Pall, USA) were purchased from VWR International (Milan, Italy). Centrifugation vials were used throughout the work for washing steps and buffers exchange during labelling procedure of antibodies.

Procedures. Mouse monoclonal IgGs anti human -AFP, -CA125, -CA19-9, -CEA and -hCG were labelled with Pr^{3+}, Ho^{3+}, Tb^{3+}, Gd^{3+} and Eu^{3+}, respectively.

Labelling of antibodies by the TCEP method. An aliquot of antibody (40 μg, 266 pmol) was washed on a cut-off filter by centrifugation (1 x 500 μL, 10000 rpm, 6 minutes) with phosphate buffer, recovered at 1 mg mL^{-1} (6.6 μM) and incubated with 6-fold molar excess (1.6 nmol, 40 μM) of TCEP at 37°C for 30 min to reduce disulphide bridges. The antibody was quickly washed (1 x 500 μL) with Tris buffer saline to remove the TCEP in solution by centrifugation and resuspended in the same buffer at 1 mg mL^{-1}. An aliquot of 0.5 μL DOTA dissolved in DMSO (24.1 mM) was added in 50-fold molar excess (12 nmol, final concentration 320 μM) and the mixture was incubated at 37°C for 1 h. Then, the mixture was washed by centrifuge with Tris buffer saline (500 μL) twice to remove excess of unreacted DOTA, and resuspended in 20 mM ammonium acetate buffer (pH 6.0). An aliquot of $LnCl_3$ was added to reach 1 mM concentration and the mixture was incubated at 37°C for 30 min. The tagged antibody was extensively washed by centrifugation with Tris buffer saline to remove excess of free metal and was recovered at a nominal concentration of 0.5 mg mL^{-1}.

Labelling of antibodies by the SATA method. An aliquot of 40 μg (266 pmol) of mAb was extensively washed by centrifugation with phosphate buffer saline (to remove NaN_3 used as preservative which could quench the subsequent reaction) and recovered at 1 mg mL^{-1}. An

aliquot of SATA solution in DMSO at a desired molar excesses of 10, 20 and 50 fold (2.6, 5.3 and 13 nmol, respectively) was added and the mixture was incubated at ambient temperature for 1 h. The SATA-modified mAb was washed by centrifugation with phosphate buffer saline on the cut-off filter and resuspended at 1 mg mL^{-1} in the same buffer. The solution was deacetylated with 1:10 *v/v* of hydroxylamine solution by incubation at room temperature for 2 h. The mixture was washed by centrifugation and the mAb was resuspended in Tris buffer saline. An aliquot of mDOTA in DMSO (24.1 mM) was added and the mixture was incubated for 1 h at 37°C. The mixture was centrifuged for wash out unreacted reagents with Tris buffer saline and resuspended in 20 mM ammonium acetate buffer (pH 6.0). An aliquot of $LnCl_3$ was added to reach the metal concentration of 1 mM and the mixture was incubated for 30 min at 37°C. The tagged-mAb was washed by centrifugation extensively with Tris buffer saline and recovered at the concentration of 0.5 mg mL^{-1}. Element tagged-antibodies were stored at 4°C in Tris buffer saline until use.

Multiplexed determination of the protein biomarkers in human serum and tissue lysates. A sample serum aliquot (30 µl) containing 0-100 international units (UI) per mL^{-1} of protein was incubated overnight at 4°C with a mixture of labelled antibodies at nominal concentration 10 µg mL^{-1} and subsequently analysed by SEC-ICP-MS.

Tissue lysates were diluted with serum to make the biomarkers concentration fall into the analytical range. Several dilutions were tested.

Size-exclusion chromatography ICP-MS. The proteins were separated on a size exclusion column Superdex™ 200 HR 10/30 (10 mm x 300 mm x 13 µm beads size) with the approximate bed volume of 24 ml and a declared linear separation range 10-600 kDa for globular proteins (GE Healthcare). The column was calibrated with a mixture of thyroglobulin (670 kDa), holo-transferrin (81 kDa), bovine albumin (66 kDa), chicken egg albumin (44 kDa), Mn superoxide dismutase (39.5 kDa), Cu/Zn superoxide dismutase (32.5 kDa), carbonate dehydratase (29 kDa) and myoglobin (16.7 kDa) using UV/VIS detection at 280 nm with baseline evaluation at 800 nm. Retention times (in min) plotted versus the logarithm of molecular mass (in kDa) gave a straight line ($y = -0.134\ x + 4.385$; $r^2=0.992$). Separations were carried out isocratically at 0.5 mL min^{-1} using 100 mM ammonium acetate (pH 6.8) as mobile phase. ICP-MS conditions were: plasma gas (Ar) 15 L min^{-1}, forward power 1500 W, carrier gas 1.05 L min^{-1}. The performance of the system was checked to respect the manufacturer indications for 1 µg L^{-1} $^{7}Li^{+}$, $^{89}Y^{+}$ and $^{205}Tl^{+}$ in 2% HNO_3, with a dwell time of 100 ms for each isotope. Double charge ion and oxide levels were opitimized on $^{140}Ce^{+}$ and were classically <2 %. The isotopes monitored (in time resolved analysis mode with a dwell time of 100 ms each) were ^{141}Pr, ^{151}Eu, ^{153}Eu, ^{157}Gd, ^{158}Gd, ^{160}Gd, ^{159}Tb, and ^{165}Ho.

RESULTS AND DISCUSSION

The principle of the analytical approach is based on the quantitative formation of a stable complex between the protein to be determined and the metal-labelled antibody added to the sample. In order to be determined by ICP-MS, the immunocomplex formed needs to be separated from any other chemical forms of the labelling metal, such as, e.g. unreacted antibody or its reaction products with other sample components. Owing to the elemental (isotopic) specificity of the ICP-MS several proteins can be determined in the same time provided that the antibody for each of them is tagged with a different element or isotope, and there are no isobaric interferences among them.

The method development has included the following steps: (i) synthesis of antibodies labelled with the different metal ions; (ii) choice of the reaction conditions of the antibodies with the target proteins in the samples to allow a linear increase of the signal as a function of the antigen concentration; and (iii) choice of the sample introduction system allowing the introduction of the antigen-labelled antibody unaccompanied by any other species of the tagging metal.

Size-exclusion LC – ICP-MS for studies of antibody labelling and reactivity. A highly cross-linked agarose and dextran gel was preferred to silica gels in order to minimize interactions of the stationary phase with metal and assure the high recovery of antibodies. The column used (10-600 kDa) allowed to obtain the separation of the unreacted antibody (150 kDa) and the high MW antibody – antigen complex used for quantitative analysis and to monitor with adequate resolution the release of the metal-bearing complex (mDOTA). The use of the ICP-MS detector allows the following of the reaction between the antibody and the antigen during the labelling reaction and the quantification of the complex. The concentration of the mobile phase (0.1 M ammonium acetate) was high enough to reduce the interactions between the proteins and the stationary phase and low enough to assure good nebulization in ICP-MS (*36*).

The feasibility of SEC-ICP-MS was first evaluated by studying a commercial rabbit anti-mouse Eu-tagged IgG antibody. The chromatogram (**Fig. 1a**) shows a number of peaks when a solution of this tagged antibody was injected. Antibody signal was found at 23 min when a theoretical value of 23.5 min was expected as calculated from column calibration curve. Another peak of same intensity was found at 40 min, falling out from column separation range (< 10kDa). This signal was identified as impurity of commercial labelled antibody in the form of free DOTA-Eu complex. To confirm this hypothesis, DOTA-Lu was injected as

pure solution as well mixed with Eu-tagged IgG antibody and its elution profile (**Fig. 1b**) fitted reasonably with the unknown peak found in the chromotogram of Eu-tagged antibody. To be note that the size exclusion LC is unable to differentiate between the compounds DOTA-Lu and DOTA-Eu, and DOTA-Lu was choosen in order to observe the eventual exchange of lanthanides between DOTA complexes. As these compounds interact with the stationary phase and elute well after the total volume of the column slight shifts in the elution volumes can be observed. This was further washed extensively by centrifugation on 30 kDa filter and the purified form was incubated with mouse ascitic fluid containing a declared 2 mg mL^{-1} of IgG in order to observe i) elimination of free DOTA-Eu, ii) formation of stable complex between the two antibodies and iii) behaviour of immunocomplex in SEC, since the separation depends from size and shape of analytes. From the chromatogram of sample incubated (**Fig. 1c**) free DOTA-Eu was found at very low level as effect of high efficiency of washing steps. In addition, the elution profile shows a new peak at 20 min corresponding to a calculated molecular mass of 300 kDa, which represents the formation of an immunocomplex involving one rabbit Eu-tagged IgG with one mouse antibody biomolecule (150 kDa each). Another peak appeared at 30 min, giving back a nominal molecular mass of 70 kDa which fit well with albumin. Since it is capable to bind free metal complex present in IgG-Eu-tagged antibody solution and it is present at high concentration in ascitic fluid, a bind of free metal complex occurs. The recovery of europium from the column was 90 %. Column was found able to discriminate between unbound/bound antibody and free residue labelling reagent and therefore suitable for our purpose.

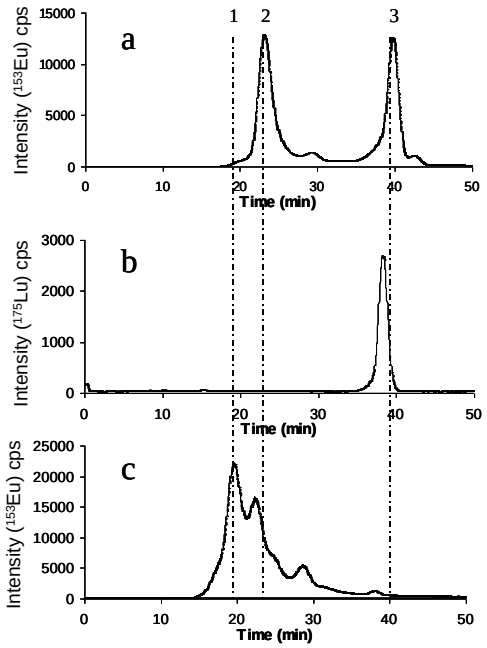

Fig. 1: Size-exclusion chromatograms of a) IgG-Eu-tagged antibody (30 μg mL^{-1}); B) DOTA-Lu 1 ppb; C) rAb-Eu tag (50 μg mL^{-1}) incubated with ascite fluid containing 2 mg mL^{-1} mouse IgG overnight at 4°C. Immunocomplex, free Eu tag IgG and free DOTA-metal complex are indicated by dotted lines 1, 2 and 3 respectively.

Effect of the incubation medium. Contrary to the existing methods, the solubility of the analytically useful immunocomplex is critically important in the liquid phase assay developed in this work. Indeed, poorly soluble immunocomplex may precipitate on the column filter

which would result in a decrease in sensitivity. The solubility of an Ab-Ag complex is known to decrease with the increasing molecular size and depends on the incubation medium.

Fig. 2 shows the effect of various incubation medium (differing in ionic strength, surface tension and protein concentration) on the signal intensity of the immunocomplex in SEC-ICP-MS. The data show that the presence of surfactant or protein has a significant effect on the signal intensity. The composition of human serum virtually stabilizes the immunocomplex, thus significantly enhancing the SEC-ICP-MS signal. Aspecific background was found to depend on the incubation medium. In the case of Tween 20, the nonspecific signal could result from the dimerization of antibody molecules as a consequence of hydrophobic interactions. By testing control human serum, cross-reactivity of the method here developed was demonstrated to be within 20%. The recoveries of the immunocomplex, measured as the ratio of the metal fraction recovered after chromatography exceeded 70% and were reproducible within 5%. Centrifugation of the sample before chromatography did not affect the recovery.

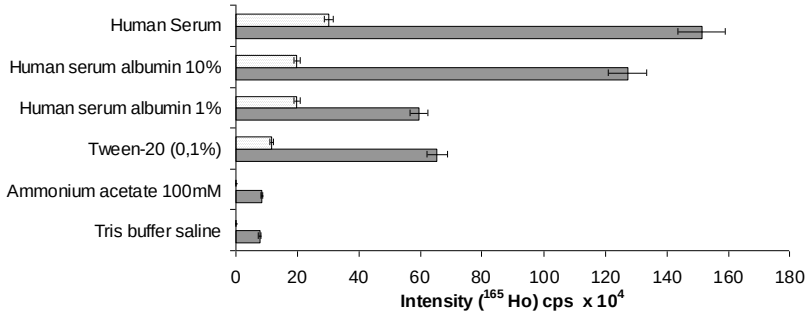

Fig. 2: Effect of different incubation media on the signal intensity of the immunocomplex of CA125 with the corresponding Ho-tagged antibody. White dotted bars: control samples (non CA125 added); shadowed bars – in the presence of CA125. Error bars correspond to three measurements.

Synthesis of metal-labelled antibodies and verification of their stability and activity. The set of 5 proteins chosen for the method development consisted of alpha-fetoprotein (AFP, MW=70 kDa), chorionic gonadotropin (hCG, MW=40 kDa), ovarian tumor antigen (CA125/MUC6, MW=500 kDa), gastrointestinal tumor antigen (CA19-9, MW=360 kDa) and carcinoembryonic antigen (CEA, MW=200 kDa). Monoclonal antibodies developed towards the whole protein molecules and tested to allow the recognition of the target protein in native state were acquired in different immunoassay formats with limited cross-reactivities, in order to be tagged with metal ions. The antibodies belong to the same class (mouse anti-human IgG), their structure consisting of two heavy chains and two light chains connected by a variable number of disulphide (S-S) bridges and supramolecular interactions (*37*).

The two methods of tagging investigated were based on the introduction of a lanthanide ion as a stable complex with the DOTA ligand. One was based on the reaction of the DOTA

maleimide pendant arm with –SH groups generated by selective reduction with tris(carboxyethyl)phosphine (TCEP) of disulphide bonds of the antibody as described elsewhere (23). The other one was based on the initial introduction of –SH groups onto the antibody surface by reacting aminogroups of lysine with *N*-succinimidyl-*S*-acethylthioacetate (SATA) followed by the hydrolysis of the thioether to generate a free thiol group. The two procedures, referred below as TCEP and SATA procedures, are schematically illustrated in Fig.3.

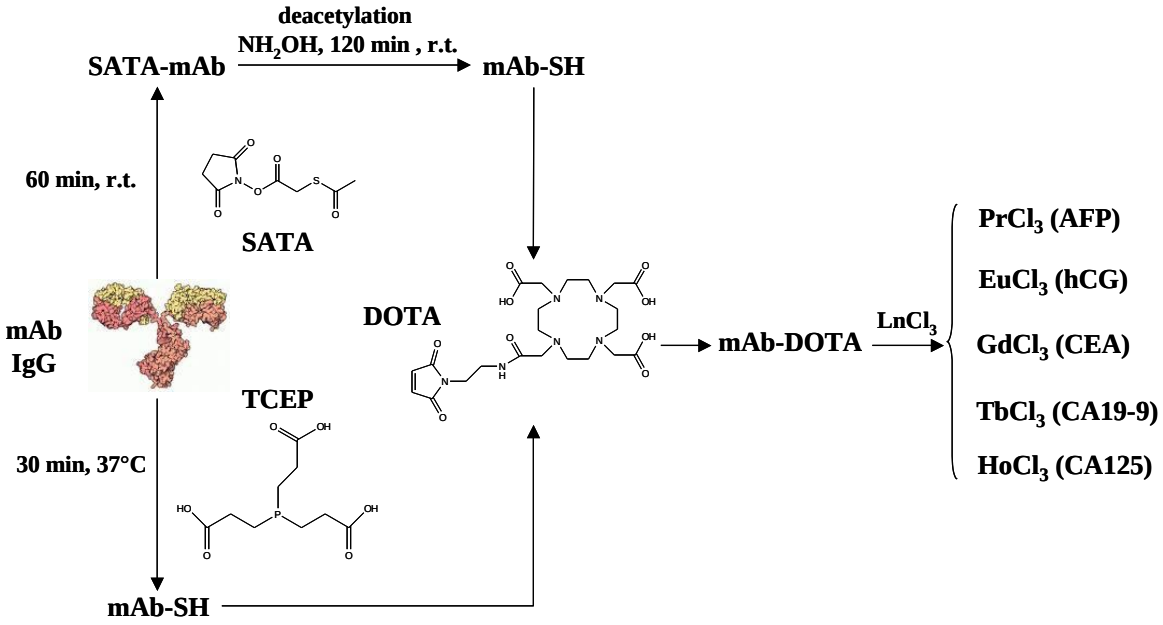

Figure 3: Scheme of the antibody labelling procedure used.

As a function of the methods chosen, different regions of the mAb which may have impact on its biological activity (reactivity) towards the antigen are modified. A trade-off between the number of metal ions introduced per antibody molecule (the more the higher the sensitivity) and the preservation of the biological activity of the antibody is necessary. The success of the mAb tagging approach was verified by checking the stability of the labelled antibody in reaction conditions useful for the possible release of the metal, and by checking its reactivity with regard to the antigen. Then the results for the TCEP and SATA procedures were compared in terms of detection limits.

Stability of the labelled antibodies. **Fig. 4** shows chromatograms obtained by the tagging of the five antibodies with the different lanthanides for the proteins studied. Regardless of the lanthanide used for tagging, they show one Gaussian peak at the same elution volume (23 min) corresponding to the molecular mass of the antibody. No trace of free DOTA reagent

(otherwise eluting at 40 min) is observed indicating that the Ab-bound lanthanide is the only chemical form of the metal introduced into the system. The chromatograms remain unchanged by incubation (at 4 °C and 37 °C) in a control serum matrix showing the stability of the metal-labelled antibody in the matrix to be analysed. Aspecific background was found negligible in most cases as human serum used for incubation was declared from healthy subject. Only in the case of AFP chromatogram, a significant aspecific background was evidenced in the appearance of a peak representing Ab-Ag_2 complex. We address this aspecific signal to the fact that AFP is thought to be the pre-albumin during fetal development and shows a high degree of sequence homogeneity with albumin. However, no aspecific background was found in the region of heavier immunocomplex used for quantification.

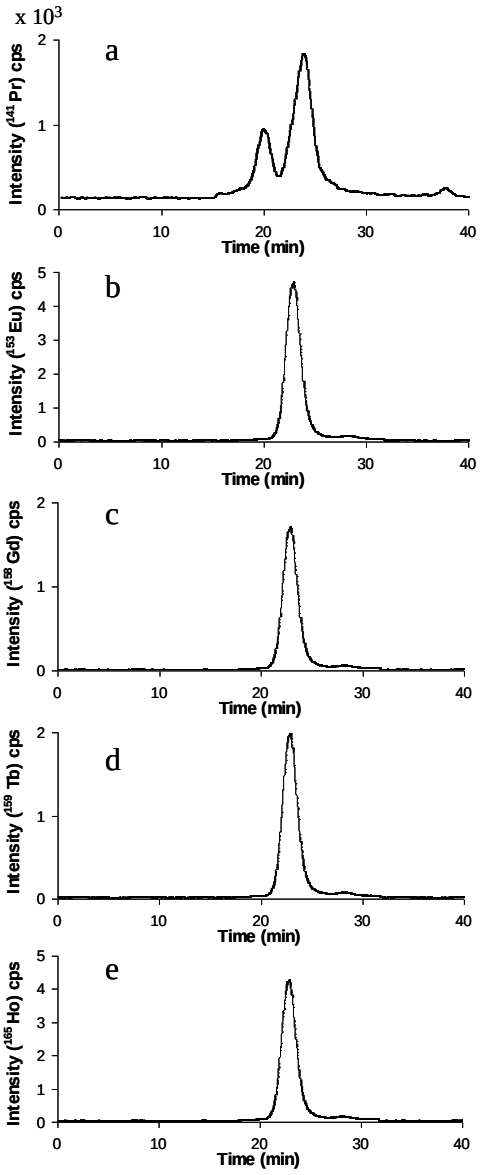

Fig 4: SEC-ICP-MS chromatograms of the labelled antibody by TCEP method. a) Pr-tagged AFP mAb; b) Eu-tagged hCG mAb; c) Gd-tagged CEA mAb; d) Tb-tagged CA19-9 mAb; e) Ho-tagged CA125 mAb.

Reactivities of the tagged antibodies with the antigens. The study of the reaction of the tagged antibodies with the proteins of interest was carried out by SEC-ICP-MS by the analysis of mixtures containing the antigen and the antibody at different concentration ratios. The equilibrium of the reaction of the mAb-Ag complex formation is strongly shifted to the right (pH 7-10) which means that the mAb-Ag2 complex should be observed when a 2-fold excess of the antibody is present unless the activity of the second binding site is reduced because of the steric hindrance. A typical set of chromatograms obtained for the proteins of interest is shown in **Fig. 5** .

They represent two types of morphologies. For small proteins [AFP (70 kDa) and HCG (40 kDa)] three peaks are present whereas for the larger three proteins [CA125 (>500 KDa), CA19-9 (>360 kDa) and CEA (200 kDa)] only two peaks are present. In all the cases a peak of the unreacted tagged-antibody can be observed, either because of the excess of the antibody

used or because of its partly deactivation during tagging. The middle peak in the case of AFP and HCG corresponds to the mAb-Ag2 stoichiometry. The most intense peak corresponds to a large immunocomplex eluting in the void corresponding to the mAb-Ag network. Because of the protein size, the mAb-Ag2 peak for the three antigens of large size co-elutes with the mAb-Ag network in the void. The mAb-Ag network peak was found to be a suitable measure of the Ag concentration in the sample. Its formation was favoured by sample incubation overnight at lower temperature (4 °C). Hence this condition was preferred to incubation at 37 °C.

Detection limits and choice of the tagging procedure. The trade-off between the tagging efficiency and the preservation of the biological activity which is the measure of the method's sensitivity was probed as a function of the molar excess of the reagent allowing the –SH available for the reaction with the lanthanide-DOTA complex. In order to optimize the TCEP procedure four aliquots of mAb α-CA125 (1 mg mL^{-1}) were reduced with 2, 4, 8, and 40-fold molar excess of TCEP, respectively. Taking into account that each S-S reduction producing two -SH groups, 4-fold molar excess of DOTA maleimide ligand was then added. Similarly, for the SATA procedure three mAb α-CA125 aliquots were made to react with the 10, 25, and 50-fold molar excess of SATA. Each of the aliquots was further subdivided, made to react with different amounts of antigen (0-100 UI mL^{-1}) in human serum and analysed by SEC-ICP-MS. The extrapolation of the peak intensity as a function of the antigen concentration allowed the calculation of the detection and quantification limits summarised in **Table 1**.

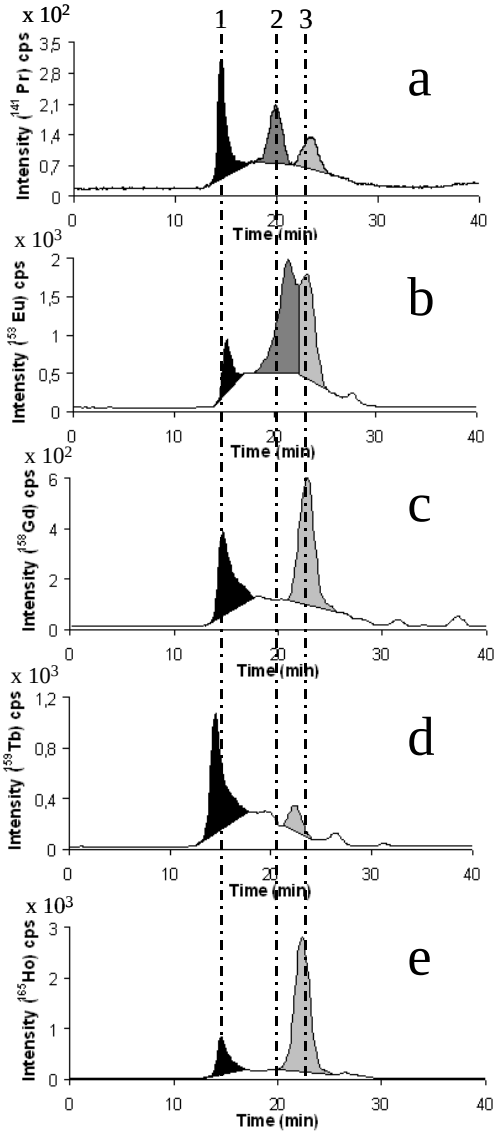

Fig. 5: SEC-ICP-MS chromatograms (one injection) obtained after incubation of a human serum sample spiked with the five proteins of interest with a mixture of the corresponding tagged antibodies. 1- high molecular weight (HMW) immunocomplex; 2 - low molecular wheight (LMW) immunocomplex (Ab-Ag2); 3 - unreacted antibody. a) Pr-channel (AFP); b) Eu-channel (hCG); c) Gd-channel (CEA); d) Tb-channel (CA19-9); e) Ho-channel (CA125).

Table 1: Comparison of TCEP and SATA effects on LOD and LOQ for CA125 antigen.

	Molar excess	LOD (UI mL^{-1})	LOQ
TCEP	2 x	24.1	80.3
	4 x	11.7	39.1
	8 x	12.3	41.0
	40 x	23.9	79.5
SATA	10 x	n.d.	n.d.
	25 x	9.1	30.4
	50 x	8.0	26.8

The data show the maximum sensitivity when a 4-8-fold molar excess of TCEP (with regard to the antibody) was used. At lower TCEP concentrations, the competition from unlabelled antibody results in a decrease of sensitivity whereas at higher TCEP concentrations a similar effect is apparently caused by the denaturation of the antibody. The excess of SATA required to obtain a similar sensitivity is much higher and not detectable immunocomplex signal is obtained at the 10-fold molar excess. At the 50-molar excess dimerization of antibody was observed. It resulted in a peak in the SEC-ICP-MS chromatogram (not showed) at the elution volume similar to that of the immunocomplex rendering data interpretation difficult. As the SATA labelling procedure was also more affected by changes in pH and temperature and required more steps, it was found less attractive and was abandoned despite slightly lower detection limits. The 6-fold molar excess of TCEP finally chosen leads theoretically to the incorporation of 12 metal atoms (DOTA complexed) per one molecule of antibody.

Optimization of the concentration of the tagged-antibody: choice of the linear response range. The optimum Me-Ab concentration should be as small as possible in order to minimize the background deriving from aspecific interaction with matrix component and at the same time be able to produce a quantificable signal for the lowest antigen concentration, but it should be large enough to assure the linear response in the range up to the highest concentration of interest. In order to determine the analytically useful working concentration of labelled antibody, solutions containing nominal concentrations of 1, 5, 10, 25, and 50 μg mL^{-1} of Me-Ab were prepared and incubated with-the minimum concentration of wished-to-be-determined

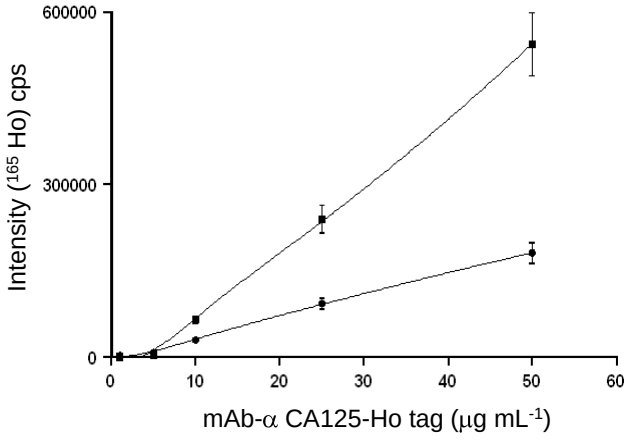

Fig. 6: determination of optimal metal tagged antibody concentration for CA125 antigen in human serum. Square: signal of unbound antibody; circle: signal of immunocomplex. Error bars correspond to three measurements.

antigen (30 UI mL^{-1}) and the maximum concentration of wished-to-be-determined antigen (500 UI mL^{-1}) respectively. **Fig. 6** shows the increasing of the immunocomplex signal (circle) and free antibody (square) at increasing concentration of the latter: a signal distinctly different from the background was obtained for 30 UI mL^{-1} of antigen at the Me-Ab concentration of 5 $\mu g\ mL^{-1}$.

This concentration was however insufficient to assure a linearly increased response above 30 UI mL^{-1} and needed to be increased twice. The Me-Ab concentration of 10 $\mu g\ mL^{-1}$ allowed the quantification of antigen concentrations up to 500 UI mL^{-1} which was satisfactory for the analytical purposes.

Linearity and figures of merit. The linearity and figures of merit were determined by establishing calibration graphs for the five biomarker proteins, each in a concentration range of interest in clinical sample analyses. The linear concentration range tested, regression data and the extrapolated limits of detection (LOD, 3σ for blank matrix/slope) and limit of quantification (LOQ, 10σ for blank matrix /slope) are given in **Table 2**.

Table 2: Linearity and limits of detection.

Antigen	Concentration range	Healthy Reference *	Linear Regression		Sensitivity		ELISA Kit **
			y = m (± S.E.) + b (± S.E.)	r^2	LOD	LOQ	LOD_{ext}
AFP	2 - 110 ng / ml	< 20 ng / ml	y = 2218 (± 143) + 9750 (± 6069)	0.9796	8.2	27.4	2
hCG	3 - 130 mUI / ml	< 5 mUI / ml	y = 3865 (± 60) + 72328 (± 3851)	0.9988	3.0	10.0	2
CEA	0.8 - 50 ng / ml	< 5 ng / ml	y = 9393 (± 430) + 114990 (± 8048)	0.9898	2.6	8.6	1
CA19-9	2 - 100 UI / ml	< 40 UI / ml	y = 9289 (± 903)+ 122069 (± 34168)	0.9378	6.0	20.0	10
CA125	2 - 100 UI / ml	< 35 UI / ml	y = 7366 (± 468) + 217974 (± 41626)	0.9649	8.5	28.3	5

* Treshold values for helathy/pathologycal antigen concentration. ** Expected declared.

The data shows that the method developed using metal-tagged antibodies across the optmized 6-fold molar excess of TCEP procedure, is able to detect tumor biomarkers down to the levels below the treshold reference values used routinely in clinical analysis to discriminate between healthy and disease states (38-39). The sensitivities are comparable with those attainable by commercially available ELISA assays (GenWay Biotech, San Diego CA) with the advantage of multiplexed analysis capacity. Virtually, no sample preparation and a sample quantity ca. 3-times less than an ELISA test is required.

Validation of the liquid-phase immunoassay for the multiplexed determination of biomarker proteins in human serum. The simultaneous determination of several biomarker

proteins is useful in preliminary cancer diagnostics and monitoring chemotherapy. **Table 3** shows the results of the validation of the multiplexed protocol developed for the 5 proteins of interest, each at three concentration levels obtained by mixing a control serum with a serum sample containing certified levels of tumor biomarkers.

The typical precision was below 5% for the antigens explored and the recovery is quantitative. The precision is slightly degraded at low and high levels of antigens which may require reoptimisation of the working concentration range in some cases.

Table 3: Validation of the multiplexed protocol.

Antigen	Accuracy			Precision
	$[Ag]_{added}$	$[Ag]_{calc}$	Recovery %	(±)
AFP	4.1	3.8	91	2.8
(ng/mL)	16.5	19.4	118	1.9
	49.4	45.9	93	5.9
hCG	3.3	2.5	77	0.2
(mUI/mL)	26.7	27.1	101	2.4
	100.0	102.8	103	14.1
CEA	1.6	1.8	110	0.4
(ng/mL)	16.3	16.5	101	1.5
	32.7	33.0	101	4.2
CA19-9	14.1	14.3	101	1.6
(UI/mL)	35.2	36.4	103	2.8
	70.5	71.5	101	8.4
CA125	11.7	12.1	103	2.7
(UI/mL)	39.0	36.4	93	2.4
	62.4	61.4	98	8.4

Application of the SEC-ICP-MS immunoassay to the discrimination between healthy and malignant tissues. The method developed was applied to the simultaneous quantitative determination of the five biomarkers in human uterine and ovarian tissue cytosols. Cytosol samples were diluted with human serum and as the biomarker concentrations were unknown different dilutions from 6 to 600 folds were tested in order to fall within the linearity range of the calibration graph. The dilution factors of 100-200 fold were eventually applied.

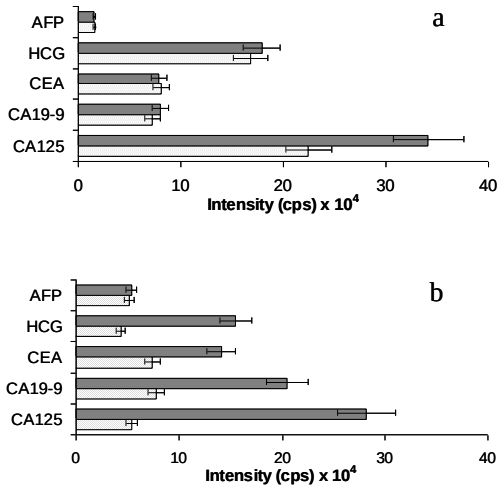

Fig. 7: a) biomarkers profile of uterus tissue lysate and b) ovary tissue lysate. White dotted bars: healthy tissue; shadowed bars: tumoral tissue.

Carcinoma and healthy tissues were analysed in parallel and the data are summarized in **Fig. 7.** In uterus adenocarcinoma samples (**Fig. 7a**) no difference between the healthy and malignant tissue cytosols was observed for AFP, CEA and CA19-9 as expected as since these proteins have not been brought in reference to this type of cancer. Despite the fact that hCG is elsewhere reported as potential biomarker in this kind of pathology, in this case similar high levels were found both in the healthy and malignant tissues.. A reliable discrimination was obtained by using CA125 concentration, this protein has been

recognised elsewhere as a useful biomarker of the uterus cancer (*38*).

Regarding the ovary carcinoma samples (**Fig. 7b)** the concentrations of all proteins except AFP are distinctly different in the healthy and malignant tissues. If hCG, CA19-9 and CA125 are well-recognised biomarkers (*38-39*), the utility of the CEA protein as been less discussed since its utility depends also from different factors which are not correlate with the tumor presence. As example, CEA is normally found at high levels in smokers (> 10 ng mL^{-1}), as well in a number of different chronic pathologies. For these reasons, its presence doesn't represent a sure discrimination between healthy/disease samples.

CONCLUSIONS

Size-exclusion chromatography – ICP-MS was proved very useful technique for the accurate and precise determination of the complexes formed by metal-labelled antibodies with target proteins. The chromatographic separation efficiency allows the analysis of a serum matrix directly upon incubation with the antibody with no additional sample preparation. The isotopic specificity of ICP-MS makes the multiplexing of the assay possible by using antibodies tagged with different isotopes for the different proteins and of general use for detection of water-soluble proteins. The sensitivity of the method developed for the simultaneous determination of five biomarker proteins characteristic of uterine and ovarian cancer is comparable with those attainable by ELISA or RIA assays with the advantage of multiplexed analysis capacity, virtually no sample preparation and sample amount consumption ca. 3-times lower than an ELISA test. Owing to the multielement detection capability of ICP-MS at the time-scale of the chromatographic peak obtained, the method developed can be extended to a larger number of proteins (at least 20 or so) to be measured at the same time provided that antibodies are available and tagged with the different isotopes.

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3.2. Determination of Labeling Stoichiometry for Elemental Tagged Monoclonal Antibodies by High Resolution- Indutively Coupled Plasma Mass Spectrometry

Abstract. The introduction of elemental labelled antibodies for multiplex determination of proteins in complex samples by ICP-MS detection, open the way for a massively multivariate analysis. Howhever, the core of immunoassay rely as first in the optimization of labeling procedure, since the succesful introduction of elements together with the retain of biological activity are the engine-drive on the global sensitivity of the method. Also if a number of procedures for antibody modification are well established, the stoichiometry of modification (i.e. the ratio metal/antibody) is not simply asses by the common spectrophotometric assays. Making full use of High Resolution-ICP-MS capability to measure sulphur overcoming the problem of polyatomic interferences, a method for the determination of labeling stoichiometry is presented. The natural sulphur content deriving from protein backbone (cysteines and methionines) is used as reference value to normalize the antibody concentration after elemental labeling and purification steps. By the simultaneous quantification of sulphur and lanthanides used for labeling purposes, the stoichiometry metal/antibody is extrapolated. The case of labeling by modification of disulphide bridges in antibody's hinge region is taken into account and the results of 3.1 ± 0.3 metal atoms per antibody, well correlate with the expected modification.

INTRODUCTION

Recently, elemental mass spectrometry, especially with inductively coupled plasma ions source (ICP-MS) has been introduced as analytical technique in the field of proteomic analysis. We developed a method for multiplexed determination of proteins in different samples (human serum and tissue lysates) performing a liquid phase immunoassay with detection of immunocomplexes by high resolution size exclusion chromatography (SEC) and ICP-MS by the use of metal labelled monoclonal antibodies. SEC was found able to discriminate between the boun/unbound forms of labelled antibodies, and also provides useful informations regarding homogeneity of labeling procedure and purity of antibody preparation. Indeed, after labeling and purification procedure the injection of antibodies onto the SEC-ICPMS system, results in a single symmetric peak at the expected corresponding to the expected antibody molecular weight and no other species containing the metal were observed. Howhever, the effect and homogeneity of labeling were checked indirectly performing an immunoassay and evaluating the effect of labeling extent on the gobal sensitivity of the method. Since labeling procedure represents the key step to retain the biological activity of antibody and therefore it determines the success of the immunoassay, the importance of sites and degree of modification is relevant. In these terms, the determination of labeling stoichiometry might provides additional information on the procedure adopted for a better rationalization of the chemistry for metal bioconjugation across DOTA ligands.

Normally, determination of sulphur free groups on biomolecule can be assess by Ellman's reagents if the concentration of protein is known (for example by common Bradford's assay in which lysine residues are involved) by a spectrophotometric tritation. In our case, the total amounts of antibody prepared after metal labeling make the application of these methods difficult. In addition both assay must be perform prior to metal loading, since complex of DOTA with lanthanides are well known to give absorbance and emission at different wavelenghts interfering with the analysis. The overlap effect with the wavelenghts use in Ellman and in Bradford assay prevent the simple determination of free sulphidryl and total protein concentration respectively, and finally the stoichiometry of labeling is not determinable.

Within this frame, we approach the stoichiometry determination by the use of High Resolution-ICPMS (HR-ICPMS) which permits the simultaneous determination of sulphur and lanthanides. The problem of sulphur determination with a common quadrupole analyzer lies in the problem of polyatomic interference due to the formation in high amounts of $^{+}O_2$ that completely cover the isotopes of sulphur, especially the most abundant ^{32}S. With the

instrumental resolution improved by the presence of a sector field, sulphur can be direct determines and therefore we decide to use the intrinisc content of sulphur from protein backbone as normalization factor to measure the protein content and stoichiometry of labelin. Since our method is based on the selective introduction of DOTA ligand after reduction of the disulphide bridges at the level of the hinge region, should be mentioned that their number is strictly dependent from the subclass of Gamma-Immunoglobulins. Indeed, IgGs are divided into four different subclasses, which exhibit a variable number of 2, 4, 11, 2 disulphide bonds into the hinge region for IgG1, IgG2, IgG3 and IgG4 respectively.

EXPERIMENTAL SECTION

Materials. *Chemicals, Salts and buffers*. A complete mixture of lanthanides elemental standard at 100 μg mL^{-1} in 4% HNO_3 and ammonium sulphate in water at a concentation 1000 μg mL^{-1} in sulphur was from Scharlau (Spain). Analytical grade HNO_3 (69-70%) was from J.T.Baker. Analytical grade ammonium acetate, sodium phosphate monobasic (NaH_2PO_4), tris-(hydroxymethyl) aminomethane hydrochloride, sodium chloride, ethylene-diaminotetraacetic acid disodium salt (EDTA) were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). Buffers used were: ammonium acetate buffer (100 mM, pH 6.8 as elution buffer and 20 mM, pH 6.0 for metal complexation), phosphate buffer (100 mM, pH 7.2, 2.5 mM EDTA), Tris buffer saline (20 mM Tris-HCl, 0,45% NaCl, pH 7.0), phosphate buffer saline (10 mM in phoshate, 150 mM in NaCl pH 7.4, 2.5 mM EDTA), deacetylation buffer (100 mM phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.4). Ultrapure water 18 MΩ cm from a Milli-Q system (Millipore, Bedford, MA) was used throughout the work.

Antibodies. Rabbit polyclonal anti-mouse (whole molecule) IgG Europium labelled (DELFIA) was from Perkin Elmer Life Science (Waltham, MA). Mouse IgG anti-human monoclonal antibodies (mAb) α-CA125 (clone X325), α-hCG (clone HCG1) and α-CEA (clone 1C11) were purchased from Abcam (Cambridge, UK). All mAbs were obtained in liquid form as IgG or protein A purified fractions. Upon reception, mAbs were divided into single working aliquots and stored at –20°C.

Labelling reagents. 1,4,7,10-tetraazacyclododecane-1,4,7-tris-aceticacid-10-maleimido-ethylacetamide (mDOTA) was purchased from Macrocyclics (Dallas, TX). Lanthanide chlorides ($HoCl_3$, $EuCl_3$, $GdCl_3$) with natural isotopic abundances were from Aldrich (Schelldorf, Germany). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was from Pierce (Rockford, IL).

Instrumentation. An Agilent model 1100 binary pump, equipped with a manual injection system with a loop of 50 μL was used as solvent delivery system an for injection of samples. The High-Resolution ICP mass spectrometer was a Thermo Scientific ELEMENT 2 XR (Thermo Fisher Scientific, Bremen, GmbH) fitted with a Meinhardt nebulizer. HR-ICPMS conditions were: plasma gas (Ar) 16 L min^{-1}, forward power 1300 W, carrier gas 1.17 L min^{-1}. The performance and calibration of the system was checked to respect the manufacturer indications daily. For size exclusion chromatography and flow injection experiments, nebulization chamber was maintained dry by set the peristaltic pump at 20 rpm (0.52 mL min^{-1}). Each isotope was acquired in chromatogram analysis mode with the instrument operating in medium resolution. Parameters set for isotopes monitoring were as follow: dwell time 20 ms for ^{151}Eu and ^{153}Eu , 10 ms for ^{157}Gd, ^{158}Gd and ^{160}Gd, 20 ms for ^{165}Ho, 50 ms for ^{32}S and 20 ms for ^{34}S, 20 ms for ^{205}Tl.

The chromatographic system was connected by means of PEEK tubing to the ICP-MS nebulizer. Nanosep centrifugation tubes with low protein membrane and a 30 kDa cut-off (Pall, USA) were purchased from VWR International (Milan, Italy). Centrifugation vials were used throughout the work for washing steps and buffers exchange during labelling procedure of antibodies.

Procedures. *Labelling of antibodies by the TCEP method*. Monoclonal antibodies anti-CA125, anti-CEA and anti-hCG were labelled with Ho^{3+}, Gd^{3+} and Eu^{3+}, respectively as described elsewhere. Briefly, an aliquot of antibody (40 μg, 266 pmol) equilibrated in phosphate buffer at 1 mg mL^{-1} (6.6 μM) was incubated with 6-fold molar excess (1.6 nmol, 40 μM) of TCEP at 37°C for 30 min to selectively reduce disulphide bridges in the hinge region. The antibody was quickly washed with Tris buffer saline to remove the TCEP in solution by centrifugation and resuspended in the same buffer at 1 mg mL^{-1}. A 20-fold molar excess of mDOTA previously dissolved in DMSO was added (12 nmol, final concentration of 320 μM) and the mixture was incubated at 37°C for 1 h. Then, the mixture was washed with Tris buffer saline to remove excess of unreacted DOTA, and resuspended in 20 mM ammonium acetate buffer (pH 6.0). An aliquot of $LnCl_3$ was added to reach 1 mM concentration and the mixture was incubated at 37°C for 30 min. The tagged antibody was extensively washed with Tris buffer saline to remove excess of free metal and was recovered at a nominal concentration of 0.5 mg mL^{-1}.

Size-exclusion chromatography HR-ICPMS. Labelling of proteins and purity (absence of free DOTA-Ln) were checked on a size exclusion column Superdex™ 200 HR 10/30 (10 mm x 300 mm x 13 μm beads size) with declared linear separation range 10-600 kDa for globular

proteins (GE Healthcare, Uppsala, Sweden). Elution was carried out isocratically at 0.5 mL min^{-1} using 100 mM ammonium acetate (pH 6.8) as mobile phase and column was connected by means of PEEK tubing to HR-ICPMS.

Flow-injection High Resultion-ICPMS. Standards mixture containing all the lanthanide ions and sulphur, as well the standard proteins and samples were prepared in HNO_3 7% containing ^{205}Tl at 10 ng mL^{-1} as internal standard. Samples were loaded in the injection valve and the 50 μL filled into the loop were transferred to the HR-ICPMS by a continuos flow of HNO_3 2% at 0.5 mL min^{-1}. Each sample was run in triplicate. External calibration curves were obtained by injecting standard solution in a range 0.5-1000 ng mL^{-1}. Antibody samples were diluted after labelling to a concentration range 10-100 mg mL-1 in antibody prior to analysis by FJ-HR-ICPMS

RESULTS AND DISCUSSION

Size Exclusion Cromatography-High Resolution ICPMS. The total content of sulphur into the antibody, represent an intrinsic property of the molecule since its value derives from the aminoacid backbone of the protein. The number of cysteines and methionine define the total content of sulphur into the investigate proteins. In respect to the common tritation methods by Ellman's reagent based on the quantification of only free sulphydryl (reduced cysteines), in this case the possibility to take into account also the fraction rising from methionine represent an automatic increase in terms of sensitivity. To perform an accurate quantification of sulphur present into the backbone of the protein the purity of the samples must be determined in terms of other sulphur free species. Indeed, presence of polluting sulphur-containing molecule will results in a misleading quantification of the true protein content.

Initially, a commercially available labeled antibody was used in order to test the feasibility of the method. A rabbit anti-mouse polyclonal IgG labelled with europium (IgG-N1) was obtained at a declared concentration of 391 μg mL^{-1} and it contain a declared average of 6-9 atoms of Eu per antibody molecule. The procedure used for the labeling of IgG-N1 reagents involves the use of an isothiocyanate-DOTA by-product, which reacts with lysine groups at basic pH. Then the quantity of sulphur into antibody is in this case increased by the presence of DOTA ligands. Furthermore, eventual residue of unreacted labelling reagent might be present in the solutions. Then, purity of IgG-N1 was checked by directly coupling size exclusion chromatography to HR-ICPMS. The baseline for ^{32}S and ^{34}S isotopes was previously checked and found stable (baseline variation < 1%) at 6000 cps for ^{32}S, when a flow of 100 mM of ammonium acetate at 0.5 mL min^{-1} was used as elution buffer. Baseline

value for ^{153}Eu and ^{151}Eu isotopes was found negligible (40 cps) since this element is not affected by interference species. A chromatogram for an injection of 100 μL of antibody at 10 μg mL^{-1} onto SEC-HR-ICPMS is shown in Fig.1.

Two peaks were observed. In Fig.1A, peak 1 at 16 min correspond to the elution of IgG-N1 antibody, with a molecular mass calculated of 150 kDa from column calibration curve. Peak 2 eluting at 28 min was previously identified as unbound Eu-DOTA complex. In our previous work the column recovery for the total Eu was calculate to exceed the 90%. Integrated signals for the two peaks were found similar, which means 50% of the elements into the solutions is given from labeled antibody, and the other 50% derives from the free DOTA-Eu complex. Moreover, after a dialysis by centrifugation on a 30 kDa filter the free DOTA-Eu complex was removed and a single peak at 16 min (Fig.1B) appears in the chromatogram from SEC-HR-ICPMS. As opposite, when mouse monoclonal antibodies where prepared by the TCEP method the extensive washing as final steps in the procedure provides a good purity of the samples, since no traces of free DOTA-Ln complexes were observed.

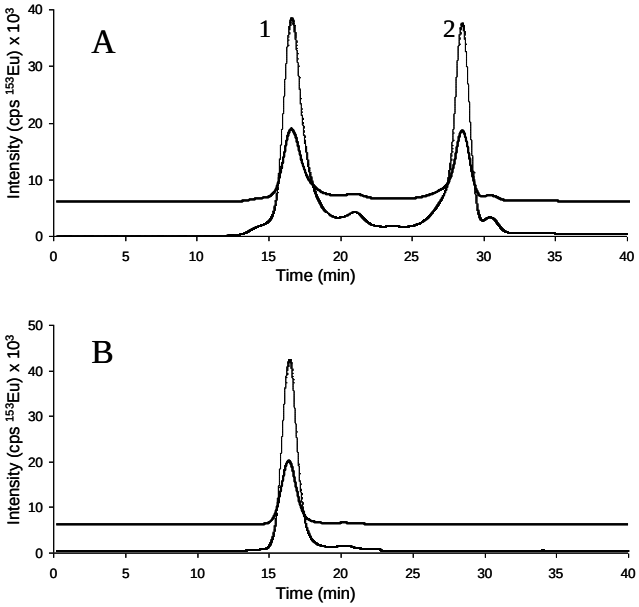

Fig.1. Chromatograms of IgG-N1 from SEC-HR-ICPMS for ^{32}S (bold line) and ^{153}Eu (thin line): A) injection of IgG-N1 as received from manufacturer and B) injection after purification by ultracentrifugation on 30 kDa cut-off filter.

Optimization of Flow injection High-Resolution ICPMS (FJ-HR-ICPMS) Samples were prepared in HNO_3 at 7% containing 10 ng mL^{-1} of Tallium (^{205}Tl) as internal standard for normalization of the signals. As first, a blank sample of HNO_3 at 7% was injected to check the purity of this matrix, with a special regards to sulphur, and variation of baseline for each isotope of interest was found absent. Then, the presence of carry-over effects in the injection valve and system of PEEK tubes connecting the solvent delivery pump to HR-ICPMS was tested by injection of a multi-analyte standard solution (containing all the lanthanides, internal standard and sulphur) at 1000 ng mL^{-1}, which represents the highest concentration used in this work for calibration purpose. Signals eluted in a time window of 20 seconds, and after additional 40 seconds the baseline value was found at the corresponding intensity measured

before the injection. An injection of 50 μL of blank HNO_3 at 7% after the sample shown the presence of residual analytes in the injection system, which disappeared completely after three washing cycles. Then, an acquisition time window for each sample was set at 60 seconds and 5 washing cycles with HNO_3 at 7% between each sample injection were done. In each case no memory effect in the nebulization chamber of the ICPMS torch was observed, since baseline came back to the initial intensity after all the injections. With the parameters set as described above, calibration with the multi-analyte standard solution in a range 0.5-1000 ng mL^{-1} was performed by injecting each levels in triplicate and the linear relationships were established.

Determination of sulphur content and stoichiometry of labelling by FJ-HR-ICPMS. Initially, the method was applied on the IgG-N1. Antibody was diluted at 2.5-5-10-20-40-80 μg mL^{-1} and samples were analyzed by FJ-HR-ICPMS. Integrated signals were transformed in molar units across the external calibration and concentration of IgG was plotted versus the calculated concentration of the single elements. The linear relationship *y=120.5x + 26913 (r^2 0,966)* obtained is reported in Fig.2. The slope of linear regression represents the ratio sulphur/IgG, which was found 120.5 for the total content of sulphur into the sample. Regarding the determination of Europium, in the same way a value of 12.3 was calculated for the ratio Eu/IgG. Taking into account the composition of the analyzed sample by SEC-HR-ICPMS, in which a 50 % of sulphur and europium derives from the free DOTA-Eu complex, both values must be reduce by an half, resulting in a total content of about 60 atoms of sulphur and 6.5 atoms of Europium for each IgG-N1 molecule. The obtained results from Europium are in good agreement with the declared average of 6-9 atoms/IgG. Regarding the content of sulpur, from an IgG aminoacid sequence from Protein Data Bank (IgG from Mus Musculus sequence number Q9D8L4 for constant region and Q5F2I8-Q5F2I1 for variable regions www.expasy.ch) a content of 51 atoms of sulphur into antibody is expected from the presence of 13 methionines and 38 cysteines. Since each incorporated DOTA, carry on an atom of sulphur, the real ratio sulphur/IgG can be estimate around 57, which fit reasonably with the 60 measured. Then, we proceeded with the determination of sulphur into the three monoclonal

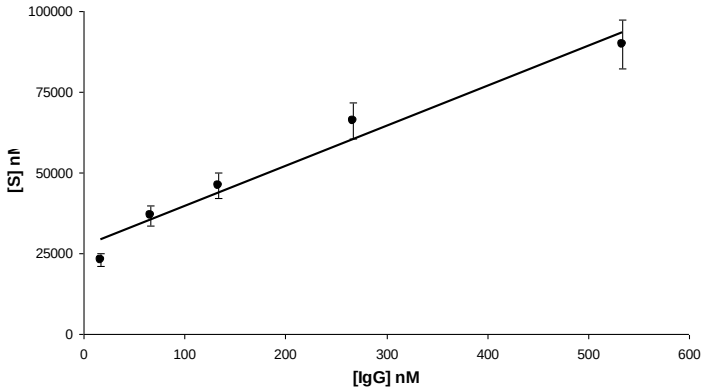

Fig.2. Linear relation for sulphur content into antibody as determined from FJ-HR-ICPMS.

unlabelled antibodies α-CA125, α-CEA and α-hCG which results in the range 57 ± 5. This latter value was used to normalize the antibody concentration in the elemental tagged antibodies injected after the entire procedure of labeling. For the antibodies tagged with Ho, Gd and Eu respectively, a stoichiometry of labeling of 3.1 ± 0.3 was calculated. Since all the antibodies analyzed were of subclass 1, two disulphide bonds are present in their hinge region which originate four free sulphidryl groups sensisble to the DOTA attachment for a maximum labeling with four lanthanide ions.

CONCLUSIONS

As first approach, can be concluded from this former study that the selective reduction of disulphide bridges in the hinge region was accomplished, since labeling stoichiometry never exceed the maximum number of free sulphidryl sites and aspecific modification of other cysteine into the biomolecule was avoided. For the first time, HR-ICPMS was demonstrate to be able to perform analysis of sulphur content into antibodies, which can in turn be use for the normalization of biomolecule concentration in unknown samples like the labelled preparation. The quantities of sample required for the analysis are lower (as total 5 μL of antibody at a declared concentration 0.5 mg mL^{-1} were used) than required for classical UV/VIS tritation methods like Bradford or Ellman assay and therefore the method developed results very useful when aliquotes of 20 μL of labelled antibody were prepared. Howhever, to check the validity of the method developed, a comparison with data obtains from classical UV/VIS tritation methods and the identification of modification sites by molecular MS are necessar. These aspects are under investigations.

3.3. Enhancement of Sensitivity in Immunoassay with Antibody-Dendrimers Conjugate and Inductively Coupled Plasma Mass Spectrometry.

Abstract. A procedure for labeling of antibodies with metals for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) based immunoassay using DOTA modified dendrimers is presented. The principal advantage given by the labeling wioth dendritic polymers rely in the possibility to insert an increased number of metal atoms for each site of modification onto antibody molecule. The derivatization of commercially polyamidoamine (PAMAM) dendrimers on the functional amine terminal groups with a DOTA moiety is investigate by the use of size exclusion chromatography and MALDI-MS. Finally, a comparison of sensitivities by liquid-phase immunoassay between the dendrimer modified antibody and a classical single DOTA modification is carried out for the determination of human chorionic gonadotropin in human serum as model system.

INTRODUCTION

The introduction of ICP-MS as analytical technique in proteomic analysis, provides several valuable features. The wide linear dynamic range, excellent sensitivity, the virtual indipendence of the signal (isotopic) from sample matrix and especially its multi-analyte capability are key characteristic to deal with quantification of proteins. Indeed, when analysis of a proteome is taken into account, the principal problem to cope with is the spread of concentrations of the analytes in a range of several orders of magnitude. Recently, we introduced a method for multiplex proteins quantification by an integrated approach of liquid-phase immunoassay and ICP-MS. The key note of this strategy lies in the versatility of introduction of *"elemental tag"* by labeling with a common DOTA ligand and further the complexation of lanthanide ions. The species-specific determination of each protein (antigen) is then assured by the insertion of an unique tag for each antibody. The use of size exclusion chromatography (SEC) allowed the chromatographic separation between immunocomplex and free form of antibody and quantification was performed by on-line coupling to ICP-MS by measuring the immunocomplex *(1)*.

Principally, the global power of ICP-MS for the quantification of antigens present into the sample is driven by two well distinct characteristic of the analytical system developed: i) the intrinsic affinity of antibody toward its antigen and ii) the sensitivity directly related to the quantity of inserted elemental tag into antibody. Regarding the former point, optimization of labeling strategy is necessary in order to limit the loss of biological activity (affinity), which increase with the grow of DOTA incorporation. On the other hand, for the latter an increase of DOTA and then final element content, give back a more sensible detection from the instrumental point of view. Across our strategy of labeling, the highest number of DOTA molecules rely from the number of disulphide bridges present into the hinge region of the antibody, ranging from 2 to 12 depending by the IgG subclass. Howhever, for higher labeling yields a suppression effect of biological activity, which results in worst global sensitivity of analysis was observed.

To overcome these contrary effects, Tanner and co-workers approach the problem disfruting the MaxPar™ linear polymers bringing multiple copies of DOTA ligands synthesized by Lou et al. *(2)*. In opposite to linear polymers, dendrimers are well-defined, spherical and low polydisperse polymeric structures which make them attractive for many novel applications including as a catalyst, as a host molecule, in analytical chemistry, in gene and drug delivery, and as a diagonistic reagent *(3-5)*. Dendrimer size and related number of surface groups available for modification depend by the generation, obtained as results of

cycles of amplification in branch. Especially poly-amido-amine (PAMAM) dendrimers were the most used since their amine terminal groups offers a good versatility for different modifications (Fig.1).

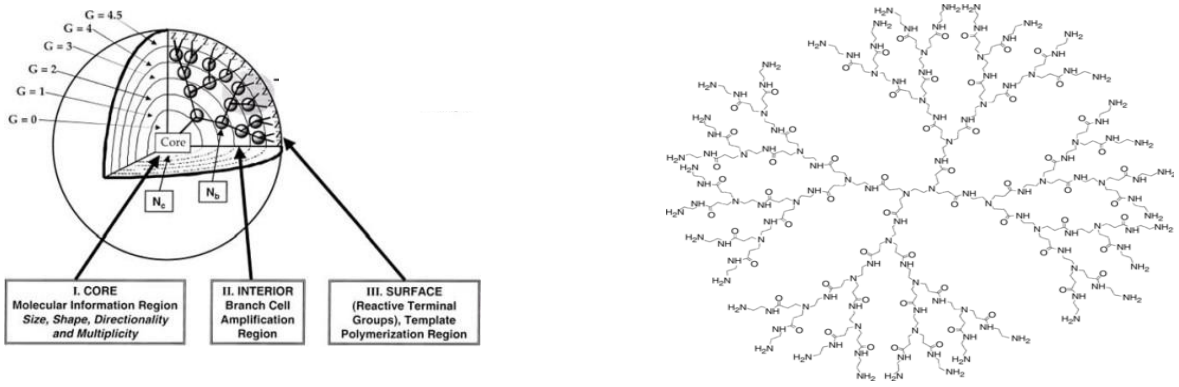

Fig.1. Left: schematic presentation of dendrimer structure with information regarding core (EDA), amplification region for generation growth and surface with terminal groups (amino). Right: molecular structure of PAMAM generation 3 with 32 amino surface groups ready for modification.

A wide variety of analytical techniques have been considered to characterize dendrimers during the last years: low angle laser light scattering, infrared (IR) spectroscopy *(6)*, capillary electrophoresis (CE) *(7,8)*, mass spectrometry (MS) *(9-11)* and high-performance liquid chromatography (HPLC) *(12)*, low angle laser light scattering (13) and ^{1}H, ^{2}H, ^{13}C nuclear magnetic resonance (NMR) *(6,9,14,15)* were used for this purpose. Across these techniques, informations regarding molecular mass, presence of synthetic defect or impurities as well structural properties can be achieved. Unfortunately, the performance of all these techniques declines with increasing size of dendrimers. In their recent works, Maiti et al. used fully atomistic simulation to a deeper understanding of denrimers characteristic like size (radius of gyration), shape tensor, asphericity, molecular surface area, spatial arrangement of branch points, terminal groups and monomer density distribution, solvent accessible surface area and molecular volumes as a function of generation *(16-18)*. Results from these simulations are in good agreement with the experimental data available in literature.

By far, size exclusion chromatography (SEC) is the most preferred method for determining the absolute molar masses and molar mass distribution of polymers. Since separation in SEC is based on the sizes of molecules, this is ideal for the characterization of different generations of dendrimers and the first precise experimental data about the rough size of dendrimers came from size exclusion chromatography. Baker and co-workers used SEC coupled with multiangle laser light scattering (MALLS), differential reflactive index and UV-visible to determine average molar mass and polydispersity, especially size-dependent dispersity of

different generation of PAMAM dendrimers *(12)*. Different stationary phase and column format for SEC, including Superdex 75 column, were explored for separation of polypropylenamine (POPAM) dendrimers by Mengerink et al. the authors observed only good separation within generations at acid condition (pH 2.5) but an uncomfortable effect of oligomers formation within dendrimers of same generation was observed in these conditions. Formation of supramolecular species was avoided only by adding organic solvent, but Superdex gel media do not support more then 30 % of organic component *(19)*.

In this context, we make full use of PAMAM dendritic polymers to increase the number of elemetal tags bring by the antibody. To the best of our knowledge is the first time that dendrimer are used for carry out an immunoassay coupled with detection by ICP-MS. The scope of this work is to check the feasibility to perform liquid phase immunoassay followed by SEC-ICPMS with denrimers-antibody conjugates aims to enhance the global sensitivity of the method. Then, basic points are explored here: i) the behaviour of different generations of PAMAM dendrimers for SEC separation; ii) the development of an opportune strategy for dendrimer modification, characterization of the products and conjugation with the antibody; iii) evaluation of the antibody-dendrimer conjugates performances in a liquid phase immunoassay based on SEC-ICPMS detection.

EXPERIMENTAL SECTION

Materials.

Chemicals. Polyamidoamine (PAMAM) dendrimers of generations 0-7 (G0-G7) were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France) in methanol solution. 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidoethylacetamide (mDOTA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra-acetic acid mono (N-hydroxysuccinimide ester) (NHS-DOTA) were from Macrocyclics (Dallas, TX). Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), 1,11-bis(maleimido) triethylene glycol ($BM(PEG)_3$) and *N*-succinimidyl-*S*-acetylthioacetate (SATA) were from Pierce (Rockford, IL). Water-dried dimethyl sulfoxide, triethylamine, 2,5-dihydroxybenzoic acid and hydroxylamine chloride (>99%) were from Fluka. Holmium chloride was from Aldrich (Schelldorf, Germany).

Antibody and proteins. Mouse IgG anti-human monoclonal antibodies (mAb) α-hCG (clone HCG1) was purchased from Abcam (Cambridge, UK). Human chorionic gonadotropin (hCG) and human serum were from Sigma-Aldrich (St. Quentin-Fallavier, France).

Salts and buffers. Analytical grade ammonium acetate, citric acid, sodium phosphate monobasic (NaH_2PO_4), tris-(hydroxymethyl) aminomethane hydrochloride, sodium chloride,

ethylene-diaminotetraacetic acid disodium salt (EDTA) were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). Buffers used were: ammonium acetate buffer (100 mM, pH 6.8 as elution buffer and 20 mM, pH 6.0 for metal complexation), citric acid buffer (100 mM, pH 2.74), phosphate buffer (100 mM, pH 7.2, 2.5 mM EDTA), Tris buffer saline (20 mM Tris-HCl, 0,45% NaCl, pH 7.0), phosphate buffer saline (10 mM in phoshate, 150 mM in NaCl pH 7.4, 2.5 mM EDTA), deacetylation solution (100 mM phosphate, 10 mM EDTA, pH 7.4). Nanosep centrifugation tubes with low protein membrane and a 30 kDa cut-off (Pall, USA) were purchased from VWR International (France). Centrifugation vials were used throughout the work for washing steps and buffers exchange during labelling procedure of antibodies. Ultrapure water 18 MΩ cm from a Milli-Q system (Millipore, Bedford, MA) was used throughout the work.

Methods.

Size Exclusion Chromatography UV-VIS. SEC experiments were performed using a Superdex™ 200 HR 10/30 and Superdex™ 75 HR 10/30 glass columns (10 mm x 300 mm x 13 μm beads size, GE Healthcare, Uppsala, Sweden), both with the approximate bed volume of 24 ml and a declared linear separation range 10-600 kDa (1000 kDa exclusion limit) and 3-70 kDa (100 kDa exclusion limit) respectively for globular proteins. An Agilent model 1200 binary pump, equipped with an autosampler, automatic injection system with a loop of 900 μL and a UV-VIS multiwave detector was used as solvent delivery system and for on-line UV-VIS analysis. Column were calibrated with an appropriate mixture of proteins and peptides as indicated by manufacturer using UV/VIS detection at 280 nm with baseline evaluation at 800 nm. Retention times (in min) plotted versus the logarithm of molecular mass (in kDa) gave a straight lines $y = -0.134\ x + 4.385$ and $y = -0.18\ x + 4.38$ (r^2=0.992) respectively for Superdex™ 200 and Superdex™ 75. 100 μL of dendrimer solution at 1 mg mL^{-1} were injected and the elution was carried out isocratically with a flow rate of 0.7 mL min^{-1}. For dendrimers characterization purposes both ammonium acetate buffer 100 mM (pH 6.8) and citric buffer 100 mM (pH 2.74) were evaluated in terms of effectiveness to reduce aspecific interaction with stationary phase and optimal resolution. In both cases dendrimers elution was monitored by absorbance at 210, 214, 254 and 280 nm with a reference baseline set at 800 nm.

Size Exclusion Chromatography ICP MS. Antibody-dendrimer conjugates ^{165}Ho labeled, were characterized by SEC-ICP MS analysis using ammonium acetate 100 mM (pH 6.8) as elution buffer at a flow rate of 0.7 mL min^{-1}. The Agilent model 1200 binary pump was used for solvent delivery purposes and automatic injection of samples. The ICP mass spectrometer

was an Agilent 7500 CE fitted with a Meinhardt nebulizer. The exit of the column was connected by means of PEEK tubing to the ICP MS nebulizer. ICP MS conditions were: plasma gas (Ar) 15 L min^{-1}, forward power 1500 W, carrier gas 1.05 L min^{-1}. The performance of the system was checked to respect the manufacturer indications for 1 μg L^{-1} $^{7}Li^{+}$, $^{89}Y^{+}$ and $^{205}Tl^{+}$ in 2% HNO_3, with a dwell time of 100 ms for each isotope. Double charge ion and oxide levels were opitimized on $^{140}Ce^{+}$ and were classically <2 %. The isotopes were monitored in time resolved analysis mode with a dwell time of 100 ms each.

MALDI-TOF of dendrimers and their DOTA derivatives. The positive-ion MALDI-TOF mass spectra were recorded on a Voyager DE mass spectrometer (Applied Biosystem) operating in reflector or linear mode with delayed extraction. Instrument calibration was performed with a mixture of known peptides for reflector mode or with a mixture of myoglobin, bovine serum albumin and gamma-immunoglobulin for the linear mode. Laser intensity was adjusted for each dendrimer generation to maximize signal to noise ratio and reduce dendrimer's fragmentation. In reflector mode, classical operating parameter were: applied voltage 20 kV, grid voltage % 85, delay time 100 ns, 100 laser shot per spectra were acquired. 2,5-dihydroxybenzoic acid (DHB) was used at 10 mg mL^{-1} in pure methanol as matrix. Dendrimer samples were diluted as necessary in pure methanol and finally mixed 50/50 (% v/v) with the matrix solution. Then 1 μL of sample was spotted onto plate by the dry-droplet methods. Classically 50-100 ng of dendrimer were deposed and the solvent were permitted to dry by air evaporation prior to analysis.

Procedures.

Derivatization of PAMAM Dendrimers (G_y). To a 50 μL of PAMAM G_y (y = 0-7) solution in dimethyl sulfoxide at a concentration 10 mM in amino terminal groups, 9 μL of trietylamine (final concentration 1 M) are added and the solution is mixed for 2 min. Then, from freshly prepared solutions in dimethyl sulfoxide of NHS-DOTA (100 mM) and SATA (43 mM), a 2-fold molar excess on PAMAM G_y amino terminal groups of NHS-DOTA (10 μL, 1 μmol) and a 0.75 molar fold respect to PAMAM G_y of SATA (i.e. for G0: 2.9 μL, 125 nmol) are added. The solution is briefly mixed and incubated at 50 °C for 15 min. The reaction is subsequently quenched by adding 10 μL of NaOH 1 M, further neutralized with 10 μL of HCl 1 M and the solvent are evaporated under vacuum to give the desired product SATA-G_y- DOTA.

Activation of sulphydryl moiety. SATA-G_y-DOTA is resuspended in 50 μL of phosphate buffer and 5 μL of deacetylation solution are added and the mixture is incubated for 2 h at room temperature to achieve the deprotection of acetylated sulphydryl. The mixture obtained containing the active PAMAM G_y DOTA derivative (SH-G_y-DOTA) for a theoretical

maximal concentration of active sulphydryl of 0.75 fold respect to initial concentration of PAMAM G_x dendrimer. The prepared solution was promptly used without further purification for conjugation with antibody and keep frozen at –20 °C for further preparations.

Conjugation of antibody α-hCG (IgG) and DOTA-G_y-SH. To 10 μL of mouse monoclonal antibody at 1 mg mL^{-1} (66.6 pmol) in phoshate buffer, a 20-fold molar excess of TCEP (1.33 nmol, 133 μM) and a 100-fold molar excess (6.6 nmol, 600 μM) of $BM(PEG)_3$ are added and the mixture is incubated for 1 h at 37 °C. Excess of $BM(PEG)_3$ and TCEP byproducts are washed away by centrifugation on 30 kDa molecular filter with 3 x 500 μL of phosphate buffer and then a 20-fold molar excess of SH-G_y-DOTA (1.3 nmol) dendrimer is added to the recovered antibody solution from the freshly prepared solution and the mixture incubated at 37 °C for 2 h. Antibody-dendrimer conjugate (IgG-G_y-DOTA) is washed by centrifugation against 5 x 500 μL of phosphate buffer on Nanosep 30 kDa molecular filter and the buffer is changed by 2 x 500 μL additional washing step with ammonium acetate 20 mM pH 6.0. $HoCl_3$ is added to a final concentration 5 mM and the sample is incubated for 30 min at 37°C. Finally, IgG-DOTA-G_y-(Ho) holmium labeled is extensively washed against 3 x 500 μL of ammonium acetate and then with 3 x 500 μL of tris buffer saline. Antibody is recovered at 0.5 mg mL^{-1} and stored at 4°C until use.

Labeling of antibody α-hCG with maleimido-DOTA (mDOTA). Four aliquots of 10 μL of antibody at 1 mg mL^{-1} in phosphate buffer are incubated with 1, 2, 6, 20-fold molar excess of TCEP in presence of a 50-fold molar excess of mDOTA for 1 h at 37 °C. Then mDOTA and TCEP byproducts are washed away by centrifugation on 30 kDa molecular filter (3 x 500 μL). The buffer is changed with ammonium acetate 20 mM pH 6.0, $HoCl_3$ is added to a final concentration 5 mM and the samples are incubated for 30 min at 37 °C. Lanthanide excess is washed by centrifugation against 3 x 500 μL of ammonium acetate 20 mM pH 6.0 and the with 3 x 500 μL of tris buffer saline. IgG-mDOTA(Ho) labeled antibodies are stored at 4°C until use at a concentration 0.5 mg mL^{-1}.

Liquid phase immunoassay and SEC-ICP MS analysis. Human chorionic gonadotropin antigen (hCG) was prepared in human serum sample of 100 μL at different concentration levels: 0-5-10-50-75-100 mUI mL^{-1}. One set of hCG samples was prepared for each preparation of mAb-mDOTA and mAb-G_y-DOTA conjugates holmium labeled. Antibody are added to a final concentration of 5 μg mL^{-1} and the samples are incubated for 1 h at 37°C. Then 50 μL of each samples are injected onto SEC and analyzed by ICP MS.

RESULTS AND DISCUSSION

Size exclusion chromatography of PAMAM dendrimers. In order to perform a liquid phase immunoassay, based on the use of antibody-dendrimers conjugates, followed by a size-exclusion separation between immunocomplex and free antibody, the behaviour of dendritic polymers in the chromatographyc system became imperative. Infact, by parity of dendrimer conjugates for antibody molecule, the content of elemental tag grows up with the increasing generation (G_x) of dendrimer. The presence of higher dendrimer G_x in the composition of the bioconjugate might affect the separation, since side-effects of aspecific interactions driven by the dendrimer part, and the final shape of the bioconjugate are both involved when a SEC is performed.

Pure amine-terminated PAMAM dendrimers were analyzed by Superdex™ 75, which exhibit a linear separation range appropriate for the pure dendrimer sizes, and by Superdex™ 200 which represent the elected column for the separation of high molecular weights molecules like immunocomplex and antibody-dendrimer conjugates. Dendrimer's elution was evaluated with two elution buffers, ammonium acetate buffer 0.1 M (pH 6.8 and pH 4.75) and citric acid buffer 0.1 M (pH 2.74), since the presence of different counter-ions and degree of amines protonation are known to influence chemical-physical characteristic of dendrimers and therefore the separation performances in SEC. When ammonium acetate buffer was used at both pH values, all dendrimer generations were eluted in a range of 20-25 min by both columns, corresponding to a mass 6-0.75 kDa and 50-10 kDa for Superdex 75 and 200, respectively. At the increase in dendrimer generation, broader and tailed peaks were registered, which indicates an effect of absorption on the stationary phase. The cause for this interaction with the stationary phase lie in the effect of pH, since at this values (pH 6.8 and 4.75) not all the amine groups are protonated. In fact, only the terminal primary amine groups are protonated (pKa ≈10), while the secondary amino groups (pKa ≈ 7) presents at each branch point of the dendrimers and the tertiary amine groups (pKa ≈ 4) at the etylenediamine (EDA) core are not. In their recent works Maiti et al. shown that denrimers branches exhibits an high molecular dynamic and a significant amount of terminal groups are back-folded in the interior of the molecule at each level of pH, in opposition of what is expected from the classical misleading two-dimensional representation of dendrimers *(17).* Then if not completely protonated, amino groups tend to interact aspecifically with the agarose based gel media of the stationary phase. At pH 2.74 of citrate buffer, all amine groups are protonated and consequently dendrimers absorption on stationary phase was completely avoided.

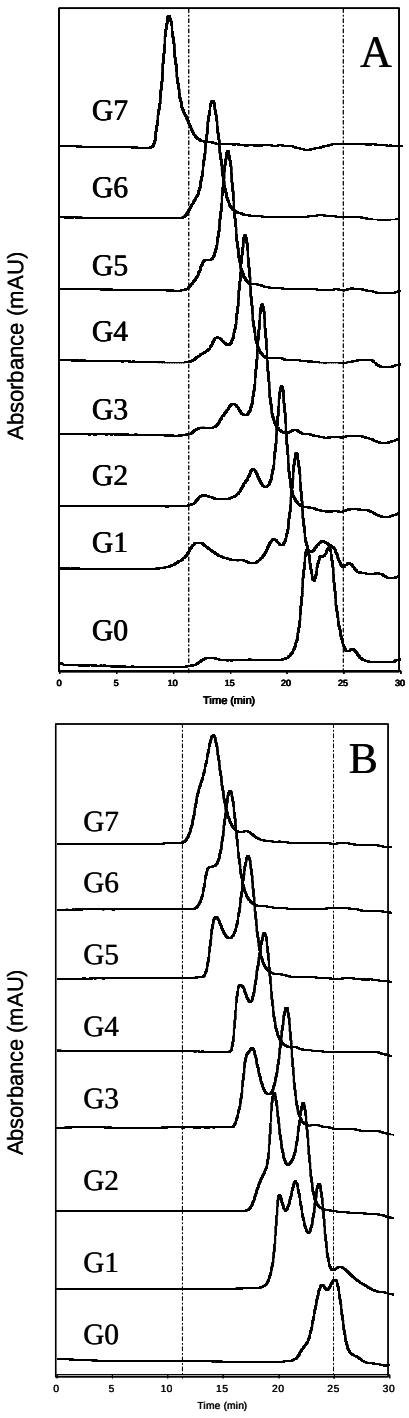

Fig.1. SEC of generation 0-7 PAMAM dendrimers eluted with citric acid buffer at pH 2.74 by Superdex 75™ (A) and Superdex 200™ (B). Detection was performed by UV/VIS monitoring the absorbance at 254 nm.

Chromatogram profiles from Superdex™ 200 and Superdex™ 75 column for each G_x of dendrimer eluted in citric acid buffer are shown in Figure1.

As appears, both columns were able to to discriminate between each dendrimer generation. Surprisingly, also dendrimer of lower generation (G0-G2) which molecular masses lie down of the lowest limit of column separation range, where disciminate within them and from the highest generation (G3-G7). Each generation was eluted with a profile of at least two peaks, indicating the formation of oligomers in this buffer conditions. Since no reference materials exhist to perform a calibration curve for dendrimers, a calibration obtained across a mixture of standard proteins was applied, and the molecular mass values calculated are reported in Table 1.

In general, from both columns the masses calculated, exceed the theoreticals for each generation of dendrimer. Furthermore, the molecular weights calculated by the two columns are not in agreement, showing a substantial mass increment for each generation when dendrimers are eluted from Superdex™ 200 respect to Superdex™ 75 column. From the latter, calculated values fit with dimer species for G1-G6, except for G7 dendrimer, which molecular mass (116 kDa) fall up to the exclusion limit (100 kDa) of the column, and therefore dimer formation could not be checked. The molecular masses calculated for the same generations when eluted from Superdex™ 200 represents the formation of extense agglomerates (tri- or tetra- forms) of single denritic polymer. These data are not in agreement with other results in literature, where dendrimers molecular weights were determined by SEC *(12)*. In this case dendrimer's sizes were calculated without apply an external calibration curve, but the absolute molecular weight was determined by multi angle

laser light scattering (MALLS), suggesting that deviation in dendrimer size in our experiments might be due partially to the calibration curve applied.

Table 1: dendrimer characteristic (surface amine terminal groups and molecular weights) and the corresponding results obtained from SEC-UV/VIS analysis after injection of 100 μL at 2 mg mL^{-1} for each generation onto Superdex™ 75 and Superdex™ 200.

Dendrimer			**Superdex 75**		**Superdex 200**	
G_x	NH_2	M_w (g/mol)	Time (min)	M_n (g/mol)	Time (min)	M_n (g/mol)
G0	4	517	23.9	1197	25.4	9581
G1	8	1430	21.7	2979	24.1	14309
G2	16	3256	19.7	6823	22.4	24177
G3	32	6909	17.9	14388	20.9	38406
G4	64	14215	16.2	29107	19.3	62922
G5	128	28826	14.7	54200	17.6	106316
G6	256	58048	13.5	89125	16.2	163757
G7	256	116493	12.6	129420	14.9	244568

In addition, the mismatch within calculated molecular weights by the two colums suggest additional features which might interfer with SEC separation of dendrimers. The recorded mass increment is not linear respect to dendrimer generations, but tends to decrease for higher generation as shown in Figure 2.

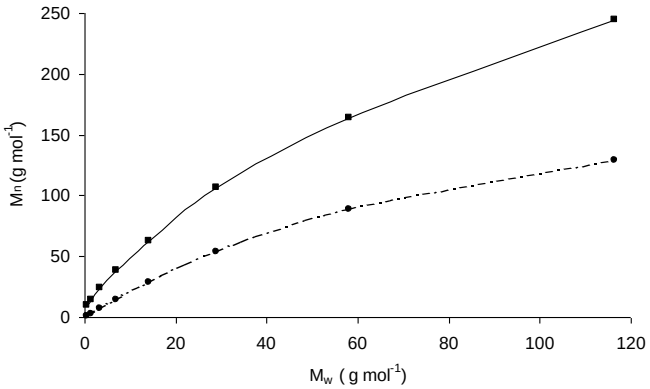

Fig.2. expected molecular wheights (Mw) for dendrimer generation are reported versus the calculated values from SEC (Mn) by Superdex 75 (circle) and Superdex 200 (square).

This observation might find partial confirmation in a recent fully atomistic study of dendrimers in water solvent *(17)*. The authors highlighted an effect of swelling due to the solvent and the counter-ion ability to penetrate into dendrimer structure, and therefore increment their size. In the same work was also pointed out the aspherical character of denrimers shape, which tends to be greater for the lower generations. As consequence, the increased sizes of dendrimers as observed in our SEC experiments, might be the reflex of swelling and aspherical shapes of polymers, since this separation technique is sensible not only to the size but also to the shape of eluted molecules. Then

dendrimers of low generation, characterized by a less spherical shape, tends to escape a correct interaction with stationary phase along the column, by a minor capacity to penetrate its pores and finally results in a faster elution. Howhever, a MALDI-TOF analysis of each peak eluted revealed only the presence of dendrimer monomers, suggesting that oligomerization depends from the solution conditions by supramolecular interactions of dendrimer molecules.

Derivatization of PAMAM dendrimers and MALDI-TOF characterization. Since sensitivity of an ICP MS based immunoassay is direct related, at least from the instrumental point of view, with the number of element bound to the antibody, dendrimers were modified to give a bioconjugate with the purpose of maximize the lanthanide atoms carried by each antibody molecule. A synthetic strategy based on the modification of terminal amine groups was optimized to obtain a bifunctional dendrimer, which brings on one hand the highest number of DOTA moieties as chelators for lanthanide ions, and on the other hand a free sulphydryl group useful for conjugation with antibody. Derivatization steps are schematically presented in Figure 3.

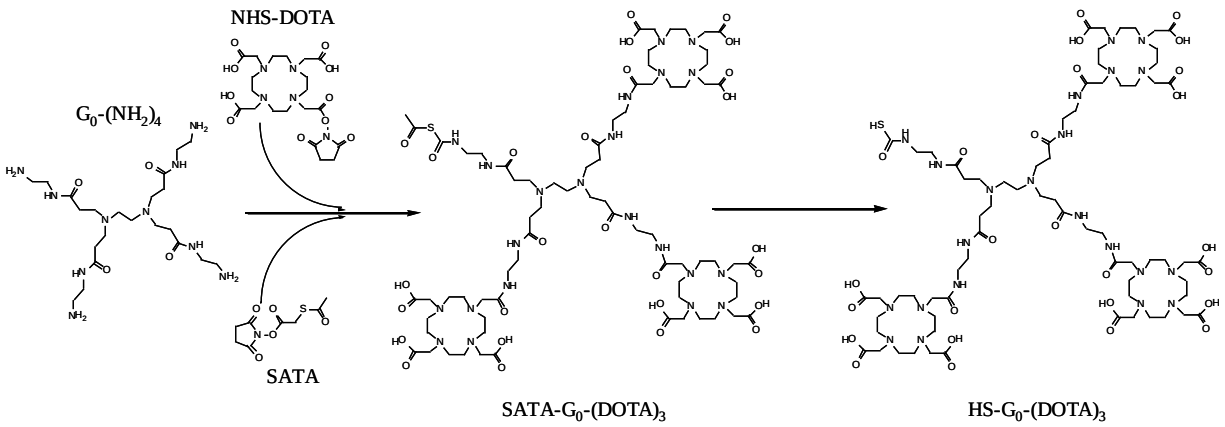

Fig. 3. synthetic strategy for preparation of G0 dendrimer active thiol free DOTA derivatives SH-G0-$DOTA_3$. The procedure was applied to each generation G_y-$(NH_2)_n$, where *n* represents the number of surface amine groups to give the corresponding SH-G_y-$DOTA_{n-1}$ products.

Both reagents used for amine terminal modification of dendrimer (NHS-DOTA and SATA), shown the same active ester group, allowing their simultaneous use in the reaction mixture. Molar ratios of both esters were optimized to reach the maximal derivatization with DOTA groups, and at the same time limit the insertion of one SATA group. The importance of avoiding insertion of more than one SATA group rise in the danger of turn the dendrimer molecule into a reactive cross-linker, which might act both between antibody molecules or dendrimers with a consequent formation of undesired agglomerates. Optimization of reaction steps was carried out on dendrimer G0, since it is an ideal model for MALDI-TOF

characterization of each component into the reaction mixture and the final products. Infact, MALDI-TOF mass spectrometry was succesfully applied for PAMAM dendrimers characterization, but resolution is lost starting from G2 PAMAM, when unresolved background signals emerge *(20,21)*. Still, for each generation of pure PAMAM dendrimer a general molecular weight distribution is well observed. Results for the optimization are reported in Figure 4 (A, B and C) by the MALDI-TOF spectra acquired after the 20 min of reaction at 50°. In each case the signal of original PAMAM G0 at m/z 517.5 was never detected.

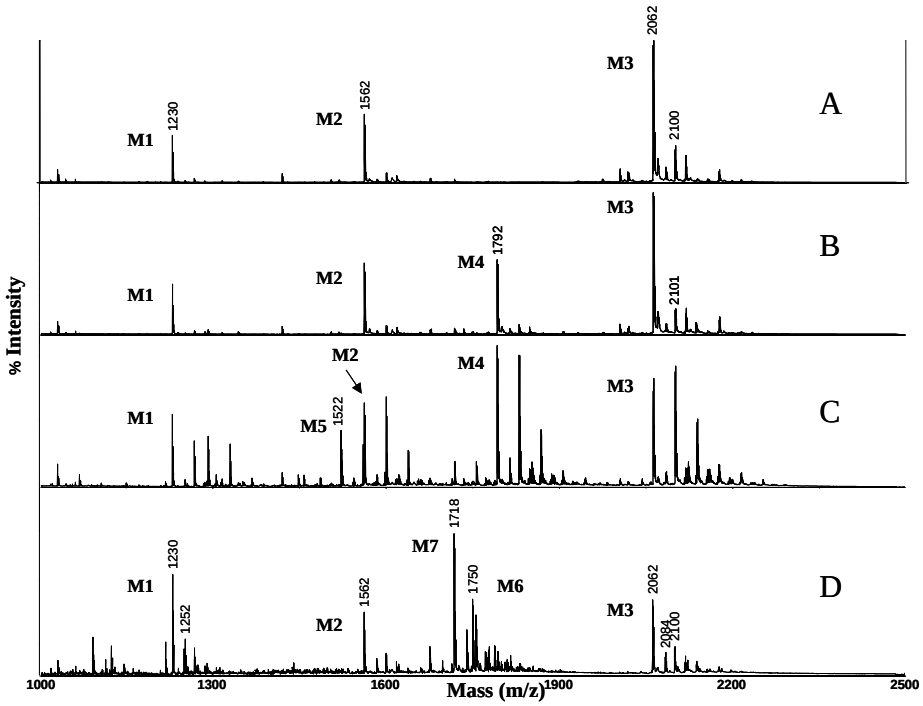

Fig.4. MALDI-TOF spectra for dendrimer G_0 preparation of active SH-G_0-$DOTA_3$ product. Spectra A, B and C for reaction mixtures at different concentration of SATA reagent respect to fix amount of NHS-DOTA and PAMAM G_0. Respectively: A) absence of SATA; B) SATA at 0.5 molar ratio to G0; C) SATA at 1 molar ratio to G0. Spectra D: analysis of mixture form spectra B after deprotection of the inserted thiol group.

As first, the required concentration of NHS-DOTA to obtain quantitative conversion of all NH_2 terminal groups of G0 PAMAM was optimized. A 2-fold molar excess of NHS-DOTA respect to NH_2 groups (8-fold molar excess respect to dendrimer G0) was found enough for this purpose as reported in Fig. 4A, where the G0-$DOTA_4$ product (M3) was found at m/z 2062. To be note, presence of other peaks in the mass spectra derive from structural deviations in the starting PAMAM dendrimer, like a missing arm (signal M1) and intra-cyclization of one dendrimer arm (signal M2) as well established by Lopp and co-workers *(20)*. Mass number exceeding the ideal mass by +22 and +38 are the corresponding Na and K salts. The use of an high excess of trietylamine was found essential, since for lower

concentrations the reaction do not proceed until complete derivatization, with a consistent signal in the MALDI spectra of original PAMAM G0. Then, insertion of SATA group was optimized by the use of different molar ratios respect to denrimer molecule, in presence of the defined amounts of NHS-DOTA. Increasing SATA reagent in the mixture from 0 to 1 equivalents respect to dendrimer, brings to a maximal derivatization to mono-SATA derivative SATA-G0-$DOTA_3$ (M4) at m/z 1792 with 0.75 equivalents as shown in Fig. 4B. By the use of an equimolar use of SATA reagent, formation of the undesired bis-adduct $SATA_2$-G0-$DOTA_2$ M5 at m/z 1522, was found. After vacuum removal of volatile components and resuspension in acqueous buffer, the deacetylation step carried out with an excess of hydroxylamine give the active SH-G0-$DOTA_3$ (M6) at m/z 1750 and its corresponding M7 at m/z 1718 from the in-source loss of the SH group. The procedure was scaled up to each generation and molecular masses of desired products were determined by MALDI-TOF until G6 and are reported in Table 2.

Table 2. calculated molecular weight for derivatized dendrimer generation (SH-Gy-$DOTA_{n-1}$) and the corresponding measured mass by MALDI-TOF analysis.

Dendrimer	**Surface groups**	**G_x**	**HS-Gx-$(DOTA)_{n-1}$**	**Maldi-Tof**
G_x	NH_2 (n)	M_w (g mol^{-1})	calc M_w (g mol^{-1})	meas M_w (g mol^{-1})
G0	4	517	1791	1791
G1	8	1430	4248	4248
G2	16	3256	9162	9000
G3	32	6909	18991	18400
G4	64	14215	38649	33600
G5	128	28826	77964	68400
G6	256	58048	156594	132000
G7	512	116493	313855	N.D.

To be note that molecular mass spectra for dendrimers of growing generation results in a lost of resolution and sensitivity due to the increased polydispersity, which is multiplied in the case of the derivatized compounds. Signals appear with a classical gaussian shape, so values reported for generations above G2 are extracted from the center of the molecular weights distribution.

SEC-ICP MS of derivatized PAMAM dendrimer. Modified PAMAM dendrimer (SH-Gy-$DOTA_{n-1}$; y= 0-7) were loaded with holmium and further injected onto SEC and analysed by ICP MS. Ammonium acetate (pH 6.8) and citric acid (pH 2.74) buffers were used, and separation on both column Superdex 75 and 200 was explored. When elemental labeled denrimers were injected in presence of citric acid as elution buffer, an increase of the baseline

value since the void volume belong the total chromatographic run was observed, due to loss of metal from its DOTA-complex form. On the other hand, use of ammonium acetate fall in the interaction between derivatized denrimer molecule and the stationary phase, as observed for the experiments with pure compounds by SEC-UV/VIS experiments. SH-Gy-DOTA$_{n-1}$ (y =0-5) were not discriminate, appearing as a single peak at 27 min in both column, but a different behaviour was observed for SH-Gy-DOTA$_{n-1}$ (y =6-7). On both column, discrimination was achieved only for derivatized dendrimer of generation 6 and 7. When injected onto Superdex 75, for which both molecules exceed the column exclusion limit, a tailed peak was observed respectively molecular characterized by molecular masses exceeding the column exclusion limit, gave a signal at 12 min. The same compound appears with multiple signals when injected onto Superdex 200.

DOTA-dendrimer- antibody conjugation. Antibody and sulphydryl free-DOTA dendrimers (SH-Gy-DOTA$_{n-1}$) were conjugate by the use of BM(PEG)$_3$, an homo-bifunctional linker which provides a centered polyethylene motif as spacer between two identical maleimido groups at the extremities. The latter are sensible for the selective coupling of two free sulphydryl groups. Bio-conjugation is schematically presented in Figure 5.

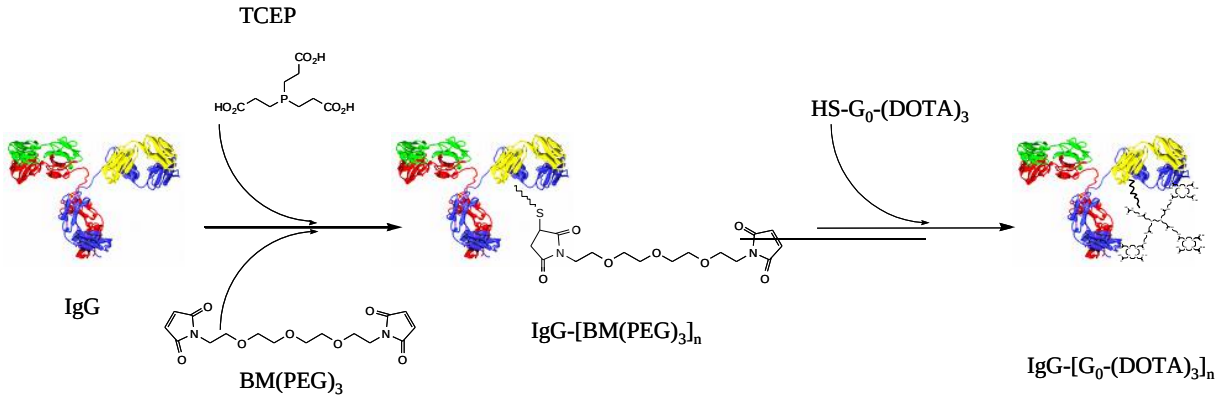

Fig. 5. Bio-conjugation scheme of DOTA-dendrimers to IgG antibody.

As first, antibody was modified with BM(PEG)$_3$ across the selective reduction of disulphide bridges in the hinge region by the use of TCEP as described elsewhere *(1)*. In order to find the optimal condition of coupling, different aliquotes of antibody were reduced with a 2-fold molar excess of TCEP in presence of an 100-fold molar excess of BM(PEG)$_3$ linker. For optimization purposes modified G0 dendrimer was used, and to each aliquote of modified antibody IgG-BM(PEG)$_3$ different amounts (20-50-100 molar excess) of prepared active SH-G0-DOTA$_3$ were added. After steps of incubation and buffer exchange, modified IgG-G0-

$DOTA_3$ were loaded with holmium. To demonstrate the dependence of labeling yield from the amount of TCEP used for reduction of disulphide bridges into IgG, other aliquotes were prepared with a 20- or 40-fold molar excess of TCEP and conjugation with increasing molar excess (20-50-100) of SH-G0-$DOTA_3$ was carried out. Labeled samples were diluted to 5 μg mL^{-1} in antibody concentration and analyzed by SEC-ICP MS.

As shown in Figure 6, the 20-fold molar excess of prepared SH-G0-$DOTA_3$ was found enough for a complete reaction of all $BM(PEG)_3$ moieties inserted, indipendently by the used molar excess of TCEP, into antibody molecule. For increasing concentrations of SH-G0-$DOTA_3$ (50 and 100 molar excess) no significant variations for the signal at 16 min related to the holmium labeled IgG-G0-$DOTA_3$ were observed. Peak at 27 min represents the free holmium labeled SH-G0-$DOTA_3$, which increase as consequence of molar excess used. Maximum labeling was obtained when a 20-fold molar excess of TCEP was used, since no difference in signal intensity at 16 min was observed when the 40-fold molar excess was employed. Then a 20 fold-molar excess of TCEP, as well a 20-fold molar excess of each SH-Gy-$DOTA_{n-1}$ were chosen for IgG modification. Labeling of each IgG-Gy-$DOTA_{n-1}$ (y = 0-7) was further checked by injection of samples diluted to5 μg mL^{-1} in IgG concentration into SEC-ICPMS system. Signal at 16 min for labeled antibody grew until the use of G2 dendrimer. Starting from G3 denrimer signal was found to decrease, and further disappeared corresponding to G5 dendrimer, when only the signal related to free labeled dendrimer was observed. This effect might be the result of an ineffectiveness availability of free thiol group inserted into dendrimers, due to an elevated back-folding of surface groups as well from a steric hindrance given by the molecular sizes of higher dendrimer generations during the reaction with antibody molecule.

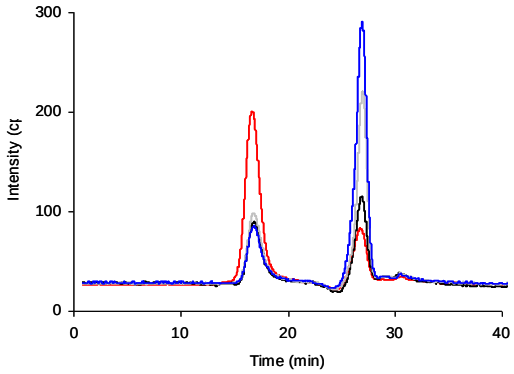

Fig.6. Overlay of injected holmium labeled IgG-G0-$DOTA_3$ at 5 μg mL^{-1}. Modified IgG signal appears at 16 min, and labeled free G0-$DOTA_3$ at 27 min. Signal for reactions carried out with a 2-fold molar excess of TCEP and increased amount (20-50-100 molar excess) of SH-G0-$DOTA_3$ are presented by lines black, grey and blue respectively. Red line represents the elution of IgG modified with a 20-fold molar excess of TCEP.

Liquid phase immunoassay. Reactivity of modified IgG-Gy-DOTA$_{n-1}$ (y = 0-4) was tested by liquid phase immunossay as described elsewhere *(1)*. Briefly, human serum samples containing human chorionic gonadotropin (hCG) antigen at different levels were incubated with the labeled IgG at 10 mg ml-1. for comparison purpose, a set of samples was incubated with a modified IgG with maleimido-DOTA (mDOTA) prepared as described in the experimental section. After incubation for 1 hour at 37°C, samples were analyzed by SEC-ICPMS. Areas of signal immunocomplex eluted at 10 min was found to increase directly with the concentration of antigen and a linear relationship was established for each set of samples. For the other sets of samples results are reported in Table 3.

Table 3. Sensitivities and limits of detection from liquid phase immunoassay for modified monoclonal antibody anti-hCG in human serum.

Labeled IgG form	Linear regression	LOD (mUI/mL)
mDOTA	y = 0.15x + 732	10.1
G0-DOTA$_3$	y = 0.66x + 367	2.4
G1-DOTA$_7$	y = 2.61x + 884	0.6
G2-DOTA$_{15}$	y = 1.38x + 1105	1.1
G3-DOTA$_{31}$	N.C.	N.C.
G4-DOTA$_{63}$	N.C.	N.C.

Sensitivity of the immunoassay tends to increase with the number of elements loaded into antibody from the use of mDOTA to G1 dendrimer, when the limit of detection was reduced of more than 10-fold. Starting from the labeling with G2 dendrimer a decrease in sensitivity was achieved, and for G3 and G4 dendrimers it was not calculable since signal of immunocomplex was not observed in the chromatograms.

The presence of dendrimers of 21.5 and 43.7 kDa respectively for G3 and G4, might affects the formation of immunocomplex, reducing the mobility necessary for a correct orientation of antibody arms carring the site for specific recognition of epitope of antigen. This effect of suppression in immunocomplex formation might be avoided by the use of a longer spacer than BMPEG$_3$, and further developments in this direction are under exploration.

CONCLUSIONS

A new general approach based on PAMAM dendrimer for elemental labeling of monoclonal antibodies was investigated, for the first time, for application in immunoassay with ICP-MS detection. The approach provides the possibility of increase the number of elemental tags inserted into the biomolecule for each site of modification. Modification of amine surface groups to obtain the active DOTA modified dendriemr was succesfully followed and characterized by MALDI-MS, which permits, at least for dendrimer of lower generation (G0-G2) the identification and consequent synthetic optimization of all species of interest. As result, after performing a liquid phase immunoassay with SEC-ICPMS method introduced recently by our work, an enhancement in sensitivity was recorded respect to the use of single labeling reagents. Howhever, various problems emerged from this former study. Regarding the effect of dimension of modified DOTA dendrimers on the antibody labeling, the coupling of dendritic reagent higher than fourth generation was found not possible. We ascribe this latter principally to two possible distinct effects as i) the steric hyndrance of dendrimer exceeding the 30-40 kDa in the insertion to the hinge region of antibody or ii) the not available free sulphidryl group onto dendrimer surface for the coupling with the cross-linker. Regard to the second point the use of a longest arm emerging from dendrimer surface might be a solution and is now under investigation. Furthermore, retain of immunoactivity was found only until labeling with second generation dendrimers, higlighting also an effect of steric hyndrance which prevent the formation of immunocomplex. These results suggest for further developments the use of dendrimer of low or half generation, if the modification of disulphide bridge for labeling purpose is taken into account.

In addition, a former study of behaviour of PAMAM dendrimers by size exclusion column based on an agarose-dextran polymeric matrix, shown peculiar features of these fascinating chemical structures. Indeed, the solution behaviour and the elution profiles support literature data from fully atomistic studies, especially in terms of pH effects and swelling due to solvent penetration into cavities of branched dendrimers.

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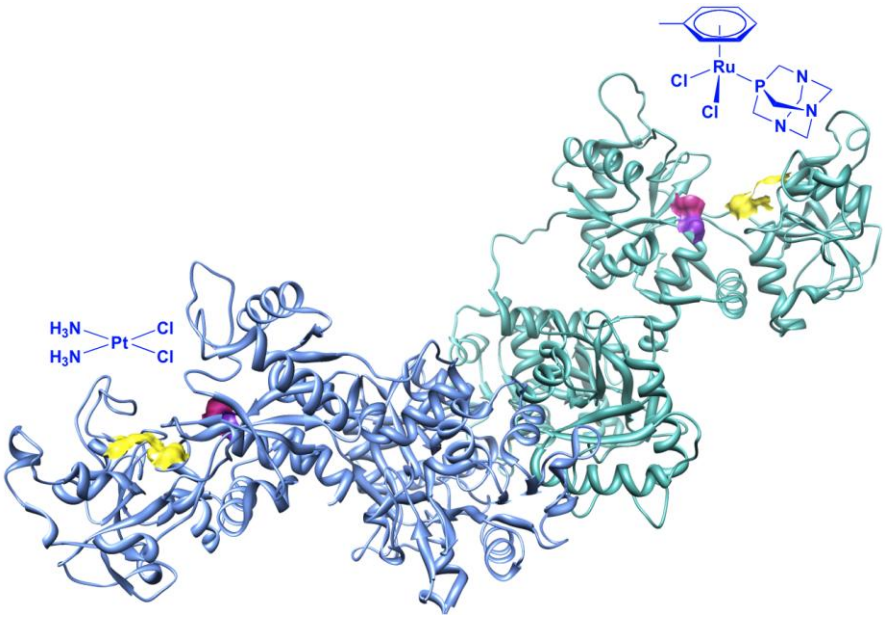
Chapter 4

The Role of ICP-MS in Metallodrugs-Proteins Interaction Monitoring

4.1. Reactivity of Anticancer Metallodrugs with Serum Proteins: New Insights from Size Exclusion Chromatoghraphy-ICP-MS and ESI-MS

Abstract. A method based on the coupling of high resolution size-exclusion liquid chromatography using a polymer stationary phase with inductively coupled plasma mass spectrometry was developed to study the interactions of two metallodrugs - cisplatin and RAPTA-T ($Ru(\eta^6\text{-}C_6H_5Me)(PTA)Cl_2$), where PTA = 1,3,5-triaza-7-phosphadamantane) - with the serum proteins albumin and transferrin. In contrast to previous approaches, the technique allowed the total recovery of the metals from the column and was able to discriminate between the different species of the metallodrug and its complexes with the proteins at femtomolar detection levels. Metal binding was found to be dependent on the protein concentration and on the incubation time of the sample. Cisplatin was found to bind the serum proteins to the same extent, whereas RAPTA-T showed marked preference for transferrin. The affinity of the ruthenium complex for holo-transferrin was higher than for the apo-form suggesting a cooperative iron-mediated metal binding mechanism. RAPTA-T binding to holo-transferrin was further investigated by electrospray mass spectrometry using both the intact protein and a model peptide mimicking the iron-binding pocket.



INTRODUCTION

Dating back to the Renaissance and to Paracelsus's alchemical studies, metals and minerals have profoundly fascinated physicians and have played a major role in the history of medicine.[1] A large number of different metal salts and compounds are still used to treat a wide range of deseases. Notably, platinum coordination complexes are extensively used to treat cancer. Indeed, following the discovery of the anticancer properties of cisplatin in 1965 by Rosenberg,[2] considerable efforts have been made to both unravel the mechanism by which it exerts its anticancer effect and to develop alternative metal-based drugs.[3-5] In recent years a vast number of non-platinum compounds have been prepared and evaluated as experimental anticancer drugs.[6, 7] Among them, ruthenium(II)-arene complexes bearing 1,3,5-triaza-7-phosphaadamantane (PTA) ligands – named RAPTA – have been found to exhibit promising antimetastatic properties *in vivo*.[8] Their mechanism of action is still largely unknown, however, there is evidence to suggest that RAPTA compounds work on molecular targets other than DNA implying a biochemical mode of action profoundly different from classical platinum anticancer drugs.[9, 10] Indeed, ruthenium compounds have been shown to directly interfere with specific proteins involved in signal transduction pathways and/or to alter cell adhesion and migration processes.[11]

An understanding of metallodrug-protein interactions is crucial since the pharmacological activity of a drug, even for drugs where DNA is the ultimate target, is usually modified upon protein binding. Metal drug-protein interactions are not only important with regard to apoptosis, but also with respect to unwanted side-effect (due to indiscriminate protein binding), drug resistance (due to binding/deactivation by metallothioneins,[12] glutathione transferases,[13, 14] etc.) and even possibly drug delivery and storage.[15] Upon intravenous application, serum proteins including albumin, transferrin and globulins are thought to play a crucial role in the transport, delivery, and storage of anticancer metallodrugs. Human serum albumin (HSA) is the most abundant protein (about 52% of total serum protein) with a concentration of 40-45 g L^{-1} in healthy humans (ca. 600 μM; M_w 66-67 kDa). It comprises a single chain of 585 amino acids organized in three similar domains, each composed of two subdomains.[16] HSA is known to bind a remarkably wide range of drugs, thereby restricting their free, active concentrations.[17] Reducing this binding affinity represent a major challenge in drug development. However, HSA can also be exploited for targeted delivery strategies, as in the case of organometallic ruthenium(II)-arene anticancer compounds, to develop compounds that can selectively accumulate in tumors cells.[18]

Human serum transferrin (Tf) is a single-chain glycoprotein containing 679 amino acids with a molecular mass of about 80 kDa and is found in blood at a concentration of about 2.5 g L^{-1} (35 μM). Tf acts as an iron transporter and is capable of binding two iron(III) ions (Fe^{3+} is bound selectively over Fe^{2+}).[19] Tf is normally only 30% saturated with iron in the body,[19] and at least 30 other metal ions can also bind to Tf.[20] Therefore, it is possible to use Tf as a metal transporter within the body and the cellular uptake mechanism *via* the Tf-transferrin receptor transport system has the potential to be exploited for site-specific delivery of various therapeutic metal ions, drugs, proteins and genes.[21] In the case of metallodrugs the resulting conjugates significantly improve the cytotoxicity and selectivity of the drugs itself. For example, anticancer Ru(III) complexes were reported not only to bind to Tf,[22, 23] but it has been shown that injection of Ru(III)-Tf conjugates resulted in high tumor uptake of the metal,[24] which suggests that Tf uptake appears to be the more important mode of transport of Ru(III) anticancer complexes to the tumor. In some cases, the Ru-Tf adducts exhibit a significantly higher antitumor activity against human cancer cells than the Ru(III) complex itself, attributable to the Tf-mediated uptake mechanism, which may also lower drug toxicity.[25]

The development of analytical methods capable of providing a better understanding of how metallodrugs interact with proteins remains challenging.[26, 27] The direct monitoring of real-time protein-mediated metabolism of anticancer agents using hyphenated (coupled, tandem) techniques, that combine chromatographic separation with sensitive and element-specific detection techniques such as inductively coupled plasma-mass spectrometry (ICP-MS) have been effectively used.[28-30] In terms of separation techniques, high performance liquid chromatography (HPLC)[31, 32] and capillary zone electrophoresis (CZE)[29,33-35] were proposed for the study of platinum and ruthenium based drugs, to assess kinetic constants of hydrolysis and subsequent reactions with a variety of physiological targets. This concept has also been transferred to study the distribution of gallium-based pharmaceuticals in human serum.[36]

Within this frame, we propose size-exclusion liquid chromatography (SEC) coupled to ICP-MS to study metal complexes-proteins interactions. We believe that SEC has certain advantages compared to other separation techniques to characterize metallodrug binding to biomolecules, and that in any case it could be used in concomitance with other hyphenated systems to obtain complementary information. In comparison to ion exchange chromatography (IEC), the main advantage of the SEC approach lies in the more "gentle" conditions, as IEC usually requires buffers with high ionic strength and extreme pH values.[37] The importance of working under more physiological-type conditions is crucial when studying metallodrugs to avoid interferences which might alter the metal-protein adducts and

the coordinative metal-ligand bonds. With respect to capillary electrophoresis (CE) the major advantage of the SEC separation lies in the ease and robustness of the coupling to ICP-MS which is considered one of the main problems of CE-ICP-MS and usually leads to higher limits of detection compared to LC-ICP-MS.[35, 38, 39]

Up to now, the SEC coupled to ICP-MS has been used to probe protein interactions with ruthenium drugs, but suffers from the poor recoveries because of the reactivity of the compounds with the column stationary phase.[40] This problem was alleviated by using a short size-exclusion column at expense of the chromatographic resolution, or it was simply used as a first separation technique to roughly discriminate between bound and unbound species prior to ion-exchange separation.[40] Therefore, the primary objective of our research was to evaluate a polymeric stationary phase with regard to the unspecific retention, reactivity and carryover of an organometallic drug RAPTA-T ($Ru(\eta^6\text{-}C_6H_5Me)(PTA)Cl_2$) (Chart 1), and its adducts, with the serum proteins albumin and transferrin, in comparison to cisplatin. The binding of RAPTA-T towards intact Tf and the possible binding sites of the metallodrugs on a model peptide mimicking one of the iron-binding pockets of Tf were also investigated using electrospray ionization-mass spectrometry (ESI-MS).

EXPERIMENTAL SECTION

Reagents. Cisplatin, human serum albumin (>99%, HSA), human holo transferrin (>98%, hTf) and human apo transferrin (>97%, apo-Tf) were obtained from Sigma-Aldrich. Sodium chloride, formic acid, Tris(hydroxymethyl)aminomethane hydrochloride, ammonium acetate and ammonium hydrogencarbonate were also purchased from Aldrich and were of analytical reagent grade. Buffers used for SEC-ICP-MS analysis were prepared as follows: ammonium acetate buffer (100 mM, pH 6.8) and Tris buffer saline (20 mM Tris-HCl, 100 mM NaCl, pH 7.2). De-ionized water (18 MΩ/cm) (Millipore, Bedford, MA) was used throughout. RAPTA-T was synthesized as described elsewhere.[8] The HPLC-purified model peptide (KDCHLAQVPSHTV) was purchased from PSL (Heidelberg, Germany).

ICP-MS instrumentation. An Agilent model 1200 binary pump, equipped with an autosampler, automatic injection system with a loop of 900 μL and a UV-VIS detector was used as the solvent delivery system and was controlled by Agilent ChemStation software. An Agilent 7500ce ICP-MS fitted with a Meinhardt nebulizer was used as the detector in SEC-ICP-MS experiments, employing the following conditions: plasma gas (Ar) 15 L min^{-1}, forward power 1500 W, carrier gas 1.05 L min^{-1}. The working conditions were optimised using a 1 μg L^{-1} solution of $^{7}Li^{+}$, $^{89}Y^{+}$ and $^{205}Tl^{+}$ in 2% HNO_3, with a dwell time of 100 ms for each isotope. For iron isotope monitoring, a collision cell was used to remove polyatomic interferences with H_2 (3-3.5 ml min^{-1}) and He (1.0 ml min^{-1}) as auxiliary gases. Double charged ions and oxide levels were minimized by using $^{140}Ce^{+}$ and were typically < 2%. The isotopes monitored (in time resolved analysis mode with a dwell time of 100 ms each) were ^{54}Fe, ^{56}Fe, ^{195}Pt, ^{196}Pt , ^{197}Pt , ^{198}Pt , ^{100}Ru, ^{101}Ru and ^{102}Ru. The ^{54}Fe, ^{56}Fe, ^{102}Ru, ^{195}Pt and isotopes were used for quantification of binding and uptake.

Size-exclusion chromatography. Samples were analyzed on a Superdex™ 200 HR 10/30 (10 mm x 300 mm x 13 μm) (GE Healthcare) exclusion column with approximate bed volume of 24 mL and a linear separation range of 10-600 kDa for globular proteins. The column was calibrated as specified by the manufacturer with a mixture of thyroglobulin (670 kDa), hTf (81 kDa), bovine albumin (66 kDa), chicken egg albumin (44 kDa), Mn superoxide dismutase (39.5 kDa), Cu/Zn superoxide dismutase (32.5 kDa), carbonate dehydratase (29 kDa) and myoglobin (16.7 kDa) using UV/VIS detection at 280 nm with baseline evaluation at 800 nm. Retention times (in min) plotted versus the logarithm of molecular mass (in kDa) showed linear correlation ($y = -0.134\ x + 4.385$; $r^2=0.992$). Elution was carried out isocratically at 0.7 mL min^{-1} using 100 mM ammonium acetate (pH 6.8) as the mobile phase. Solvent flow from

the chromatographic system was introduced into the ICPMS nebulizer by means of PEEK tubing.

Sample preparation for SEC-ICP-MS. Cisplatin and RAPTA-T stock solutions were prepared by dissolving the compounds in ammonium acetate buffer or in Tris buffer. Proteins were dissolved in ammonium acetate at a concentration 0.5 mM. To investigate the interactions between serum proteins and the metal complexes, samples were initially prepared with various protein concentrations (1, 50 and 250 μM) and adding the appropriate amounts of metal complex to achieve different protein:metal stoichiometric ratios (1:1, 1:2 or 1:5) at each protein concentration. In the case of 250 μM protein samples only a maximum two-fold molar excess of the metal complex was applied due to protein precipitation phenomena for transferrin. Samples were then incubated for 1 hour at 37°C and then diluted in ammonium acetate buffer to yield a final protein concentration of 1 μM prior to injection into the SEC column. Alternatively, for the evaluation of maximum metal binding, the protein concentration was fixed at 50 μM, while each metal complex was added in stoichiometric amount or in 2, 5, 10 and 18-fold excesses. Metal complex solutions were added freshly prepared (sample Set A) or 24 h after preparation (sample Set B; solution stored at 4°C) in ammonium acetate buffer. Samples were then incubated for 1 h at 37 °C and diluted to 1 μM protein before injection. Quantification of the metal content in each chromatographic fraction was calculated directly from the integrated signal from the SEC-ICP-MS chromatograms using the formula $c_b = M_b \times c_{inj} \times D$, where c_b represents the initial concentration of metal in the original sample, M_b is the fraction of metal bound to protein in the chromatogram (calculated as a percentage of bound metal signal with respect to the total sum of signals), c_{inj} is the concentration of metal injected for analysis and D is the dilution factor applied to the samples prior to injection.

ESI-MS analysis. For ESI-MS experiments with the model peptide, samples were prepared with a metallodrug:peptide ratio of 1:1 (25 μM) in 20 mM carbonate buffer (pH 7.4) and incubated for 24 h at 37° C prior to analysis. Samples were then mixed with acetonitrile and formic acid (final concentration 30% and 0.1%, respectively) and placed into a 96-well plate in an Advion TriVersa robot (Advion Biosciences, Ithaca, NY) equipped with a 5.5 μm-nozzle chip. The ESI robot was controlled with ChipSoft v7.2.0 software employing the following parameters: gas pressure 0.45 psi; voltage 1.7 kV. The samples were analyzed in positive ion mode using a LTQ XL mass spectrometer (ThermoFisher Scientific, Bremen, Germany). The Xcalibur software bundle was utilized for data acquisition and data analysis.
ESI-MS measurements of apo transferrin-metallodrug interactions were carried out on an Ultima II q-TOF mass spectrometer (Waters, Manchester, UK) operated in positive mode.

Data acquisition and analysis were carried out using the MassLynx software bundle (Waters). The instrument was calibrated daily using a 0.01% phosphoric acid solution in 50% acetonitrile. Samples were prepared with a metallodrug:protein ratio of 5:1 in 20 mM carbonate buffer (pH 7.4) and incubated for up to 24 h at 37° C. Prior to analysis, samples were ultracentrifuged using 30kDa cut-off filters (VWR, Switzerland) to remove unbound metallodrugs and injected into the ESI-MS system after dilution with acetonitrile and formic acid (final concentrations 25% and 0.05%, respectively).

RESULTS AND DISCUSSION

Size-exclusion chromatography of cisplatin and RAPTA-T.

SEC-ICP-MS system was initially used to evaluate solutions of the two metal drugs, cisplatin and RAPTA-T (Chart 1), in the absence of any protein in order to establish the evolution of metal species. Based on the molecular weight of the complexes (300-400 Da) an expected single peak with a total bed column volume (≈ 24 ml), corresponding to 35 min at flow rate of 0.7 mL min^{-1}, might be expected to elute from the column. Two different buffers, namely Tris buffer saline (TBS) and ammonium acetate (pH ~ 7), were evaluated as incubation media and eluent for cisp latin and RAPTA-T hydrolysis. For method development, 50 µl of 1 µM cisplatin (195ng mL^{-1}, 50pmol of Pt) or RAPTA-T (102ng mL^{-1}, 50pmol of Ru) in the respective buffers were injected into the SEC column either freshly prepared or pre-incubated for 12 hours at 37 °C. Elution profiles for the two drugs in TBS are shown in Figure 1. In the case of cisplatin (Figure 1, top spectrum), freshly prepared solution gave signals at 32 and 42 min, while for the sample obtained after 12 hours incubation an additional peak appeared at 28 min. In both cases the peak at 42 min (intact cisplatin) was the most intense. Peaks at 32

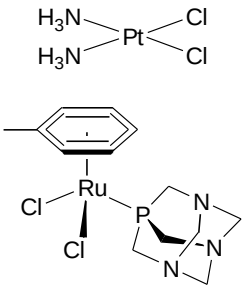


Chart 1. Structures of cisplatin (up) and RAPTA-T (bottom).

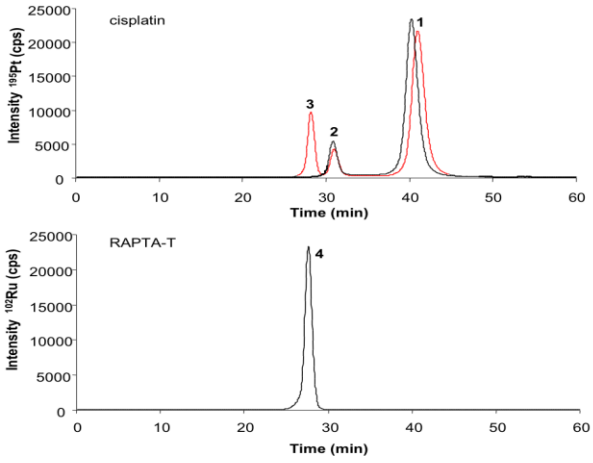


Figure 1. SEC-ICP-MS elution profiles of the investigated metallodrugs. The upper chromatogram compares a freshly prepared cisplatin solution (black trace) to a cisplatin solution after 12 h incubation at 37°C (red trace): peak 3 at 28 min: diaqua complex; peak 2 at 31 min: monoaqua complex; peak 1 at 40 min: intact cisplatin. The bottom chromatogram shows the single elution peak (peak 4) of RAPTA-T at 28 min.

and 28 min corresponded to cisplatin hydrolysis products (mono- and bis-aqua species, respectively) as confirmed by ESI-MS analysis. In ammonium acetate buffer, these compounds were detected at the same elution times, but more extensive hydrolysis of cisplatin was observed due to the absence of chlorido ions in the buffer. To be note, the separation of different forms of metallodrugs arise from aspecific interactions with the stationary phase, since no differentiation is possible based on their low molecular wheights. Howhever, elution times were found constants after multiple injection of metallodrug. Only for peak eluting at 40 min well after a total column volume, slight shifts of ± 2 min in its elution time were observed. When RAPTA-T was injected, either freshly prepared or after 12 hours of incubation, only a single symmetric peak at 28 min of the chromatographic run was observed regardless of the buffer employed (Figure 1, bottom spectrum). This peak probably corresponds to the intact complex or to the mono-acqua $[Ru(\eta^6\text{-}C_6H_5Me)(PTA)ClH_2O]$ species as suggested by ESI-MS analysis and UV-visible spectrophotometry (data not shown). Recovery experiments, based on the comparison of the metal content in the injected sample and the eluate, showed that the metals were not retained on the stationary phase. Indeed, recovery values for cisplatin and RAPTA-T were 100 ± 2% and 93 ± 3% respectively, in both buffers. The baseline remained stable during the analyses and a post-analysis injection of HSA gave a blank chromatogram.

Interaction of metallodrugs with serum proteins.

The binding of cisplatin and RAPTA-T to transferrin - both holo (hTf) and apo-transferrin (apo-Tf) - and human serum albumin was investigated by SEC-ICP-MS. Since no differences in the chromatographic separation of the complexes were observed as a function of the buffer, ammonium acetate was selected as it exhibited sufficient ionic strength to limit the interactions of the proteins with the stationary phase and is better tolerated in ICP-MS.[41] The two proteins investigated in this study have a molecular mass which is not expected to be separated by this SEC column. In fact, calculated elution times for transferrin and albumin from column calibration curve are 18.5 and 18.9 minutes when molecular weights of 80 and 70 kDa are taken into account.

Holo- and apo-transferrin were analyzed first in order to establish their elution time; note that hTf carries iron which can be used for ICP-MS analysis. Figure 2 shows the chromatograms ($^{56}Fe^+$) of 1 μM hTf and apo-Tf samples in comparison to the ion chromatograms ($^{102}Ru^+$, $^{195}Pt^+$) of the metallodrugs to demonstrate the baseline separation of the iron-containing protein peak from the other metal species present in solution. The results show that the apo-Tf is not completely iron-free. To establish the amount of iron present in

holo- and apo-transferrin, the proteins were digested and analyzed by ICP-MS. The results reveal a stoichiometric metal:protein ratio of 1.22 ± 0.12 for hTf and 0.20 ± 0.05 for apo-Tf. Notably, there is no change in retention time for both Tf and HSA keep upon adduct formation with the metallodrugs, since the resulting increase in the molecular mass is negligible. It is worth mentioning that in the case of the incubation mixtures between Tf and HSA with both drugs, recovery experiments shown values as calculated for the free compounds. Due to the high resolution separation of the metal-containing species and their excellent recovery rates, quantitative data on the amount of drug binding could be extracted directly from the chromatograms. With the optimized method two types of experiments were set up. These experiments aimed at, first, observing the effect of different protein concentrations and relative protein:metal ratios on the adduct formation, and second, quantitating the extent of adduct formation at increasing metallodrug concentrations.

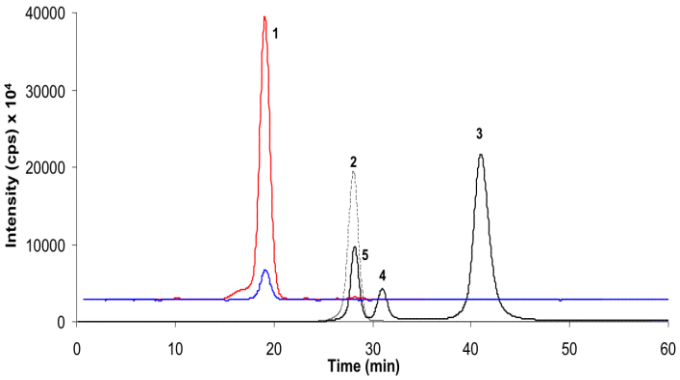


Figure 2. Overlay of selected ion chromatograms for both holo-transferrin (red trace) and apo-transferrin (blue trace) eluting at 19 min (peak 1: Tf; $^{56}Fe^{+}$) with chromatograms of both RAPTA-T (dotted line in peak 2: free complex; $^{102}Ru^{+}$) and cisplatin (peak 3: intact complex, peak 4: monoacqua complex, and peak 5: diaqua complex; $^{195}Pt^{+}$).

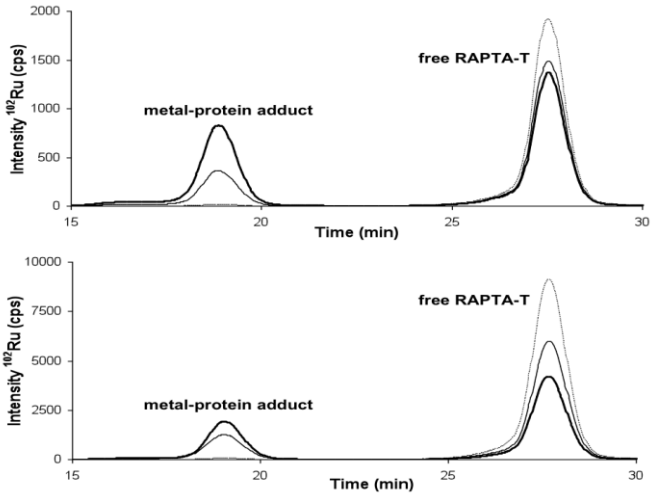


Figure 3. SEC-ICP-MS chromatogram of HSA incubated with RAPTA-T at different protein:metal ratios for 1 h at 37°C. 1:1 (top) and 1:5 (bottom). Protein concentrations are 1 μM (dotted line), 50 μM (thin line) and 250 μM (bold line). All samples were diluted to 1 μM (protein) prior to injection into the SEC-ICP-MS system. The peak at 19 min corresponds to the HSA-RAPTA-T adduct, the peak at 28 min to free RAPTA-T.

Effect of protein concentration on adduct formation. Different concentrations (1, 50 and 250 μM) of single serum proteins were incubated with each metal complex at different protein:metal ratios (1:1, 1:2 and 1:5) for 1 hour at 37°C. Representative SEC-ICP-MS chromatograms for samples of HSA incubated with RAPTA-T are given in Figure 3, the

whole dataset for interactions of the serum proteins with RAPTA-T and cisplatin is summarized in Table 1. The data indicate that RAPTA-T can bind equally to both hTf and apo-Tf but to a significantly greater extent compared to cisplatin, regardless of the applied concentrations and metal complex:protein ratios.

Table 1. Evaluation of metal binding by SEC-ICP-MS for samples with different metallodrug:protein ratios, incubated for 1 h at 37°C. Results are the average of three independent experiments with S.D. < 2%.

Protein (μM)	Drug (μM)	Molar ratio metal:protein	Metal bound (%)	
			cisplatin	RAPTA-T
hTf				
250	250	1	17.3	38.4
250	500	2	16.0	30.4
50	50	1	5.9	19.0
50	250	5	5.7	16.9
1	1	1	0.2	1.1
1	5	5	0.3	0.8
apo-Tf				
250	250	1	14.5	37.2
250	500	2	14.8	36.6
50	50	1	2.9	10.1
50	250	5	3.2	9.7
1	1	1	0.2	0.7
1	5	5	0.2	0.7
HSA				
250	250	1	21.1	18.7
250	500	2	20.8	18.1
50	50	1	6.6	7.5
50	250	5	5.3	6.8
1	1	1	0.5	0.3
1	5	5	0.6	0.4

In contrast, similar metal binding of both metallodrugs with HSA was observed. In particular, since the short incubation time does not allow reaching the thermodynamic equilibrium, a kinetic preference of RAPTA-T for binding to Tf was detected with respect to HSA. However, it must be considered that HSA is about 20-times more concentrated than Tf in blood and therefore favoured for metal binding, as shown for the ruthenium(III) anticancer compound KP1019.[42] Interestingly, the incubation concentrations affect the formation of protein-metal adducts, metal binding being favoured at higher concentrations of both the metallodrugs and the proteins.

Influence of hydrolysis on protein binding.

Further experiments on protein binding and relative binding affinities of the metallodrugs were performed using a fixed concentration of proteins, 50 μM, (which represents a concentration similar to that of Tf in blood) incubated with different amounts of the metallodrugs. Quantitative metal binding data were calculated based on the fraction eluted at 19 minutes in the SEC-ICP-MS profiles. In Table 2, results from each protein sample treated with RAPTA-T or cisplatin and incubated for 1 hour at 37 °C are shown. Dataset A represents data for samples with freshly prepared solutions of the metal complexes and Dataset B for samples with metal complex solutions pre-incubated for 24 hours prior addition to the protein to study the effect of metallodrug hydrolysis on protein binding.

The most notable difference between the two preparations corresponds to the availability of the different forms of the metallodrugs. For instance, in Dataset B higher contents of hydrolyzed products are present for cisplatin with respect to Set A. Since cisplatin hydrolysis products carry a positive charge, their interaction with negatively charged amino acid residues on the protein chains (possible under these conditions considering calculated p*I* values are 5.92 and 6.81 for HSA and Tf, respectively)[43] potentially increases interactions compared to the neutral starting compound. Moreover, the hydrolized product is more reactive as the aqua ligands are more labile than chlorido ligands. In Set B, platinum adducts are formed to a

Table 2. Quantification of adduct formation between cisplatin and RAPTA-T with the serum proteins hTf, apo-Tf and HSA. Protein concentration was fixed at 50 μM, the metal:protein ratio varied between 1:1 and 18:1. Samples were incubated for 1 h at 37°C (Set A: freshly prepared metal complex solution added. Set B: 24 h pre-incubated metal complex solution added.). Results are the average of three independent experiments with S.D. < 2%.

Compound	Molar ratio metal:protein (theoret.)	Set A (μM)	Molar ratio metal:protein (experimental)	Set B (μM)	Molar ratio metal:protein (experimental)
			hTf		
Cisplatin	1	1.4	0.03	1.7	0.03
	2	2.7	0.05	3.3	0.07
	5	6.4	0.13	7.5	0.15
	10	8.1	0.16	14.4	0.29
	18	11.4	0.23	16.9	0.34
RAPTA-T	1	8.5	0.17	8.2	0.16
	2	14.7	0.29	15.3	0.31
	5	33.1	0.66	32.3	0.65
	10	56.8	1.14	57.0	1.14
	18	70.9	1.42	69.4	1.39
			apo-Tf		
Cisplatin	1	1.0	0.02	1.7	0.03
	2	2.0	0.04	3.1	0.06
	5	4.4	0.09	7.0	0.14
	10	7.6	0.15	14.0	0.28
	18	12.2	0.24	19.2	0.38
RAPTA-T	1	3.2	0.06	4.6	0.09
	2	7.8	0.16	8.8	0.18
	5	16.0	0.32	18.5	0.37
	10	26.1	0.52	34.7	0.69
	18	33.1	0.66	52.9	1.06
			HSA		
Cisplatin	1	2.1	0.04	3.9	0.08
	2	4.3	0.09	6.8	0.14
	5	7.3	0.15	11.8	0.24
	10	10.0	0.20	16.7	0.33
	18	11.2	0.16	17.4	0.35
RAPTA-T	1	3.3	0.07	3.8	0.08
	2	6.7	0.13	7.1	0.14
	5	13.3	0.27	15.9	0.32
	10	20.3	0.41	30.4	0.61
	18	24.2	0.48	32.0	0.64

greater extent with respect to Set A at any concentration. In general, for both datasets, cisplatin shows a slightly increased affinity towards HSA at lower molar excess of drug and no apparent preference for the two forms of transferrin. Upon increasing the drug excess, the reaction proceeds more rapidly and, as expressed by an increased number of metal ions bound per protein molecule, the proteins are metallated to a higher degree.

For RAPTA-T, a higher affinity is found for hTf with respect to apo-Tf and HSA, and the amount of ruthenium bound to hTf is similar in Set A and Set B. For apo-Tf and HSA, increased binding is observed for the Set B samples, especially at high metal:protein ratios. Based on the ratio of the metal concentration of the bound fraction, for cisplatin maximum binding was in the range from 0.2 to 0.35 for all three proteins. For RAPTA-T the highest metal binding was observed for hTf, with an average of ca. 1.4 ruthenium ions per protein. For apo-Tf and HSA an average 0.5 to 1.0 ruthenium ions bind per protein, which is higher than observed for cisplatin. In comparison to previously reported data using CZE-ICP-MS, which shows extensive binding of cisplatin toward the protein (up to 4 Pt ions per molecule of HSA for a sample containing a 10-fold excess of metal complex after 24 hours incubation in PBS buffer), the values obtained for cisplatin-HSA adduct formation in our experiments were unexpected.[33] Therefore, protein samples incubated for 24 hours with a 10-fold excess of cisplatin or RAPTA-T were studied. For all the three proteins increased metal binding for both metallodrugs was detected (Table 3), although not to the same levels observed by CZE-ICP-MS.

Table 3. Influence of different incubation times (1 and 24 h) on the extent of adduct formation between metallodrugs and the serum proteins. Protein concentration was fixed at 50 μM with a metal complex:protein ratio = 10:1. Results are the average of three independent experiments with S.D. < 2%.

Incubation time		**Cisplatin**		**RAPTA-T**	
		metal bound (μM)	**metal:protein ratio**	**metal bound (μM)**	**metal:protein ratio**
1 hour	hTf	8.1	0.16	56.8	1.14
	apo-Tf	7.6	0.15	26.1	0.52
	HSA	10.0	0.20	20.3	0.41
24 hours	hTf	31.6	0.6	93.5	1.9
	apo-Tf	25.6	0.5	50.8	1.0
	HSA	36.6	0.7	46.3	0.9

Overall, the low detection limits of the applied method allowed analysis of samples containing 400 fmol of metals bound to the proteins, with as little as 2 pmol of protein injected into SEC column. In the case of RAPTA-T, the higher amount of ruthenium bound to hTf with respect to apo-Tf suggests that the presence of iron bound to protein might influence the formation of metal complex adducts, most likely maintaining a protein folding which favours metallodrug binding.

Metallodrug binding versus iron loss in holo-transferrin.

Despite the higher affinity of RAPTA-T than cisplatin for hTf, no differences in terms of the released iron were observed. Figure 4 shows the binding behaviour of both metal complexes for hTf, together with the decrease of the iron signal recorded by ICP-MS, for each concentration of metal complex. The graph shows that the iron is not released in a linear fashion. In fact, the iron signal decreases only slightly until a 10-fold molar excess (500 μM) of the metallodrug and then immediately drops to the half of its original value.

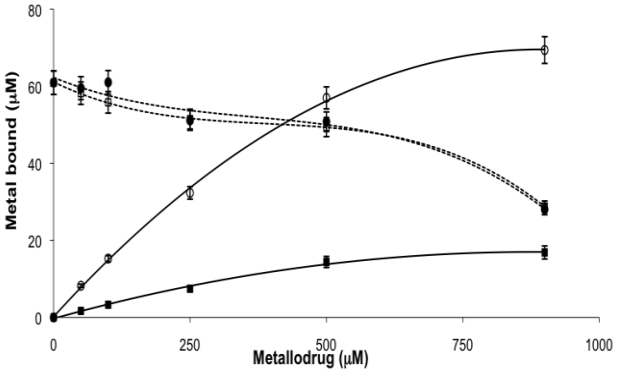


Figure 4. Concentration dependent binding of cisplatin and RAPTA-T towards holo-transferrin.
Pt = square; Ru = circle. Dotted traces represent the corresponding iron signal.

As the employed hTf was only ca. 60% loaded with iron, these data suggest that initially metallodrug binding occurs at the iron-free protein active sites and therefore does not affect the iron release. Afterwards, in the presence of excess of metallodrug, significant iron release may result from the partial denaturation or conformational changes of Tf upon addition of excess of metal, in agreement with previous observation on cisplatin binding to hTf by Hoshino *et al.*.[33]

ESI-MS studies with transferrin and an iron binding site-model peptide.

As the interactions of cisplatin with intact transferrin studied by ESI-MS have already been reported earlier,[44] only the binding of RAPTA-T to transferrin was further investigated by ESI-MS in this study. Figure 5 shows the spectrum of apo-Tf treated with a 5-fold excess of RAPTA-T and incubated for 1 hour at 37° C, in comparison to the pure protein. The spectra of the RAPTA-T treated samples show additional signals corresponding to adduct formation with the ruthenium compound. Based on the mass difference, these signals are attributable to RAPTA-T after loss of both chlorido and PTA ligands and subsequent addition of a carbonate

anion. The fact that only the arene ligand stays attached to the metal centre is in good agreement with data obtained for interactions of RAPTA compounds with cytochrome c,[45] and the coordination of carbonate is also not surprising under the conditions employed. It is worth noting that a synergistic carbonate anion interaction is also crucial for stabilizing the binding site to ensure efficient binding of iron to transferrin.

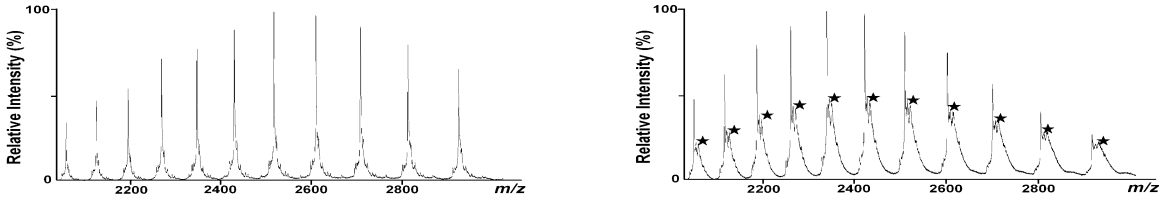


Figure 5. ESI-MS spectrum of pure apo-transferrin (left) and incubated with RAPTA-T for 1 h at 37° C at a metallodrug:protein ratio of 5:1 (right). Stars indicate the additional peaks caused by the adduct formation.

Furthermore, the exact binding mode of cisplatin and RAPTA-T towards Tf and the nature of the species that bind to the protein as well the identification of the amino acids involved were probed by ESI-MS utilizing a model peptide consisting of the active site residues ^{239}KDCHLAQVPSHTV^{251} of Tf. This peptide contains the His249 residue, which is involved in the iron binding in Tf, but also threonine and cysteine, residues which are possible binding partners.[44, 46, 47] The model peptide was incubated with the metallodrugs under simulated physiological conditions (pH 7.4, 37 °C) for 24 hours and then analysed. The bound species were identified as RAPTA-T and cisplatin after loss of the chlorido ligands, indicating a bidentate binding to the peptide. Although loss of a chlorido ligand could also be induced during ionization, a multidentate binding mode seems more probably due to hydrolysis of the compounds under the used incubation conditions. Different amino acids

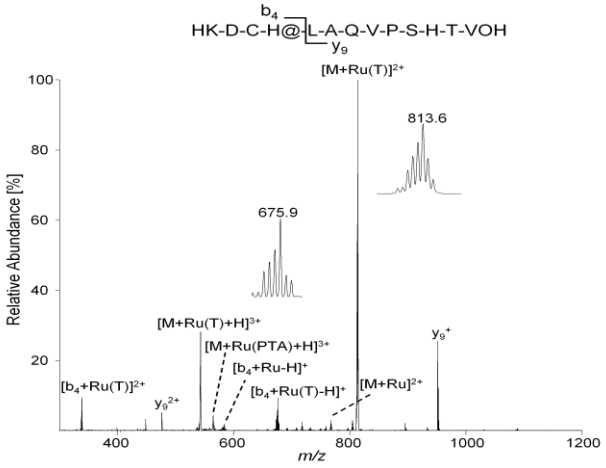


Figure 6. MS/MS spectra of the transferrin iron-binding site model peptide after incubation with RAPTA-T revealing histidine as the binding partner. The insets show the $[M+Ru(T)]^{2+}$ (T = toluene) ion at 813.6 *m/z* as well as the $[b_4+Ru(T)-H]^{+}$ ion at 675.9 *m/z* with an isotopic distribution characteristic for ruthenium. The fragmented parent ion corresponds to $[M+Ru(PTA)(T)+H]^{3+}$.

depending on the metal complex were identified as binding partners upon collision induced dissociation (CID) of the modified peptide, as can be seen from Figures 6 and 7. The insets show signals of metallated ions, exhibiting characteristic isotopic patterns induced by the ruthenium and platinum, which facilitate the identification of modified residuies. In general, the remaining ligands of the metallodrugs (amino for cisplatin, toluene and PTA for RAPTA-T) were dissociated more easily than the peptide bonds under fragmentation conditions, indicating the formation of strong bonds between the amino acids and the metals. For RAPTA-T (Figure 6), the first histidine residue appears to be the major binding partner, as evidenced by the presence of the modified b_4 ion in contrast to the unmodified y_9 ion following CID. This is not surprising since nitrogen donor ligands are known to form strong complexes with ruthenium. Interestingly, no binding towards the histidine residue in position 11 of the peptide (iron binding His249 in transferrin) could be detected for the ruthenium compound, indicating that the cysteine residue might also be involved in the binding of RAPTA-T. It also worth mentioning that cleavage of the peptide occurs predominately next to the metal binding site, resulting in low relative abundances of other b and y fragment ions.

In the case of cisplatin (Figure 7), the sulphur containing cysteine residue seems to be responsible for the binding. CID resulted in a more extensive fragmentation of the peptide as compared to RAPTA-T, and unmodified b_2 and y_{10} in contrast to modified b_3 and y_{11} ions allow unambiguous identification of the binding site. Although these data are in agreement with previous studies which show that cisplatin readily reacts with sulphur containing biomolecules such as glutathione or metallothionein within the cell,[48] caution should be applied to the validity of the model peptide in terms of comparability to intact proteins. As it has been shown in earlier proteomic studies of transferrin-cisplatin interaction[44] as well as on samples extracted from *E. coli* upon incubation with a ruthenium-arene compound and cisplatin,[49, 50] oxygen atoms in the carboxy- and hydroxyl-functions of certain residues (e.g. aspartate, serin, threonine) may also serve as binding partners in biological systems.

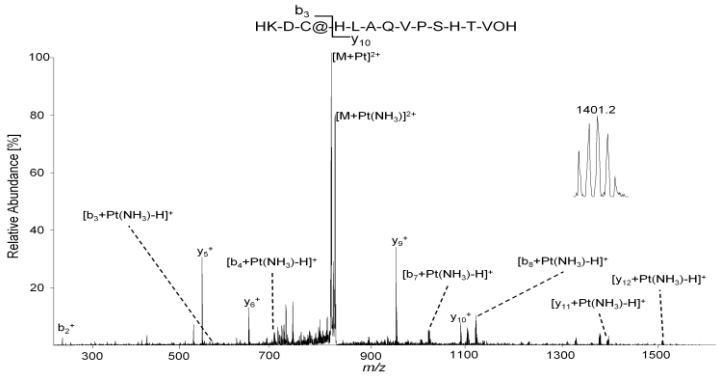


Figure 7. MS/MS spectra of the transferrin iron-binding site model peptide after incubation with cisplatin revealing cysteine as the binding partner. The insets shows the $[y_{11}+Pt(NH_3)-H]^+$ ion at 1401.2 *m/z* with an isotopic distribution characteristic of platinum. The fragmented parent ion corresponds to $[M+Pt(NH_3)_2+H]^{3+}$.

Consequently, protein conformation has a strong influence on the site and type of adduct formed as exposed residues might be more easily accessible and therefore more reactive than those buried in the folded protein. Although such structural features cannot be mimicked by a short, linear model peptide, the general reactivity of a compound in the presence of several possible binding amino acids can be assessed.

CONCLUSIONS

High resolution SEC-ICP-MS based on a polymer stationary phase was demonstrated, for the first time, to be a powerful tool for the analysis of metal drugs with serum proteins. The approach provides information on the affinity of each metallodrug for the selected proteins, quantification of metal uptake and on the stoichiometry of the metal-protein complexes. The primary advantage of this approach over the previous SEC-ICP-MS methods is that all the metal species are quantitatively recovered under the separation conditions. Although the SEC system was not suitable for the baseline separation of samples containing both HSA and Tf, it proofed to be an efficient tool to analyze the binding of metallodrugs to single proteins and in principle could be developed to investigate complex protein mixtures. Indeed, recently the use of SEC hyphenated to ICP atomic-emission spectrometry has been successfully applied for metalloproteome studies of human plasma.[51] The reported results highlight the different reactivity of cisplatin and the antimetastatic ruthenium-based drug RAPTA-T. The latter compound showed a preferential binding for Tf with respect to HSA, while cisplatin was less selective, being able to bind both proteins to a similar extent. Differences in the selectivity of cisplatin and RAPTA compounds were already identified using an ESI-MS based approach to study protein mixtures.[52, 53] In particular, cisplatin was found to be moderately reactive towards the protein without any discrimination/selectivity, whereas the RAPTA compound was considerably more reactive and could also bind selectively. Such selectivity has important implications for the mode of action of the metallodrugs in the cell, leading to the efficacy of the compound towards specific tumor types, and presumably also for their toxic side effects.

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During the Ph.D. work, the author faced up with the problems involves in the development of analytical methods able to detect and quantify multiple proteins in complex systems under investigation. The scope relies in the idea to reach, one day, the ability to check the state of a given system, by the systemic knowledge of its components. As personal point of view, this is particular important for living system, since their functional properties emerges only at various level of organization in complex networks. Taking into account the role of proteins as expression of all the activities at each level of organization in living systems, the knowledge and the ability to identify, quantify and establish the correct relationships between them and with their environment, represents a possible approach to understand the whole process at the base of life.

Until now, promising steps have been done towards this purpose and proteomics, relates as the study of whole (or well defined) proteomes, play a central role in this task and mass spectrometry, seems nowadays the only technique able to address the way. At this point, is necessary to underline the non availability of a preferential mass spectrometric techniques, since the investigation of complex systems requires versatile tools and taking into account the variety of the proteome, one size does not fit at all. Laws of chemistry, physic or biology can be succesfully apply to explain in detail the characteristic of single components, but in opposite we cannot derive simply from them the behaviour of complex systems, where repetition is just an exception.

After these three years of research in this growing field, a renew enthusiasm surround the idea in my mind regarding the possibility to have access to the knowledge of how simple molecules can talk together and find a such agreement between them to maintain us alive.

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