Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1093 (2005) 36–46
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Screening for metal ligands by liquid chromatography–ligand-exchange–electrospray mass spectrometry

J.G. Krabbe *, H. Lingeman, W.M.A. Niessen, H. Irth

Faculty of Sciences, Division of Chemistry, Department of Analytical Chemistry and Applied Spectroscopy, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

Received 26 April 2005; received in revised form 21 June 2005; accepted 4 July 2005
Available online 12 September 2005

Abstract

Electrospray ionization mass spectrometry is applied for the selective detection of metal ligands after a post-column continuous-flow ligand-exchange reaction. The detection is based on the specific release of a reporter ligand from a metal–reporter ligand complex by a high affinity ligand. Constant infusion and direct-injection experiments are performed to optimize the method. The on-line coupling of a liquid chromatographic separation prior to the continuous flow ligand-exchange reaction enables the screening for high affinity ligands in complex samples. The feasibility of the method is demonstrated by using several ligands with a different affinity for either Cu(II) or Zn(II) ions. The selectivity of the ligand-exchange detection method can be tuned by the choice of the reporter ligand. This is demonstrated by using either 2,2′-bipyridyl or 5-methyl-1,10-phenanthroline as reporter ligands.

Keywords: Mass spectrometry; Metal ligands; Ligand-exchange; Screening; Liquid chromatography

1. Introduction

The importance of electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry in the characterization of metal complexes and organometallic compounds is readily recognized [1]. The adduct formation of analytes with various metal ions is also studied in order to either influence the sensitivity, as in coordination ESI [2,3], or to change the fragmentation reactions of the analytes involved [4,5]. ESI-MS also plays an important role in the study of host–guest complexes, relevant for instance in understanding molecular recognition and in the field of supramolecular chemistry [6–8].

However, since ESI is a liquid-based ionization technique, it can also be used for the continuous monitoring of liquid-phase reactions, e.g., ligand-exchange reactions with metal ions, e.g., see [9]. In principle, this enables the study of ligand–metal interactions in complex (biological) systems. A large variety of biological processes rely on the (non-) reversible binding and dissociation of specific ligands to metal ions, i.e., on ligand-exchange reactions. An example, crucial to life, is the uptake, transport and delivery of oxygen and carbon dioxide by hemoglobin, containing an iron–porphyrin active site.

In fact, the study of ligand-exchange reactions by ESI-MS opens a number of research opportunities. A continuous-flow ligand-exchange reaction can be applied to assess (relative) affinities of various ligands to a certain metal ion, or alternatively the (relative) affinities of various metal ions for a particular ligand. While this may be useful for fundamental purposes, it also gives way to various more practical applications. For instance, it allows the development of screening and analysis methods based on ligand-exchange reactions. Recently, we demonstrated the selective detection of phosphorylated peptides in peptide mixtures separated by liquid

* Corresponding author. Tel.: +31 205987537; fax: +31 205987543.
E-mail address: jg.krabbe@few.vu.nl (J.G. Krabbe).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.07.022

chromatography, based on the high affinity of a phosphogroup to Fe(III) metal ions [10]. Upon mixing the column effluent in a continuous-flow reactor with an Fe(III)-reporter ligand complex, the presence of a high-affinity phosphorylated peptide in the effluent will release the reporter ligand from its complex with Fe(III). Using ESI-MS, not only the change in the free reporter ligand concentration can be measured and quantitated, but also the identity of the phosphorylated peptide involved may be established [10].

The ability to monitor (relative) affinities of ligands to metal ions may also be applied in the discovery of new drugs targeting metalloproteins [11,12] as well as in the optimization of other systems of homogeneous catalysis [13]. The biological activity of a variety of enzymes is based on catalytic metal–ligand complexes, for instance the cytochrome P450 enzyme complex [14], in which the iron–porphyrin active site catalyses the oxidation of endogenous and exogenous molecules in the cell, and is thus heavily involved in phase I drug metabolism.

A more common approach to analyze ligands with a certain affinity for metal ions is immobilized metal affinity chromatography (IMAC) [15,16]. A major drawback of this approach is that all ligands are exposed to the immobilized metal ion prior to separation, resulting in the possibility that a high concentration of an high affinity ligand will exclude the simultaneous enrichment of low affinity ligands by occupying all available coordination sites.

In the present study, the potential of ESI-MS in monitoring ligand-exchange reactions is explored from a more general point of view. Competition between various ligands for the metal ions Cu(II) and Zn(II) is investigated in a continuous-flow ligand-exchange reactor coupled to ESI-MS. The ligands can be introduced into the reactor by means of either flow injection or after separation by liquid chromatography. The latter opens the possibility to screen mixtures of potential ligands for their (relative) affinity to a particular metal–ligand complex. Since the reporter ligand can be chosen more freely in ESI-MS, in contrast to for instance in fluorescence based methods, this method opens a way to tune for selectivity of the method. Additionally, upon introduction of ligands MS provides a possibility to monitor not only the reporter ligand, but also dissociation of metal–reporter ligand complexes and formation of metal–ligand of interest complexes. The approach further provides insight in the identity of the complexes formed under different experimental conditions, e.g. with respect to solution pH, organic modifier content, and mixing ratios between metal-ions, reporter ligand, and the ligand investigated.

2. Experimental

2.1. Materials

Acetonitrile and methanol were purchased from Baker (Deventer, The Netherlands) and were purified over a 0.45 μm Millipore filter. The nitrate salts of Cu(II) and Zn(II) and all ligands were purchased from Sigma–Aldrich (Steinheim Germany), except for nicotinamide, 2-(aminomethyl)pyridine and 4-picoline which were purchased from Acros Organics (Geel, Belgium).

2.2. Solution preparation

Stock solutions of 10 mM of the ligands were prepared in methanol, except for 4,7-dimethyl-1,10-phenanthroline which had a stock concentration of 4 mM. Cu(II) and Zn(II)-solutions were prepared in milli-Q water. The reagent solution was prepared by adding a stock solution of metal ion to a solution of ligand in ammonium formate solution (5 mM; pH 6.3) containing 50% methanol. The actual concentrations of the reagent solutions used are indicated either in the text or in the legend of the figure.

2.3. Setup for infusion experiments

The setup for the preliminary infusion experiments on the on-line mixing of solutions of metal salts and ligands is shown in Fig. 1.

The system consists of a Shimadzu (‘s Hertogenbosch, The Netherlands) LCMS-2010A, and a separate Shimadzu LC-10Ai pump (pump 2). The LCMS-2010A consisted of two LC-10ADvp pumps (pumps 1a and 1b), a SCL-10ADvp system controller, a SIL-10ADvp autosampler, a CTO-ACvp column oven and a single quadruple mass spectrometer equipped with an ESI-probe. The binary pumps 1a and 1b are used to generate either a metal ion or a ligand concentration gradient at 100 μL/min. Thus, pump 1a is pumping the running buffer consisting of methanol/5 mM ammonium formate (50/50, v/v) and pump 1b is pumping a certain concentration of either metal ions or ligand in running buffer. The LC-10Ai pump (pump 2) delivers at 100 μL/min either the ligand or the metal ion solution at a fixed concentration. The total flow into the home-made 40 μL, 0.18 mm I.D. knitted PEEK reaction coil and the ESI-MS was 200 μL/min. The reaction coil is thermostated at 60 °C by means of a water bath.

2.4. Setup for direct-injection and LC–ligand-exchange–ESI-MS

The general set-up applied in experiments involving direct-injection and LC in combination with the continuous-flow post-column ligand-exchange reaction coupled to ESI-MS is very similar to the direct infusion system. An autosampler and an LC column are placed between the binary gradient pump 1 and the T-piece for the mixing of the solution from pump 2 (see Fig. 1). For direct-injection experiments, the LC column is replaced by a short PEEK tubing.

In the continuous-flow post-column detection system, the metal/reporter ligand solution (100 μL/min) is continuously mixed with a sample carrier flow (100 μL/min), in which

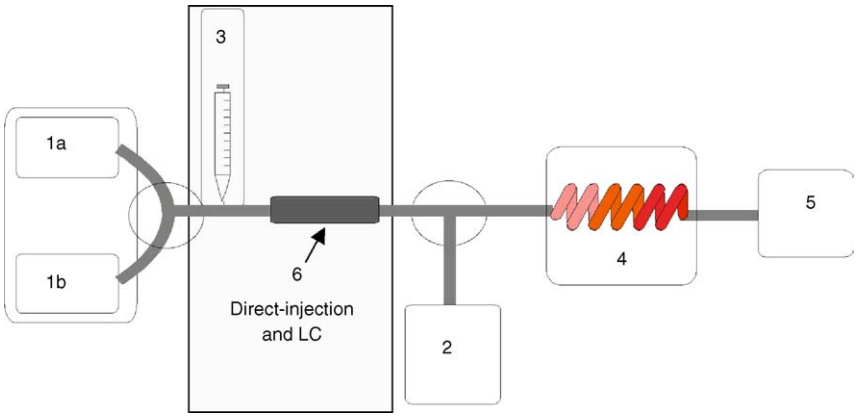

Fig. 1. Schematic drawing of the set-up used in infusion, direct-injection and LC experiments. For the direct infusion experiments, pumps (1a and 1b) generate a concentration gradient of either metal ions or a ligand at a flow-rate of 100 μL/min (unless otherwise reported), pump (2) continuously delivers the ligand or metal ion of interest at 100 μL/min (unless otherwise reported), (4) a 40 μL thermostated reaction coil, and (5) the ESI-MS instrument. For the direct infusion and LC-experiment additionally (3) an autosampler and column (6) are added to the system. In these cases pumps (1a and 1b) generate a sample carrier-flow or a gradient (100 μL/min LC-gradient or running buffer unless otherwise reported); and (2), the reagent pump provides 100 μL/min metal ion-reporter ligand solution (unless otherwise reported)).

either pure compounds are directly injected, or the column effluent from the LC. In the LC-experiments with Cu(II), a 100 mm × 2.1 mm I.D. Luna Phenomenex C8(2) reversed-phase LC column (3 μm particles) is applied, and in the LC-experiments with Zn(II), a 50 mm × 1 mm I.D. Luna Phenomenex C18(2) reversed-phase LC column (3 μm particles). The gradients used in the LC-experiments are reported in text and in the legend of the figure. The mixing of the two flows is improved by a home-made 40 μL, 0.18 mm I.D. knitted PEEK reaction coil placed in a thermostated bath of 60 °C.

2.5. Mass spectrometry settings

MS detection was performed in positive-ion ESI. The probe voltage was 4 kV. Nitrogen (99.999 % purity, Praxiar, Oevel, Belgium) gas flow was set at 1.5 L/min. A nitrogen counter gas flow was set at 0.06 MPa. It was applied for better solvent evaporation. The curved desolvation line (CDL) temperature and the block temperature were set at 200 °C. ESI-MS data were acquired by switching between full-spectrum mode and selected-ion monitoring (SIM), detecting a number of specific *m*/*z*-values related to the reporter ligand, the metal–reporter ligand complex, the ligand of interest and/or complexes of the metal-ion and the ligand of interest. In addition to the ligand and complex *m*/*z*-values, a system monitoring compound (SMC) was continuously detected in SIM during the direct injection and LC-experiments.

3. Results and discussion

3.1. General considerations

The setup of the continuous-flow ligand-exchange system (see Fig. 1) is based on the continuous monitoring by means of ESI-MS of the ligand-exchange reaction between a metal–reporter ligand complex and a ligand of interest. If the ligand of interest has a high affinity to the metal ion, it forms complexes with the metal ion and thereby releases the reporter ligand. As a result, the free reporter ligand concentration increases and the concentration of metal–reporter ligand related complexes decreases.

Cu(II) and Zn(II) were chosen as metal ions since they play an important role in various processes in biochemistry [14,17,18], and because they exhibit a distinct isotope patterns, which simplifies the detection of complexes in the mass spectra. A variety of ligands were chosen in order to have a wide range of affinities between the ligands and the metal ions (see Table 1). Since the complex formation with these metal ions and ligands is almost instantaneous and mainly dependent on mixing, the reaction coil volume, which was added for better mixing can be kept small. This is favorable with respect to the reduction of band broadening.

Preliminary experiments demonstrated that the presence of methanol or acetonitrile as an organic modifier did not significantly influence the ligand-exchange reaction. In this case methanol was added to achieve better electrospray ionization conditions.

Buffering of the solution pH must be performed in order to achieve stable complex formation and ionization conditions. In most cases, the complex formation constants are pH-dependent, because at a pH below the pK_a of the ligand the complex formation is influenced by competition for the ligand between H^+ and the metal ion. Generally, a low pH is favorable in positive-ion ESI-MS, because it provides better protonation of most ligands. On the other hand, at pH higher than 7, insoluble metal hydroxides may be formed. A pH of 6.3 (pH before mixing with methanol) resulted in a good compromise between the ligand-exchange reaction and ESI-MS response.

In attempt to identify unexpected ion suppression upon analyte injection, pyridine was continuously introduced and

Table 1
Stability constants of ligands used in this research (obtained from NIST Database 46; Critically selected Stability Constants of Metal Complexes, Version 8 (2004))

Ligand	pK_a	m/z $[M+H]^+$	Metal ion	K1	β2	β3
Pyridine	5.24	80	Cu(II)	2.54	4.38	5.7
			Zn(II)	1.05	1.45	
4-Picoline	6.05	94	Cu(II)	2.88	5.07	6.77
			Zn(II)	1.40	2.11	2.85
2-Aminomethyl-pyridine	8.65	109	Cu(II)	9.50	17.2	
			Zn(II)	5.28	9.44	12.3
Benzimidazole	5.56	119	Cu(II)	3.43	6.41	8.92
			Zn(II)	1.61		
Nicotinamide	3.31	123	Cu(II)	1.65	2.69	3.19
			Zn(II)	0.78	1.15	
2,2′-Bipyridyl	4.41	157	Cu(II)	8.12	13.63	17.0
			Zn(II)	5.34	9.96	14.0
1,10-Phenanthroline	4.92	181	Cu(II)	9.13	15.84	21.0
			Zn(II)	6.38	12.08	17.3
5-Methyl-1,10-phenanthroline	5.27	195	Cu(II)	8.55	15.0	20.1
			Zn(II)	6.62	12.6	18.3
4,7-Dimethyl-1,10-phenanthroline	5.95	209	Cu(II)	8.76	16.0	22.0
			Zn(II)	6.90	13.1	19.1

monitored as a system monitoring compound (SMC) [10,19]. Pyridine has similar ionization characteristics as most of the ligands studied, but shows low affinity to Cu(II) or Zn(II) and does not interfere in the ligand-exchange reaction.

3.2. Preliminary infusion experiments

Initially, infusion experiments were performed, using pumps 1a and 1b, see Fig. 1, for either a flat concentration gradient of a metal ion with a fixed concentration of ligand via pump 2, or vice versa. From a fundamental point-of-view, a general concern in developing a continuous-flow ligand-exchange reaction with ESI-MS detection is that the complexes observed in the electrospray mass spectrum may not represent the complexes formed in solution, e.g., due to in-source fragmentation or selective ion suppression. Although good agreement between the complexes observed in the mass spectrum and the complexes formed in solution was demonstrated in various cases [20,21], we decided to perform some additional studies, taking advantage of the production of the complexes in a continuous-flow reactor with continuous monitoring of the complexes formed by means of ESI-MS. We also considered that the picture on complex formation might be obscured by issues related to different ionization efficiencies of ligands and metal–ligand complexes with different composition ratios. Therefore, a computer program was developed enabling the prediction of the (relative) concentrations of the various complexes under the prevailing conditions in the metal ion or ligand concentration gradient system. These theoretical complex formation profiles were compared with experimental ones, acquired in the infusion experiments. In general, the agreement of experiment with theory was good, as demonstrated by a typical example of Zn(II) and 5-methyl-1,10-phenanthroline in Fig. 2a (experimental) and Fig. 2b (theory), respectively. These experiments also enabled us to have a detailed look at the various complexes formed under these conditions. Fig. 2c shows the result of the reversed experiment where the concentration of ligand is kept constant and a metal concentration gradient is applied in time. One of the interesting issues revealed in such experiments between Cu(II) and 2,2′-bipyridyl is the observation of a Cu(I)–2,2′-bipyridyl complex, see trace 4 in Fig. 2c. It is known [22], that in the presence of methanol Cu(II)-ions are reduced to Cu(I)-ions. The reduction is slow in solution, but quite fast in electrospray ionization. The infusion experiments provide information about the possibility to use the specific ligand as a reporter ligand. Furthermore, also certain m/z-traces can be obtained, which can function as additional reporter traces, and can be correlated to metal–ligand complexes.

When studying metal–ligand interactions in general the kinetics of complex formation and dissociation should be kept in mind. One can think for instance of a ligand with a high affinity for the metal ion but slow formation kinetics. Despite the high affinity of the ligand to the metal ion, complex formation will not be observed when using a continuous flow set-up described in this paper.

3.3. Direct-injection–ligand-exchange–ESI-MS system

The direct injection of ligands of interest in a continuous flow ligand-exchange–ESI-MS detection provides the possibility to monitor specific m/z-values in SIM mode, enabling the simultaneous detection of the reporter ligand, the

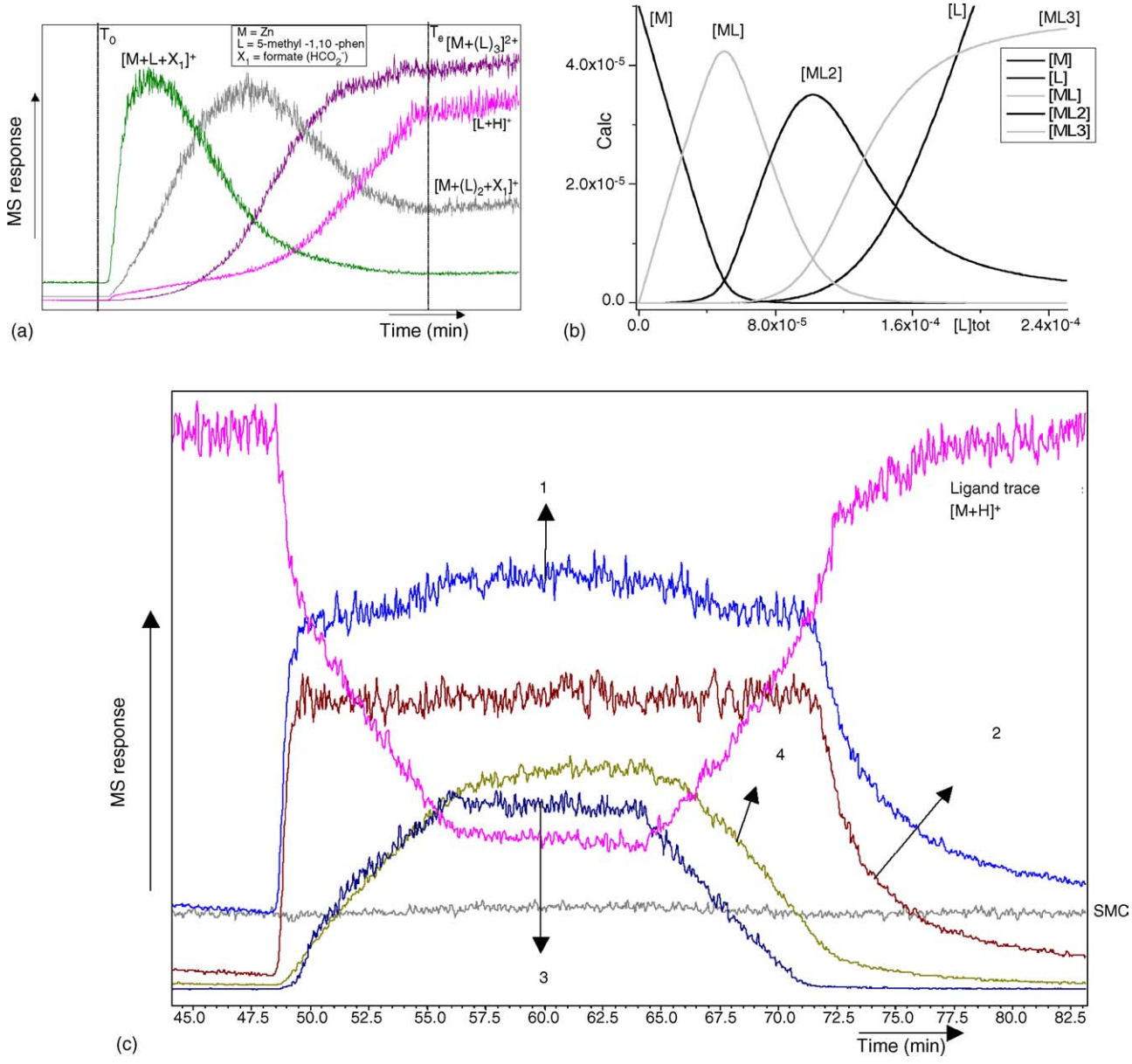

Fig. 2. (a) Complexation profile of Zn(II) and 5-methyl-1,10-phenanthroline. 50 μM of Zn(II) is continuously pumped at 100 μL/min. The gradient starts (T_0) at after 5 min from 100% running buffer in 60 min upto 100% 5-methyl-1,10-phenanthroline solution (250 μM) (T_e). (b) Theoretical calculations for Zn(II) and metal ion and 5-methyl-1,10-phenanthroline. For comparison with (a). The calculated concentration of the different complexes ([Calc] μM) are plotted against the total concentration of introduced ligand ([L_{tot}] μM). (c) Reversed complexation profile of Cu(II) and 2,2′-bipyridyl. 100 μM 2,2′-bipyridyl in running buffer (methanol/5 mM ammonium formate (50/50, v/v)) is pumped continuously at 100 μL/min (pump 2). The gradient starts at 48.5 min from 100% running buffer in 8 min upto 100% Cu(II) solution (50 μM) followed by a continuous flow of 100% Cu(II) solution for 8 min and than decreasing again to 100% running buffer in 8 min. (1) $[Cu(II)(bipy)(HCOO^-)]^+$; (2) $[Cu(II)(bipy)_2(HCOO^-)]^+$; (3) $Cu(II)(bipy)_2(NO_3^-)]^+$; and (4) $[Cu(I)(bipy)_2]^+$.

metal–reporter ligand complex, the ligand of interest and/or complexes of the metal-ion and the ligand of interest. In analyzing mixtures of unknown compounds for their affinity to a metal ion, the ESI-MS system may provide additional structural information about the unknown ligands as well.

Low-affinity ligands show low or no complex formation, whereas high-affinity ligands form complexes with the metal ion. Therefore, the relative affinity of a ligand for the metal ion can be derived from the changes in the ESI-MS trace of the free reporter ligand concentration or the dissociation of the reporter–ligand-complex traces. It is a relative affinity, as it relates to the affinity of the reported ligand for the metal ion. This means that one can actually tune the ligand-exchange reaction, depending on the application (see below). Several parameters influence the response of the system [10], e.g., the solvent pH, as discussed above, and the ratio between the metal ion and reporter ligand concentrations. A significant excess of reporter ligand increases the background signal resulting in a higher noise. An excess of metal ion results in unoccupied ligand binding sites leading to a reduced release

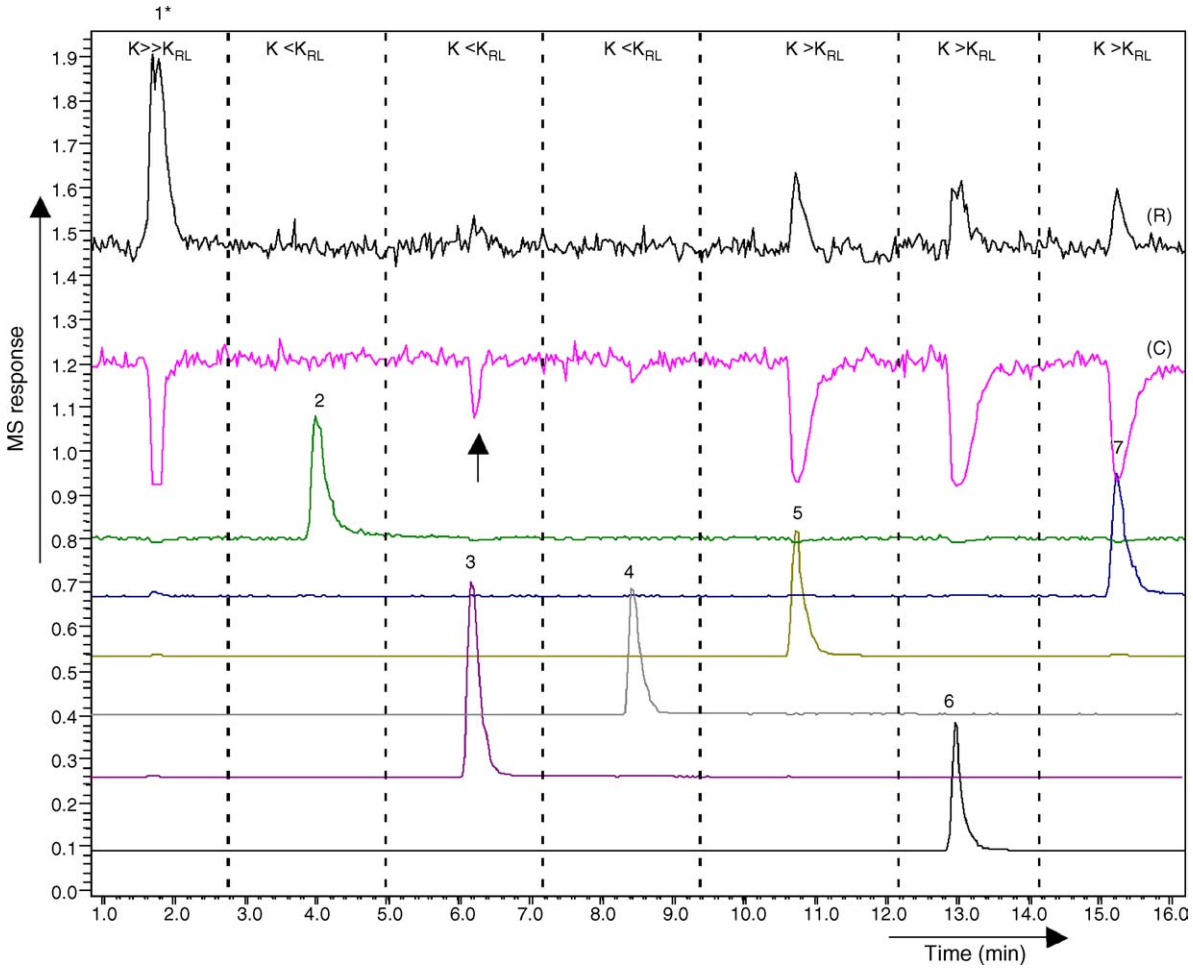

Fig. 3. Direct-injection–ligand exchange–MS experiments with specific ligands. Numbers indicate the traces of protonated ligands injected. (1) EDTA*; (2) 4-picoline; (3) benzimidazole; (4) nicotinamide; (5) 1,10-phenanthroline; (6) 5-methyl-1,10-phenanthroline; (7) 4,7-dimehtyl-1,10-phenanthroline. R, indicates the reporter ligand trace (2,2′-bipyridyl, $[M + H]^+$; *m/z* 157) and C, indicates the complex related trace ($[Cu(II)(2,2'\text{-bipyridyl})(HCO_2^-)]^+$; *m/z* 264). Reagent conditions; 50 μM Cu(II)-$(2,2'\text{-bipyridyl})_2$ in methanol/5 mM ammonium formate (50/50, v/v); flow-rate 200 μL/min. Mass spectrometric conditions; SIM and scan in the positive mode. All ligands had a concentration of 100 μM (absolute introduced amount 1 nmol). *Since EDTA has an overall negative charged under these conditions, it is not observed in positive electrospray mass spectrometry.

of reporter ligand in the ligand-exchange reaction. For optimum response, bidentate reporter ligands were mixed with metal ions in a concentration ratio of two to one.

3.4. Direct-injection–ligand-exchange–ESI-MS with Cu(II)

As a first test of the system, direct injection of some blank solvent, of EDTA, which is a known strong complexing agent for Cu(II), and some other ligands of interest were performed. The ligand 2,2′-bipyridyl was used as reporter ligand. Some results are summarized in Fig. 3. Upon injection of EDTA, a positive response is observed in the reporter ligand trace (at *m/z* 157), indicating that a ligand-exchange reaction occurred. At the same time, a loss of response was observed in the trace related to the Cu(II)-2,2′-bipyridyl complex trace (at *m/z* 264). No response change was observed upon injection of the blank.

Another important issue, demonstrated in Fig. 3, is that not all of the injected ligand forms complexes with the metal ions. A fraction of the ligand continues to exist as a free ligand in solution, and can thus be monitored by ESI-MS. Some relevant traces of the protonated molecules of injected ligand are also shown in Fig. 3. When dealing with unknown ligands, this feature can be used for identification purposes.

In general, injection of ligands with an affinity to the metal ion comparable to or higher than the reporter ligand, e.g., 1,10-phenanthroline, 5-methyl-1,10-phenanthroline, and 4,7-dimethyl-1,10-phenanthroline, resulted in a positive response in the reporter trace and a negative response in the complex related trace, whereas the low affinity ligand, e.g., 4-picoline, and nicotinamide, did not show any response in either the reporter ligand trace or in the complex related trace (cf. Table 1). Benzimidazole does show a negative response, however, the negative signal is related to ion suppression, since a negative response is also observed in the SMC-trace.

Both low and high affinity ligands are observed at their specific *m*/*z*-value as protonated molecules. Additional increasing responses could be observed at *m*/*z*-values not related to the Cu(II)–2,2′-bipyridyl complex, but in fact related to the formation of a complex between the injected ligand and Cu(II) (or Cu(I), see above). It must be mentioned, that some complexes formed, e.g., the Cu(II)–EDTA complex, are not observed in positive-ion ESI-MS, because they carry an overall neutral or negative charge.

Compared to other high-affinity ligands tested, EDTA gives a higher reporter ligand response but a lower response change of the metal–reporter ligand complexes. A possible explanation is that the hexadentate EDTA upon complex formation with a metal ion releases one, two or possibly even three bidentate reporter ligands. On the other hand, the bidentate 1,10-phenanthroline ligand, for instance, displaces only one reporter ligand. This implies that the choice of the reporter ligand can also have a distinct influence on the sensitivity of the system as a detector for the injected ligands. In this respect, the number of coordination sites of the metal ion and especially the nature of the reporter ligand as a monodentate or polydentate ligand is very important. With a monodentate reporter ligand, a bidentate ligand of interest may (depending on kinetics and steric hindrance) in the ligand-exchange reaction release two reporter ligands and therefore enhance the response of the ligand-exchange detection, measured from the change in the reporter ligand concentration, by a factor of two.

3.5. Tuning selectivity

In the past, ligand-exchange reaction detection systems based on fluorescence detection were described [23,24]. Compared to such a system, the proposed method based on ESI-MS has major advantages in a greater freedom in choosing the reporter ligand. The ability to protonate or deprotonate a ligand for ESI-MS detection is more common than the presence of a fluorophore in the ligand. In applications of the ligand-exchange reactor for the monitoring of biological reactions, this advantage will be even more pronounced, because in such cases often derivatization had to be applied to enable fluorescence detection of the relevant reporter ligands [19].

The greater flexibility in the choice of the reported ligand can actually be exploited to tune the selectivity of the ligand-exchange detector to include or exclude ligands with relatively low affinities for the metal ion. For instance, when a reporter ligand with a moderate affinity to the metal ion is chosen, all injected ligands with an affinity higher than the reporter ligand (at a certain concentration) will give a response in the ligand-exchange detector. On the other hand, when a high-affinity reporter ligand is chosen, some of the ligands giving response in the system with the moderate-affinity reporter ligand, are not detected anymore by the ligand-exchange detection system. This opens the possibility to actually determine relative binding affinities of new ligands by injection in ligand-exchange systems with different reporter ligands.

To demonstrate this in practice, two ligands with different affinity were chosen to serve as reporter ligands. In each system, a set of ligands with different affinities were introduced. Some results of monitoring both the reporter ligand at its specific *m*/*z* and its complex with the metal ion are shown in Fig. 4. The data clearly show that the complex traces related to the metal–reporter complex give different responses with different reporter ligands. In Fig. 4a, 5-methyl-1,10-phenanthroline is used as a ligand of interest and 2,2′-bipyridyl as reported ligand. Since 5-methyl-1,10-phenanthroline has a higher affinity to Cu(II) than 2,2′-bipyridyl, a positive response is seen in the reporter ligand trace. On the other hand, when 2,2′-bipyridyl is used as a ligand of interest, no response is observed in the 5-methyl-1,10-phenanthroline (reporter ligand) trace (see Fig. 4b).

Upon injection of benzimidazole as a ligand, a negative response was observed in the metal–reporter trace, as indicated by the arrows in Fig. 4a and b. This decrease in response is not corresponding with ligand-exchange, but is due to ion suppression. A similar decrease was observed in the trace of the system monitoring compound (data not shown).

It is tempting to qualitatively or even quantitatively correlate the relative intensity of the response with the relative stability constants, i.e., to investigate whether a ligand with a higher affinity results in a higher release of reporter ligand. Table 2 displays the limit of detection (LOD) for the different injected ligands with both ligand-exchange reactions. It is clear that under the given conditions similar concentrations of low affinity ligands will not give a response in the ligand-exchange detection method, whereas the high affinity ligands do. While a qualitative correlation appears to be observed, drawing conclusions must be done with great care, because the actual response is the result of various interrelated parameters. As indicated above, the relative release of reporter ligands is determined by the character of the injected ligand (mono- or polydentate) and of the reporter ligand. In addition, the ESI-MS response of a certain concentration of the reporter ligand might be influenced by other components in the mixture, i.e., a certain injected ligand might result in a relative suppression of the reporter ligand response without acting as a clear ion suppression agent. In addition, some of the complexes formed will have no net positive charge and are not detected by positive-ion ESI-MS at all. A complete quantitative assessment of the responses obtained in the ligand-exchange reactor is beyond the scope of the present study.

3.6. LC–ligand-exchange–ESI-MS with Cu(II)

A possible application area of the ligand-exchange ESI-MS detection system is in the screening of (complex) mixtures, e.g., combinatorial libraries, biological samples, or natural extracts, for ligands with particular affinities to certain metal ions. This might for instance be useful to screen

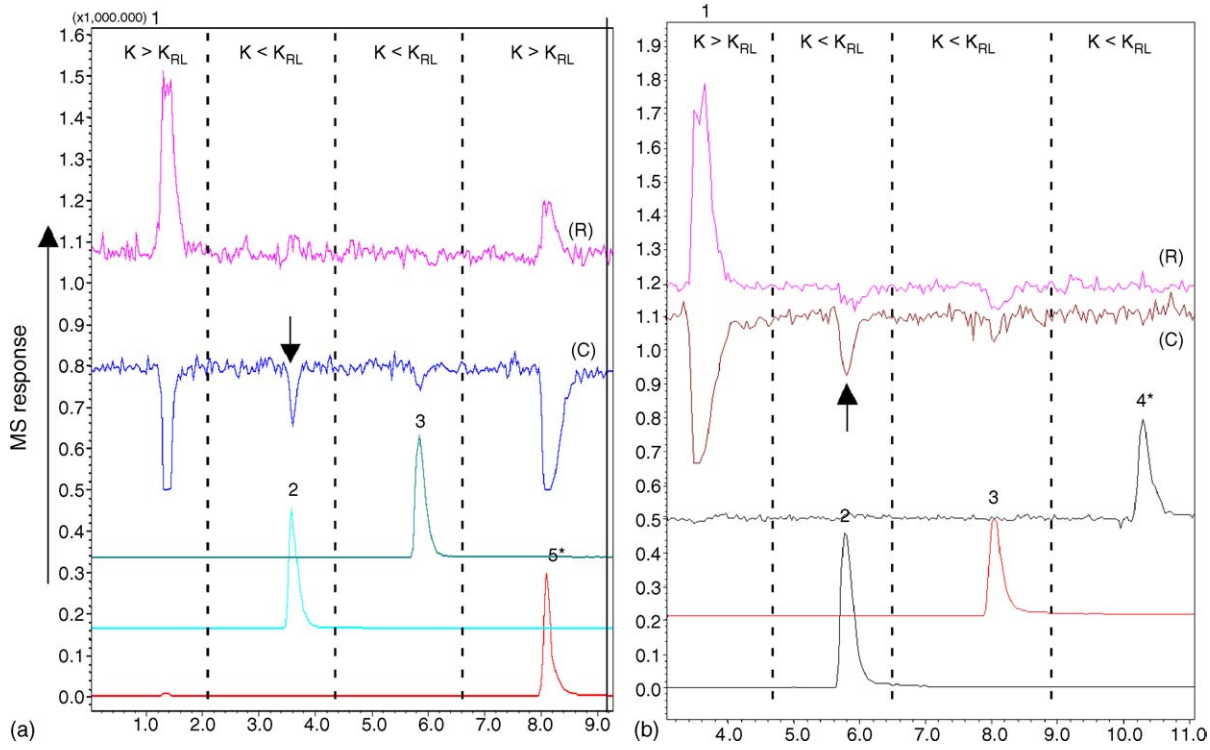

Fig. 4. Responses of the ligand-exchange detection of injections of ligands with different affinities for Cu(II). Numbers indicate the traces of protonated ligands injected. (1) EDTA ($K \gg K_{RL}$), (2) benzimidazole, (3) nicotinamide, (4) 2,2′-bipyridyl, (5) 5-methyl-1,10-phenanthroline. Two different ligands are chosen as reporter ligand (RL). (a) Reagent conditions; 50 μM Cu(II)–$(2,2'\text{-bipyridyl})_2$ in methanol/5 mM ammonium formate (50/50, v/v); (R) = RL = 2,2′-bipyridyl ($[M+H]^+$; *m/z* 157); (C) = complex related trace $(Cu(II)(2,2'\text{-bipyridyl})(HCO_2^-))^+$. (b) Reagent conditions; 50 μM Cu(II)–$(5\text{-methyl-1,10-phenanthrolinel})_2$ in methanol/5 mM ammonium formate (50/50, v/v); (R) = RL = 5-methyl-1,10-phenanthroline ($[M+H]^+$; *m/z* 195); (C) = complex related trace $(Cu(II)(5\text{-methyl-1,10-phenanthroline})(HCO_2^-))^+$;*reporter ligand in the other experiment. All ligands had a concentration of 100 μM (absolute introduced amount 1 nmol).

for inhibitors of metal-containing enzymes or other metal-based homogeneous catalytic systems. Screening of complex mixtures can be performed via an on-line coupling with LC in order to separate the mixture prior to introduction into the ligand-exchange reactor. Because a fraction of the ligand introduced in the reactor is not involved in the ligand-exchange reactor, on-line ESI-MS and MS–MS detection enables the identification of the unknown compounds in a mixture with affinity to the metal ion. This can be performed in the same run.

To test the efficacy of on-line LC–ligand-exchange–ESI-MS detection, various experiments were performed. Gradient elution, with either methanol or acetonitrile as organic modifier, was used to obtain an acceptable separation of a test mixture of ligands. Although both acetonitrile and methanol can be used as organic modifiers, methanol is used in further on-line LC experiments.

Fig. 5 displays the chromatogram obtained when a test mixture of ligands with different affinities to Cu(II) is analyzed by means of the LC–ligand-exchange–ESI-MS detec-

Table 2
Estimated limit of detection (LOD) (signal-to-noise ratio (S/N) = 3) for ligand-exchange detection of the different ligands using two different reporter ligands with corresponding different affinities

	R.L.: 2,2′-bipyridyl		R.L.: 5-methyl-1,10-phenanthroline	
Introduced ligands	S/N (100 μM)	Estimated LOD (μM)	S/N (100 μM)	Estimated LOD (μM)
4-Picoline	N.D.	–	N.D.	–
Benzimidazole	N.D.	–	N.D.	–
Nicotinamide	N.D.	–	N.D.	–
2,2′-Bipyridyl	R.L.	–	N.D.	–
1,10-Phenanthroline	5.17	57	4.57	66
5-Methyl-1,10-Phenanthroline	4.62	65	R.L.	–
4,7-Dimethyl-1,10-Phenanthroline	4.13	73	3.68	81
EDTA	13.2	20	19.8	15

R.L., reporter ligand; N.D., not detectable. The LODs are based on the response of the reporter ligand.

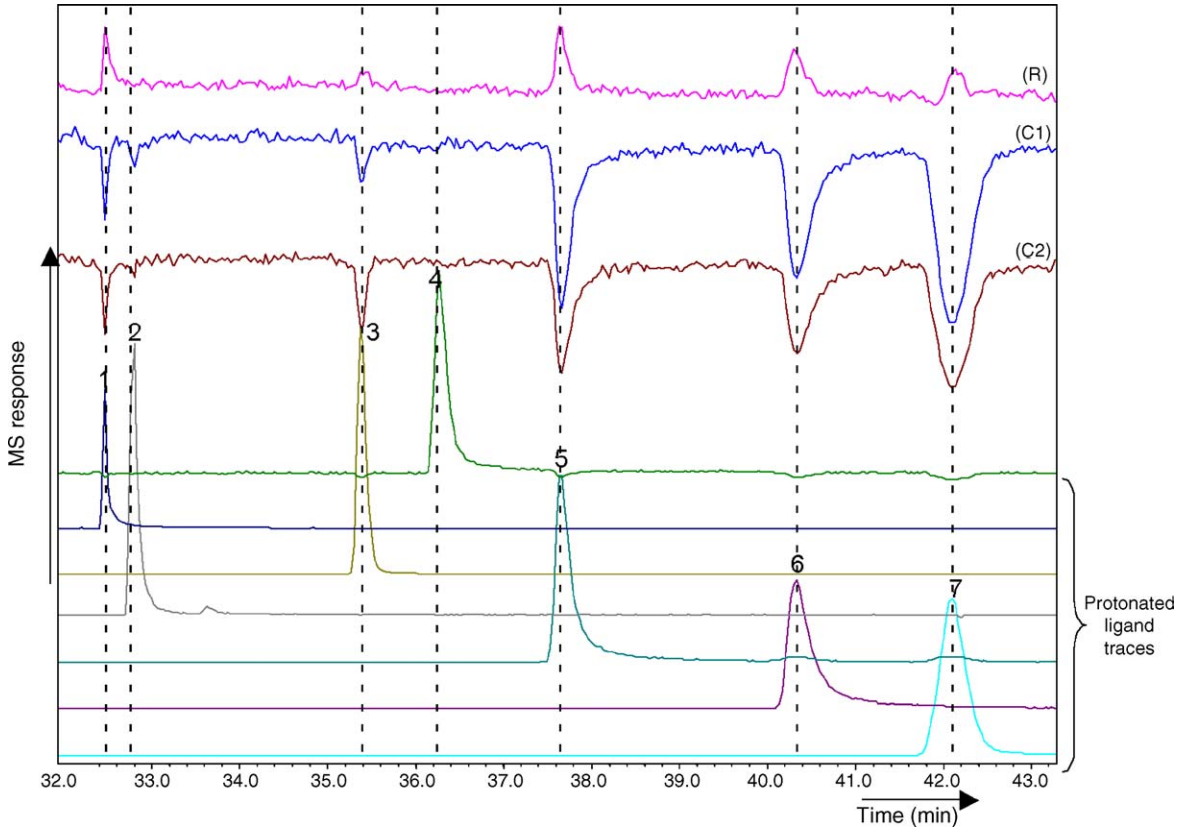

Fig. 5. LC–ligand-exchange–ESI-MS chromatogram for ligands with an affinity for Cu(II). (R), reporter ligand trace ($[M+H]^+$; *m/z* 157); (C1), $[Cu(II)(2,2'\text{-bipyridyl})(HCO_2^-)]^+$ and (C2), $[Cu(II)(2,2'\text{-bipyridyl})_2(HCO_2^-)]^+$. LC conditions; 100 mm × 2 mm I.D. C8 (3 μm particles). A gradient ran from 100% A to 100% B in 2 min and remained stable at 100% B for 10 min at a total flow of 100 μL/min. A, methanol/5 mM NH_4HCO_2 (pH 6.3) (35/65, v/v); B, methanol/5 mM NH_4HCO_2 (pH 6.3) (50/50, v/v). Reagent conditions; 50 μM $Cu(II)(2,2'\text{-bipyridyl})_2$ in B. The injected mixture contained: (1) 2-(aminomethyl)-pyridine; (2) nicotinamide; (3) benzimidazole; (4) 4-picoline; (5) 1,10-phenanthroline; (6) 5-methyl-1,10-phenanthroline; and (7) 2,9-dimethyl-1,10-phenanthroline. All ligands had a concentration of 100 μM (absolute introduced amount 1 nmol).

tion method. In this experiment, 2,2′-bipyridyl was chosen as the reporter ligand.

In the top trace in Fig. 5, the reporter ligand trace is given, showing response changes upon elution of ligands with higher affinity to Cu(II). In the middle, a trace related to the Cu(II)-reporter ligand complexes, also showing response changes upon elution of ligands with higher affinity to Cu(II). The lower traces show a 'conventional' LC–ESI-MS chromatogram of the separated ligands in the mixture. Peaks that show up in the lower trace, but do not appear in the upper two traces, correspond to compounds with a low affinity to Cu(II), e.g., nicotinamide and 4-picoline (cf. Table 1).

3.7. LC–ligand-exchange–ESI-MS with Zn(II)

Most of the results discussed in this paper were obtained using Cu(II) as metal ion. Obviously, the ligand-exchange detection principle is not limited to the use of Cu(II) alone. LC–ligand-exchange–ESI-MS detection based on reactions of phosphopeptides with Fe(III)-MCB (methylcalcein blue) complexes was reported earlier [10]. Some of our results with Zn(II) are discussed in this section. Zn(II) is a common Lewis acid in biological systems and is involved in all kind of biochemical reactions [17]. Compared to Cu(II)-complexes, Zn(II)-complexes are generally weaker, which might pose a challenge in the ligand-exchange detection.

The setup used in monitoring Zn(II)-related ligand exchange is identical to the one used in the detection for Cu(II)-related ligand exchange (see Fig. 1). Like with Cu(II), also Zn(II)-complexes can be easily recognized by means of their specific isotope pattern. Preliminary experiments, such as a study of the complex formation profiles and direct injections of ligands with different affinities for Zn(II), were performed (data not shown). This demonstrated that a system based on Zn(II) can be developed with the same ease as a system based on Cu(II).

Fig. 6 shows a chromatogram obtained after LC separation of a test mixture of ligands and a Zn(II) ligand-exchange–ESI-MS detection. The same type of traces are shown as in Fig. 5 for the Cu(II) system. Again, we only detect a positive increase in the reporter ligand trace and an decrease in signal in the metal–reporter ligand complex trace when a ligand of interest was present which had a higher affinity to Zn(II). Under those conditions a ligand-exchange reaction occurred.

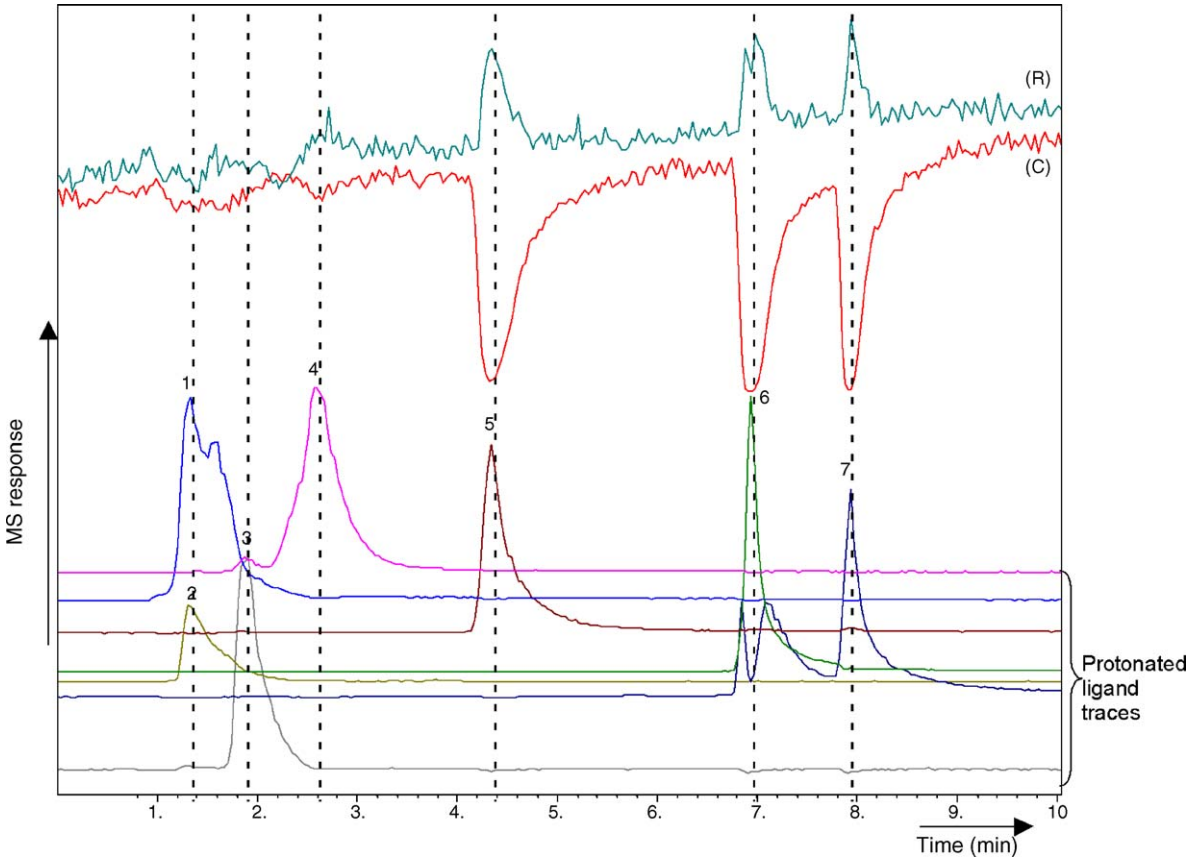

Fig. 6. LC–ligand-exchange–ESI-MS chromatogram for ligands with an affinity for Zn(II). (R), reporter ligand trace ($[M+H]^+$; m/z 157); (C), complex related trace [Zn(II)–(2,2′-bipyridyl)(HCO_2^-)]$^+$. LC conditions; 5 mm × 1 mm I.D. Phenomenex Luna C18(2) (3 μm particles). A gradient was operated at 50 μL/min between pumps 1a and 1b. The gradient ran from 20% B upto 85% B in 6 min where A was, methanol/5 mM NH_4HCOO (pH 6.3) (20/80, v/v); B, methanol/5 mM NH_4HCOO (pH 6.3) (80/20, v/v). Reagent conditions; 50 μM Zn(II)–$(2,2'\text{-bipyridyl})_2$ in methanol/5 mM NH_4HCOO (pH 6.3) (50/50, v/v). The injected mixture contained: (1) 2-(aminomethyl)-pyridine; (2) nicotinamide; (3) pyridine (also SMC); (4) 4-picoline; (5) 1,10-phenanthroline; (6) 5-methyl-1,10-phenanthroline; and (7) 4,7-dimethyl-1,10-phenanthroline. The concentration of the ligands was 100 μM (absolute introduced amount 1 nmol).

In general, the LC mobile-phase composition might interfere with the ligand-exchange detection. For instance, LC performed under acidic conditions can interfere since a ligand may be protonated under these conditions. Buffer comaptibility issues may be overcome by choosing a suitable buffer in the ligand-exchange reaction to control the pH of the ligand-exchange reaction. Of course, the current methodology requires compatibility of the buffer used with electrospray MS detection.

4. Conclusions

In the present paper, we have demonstrated that by on-line coupling of the ligand-exchange–ESI-MS detection with an LC separation, it is possible to screen mixtures of ligands for their relative affinity to a particular metal–reporter ligand complex. Although the interactions of selected ligands with Cu(II) and Zn(II) was investigated, the approach can be considered as a template to study selective interactions between other ligands and other metal ions in a similar way.

The use of ESI-MS for detection allows a free choice of reporter ligand, and therefore the affinity of the reporter ligand to the metal ion, compared to other approaches, e.g., based on fluorescence detection. Additionally, MS provides the possibility to monitor not only the reporter ligand, but also dissociation of metal–reporter ligand complexes and formation of metal–ligand of interest complexes. Moreover information about the ligand of interest can be obtained This is especially interesting when dealing with unknown ligands.

During method development, direct-infusion experiments provide insight in the identity of the complexes formed under different experimental conditions, e.g. with respect to solution pH, organic modifier content, and mixing ratios between metal-ions, reporter ligand, and the ligand investigated. The selectivity of the ligand-exchange detection method can be tuned by the choice of the reporter ligand. This is demonstrated by using either 2,2′-bipyridyl or 5-methyl-1,10-phenanthroline as reporter ligands. In case that the system should be used for the screening of low affinity analytes it is preferable to use a low affinity reporter ligand for detection. The same strategy should be followed when

screening of low concentrations of high affinity ligands is considered.

The most significant improvement with respect to the more commonly used method IMAC lies in the possibility to screen a mixture of metal ligands with a large range of affinities. In IMAC, where all ligands are exposed to the immobilized metal ion prior to separation, a large concentration of a high affinity ligand would exclude the simultaneous enrichment of low affinity ligands by occupying all available metal coordination sites. In the current method, ligands are separated prior to the interaction with the metal ion.

Applications of this approach in the screening of (complex) mixtures, e.g., combinatorial libraries, biological samples, or natural extracts, for possible new drugs, catalysts or other ligands with particular affinities to certain metal ions are currently under development.

Acknowledgements

The late Dr. M.W.G. de Bolster from the section of organic chemistry and inorganic chemistry of the Vrije Universiteit Amsterdam is acknowledged for his helpful discussion. Shimadzu Benelux (‘s Hertogenbosch, The Netherlands) is acknowledged for the loan of equipment.

References

[1] J.C. Traeger, Int. J. Mass Spectrom. 200 (2000) 387.
[2] J.R. Seal, C.M. Havrilla, N.A. Porter, D.L. Hachey, J. Am. Soc. Mass Spectrom. 14 (2003) 872.
[3] M. Gaub, A. von Brocke, G. Roos, K.A. Kovar, Phytochem. Anal. 15 (2004) 300.
[4] M. van Stipdonk, V. Anbalagan, W. Chien, G. Gresham, G. Groenewold, D. Hanna, J. Am. Soc. Mass Spectrom. 14 (2003) 1205.
[5] C.P.G. Butcher, B.F.G. Johnson, J.S. McIndoe, X. Yang, X.B. Wang, L.S. Wang, J. Chem. Phys. 116 (2002) 6560.
[6] M. Vincenti, J. Mass Spectrom. 30 (1995) 925.
[7] N.G. Tsierkezos, D. Schroder, H. Schwarz, Int. J. Mass Spectrom. 235 (2004) 33.
[8] M. Satterfield, J.S. Brodbelt, Inorg. Chem. 40 (2001) 5393.
[9] M.L. Reyzer, J.S. Brodbelt, A.P. Marchand, Z.B. Chen, Z.L. Huang, I.N.N. Namboothiri, Int. J. Mass Spectrom. 204 (2001) 133.
[10] J.G. Krabbe, H. Lingeman, W.M.A. Niessen, H. Irth, Anal. Chem. 75 (2003) 6853.
[11] C. Matsumoto, O. Ohmori, T. Shinkai, H. Hori, J. Nakamura, Psychiatr. Genet. 14 (2004) 209.
[12] N. Farrell, Coord. Chem. Rev. 232 (2002) 1.
[13] P. Chen, Angew. Chem. Int. Ed. 42 (2003) 2832.
[14] C. de Graaf, N.P.E. Vermeulen, K.A. Feenstra, J. Med. Chem. 48 (2005) 2308.
[15] J.W. Wong, R.L. Albright, N.H.L. Wang, Sep. Purif. Methods 20 (1991) 49.
[16] L.D. Holmes, M.R. Schiller, J. Liq. Chromatogr. Relat. Technol. 20 (1997) 123.
[17] T. Dudev, C. Lim, J. Am. Chem. Soc. 122 (2000) 11146.
[18] V.E. Vandell, P.A. Limbach, J. Mass Spectrom. 33 (1998) 212.
[19] A.R. de Boer, T. Letzel, D.A. van Elswijk, H. Lingeman, W.M.A. Niessen, H. Irth, Anal. Chem. 76 (2004) 3155.
[20] M.J. Greig, H. Gaus, L.L. Cummins, H. Sasmor, R.H. Griffey, J. Am. Chem. Soc. 117 (1995) 10765.
[21] E.C. Kempen, J.S. Bordbelt, Anal. Chem. 72 (2000) 5411.
[22] A.L. Chaparro, R.W. Vachet, J. Mass Spectrom. 38 (2003) 333.
[23] H. Irth, M. Lamoree, G.J. de Jong, U.A.T. Brinkman, R.W. Frei, R.A. Kornfeldt, L. Persson, J. Chromatogr. 499 (1990) 617.
[24] C.E. Werkhoven-Goewie, W.M.A. Niessen, U.A.T. Brinkman, R.W. Frei, J. Chromatogr. 203 (1981) 165.