ELECTROSPRAY IONIZATION USING MAGNETIC SECTOR MASS SPECTROMETERS

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SUMMARY

Electrospray Ionization (ESI) has become a routine technique of enormous importance for analysis of biomolecules like proteins and peptides. Application of ESI to biological problems can benefit from the mass resolution and mass range of double focusing magnetic-sector mass spectrometers because of their high resolving power and sensitivity. One

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particularity of ES sectors is the complexity of the interface design due to high potentials of sector instruments. Detection limits are related to the mass spectrometer transmission and the detector sensitivity. In the case of sector instruments with array detection, the problem of dispersion can partially be removed. For example, hen egg-white lysozyme was detected at concentrations down to 500 attomol/µl and a high quality spectrum was obtained for only 10 femtomol of sample consumed. Nevertheless, high resolution work on magnetic sector instruments requires very intense and stable ion currents, often not easily achievable when sample amounts are limited. Several improvements have been suggested, on one hand by increasing the efficiency of ion transmission from the source to the analyzer region, on the other by miniaturizing the spray process. In conclusion, magnetic sector instruments seem to have great potential for ESI-MS.

INTRODUCTION

Electrospray Ionization (ESI) has become a routine technique of enormous importance for analysis of biomolecules like proteins and peptides. It has great potential for the sensitive and specific analysis of a wide range of large molecules (e.g. those with molecular weights up to 250 000). The ES phenomenon is described as a process which creates a spray of fine, highly charged droplets in the presence of a strong electric field. The solution, with an electrolyte concentration not exceeding 10^{-3} M, is infused via a stainless steel capillary held to a high voltage (3000V and more) and droplets charging is initiated by the appearance of the Taylor cone /1/. At an appropriate condition of applied potential, the cone tip comes to extend out to form a thin liquid "filament" /2/ which breaks up by successive Coulombic explosions to form individual charged droplets, and desorption of ions occurs in the gas phase towards the analyzer. Several mechanisms of ion desorption from charged droplets have been proposed. In 1968, Dole first had the idea of using ES in mass spectrometry /3/ (MS) and developed the "charged-residue" model. Smith and Huang performed the subsequent applications /4,5/. Some other literature is available /6/ but the mechanism of ionization during the ES process is still undergoing elucidation. Droplets are usually produced at low flow rates comprised in the 1 to 10 µl/min range. The choice of the flow rates depends on the ES ion source design. A

"micro-electrospray" source operates using capillary LC columns with nanoliter flow rates, for example in the 300 to 800 nl/min range /6-8/.

ES sources /3,9-15/ have been successfully interfaced on quadrupole mass analysers /11-16/. ESI of polar biomolecules are observed normally in the m/z range 300 to 2000 Da because of the high charge state, and therefore many applications have been performed using an ES-quadrupole instrumentation, so far the most popular ESMS system. Indeed, they are relatively inexpensive, easy to use, and possess a good mass accuracy. For instance, a mass accuracy of relative molecular mass 0.2 has been obtained for myoglobin (relative mass: 16950.5) using a quadrupole instrument /16/. Mass range, resolution and mass accuracy depend on the instrument used. Problems of dispersion and moderate resolution for some applications constitute the two shortcomings of quadrupole instruments. The ESI sources have been interfaced with other mass analyzers such as Time-of-Flight /17,18/, ion trap /19/, Fourier transform /20/ and magnetic sector mass spectrometry /21-31/. Application of ESI to biological problems can benefit from the mass resolution and mass range of double focusing magnetic-sector mass spectrometers because of their high resolving power and sensitivity.

The first ESI magnetic sector instrument was constructed by Nikolaev /31/ and was only used for small molecules. Good sensitivities were obtained. One particularity of ES sectors is the complexity of the interface design due to high potentials of sector instruments. Detection limits are related to the mass spectrometer transmission and the detector sensitivity. In the case of sector instruments with array detection, the problem of dispersion can partially be removed /32,33/. For example, hen egg-white lysozyme was detected at concentrations down to 500 attomol/µl and a high quality spectrum was obtained for only 10 femtomol of sample consumed /32/. By reducing the pressure in the region of high voltage ion acceleration, high resolution, good mass accuracy, and high sensitivity can be achieved with an ES ion source interfaced with a high performance magnetic sector mass spectrometer /25/. Woolfitt /34/ described a new high sensitivity ES/Atmospheric Pressure Chemical Ionization interface where an RF-only hexapole lens is incorporated in the intermediate pumping stage.

HYPHENATED TECHNIQUES

Perkins and Tomer /35/ have been interested in the development of low flow rate (nl/min) separations, like Capillary Electrophoresis (CE) in conjunction with MS. Their studies showed that CE, interfaced with ESI-MS on a sector instrument, provided additional resolution for superior mass assignment of multiple charged species of similar mass to charge ratios, as compared to the quadrupole instrumentation. Recently, CE/ESI-MS allowed both analysis of simple peptide mixtures and a mixture of polypeptides and proteins[36].

There are several general reviews on the CE-MS combination /37,38/. Most of the work on CE/MS has been carried out by using quadrupole instruments /39,40/ and only a few by using sector /36,41/ or other analyzers /42/. There are two main methods to interface CE with the electrospray system: the direct coupling method /43/ and the liquid junction method /44/. The first method is easier to implement because it does not need any special device. This method requires the work with a microESI interface or with a three-layer interface allowing the use of a sheet liquid.

In order to overcome the sensitivity limitations due to the small volumes that can be injected in the CE system, several on-line sample concentration methods were considered. In recent years several authors developed methodologies for sample concentration in CE, including capillary isotacophoresis or double stacking methods. These methods imply the change of the inlet and outlet buffers or the pressurization of the capillary outlet. In 1991 Guzman *et al.* /45/ reported for the first time the use of capillary fused silica columns for sample preconcentration on CE. This idea has been taken up again recently by Naylor and coworkers /46,47/, who use a short Teflon line filled with HPLC sorbents on line with the fused silica capillary of the electrophoretic system for sample concentration.

TANDEM MASS SPECTROMETRY

Information on the structure of a parent ion and reaction monitoring are obtained with ESI/MS/MS studies. There are two basic types of tandem mass spectrometry experiments:

1. low energy collisions (<100 eV) as exemplified by triple quadrupole and hybrid instruments, and

2. high energy collisions (1-10 keV) characteristic of experiments carried out in four-sector instruments.

Triple quadrupole and hybrid instruments differ from each other only in the first stage in which the ions that are to undergo collisions are selected by the first quadrupole mass filter in a triple quadrupole instrument (unit resolution) or by a sector mass spectrometer in an hybrid instrument (higher resolution if sensitivity allows). In the latter case the ions pass through a retarding lens to reduce translational energy typically to below 100eV before they enter the collision cell /48/. MS/MS data for compounds with molecular masses above 800 are usually difficult to get on hybrid instruments, as high collision energies are needed. The great advantage of hybrid instrument is versatility associated with the presence of more than one possible decomposition case.

DESCRIPTION OF A MAGNETIC SECTOR ESI-MS/MS

An example of such a hybrid ESI-MS/MS is the VG 70-SEQ hybrid mass spectrometer (EBqQ geometry) of the Joint Research Center in Ispra. It is equipped with an electrospray interface (VG Analytical, Fisons). The instrument is based on a 189 mm radius 70° electrostatic sector, followed by a 305 mm radius 35° magnetic sector. This magnetic sector is a high field "SE- type" laminated magnet. The magnet core is similarly constructed with the exception of the high saturation induction permandur pole tips, which extend the maximum field strength to 2.4 Tesla. This gives a mass range in excess of 3000 daltons at 8 keV ion energy.

The ESI interface is described in Figure 1. Highly charged droplets formed in the ES housing were assisted by a warm flow of nitrogen at 2.5 l/min with the source temperature set to 80 °C. The curtain of nitrogen is fed through baffles in the entrance body of the probe. Two differentially pumped intermediate regions between the ES atmospheric pressure chamber and high vacuum are employed. A rough vacuum stage inserted between the sampling cone and the second skimmer (orifice: 1.5 mm) is maintained at approximately 10-1 mbar by a rotary pump (Edwards, model E1M-18, England). The region between the second skimmer and the ring electrode is pumped at about 1.10-3 mbar by a turbo pump (Varian, model 969-9001, Italy).

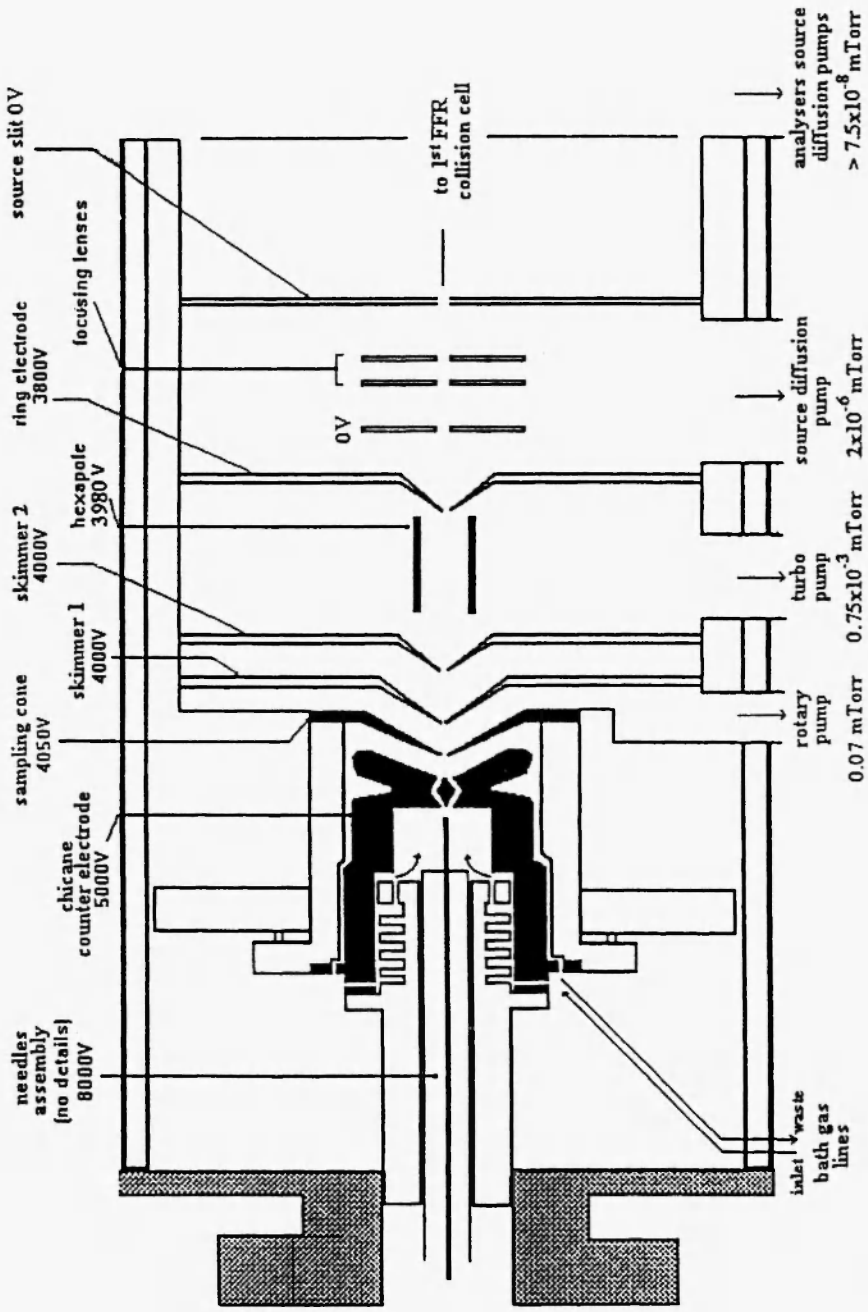


Figure 1. Schematic representation of the electrospray ionization source

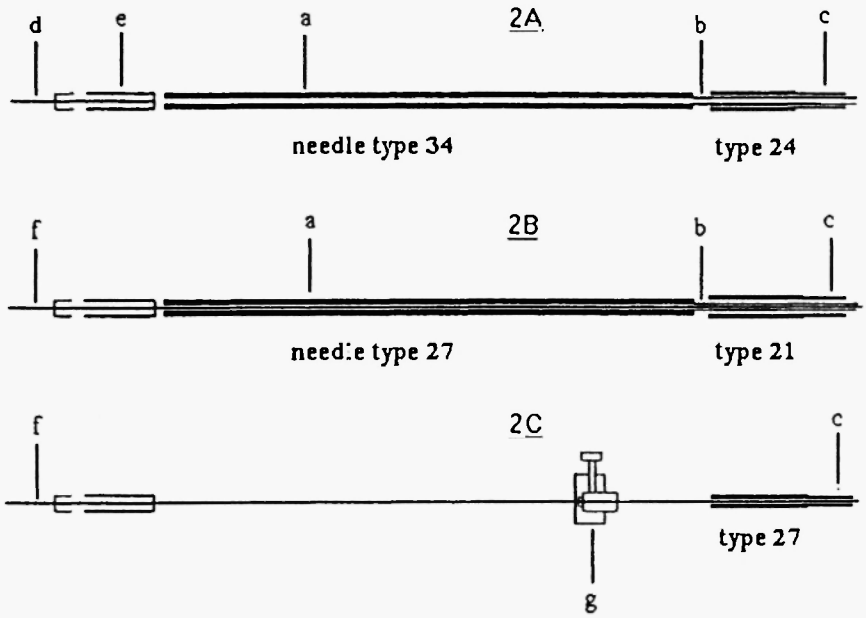


Figure 2. Schematic description of the needles assemblies in the electrospray probe;
2A- double - layered ES needle with nebulizer option;
2B- Triple layer with nebulizer and sheath flow option;
2C- A double layer design;
(a) stainless steel (ss) capillary; (b) needle tip in tungsten; (c) outside ss tee for SF6 flow; (d) silica tubing (any o.d.); (e) Teflon sheath.; (f) fused silica capillary (50 mm i.d. and 150 mm o.d.; about 40 cm long); (g) specific gripper.

The potential on the needle is about 3-4kV with respect to the counter electrode or 7-8 kV with respect to ground. Ions are sampled through the orifice in the sampling cone (size of its orifice 0.2 mm) which is held at a potential of around 4.2 kV. High voltages applied on the other focusing elements behind the sampling cone are decreasing until entrance in the high vacuum part. The ring electrode orifice is 1.5 mm. The effective ion acceleration potential used was 4 kV, which gives a convenient m/z range of 0-3000 Da on this instrument (maximum acceleration potential at 6 kV).

The ES needle assembly used is shown in Figure 2. In both cases (2A, 2B), the needle tip is of tungsten (2.5 cm long). In the double layer design,

the spraying needle is in stainless steel (type 34; 0.127 mm i.d., 0.229 mm o.d.) and the tip is sheathed with a stainless steel needle (type 24; 0.31 mm i.d., 0.56 mm o.d.), and allows for the introduction of an auxiliary gas SF_6 (nebulizer gas) in the vicinity of the tip to inhibit corona discharges. In the triple layer assembly, the sample flows through a fused silica capillary (50 mm i.d.) inserted in a needle sheath (type 27; 0.20 mm i.d., 0.41 mm o.d.) in stainless steel which allows for the circulation of the sheath liquid. The tip is a stainless steel needle (type 21; 0.51 mm i.d., 0.81 mm o.d.). The stainless steel capillary ensures immediate electrical contact with the solution flowing out of the fused silica capillary. The distance between the needle tip and the counter electrode is between 4 and 10 mm depending on the flow rate used.

The needle assemblies are arranged in an ES probe. The latter is supplied with an injector bracket on which a range of Valco and Rheodyne injection or switching valves and also a pressurized vial system can be mounted. The probe is fitted with a microswitch to ensure that the high voltage is only applied when the probe is fully inserted in either the ES housing or the spray tester. High voltage is supplied into the socket at the back of the ES probe.

The source is equipped with a RF only hexapole lensing system, which replaced the electrostatic lenses used previously. This novel ion optical arrangement is designed to collimate the ions beam in a narrow field in order to optimize ion transmission before exit from the source. This increases sensitivity of the system by a factor of at least 100 for masses below 1000 Da and a factor of 10 for multiply charged and high mass ions above 1000 Da.

The ESI interface is singular in possessing a chicane counter electrode (or "pepperpot" system) for analyses at high flow rates (usually equal to 10 ml/min or higher). The latter is shown in Figure 3.

The instrument can be operated in both negative and positive mode. In negative ions mode, a mixture of methanol and water 1/1 with the addition of 0.1-1% of ammoniumacetate (saturated aqueous solution) is used, while in positive ions mode a mixture of methanol and water 1/1 with the addition of 0.1% of acetic acid is giving good results depending on the type of application. Only high purity reagents and HPLC grade solvents should be used in order to minimize background noise. All mobile phases are degassed by vacuum filtration.

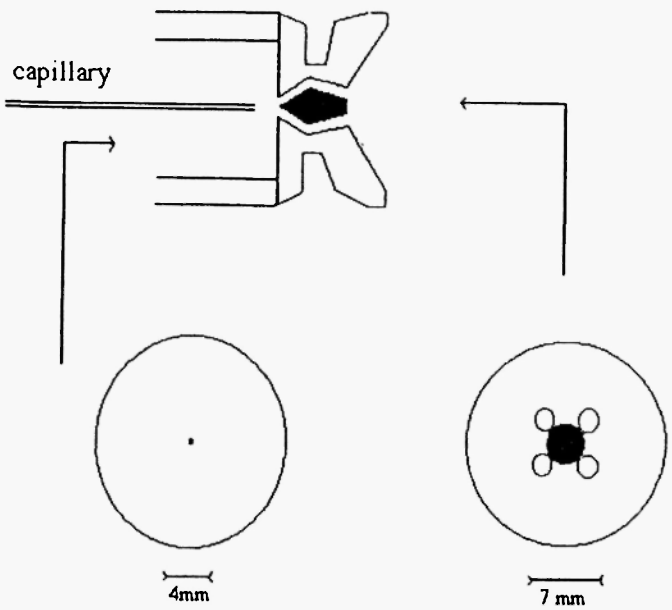


Figure 3.
A rough schematic representation of the chicane design from Fisons Instruments

In direct infusion experiments, sample solutions are supplied to the ES source at 0.3-10 ml/min using a Harvard apparatus Model 22 syringe pump. The syringe used for infusion is connected to the ES source via PEEK tubing with an outer diameter of 150 mm. Sheath liquid (typically same as solvent) is introduced via PEEK tubing by a micro flow syringe pump (Kontron, mLC-500, Milan) at a flow rate of 0.5-1.0 ml/min.

The spectra are recorded in the continuum mode with a magnet scan rate of 0.1 s/decade at a resolution of 1000 M/ΔM (10% valley definition) and acquired on a dual conversion dynode photomultiplier ion detector comprising +/-10kV conversion dynodes for positive and negative ion detection. Another detector of the same type is situated after the second quadrupole to detect the products of MS/MS experiments. The spectra shown are an accumulation of several scans. Mass spectra are acquired on a VG OPUS workstation, Opus software version 3.OA/cX. This VG data system is based upon the VAX station range of computers and the VMS operating system from Digital Equipment Corporation (DEC).

To demonstrate the basic sensitivity of the ES interface, we report here spectra of the following test compounds: lysozyme (+ve ions), raffinose and a ribonucleotide trimer (-ve ions). Analyses are done both in negative and positive ions modes.

Lysozyme (MW: 14305) analysis is performed on the chicane counterelectrode design. Stainless steel double layer needles are used (figure 2A) and the distance between the capillary tip and the counter electrode is about 5 mm. The spectrum is acquired with a 500 fmol/ml solution of Lysozyme in 1:1 methanol:water with the addition of 0.1% of acetic acid at a constant flow rate of 10 ml/min. 0.1% ammonium acetate solution in 50:50 methanol: water, containing Raffinose (MW: 503) at 50 ng/ml, has been used at a constant flow rate of 10 ml/min with the chicane design counter electrode. A solution of 480 pmol/ml of the r(ApApCp) trimer ribonucleotide in a mixture of methanol:water 1:1 with 0.1% of NH_4Ac (saturated aqueous solution) has been electrosprayed using four different designs of the needle assemblies:

- double layer with the all stainless steel needle as shown in Figure 2A
- double layer geometry with a fused silica capillary fixed with a gripper (Figure 2C).
- a fused silica capillary depolyimided and coated with gold /49/.
- a triple layer needle (Figure 2B)

The triple-layered needle assembly has been used for optimization (Figure 2B). A solution of the RNA trimer in 1:1 MeOH:H_2O with 0.1% of NH_4Ac has been electrosprayed at flow rates lower or equal to 1 µl/min at 480 pmol/µl and ten-fold diluted, using also ammonia (30% (aq)) and tetraethylammonium acetate as sheath solutions (1:1 MeOH:H_2O, 1% TEA). Ammonium ions appear to be less tightly bound to the nucleotide when ionized in the gas phase.

The ESI mass spectrum of Lysozyme measured at a resolution of 1000 has been accumulated on four scans (Figure 4). Good performance in the femtomole range can be reached at a signal to noise ratio of 70:1.

The ribonucleotide trimer r(ApApCp) spectrum is shown in Figure 5. A change in needle voltage results in a linear response until a critical value where intensity reaches a maximum value with this design (Figure 6).

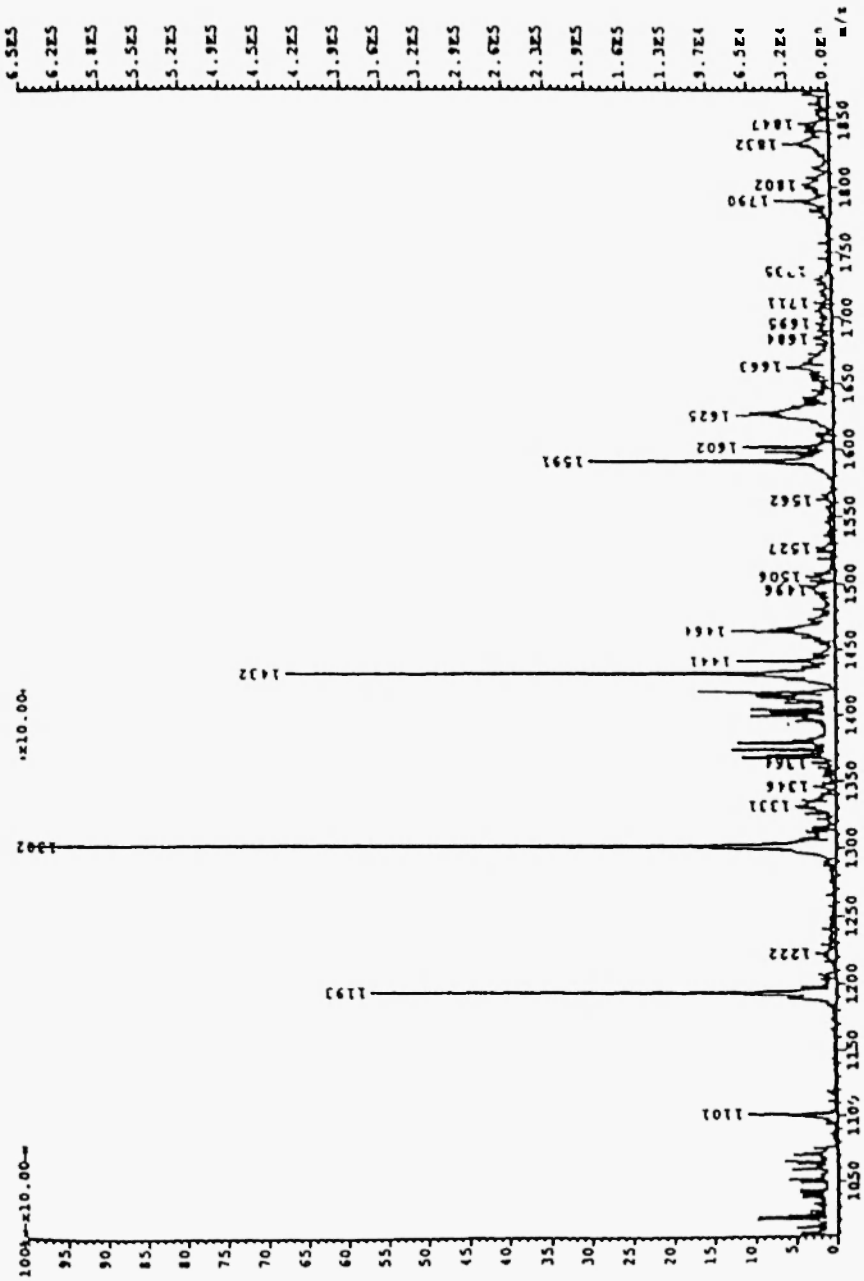


Figure 4. ES spectrum of 500fmol/µl Lysozyme (MW 14305) in 50:50 methanol:water with 0.1% acetic acid - flow rate 10 µl/min- 6.5 pmol consumed over four scans

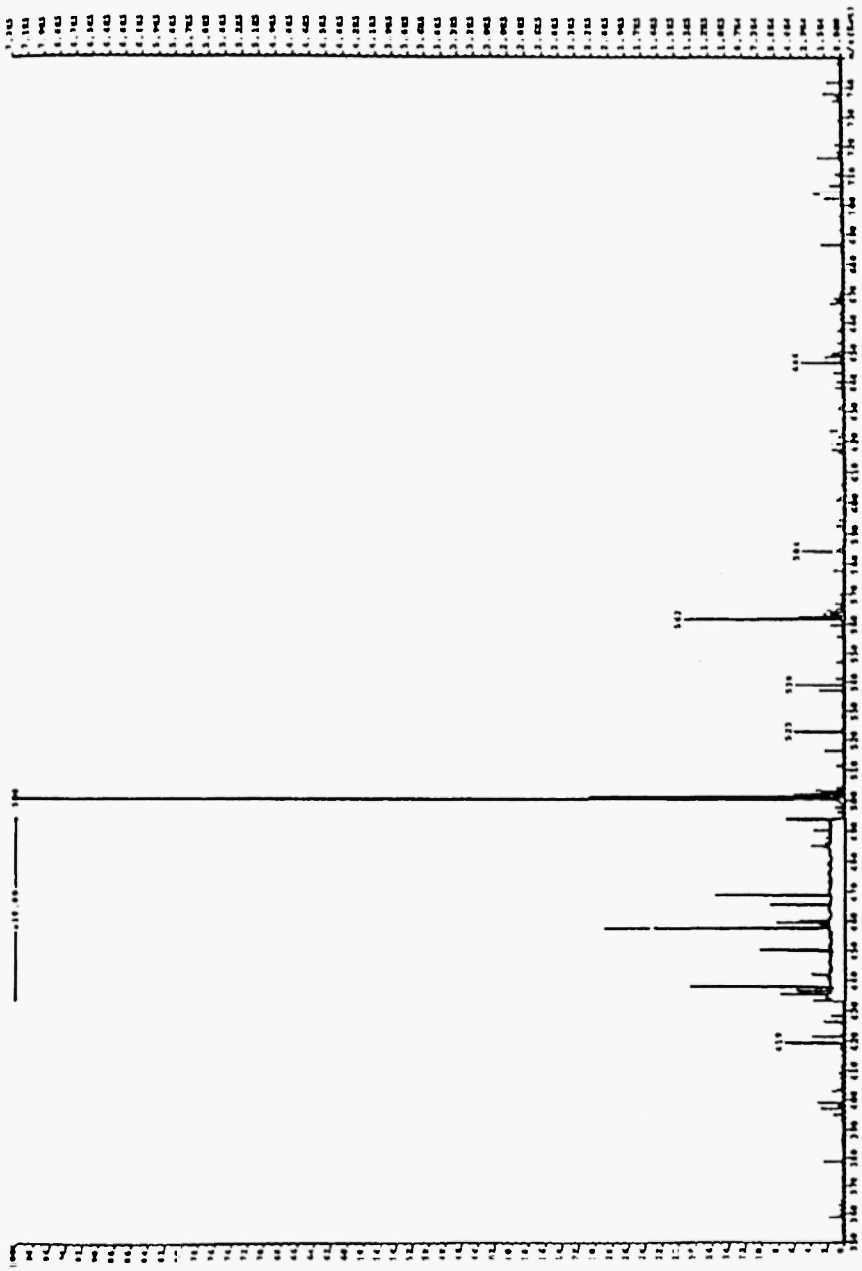


Figure 5. ES spectrum of 100 pmol/µl raffinose (MW 305) in 50:50 methanol:water with 0.1% NH_4Ac - flow rate 10 µl/min- 770 ng consumed over four scans

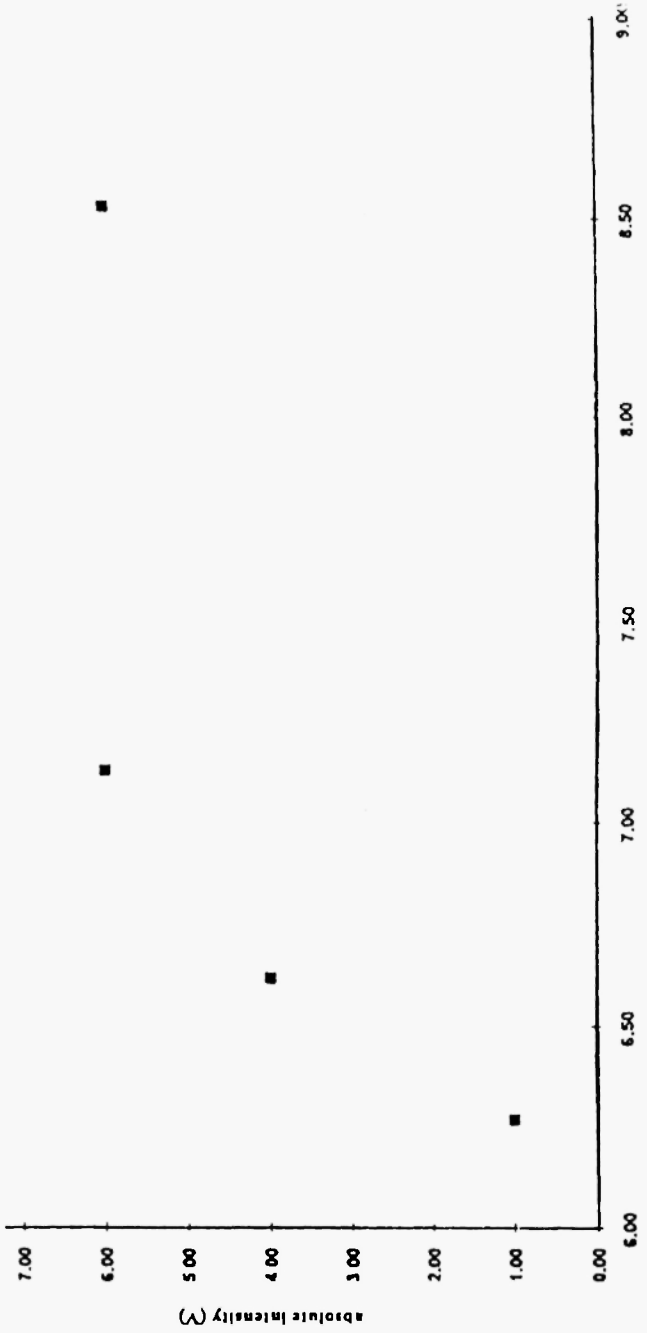


Figure 6. Absolute intensity of $[r(AAC)\text{-}H]^-$ against needle high voltage in ESI/MS, ss double- layered needle , 2 µl/min, chicane counter electrode, Fast Amplifier 3 x 10^{-6}

It is possible to improve this performance with the design of a "tapered" capillary tip commercially available. An example of a spectrum is shown in Figure 7. 144 pmol were consumed for 1 minute acquisition time. Main peaks are $[M-H]^-$ (MW: 900) with sodium adducts (M+23). The highly charged nature of oligonucleotides results in the binding of sodium ions even if present in trace amounts. A 5'-terminus base elimination of Adenine (m/z: 766) and two fragments (m/z: 571 and 595) can be observed.

The use of triple layer geometry with triple layer run allows a good sensitivity to be reached (Figure 8 (a)).

Removal of salt ions is achieved with replacement of sodium ions by ammonium ions /50/. Partial displacement of sodium is observed when adding ammonium but with a sensitivity five times lower.

The results presented are mainly based on the analysis of a small trimer oligonucleotide. A triple layered needle is the most effective for ribonucleotide analysis. In practice, a double layer geometry with all stainless steel needles is easier to use. A small quantity of silver paint /51/ can be used to ensure good electric contact between the capillary and the surrounding needle tee.

Previous works performed in ESI on this sector instrument allowed the use of a 0.2×10^{-9} mol quantity of this same trimer for determination of molecular weight and fragment analysis /52/. Moreover, a thirteen-fold higher total concentration has been used in analysis by FAB and FAB-MS/MS on the same instrument /53/. Integration of the hexapole lens /34/ into the interface allowed a sensitivity to be reached almost 100-fold higher than the original interface design with electrostatic lenses.

Larsen *et al.* /24/ have shown performances reached with an ES ion source from Analytica of Branford coupled to a magnetic sector instrument. Their experiments, first performed with two stages of mechanical pumping with removed baffles, consisted in the reduction of ion-molecule collisions in the kVolt ion acceleration region by reducing the pressure through more efficient pumping. Finally, this reduction provided an improvement to 60 finol of Lysozyme consumed (60 fmol/µl). They also experimented on a single stage of mechanical pumping with a more restrictive skimmer orifice (0.36mm instead of 0.56mm) in order to reduce the pressure further. They could reach sensitivities down to 15 fmol. They demonstrated the advantages of a high performance magnetic sector MS over quadrupole instruments that can be achieved with a relatively simple ESI interface.

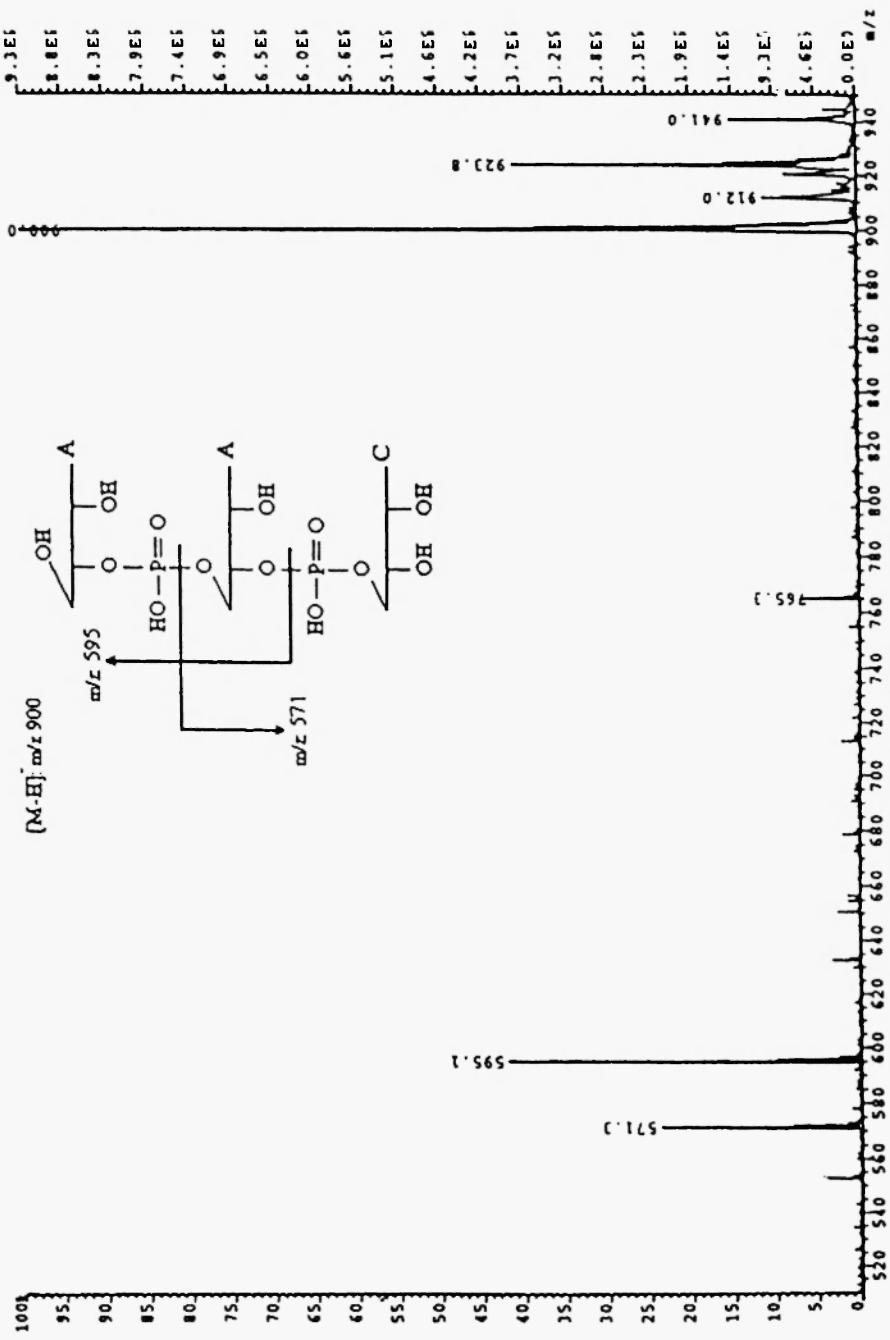
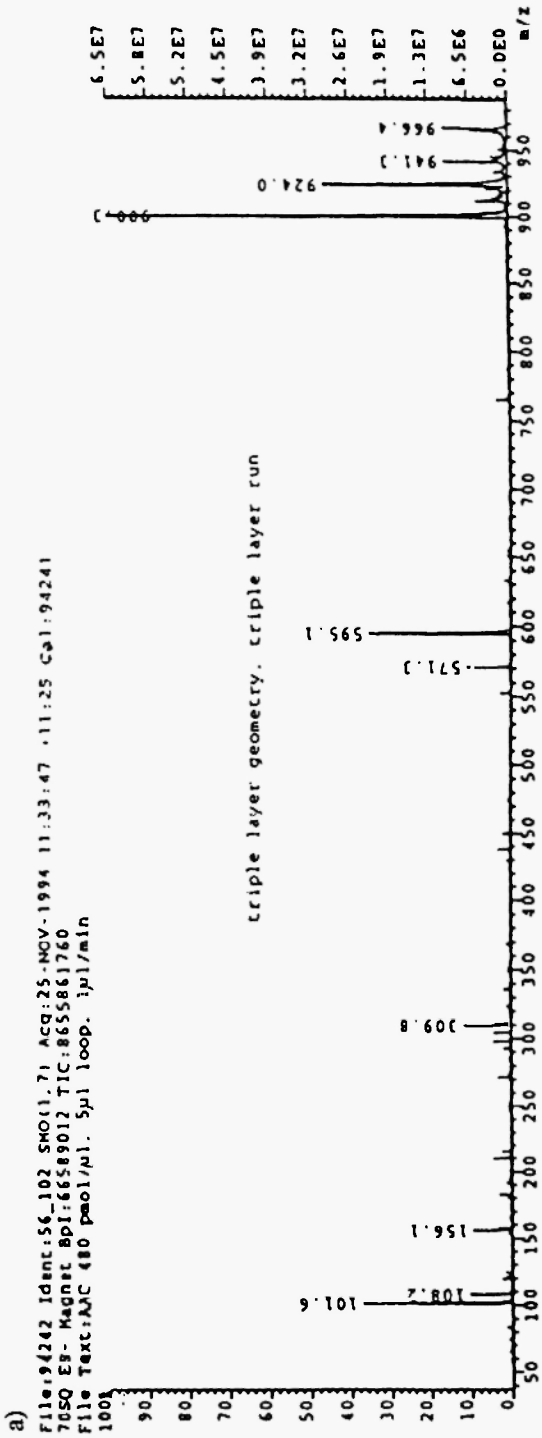


Figure 7. ESI/MS spectrum of the trimer RNA with a triple layer needle; 480 pmol/µl in 1:1 methanol:water and 0.1% NH_4Ac; sample solution infused at 0.3 µl/min, sheath solvent at 1 µl/min


a)
File:94242 Ident:56_102 SMO(1,7) Acq:25-NOV-1994 11:33:47 .11:25 Cal:94241
70SQ ES- Magnet BPI:66558912 TIC:865586760
File Text:AAC 480 pmol/µl. 5µl loop. 1µl/min
triple layer geometry, triple layer run
m/z
101.6
108.2
156.1
309.8
571.3
595.1
924.0
941.3
966.4

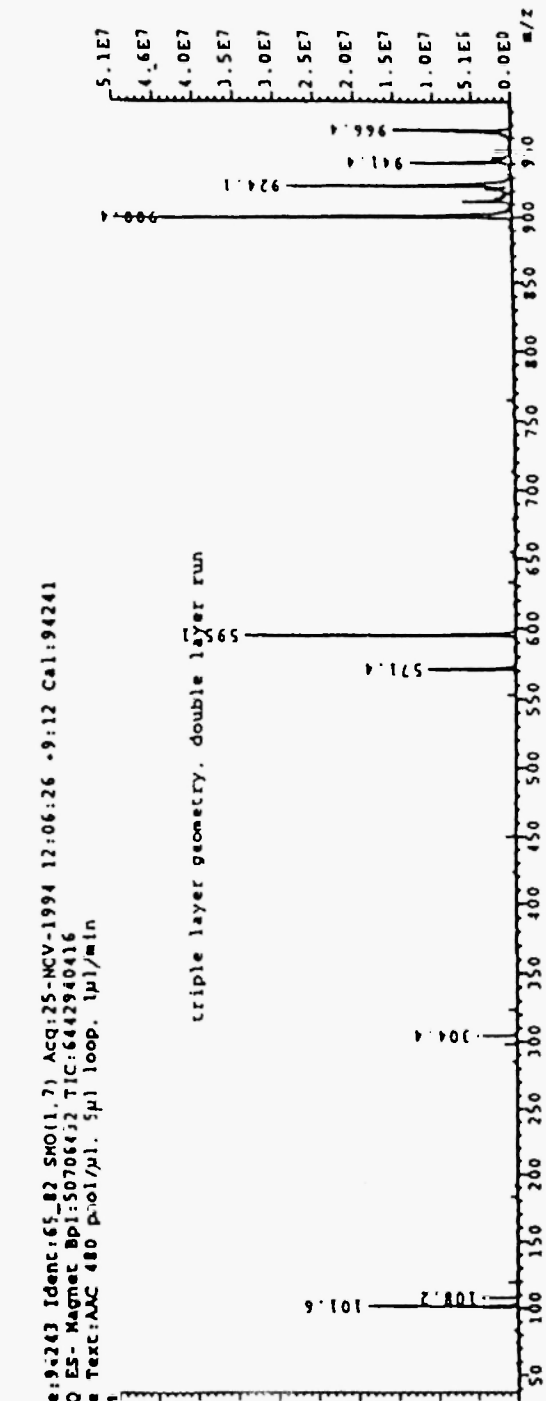


Figure 8. ESI/MS spectrum of ribonucleotide trimer r(AAC), 480 pmol/µl, negative ions mode; 5µl loop injection at 1µl/min; 1:1 methanol:water and 0.1% of Triethylammoniumacetate both as solvent and sheath liquid at 0.5 µl/min; (a) triple layer geometry, triple layer run; (b) triple layer geometry, double layer run

Instrument stability is an essential feature for ESI because fewer reference compounds are available than for conventional ionization modes. This feature ensures extended operation times without the need to recalibrate. In terms of ease of use, the quadrupole is still superior for routine applications because only one calibration is needed for all scan ranges and speeds.

The choice of an instrument partially depends on the final purposes of the user.

Problems of compatibility can occur when packed capillary LC columns, which typically run to about 500 nl/min, are used. A sheath solution is added to get a flow rate in the microliter range per minute and obtain a stable spray.

We can emphasize that our ESI source allows to work with a large range of flow rate from 1 µl/min to 1 ml/min, especially with LC/MS and CE/MS.

CONCLUSIONS

Magnetic sector instruments are known for their utility for accurate mass measurements and elemental composition calculations. Various groups have sought to extend the resolution available from the mass analyzer to improve the mass accuracy for ESI-MS analysis and to determine the number of charges on a given species from the mass-to-charge ratios of resolved isotope peaks. Accuracies as high as 0.003% have been reported for measurements of proteins made by using a magnetic sector mass spectrometer /24/. ESI spectra acquired at resolutions exceeding 10000 (10% valley definition) have been reported /24,25/. Nevertheless, high resolution work on magnetic sector instruments requires very intense and stable ion currents, often not easily achievable when sample amounts are limited. Several improvements have been suggested, on one hand by increasing the efficiency of ion transmission from the source to the analyzer region, on the other by miniaturizing the spray process. Higher transmission has been achieved mainly by the introduction of rf-only hexapole or octapole ion focusing devices in the transfer region from the source to the analyzer. Miniaturization has produced novel techniques like micro-electrospray /7/ and nano-electrospray /49/ that allow total sample volumes of around 1µl to be handled without significant wastage, and steady, stable signals at flow rates of around 25 nl/minute to be obtained. This technique has been also

successfully applied to magnetic sector instruments. More intense and more stable ion currents have also greatly improved the capability to perform MS/MS experiments on magnetic sector instruments, either on hybrid or multisector instruments[54].

In conclusion, magnetic sector instruments seem to have great potential for ESI-MS. Nevertheless there seems to be a growing interest in competitive technologies, like Fourier Transform Mass Spectrometry (FT-MS), for high-resolution tandem mass spectrometry above 10kDa /55/. Only time will tell if ESI-MS on magnetic sector mass spectrometers is a viable analytical technique, or just an upgrade to existing instrumentation.

ACKNOWLEDGEMENTS

This work has been carried out with financial support from the European Commission (DG XII, Science and Technology, Division XII-H-1), in the framework of the Human Capital and Mobility Programme, as part of the network project "Hyphenated Analytical Chemistry for Environmental and Public Health Research in the European Union".

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