Ion Chromatography Coupled to MS for Metabolomic Analysis

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The fields of metabolomics and metabonomics attempt to phenotype and quantify the vast array of metabolites present in biological samples. Reverse phase HPLC coupled to mass spectroscopy is a valuable tool in the separation and identification of these compounds. Reverse phase HPLC techniques cover a wide range of compounds, however ionic and polar compounds such as organic acids, carbohydrates, nucleotides and amino acids are difficult to separate or even retain on traditional reverse phase columns. Ion exchange chromatography can prove to be a far better separation process for these compounds. The problem with the separation mode in this application relates to the salt eluents employed in ion exchange being incompatible with mass spectrometers. Here we describe an ion exchange system which can achieve good separation of these polar compounds with on-line desalting to allow MS detection. This technique allows studies of key metabolites which do not separate well on traditional reverse phase methods. Some of these ionic metabolites may merely be positional isomers adjacent to each other in a synthetic pathway and as such, isobaric. Mass Spectroscopy alone in these cases would not be enough to give a positive identification between them. There is clearly a requirement for good separation of isomeric polar metabolites.

Key words; Metabolomics, Metabonomics, Eluent Suppression, Mass Spectroscopy

Ion Chromatography

Ion Chromatography (IC) was developed with eluent suppression techniques in 1978 by Small et al. [1]. It has grown to be the primary analysis technique for the analysis of small anions and a strong method choice for inorganic cation analysis.

It is also a very efficient method for the analysis of small polar compounds such as organic acids and amines [2] all detected by conductivity. Larger charged biomolecules, such as peptides, nucleotides and carbohydrates, are successfully separated with IC but normally without the use of suppression techniques [3,4]. Suppression is routinely linked to conductivity detection of the analyte. In the case of peptides, UV detection is used and carbohydrates can be detected electrochemically with pulsed amperometic detection. However, it is the eluent suppression techniques that are interesting to Mass Spectroscopy [MS] as it converts the high salt eluents from Ion Exchange Chromatography into MS compatible pure water. So the ion exchange separation of these metabolites can still be utilized and coupled to suppression to allow detection by MS. The field of IC covers an impressively wide range of compounds with an array of column chemistries and detection techniques utilized in addition to suppressed conductivity [5]. The implications of making all these analytes accessible to a MS based metabolomics study, with the aid of an on-line desalting technique are quite evident.

Ion Chromatography / Mass Spectroscopy System

In today's analytical world, MS is increasingly turned to as a preferred detection choice. This is not only because of the additional information acquired to confirm the identity of the compounds but also due to the additional separation and sensitivity it bestows on the analysis. The coupling of Ion exchange chromatography to MS has never really been explored due to the high salt concentrations used in the elution of compounds from the ion exchange column. Although eluent suppression techniques have been available for some time that which effectively converts high conductance sodium or potassium hydroxide eluent back to pure water via an ion exchange substitution with H+ ions, IC has remained associated with the analysis of small inorganic ions using conductivity detection.

Figure 1 outlines the chemistry inside an anion electrolytic suppressor. The cation suppressors work in a similar manner with opposite charges. The suppressor devices use a platinum electrode for the hydrolysis of water as a source of the H+ required to keep the charged semi-permeable membrane in the H+ form. As KOH enters the suppressor it is converted to H2O in an exchange reaction on the membranes. The use of membrane screens in the suppressor allows a large surface area for exchange whilst keeping the dead volume to a minimum. In this way eluent is continuously converted to water and the membranes permanently charged for continuous use.

As the eluent from the analytical ion exchange column is transformed to pure water from both anion and cation exchanger's in suppressed IC there is no reason why direct coupling to MS cannot be done. In addition to this, all the compounds which bind and elute from ion exchange columns are naturally ionic

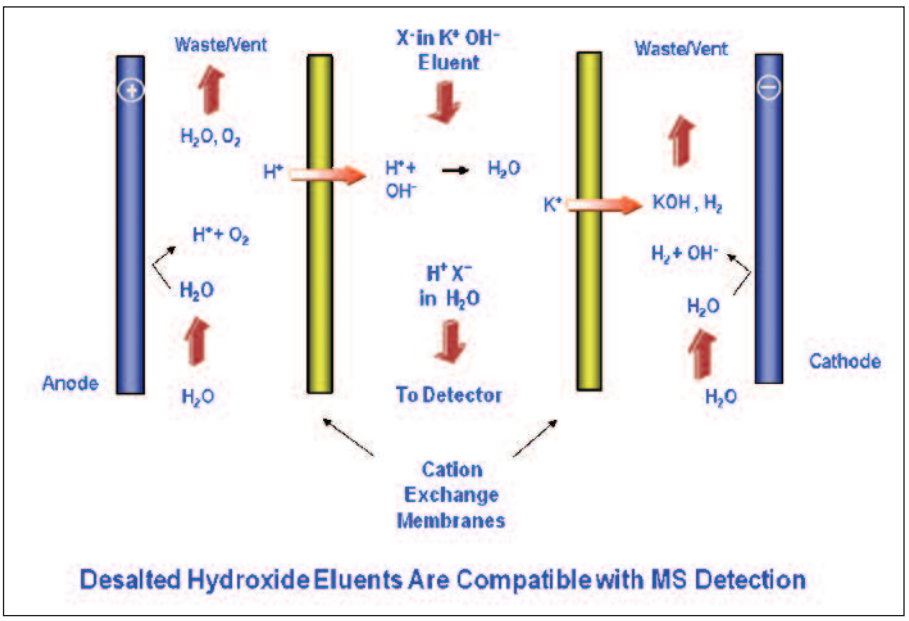

Fig 1 Conversion of a KOH eluent to water using a modern electrolytic anion suppressor

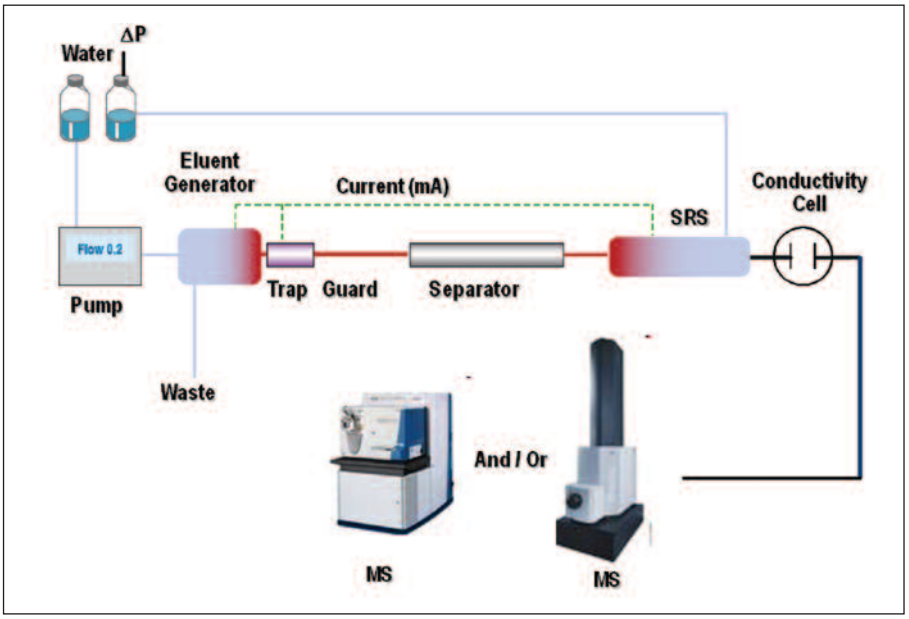

Figure 2. Schematic of a modern IC-MS system.

or highly polar and so ideal candidates for electrospray ionization. Highly polar and ionic compounds which tend to come elute straight through reverse phase columns without separation are more likely to chromatograph well on ion exchange. This opens up many classes of compounds to IC-MS analysis which have proven difficult to measure with classical reverse phase MS.

Continuous On-Line Desalting

The coupling of IC to MS has now become commercially available and the viability of the technique across several analytical areas is being explored. The modern system provides a controlled delivery of ultra pure water to an electronic eluent generator to electrolytically produce potassium hydroxide (KOH) in a very pure form at the head of the column. This is done in a similar manner to the suppressor by generating OH- from water at a platinum electrode. The amount of OH- produced is directly proportional to the flow rate and the current applied to the electrode. The K+ is added across a semi-permeable membrane as a counter ion from a reservoir. In this way, very pure and accurate salt gradients can be produced with virtually no delay volume. The KOH gradient produced is used to separate the sample on a high efficiency 2mm i.d. anion exchange column. A low dead volume, continuous, electrolytic suppressor is used post column to create a compatible effluent for conductivity and MS detection. In this way, the effluent supplied to the mass spectrometer is kept in a very pure form to reduce the background and increase sensitivity. The conductivity is monitored and is usually below 1.0 µs.cm-1 demonstrating the purity of the effluent at all times. The separated ionic metabolites are then detected and identified using a high resolution mass spectrometer. A schematic of a typical IC system coupled to a high resolution mass spectrometer is shown in Figure 2.

IC-MS Schematics

Practical IC-MS

Publications surrounding IC coupled to MS are emerging in the literature. Applications looking at organic acids in beverages [6], agricultural chemicals and water pollutants [7,8] have been published. There are now publications which show the use of suppressed IC for carbohydrate analysis with MS detection [9]. Carbohydrate analysis by anion exchange chromatography is usually coupled to electrochemical detection and so does not normally use the on-line desalting provided by a suppression system. Here, the reduction of the KOH/acetate eluents to volatile acetic acid in the suppression system is used to allow the coupling of a very efficient separation to on-line electrospray-MS. In spite of these key applications now being coupled to MS, very little has been seen using this technique in the field of biological metabolites where the sample matrix can be very complicated and the numbers of potential ionic metabolites are vast. Ionic and polar compounds, such as organic acids, carbohydrates, nucleotides and amino acids, are difficult to separate on traditional reverse phase columns, yet an example of virtually all these compounds being separated on an anion exchange column with a simple KOH gradient can be seen in Figure 3. This shows the applicability of ion exchange to this range of metabolites classes. An ion exchange system which can give good separation of these polar compounds with on-line desalting to allow MS detection can provide a new analytical platform to help study metabolism.

Gradient used was 2 to 12 mM NaOH in 15 min, to 20 mM in 25 min, to 70 mM in 35 min. Column was a Dionex IonPac AS11 Anion exchange. Upper trace is suppressed conductivity and the lower trace is UV 260nm.

The mode that we have used the most is an anion exchange system to target organic acid based metabolites, looking for carboxylated, phosphorylated or even sulphonated species. An added advantage of the high pH KOH eluents used in this system is that compounds such as sugars which contain OH groups, ionize to form anionic species and so they also have interaction on the ion exchange column. There is also the possibility of using a cation exchange system to target amine containing compounds. This has been shown with work in

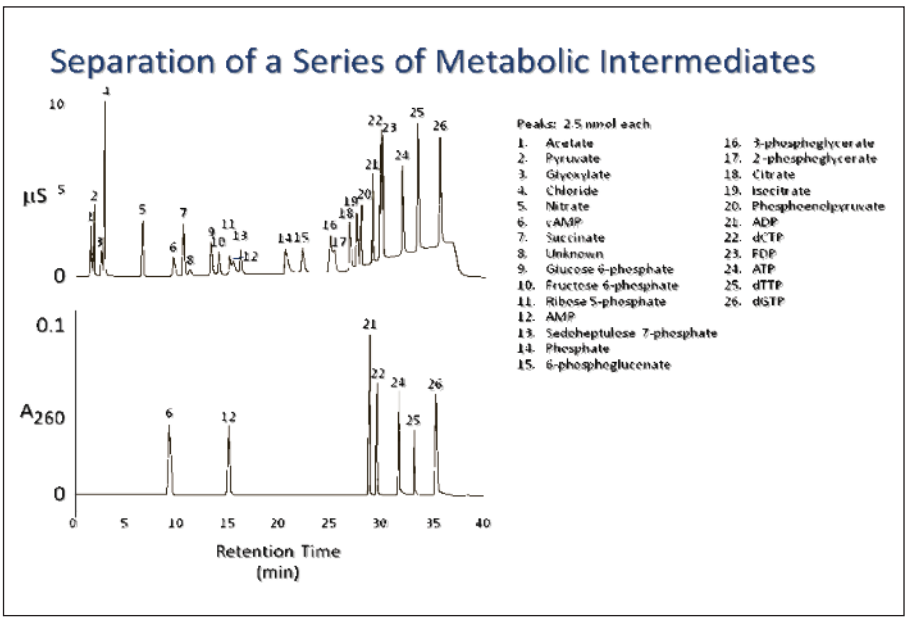

Figure 3. Separation of metabolites on an anion exchange system with a hydroxide gradient.

the analysis of bio-genic amines [10]. The system layout is identical to that outlined in Figure 2 but the columns and suppressor would be cationic versions and the eluent generator would create methane sulphonic acid with a cation generation cartridge.

Metabolomics/Metabonomics Analysis

The study of metabolic pathways using LC-MS based methodology is also still very much in a development stage. The chromatography methods are still being improved alongside the mass spectrometry, metabolite databases, search engines, and pathway analysis software. All of these need to be brought together to allow biological samples to be analysed in detail, then suggested conclusions can be given to the state of the metabolic pathways under study. Different chromatography conditions, such as high performance reverse phase and HILIC chromatography, have been used and compared already to try to increase the coverage of the metabolites seen in biological samples [11].

Metabolomics presents the challenge of cataloguing and quantifying vast numbers of small molecule components of biological fluids and cell extracts [12]. Metabonomics has an even greater problem of quantification in response to stimuli or genetic modification [13]. Control and test samples have to be carefully prepared, separated, and identified for changes in a few compounds inside an extremely large haystack of unaltered metabolites. Separation of these components to allow decent quantification and identification will require more than one chromatography technique. Gas Chromatography (GS) coupled to MS can be used for the volatile compounds with very high resolution. Liquid Chromatography (LC), however, opens up a wider range of analytes and the advent of high resolution systems adds more power to the resolving capabilities in the reverse phase mode. The proposed use of Ion Exchange Chromatography will open up the analysis of compounds not retained on reverse phase columns, and targets a different class of compounds to hydrophilic interaction (HILIC) chromatography.

The use of orthogonal separation modes before MS identification increases the coverage of the metabolic classes present in these mixtures. C18 chromatography covers the hydrophobic compounds, HILIC chromatography selects the hydrophilic components, whereas the ion exchange system will target the ionic compounds.

Identification and Classification of Metabolites

Currently, most identification of metabolite information obtained from complex LC-MS data is performed by accurate mass alone. XCMS, MZMINE, Sieve, Masstrix and MetExplore are all databases from different manufacturers which can be used for this purpose. There are moves to improve the reliability of identification by the incorporation of isotope pattern (TargetAnalysis [Bruker Daltonics]), and fragmentation data. Retention time is a key additional dimension of information that can be applied to significantly improve identification confidence.

Identification of individual metabolites of interest is significantly more robust, as standards may be available, and more rigorous analysis may be performed. Identifications of metabolites are normally performed against a large metabolite database, such as the human metabolome database (HMDB), Kyoto encyclopaedia of genes and genomes (KEGG) or ChemSpider. In addition, metabolites are commonly placed in a systems context with the use of pathway analysis software, such as Masstrix or MetExplore. Both applications attempt to match observed masses to components of known biological pathways (from KEGG and BioCyc respectively), providing a schematic of the relationships between metabolic intermediates.

Information Gained From the Inclusion of ICMS in Metabolite Studies

Reverse phase, HILIC, and anion exchange chromatography were used with high-resolution MS to investigate the metabolic profile of biological samples. The classes of compounds identified with each technique were compared to qualify the usefulness of IC in a metabolomics environment.

Investigation of urine analysis shows that there is the expected overlap between the different analytical methods in terms of compounds identified (Figure 4). It also shows there would be a significant drop in the coverage of metabolites identified if any of the three techniques were dropped.

To concentrate on the contribution of ion exchange, several classes of compounds increase in visibility through using this technique. We are now seeing a greater number of amino acids, aromatic acids, keto acids, carboxylic acids, nucleosides, purines, carbohydrates and glucuronides. These classes of compounds are members of several important biological pathways. High coverage of intermediates involved in synthetic pathways will naturally lead to a better understanding of the control mechanisms and disease states. IC-MS data has been extremely useful in identification and quantification of components of the Glycolytic pathways. This would be expected with the intermediates containing carboxylic acid and phosphate groups. These negatively charged metabolites chromatograph well on ion exchange and are already charged for electrospray analysis. Similar improvements were seen in the identification coverage of amino acid synthesis pathways. With this combined approach, 379 out of 652 intermediates of several pathways were reliably identified with minimum effort in optimization.

Studies using cell extracts from Trypanosoma Brucei have greatly increased the coverage of several biological pathways including oxidative phosphorylation, nucleotide, and amino acid metabolism (Figure 5). In the tryptophan pathway, 24 out of 80 components were identified by IC-MS alone.

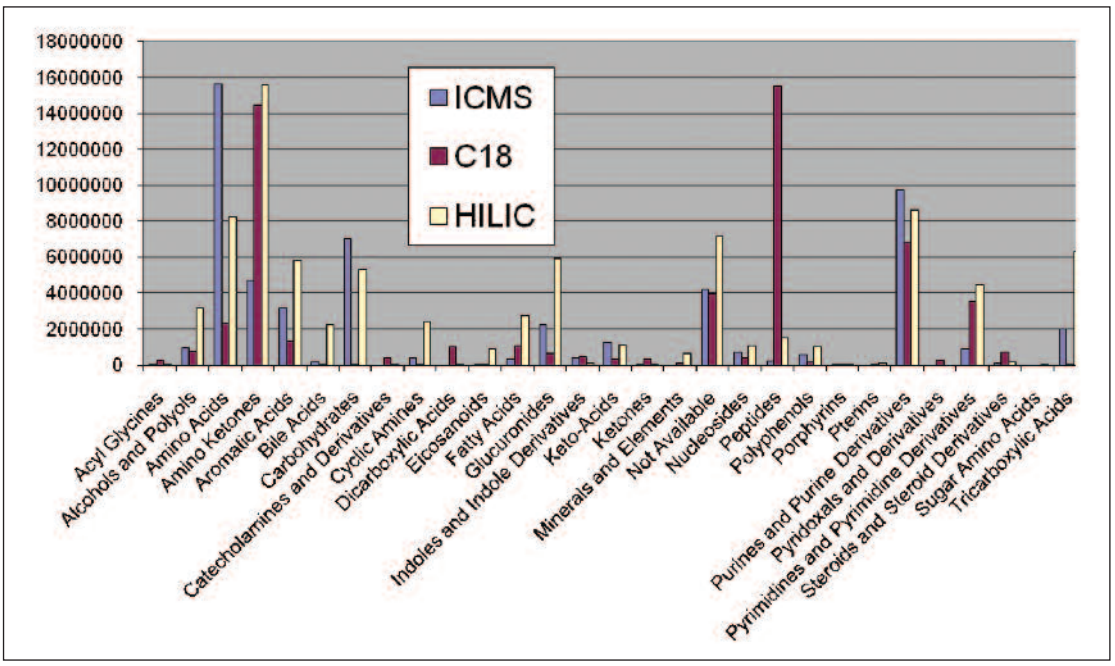

Figure 4. Classes of compounds identified by Ion Exchange, Reverse Phase and HILIC.

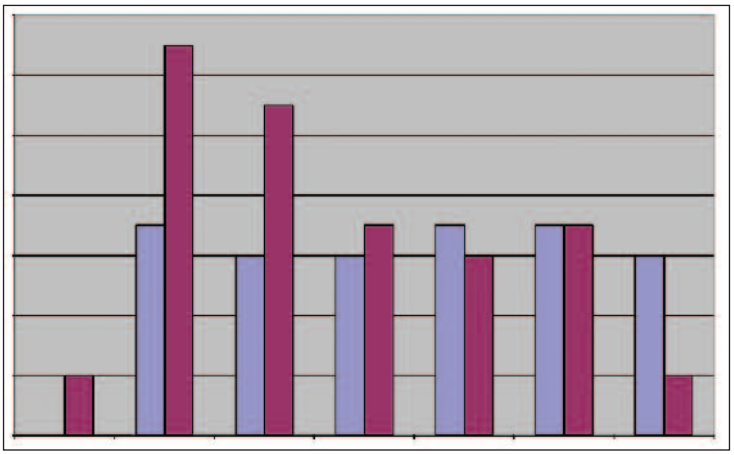

Figure 5. Pathways Identified in Trypanosoma Brucei. Those in red were identified by IC-MS and those in blue using HILIC.

Conclusions and Future developments

The studies so far have just included anion exchange chromatography. This will miss the cationic metabolites. There will be an effort to look at the contribution that cation exchange chromatography can make towards increased coverage of biological pathways. Full coverage will probably never be achieved in some biological pathways due to the extremely short half-life and turnover of some intermediates in these pathways. Studies of biological markers using HILIC and Reverse Phase chromatography proved that the markers identified using the two techniques where were different in each case [11]. This provides a good indication that important biological markers for certain disease states could be found more easily with a targeted approach to the analysis. Changes in the metabolic phenotype in response to clinical conditions and drug treatment could be more easily seen by specifically looking at the metabolite class most likely to be effected, using the most appropriate separation technique for those compounds a significant increase in the coverage of those pathways can be predicted.

Systems biology approaches will also benefit from targeted analysis of metabolite classes. Changes in the proteome or genome should be verifiable by an intelligent approach to find the expected differences in the metabolome. In addition, the metabolome is often an amplified response to small changes in the proteome and so theoretically easier to see. A relatively minor change in the proteome could lead to a dramatic change in activity of the rate limiting enzymes present in a biological pathway. The proteome change could be difficult if not impossible to see, however, the change imparted at the metabolite level could be dramatic.

A directed approach to bio-marker discovery and metabolic phenotyping with intelligent choices to give the correct coverage of metabolites would also remove some uninteresting metabolite classes which are only interfering with the analysis. Many hydrophobic metabolites would not be retained on an ion exchange column and so be removed from a study of the charged species in pathways, such as amino acid metabolism or the glycolytic pathway.

The availability of high resolution reverse phase, HILIC, and ion exchange chromatography gives a great deal of choice and breadth in the chromatography. There are also new developments in capillary IC using much smaller column technology which will increase sensitivity and allow the analysis of much smaller samples volumes [14]. Mass spectrometers are now much more sensitive, faster and with higher resolution so the physical tools for separation, detection, and identification have improved dramatically in metabolomics. Future studies in this field should now yield some very interesting results.

References

*Papers of particular interest have been marked ***

[1] Small, H. Stevens, T.S. and Bauman, W.C. Anal. Chem. 47 [1975] 1801.

[2] De Borba, B. M.; Rohrer, J. S. J. Chromatogr., A 2007, 1155, 22–30.

[3] Lyubarskaya, Y.; Houde, D.; Woodard, J.; Murphy, D.; Mhatre, R. Anal. Biochem. 2006, 348, 24–39.

[4] Thayer, J. R.; Rao, S.; Puri, N.; Burnett, C. A.; Young, M. Anal. Biochem. 2007, 361, 132–139.

*[5] Weiss, J. , "Ion Chromatography," VCH Weinheim, 1995. ** An excellent review of all IC techniques for the analysis of ionic and polar molecules*

[6] Wang, L.J. Schunte, W.A. LG-GC, Sep 2, 2009

[7] Mohsin,S.B. J.Chromatogr. A. July 2000, 884, 23 – 30.

[8] Bauer KH, Knepper TP, Maes A, Schatz. V, Voihsel M (1999) J Chromatogr A. 837:117–128

*[9] Bruggink,C. Maurer.R, Herrmann. H, Cavalli S, Hoefler. F J Chromatogr. A, 1085 (2005) 104–109 ** Early use of an IC-MS system for the analysis of carbohydrates from biological samples*

[10] Saccani, G. et. al. J. Chromatogr. A, 2005, 1082, 43–50.

*[11] Gika,H.G. Theodoridis, G.A. amd Wilson, I.D. J. Sep. Sci. 2008, 31, 1598 – 1608 ** An interesting example of a metabolomic study comparing the use of different chromatographic techniques before mass spectrometry*

*[12] B. Daviss, "Growing pains for metabolomics," The Scientist, 19[8]:25-28, April 25, 2005 ** Insight into the difficulties faced in the field of metabolomics*

[13] Lindon, J.C., Holmes, E., Bollard, M.E., Stanley, E.G., and Nicholson, J.K. (2004) Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. Biomarkers. Vol. 9, No. 1. p.1-/31

[14] Bruggink,C. Wuhrer,M. Koeleman,C.A.M. Victor Barreto, Yan Liu, Chris Pohl, Arnd Ingendoh, Cornelis H. Hokke and André M. Deelder. J.Chromatogr. B, 829 (2005) 136–143