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Evaluation of multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) for sulfur metabolic studies using ^{34}S-labelled yeast.

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Abstract

A multi-collector ICP-MS instrument was evaluated for the on-line measurement of sulfur isotope ratios during the Liquid Chromatography separation of sulfur metabolites in mouse urine after the oral administration of ^{34}S-labelled yeast. The multi-collector instrument used was equipped both with Faraday cups and ion counters at positions L4 (32), C (33) and H4 (34). For the optimisation of the resolution, cup configuration and measurement conditions an artificial mixture of natural sulfur and highly enriched ^{33}S and ^{34}S was prepared. The results were compared with those obtained using a sector field single collector instrument from the same manufacturer. Both instruments provided good accuracy in the measurement of sulfur isotope ratios but better precision (<0.01%) was obtained with the multi-collector instrument. For HPLC coupling a nebulization-desolvating system (Aridus II) was used to eliminate the high amounts of methanol present in the mobile phases and maintain the sensitivity for sulfur

during the gradient separation of urinary metabolites. Preliminary sulfur metabolism studies were carried out feeding healthy and prostate cancer mice with one dose of yeast enriched with ^{34}S and measuring the sulfur metabolites in urine at different times using a ^{33}S solution as post-column spike. Chromatograms obtained showed different sulfur isotope enrichments for several metabolites in healthy and diseased mice. However, the variability in retention times from sample to sample hindered the interpretation of the results. Additionally, the number of samples is not enough to draw any metabolic conclusions at this stage.

Keywords: Sulfur metabolism, stable isotopes, isotope ratio measurements, Liquid chromatography, MC-ICP-MS.

Introduction

Sulfur is an essential trace element present in multitude of biologically important compounds. In particular, sulfur is present in two essential amino acids, methionine and cysteine. These amino acids, either one or both, are present in almost 98% of human proteins. Tracer studies on sulfur metabolism have been carried out traditionally using radioactive ^{35}S with a half-life of 87.5 days.[1] However, stable sulfur isotopes, such as ^{33}S or ^{34}S, could be employed to study sulfur metabolism in combination with ICP-MS detection[2] with the added advantage that a chromatographic separation could be coupled to the ICP-MS to achieve speciation information. So, for the application of enriched stable isotopes of sulfur in metabolism/speciation studies, the accurate and precise measurement of sulfur isotope ratios in transient signals obtained after a chromatographic separation is required. Unfortunately, the detection of sulfur by ICP-MS is hampered by its low ionization efficiency and the occurrence of serious spectral interferences. The ionization efficiency of sulfur in the argon plasma is only ca. 10%, due to its high first ionization potential (10.36 eV), and leads, in general, to low sulfur sensitivity. Additionally, when organic modifiers are employed for the separation of sulfur containing compounds by liquid chromatography the sensitivity in the ICP-MS reduces further.[3] On the other hand, all sulfur isotopes are interfered by polyatomic ions in the ICP-MS. For example, the most abundant sulfur isotope ^{32}S is interfered by the highly abundant oxygen dimer ion $^{16}O_2^+$. The introduction of the double focusing sector field ICP-MS with high mass resolution has allowed to resolve all major spectral interferences that affect the measurement of sulfur by ICP-MS[4,5] and provide high sensitivity sulfur detection. In our laboratory we have developed a

procedure for the study of sulfur metabolism based on the synthesis of ^{34}S-labelled yeast,[6] its characterisation by HPLC-ICP-MS[7] and its application to the study of sulfur metabolism in laboratory animals.[8] However, the single collector instrument employed provided low precision isotope ratios which precluded the accurate identification and quantitation of low enrichment sulfur-containing compounds. It was clear that, to get a much better picture of sulfur metabolism, the use of a multicollector ICP-MS was required.

During the last years, the use of multicollector ICP-MS instruments has become increasingly popular for the measurement of elemental isotope ratios when high precision and accuracy are required.[9] In the case of sulfur, its isotope abundances can vary substantially in nature[10] and MC-ICP-MS instruments have been applied successfully to distinguish among different sulfur sources[11,12,13] with instrumental precisions between 0.1% and <0.005% depending on sulfur concentration. Additionally, the on-line coupling of a separation technique to the MC-ICP-MS instrument has been described for sulfur isotope ratio measurements using both LC[11] and GC[14,15] separations. In these applications a high concentration of sulfur is usually required to obtain precise isotope ratios as low sensitivity Faraday cups are normally employed as detectors. Unfortunately, the low concentration of some sulfur metabolites in urine may require the use of the most sensitive ion counting detectors under a multicollector configuration. In this work we evaluate a multicollector ICP-MS instrument equipped both with Faraday cups and three ion counters for the precise measurement of sulfur isotope ratios during metabolic studies and the results are compared with the single collector instrument employed previously.[8] To perform the metabolic studies, a single dose of a yeast slurry enriched in ^{34}S

has been delivered to healthy and prostate cancer mice. Urinary metabolites have been separated by reverse phase HPLC and detected by ICP-MS using a membrane desolvating system. Sulfur isotope ratio data was employed to calculate the tracer/trace ratios for the different sulphur compounds detected and a simultaneous quantification of sulfur in the different chromatographic peaks was carried out by post-column isotope dilution using a ^{33}S enriched spike as described previously.[8]

Experimental

Reagents

A stock solution of 1000 mg/L of natural abundance S (as sulfuric acid in water) was purchased from Merck (Darmstadt, Germany). Further dilutions of this stock solution were made using ultra-pure water obtained from a Milli-Q system (Millipore Co., Bedford, MA, USA) to prepare the different working aqueous standard solutions as required. Enriched ^{33}S and ^{34}S were supplied from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder. Stock solutions of about 1000 mg/L were prepared and characterized in terms of concentrations and isotopic abundances as described previously.[6] The natural abundance and ^{33}S and ^{34}S spike solutions were kept refrigerated at 4°C. A mixture of the different sulfur isotopes, containing ca. 30 mg/L of ^{33}S, ^{34}S and natural sulfur, was made by diluting the stock solutions using ultra-pure water. This mixture was employed for the optimisation of conditions in the multicollector instrument.

Yeast labelled with ^{34}S (66% isotopically enriched)[8] was prepared previously in our laboratory by yeast grown on a ^{34}S-enriched medium. Enriched yeast was characterized in terms of isotope enrichment and total sulfur concentration using a MC-ICP-MS instrument as described previously.[6,8] Yeast was kept frozen at -20ºC until use. BAX Inhibiting peptide V-5 was employed to correct for retention time variability and was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

Ammonium acetate was purchased from Fluka Analytical (Buchs, Suiza) and HPLC-grade methanol from Merck. Both reagents were employed in the chromatographic mobile phase.

Instrumentation

The double focusing inductively coupled plasma mass spectrometer (DF-ICP-MS) used was an Element II from ThermoFisher Scientific (Bremen, Germany), and was operated at the medium resolution mode ($m/\Delta m = 4000$). All measurements were made with the standard sample introduction configuration of the instrument, that is, a Scott-type spray chamber working at room temperature, a Meinhard concentric nebulizer and a Fassel torch. The multicollector inductively coupled plasma mass spectrometer (MC-ICP-MS) used was a Neptune Plus from ThermoFisher Scientific (Bremen, Germany) and was operated at the pseudo high resolution mode. The instrument was equipped with 9 Faraday cups and 3 ion counters located at positions L4 (IC2), C (IC1) and H4 (IC3). The sample introduction system was a PFA-100 microconcentric nebulizer and a cyclonic spray chamber. The optimum

instrumental settings for the measurement of sulfur isotope ratios in both instruments are summarized in Table 1. The nebulizer gas flow rate, torch position and ions lens settings were optimized for higher sensitivity and the acquisition parameters were optimized for better precision of the measurements of the sulfur isotope ratios.

Liquid Chromatography separations were performed on a Surveyor LC Pump Plus (ThermoFisher Scientific, Bremen, Germany) using a Discovery BIO Wide Pore C18 reverse phase column (15 cm X 2.1 mm, 5 µm particle size, Supelco, Bellefonte, Pennsylvania, USA). A peristaltic pump Minipuls 3 (Scharlab, Barcelona, Spain) and a T-piece were used to continuously mix the eluent from the chromatographic column with the ^{33}S isotope enriched solution at 20 µl/min before the sample introduction system of the ICP-MS. A desolvating microconcentric nebulizer Aridus II (CETAC Technologies Inc., Nebraska, USA) was used to coupled the chromatographic system to the MC-ICP-MS instrument. The instrumental parameters employed in the chromatographic and desolvating systems are summarized in Table 2.

All standard solutions were prepared gravimetrically using an analytical balance model AB204-S (Mettler-Toledo GmbH, Greifensee, Switzerland). A centrifuge (Fisher Scientific, Waltham, MA, USA) was used for the separation of the suspended solids from the urine samples.

Procedures

Metabolic studies. Four c-57 mice (two of them healthy and two with advanced prostate cancer) were hosted in a metabolic cage. After a short period of

acclimatization, mice were fed once with 0.6 g of ^{34}S enriched yeast as slurry. Urine samples were collected at different time intervals after administration (0, 12 and 24 hours), centrifuged at 5000 g during 10 minutes to separate suspended solids and then stored at -20°C.

Chromatographic separation and desolvating system. The separation of the sulfur metabolites present in urine was carried out by injecting 5 µL of the undiluted sample in the chromatographic system using the conditions shown in Table 2. The flow exiting the column was mixed with a flow of 20 µL/min of a 1 µg/g ^{33}S-enriched standard solution. Then, the mixture was nebulized into the PFA spray chamber of the Aridus II system using a PFA-100 microconcentric nebulizer and an argon gas flow of 0.9 $L.min^{-1}$. The spray chamber was heated at 110 ºC to reduce de formation of solvent droplets and the microporous PTFE tubular membrane was heated at 160 ºC. The solvent vapour was removed by an external flow of Ar (sweep gas) of 0.5 L/min.

Data treatment procedure. Data treatment is essentially the same as that described previously.[7] In brief, intensity chromatograms were converted into isotope ratio chromatograms by dividing the signals at masses 32 and 34 by the signal at mass 33 in each point of the chromatogram. Then, isotope ratios were corrected for mass bias using the exponential model[7] and a 100 ppb natural sulfur standard solution, measured daily before the samples, and transformed into isotope abundances. Finally, the contribution of natural abundance sulfur, ^{34}S-enriched sulfur and ^{33}S-enriched sulfur to the observed isotope abundances, the molar fractions, were determined by Isotope Pattern Deconvolution (IPD). The relative molar flow chromatograms for ^{34}S and natural abundance sulfur were calculated by dividing the obtained molar fractions of ^{34}S

and natural sulfur by those of ^{33}S at each point of the chromatogram. Using this procedure no blank correction is necessary as the possible sulfur in the blank is taken as part of the natural abundance sulfur in the urine matrix.

Results and discussion

Cup configuration and spectral interferences

The multicollector instrument installed at the University of Oviedo is equipped with three ion counters, IC2, IC1 and IC3, situated at positions L4, C and H4. Both ion counters at L4 and H4 can be moved with the corresponding Faraday cups in the same position. So, in order to compare the Faraday cups with the ion counters using the same cup configuration the isotope 33 of sulfur was set at the central cup while isotopes 32 and 34 were collected in positions L4 and H4 respectively as shown in Figure 1. Due to the low natural abundance of ^{33}S, we prepared a "calibration" solution containing all ^{32}S, ^{33}S and ^{34}S at approximately the same concentration as described in the experimental section. Figure 2A shows the pseudo-high resolution spectra obtained for the calibration solution in the Faraday cups at 30 µg/g of total sulfur while Figure 2B shows the spectra obtained with the ion counters at a much lower concentration of total sulfur (150 ng/g). As can be observed in Figure 2A all three spectra show a wide area, around mass 32.965 u for the centre cup, where sulfur can be detected without interferences, as expected.[6] For example, for ^{33}S in the central cup, the lower mass plateau corresponds to the sulphur isotope while the middle plateau corresponds to the sulphur isotope plus polyatomic

interferences; the final plateau at the higher masses corresponds only to the interferences. The same can be said for the other sulphur isotopes at collectors L4 and H4. By careful selection of the cup position we can measure all isotopes free of spectral interferences as shown by the arrow included in Figure 2A. In the case of Figure 2B for the ion counters the mass spectra show a very different behaviour. First, the ion counting detectors go into protection mode when the signals are too high (above ca. 10^6 cps). Consequently, only the signals corresponding to the sulfur isotopes are observed in the spectra while the signals corresponding to the interferences, much higher in intensity, are suppressed as the ion counters get saturated. It can be understood that, under these conditions, it is very difficult to set the ion counters to measure sulfur isotope ratios. The ion counters get saturated when the cups are not in the right position and no signal is detected. The way round this problem was to set first the Faraday cups L4 and H4 with the calibration solution containing all three ^{32}S, ^{33}S and ^{34}S isotopes at approximately the same concentration and then apply the deflection voltage to move the ion beams from the Faradays to the ion counters. Once the cup configuration was set and saved it could be recalled on a different day without further complications. In Figure 2B we can also see that the signal for ^{32}S is higher than the signal for ^{34}S which is the opposite of that observed in Figure 2A. This is due to the fact that, at the very low concentration of sulfur in the calibration solution in Figure 2B, the blank contribution of natural abundance sulfur increased substantially which is why the signal for ^{32}S is now the predominant in the mass spectra.

Linear range, blanks and limits of detection

The linear range of the Faraday cups and the ion counters in the multicollector instrument was evaluated by measuring a series of standards of natural abundance sulfur (Merck standard) at increasing concentration levels from 0 up to 10000 ng/g. The ion counters showed a linear range for isotope 32 up to only 100 ng/g of sulfur while the Faraday cups where linear up to, at least, 10 µg/g. For comparison purposes the linear range of the single collector instrument, working at medium resolution, was evaluated as well using the same solutions. It was observed that the calibration was linear up to 5 µg/g and then curved due to detector dead time. The instrumental limits of detection were calculated using the calibration data for isotope ^{32}S obtained between 0 and 100 ng/g using the criteria of three times the standard deviation of the intercept divided by the slope of the calibration graph. The limits of detection were lowest for the multicollector instrument using the ion counters, 4 ng/g of sulfur, and highest for the Faraday cups, 38 ng/g of sulfur. The results for the single collector instrument were in the middle with a limit of detection of 23 ng/g. The concentration of natural abundance sulfur in the milli-Q water blank was estimated to be ca. 20 ng/g by transforming the calibration data into a standard addition curve.

Isotope ratio measurements

Isotope ratio measurements were performed at different concentration levels using natural abundance sulfur and the results compared with those obtained with the single collector instrument. The range of concentrations tested was always within the linear working range for each instrument and cup configuration tested. The results were evaluated only in terms of reproducibility

of the $^{34}S/^{32}S$ isotope ratio using the constant nebulisation of sulfur standards. For the multicollector instrument data were acquired as 5 blocks of 10 cycles each. For the single collector instrument the data were acquired as 5 runs of 200 passes each. In both cases the isotope ratio, R, was calculated from the slope of the line "y=Rx", where x was the intensity for the isotope 32 and y was the intensity for the isotope 34, using the raw data points as described previously.[16] The standard error of the isotope ratio was taken as the standard deviation of the slope and corresponds to the standard error of the mean.[16] The results obtained for the $^{34}S/^{32}S$ isotope ratio in the natural abundance sulfur standard are shown in Figure 3. As it can be observed, the precisions obtained with the multicollector instrument were much better than those obtained with the single collector instrument at high sulfur concentrations reaching relative standard errors below 0.01% in the Faraday cups for the higher concentrations tested. On the other hand, the precision obtained with the ion counters were below 0.1% for sulfur concentrations between 5 and 100 ppb ng/g. Such precisions could not be achieved using the single collector instrument (a factor of ca. 4 worse) or the multicollector instrument with the Faraday cups at this concentration levels. We can conclude that the ion counters provide very good isotope ratio precisions at low sulfur concentration levels. Unfortunately, the ion counters get saturated easily and then the signal drops suddenly due to self-protection.

The values shown in Figure 3 correspond to the standard error of single measurements. No attempt to calculate combined uncertainties was performed in this comparison of instrumental configurations.

Separation and detection of sulfur metabolites in urine

Different chromatographic types (anion and cation exchange, ion pair and reverse phase chromatography) were assayed for the separation of the sulfur metabolites present in the urine of c-57 mice for its detection by multicollector ICP-MS. Better separations were found using reverse phase chromatography and this mode was selected for further experiments. The main problem in the detection of sulfur compounds in urine by multicollector ICP-MS was the presence of a high peak of inorganic sulphate which appeared at the beginning of the chromatogram. This early eluting peak prevented us to use the ion counters for sulfur detection as the ion counters went into protection mode for most of the chromatogram even after the elution of the main sulfur peak. So, for the chromatographic study, only the Faraday cups were employed.

Figure 4 shows a typical chromatogram obtained for the direct injection of a control mouse urine (healthy c-57 mice) for isotopes 32 (black line, left axis) and 33 (open points, right axis) of sulfur using the chromatographic conditions shown in Table 2. About 20 different sulfur-containing compounds were detected under the conditions employed with relatively strong signals for the multicollector ICP-MS using the Faraday cups. The main peak, off-scale, corresponds to sulphate and eluted near the dead-volume of the column. The identity of most of the other sulfur-containing peaks in Figure 4 is unknown to date; methionine and glutathione eluted in less than ten minutes under the conditions tested (7.0 and 5.7 min respectively) while no other peak in the chromatogram matched the retention time of other possible metabolites evaluated (Cysteine, Cystine, Taurine, Homocysteine, Cistathione and Cisteinil-glycine). The last peak at ca. 58 minutes retention time corresponded to BAX

Inhibiting Peptide V-5 which was added to all samples as internal standard to check for retention time variations and to evaluate the mass bias correction procedure. The mass bias correction was performed using the exponential model and the magnitude of the mass bias factor was between 1-2% in all cases.

The signal at mass 33 in Figure 4 is due to the post-column addition of highly enriched ^{33}S for quantitative purposes.[7] As it can be observed, the signal for ^{33}S is almost constant for most of the chromatogram (up to 50 minutes) with a small reduction after this time because of the introduction of a 30% methanol mobile phase (see Table 2 for the gradient elution program). It is important to note here that, when the desolvating nebulizer was not employed, the signal for ^{33}S was reduced to less than 10% of that at the beginning of the chromatogram because of severe sulfur ionisation suppression in the ICP. So, the late eluting sulfur-containing peaks went almost undetected by direct nebulisation. On average, the sulfur sensitivity improved by a factor of 5 at high levels of methanol in the mobile phase when the desolvating nebuliser was employed. Additionally, no band broadening effects were noticeable when employing the desolvating nebuliser.

Figure 5 shows the $^{34}S/^{32}S$ isotope ratio chromatogram obtained in the multicollector instrument for the same healthy c-57 mouse before (white points) and 6 hours after (grey points) the oral administration of the ^{34}S-labelled yeast. As it can be observed, there are several peaks, particularly those two at 26.8 and 30.2 minutes retention time, which show clear isotope enrichment for ^{34}S. These two peaks are observed in all chromatograms either for healthy or

diseased mice and their isotope enrichment changes drastically with the time of urine collection after the administration of the ^{34}S-labelled yeast.

Sulfur metabolic studies in c-57 mice

For these experiments four c-57 mice were employed: two healthy and two with advanced prostate cancer. Each mouse was allocated for ca. 48 hours in a single-mouse metabolic cage which was cleaned after each use. The mice were set in the cage in the evening of the first day and, next day, the urine collected during the night was taken as control sample. Then, a dose of ^{34}S-labelled yeast was given orally to the mice, as slurry, and the urine excreted during the next 12 hours collected. Finally, a third urine sample was collected ca. 12 hours later. All urine samples were kept at -20ºC until analysis. Finally, the mice were returned to their original breading quarters without any apparent damage.

For analysis the samples were thawed, an aliquot of the BAX Inhibiting Peptide V-5 added, and the chromatograms at masses 32, 33 and 34 measured in the multicollector instrument. The quantification procedure followed is indicated in Figure 6 for one of the prostate cancer mice at 12 hours after the administration of the yeast. The intensity chromatograms (Figure 6A) were corrected for mass bias and converted into molar fraction chromatograms by solving the multiple linear regression equation shown below for each data point in the chromatogram.

$$\begin{bmatrix} A_{32} \\ A_{33} \\ A_{34} \end{bmatrix} = \begin{bmatrix} 0.9493 & 0.0001 & 0.0021 \\ 0.0076 & 0.9970 & 0.0042 \\ 0.0429 & 0.0029 & 0.9937 \end{bmatrix} \times \begin{bmatrix} x_{nat} \\ x_{33} \\ x_{34} \end{bmatrix}$$

In this equation A_{32}, A_{33} and A_{34} are the relative contribution of the intensities at masses 32, 33 and 34 measured (e.g. $A_{32}= I_{32}/[I_{32}+I_{33}+I_{34}]$). The numerical values correspond to the isotopic composition of natural sulfur, the ^{33}S-enriched sulfur and the ^{34}S-enriched sulfur respectively[6] while the unknowns are the molar fractions of natural, ^{33}S-enriched and ^{34}S-enriched sulfur contributing to the observed isotope distribution. The obtained molar fraction chromatogram is shown in Figure 6B. As can be observed, the contribution of the ^{34}S-enriched sulfur is very low except for a few small peaks between 20 and 30 minutes retention time. Then, the molar fraction chromatogram is transformed into a relative molar flow chromatogram by dividing the molar fractions x_{nat} and x_{34} by that of x_{33} in each point of the chromatogram. A full theoretical description of the equations involved can be found elsewhere.[17] The results shown in Figure 6C indicate that most of the peaks obtained show a contribution from both sources of sulfur (natural and ^{34}S-enriched sulfur). The peak at a retention time of ca. 62 minutes corresponds to the BAX peptide and shows natural isotopic composition while the two peaks at 25.2 and 29.3 minutes are clearly enriched in ^{34}S. These two peaks are the same as those shown in Figure 5 for the ^{34}S/^{32}S isotope ratio with a small change in retention times.

Finally, the relative concentration of ^{34}S-enriched and natural sulfur in each chromatographic peak, the tracer/tracee ratio, was obtained by two alternative procedures: i) by integrating the relative molar flow chromatograms and dividing the peak area obtained for the ^{34}S-enriched by that obtained for natural sulfur and ii) by dividing the molar fraction for ^{34}S by that of natural sulfur during the chromatogram. The results for this second alternative are shown in Figure 6D.

As can be observed, apart from the high tracer/tracee ratios for the two primary metabolites (off-scale) there are a number of other peaks showing significant enrichment in ^{34}S. This second alternative proved to be the best for data evaluation as, for some peaks, it was impossible to integrate the chromatogram shown in Figure 6C. For example, the peak obtained at 25.2 minutes retention time shown on the ^{34}S relative molar flow chromatogram has no counterpart in the natural sulfur chromatogram. So, it was not possible to calculate the tracer/tracee ratio in this peak by area integration. Additionally, for the peaks that could be integrated in both mass flow chromatograms, their tracer/tracee ratios were very close to those shown in Figure 6D at the maximum of the corresponding peaks.

The tracer/tracee ratio chromatograms obtained for the two healthy mice (A and B) and the additional prostate cancer mouse (C) are shown in Figure 7. When we compare the data for the four mice, including Figure 6D as prostate cancer mouse, we can see three main features:

1. The two primary metabolites are always the dominating peaks in each chromatogram. Their tracer/tracee ratios are different for each mouse and depend strongly on the time of urine sampling. The samples collected after 24 hours after the yeast administration showed only traces of these two peaks for all the mice. So, there is a need for normalization of the tracer/tracee data both in terms of biological variability and temporal variability.

2. The retention times for the main peaks in all chromatograms of mice urine changed from sample to sample. This problem was not observed for the sulphur aqueous standards that we had previously tested such as methionine or glutathione. Different attempts changing the chromatographic conditions were

assayed to obtain reproducible retention times, including 1 hour of column equilibration after each chromatogram. Unfortunately, the problem remained in all cases. It was observed that the retention times for the same urine sample did not change when it was analysed on different days so the problem could be attributed to differences in the urine matrixes which eventually affect the chromatographic separation. Thus, we decided to add to all urine samples the BAX Inhibiting Peptide V-5, which eluted at the end of the chromatogram, to help correct for retention time drift and improve peak alignment. To do that, the retention time for three peaks (the initial sulphate peak, the main peak at ca. 30 minutes retention time and the final BAX peptide peak) were used as reference and all retention times were normalised using a quadratic regression. The normalised chromatograms showed that the retention times for all sulfur compounds agreed much better for different urine samples. As an example, Table 3 shows the experimental and normalized retention times for 6 peaks in the chromatograms shown in Figures 6 and 7. As can be observed, after normalisation the retention times agree much better between the different urine samples..

3. There are no obvious similarities and/or differences between the healthy and prostate cancer mice with regards to tracer/tracee ratios in sulfur metabolites. It is clear that more experiments would be needed to study these differences in combination with multivariate data treatment. In our laboratory we have started an experiment in which 10 mice (5 healthy and 5 with genetic predisposition to develop prostate cancer) will be followed during ca. 50 weeks.

CONCLUSIONS.

The comparison of ICP-MS instruments developed in this work has demonstrated that the multicollector ICP-MS instrument using Faraday cups show better precisions in the measurement of sulfur isotope ratios at relatively high sulfur concentrations. However, at concentrations below 0.1 ppm, the double focusing instrument and, particularly, the multicolector instrument using ion counters have better precisions. In the latter case, sulfur isotope ratio precisions as good as 0,1% could be achieved at concentrations as low as 0,01 ppm. Unfortunately, the concentrations of sulfur in biological samples are too high so ion counters were not used in metabolic studies.

The separation of the sulfur metabolites present in the urine of mice by reverse phase HPLC required the use of a gradient of methanol which is incompatible with the argon ICP. Fortunately, the used of a desolvation unit (Aridus II) allows the removal of methanol prior to its introduction into the plasma. Thus, the eventual plasma extinction and the signal depression are avoided improving the sulfur metabolites separation and detection. Problems with variable retention times in the urine samples were solved by adding the BAX Inhibiting Peptide V-5 to every sample and a simple quadratic normalization using three peaks as reference.

Metabolic studies were performed feeding mice with a single dose of ^{34}S enriched yeast. The amount of natural sulfur and sulfur from the ^{34}S enriched yeast in each metabolite could be calculated using an IPD mathematical tool. It was observed that several sulfur metabolites show a high enrichment after 12 hours. However, the compounds and the level of enrichment were not very different in healthy mice and in mice with prostate cancer. These results indicate that further studies are necessary to study such differences at different

stages of the disease. Also, other types of cancer will be investigated in order to evaluate the proposed methodology in the early diagnosis of such diseases.

ACKNOWLEDGEMENTS.

The authors are grateful for financial support from Spanish Ministry of Economy and Competitiveness through Projects Ref. CTQ2009-12814 and Ref. CTQ2012-36711 (co-funded by FEDER). The UE is acknowledged for the provision of FEDER funds for the purchase of the MC-ICP-MS instrument. Oscar Galilea San Blas acknowledges his doctoral grant to the University of Oviedo, Spain. Provision of mice from experimental therapies group (Juan Carlos Mayo, University of Oviedo) is deeply appreciated. Teresa Fernández and Agustín Brea from the Biotery of the University of Oviedo are also gratefully acknowledged.

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Table 1- Instrumental operating conditions and acquisition parameters employed for the different configurations.

Parameters	Element II	Neptune Plus (Faraday cups)	Neptune Plus (Ion counters)
Rf power	1350 W	1200W	1200W
Cool gas flow	14 L min^{-1} Ar	14 L min^{-1} Ar	14 L min^{-1} Ar
Auxiliary gas flow	0.95 L min^{-1} Ar	0.9 L min^{-1} Ar	0.9 L min^{-1} Ar
Sample gas flow	0.91L min^{-1} Ar	0.89 L min^{-1} Ar	0.89 L min^{-1} Ar
Acquisition method	5 runs, 200 passes, 0.01 s sample time, 10 samples per peak, 3 s setting time	5 blocks, 10 cycles, 4.194 s integration time, 3 s idle time	5 blocks, 10 cycles, 4.194 s integration time, 3 s idle time
Cup/ion counter Configuration		L4 ^{32}S	IC2 ^{32}S
		C ^{33}S	IC1 ^{33}S
		H4 ^{34}S	IC3 ^{34}S

Table 2- Instrumental parameters used in the chromatographic and desolvating systems.

HPLC Conditions	
Column	Discovery BIO Wide Pore C18
Injection Loop	5 µL
Flow rate	80 µL/min
Mobile phases	A)Ammonium Acetate 75 mM, pH 7,4 , 2% MeOH
	B) Ammonium Acetate 75 mM, pH 7.4, 30% MeOH
Gradient	2 min 100% A, 30 min 50% A, 40 min 100% B, 50 min 100% B, 60 min 100% A
Post-column flow rate	20 µL/min
Desolvation-nebulization conditions (Aridus II)	
Nebulizer gas flow	0.9 $L.min^{-1}$
Sweep gas flow	0.5 $L.min^{-1}$
PFA spray chamber temperature	110ºC
Desolvating membrane temperature	160ºC

Table 3.- Experimental and normalized retention times (min) for 6 different peaks in the real urine samples for healthy and prostate cancer mice.

Healthy 1		Healthy 2		Prostate 1		Prostate 2	
Exp.	**Norm.**	**Exp.**	**Norm.**	**Exp.**	**Norm.**	**Exp.**	**Norm.**
11.3	12.1	11.9	12.0	11.3	12.3	12.5	12.0
22.7	24.2	24.0	24.1	21.8	24.1	25.3	24.1
25.6	27.6	28.3	27.9	25.1	27.5	29.2	27.9
30.0	31.7	31.9	31.8	29.3	31.7	32.3	31.4
30.9	32.6	32.9	32.8	30.3	32.7	34.1	32.6
48.8	49.4	50.3	49.4	48.7	49.4	51.2	49.4

LEGEND OF FIGURES

Figure 1- Cup configuration employed in the multicollector instrument.

Figure 2.- A mass scan showing the simultaneously measurement of ^{32}S, ^{33}S and ^{34}S in the Neptune MC-ICP-MS instrument using: (A) Faraday cups (5 ppm) and, (B) ion counters (50 ppb).

Figure 3- Relative standard deviation of the slope for the $^{34}S/^{32}S$ isotope ratio as a function of the concentration of sulfur in the single collector instrument (black points) and the multicollector instrument using the Faraday cups (white points) or the ion counters (grey points).

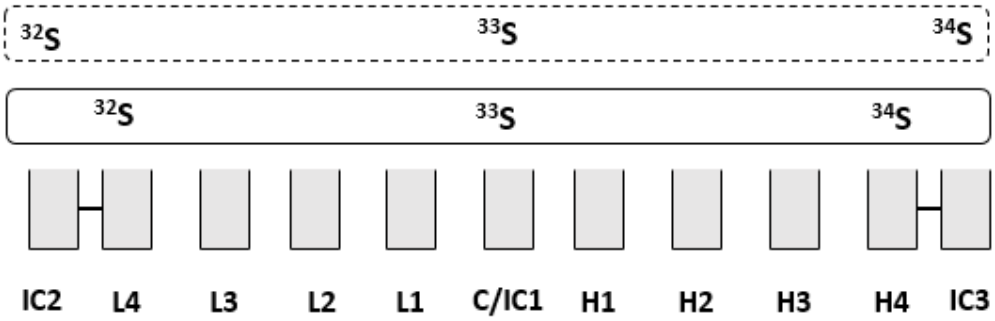
Figure 4- Typical chromatographic separation of urine metabolites with sulfur detection at isotopes 32 (black line) and 33 (white points) with the multicollector ICP-MS instrument.

Figure 5- Isotope ratio chromatograms ($^{34}S/^{32}S$) for a healthy mouse urine before (white points) and 6 hours after (grey points) the administration of the ^{34}S-labelled yeast.

Figure 6- Illustration of the data treatment procedure for the prostate cancer mouse 1: A) Intensity chromatogram. B) Molar fraction chromatogram. C) Relative molar flow chromatogram. D) Tracer/tracee ratio chromatogram. Black line: ^{32}S or natural sulfur. Grey points: ^{34}S. White points: ^{33}S.

Figure 7- Tracer/tracee ratio chromatograms of: A) Healthy mouse 1. B) Healthy mouse 2. C) Prostate cancer mouse 2.

Figure 1:



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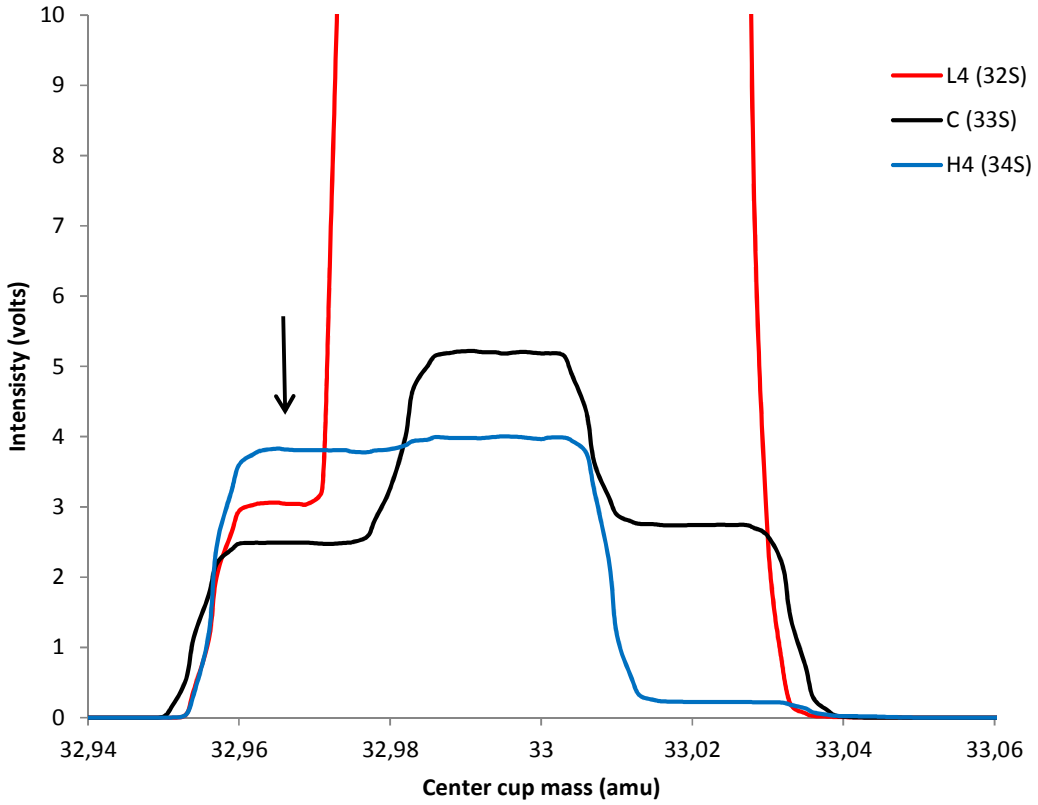

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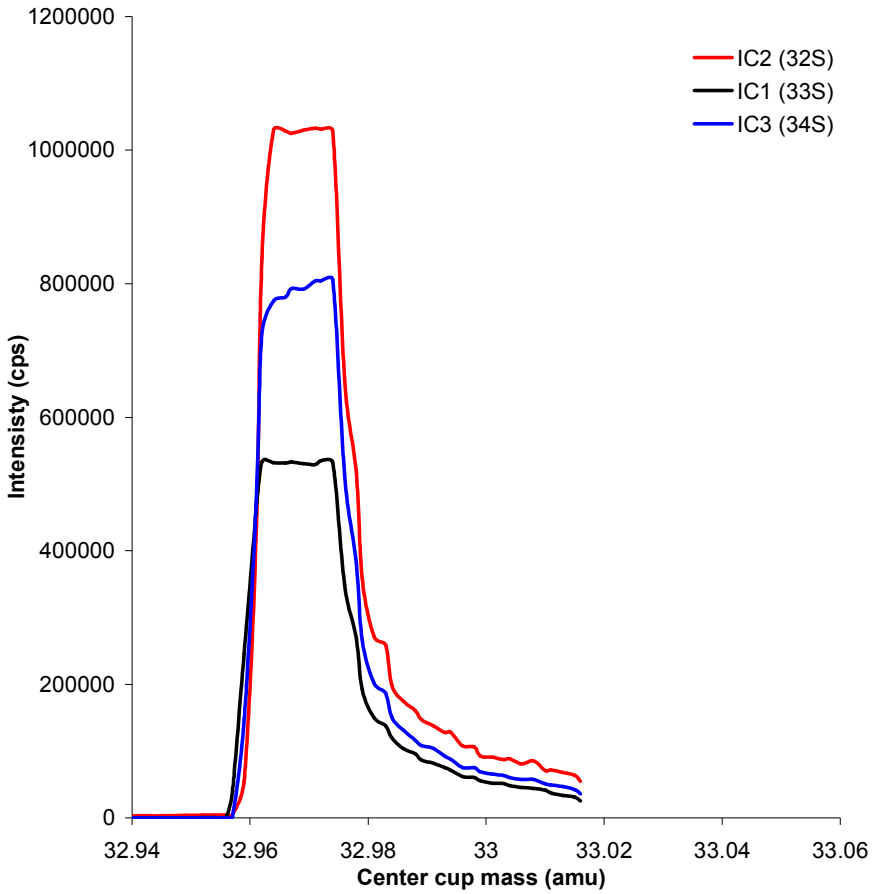

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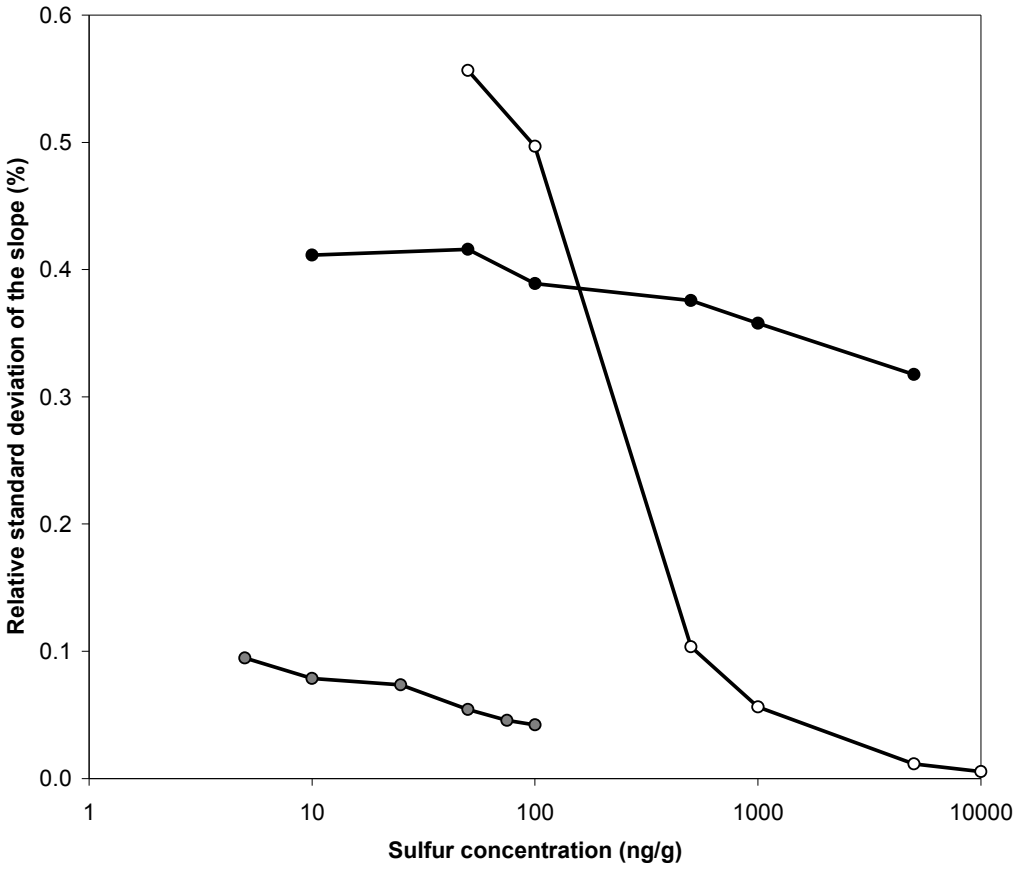

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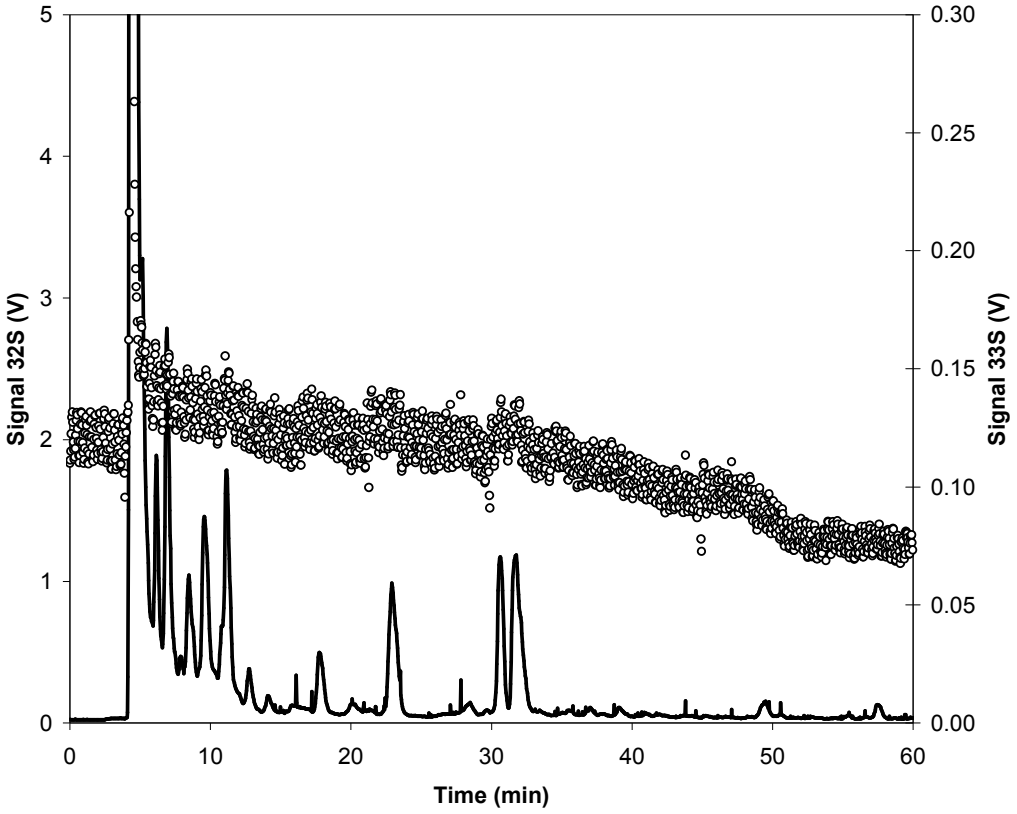

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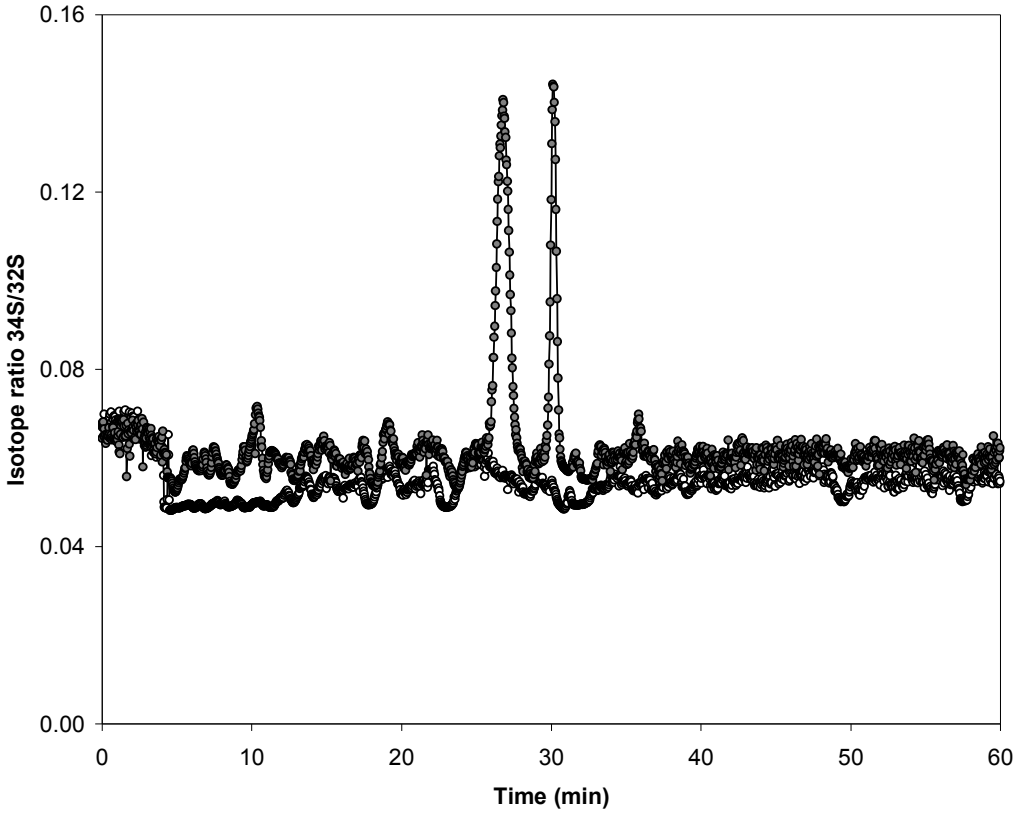
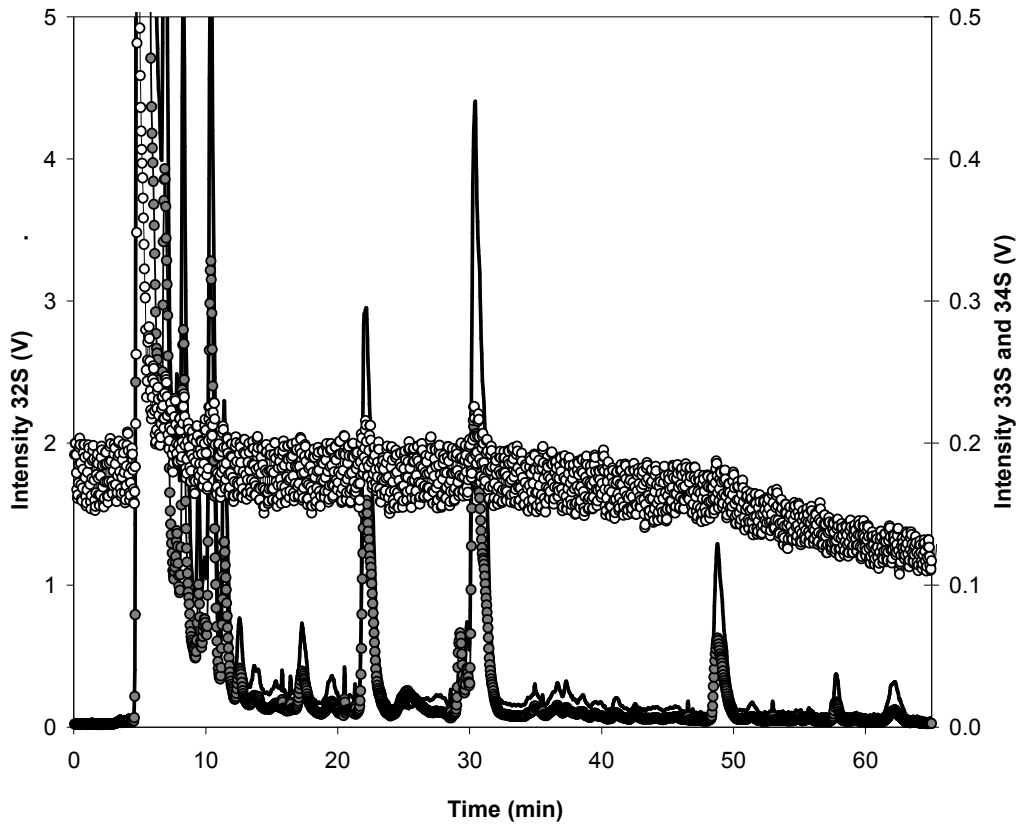


Figure 6

A)



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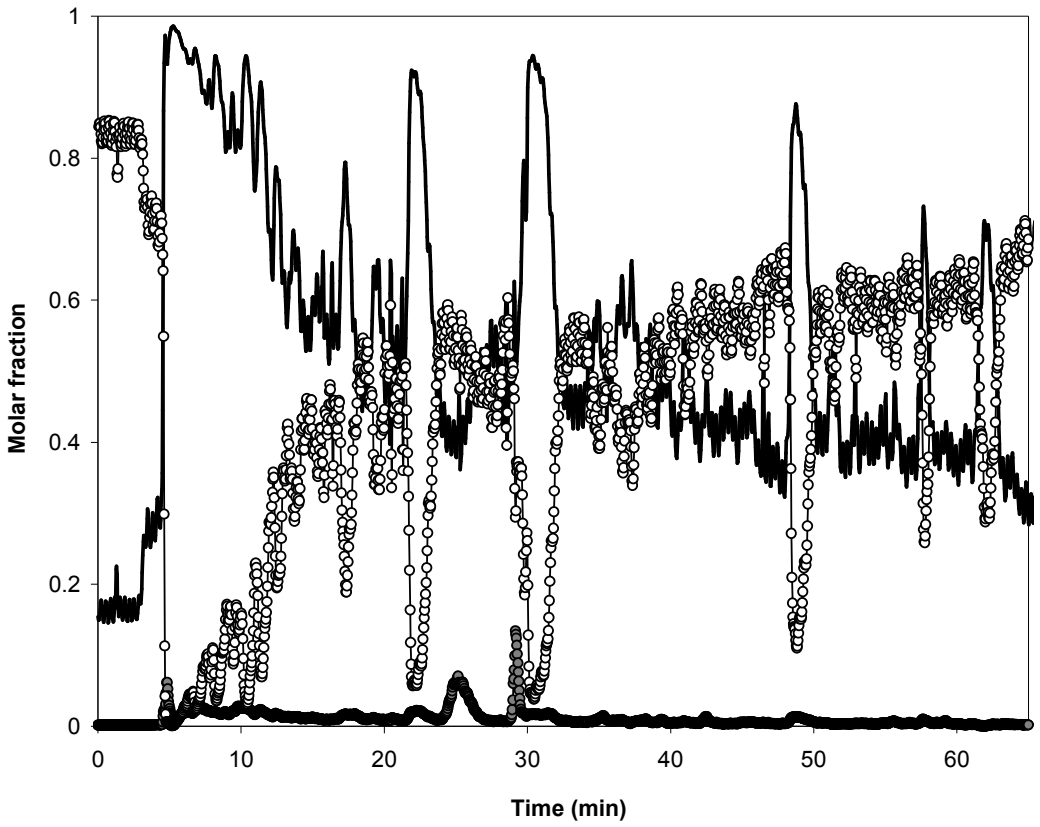

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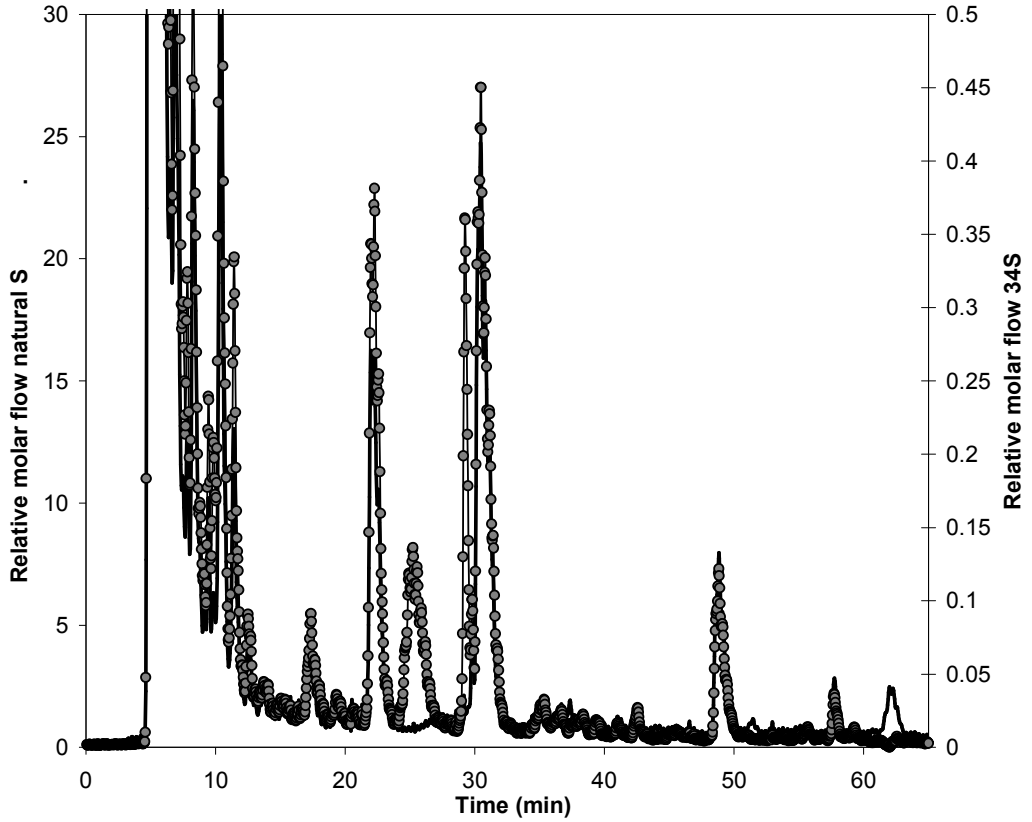

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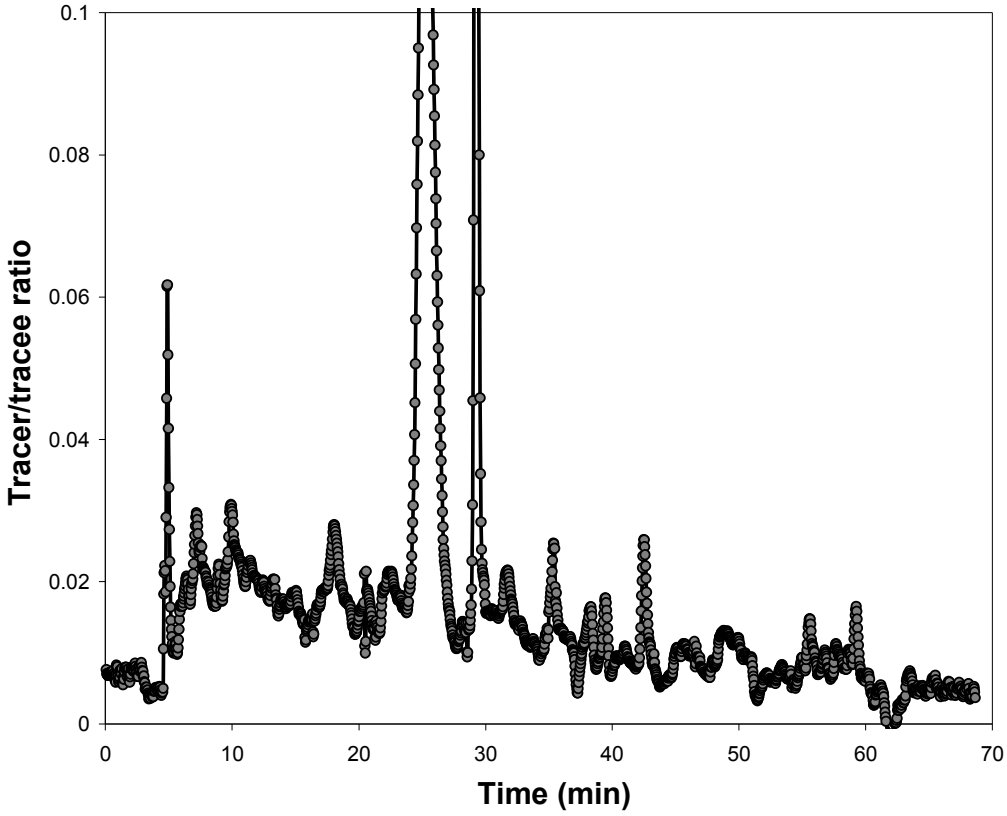
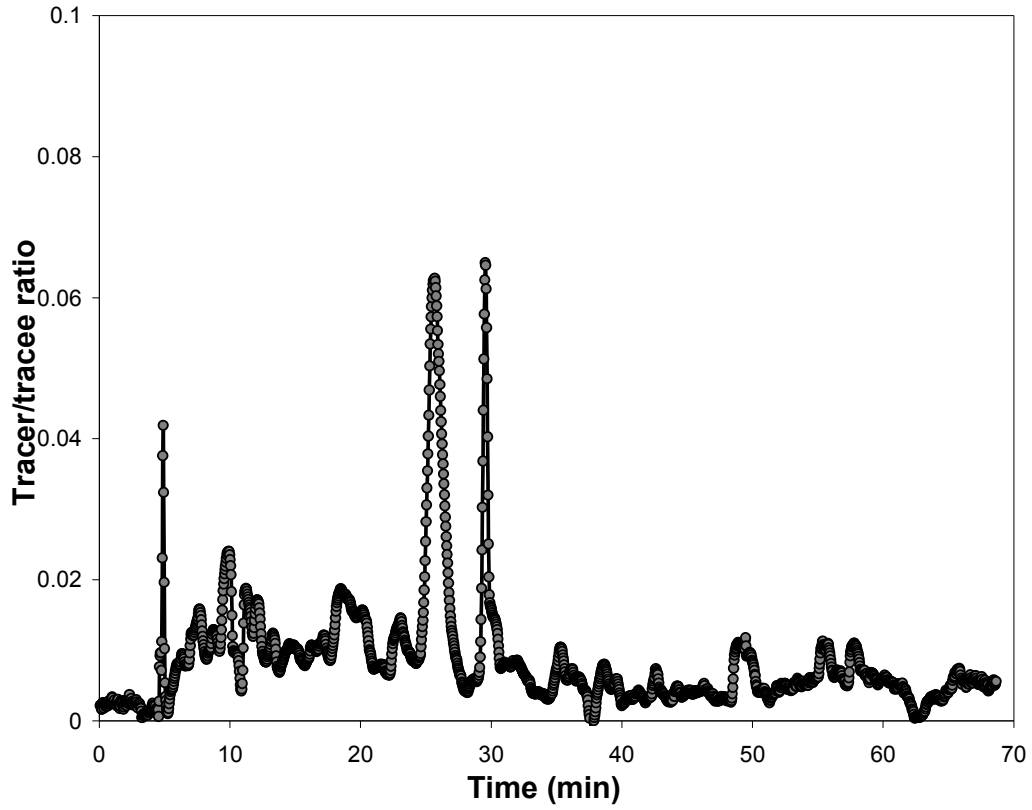


Figure 7

A)



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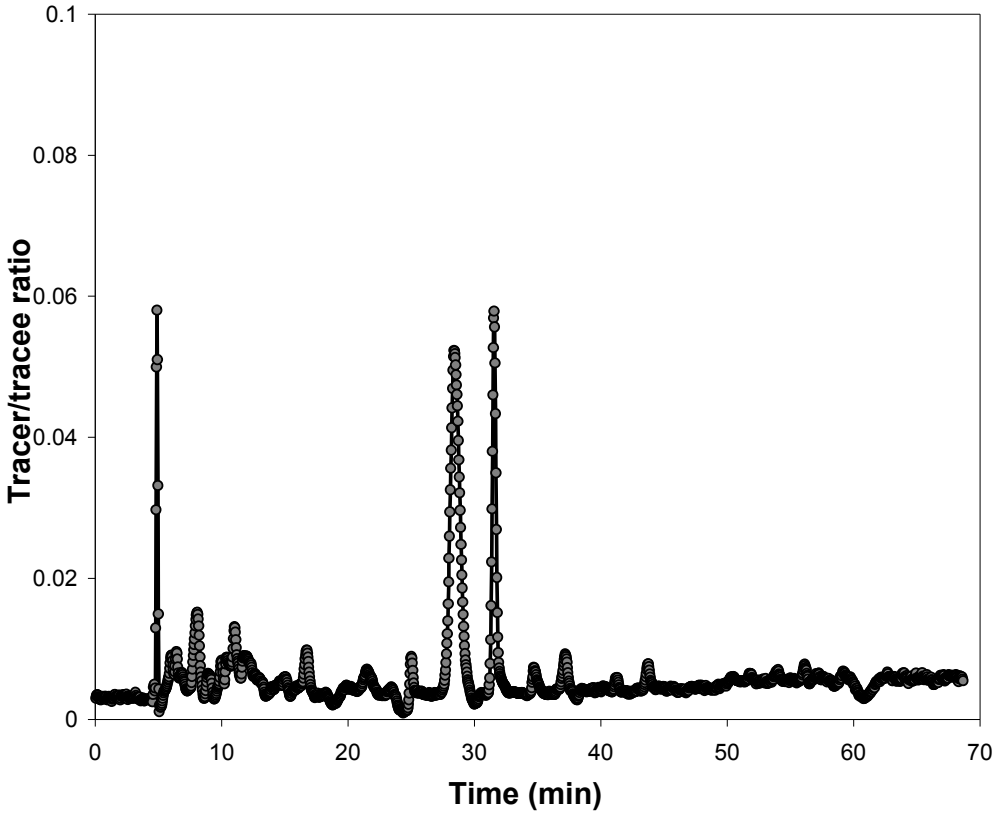

C)