Metabolite Identification Using a Nanoelectrospray LC-EC-array-MS Integrated System

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Abstract

A novel approach to the parallel coupling of normal-bore high-performance liquid chromatography (LC) with electrochemical-array detection (EC-array) and nanoelectrospray mass spectrometry (MS), based on the use of a nanosplitting interface, is described where both detectors are utilized at their optimal detection mode for parallel configuration. The dual detection platform was shown to maintain full chromatographic integrity with retention times and peak widths at half-height between the EC-array and MS displaying high reproducibility with relative standard deviations of <2%. Detection compatibility between the two detectors at the part per billion level injected on-column was demonstrated using selected metabolites representative of the diversity typically encountered in physiological systems. Metabolites were detected with equal efficiency whether neat or in serum, demonstrating the system's ability to handle biological samples with limited sample cleanup and reduced concern for biological matrix effects. Direct quantification of known analytes from the EC-array signal using Faraday's law can eliminate the need for isotopically labeled internal standards. The system was successfully applied to the detection and characterization of metabolites of phenylbutyrate from serum samples of Huntington's disease patients in an example that illustrates the complementarity of the dual detection nanoelectrospray LC-EC-array-MS system.

Metabolomics is considered a comprehensive study of metabolites, typically of molecular weight (MW) less than 1000, found in biological systems. Metabolomics seeks to aid comprehension of the important processes of an organism, organ system, cell type, cell, subcellular system and so on.[1,2] The complexity of a given metabolite sample can be quite great, requiring the ability to analyze a large spectrum of compound class types over an even greater dynamic range in concentration, while present in biological matrixes such as blood, plasma, and urine. The most common means to cover large types of compounds and eliminate any assay interferences from such complex matrixes is by using hyphenated techniques and complementary instrumentation in parallel. Generally, a separation technique such as solid-phase extraction (SPE), high-pressure liquid chromatography (HPLC), gas chromatography,

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thin-layer chromatography, and capillary electrophoresis is used before the employment of a sensitive detection technique such as mass spectrometry (MS), electrochemical or coulometric array detection (EC-array), nuclear magnetic resonance, Fourier transform infrared, or Raman spectroscopies.[3,4]

HPLC coupled with electrochemical detection (EC) has proven to be a very sensitive technique for analyzing and quantifying redox-active compounds down to picomolar concentrations[5,6] while also having the ability to analyze more than 1000 metabolites in a given HPLC chromatographic run.[7,8] In addition to being sensitive and highly precise,[9-11] use of EC cells in an array (EC-array)[12] allows differences in oxidation potentials to resolve coeluting species, adding an element of specificity to the analysis. However, despite this high specificity, the inability to elucidate the structures of such species is a major limitation of EC-array detection. HPLC-EC-array metabolomics profiling has been used to uniquely separate categories of lower motor neuron disease from controls[13] and diagnose Parkinson's disease from control.[14] However, the strongest discriminating compounds are often structurally unknown. One way to overcome this limitation is via the parallel coupling of EC-array with MS detection.[15-18]

The effective coupling of these two complementary detectors requires the consideration of several analysis parameters. For example, the redox activity of a compound is not only dependent on its chemical class and structure but also on the conditions under which it is being assayed. Solvent properties such as pH and supporting electrolyte (ideally >20 mM buffer) as well as LC flow rate need to be optimized for simultaneous EC-array and MS detection without compromising their respective sensitivities.[19] The high-concentration salt buffers typically employed in EC-array analysis are detrimental to ESI-MS analysis, creating analyte adducts and causing ion suppression often rendering analytes of interest undetectable.[20] Nanospray-ESI-MS (flow rates <200−300 nL/min) has been proposed as an alternative to overcome many of the latter problems as it has been proven to increase ionization, desolvation, and ion-transfer efficiency over ESI conducted at higher flow rates[19] while also decreasing ion suppression due to matrix effects. This is in sharp contrast to HPLC-EC-array, which is normally operated in combination with normal-bore (4.6-mm-i.d.) columns and in a flow regime of the order of 1 mL/min.[21,22] Although coupling nanospray ESI-MS with EC-array detection appears to be a logical approach for global metabolomics analyses, several obstacles have to be overcome, in particular appropriate adjustment of mobile-phase composition and flow conditions to maintain the chromatographic integrity of the dual detection system while also maintaining optimal performance of each detector.

In view of the aforementioned mismatch in the detection requirements of MS and EC-array, it is advantageous to design a flow-splitting interface that would accommodate the integration of the two detectors into a common HPLC system. The nanoSplitter interface, developed previously in our laboratory and that delivers a very small fraction (<0.1%) of the HPLC eluent into the MS via a concentric split design, has demonstrated significant improvements in MS sensitivity when compared to a conventional LC-ESI-MS system for both in vitro and in vivo metabolism studies.[23] These improvements ranged from 1.8- to 40-fold increases in analyte peak area, dependent on analyte and gradient elution profile. The most significant improvements were demonstrated by polar analytes, eluting under high aqueous conditions.[24] Also, and most important for incorporating EC-array with nanospray-ESI-MS, the nanosplitter allows for the use of large-bore HPLC columns and high flow rates, while also having the ability to take advantage of the sensitivity of nanospray-ESI- MS.

In this paper, we present a novel approach to the parallel coupling of normal-bore HPLC with EC-array and nanospray ESI-MS based on the use of a nanosplitting interface, in which both detectors are utilized at their optimal detection mode for this parallel configuration while also maintaining the full chromatographic integrity of the system.

EXPERIMENTAL METHODS

LC-EC-Array-MS Instrumentation

Gradient LC-MS analyses were performed using an Agilent 1100 binary HPLC pump (Wilmington, DE) and an ESA model 6210 CoulArray detector (Bedford, MA) equipped with four electrochemical cells coupled online to a ThermoFinnigan TSQ700 triple quadrupole mass spectrometer or LCQ classic ion trap MS (San Jose, CA). Metabolite mixtures were separated on a 4.6 mm × 150 mm LC column (Agilent Zorbax C_{18} SB_{aq}, 3.5 μm).

In order to achieve nanoflow conditions into the mass spectrometer, a home-built concentric nanosplitting device was used and has been described elsewhere in detail.[20,23] The flow rate through the LC column was held at 1.0 mL/min and then split postcolumn; using a conventional T split providing 0.8 mL/min to the CoulArray and 0.2 mL/min to the nanosplitter, where the MS flow was split again and 300 nL/min entered the mass spectrometer. Flow rates into the MS were determined at 50% of the gradient flow with the voltage disconnected from the nanosplitter. A stopwatch and a glass microcapillary scored in 1-μL increments were used to determine the amount of LC flow out of the tip at a given time period.

Serum Extracts Preparation

A 9-mL serum sample was precipitated with 9 mL of acetonitrile (ACN)/0.4% glacial acetic acid at −80 °C, vortexed for 20 s, and centrifuged for 30 min at 12000g at −2 °C. The supernatant was transferred and aspirated to dryness under vacuum in a CentriVap & Concentrator (Labconco). The dry precipitate was dissolved in 200 μL of mobile phase. An aliquot (see below, 24.5 μL) of each sample was injected manually into the HPLC system.

Mass Spectrometry and HPLC Conditions on Extracts

Solvent A was 2% ACN/25 mM ammonium formate (pH 3.1), and solvent B was 80% ACN/ 25 mM ammonium formate/0.3% formic acid with conditions held at 0% B for 4 min then ramped to 85% from 5−25 min.

The mass spectrometric conditions were as follows: Full-scan mass spectra acquired in the positive mode with Q1 scanning the range from m/z 125 to 500, a total scan time of 0.5 s, and the electron multiplier set to 1080 V. SRM transitions were determined by infusion of a 1 μg/ mL concentration of analyte into the nanosplitter at 15 μL/min using a syringe pump and further split to 300 nL/min and delivered to the MS. The MS was then operated in product ion scanning mode, where Q1 was used to isolate the ion of interest, Q2 was used as a collision cell, and Q3 scanned between m/z 100 and 300. Multiple reaction monitoring (MRM) analyses were done using an ICL program written to adjust the SRM transition monitored based on the scan time of the instrument and the elution time of each analyte so each SRM was monitored individually during a given scan window. All SRMs used a total scan time of 0.4 s with varied collision voltages depending on analyte chemical composition and the electron multiplier set to 1300 V. For all MS analyses, the capillary temperature was set to 190 °C and the capillary voltage was held at 2.5 kV. No sheath gas was used due to the low flow rate.

Phenyl Butyrate Patient Sample Preparation and LC-EC-Array Profiling Conditions

The 250 μL of plasma from stage II Huntington's disease patients administered the drug phenyl butyrate was precipitated with 1 mL of ACN/0.4% glacial acetic acid, vortexed for 20 s, and centrifuged for 30 min/12000g at −2 °C. The supernatant was transferred and evaporated to dryness under vacuum in a CentriVap & Concentrator (Labconco). The dry precipitate was dissolved in 100 μL of mobile phase. An ESA model 5240 system equipped with 12 EC-array cells, a UV cell, and a fluorescence cell was used to screen the samples. Each analysis proceeded from 0−55% ACN with 100 mM lithium phosphate in a linear 35-min gradient.

Preparation of Serum Fractions for LC-EC-Array-MS Metabolite Identification

Four milliliters of serum from patients receiving the therapeutic drug sodium phenyl butyrate was precipitated with 16 mL of ACN/0.4% glacial acetic acid, vortexed for 20 s, and centrifuged for 30 min/12000g at −2 °C. The supernatant was transferred and evaporated to dryness under vacuum in a CentriVap & Concentrator (Labconco). The dry precipitate was dissolved in 300 μL of deionized water, and SPE was preformed using a 500-mg C_{18} SPE column (Diazan). The SPE column was eluted with 1 mL of dionized water, 10, 20, 30, 40, and 100% ACN. The 1-mL fractions were collected and subsequently evaporated to 100 μL of sample. Fractions were diluted in a 1:1 ratio with HPLC grade water prior to nanoelectrospray LC-EC-array-MS analysis.

Sodium Phenyl Butyrate Serum Sample LC-EC-Array-MS Instrumentation

Analyses were conducted using a Waters 717 plus autosampler (Milford, MA), an Agilent 1100 binary HPLC pump, and an ESA model 6210 CoulArray detector equipped with four electrochemical cells all coupled online to a ThermoFinnigan LCQ classic ion trap mass spectrometer. Separations were conducted on a 4.6×250 mm Atlantis T3 5-μm HPLC column (Waters). HPLC flow was split between the two detectors and calibrated into the MS in the same manner as described above.

Sodium Phenyl Butyrate Serum Sample Mass Spectrometry and HPLC Conditions

Solvent A was 2% ACN 25 mM ammonium formate (pH 3.1), and solvent B was 80% ACN/ 25 mM ammonium formate/0.3% formic acid. A 35 min linear gradient from 0−100% B was run.

The mass spectrometric conditions were as follows: Full-scan mass spectra were acquired using data-dependent fragmentation in the negative ion mode. The mass spectrometer was tuned and optimized in negative ion mode using a solution of 2-hydroxyphenylacetic acid. Ions were sampled into the mass spectrometer at a maximum injection time of 300 ms. The first scan event was operated in full-scan mode ranging from 100 to 500 Da. The second scan event was set as an MS/MS-dependent scan on ions with an intensity minimum of 1×10^4, using relative collision energy set to 40% and isolation width of 4 Da. The capillary temperature was set to 190 °C, and the voltage was held at 2.0 kV. No sheath gas was used due to the low flow rate.

RESULTS AND DISCUSSION

Figure 1 shows a schematic of the integrated nanoelectrospray LC-EC-array-MS detection system. The system consists of a binary HPLC pump connected to a large-bore, 4.6-mm-i.d. column followed by a zero dead volume T union used to split the flow 80:20 to the EC-array and nanosplitter, respectively. The nanoSplitter expanded view in Figure 1 illustrates how the remaining 200 μL/min of liquid is split concentrically down to 300 nL/min of eluent delivered to the mass spectrometer. As noted earlier, it is important that there is a reproducible agreement of retention times between the EC-array and MS in order to confidently compare and identify analytes between both instruments. In addition, chromatographic integrity must be retained for the most favorable evaluation of metabolites in solution and optimization of both detection techniques. Although, it has been demonstrated previously that online incorporation of an LC-EC-array with MS is possible, the high flow rates necessary for the EC-array analysis compromise the MS detection, and performing aggressive splits on a 1 mL/min solution to submicroliter flow rates will irrevocably destroy the chromatography leading to diffusion of analytes, shifts in retention, and poor nanoelectrospray-ESI-MS analysis.

Evaluation of System Performance

i. Chromatography—In order to establish the utility of the integrated nanoelectrospary LC-EC-array-MS detection system, several parameters concerning chromatographic integrity, such as retention time and peak width at half-height, were compared and contrasted. A solution of the neurotransmitter dopamine (DA), MW = 153, and its metabolite 3-methoxytyramine (3MT), MW = 167, were assayed using MRM scanning as described above in the Experimental Methods. The spectra of both molecules showed an abundant ion of [M + H − 17]; therefore, the transitions $154 \rightarrow 137$ and $168 \rightarrow 151$ m/z were monitored for DA and 3-MT, respectively. These two molecules were chosen for analysis because of their different chromatographic retentions, their relation to each other in terms of metabolism, and their strong EC-array as well as MS responses.

Table 1 details the chromatographic properties evaluated, showing comparisons between EC-array and MS for both analytes. Both detectors showed reproducible run-to-run retention times with relative standard deviations (RSDs) less than 2%, and those RSDs remained less than 2% when the retention times were compared between the detectors. Assurance of identical analyte retention times allows for accurate identification of compounds between both detectors, which is especially useful in the analysis of unknown peaks. Additionally, as seen through the comparison of peak width at half-height, the chromatographic efficiency is maintained through the entire system. Comparisons of these chromatographic values verify that through two aggressive splits there is limited sample diffusion and well-maintained separation efficiency essential for accurate analysis. These results prove the efficiency of the dual detection system for analyte comparison between detectors.

ii. EC-Array and MS Detector Comparison—The utility of the integrated nanoelectrospray LC-ECarray-MS detection system is also dependent on identifying the relative responses of the two detectors under the flow split conditions used. Due to the variety of compounds and concentrations commonly found in a given metabolomics sample, it is often difficult to match limits of detection throughout chemical classes of compounds. However, if the two detectors are to be used in a complementary fashion, it is important to identify their respective sensitivities under the system's flow split conditions in order to use the data in a comprehensive manner.

The molecules DA and 3-MT were used again in order to compare the limits of detection of the integrated nanoelectrospray LC-EC-array-MS system. As indicated in Table 1, the assay is more sensitive for the neurotransmitter DA, detecting reproducibly down to 25 ng/mL, which translates to 0.625 ng of material on column of which 0.13 and 500 pg are delivered to the MS and EC-array, respectively. These mass delivery numbers reflect the initial 80% of the sample diverted to the EC-array after the first split and the eventual mass transferred to the MS via the nanoSplitter. Although, it is commonly found that the EC-array is more sensitive than the MS, in this instance, when splitting the HPLC flow in the manner done here it is found that both detectors reproducibly detect down to a concentration of 25 ng/mL with a signal-to-noise ratio (S/N) of 3. In this case the MS, while analyzing the same concentration of sample, actually detected 3 orders of magnitude less sample mass than the EC-array.

As can be seen in Table 1, for both DA and 3-MT, the concentration detection limits for both the EC-array and MS detectors were comparable, although differing analyte masses were delivered to each. This illustrates their compatibility when working in a parallel mode in this configuration. It is understandable that, in this specific case, the MS proved to be more mass-sensitive than the EC-array since it was operated in the MRM mode, the most sensitive and selective triple quadrupole scanning mode. It should also be noted that the mobile-phase composition, such as pH and ionic strength, has a great effect on performance of the EC-array. The mobile-phase makeup is a compromise of salt concentrations that would allow the EC-

array to work efficiently without completely destroying the MS signal. For example, it has been reported by Alvarez et al. that both DA and 3-MT could be detected down to the low-picogram level using the EC-array.[25] However, these detection limits were achieved by using a mobile phase consisting of 25 mM potassium dihydrogen phosphate, 0.4 mM heptanesulfonic acid, and 50 mM EDTA and adjusted to pH 2.5 with 85% phosphoric acid, conditions not compatible with MS even while operating in nanoelectrospray mode. Thus, we recognize that while the optimal conditions for EC-array or MS alone may be greatly different, the nanoelectrospray LC-EC-array-MS platform described here provides optimal performance conditions when used in parallel.

iii. Quantification Using EC-Array—A definitive advantage of EC-array detection is its ability to use Faraday's law to directly quantify the amount of material being oxidized or reduced without the need of internal standards or response factors. In EC-array detection, a porous graphite working electrode is used where 100% of the LC eluent is passed through, and subsequently, 100% of each analyte is oxidized when monitored at its optimal oxidation potential. Faraday's law, $Q = nFN$, can then be applied. Here Q is the amount of charge transferred in the reaction equal to the integrated area under a chromatographic peak, n is the number electrons transferred in the reaction and is unique to each analyte, F is Faraday's constant of 96 500 C, and N is the moles of analyte oxidized.

Faraday's law was then applied to calculate the amount of DA and 3-MT oxidized by the EC-array in the integrated system and compared to the amount actually delivered to the EC-array for analysis. At the concentration of 25 ng/mL, 625 pg of each was injected on column and 80% or 500 pg was directed to the EC-array. The EC-array was set to potentials of 650, 700, 750, and 800 mV, respectively. The highest oxidation potential for DA was observed at 650 mV and for 3-MT was at 800 mV. The oxidation of DA is a two-electron-transfer process; therefore $n = 2$, and at 25 ng/mL and 650 mV, 540 nC of charge was transferred. Applying Faraday's law, 430 pg of DA was calculated as being oxidized. For 3-MT at the same concentration, 285 nC of charge was transferred in the one-electron oxidation process, equating to 496 pg.

The calculated values were within 15% of the theoretical amount delivered to the detector without the need for an internal standard or calibration plot. This feature is very useful, especially in comparison to mass spectrometry, where deuterated internal standards are needed for quantitative analyses. Direct quantification can then be done using the EC-array free of standards and without compromising the identification and characterization properties of nanoelectrospray MS. These calculations were done post LC-EC-array-MS analysis of the mixtures. Therefore, in the context of unknown analysis, compound quantification could be assessed retrospectively, after MS identification and the determination of its oxidation mechanism.

iv. Analysis in a Biological Matrix—As discussed in the introduction, detection systems in metabolomic analysis should be able to cover different classes of chemical compounds encountered in blood, plasma, urine, etc., over a range of concentrations. Thus, in order to determine the general applicability of the dual EC-array-MS detection system, an eight-compound mixture representative of the diversity typically encountered in such physiological systems was analyzed both in neat solution and in a serum matrix. These specific analytes were selected due to their differences in chromatographic retention, their strong EC-array and MS responses, and their penchant for being found in urine and plasma samples.

After obtaining the product ion spectra for each of the eight compounds, eight SRM transitions were identified for selective monitoring of the analytes. Next, by comparing retention times from our preliminary nanoelectrospray LC-EC-array-MS analysis where the MS was operated

in full-scan mode in parallel with the EC-array, the elution times of the compounds were determined. This information was then used to write a Unix system-based ICL program that allowed the MS to monitor a specific SRM transition with collision energies optimized for each analyte over a given time period measured in MS scan events.

Actual LC-EC-array-MS chromatograms of the mixture at a concentration of 250 ng/mL both neat solution and spiked into serum matrix following protein precipitation are shown in Figure 2 and Figure 3. As with the previous two-component mixture of DA and 3-MT, it is significant to note the excellent reproducibility in MS and EC-array retention times of all compounds both in the neat solution and in the serum sample. Through a comparison of analyte retention times and signals observed, it is evident that, in the spiked serum sample EC-array chromatogram (Figure 3), several new and, often, coeluting peaks can be monitored. At a retention time of 4.45 min and potential of 800 mV, a large peak not present in the neat solution is observed. This peak, only evident in the matrix sample, distorts the tyramine (RT 4.3 min) EC-array signal while its MS signal remains essentially constant. Presumably, the use of nanospray ESI, and the selectivity associated with the SRM scanning mode of the MS, results in minimal matrix effects on the analyte signals when monitored by MS as opposed to by EC-array.

The eight-metabolite mixture was analyzed both neat and in serum over four concentrations (250, 125, 62, and 31 ng/mL), the lowest of which is close to the MS limit of detection (S/N = 3) determined for DA (RT 3.22 min) in neat solution. The effects of the serum matrix on both the EC-array and MS detectors were examined by comparing the absolute signal observed for the eight analytes in neat and serum solution and are summarized in Table 2. It should be noted that limited sample preparation was done concerning the serum samples. Merely a protein precipitation was performed before spiking the analytes and conducting the analysis.

The results in Table 2 show good reproducibility in MS response at all concentrations for both the neat and serum samples with RSD values generally below 30% and, as expected, lower variance in the analysis of the neat solutions. Moreover, with the exception of tryptophan, which is a major constituent of serum and therefore yielded major differences in analyte signal (Figure 4), the MS response for all analytes at a given concentration showed much less variability between the neat solutions and the serum samples. A similar trend was also observed with the EC-array detection except that, in addition to tryptophan, a significant signal increase was observed for DA (RT 3.22 min) when spiked into serum. A coeluting serum component observed at essentially the same retention time as DA was (RT 3.27 min; Figure 4) probably responsible for the signal enhancement. While the maximum oxidation potential for the interfering peak is at 950 mV, the signal of DA at 800 mV was not the maximum signal of DA was at 800 mV (Figure 2 and Figure 3). Upon inspection of the serum blank EC-array chromatogram (Figure 4), a limited signal was evident at 800 mV, contributing to the increases seen in Table 2. However, the lack of MS signal and obvious difference in oxidation profile suggests it is merely a coeluting serum compound. The results from the above comparisons suggest that metabolites in a biological sample can be accurately assayed with limited cleanup using the nanoelectrospray LC-EC-array MS system. The two detectors functioning in a complementary fashion allow for the differences in each chromatogram to be used together successfully to assess the components of the mixture.

Identification of Unknown Sodium Phenyl Butyrate Metabolites: Following confirmation of the efficiency of the nanoelectrospray LC-EC-array-MS platform, we examined next its practical utility toward the identification of unknown metabolites of the pro-drug sodium phenylbutyrate (PB) in patient serum. PB is known to be highlyeffectiveforthetreatmentofpatientswithhyperammonemia,[26-28] as well as showing promise in the treatment of cystic fibrosis,[29,30] sickle cell anemia,[31] and thalassemia. Its clinical effectiveness, however, is limited by known occasions of toxicity from bodily

metabolism of the drug.[32,33] It is currently being investigated for treatment of Huntington's disease (HD) patients and undergoing patient tolerability and efficacy trials.

To assess levels and structures of metabolites, samples were obtained from a multicenter safety and tolerability study in which 15 g of phenylbutyrate was given daily to 60 early symptomatic subjects with HD, in collaboration with the HSG. Following an initial one-month-long randomized placebo-controlled exposure, subjects received open-label treatment for 12 additional weeks. Assessments included clinical measures of tolerability, the UH-DRS, standard safety laboratory studies, and a blinding assessment. Supplementary biological measures included fetal hemoglobin, plasma glutamine, histone acetylation and gene expression in lymphocytes, plasma levels of phenylbutyrate and metabolites, and metabolomic profiling.

An initial patient serum screening, where possible metabolites were initially detected, was performed and optimized using a 12-channel HPLC-EC-array system. Baseline patient serum, prior to PB treatment, and serum taken post PB treatment were compared to detect profile changes between the patient time points. These analysis conditions were not directly compatible with the nanoelectrospray LC-EC-array-MS system. Consequently, fraction collection and analysis conditions for evaluation of compounds identified as sodium phenylbutyrate metabolites had to be transferred to an MS-compatible formate buffer system. Patient serum underwent the same protein precipitation for LC-EC-array-MS analysis but then was subjected to SPE fraction collection using the MS-compatible elution solvents of water and ACN. The unknown metabolite peaks, indicated in Figure 5, were collected in the 30% ACN SPE elution fraction isolated from patient serum and were then analyzed using the integrated nanoelectrospray LC-EC-array-MS system. The EC-array was held at potentials of 700, 800, 900, and 1000 mV, respectively, and the LCQ ion trap MS was operated in negative ion detection mode using data-dependent full scans.

Figure 6 is an LC-EC-array-MS chromatogram where the bottom panel shows that at 800 mV the LC-EC-array detected three distinct peaks occurring at retention times of 17.0, 18.3, and 19.3 min. These retention times coincide with the extracted ion chromatograms (XIC) of three molecular species of m/z 179, presumably isomeric [M – H]$^-$ ions of a 180 Da molecule, shown in the top panel of Figure 6. The matching retention times confirm that the peaks monitored in the MS are indeed the unknown metabolites detected by the LC-EC-array detector. In addition to the aforementioned three isomers, the XIC of a fourth compound with molecular mass of m/z 339 ([M − H$^-$]) not detected by EC-array was observed by the MS detector at 18.4 min. The MS/MS spectra of the major isomeric metabolites of m/z 179 and that of the m/z 339 ion are presented in Figure 7A and B, respectively.

The 179-Da mass of the [M – H$^-$] ion of the three isomeric metabolites, shown in Figure 7A, represents a 16-Da increment over that of the parent drug and is consistent with a hydroxylation, presumably at the aromatic ring of the compound. This ring hydroxylation is further supported by the EC-array signal, which is consistent with a redox-active aromatic hydroxyl group. The MS/MS spectra also confirm this assignment although in the absence of reference compounds it is not possible to distinguish among the different isomers.

In Figure 7B, the MS/MS spectrum of the compound eluting at 18.4 min (m/z 339 [M – H$^-$]), shows an abundant fragment ion of m/z 163, strongly suggesting the presence of a PB moiety in the molecule. The inset in the figure shows the MS/MS spectrum of the m/z 339 ion and its subsequent fragments of m/z 193, 175, 163, and 113. This fragmentation pattern may be explained by the presence of a glucuronide metabolite, as shown. This assignment is further supported by the data of Bruengraber et al., who recently reported on the formation of a phenylbutyryl-β-glucuronate metabolite indirectly by incubating PB patient urine with β-

glucuronidase and monitoring the increase in PB concentration.[34] Their experiment indicated the probable formation of the phenylbutyryl-*β*-glucuronate metabolite of PB, through an indirect assessment, along with several other secondary PB metabolites in both humans and rats. The proposed glucuronide metabolite structure also explains the absence of any EC-array signal at 18.4 min since its functional groups are not expected to undergo redox reaction at the voltages employed.

It should also be pointed out that the elution range of ~16−20 min for the four metabolites in Figure 6 is earlier than that of the parent drug, which was observed at 20.74 min when translated to the formate buffer system (data not shown). This behavior is consistent with the more polar character of these metabolites compared to the PB pro-drug and further supports the structural assignments. The ability to confidently compare signals between detectors for unknown peaks greatly facilitated the possible structural identification of unknown PB metabolites. Additionally, MS can be utilized to find metabolites that are not EC active, adding an additional dimension to the analysis. This experiment clearly demonstrates the utility of the nanoelectrospray LC-EC-array-MS system for metabolite identification in biological matrixes.

CONCLUSION

An underlying objective in any 'omics-inspired research is to identify and characterize any change in biological makeup as a result of disease, xenobiotic exposure that can cause disease, [35] or drug metabolism. Once these changes are identified and characterized, they can be used as biomarkers to track disease or drug efficacy progression. HPLC in combination with EC-array detection has been shown to be highly effective in metabolite profiling and screening of urine, plasma, or CSF matrixes requiring minimal or even no sample cleanup. Sophisticated pattern recognition software can then be used to identify significant variations in the profiles and locate potential biomarkers for both the diagnosis of disease and monitoring disease progression.[13,14] Additionally, the sensitivity of EC-array is oftentimes superior in low-level analyte detection. Despite these significant features, a fundamental drawback of the technique is the inability to generate definitive structural information on these markers, especially when dealing with unknown compounds. The parallel use of mass spectrometry with EC-array detection can address this problem.

Previous HPLC-EC-array-MS combinations have been shown to compromise the performance of the MS detector with high flow rates and biological matrix effects. The platform discussed here permits operation of the MS under optimal nanoelectrospray conditions while also maintaining full chromatographic integrity and essentially perfect correspondence of band retention times between the two detectors. The nanoSplitter's ability to use a large-bore high flow rate HPLC in a nanoelectrospray-MS analysis also allows the direct coupling of this technique to the sensitive EC-array technology. The novel approach to the utilization of these two techniques allows us to capitalize on the sensitivities of both detectors for metabolomics studies for both targeted analysis and unknown metabolite identification and characterization. The results presented clearly show the utility of the combined detection system to simultaneously monitor several metabolites both in and out of a biological matrix reproducibly and down to levels commonly found in biological samples. Also, the system's ability to detect and structurally identify unknown metabolites in a real biological sample, using the combination of MS and EC-array, has been clearly demonstrated. This proof of concept was established using a basic four-cell EC-array system with limited ability to discern between varying oxidation potentials. Significant improvement in the overall nanoelectrospray LC-EC-array system would be possible by incorporation of a complete 16-cell EC-array system where the full advantage of EC-array's powerful selectivity could be realized.

In summary, the ability to simultaneously and reproducibly detect several analytes in a biological matrix using an integrated nanoelectrospray LC-EC-array-MS detection scheme with limited sample cleanup and full retention of chromatographic integrity between the two detectors is clearly detailed. The nanoelectrospray LC-EC-array-MS detection system has been shown here to be able to characterize possible PB metabolites in a HD patient serum sample proving its utility in drug metabolism studies. In this case, elucidation of these possible PB metabolites could potentially give much needed insight into the drug's metabolism and efficacy for HD patient treatment with the possibility to be used to further facilitate HD biomarker identification.

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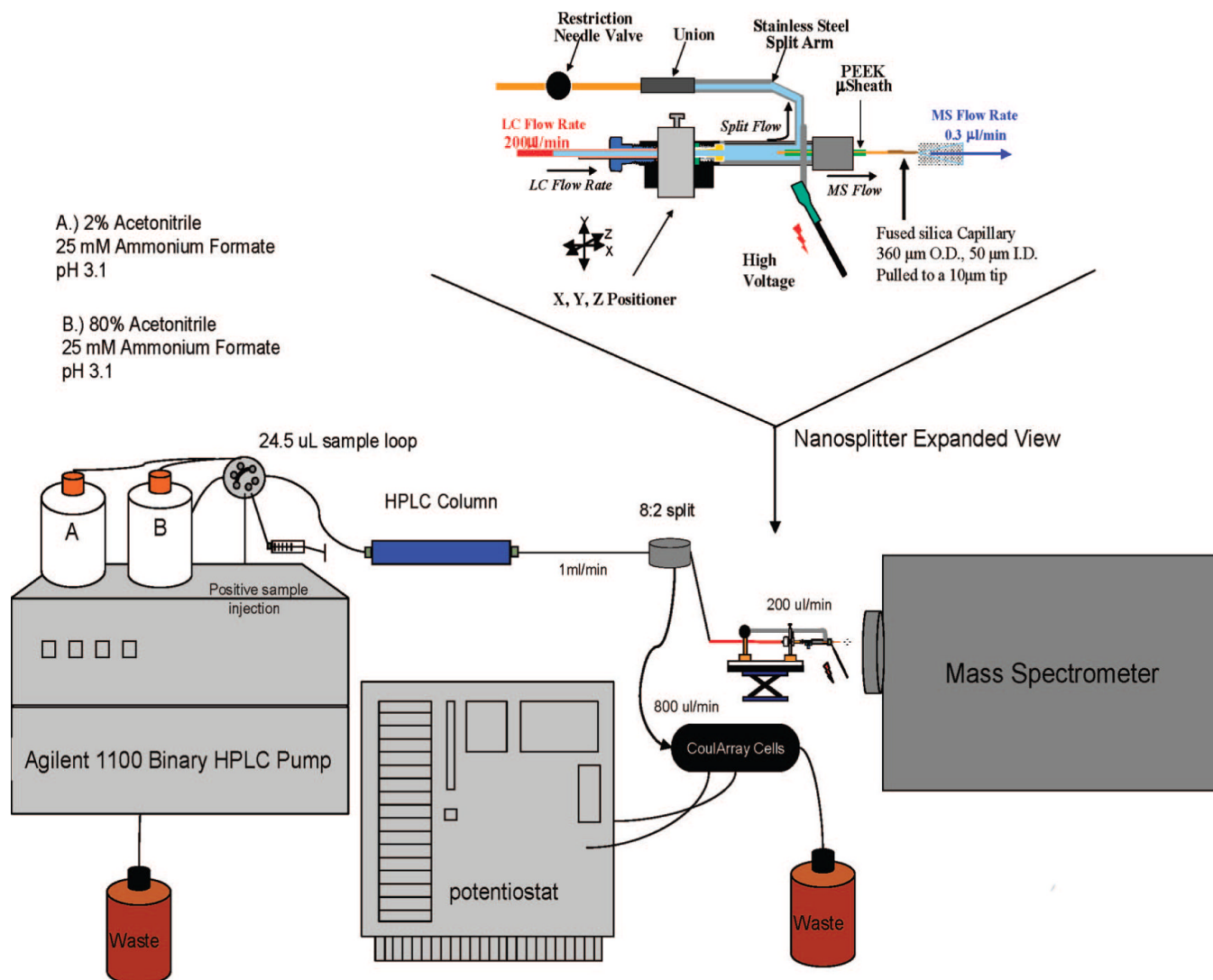35. Castrillo JI, Oliver SG. Metabolome Anal 2005:9–29.

Figure 1.
Experimental setup of nanoelectrospray LC-EC-array-MS system as it would be configured to any mass spectrometer including nanosplitter interface. The expanded view shows the nanosplitter in detail and the means by which it achieves concentric split ratios of up to 5000:1, with a stainless steel split arm allowing bulk flow not sampled by the capillary tip emitter to be taken to waste and a restriction needle valve controlling the eluent flow into the mass spectrometer.
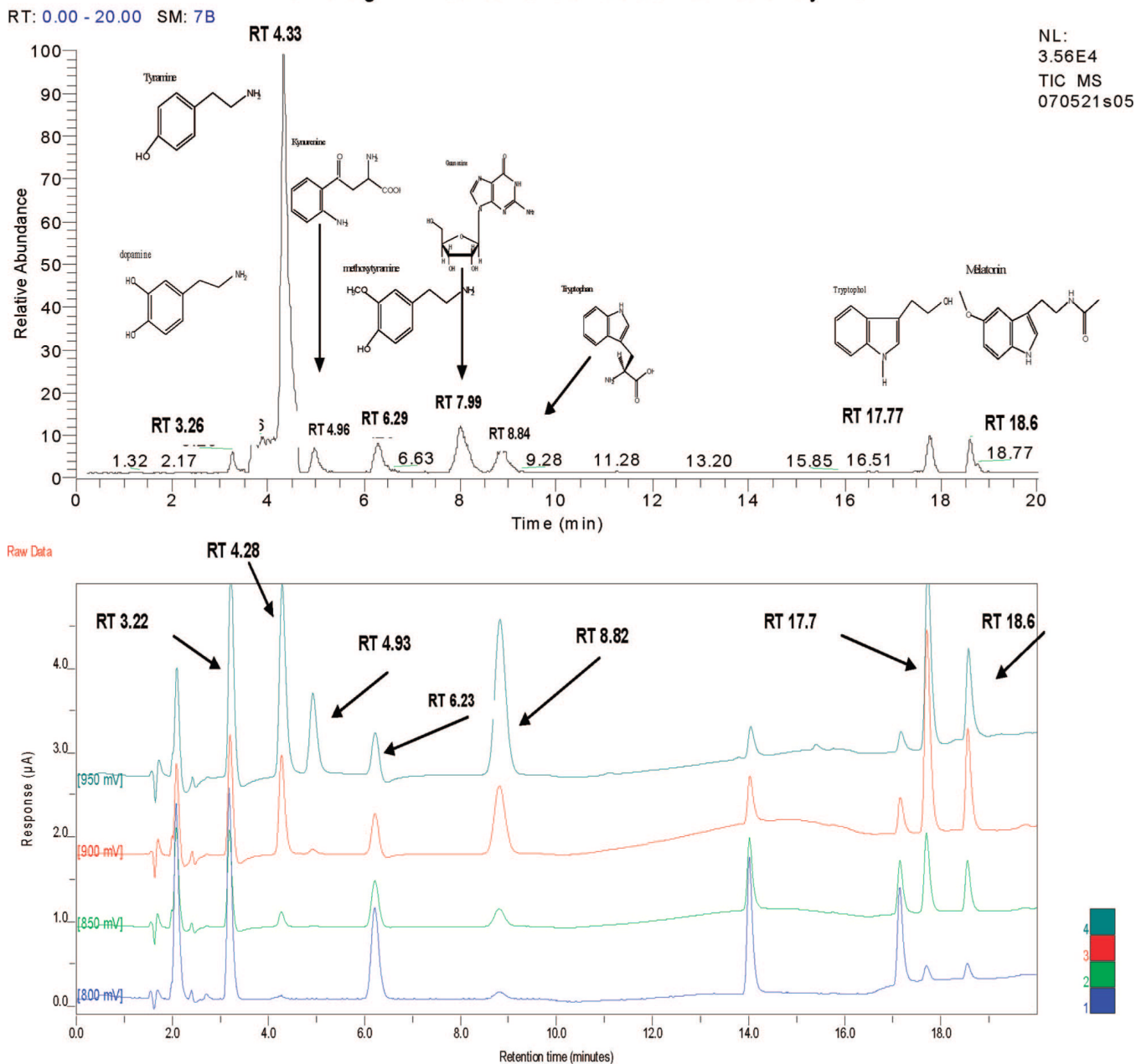
Figure 2.
(top) MRM TIC chromatogram and (bottom) an EC-array chromatogram, for the analysis of a 250 ng/mL mixture (6.25 ng injected on column) of eight metabolites in neat solution. The MS MRM chromatogram shows detection of all eight metabolites, where the CA chromatogram only shows seven, due to guanosine (MS retention time 7.99 min) requiring a much larger potential in order to yield an oxidation response.
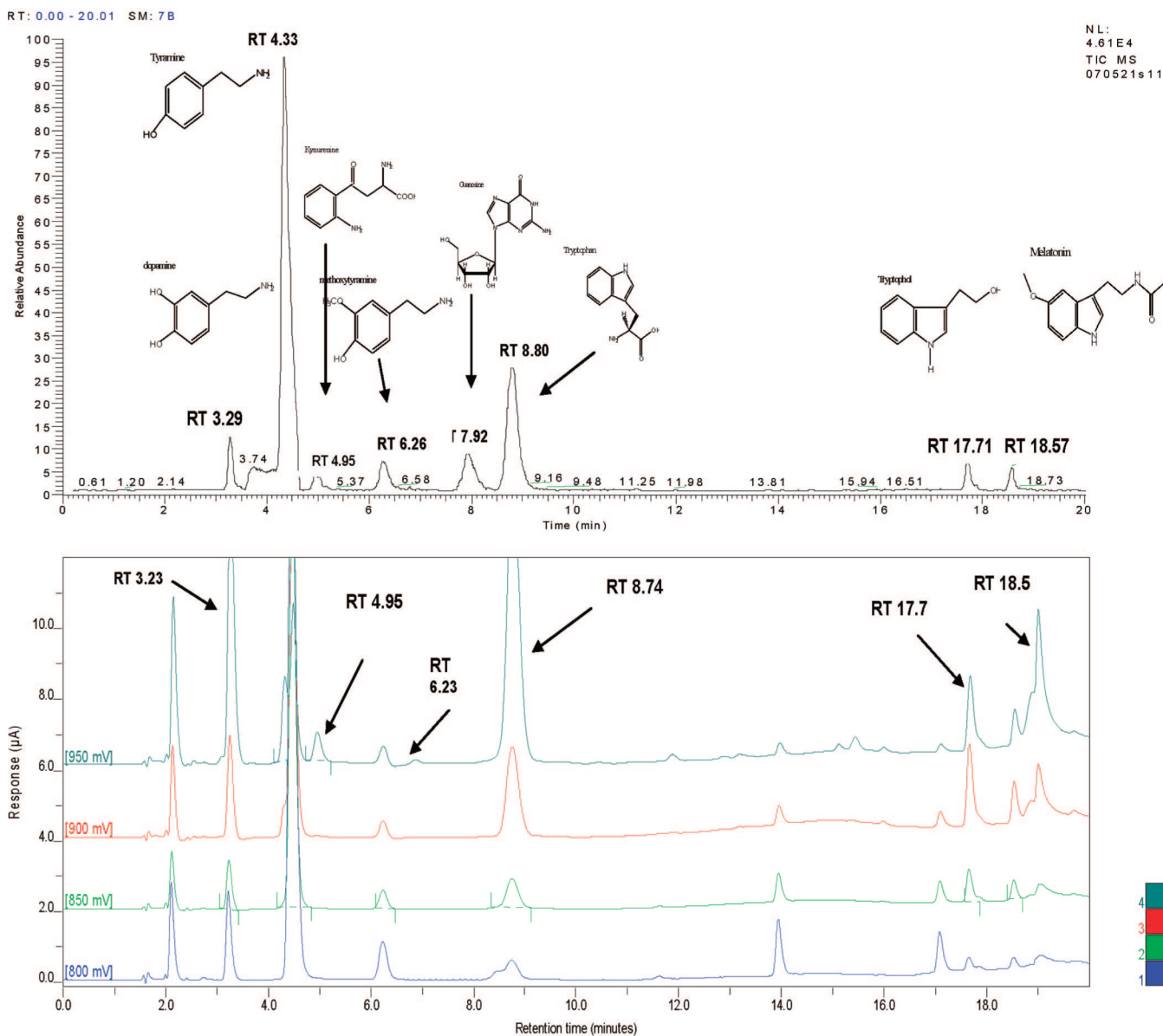
Figure 3.
(top) MRM TIC chromatogram and (bottom) an EC-array chromatogram, for the analysis of a 250 ng/mL (6.25 ng injected on column) mixture of eight metabolites in serum matrix. The matrix minimally affects the MS MRM transition peak intensities while having a greater effect on the detection of EC-array peaks due to matrix signals overlapping with analyte signals. Metabolite tyramine, at RT 4.33 min, is clearly detected in the MS chromatogram; however, its signal is suppressed in the EC-array due to a matrix peak at 4.5 min.
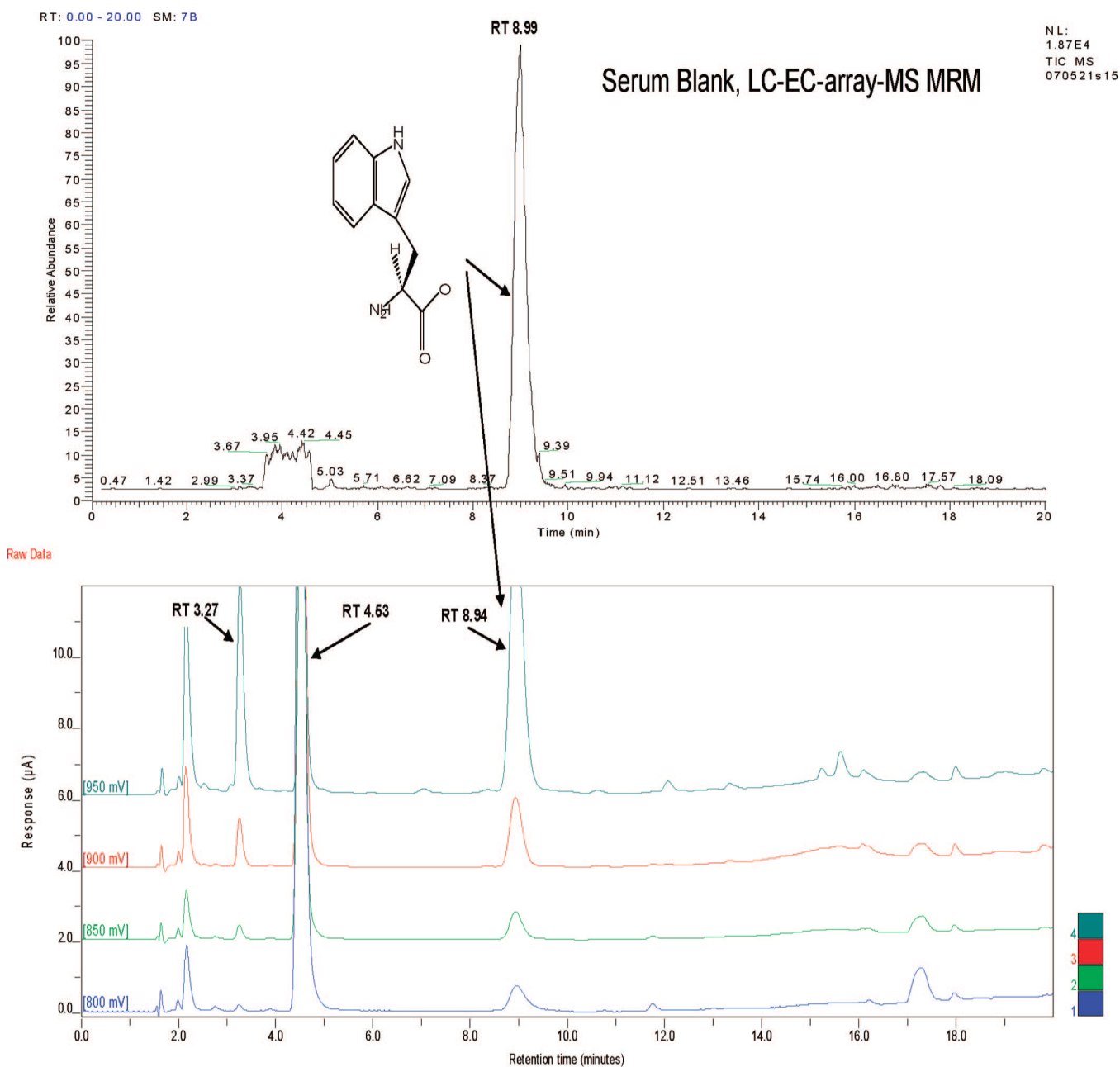
Figure 4.
(top) MRM TIC chromatogram and (bottom) an EC-array chromatogram, for the analysis of a serum blank. An intense peak at 8.99 min is observed in each chromatogram indicating tryptophan is present in the blank. The peak at 3.27 min elutes at the same time as dopamine, although with a different oxidation profile. These matrix peaks in the EC-array interfere with the signals from spiked analytes, making accurate identifications complicated without MS.

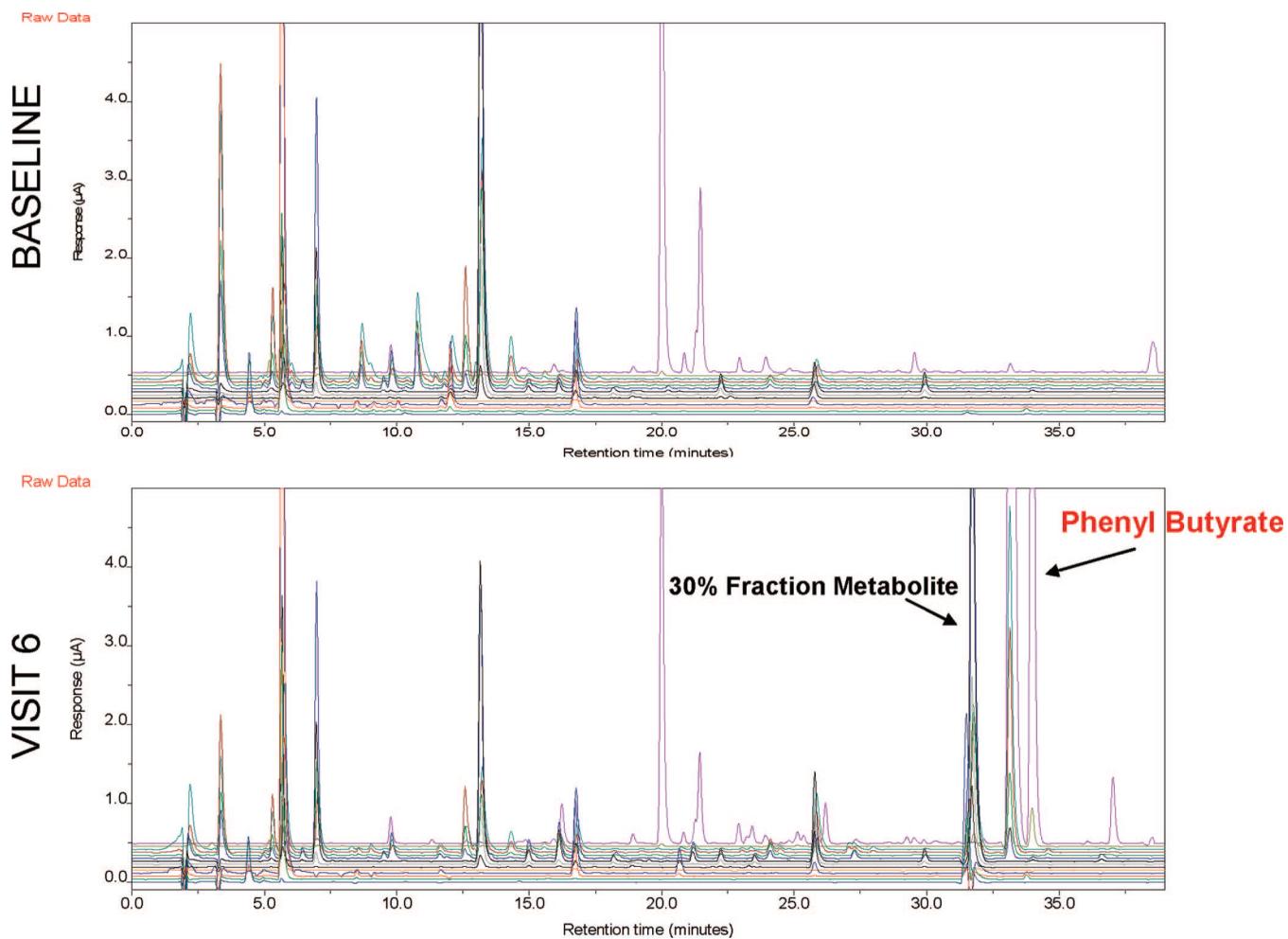**Figure 5.**
(top) HD patient serum prior to PB administration. (bottom) HD patient serum at the patient's sixth visit to be administered PB. Several changes in the chromatograms are evident; however, indicated is the metabolite collected in 30% SPE fractionation and the PB parent drug peak. Both chromatograms were acquired on a 12-channel EC-array system equipped with UV and fluorescence detection.

Figure 6.
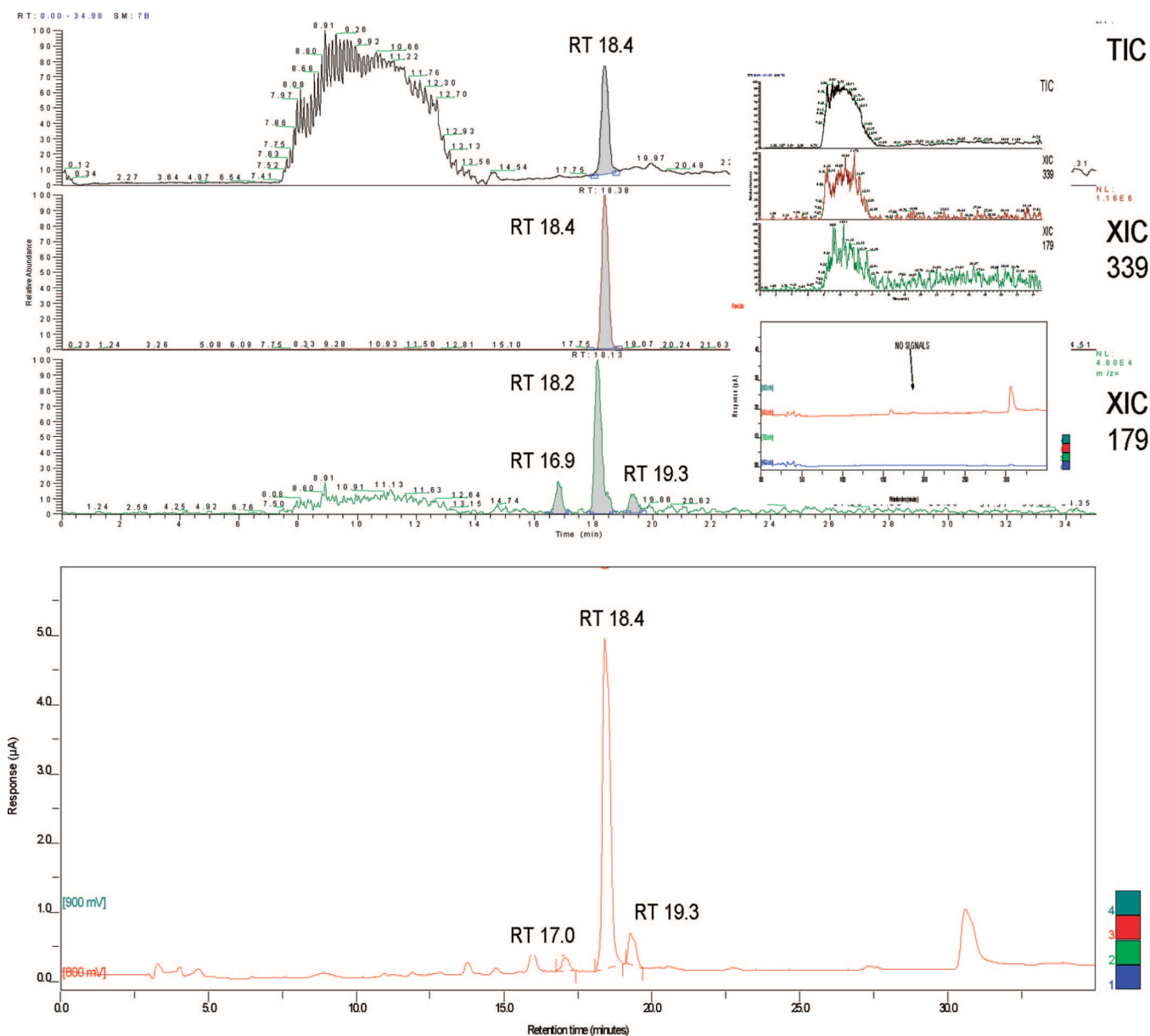(top) Full-scan MS chromatogram, as well as two XIC for 339 and 179 *m/z* corresponding to the metabolite masses observed in the 30% fraction. (bottom) EC-array chromatogram from the analysis with a large peak at the positional 800 mV and 18.4 min. Two smaller EC-array peaks, representing isomers of the 179 *m/z* ion are also observed. The inset shows a water blank analysis with the same TIC and XIC chromatograms.
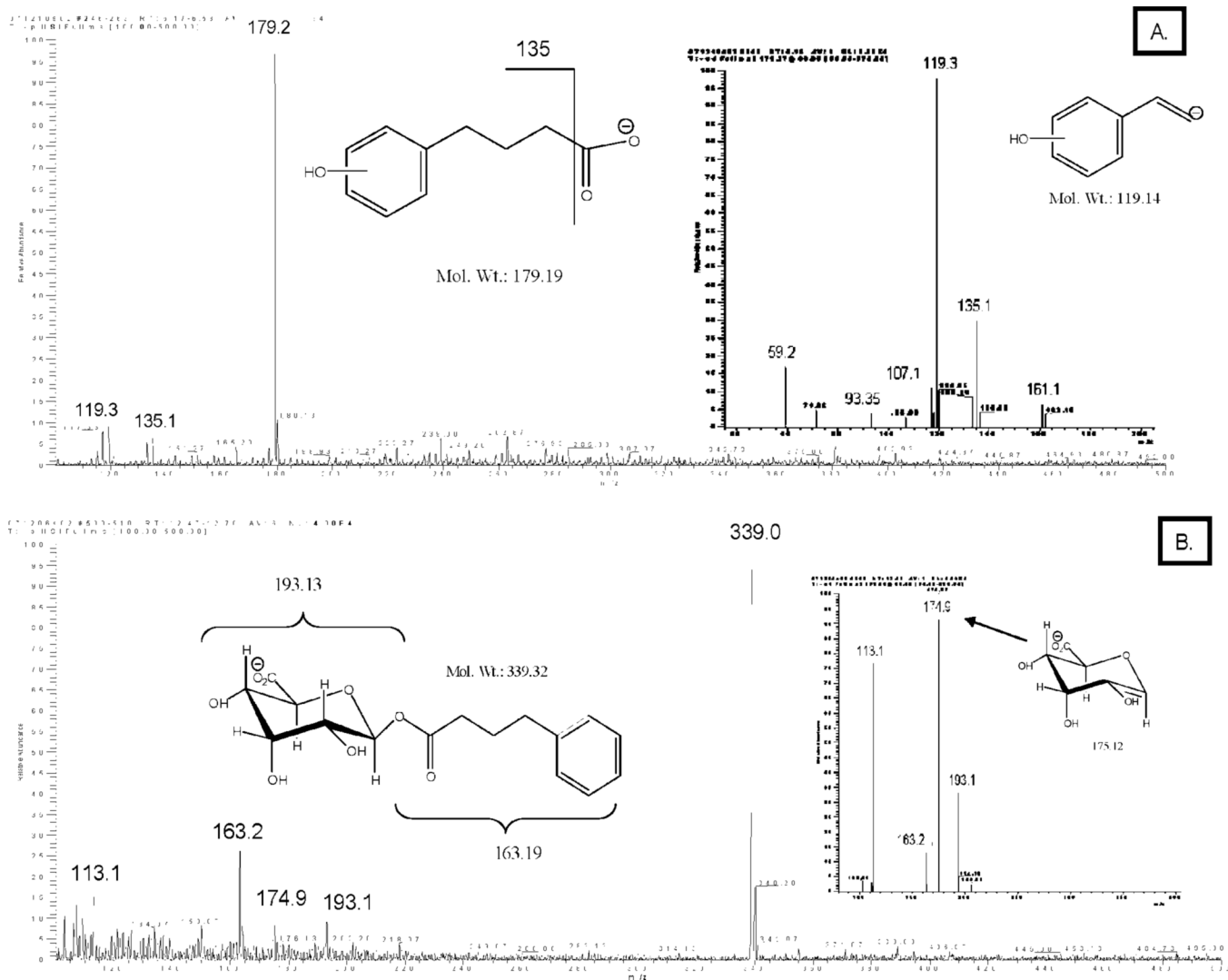
Figure 7.
(A) Shows the mass spectrum between 100 and 500 *m/z* for the peak at 18.2 min. The inset is its data-dependent MS/MS scan giving fragments of 135, 119, and 59 *m/z* indicating fragments of the proposed hydroxyphenylbutyric acid metabolite shown. (B) shows the mass spectrum between 100 and 500 *m/z* observed under the peak at 18.4 min observed in Figure 6. The inset is the data-dependent MS/MS scan of the same peak, giving fragment ions of 193, 175, and 163 *m/z* corresponding to the indicated portions of the proposed phenylbutyryl-*β*-glucuronate metabolite shown.

Table 1

[a]

dopamine

conc (ng/mL)	amt inj (ng)	MS				EC-array			
		pg to MS	RT	Peak width	Signal to noise	ng to CA	RT	Peak width	Signal to noise
400	10	2.00	3.33	0.10	12	8	3.32	0.11	16
200	5	1.00	3.44	0.09	9	4	3.32	0.11	13
100	2.5	0.50	3.42	0.09	7	2	3.35	0.1	8
50	1.25	0.25	3.41	0.11	4	1	3.32	0.1	5
25	0.625	0.13	3.41	0.11	3	0.5	3.32	0.1	3
		average	3.40	0.10		average	3.33	0.10	
		RSD (%)	1.24	10.00		RSD (%)	0.40	5.27	

methoxytyramine

conc (ng/mL)	amt inj (ng)	MS				EC-array			
		pg to MS	RT	Peak width	Signal to noise	ng to CA	RT	peak width	signal to noise
400	10	2.00	6.45	0.2	5	8	6.45	0.17	6
200	5	1.00	6.54	0.17	4	4	6.43	0.17	4
100	2.5	0.50	6.46	0.15	3	2	6.38	0.16	3
50	1.25								
25	0.625								
		average	6.48	0.17		average	6.42	0.17	
		RSD (%)	0.76	14.52		RSD (%)	0.56	3.46	

[a]The top block compares the chromatographic parameters of retention time, peak width at half-height, and signal-to-noise values for both the MS and EC-array detector over 5 concentrations of dopamine. The bottom block does the same for the dopamine metabolite methoxytyramine. Additionally, the amount injected onto the system as well as the mass amount delivered to each detector is also given. Retention time and peak width at half-height averages and RSD values show the reproducibility of these two parameters between the detectors for each concentration.

Table 2

Comparison of Four Concentrations of the Eight Metabolites Simultaneously Analyzed via MRM and EC-Array Both in Neat Solution and in a Serum Matrix[a]

| | | neat solution | | | | serum sample | | | |
| | | MS | | CA (nA) | | MS | | CA (nA) | |
conc (ng/mL)	amt inj (ng)	area	RSD (%)	area	RSD (%)	area	RSD (%)	area	RSD (%)
dopamine									
31	0.78	2462	27.55	235	7.51	3953	8.77	3413	12.12
62	1.55	2528	5.55	647	5.98	7859	4.64	3633	15.16
125	3.13	5577		1350		12652		5130	
250	6.25	11087		2690		37745		8920	
tyramine									
31	0.78	60526	28.26	178	15.1	85213	11.04	155	8.7
62	1.55	123486	11.88	588	2.87	144834	11.04	ns[b]	
125	3.13	14500		1160		227334		2200	
250	6.25	321510		2290		471092		2390	
kynurenine									
31	0.78	1970	27.11	33	7.92	4524	22.53	29	4.69
62	1.55	4345	5.98	221	8.14	6640	35.79	87	6.72
125	3.13	8673		474		13703		435	
250	6.25	23372		948		18326		811	
methoxytyramine									
31	0.78	5504	19.51	107	4.65	5326	11.96	114	2.03
62	1.55	9508	13.78	269	5.68	9723	20.26	191	8.92
125	3.13	14310		548		19721		543	
250	6.25	28196		1080		36575		1090	
guanosine									
31	0.78	9672	18.77	ns		11003	22.53	ns	
62	1.55	19856	5.98	ns		23906	35.79	ns	
125	3.13	37243		ns		32980		ns	
250	6.25	63202		ns		60909		ns	
tryptophan									
31	0.78	4321	17.49	208	1.36	199073	4.67	4617	5.9
62	1.55	9797	9.48	472	3.87	198004	9.32	4597	11.6
125	3.13	19590		938		185457		5830	
250	6.25	38227		1840		201128		8510	
tryptophol									
31	0.78	3373	17.9	262	1.62	2673	19.3	260	4.95
62	1.55	3626	13.8	635	6.05	5153	35.09	482	8.02

conc (ng/mL)	amt inj (ng)	neat solution				serum sample			
		MS		CA (nA)		MS		CA (nA)	
		area	RSD (%)	area	RSD (%)	area	RSD (%)	area	RSD (%)
125	3.13	12500		1270		11241		1230	
250	6.25	27706		2420		22681		2300	
melatonin 31	0.78	3407	13.93	115	6.79	2446	21.67	118	2.45
62	1.55	7647	23.39	295	5.08	4453	36.32	225	5.64
125	3.13	12385		610		15563		567	
250	6.25	25684		1210		15939		1110	

[a] MS and EC-array area values are from manual integration of each analyte peak. RSD values are given for each metabolite at the concentrations 31 and 62 ng/mL for both detectors in both neat solution and serum matrix. The concentrations of 125 and 250 ng/mL were not done in replicate; therefore, no statistics were done on the values.

[b] ns, refers to the lack of guanosine to be oxidized and yield an EC-array signal.