Metabolomic Applications of Electrochemistry/ Mass Spectrometry

Paul H. Gamache, David F. Meyer, Michael C. Granger, and Ian N. Acworth
ESA Inc., Chelmsford, Massachusetts, USA

Analytical techniques used for multivariate analysis of endogenous metabolites in biological systems (e.g., metabolomics, metabonomics) must be capable of accurately and selectively monitoring many known and unknown molecules that span a diverse chemical spectrum and over extremely large dynamic concentration ranges. Mass spectrometric (MS) and electrochemical array (EC-Array) detection have been widely used for multi-component analysis with applicability to low-level (fmol) metabolites. Described here are practical considerations and results obtained with the combined use of EC-Array and MS for HPLC-based multivariate metabolomic analysis. Data presented include the study of changes in rat urinary metabolite profiles associated with xenobiotic toxin exposure analyzed by HPLC using water:acetonitrile binary gradient conditions and post-column flow splitting between EC-Array and MS detectors. Results show complementary quantitative and qualitative analysis and the ability to differentiate sample groups consistent with xenobiotic-induced histopathological changes. The potential applicability of this hyphenated technique for biomarker elucidation through measurement of redox active compounds that are commonly associated with disease pathology and xenobiotic toxicity is discussed. The use of EC reactor cells in series with MS is also presented as a means of producing likely metabolites to facilitate structural elucidation and confirmation. (J Am Soc Mass Spectrom 2004, 15, 1717–1726) © 2004 American Society for Mass Spectrometry

The metabolome can be defined as the entire cellular complement of endogenous low molecular weight (typically <1000 Da) biomolecules [1]. Metabolomics research is commonly performed in the context of systems biology [2], which involves integrated study of the genome, transcriptome, proteome, and metabolome. These investigations, typically conducted in plants [3] and individual cells or cell types [4], are often geared toward understanding metabolic regulation (i.e., homeostasis) and fluxes associated with a given genetic, physiological, or developmental state. A similar term, metabonomics [5], more typically refers to analysis of dynamic changes in metabolite profiles in higher organisms, which have a high degree of biomolecular compartmentalization. In this context, endogenous metabolic profiles, typically measured in animal tissue or bio-fluids, are often viewed as fingerprints that are reflective of highly complex anatomically and spatially integrated metabolic networks. In practice, the distinction between metabolomics and metabonomics is not always clear, and, as is common, these terms will be used interchangeably within this text. Regardless of terminology, the comprehensive analysis of metabolites in biological systems is an important aspect of studies that are aimed toward further understanding complex processes such as those associated with aging and development [6, 7], disease [8], drug efficacy [9], and xenobiotic toxicity [10, 11]. Metabolomics is viewed as holding great promise, for example, in specific biomarker discovery for clinical diagnostics, drug discovery, and development.

The metabolome includes, but is not limited to, amino acids, carbohydrates, lipids, peptides, purines, pyrimidines, vitamins, and numerous metabolites involved in biosynthesis and biodegradation pathways and serving various functions [e.g., antioxidants, cofactors, intra- and intercellular regulatory and signaling molecules (i.e., hormones, enzyme inhibitors, neurotransmitters, etc.)]. Bioanalytical techniques used for metabolomics should thus be capable of accurately monitoring numerous known and unknown molecules that span a diverse chemical spectrum and large dynamic concentration range. Biological variability and environmental factors such as the presence of foreign compounds (e.g., dietary, exposure, therapeutics), their metabolites, and systemic interactions further challenge metabolomics research. Also, the distinction between homeostatically controlled and foreign molecules is

Published online October 26, 2004
Address reprint requests to Dr. P. H. Gamache, ESA Inc., 22 Alpha Road, Chelmsford, MA 01824, USA. E-mail: pgamache@esainc.com

1044-0305/04/$30.00
doi:10.1016/j.jasms.2004.08.016

Received June 18, 2004
Revised August 24, 2004
Accepted August 25, 2004

rarely obvious as molecules that are often imperceptibly related to homeostasis (e.g., resulting from non-enzymatic processes) may frequently provide the most useful insight to an organism's response to a given perturbation (e.g., markers of oxidative stress [12–14]).

Several analytical techniques are useful for metabolomics including gas chromatography with mass spectrometry (GC-MS) [15, 16], liquid chromatography with coulometric array electrochemistry [17–21] (LC-EC-Array), LC-MS [22–24], and nuclear magnetic resonance spectroscopy (NMR) [5, 8, 10, 25]. The qualitative characteristics of techniques, such as MS and NMR, provide essential data for chromatographic peak identification and purity, structural elucidation, and for normalizing multivariate data. While NMR allows the study of any molecule that contains an active nuclide (e.g., ^{1}H and ^{13}C), it is generally limited to detection and analysis of highly abundant metabolites (typically μg quantities). MS data obtained with and without fragmentation of individual metabolites and accurate mass measurements with high-resolution instruments are key advantages to LC-MS based metabolomics research. MS techniques are very sensitive and versatile but the choice of ionization technique, mass analyzer, acquisition mode, and instrumental parameters for a given analysis is very dependent on experimental goals, which may range from purely qualitative and exploratory to quantitative and specifically targeted. LC-EC-Array has been previously used for multivariate profiling of endogenous metabolites with reported detection of several hundred metabolites in a single analysis [18], fmol limits of detection and greater than four orders of magnitude dynamic range [17, 21]. The applicability of LC-EC-Array to redox active species (e.g., hormones, neurotransmitters, anti-oxidants, markers of oxidative stress) has led to its widespread use to study oxidative metabolism and redox biochemical processes, including those related to aging [26, 27], immune response [28], inflammation [29, 30], and many pathological processes [30–34]. However, EC-Array detection is limited to so-called "redox active" compounds (i.e., species easily capable of undergoing oxidative or reductive reactions) and, although useful qualitative (viz., voltammetric) data is obtained, its utility for structural elucidation is limited. Recognizing that no single technique can be expected to meet all of the field's diverse challenges, many metabolomics research programs employ several complementary analytical techniques.

Described below are two general approaches that utilize EC in combination with MS for metabolomics research, parallel EC-Array-MS for LC-based multivariate analysis and serial EC-MS to generate and study metabolites. The use of parallel detection for exploratory multivariate profiling is exemplified by studies of xenobiotic toxin exposure in animals with an overall focus on complementary analysis with regard to chemical scope, dynamic range, resolving capacity, and qualitative information obtained. To our knowledge, this is the first report of parallel EC-Array-MS detection.

The use of decoupled EC flow cells in series with MS ion sources has been previously described [35] and is further presented in articles within this issue. Of particular relevance to metabolomics is the use of on-line EC reactions to produce likely metabolites using known metabolites as reactants [36, 37]. Redox reactions conducted in combination with analytical and preparative scale separations and qualitative techniques such as MS or NMR may be a useful route to isolation of sufficient quantities for structural elucidation and confirmation. Presented are our initial investigations of the use of serial EC-MS for identification of potential metabolites observed in biological samples.

Experimental

Chemicals

All solvents were HPLC grade or better. Formic acid was obtained from EM Science (Gibbstown, NJ). Ammonium formate and ammonium acetate were Fluka brand from Sigma-Aldrich (St. Louis, Mo). Chemicals were high purity and used without further purification.

Instrumentation and Software

In our basic investigations of coupling EC in series and parallel with various MS instruments we found that the general considerations (e.g., mobile phase, fluidic, electrical, data acquisition), discussed below, were common among devices and were primarily a function of the ionization conditions. Since a variety of MS technologies are needed to address the many aspects of metabolomics research, we present data from single quadrupole MS (1100 MSD, Agilent Technologies, Palo Alto, CA) with electrospray (ESI) and atmospheric pressure chemical ionization (APCI) as a basic representation of this hyphenated EC-MS approach.

A Model 5021 EC cell (ESA Inc., Chelmsford, MA) was used for serial EC-MS configurations. For parallel detection, an EC-Array detector (CoulArray, ESA Inc.) was used with up to 16 serial coulometric cells maintained at individual constant potentials (all reported potentials are vs. Pd quasi-reference electrode). Figure 1 illustrates both serial and parallel EC/MS configurations used. Data acquisition with Agilent ChemStation and ESA CoulArray software was synchronized using a contact closure. Pirouette (Infometrix, Inc., Woodinville, WA) was used for post-acquisition exploratory chemometric analysis.

Analytical Conditions

Data presented are based on reversed-phase HPLC with water:acetonitrile binary gradient and various conditions (e.g., flow rate, column, gradient profiles) and analytical run times of 6 to 15 min. Supporting electrolytes were premixed in each mobile phase to provide sufficient conductivity for EC-Array detection and to

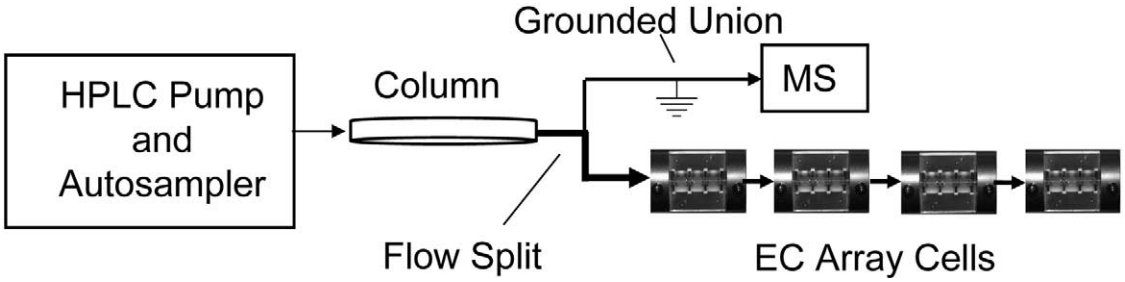

Figure 1. Diagram of analytical EC/MS system showing both parallel and serial configurations. *Pre-column cell was only present when required for on-line reaction of pure metabolite compounds.

minimize detector baseline drift and noise. Either an Agilent 1100 or ESA LC system was used for solvent and sample delivery. Post-column flow was split between EC and MS detectors with split ratios optimized for MS performance. Specific conditions are described with results.

Biological Samples

Samples from studies of acute exposure of animals to xenobiotic compounds were kindly provided by Dr. Timothy Maher (Mass College of Pharmacy, Boston, Massachusetts) and by Dr. Elaine Holmes (Imperial College, London). In one study, six groups (n = 5 per group) of adult male Sprague-Dawley rats, receiving Purina 5001 rodent laboratory chow and water ad libitum, received single oral doses of acetaminophen (20, 200, or 300 mg/kg), acetylsalicylic acid (200 mg/kg) or vehicle. Urine was collected for the first 8 h. and subsequent 24 h periods and diluted 10-fold in water prior to analysis. In a second study, adult male Fisher 344 rats were administered single intraperitoneal doses of maleic acid (300 mg/kg), chloroethanamine (750 mg/kg) or vehicle. Histopathological data showed maximal renal cortical and renal papillary toxic response in animals in the second day after receiving maleic acid and chloroethanamine, respectively, with complete recovery by the fifth day. Urine was collected over 24 h periods and samples from Day 2 and Day 5 were diluted 10-fold in water prior to analysis. Further details of the animal experimental protocols are described elsewhere [10, 38].

Practical Considerations for Parallel and Serial EC-MS

The described EC cells utilize three electrodes (reference, working, and auxiliary) for controlled-potential amperometry and require a minimum level of solution (i.e., mobile phase) conductivity for optimal functioning of the potentiostatic circuit. Commonly used additives for LC-MS [e.g., 0.1% (23.6 mM) formic acid or <10 mM ammonium acetate)] are often inadequate for LC-EC detection as they lead to low response and peak tailing. In our investigations, an optimal EC response was obtained with, for example, 20 mM ammonium acetate or formate buffer (10 mM ammonium formate:50 mM formic acid) with no significant adverse effects on MS detection (e.g., ionization suppression, adduct formation, background noise). Adding supporting electrolyte after the flow split may be an alternative approach particularly when incorporating EC-Array detection capabilities to an established LC-MS method.

MS ion source emitters used at high voltage, such as ESI, can lead to current flow through a conductive fluid, which may affect the interfacial potential of distal, upstream, wetted components (e.g., injector and analytical column). Since this can lead to uncontrolled redox reactions, thereby compromising analyte integrity [39], a stainless steel fluidic union was connected to the ground of the ion source/interface high voltage power supply as previously described [35].

Based on volume and pressure considerations, the flow rates used with EC-Array detection are typically between 0.25 to 2.0 mL/min. For parallel EC-Array and MS detection, passive flow splitting was used with flow ratios optimized for the specific MS interface used and to remain within the above flow rate range for the EC cells.

EC-Array Detection

The general concepts of EC-Array detection are described in detail elsewhere [17, 20, 21]. Briefly, this technique employs up to 16 series EC cells with porous graphitic carbon-based working electrodes (WE). Each cell is typically poised at a different potential, thereby spanning a wide potential window to allow detection of a wide scope of redox active metabolites. Efficient electrolysis obtained with high surface area WE allows selective detection and resolution of co-eluting analytes based on differences in their relative ease of oxidation and/or reduction. Hydrodynamic voltammograms (HDV) for each redox active metabolite are obtained from the response across adjacent EC-Array sensors. These data are a reflection of the kinetic and thermodynamic components of electron transfer (ET) reactions with demonstrated use for peak identification and peak

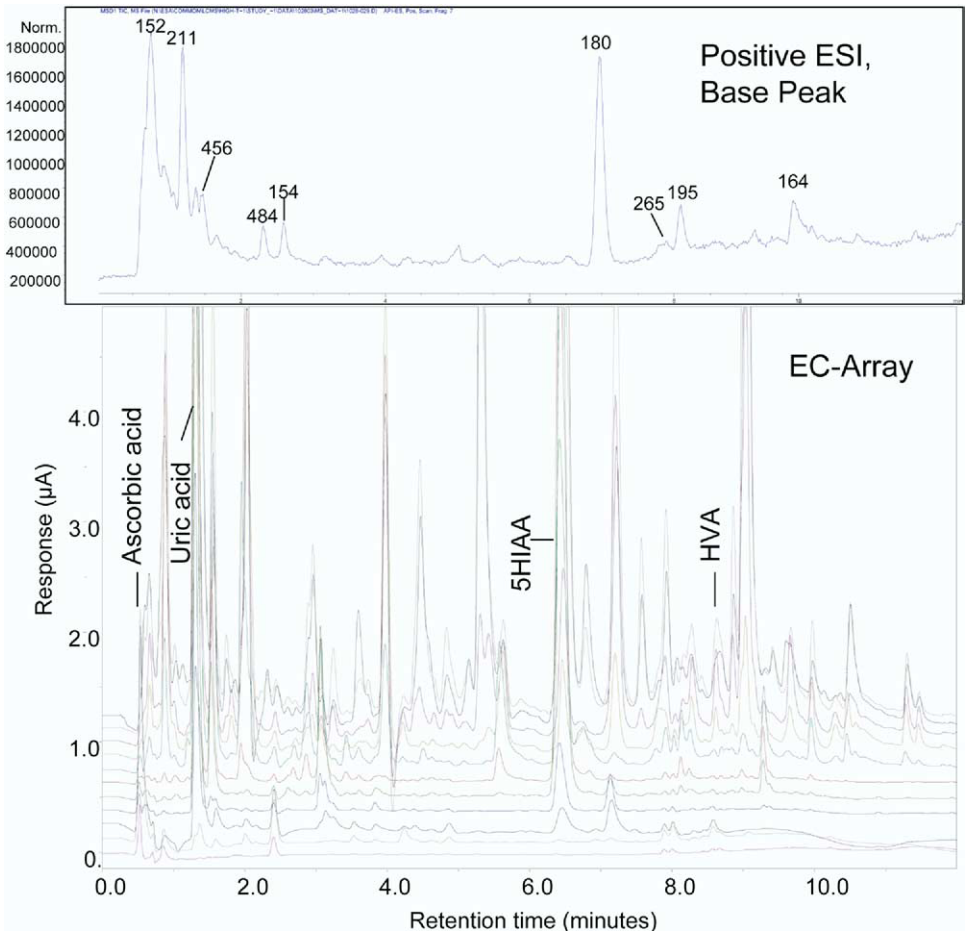

Figure 2. MS base peak chromatogram (top) and EC-Array multi-channel chromatogram (11 of 16 channels shown for clarity). MS chromatogram is labeled with base peak *m/z*. Analytical conditions: 20 μL injection of 10-fold diluted urine. Gradient elution 1 to 100% aqueous acetonitrile with 10 mM ammonium formate and 50 mM formic acid; flow rate 1.5 mL/min; Shiseido C18, 3 μm, 75 mm $\times$ 4.6 mm i.d. column; 4:1 passive post-column flow split to EC-Array: MS, respectively. EC-Array potentials 0 to 1050 mV in increments of 70 mV. ESI positive mode, capillary voltage 3500 V, fragmentor voltage 70 V scan range *m/z* 50–850, scan speed 1.2 s/cycle.

purity assessment and with applicability to trace level (fmol) metabolites [17].

Results and Discussion

The following data, although from different experiments, are described within the context of an overall scheme that progresses from exploratory to targeted analysis. This includes: (1) generating HPLC EC-Array-MS multivariate fingerprints with rapid LC methods; (2) investigating the presence of natural sample groupings based on exploratory chemometric analysis; and (3) interrogating stored EC and MS data to characterize metabolite peaks, or peak patterns, that significantly contribute to interesting sample groupings. The initial use of rapid methods is geared for medium throughput (6 to 15 min run times) with some tolerance for chromatographically unresolved peaks. The results from these exploratory studies are used to guide further analysis of stored data and, when appropriate, more targeted instrumental analysis for quantitation, structural elucidation and for further characterizing interesting metabolite patterns. Serial EC may then be used to produce possible metabolites to aid in structural confirmation. We have found this approach to be one way of efficiently and effectively using the described analytical tools to study complex biological processes and toward biomarker discovery.

Xenobiotic Toxicity Studies

A representative chromatogram of rat urine analyzed by parallel EC-Array-MS is shown in Figure 2. As expected, the MS ion chromatogram shows relatively few directly visible metabolite peaks. Mass spectral data from full scan exploratory metabonomic studies are typically processed by extracting discrete signals each defined by a particular retention time and *m/z*, and using algorithms to help distinguish analytical signal from background noise [23]. The resulting data, often

Table 1. Representative metabolites detectable by EC-Array

Chemical structure or metabolite class	Examples
Amino acids, related peptides	Cysteine, enkephalins, homocysteine, methionine, tryptophan, tyrosine
Aryl amine	Kynurenine, 3-hydroxykynurenine
Biogenic amine	Dopamine, epinephrine, norepinephrine, serotonin
Chroman	α-, β-, δ-, γ-Tocopherol, 5-nitro-γ-tocopherol
Hormone and metabolite	Estrogens, thyroxines
Conjugated polyenes	Carotenoids, retinoids, vitamin D
Pterin and Pteridine	Tetrahydrofolate, tetrahydrobiopterin
Purines	Guanine, 2′-deoxyguanosine, hypoxanthine, uric acid
Pyridine	Pyridoxal, pyridoxine
Quinone/hydroquinone	Coenzyme Q_{10}, plastoquinone, vitamin K_1
Sulfide	Glutathione, glutathione disulfide, homocysteine
Vitamin	A, B6, folic acid, C, D, E, K

consisting of hundreds of discrete signals, are then typically processed by chemometric techniques. Chromatographic variability, ionization suppression, adduct formation, and in-source oxidation with MS and electrode adsorptive and other non-Faradaic processes with EC are important factors to consider in these multivariate analyses. Our data show that the concurrent acquisition of EC-Array and MS data for each metabolite peak helps to address these potential issues. For example, the observation of a particular redox active metabolite peak (Figure 2) allows the analyst to conduct a more informed and targeted interrogation of corresponding MS data. Likewise, specific MS data are useful to normalize for retention time variability observed with both MS and EC-Array data.

The EC-Array multi-channel chromatogram in Figure 2 is shown at 5 μamperes full scale to illustrate the most abundant redox active metabolites. This scale is approximately a factor of 10^3 above the average baseline noise. Using CoulArray software algorithms set for detection of peaks with signal greater than 10 times the baseline noise, an average of 168 $\pm$ 34 distinct redox active species (peak clusters) were detected in rat urine samples (n = 22 inclusive of several treatment groups) within a 12 min chromatographic run time. The detection scope of EC-Array includes compounds with chemical substituents such as aryl alcohols, aryl amines, secondary and tertiary aliphatic amines, sulfides, and conjugated dienes. Table 1 is a brief list of redox active chemical or metabolite classes for which EC-Array has been used for targeted quantitative bioanalysis. It should be noted that metabolites not detected by EC-Array include many carbohydrates, lipids, amino acids, and peptides that lack these redox active substituents. However, considering that there are tens to hundreds of known endogenous metabolites with the above redox active substituents, exploratory analysis with high sensitivity detection typically results in information-rich profiles as seen in these and other studies [18].

With the described conditions, relatively few analytes responded on both MS and EC-Array detectors. This may, in part, be attributed to the expected low sensitivity of quadrupole-based exploratory *m/z* scanning. In addition, our results indicate that many redox active urinary metabolites exist as solution phase neutral species under a variety of reversed-phase chromatographic conditions. For example, peaks annotated in Figure 2 are ascorbic acid (AA), uric acid (UA), 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA). Of these metabolites, only uric acid was detected from extracted ion chromatograms (above baseline noise) as its protonated or adducted molecule [e.g., M + X (X = Na^+, K^+, NH_4^+)]. It should be noted that since these analyses are, by design, non-targeted, changing conditions to favor ionization of specific metabolites (e.g., post-column addition of base for gas-phase negative ion formation) is not a primary consideration. The described orthogonal nature of EC and MS detection was also observed when using alternate conditions, including several combinations of ESI, APCI, positive and negative ionization using neutral or acidic mobile phase conditions, and even with targeted selected ion monitoring (additional data not shown). The combined detection scope of MS and EC-Array thus provided higher coverage in a single analysis of the wide dynamic range and broad chemical diversity of urinary metabolites.

Based on external calibration standards, peaks evident in Figure 2, displayed at 5 μA full scale, correspond to approximately 2.0 to 40 ng on-column or 1 to 20 μg per mL of urine. Figure 3 is an overlay of a single EC detector channel (240 mV) for eight samples representing 20-fold lower metabolite levels. These signals are due to pg quantities of analyte on column and are consistent with reported 10 to 1000-fold lower limits of detection obtained with EC-Array when compared to photodiode array detection [19, 40].

Our studies have primarily utilized EC-Array data for chemometric analyses and MS data primarily for subsequent qualitative and confirmatory characterization of specific interesting variables revealed. A CoulArray software utility was used to adjust for chromatographic variability followed by conversion of otherwise raw EC-Array data into a generic format (text file) for pattern recognition analysis. This approach allowed rapid data processing—typically <5 min for 100 sam-

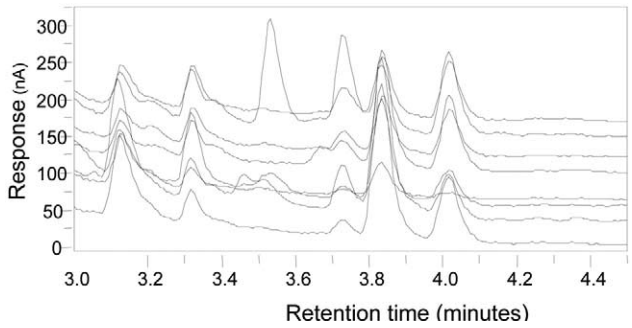

Figure 3. Overlay of amplified single channel chromatograms from EC-Array detection of urinary metabolites from several animals. Analytical conditions: 20 μL volumes of 10-fold diluted urine; binary gradient 1.0 to 80% aq. CH_3CN (vol/vol) in 6 min, with 2 min hold at 80% CH_3CN and 2 min re-equilibration; supporting electrolyte 20 mM ammonium acetate; flow rate 1.0 mL/min; Shiseido CapCell Pak C18 MG 3 μm, 7.5 cm $\times$ 4.6 mm i.d. column; post-column flow split (4:1) EC: ESI-MS; EC-Array potentials (0 to 840 mV in increments of 120mV).

ples—for subsequent exploratory pattern recognition analysis.

Samples obtained from an NMR-based metabonomic toxicity study were analyzed by LC-EC-Array-MS. Histopathological data showed maximal renal cortical and renal papillary toxic response in animals in the second day after receiving MA (300 mg/kg) and CE (750 mg/kg), respectively, with complete recovery by the fifth day. A scores plot (Figure 4) from principal components analysis (PCA) of EC-Array data from these samples showed clear differentiation between sample profiles obtained from MA and CE treated animals (MA and CE Day 2) as compared to controls and recovered animals (Day 5, Figure 4). Also, a higher degree of variability was found among controls when compared to pathological or recovered animals. Repeated LC/EC/MS analyses of these samples using several chromatographic conditions resulted in very similar PCA results. These results, including the relatively higher degree of variability among controls, are in good qualitative agreement with NMR-based metabonomic analysis of these samples (personal communication, Dr. Elaine Holmes, Imperial College, London).

PCA allows the reduction of highly complex data into a smaller number of vectors that can be graphically visualized to discern sample groupings. When sample groupings correspond, in this case, to pathological observations, eigenvector analysis can be used to discern which variables (peaks) contribute the most to a given vector. Furthermore, when multiple correlated metabolite peaks contribute significantly to a given vector, this may provide information on the involvement of whole or multiple pathways or specific processes (e.g., immune response). In this approach we have utilized EC-Array data to reveal these discriminator peaks or peak groupings with the intention to then interrogate the associated MS data for qualitative infor-

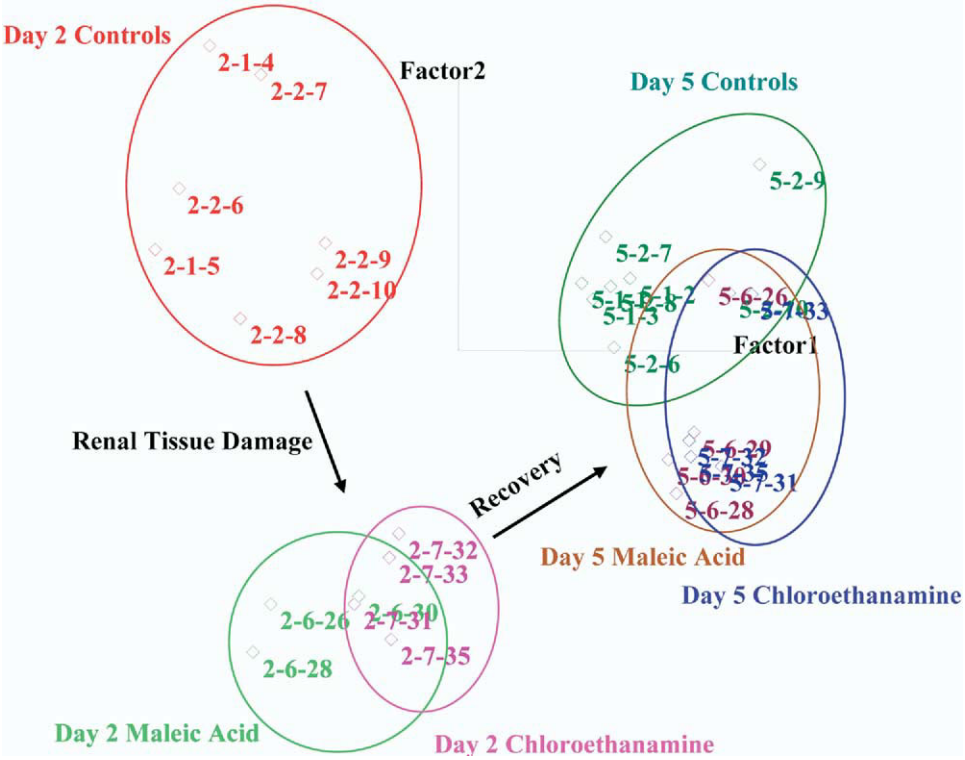

Figure 4. PCA scores plot generated from EC-Array data showing differentiation of sample groups having evidence of toxin-induced renal histopathology (MA Day 2 and CE Day 2) from control (Ctrl) and recovered animals (MA and CE Day 5). Analytical conditions as in Figure 2.

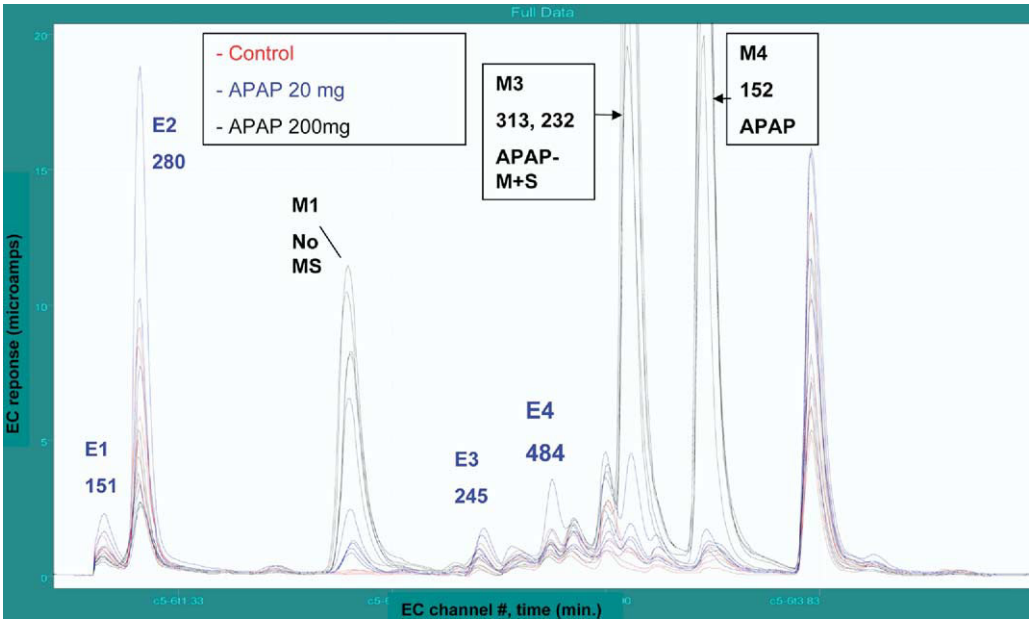

Figure 5. Overlay of EC-Array data from urine of rats administered (i.p.) vehicle, 20 mg/kg or 300 mg/kg APAP (n = 5 each group). Peaks labeled E indicate endogenous metabolites while peaks labeled M indicate drug metabolites. Base peak *m/z* ratios as determined from corresponding MS data are shown. APAP-M + S indicates coelution of sulfate and mercapturate metabolites of APAP. Analytical conditions as in Figure 3.

mation. Given the strong association of redox processes to many aspects of metabolism, toxicity and disease pathology, PCA of redox profiles in conjunction with structurally informative MS data may provide useful mechanistic insight.

In a model study of APAP-induced hepatotoxicity, results from PCA of EC-Array data showed consistent differentiation of high dose APAP (200 and 300 mg/kg, 0–8 h collection) from control, low dose (20 mg/kg) APAP, and high dose (200 mg/kg) acetylsalicylic acid. This differentiation was observed both exclusive and inclusive of xenobiotic metabolite variables. From analysis of eigenvector loadings, possible endogenous (E1-E4) marker peaks and xenobiotic metabolites (M1, M3, M4) are shown in Figure 5.

These data further demonstrate the complementary nature of EC and MS detection. For example, MS data, along with prior knowledge of a parent compound's analytical behavior and informed prediction of biotransformations, was used to remove xenobiotic data. This allowed more direct study of changes in endogenous metabolite profiles by PCA. Also, EC-Array data showed clear evidence of an APAP metabolite that was not detected by MS (peak M1, Figure 5). Furthermore, MS data indicated that peak M3 consisted of two major components having *m/z* 313 and 232. These *m/z* values are consistent with the commonly observed O-sulfated metabolite and the less frequently observed mercapturic acid metabolite of APAP, respectively. This is supported by EC-Array voltammetric data where the putative O-sulfated metabolite oxidized at a higher potential (840 mV) than parental APAP (600 mV), which can be expected based on phenol substitution. Furthermore, the similarity in voltammetric response between parental APAP and the peak corresponding to *m/z* 232, suggests that the easily oxidized *ρ*-amidophenol group is intact. These data are consistent with the commonly observed ring thioether metabolites associated with high-dose APAP. APAP-related hepatotoxicity is believed to be subsequent to CYP-450 oxidative metabolic activation to form reactive quinoneimine species with subsequent covalent binding, scavenger glutathione (GSH) depletion and further metabolism to APAP mercapturate [41]. Since APAP-mercapturate is a recognized marker of the so-called toxic pathway of APAP, the described approach of exploratory multivariate analysis and targeted interrogation of EC and MS data was an efficient and effective way to determine relevant changes associated with high dose APAP. These results demonstrate that the combined use of EC-Array and MS provided useful insight to these metabonomic toxicity studies by expanding the scope of detection, selectivity and qualitative information obtained in a single analysis.

On-Line EC Synthesis

The use of upstream decoupled EC flow cells in series with MS has been previously described as a means of synthesizing and characterizing likely drug metabolites [36, 37, 42]. Our studies have also included the synthesis of endogenous compounds using endogenous metabolite precursors [43]. EC reactions proceeded at specific

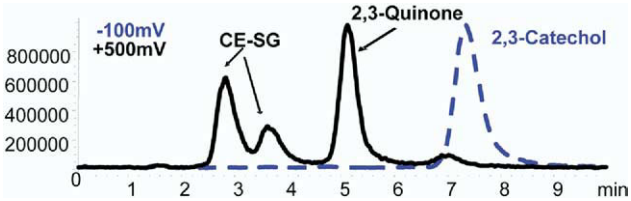

Figure 6. Combined ion chromatograms from selected ion monitoring of *m/z* values corresponding to protonated catechol (2HE, *m/z* 289), quinone (2HEQ, *m/z* 287), and glutathionyl adduct (2HE-SG, *m/z* 594) forms of estradiol. The dashed chromatogram shows the unreacted 2,3-catechol, 2HE, with the pre-column EC cell at −100 mV. The solid chromatogram shows the quantitative conversion, at 500 mV, of 2HE to products whose *m/z* correspond to the reactive quinone and isomeric forms of the mono-substituted glutathionyl adducts. Analytical conditions as in Figure 2.

potentials including aromatic hydroxylation of estradiol (E) at 1000 mV, O-dealkylation of 2- and 4-methoxyestradiol (2ME and 4ME) at 600 mV, and dehydrogenation of 2- and 4-hydroxyestradiol (2HE and 4HE) at 300 mV. Furthermore, oxidation in the presence of glutathione (GSH) showed evidence of thioether conjugate formation at the same potentials as above. For example, using analytical scale HPLC with pre-column EC oxidation (Figure 1), ion chromatograms (Figure 6) suggest the EC formation of the electrophilic estradiol-2, 3-quinone (2HEQ) and two isomeric forms of 2,3-catecholestrogen-S-yl-glutathione (2HE-SG). Our data indicate that the most abundant ions produced from E, 2HE, 4HE, 2ME, and 4ME in the presence of GSH all correspond to the reactive electrophilic quinone and catecholestrogen glutathionyl conjugates (CE-SG). These results demonstrate that this technique is capable of very closely simulating the proposed biotransformation reactions related to estrogen-dependent carcinogenesis [44]. It must be recognized that, when compared with enzymatic oxidation (e.g., cytochrome P450), EC oxidation leads to the formation of similar products for those enzymatic catalyzed reactions that are supposed to proceed through a mechanism initiated by a one-electron transfer oxidation [36, 37]. Reactions initiated by hydrogen atom abstraction such as hydroxylation of aromatic rings without electron donating groups are generally not mimicked by EC oxidation. Therefore, pre-column EC can be used to produce some possible metabolites that result from in vivo redox processes using the same conditions as described for analysis of biological samples. Our approach is, therefore, to use analytical scale EC-LC-MS to generate ng quantities of redox reaction products from endogenous precursors for direct comparison of chromatographic, MS (e.g., MS/MS, MS^n, accurate mass) and EC voltammetric data between EC-produced and biological metabolites. When EC products correspond to unknown metabolite peaks, initial structural information is gleaned from knowledge of precursor, prediction of EC reactions, MS, and voltammetric data. Furthermore, recent studies have demonstrated the feasibility of scaling up to produce sufficient quantities for structural confirmation by NMR [45]. The simplicity and speed of on-line EC-LC-MS may thus provide an effective means of characterizing some of the many unknown metabolites encountered in multivariate profiling of endogenous metabolites.

Conclusions

EC-Array and MS were used for parallel HPLC detection by flow splitting, including supporting electrolyte in the mobile phase and grounding the fluidic line upstream of the MS inlet. The concurrent acquisition of EC-Array and MS data showed several advantages in exploratory multivariate profiling, including broader coverage of the chemical diversity and concentration range of endogenous metabolites. Furthermore, EC-Array facilitated more targeted interrogation of corresponding MS data. When combined, the qualitative information from both techniques was useful for data normalization, peak purity, and structural elucidation studies. Results from several experiments were presented within the context of a general scheme that involves multivariate profiling, exploratory chemometrics, targeted analysis, and EC synthesis to aid in structural elucidation. Chemometric analysis of raw EC-Array profiles demonstrated the ability to differentiate sample groups, consistent with xenobiotic-induced histopathological changes. These results were reproducible even when using different chromatographic methods and were in agreement with NMR-based metabonomic analyses. As many organic chemicals are thought to exert toxicity via redox processes, the acquired redox profiles may be particularly useful for tissue and regio-specific modeling, diagnostic marker identification, and mechanistic insight to xenobiotic-induced toxicity.

The feasibility of electrochemically synthesizing endogenous metabolites was demonstrated for estrogen metabolites, including those proposed as potential biomarkers of hormonal carcinogenesis. The simplicity of forming reaction products on-line with LC-MS, using the same conditions as biological sample analysis, provides a potentially efficient means of characterizing some of the many unknown metabolites encountered in metabolomics.

Ongoing studies include the use of EC-Array with MS/MS and high resolution MS to assist with substructural analysis, selectivity, scan sensitivity, and for determination of elemental formula.

Acknowledgments

The authors thank Dr. Elaine Holmes, Imperial College, London, and Dr. Timothy Maher, Massachusetts College of Pharmacy, Boston, Massachusetts for their helpful correspondence and for providing biological samples used in the described studies.

References

1. Nobeli, I.; Ponstingl, H.; Krissinel, E. B.; Thornton, J. M. A Structure-Based Anatomy of the *E. coli* Metabolome. *J. Mol. Biol.* **2003,** *334*, 697–719.
2. Castrillo, J. I.; Oliver, S. G. Yeast as a Touchstone in Post-Genomic Research: Strategies for Integrative Analysis in Functional G Enomics. *J. Biochem. Mol. Biol.* **2004,** *37*, 93–106.
3. Weckwerth, W. Metabolomics in Systems Biology. *Annu. Rev. Plant Biol.* **2003,** *54*, 669–689.
4. Maharjan, R. P.; Ferenci, T. Global Metabolite Analysis: The Influence of Extraction Methodology on Metabolome Profiles of *Escherichia coli*. *Anal. Biochem.* **2003,** *313*, 145–154.
5. Nicholson, J. K.; Lindon, J. C.; Holmes, E. "Metabonomics": Understanding the Metabolic Responses of Living Systems to Pathophysiological Stimuli via Multivariate Statistical Analysis of Biological NMR Spectroscopic Data. *Xenobiotica* **1999,** *29*, 1181–1189.
6. Raamsdonk, L. M.; Teusink, B.; Broadhurst, D.; Zhang, N.; Hayes, A.; Walsh, M. C.; Berden, J. A.; Brindle, K. M.; Kell, D. B.; Rowland, J. J.; Westerhoff, H. V.; van Dam, K.; Oliver, S. G. A Functional Genomics Strategy that Uses Metabolome Data to Reveal the Phenotype of Silent Mutations. *Nat. Biotechnol.* **2001,** *19*, 45–50.
7. Williams, G. M.; Iatropoulos, M. J. Alteration of Liver Cell Function and Proliferation: Differentiation Between Adaptation and Toxicity. *Toxicol. Pathol.* **2002,** *30*, 41–53.
8. Robertson, D. G.; Reily, M. D.; Albassam, M.; Dethloff, L. A. Metabonomic Assessment of Vasculitis in Rats. *Cardiovasc. Toxicol.* **2001,** *1*, 7–19.
9. Nicholson, J. K.; Connelly, J.; Lindon, J. C.; Holmes, E. Metabonomics: A Platform for Studying Drug Toxicity and Gene Function. *Nat. Rev. Drug Discov.* **2002,** *1*, 153–161.
10. Holmes, E.; Nicholls, A. W.; Lindon, J. C.; Connor, S. C.; Connelly, J. C.; Haselden, J. N.; Damment, S. J.; Spraul, M.; Neidig, P.; Nicholson, J. K. Chemometric Models for Toxicity Classification Based on NMR Spectra of Biofluids. *Chem. Res. Toxicol.* **2000,** *13*, 471–478.
11. Bailey, W. J.; Ulrich, R. Molecular Profiling Approaches for Identifying Novel Biomarkers. *Expert Opin. Drug Safety* **2004,** *3*, 137–151.
12. Strickland, P. T.; Routledge, M. N.; Dipple, A. Methodologies for Measuring Carcinogen Adducts in Humans. *Cancer Epidemiol. Biomarkers Prev.* **1993,** *2*, 607–619.
13. Hensley, K.; Williamson, K. S.; Floyd, R. A. Measurement of 3-Nitrotyrosine and 5-Nitro-γ-Tocopherol by High-Performance Liquid Chromatography with Electrochemical Detection. *Free Rad. Biol. Med.* **2000,** *28*, 520–528.
14. Shigenaga, M. K.; Ames, B. N. Assays for 8-Hydroxy-2′-Deoxyguanosine: A Biomarker of in Vivo Oxidative DNA Damage. *Free Rad. Biol. Med.* **1991,** *10*, 211–216.
15. Jonsson, P.; Gullberg, J.; Nordstrom, A.; Kusano, M.; Kowalczyk, M.; Sjostrom, M.; Moritz, T. A Strategy for Identifying Differences in Large Series of Metabolomic Samples Analyzed by GC/MS. *Anal. Chem.* **2004,** *76*, 1738–1745.
16. Verdonk, J. C.; Ric de Vos, C. H.; Verhoeven, H. A.; Haring, M. A.; van Tunen, A. J.; Schuurink, R. C. Regulation of Floral Scent Production in Petunia Revealed by Targeted Metabolomics. *Phytochemistry* **2003,** *62*, 997–1008.
17. Gamache, P.; Ryan, E.; Svendsen, C.; Murayama, K.; Acworth, I. N. Simultaneous Measurement of Monoamines, Metabolites, and Amino Aacids in Brain Tissue and Microdialysis Perfusates. *J. Chromatogr.* **1993,** *614*, 213–220.
18. Shi, H.; Vigneau-Callahan, K. E.; Shestopalov, A. I.; Milbury, P. E.; Matson, W. R.; Kristal, B. S. Characterization of Diet-Dependent Metabolic Serotypes: Proof of Principle in Female and Male Rats. *J. Nutr.* **2002,** *132*, 1031–1038.
19. Devanesan, P.; Todorovic, R.; Zhao, J.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. Catechol Estrogen Conjugates and DNA Adducts in the Kidney of Male Syrian Golden Hamsters Treated with 4-Hydroxyestradiol: Potential Biomarkers for Estrogen-Initiated Cancer. *Carcinogenesis* **2001,** *22*, 489–497.
20. Todorovic, R.; Devanesan, P.; Higginbotham, S.; Zhao, J.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. Analysis of Potential Biomarkers of Estrogen-Initiated Cancer in the Urine of Syrian Golden Hamsters Treated with 4-Hydroxyestradiol. *Carcinogenesis* **2001,** *22*, 905–911.
21. Gamache, P.; Freeto, S. M.; Acworth, I. N. Coulometric Array HPLC Analysis of Lipid Soluble Vitamins and Antioxidants. *Am. Clin. Lab.* **1999,** *18*, 18–19.
22. Buchholz, A.; Hurlebaus, J.; Wandrey, C.; Takors, R. Metabolomics: Quantification of Intracellular Metabolite Dynamics. *Biomol. Eng.* **2002,** *19*, 5–15.
23. Plumb, R.; Granger, J.; Stumpf, C.; Wilson, I. D.; Evans, J. A.; Lenz, E. M. Metabonomic Analysis of Mouse Urine by Liquid-Chromatography Time of Flight Mass Spectrometry (LC-TOFMS): Detection of Strain, Diurnal, and Gender Differences. *Analyst* **2003,** *128*, 819–823.
24. Lafaye, A.; Junot, C.; Ramounet-Le Gall, B.; Fritsch, P.; Tabet, J. C.; Ezan, E. Metabolite Profiling in Rat Urine by Liquid Chromatography/Electrospray Ion Trap Mass Spectrometry. Application to the Study of Heavy Metal Toxicity. *Rapid Commun. Mass Spectrom.* **2003,** *17*, 2541–2549.
25. Shockcor, J. P.; Holmes, E. Metabonomic Applications in Toxicity Screening and Disease Diagnosis. *Curr. Top. Med. Chem.* **2002,** *2*, 35–51.
26. Cadenas, S.; Barja, G.; Poulsen, H. E.; Loft, S. Oxidative DNA Damage Estimated by oxo8dG in the Liver of Guinea Pigs Supplemented with Graded Dietary Doses of Ascorbic Aacid and α-Tocopherol. *Carcinogenesis* **1997,** *18*, 2373–2377.
27. Yanagawa, K.; Takeda, H.; Egashira, T.; Matsumiya, T.; Shibuya, T.; Takasaki, M. Changes in Antioxidative Mechanisms in Elderly Patients with Noninsulin-Dependent Diabetes Mellitus. Investigation of the Redox Dynamics of α-Tocopherol in Erythrocyte Membranes. *Gerontology* **2001,** *47*, 150–157.
28. Bugianesi, R.; Serafini, M.; Simone, F.; Wu, D.; Meydani, S.; Ferro-Luzzi, A.; Azzini, E.; Maiani, G. High-Performance Liquid Chromatography with Coulometric Electrode Array Detector for the Determination of Quercetin Levels in Cells of the Immune System. *Anal. Biochem.* **2000,** *284*, 296–300.
29. Christen, S.; Jiang, Q.; Shigenaga, M. K.; Ames, B. N. Analysis of Plasma Tocopherols α, γ, and 5-Nitro-γ in Rats with Inflammation by HPLC Coulometric Detection. *J. Lipid Res.* **2002,** *43*, 1978–1985.
30. Hensley, K.; Maidt, M. L.; Pye, Q. N.; Stewart, C. A.; Wack, M.; Tabatabaie, T.; Floyd, R. A. Quantitation of Protein-Bound 3-Nitrotyrosine and 3,4-Dihydroxyphenylalanine by High-Performance Liquid Chromatography with Electrochemical Array Detection. *Anal. Biochem.* **1997,** *251*, 187–195.
31. Collins, A. R.; Gedik, C. M.; Olmedilla, B.; Southon, S.; Bellizzi, M. Oxidative DNA Damage Measured in Human Lymphocytes: Large Differences Between Sexes and Between Countries, and Correlations with Heart Disease Mortality Rates. *FASEB J.* **1998,** *12*, 1397–1400.
32. Sofic, E.; Lange, K. W.; Jellinger, K.; Riederer, P. Reduced and Oxidized Glutathione in the *Substantia nigra* of Patients with Parkinson's Disease. *Neurosci. Lett.* **1992,** *142*, 128–130.
33. Russell, I. J.; Vaeroy, H.; Javors, M.; Nyberg, F. Cerebrospinal Fluid Biogenic Amine Metabolites in Fibromyalgia/Fibrositis Syndrome and Rheumatoid Arthritis. *Arthritis Rheum.* **1992,** *35*, 550–556.
34. Beal, M. F.; Matson, W. R.; Storey, E.; Milbury, P.; Ryan, E. A.; Ogawa, T.; Bird, E. D. Kynurenic Acid Concentrations are

Reduced in Huntington's Disease Cerebral Cortex. *J. Neurol. Sci.* **1992,** *108*, 80–87.
35. Zhou, F.; Van Berkel, G. J. Electrochemistry Combined On-Line with Electrospray Mass Spectrometry. *Anal. Chem.* **1995,** *67*, 3643–3649.
36. Jurva, U.; Wikstrom, H. V.; Bruins, A. P. In Vitro Mimicry of Metabolic Oxidation Reactions by Electrochemistry/Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2000,** *14*, 529–533.
37. Deng, H.; Van Berkel, G. J. A Thin-Layer Electrochemical Flow Cell Coupled On-Line with Electrospray-Mass Spectrometry for the Study of Biological Redox Reactions. *Electroanalysis* **1999,** *11*, 857–865.
38. Nicholls, A. W.; Holmes, E.; Lindon, J. C.; Shockcor, J. P.; Farrant, R. D.; Haselden, J. N.; Damment, S. J.; Waterfield, C. J.; Nicholson, J. K. Metabonomic Investigations into Hydrazine toxicity in the Rat. *Chem. Res. Toxicol.* **2001,** *14*, 975–987.
39. Liu, S.; Griffiths, W. J.; Sjovall, J. On-Column Electrochemical Reactions Accompanying the Electrospray Process. *Anal. Chem.* **2003,** *75*, 1022–1030.
40. Ferruzzi, M. G.; Sander, L. C.; Rock, C. L.; Schwartz, S. J. Carotenoid Determination in Biological Microsamples Using Liquid Chromatography with a Coulometric Electrochemical Array Detector. *Anal. Biochem.* **1998,** *256*, 74–81.
41. Nelson, S. D. Molecular Mechanisms of the Hepatotoxicity Caused by Acetaminophen. *Semin. Liver Dis.* **1990,** *10*, 267–278.
42. Jurva, U.; Wikstrom, H. V.; Weidolf, L.; Bruins, A. P. Comparison Between Electrochemistry/Mass Spectrometry and Cytochrome P450 Catalyzed Oxidation Reactions. *Rapid Commun. Mass Spectrom.* **2003,** *17*, 800–810.
43. Gamache, P.; Smith, R.; McCarthy, R.; Waraska, J.; Acworth, I. N. ADME/Tox Profiling Using Coulometric Electrochemistry and Electrospray Ionization Mass Spectrometry. *Spectroscopy* **2003,** *18*, 14–21.
44. Devanesan, P.; Santen, R. J.; Bocchinfuso, W. P.; Korach, K. S.; Rogan, E. G.; Cavalieri, E. Catechol Estrogen Metabolites and Conjugates in Mammary Tumors and Hyperplastic Tissue from Estrogen Receptor-α Knock-Out (ERKO)/Wnt-1 Mice: Implications for Initiation of Mammary Tumors. *Carcinogenesis* **2001,** *22*, 1573–1576.
45. Gamache, P.; Solomon, M.; Acworth, I. N.; Cole, R. Rapid On-Line Electrochemical Synthesis of Pharmaceutical Degradants and Metabolites for Profiling, Identification, and Quantitation. *Pittcon* **2004**.