Endocrine Disruptors from Combustion and Vehicular Emissions: Identification and Source Nomination

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Abstract

During the last decade, concerns have been raised regarding the possible harmful effects of exposure to certain chemicals that are capable of modulating or disrupting the function of the endocrine system. These chemicals, which are referred to as endocrine disrupting chemicals (EDCs), have the capability to interfere with the production, release, transport, metabolism, or elimination of the natural hormones in the body responsible for the regulation of developmental processes. Currently, exhaust samples from potential combustion and vehicular sources are being analyzed to provide initial identification of EDCs. The intent of this screening effort is to provide discerning evidence for nominating sources for further EDC characterization. Conventional sampling, advanced analytical methods, and bioassays are being used to provide initial characterization of these samples for their compound identity and EDC activity. Our intent is to sample and chemically characterize multiple combustion sources to determine whether EDCs are emitted from combustion sources and in what quantity.

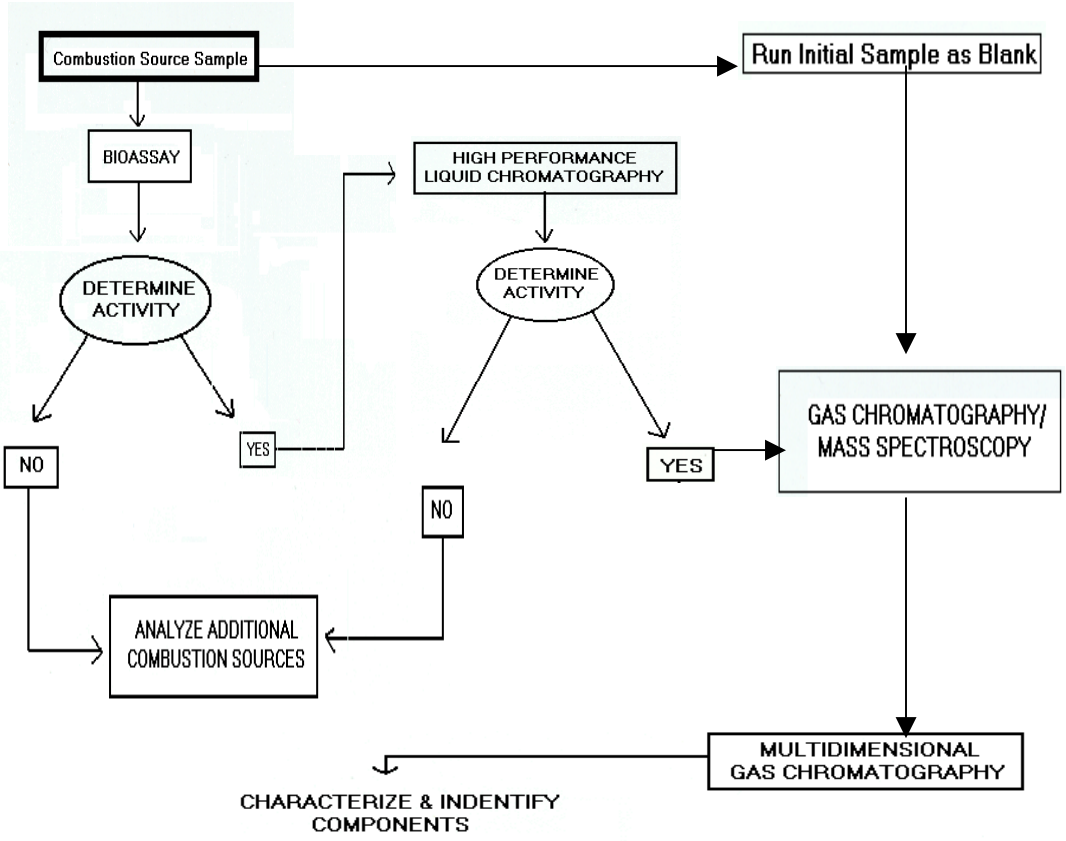
Introduction

During the last decade, concerns have been raised regarding the possible harmful effects of exposure to certain chemicals that are capable of modulating or disrupting the function of the endocrine system. These chemicals which are referred to as endocrine disrupting chemicals (EDCs) have the capability to interfere with the production, release, transport, metabolism, or elimination of the natural hormones in the body responsible for the regulation of developmental processes.[1] While the endocrine disrupting activity of industrial chemicals has been recognized since the late 1960s, it has been only recently that studies relating environmental contaminants to reproductive impairment have been conducted. A number of the compounds produced are mutagenic and have the ability to form DNA and protein adducts with the ability to activate aryl hydrocarbon receptor (AhR)-mediated activity, or interfere with estrogen receptor (ER)-mediated signaling.[2]

Recent work suggests that effluent from pulp mills may contain such chemicals, although their origin and identity are unknown.[3] It has been suggested that the substances are naturally occurring in trees, which include phytosterols such as β-sitosterol and their degradation byproducts. Sterols occur naturally in a variety of wood species and during bleaching with chlorine dioxide, unsaturated sterols such as β-sitosterol are oxidized, while saturated sterols are unaffected. A portion of the sterols apparently passes through biotreatment, and recently, it has been reported that β-sitosterol causes some of the same responses in fish as those observed in fish exposed to biotreated whole pulp mill effluent. Recently in Japan, the authors determined the estrogenic activity of chemicals in diesel exhaust particles and provide an initial identification of EDCs from combustion or vehicular emissions.[4] After sample extraction and fractionation, 4,6-dimethyldibenzothiophene was identified and confirmed as a compound emitted by diesel engines that possessed estrogenic activity.

The overall approach of this research is to conduct analogous bioassay-directed chemical analysis experiments using a cell line stably expressing an androgen-responsive reporter gene and to evaluate its ability to screen compounds responsible for androgenic activity. Bioassay-directed fractionation was developed in the late 1970s to identify chemical mutagens in diesel particulate extract.[5,6] The technique involves the separation of sample extracts into fractions followed by the determination of their toxicity by incorporating bioassays as described in **Diagram 1**. The results obtained are used to direct attention to detailed chemical analysis of the toxic fractions. Multiple processes may be completed in the fractionation until the chemical complexity of the fractions is dramatically reduced. The parent cell line for this experiment, MDA-kb2 human breast cancer cells, was chosen for its ability to express high levels of functional, endogenous androgen receptor (AR) and has been stably transfected with androgen responsive MMTV-luc promoter reporter genes.[7,8] To evaluate the cell lines capability with variable combustion sources, the model compound dihydrotestosterone (DHT) is used as a control for its known ability to act as an AR agonist. In addition, binding assays were performed in COS cells that were transfected with the human AR. This assay is used to help determine if the combustion samples affect receptor DNA binding.

Diagram 1. Schematic diagram of bioassay directed fractionation and characterization.



Materials and Methods

Chemicals. Dihydrotestosterone (minimum purity >99%) was purchased from Sigma Chemical Co. (St. Louis, MO). Hydroxyflutamide (OHF) was provided by R.O. Neri, (Schering Corp., Bloomfield, NJ). Solvent grade Ethanol was provided by LabCore Chemicial supply (Durham, NC).

COS Whole-Cell hAR Binding Assay

The COS whole cell binding assay was used to determine if components in the combustion samples would compete with [3H] 5 nM R1881, a synthetic ligand, for binding to the hAR. Combustion samples were tested as either 5 μL or 10 μL / 1 ml media. In 4 replicates, COS cells (monkey kidney line, ATCC) were transiently transfected with the human AR expression vector pCMVhAR (from Dr. Elizabeth Wilson, UNC at Chapel Hill) using diethylaminoethyl dextran. Twenty-four hrs later, cells were exposed to 5 nM [^{3}H] R1881 in the presence and absence of varying doses of

unlabeled compounds (2h, 37^{0}C). Nonspecific binding was determined by adding 100-fold molar excess of unlabeled R1881. Cells were washed in phosphate-buffered saline, lysed in 200 µL ZAP (0.13 M ethyldimethylhexadecylammonium bromide with 3% glacial acetic acid). Radioactivity of the lysate was determined by liquid scintillation counting.

Stable MDA-kb2 Cell Transcriptional-Activation Assay

MDA-kb2 Cells, which contain endogenous hAR stably transfected with the MMTV.neo.luciferase gene construct were used to assess the ability of combustion samples to induce transcription of androgen dependent genes. Cells were maintained in L-15 medium-10% FBS at 37^{0}C-with no CO_2. For each replicate, cells were plated at 10,000 cells/well in luminescent 96-well plates. Dosing solutions were prepared from stock solution in ethanol at the time of dosing, by aliquoting 2 or 3 µl of stock solution into 1 ml of medium. Control wells contained 100 µl/well (1 µl of ethanol/ml of medium) all in quadruple. Cells were incubated overnight at 37^{0}C. After 24h, medium was removed from the plate by shaking, cells were washed once with 25 µl of phosphate-buffered saline and harvested with 25 µl lysis buffer (Promega) at room temperature. Relative lights units were determined using a microtiter plate luminometer (Dynex, Chantilly, VA).

Combustion Samples. The combustion samples were a mixture of biomass extracts from mixed hardwood forest foliage (MHFF), Florida palmetto and slash pine (FPSP), and wiregrass and longleaf pine (WGLP) and combined together to increase the concentration of the sample. The biomass combustion samples were collected on quartz fiber filters and sequential extractions were performed with hexane, benzene, and an isopropyl alcohol mixture. The combustion samples stored in the hexane/benzene/isopropyl alcohol solvent were solvent exchanged into ethanol (EtOH), because of hexane/benzene/isopropyl alcohol's high toxicity with bioassays. In the procedure used for solvent exchange, the hexane/benzene/isopropyl alcohol is removed by rota-vaporizing to near dryness at room temperature and placed inside a glass screw-top vial. A small aliquot of EtOH is added to the vial and residual solvent is evaporated under a gentle stream of dry nitrogen. All organic concentrates were stored and refrigerated until further analysis.

Gas Chromatography/Mass Spectroscopy

GC/MS (HP6890/5973, Agilent, Palo Alto, CA) analysis was performed using an automated injections system, electronic pneumatic control, an ultralow capillary column with diphenyl (5%)-dimethylsiloxan (95%) copolymer stationary phase (30 m length, 0.25 mm i.d, and 0.25 mm film thickness; oven temperature programming (65 ^{0}C for 10 min to 300 ^{0}C for 45 min at 10 ^{0}C/min) The MS detector was scanned from 50 to 500 amu (3 scans/s). Chemstation software was used for instrument control and data acquisition. Quantification of target analytes with GC/MS was achieved by creating a calibration database of concentration and response ratios for the certified authentic

compounds and isotopically labeled internal standard mixtures. Individual compounds targeted were characteristic of *n*-alkane, PAH, oxy-PAH, *n*-alkanoic acid, *n*-alkanedioic acid, *n*-alkeneoic acid, resin acid, sugar, methoxyphenol, and phytosterol compound classes. Targeted sample analytes were identified using the following compound classification approach: (a) positive sample retention time and mass fragmentation pattern identically match those of an authentic primary standard and a National Institute of Standards and Technology (NIST) library mass spectrum; and (b) probables same as (a) except only secondary standards or similar compounds were available. For case b, concentration estimates of sample analytes were determined using response data of an analogous compound for which an authentic primary standard was available. Several other species were tentatively identified but were not quantified and are not reported.

HPLC Fractionation

The HPLC system consisted of a basic sample injector, gradient pump, and a diode array UV detector monitoring at 254 nm. The HPLC (Beckman Coulter, Palo Alto, CA) is controlled using the computer software Gold. For separation, 100 μL of the sample extract was injected onto a 125 x 4 mm Chromosphere 100 RP-18 column (5 mm, Merck.) The gradient profile initially started with 100% water (H_2O) and slowly ramped to 100% acetonitrile (ACN) over a linear period of 50 min, followed by a 10 min hold with 100% acetonitrile. Fractions were collected every 5 min, with a flow of 1 mL/min resulting in ten fractions. The column temperature was 25 ^{0}C.

Results and Discussion

In **Figure 1**, the positive control 0.1 nM DHT expressed a 3-fold induction of luciferase activity expressed over media. Because of the complexity or numerous by-products produced during the combustion process, we recommended pre-screening the androgen assay with pooled or combined samples before proceeding with individual sample analysis. In the pooled samples, there is considerable androgenic activity compared to the positive control DHT. There is an equal luciferase activity response to the control for pooled samples 30-32, 33-35, and 36-38. The most potent androgenic activity was from pooled samples 39-40 expressing a maximum 1.6 fold increase compared to DHT. Also, to verify AR specific activity, the luciferase response is reduced in the presence of the anti-androgen hydroxyflutamide (data not shown). Another suitable experiment to verify AR specific activity is to complete the COS AR binding assay with the pooled samples.

Figure 1. The MDA-kb2 androgenic assay evaluated with pooled biomass combustion samples. The positive control, dihydrotestestorone, expressed a 3-fold induction of luciferase activity expressed over media.

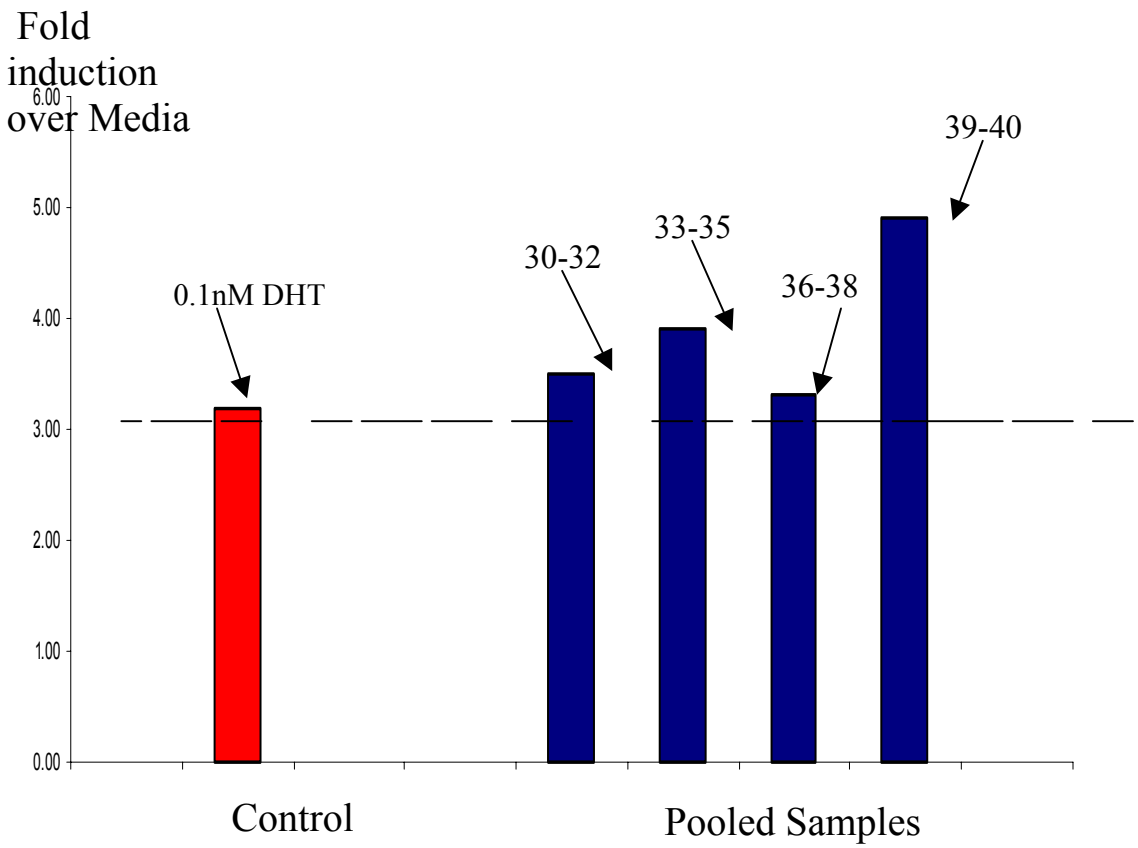
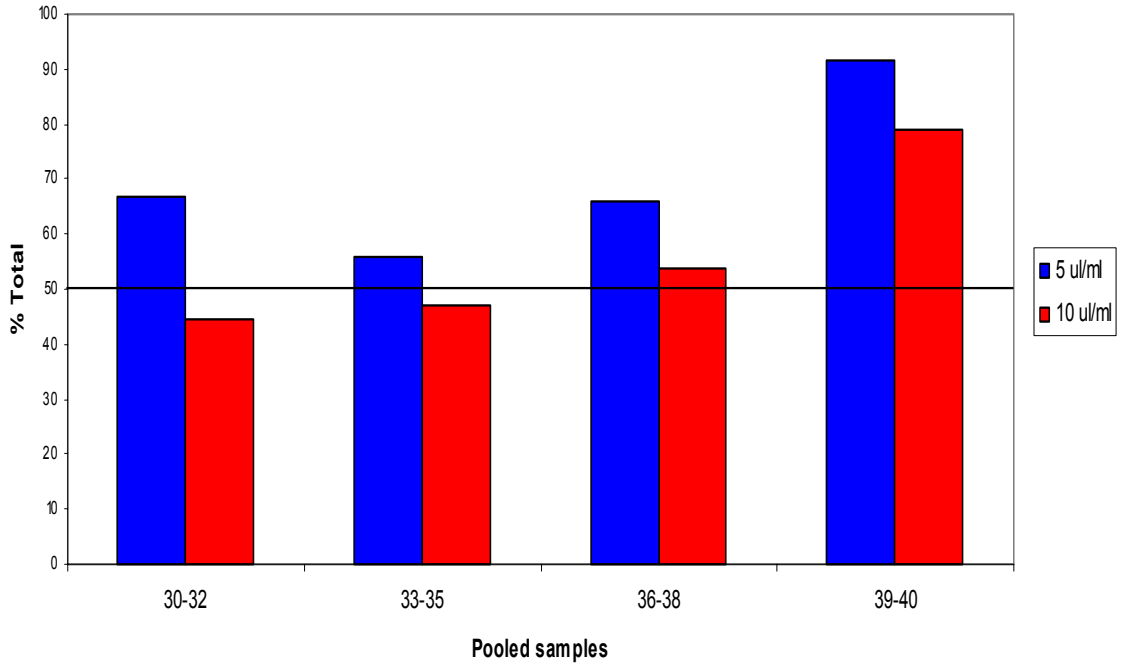
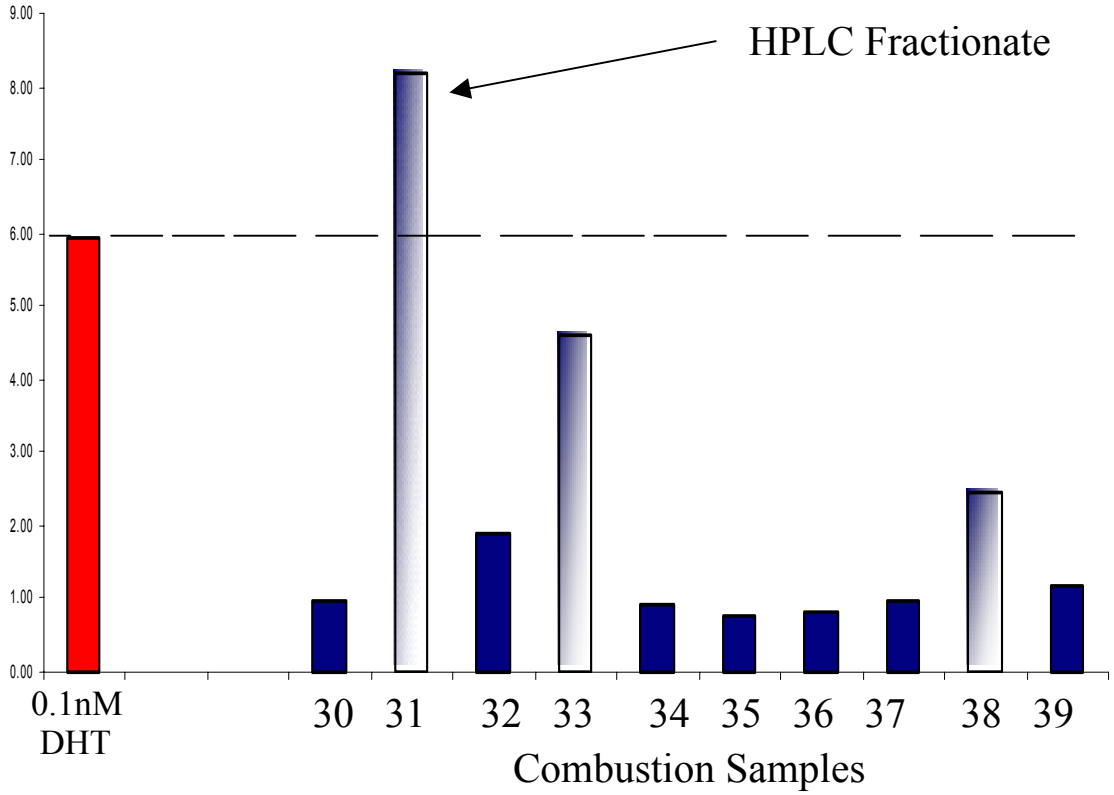


Figure 2. The COS competitive binding assay evaluated with pooled biomass combustion samples.



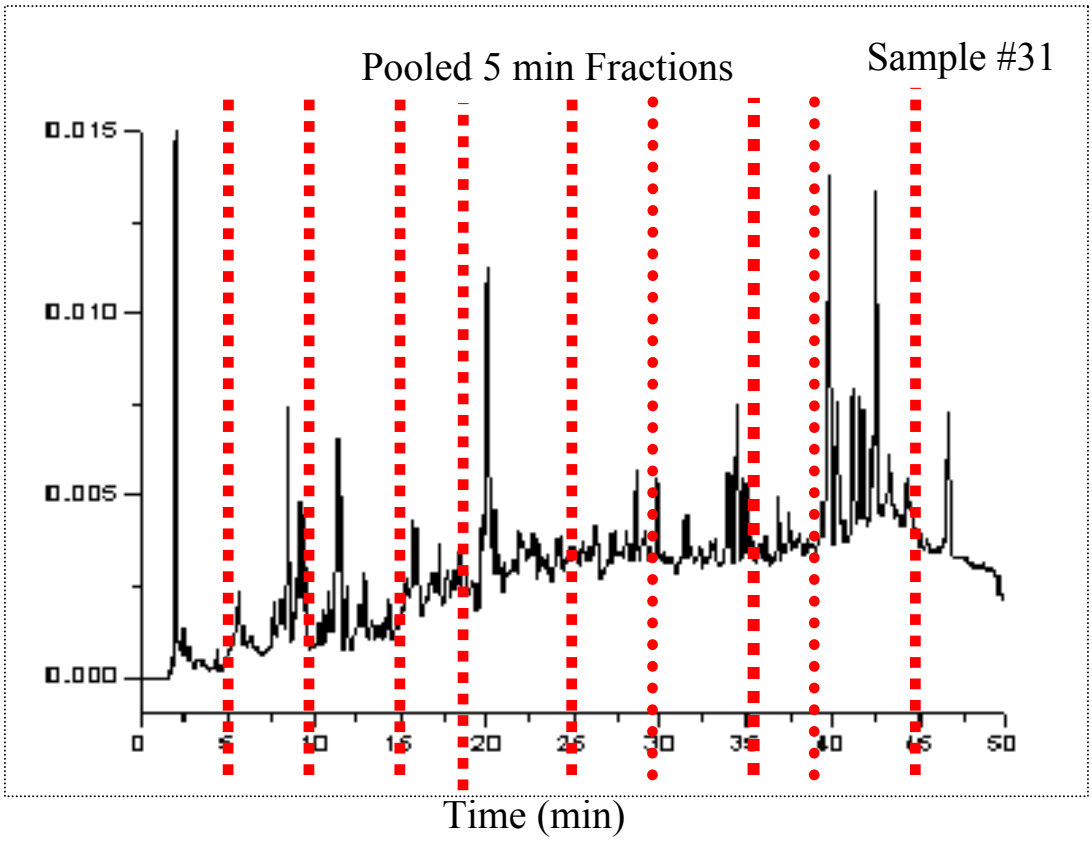
For the COS competitive binding assay in **Figure 2**, cells were transfected with the human AR. The next day, the cells are incubated with the radiolabeled ligand R1881 which is a synthetic androgen ligand and the pooled combustion samples. The labeled ligand and the unknown compounds contained in the biomass combustion compete for binding to the hAR. After 2 hours, the cells are washed to remove any unbound labeled ligand and then the cells are lysed. The lysate is added to scintillation fluid and the amount of radioactive ligand that bound to the receptor sites is tabulated. When the substances in the unknown **do not** bind to the AR, more of the labeled ligand can bind, producing higher counts. When the substances in the unknown **do** bind, they occupy the receptor sites so that less of the labeled ligand binds producing lower counts. The pooled fractions labeled 30-32 and 33-35 significantly inhibited R1881 AR binding by approximately 40-50% compared to radioligand total binding (i.e., 100% bound). In contrast, the pooled samples 36-38 and 39-40 reduced binding of the R1881 to a lessor extent as compared to the previous combustion samples. In the previous experiment with the MDA-kb2 assay shown in **Figure 1**, it was revealed that the pooled samples 36-38 and 39-40 showed agonist activity. However, in the COS binding assay, the samples did not compete effectively for binding to AR. This may have been attributed to the fact that the compounds or chemicals contained in the pooled samples may not show AR specific induction, but alter AR gene expression via an alternative mechanism.

Figure 3. The MDA-kb2 assay analysis of the 10 biomass combustion samples individually tested.



The results of the MDA-kb2 assay for the resulting ten biomass combustion samples that were individually tested are summarized in **Figure 3**. In the graph, the bars depict the relative induction fold compared to media. The bars typically absorbing relative light units less than the positive control dihydrotestosterone (DHT) indicate no androgenic activity, whereas samples above control indicated androgenic activity. The positive control produced to a six-fold induction of luciferase activity compared to media response. After a series of triplicate runs, the maximum luciferase response was noted for sample 31 producing a 1.3-fold induction compared to 0.1 nM DHT and the individual samples 30-39 analyzed. Because of the high response we decided to complete HPLC fractionation with this particular sample.

Figure 4. HPLC Chromatogram of Androgenic biomass combustion sample.



The reverse phase HPLC column will bind and separate a broad range of compounds. These compounds are eluted according to their polarity, enabling the selective removal and concentration of organic compounds from complex mixtures. By controlling the elution sequence of the compounds retained on the column, the biologically active components can be isolated within discrete fractions appropriate for higher resolution procedures. In **Figure 4**, the compounds retained on the HPLC were eluted using a gradient elution of variable volumes of acetonitrile/water mixtures (0%, 25%, 50%, 75%, 85%, 95%, and 100% acetonitrile). The samples were pooled in 5 minute portions and collected in separate vials. These fractions were rota-vaporized to dryness and re-suspended in 200µL of ethanol and subsequently assayed for androgenic activity. From the HPLC chromatogram of the biomass sample #31, you can see there are numerous peaks or compounds eluting from the column. In an instance where the HPLC is unable to separate compounds co-eluting in a specific collected fraction, further separation of that active fraction will be analyzed by re-injecting that specific collected fraction using the same solvent gradient.

Figure 5. The MDA-kb2 assay of the 5 minute pooled HPLC fractions.

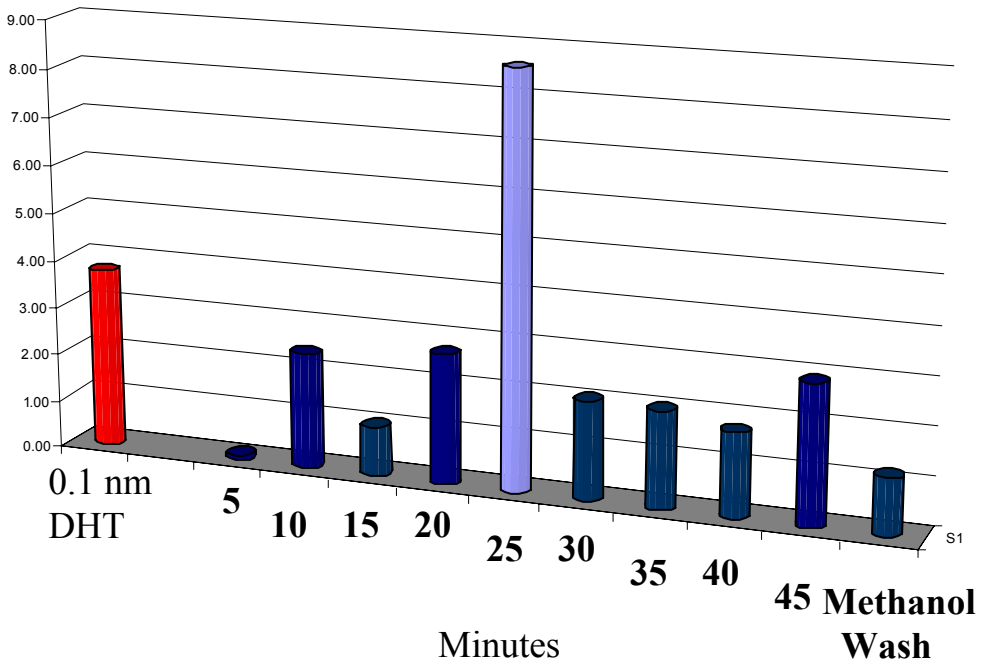


Figure 5 illustrates HPLC C18 elution profile produced when the compounds retained on the column were sequentially eluted using solvents of decreasing polarity. **Figure 5** shows the majority of androgenic activity eluted from the column between 20 and 25 minutes. There is a two-fold greater induction of luciferase activity compared to the positive control DHT. The androgenic activity determined in the fraction may be produced from a single compound or set of closely related compounds that were eluting using the gradient method employed.

The next step is to analyze the androgenic components present in the HPLC fraction by GC-MS. Before identification, it maybe impossible to determine whether the activity within the fraction could be due to a highly abundant component or whether it was due to minor components concealed with the UV absorbance chromatogram. Another possible problem with identification may be contributed by the numerous by-products produced mechanistically from combustion. Many of the compounds contained in the database of the National Institute of Standards and Technology and Wiley mass spectral library may not match the conformation of the actual compound based on physical data (e.g., boiling point and retention time).

Some of the compounds contained in the biomass combustion sources have previously been reported by GC-MS. It has not been confirmed that the compounds reported contained the androgenic activity, but where some of the major components identified in the un-fractionated sample.[9] The methoxyphenols contained in the sample are thought to be pyrolytically formed from the lignin precursor coniferyl alcohol. The partially altered and unaltered resin acids and retene (1-methyl-7-isopropylphenanthrene) appear to also be products determined in the biomass combustion. The polycyclic aromatic hydrocarbons (PAHs) and steroid components campesterol, sitosterol, and stigmasterol appear to be prevalent as well. Levoglucosan (1,6-anhydro-,-D-glucopyranose), in particular, is the most abundant compound present and demonstrates significant potential as a molecular biomarker for combustion of most types of cellulosic biomass. Unsaturated and polyunsaturated fatty acids were determined because of they are common constituents in plant oils.

Conclusions

We believe that our approach may be useful for screening combustion sources for androgenic activity. This study demonstrates the usefulness of bioassays to characterize complex combustion emissions for the purpose of comparing the relative health risks posed by combustion. Bioassy-directed chemical fractionation analysis can be a highly effective way to combine biological and chemical analysis in order to characterize the potentially harmful components of complex combustion emissions. Using the MDA-kb2 cells, we were able to possibly identify androgenic activity from the combustion of biomass only in vitro activity. Our next step is to identify and confirm the structure of the compound or compounds that present the androgenic activity.

References

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