Chromatographic Separation Strategy for Isolating and Characterizing Compounds with High Bioassay Response

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Introduction

In evaluating environmental samples that show a high bioassay response, such as dioxin-like activity, it is difficult to resolve whether some portion of their response is due to trace quantities of unknown compounds that have a high relative potency (REP) or toxic equivalency factor (TEF). As a strategy for finding such high-response compounds – or ruling out their existence – we propose a modified form of liquid chromatography-mass spectrometry (LC/MS) wherein the LC eluent flow is split by a "Y" connector. One portion of the split flow would proceed directly to the MS while another portion would be diverted onto a moving belt such as filter paper or polyimide film. The latter portion of the eluent, deposited on the filter paper or film in a continuous linear sequence that mirrors its chromatographic separation, can be physically fractionated by cutting the strip of filter paper or film into arbitrarily short lengths. Each such fraction of the original sample, corresponding to a known range of LC retention times, can be reextracted for bioassay testing and can also be subjected to further separation and testing to isolate and identify the source(s) of the high REP of the original sample. This strategy is expected to be useful for investigating individual environmental samples that show high bioassay response, but it is intended to be more than just a sample-specific analysis method. It is also an efficient way to screen and search for unknown high-REP compounds and, if such compounds exist, to characterize them as fully as possible.

Methods and Materials

It is well-known that bioassay methods such as CALUX can provide quick and reasonably accurate measurements of chemical toxicity within certain classes, particularly dioxin-like compounds, PAHs, and endocrine disruptors. For these chemotoxic classes, the bioassay response effectively *integrates* the contributions of chemicals that produce the response. Bioassays are therefore especially useful as screening methods for environmental samples that contain complex mixtures of chemical compounds.[1,2] The bioassay test results are usually expressed as a dose that is normalized to the dose of a high-response compound in the class (such as 2,3,7,8-TCDD for the class of dioxin-like compounds). The contributions of individual compounds in a bioassay sample cannot generally be distinguished but, in the absence of specific evidence of synergism or antagonism, are thought to be additive.

In most cases, bioassays serve as no more than a rapid screening method. Bioassays may also play a more precise scientific role in estimating REP or TEF values for individual compounds. The dose of a given compound needed to elicit a given bioassay response, expressed as an inverse ratio to the dose of 2,3,7,8-TCDD needed to elicit the same response, is the *relative potency* (REP) of that compound. However, it is important to note that potency in an in vitro bioassay may not accurately reflect in vivo potency in terms of TEFs, which integrate the in vivo pharmacokinetic and pharmacodynamic activity of an agent.

Bioassay testing of highly purified compounds has been a useful way to investigate the dose-response relationships of a wide range of additional compounds. However, this method has certain limitations. A highly purified compound, for example, may not be sufficiently pure. If a 99.9% pure sample of compound C contains 1 ppm of compound X whose relative potency is several orders of magnitude higher than the REP of compound C, then the high REP of compound X will create an falsely high bioassay measurement of the REP of compound C. This type of error due to impurities can be minimized but cannot easily be ruled out.[3] In addition, it is expensive to produce highly purified samples of large numbers of individual compounds, and it would be very tedious to carry out a systematic search for the most likely candidates for high REP or high TEF (i.e., high toxicity) from among the full range of possible

compounds.

These difficulties would be largely avoided by the strategy outlined here, wherein LC/MS is coordinated with "LC/bioassay" testing. The strategy is intended to identify chemical-specific dose-response relationships (i.e., REP or TEF values), and may also elucidate synergistic and antagonistic relationships, for a broader range of chemical compounds than those tested to date. The sample is separated by LC, and a "Y" connector splits the LC eluent into two flow streams of nominally identical chemical composition. The smaller flow goes directly to the MS, which consists of electrospray and MS/MS in the configuration we are setting up but may alternatively use other ionization methods such as MALDI and other MS methods such as TOF. The larger portion of the split LC eluent is deposited onto a moving belt made of a material such as filter paper or polyimide film. Prior methods of belt interface have used a recirculating belt to couple LC to MS.[4] In our application, the belt does not deliver material to the MS and is used only once (not recirculated) as it is cut by the researcher into arbitrarily short segments, each of which contains a chromatographically separated fraction of the original sample. Each such fraction, corresponding to a known range of LC retention times, can then be extracted from the belt for bioassay and other testing, or, alternatively, can be archived for future reference.

The strategy would allow researchers to start building a catalog of high-response fractions of chemical mixtures, meaning those fractions that produce high bioassay responses, where the fractions are a) reasonably well separated by chromatographic methods, b) available in sufficient aliquots for bioassay testing and for further separation and characterization, c) assigned a TEQ value based on their bioassay response, and d) well documented by MS spectra. Ideally, the fractions would be separated down to individual compounds, but in some cases the fractions may contain compounds whose spectra and fragmentation characteristics cannot be fully distinguished without additional detective work.

This strategy would typically be applied to *environmental samples* that show a high bioassay response and may be particularly useful in cases of bioassay "overprediction," where a sample's high response cannot be explained fully in terms of its analytically determined constituents. In any case, the fractions of high-response samples tend to be the fractions of interest on which further work will be done. Fractions with low response in the bioassay are of little value for the present purpose and may be discarded. However, the *sum* of the bioassay responses for all fractions combined may be of interest. This sum should equal the initial bioassay response of the original environmental sample, assuming no sample loss in the LC. Any substantial mismatch between the bioassay response of the original sample and the sum of the responses of its fractions may indicate synergism or antagonism among components in the original sample.[5]

Results and Discussion

There are two advantages in using environmental samples such as contaminated sediments rather than purified compounds as the starting-point for this strategy. First, environmental samples tend to be complex mixtures that are relatively likely to show high bioassay responses. The second reason for favoring environmental samples over purified chemical samples is the low likelihood that environmental samples will consistently show the same proportions of coeluting compounds. This may be important for any compounds that are not readily identifiable from library spectra. Sample-to-sample differences among environmental samples may prove useful in isolating and identifying unknown coeluting constituents, as described below.

If a given LC fraction contains n unknown coeluting compounds, tests can be performed on m different environmental samples, where $m \geq n$, to obtain partial identifications of the compounds and their REPs. This can be done by incorporating both MS and bioassay data, then solving m independent equations in n unknowns for an exact or best solution. Other techniques such as Principal Components Analysis may also be applied. As a simple example using simultaneous equations, suppose that $m = n = 3$ and that the three unknown compounds are dioxin-like. We assume that the three unknown dioxin-like compounds X, Y, and Z are present at the following concentrations C in the given fraction of each of three different environmental samples:

	Given fraction of sample 1	Given fraction of sample 2	Given fraction of sample 3
Unknown compound X	$C_{X1} = 6$	$C_{X2} = 1$	$C_{X3} = 30$

Unknown compound Y	$C_{Y1} = 15$	$C_{Y2} = 75$	$C_{Y3} = 5$
Unknown compound Z	$C_{Z1} = 25$	$C_{Z2} = 15$	$C_{Z3} = 2$

In this simple example, we assume that the researcher knows that $n = 3$, so that three sets of peaks are expected in the MS spectra, although in reality the value of n will initially be unknown. The researcher does *not* initially know the concentrations C_{X1} ... C_{Z3} but can infer the following relative concentrations from the spectral peak areas: $C_{X2} = (1/6) C_{X1}$; $C_{X3} = (30/6)C_{X1}$; $C_{Y2} = (75/15)C_{Y1}$; $C_{Y3} = (5/15)C_{Y1}$; $C_{Z2} = (15/25)C_{Z1}$; and $C_{Z3} = (2/25)C_{Z1}$.

The researcher does not initially know the REPs of the three unknown compounds (REP_X, REP_Y, REP_Z). However, the bioassay responses of the fractions are known. These responses have been measured for the three given fractions and are expressed as TEQ_1, TEQ_2, and TEQ_3. Based on the above relationships and the TEF-REP concept, it is evident that:

$$C_{X1}REP_X + C_{Y1}REP_Y + C_{Z1}REP_Z = TEQ_1$$

$$C_{X2}REP_X + C_{Y2}REP_Y + C_{Z2}REP_Z = (1/6)C_{X1}REP_X + (75/15)C_{Y1}REP_Y + (15/25)C_{Z1}REP_Z = TEQ_2$$

$$C_{X3}REP_X + C_{Y3}REP_Y + C_{Z3}REP_Z = (30/6)C_{X1}REP_X + (5/15)C_{Y1}REP_Y + (2/25)C_{Z1}REP_Z = TEQ_3$$

Treating $p_X = C_{X1}REP_X$ as one unknown, $p_Y = C_{Y1}REP_Y$ as a second unknown, and $p_Z = C_{Z1}REP_Z$ as a third unknown, the three above equations can be solved. In this manner, values are obtained for $p_X = C_{X1}REP_X$, $p_Y = C_{Y1}REP_Y$, and $p_Z = C_{Z1}REP_Z$. Note that each such value is a *product of unknown REP and unknown concentration*; neither the REP nor the concentration is known separately. The products $C_{X2}REP_X$, $C_{Y2}REP_Y$, $C_{Z2}REP_Z$, $C_{X3}REP_X$, $C_{Y3}REP_Y$, and $C_{Z3}REP_Z$ can likewise be determined from the above relationships. If the concentrations can be measured, or reasonably estimated by some other method such as detection limits, then the REPs will be determined within reasonable limits.

As needed, *additional* characterization may be done by further separation and testing of any given aliquot present on the filter paper or film. For any chromatographically separated fraction which is of particular interest, the fraction can be either tested directly by other methods or extracted from the filter paper or film and subjected to additional methods of separation. Any resulting subfractions can then be tested by bioassay and/or standard analytic methods to help identify constituents within the fraction of interest. Again, absent any synergism or antagonism, the bioassay response for the entire fraction should equal the sum of the contributions of any subfractions into which it is divided.

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