# EMERGING HYPHENATED ANALYTICAL TECHNIQUES FOR POPS

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## Background

The 'Quest for the Holy Grail' in the 'dioxin' analysis area is dedicated to the development of reliable procedures that can offer congener-specific results on a short time scale, at a low cost, while avoiding down time issues. Such a procedure obviously has to fulfil strict QA/QC requirements such as the ones listed in Eurachem analytical guidelines and EU or other Directives, but also has to comply with ISO17025 and/or GLP procedures. Each part of such a procedure, namely extraction, clean-up, fractionation, chromatographic separation, and physico-chemical (or biological) measurement, has to be fine tuned to its optimum capabilities.

Whatever the measurement method used, either physico-chemical or biological, the sensitivity has to be at the parts-per-quadrillion (ppq, $10^{-15}$) level. This represents an extreme case of ultra-trace analysis and a real challenge in terms of analytical chemistry. In his book 'Our Stolen Future', T. Colborn used the following comparison to illustrate the parts-per-trillion (ppt, $10^{-12}$) level : 'One can begin to imagine a quantity so infinitesimally small by thinking of a drop of gin in a train of tank cars full of tonic. One drop in 660 tank cars would be one part in a trillion; such train would be six miles long.'[1] The extension of this description to ppq would make the train 60 miles long…

Because of the ultra-trace level of target analytes, large sample sizes have to be processed and extremely large amounts of matrix-related interferences have to be removed before one can think about measurement.

## Sample preparation

The more complex the sample, the more complex the method to be used… The extraction of target analytes from the matrix and their purification from undesirable interferences take place through an expensive and time consuming multi-step approach. Although Soxhlet and liquid-liquid extraction (LLE) are still used for solid and fluid matrices, respectively, more recent and specific extraction methods exist. The major ones are supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), and solid-phase extraction (SPE).

Independently of the extraction method used, highly efficient clean-up procedures are required to purify samples issued from the extraction step prior the final analysis and quantification. Automated solid-liquid adsorption chromatographic separations, based on sorbents such as silica, alumina, Florisil, and activated carbon, are often used to ensure high sample throughput[2]. Another important aspect of the clean-up is the separation of the planar dioxins, furans and PCBs from the non-planar species. In practice, this fractionation results in a simplification of the gas chromatographic (GC) separation requirement prior to mass spectrometric (MS) analysis. For example, in dioxin analysis, a first fraction contains 17 PCDD/Fs and 4 non-*ortho*-PCBs, and a second fraction contains the 8 mono-*ortho*-PCBs, as well as a group of 6 indicator PCBs (Aroclor 1260) that has also to be monitored because of their significance. The 2 fractions are normally subjected to GC-MS separation and analysis separately.

A strategy for efficient integrated extraction, clean-up and fractionation rests on the use of online automated system. A possible approach is to combine either the SPE or the PLE with multi-column clean-up. Figure 1 represents the plumbing diagram for such a system in the PLE version. In the PLE version, samples (meat, serum,…) are placed inside the extraction cell and extracted at elevated temperature and pressure. The eluant from extraction is directed to the clean-up columns where the fractionation also takes place after inline solvent reduction. Separate fractions are collected and further evaporated prior GC-MS injection. For the SPE version, fluid samples are directly loaded on the SPE column, dried under nitrogen flow and then eluted on the clean-up column. QA/QC data and the performance of the integrated system have been previously reported for various low level matrices.

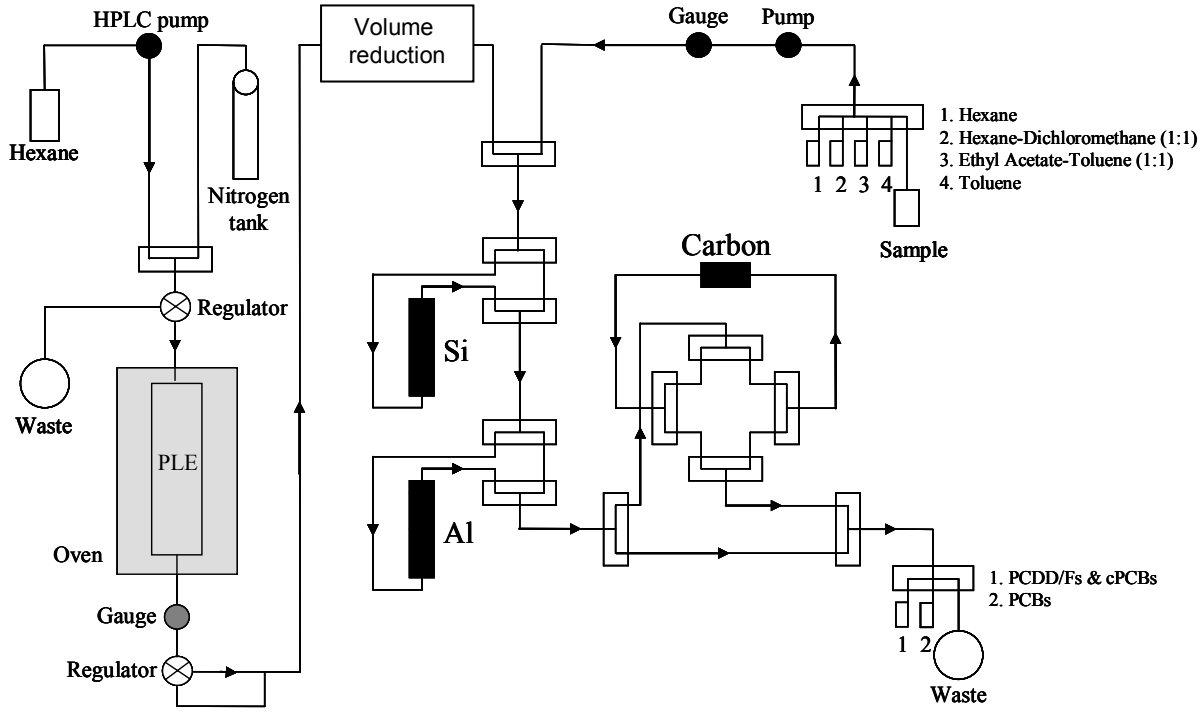


**Figure 1 :** Plumbing diagram of the so-called integrated PLE and clean-up system.

**Measurement**

Back in the mid 1970's Baughman and Meselson reported on the use of electron impact (EI) time-averaged high resolution mass spectrometry (HRMS) for the measurement of ppt levels of TCDD[3]. They were using $^{37}$Cl-TCDD for quantification purposes. Since then, the apparition of capillary gas chromatography (GC), the availability of isotopically labeled standards, and the improvement of the MS instrumentation conducted to what is now know as GC-IDHRMS (ID = isotope dilution, with $^{13}$C-labeled internal standards) for the measurement of dioxins and other selected POPs. Performing the mass acquisition in selected ion monitoring (SIM) mode allows to lower the sensitivity of modern instruments to 10 fg injected. This sensitivity is accompanied by good selectivity due to the high mass resolution provided by the sector instrument. Today, this technique is the 'gold standard' and is a requirement in EU, US and other regulations to perform 'dioxin' target analysis[4-7].

If we think in terms of analytical dimensionality, one can 'segment' GC-HRMS in 3 dimensions, GC for separation by mainly volatility, MS for separation in mass, and also the HR effect that allows to directly identify molecules based on exact mass. Therefore, any alternative tool should at least offer the same number of analytical dimensions to, at least theoretically, be considered as a viable alternative (those dimensions being the same or different).

**GC coupled to low resolution quadrupole ion storage MS operating in tandem mode (GC-LRQISTMS/MS)**

If we go for low resolution MS, we have to offer an extra dimension to the system to maintain the required dimensionality. An option is to perform tandem MS (MS/MS) and monitor daughter ions specifically formed through collision induced dissociation (CID) with buffer gaz. This target analysis approach has been exploited with quadrupole ion storage MS since mid 1990's[8], based on the reported specific $COCl^{\cdot}$ loss for dioxins[9]. Sub-ppt levels can be attained in terms of instrument detection limits (IDLs). IDL of 200 fg $\mu l^{-1}$ injected with a signal-to-noise ratio of 5:1 for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) can be obtained using large volume programmable temperature vaporizer (PTV) injection[10].

**Comprehensive two-dimensional GC coupled to low resolution Time-of-Flight MS (GCxGC-LRTOFMS)**

The basics of this technology and its application for POP analysis are presented in previous reports[11]. In GCxGC, the sample is successively in contact with 2 different GC phases connected together by a so called modulator responsible for preserving the separation attained in the first column (the first dimension, $^{1}D$) when transferred to the second column (the second dimension, $^{2}D$). The GC parameters are tuned to ensure a certain degree of independency (orthogonality) between the two dimensions. The major advantage of the methodology is to increase the peak capacity (the number of peak that can be separated in the separation space), opening the door for the GC separation of more analytes in a single injection, e.g. PCDDs, PCDFs, PCBs, OCPs, PBDEs.

Because the modulation process, the GC peaks are very narrow (50-200 ms) and a high sampling rate detector is required for peak characterisation. HRMS can normally not be used because of scanning rate limitations, therefore, non-selective detectors like micro electron-capture detector (μECD) and flame ionisation detector have to be used. An extra dimension can be added to the system if TOFMS is used. Compared to HRMS, this is a low mass resolution analyser, but has no scanning rate limitations. However, as the two GC dimensions can be seen as nearly independent, the final combination of GCxGC and TOFMS offers 3 dimensions.

GCxGC-TOFMS using isotope dilution has been shown to accurately correlate to GC-IDHRMS for low level matrices[12]. The major limitation of this approach is the lack of sensitivity at sub-picogram level, mainly due to ion source design of the major commercially available instrument. An advantage of GCxGC-TOFMS is however its capacity to collect all masses of the operating mass range, making possible the screening of any compounds (known or not) that has survived the sample preparation procedure. This truly makes the analysis comprehensive, compared to classical target analysis. The latest development in TOFMS hardware tend to indicate that better mass resolution and better sensitivity are just around the corner for comprehensive analysis.

**GCxGC coupled to HRMS (GCxGC-HRMS)**

Although it was just said that HRMS was not capable to characterise narrow GCxGC peaks, there is still one approach where HRMS can be involved. Next to the peak capacity enhancement advantage, GCxGC also produce refocusing and zone compression of GC peaks. This results in a significant improvement of the signal to be recorded at the detector, lowering the instrumental limits of detection (iLODs). Since the early attempts to couple GCxGC to the already very sensitive HRMS for further lowering of the LODs[13], GCxGC modulators have amazingly evolved to become stable and robust pieces of equipment. The cryogenic modulator allows reproducible peak refocusing to widths of as low as 50 ms. This produces a net increase of at least one order of magnitude in terms of signal

intensity, compared to regular GC. Obviously, if one wants to use HRMS as the detector for those very narrow peaks, we have to transform a slow scanning instrument (less than a scan per sec) into a faster detector. In those conditions, compared to the comprehensive analysis provided by the GCxGC-LRTOFMS, only target analysis (SIM) can be performed.

The point to make is to tune the sector instrument to detect what is not by classical GC-HRMS. Because of sample size limitation due to specimen availability, it is most of the time impossible to detect 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD in human serum of non-exposed population by GC-HRMS. GCxGC-HRMS should thus focus on those congeners. Therefore, reducing the number of SIM windows, reducing the number of monitored ions (thus increasing the time spent on the ion of interest to acquire data), reducing the lock mass time and optimising all electrical parameters can decrease the acquisition cycle time to around 50 ms. Peaks of 300-400 ms width can then be detected and reconstructed on the basis of 6 to 8 data points across the Gaussian with a scan rate of 20 Hz.

GCxGC-HRMS showed very low LODs at the level of 250 ag ($10^{-18}$, parts-per-quintillion) with a signal to noise ratio of 200[14]. It is important to mention that, at those levels, deviations of isotope ratio are observed compared to theoretical values. This can probably be partially due to the limited number of molecules that actually hit the ion detector after being ionized, separated, and transmitted and can potentially not be in sufficient amounts for the respect of ions statistics. It is therefore still a decision to take to see if a GCxGC-HRMS system should be considered as a 3 or 4 dimension system, under the definition stated earlier.

**GCxGC coupled to low resolution quadrupole MS (GCxGC-LRqMS)**

Quadrupole MS is, like HRMS, a scanning instrument, that is not a detector of choice for GCxGC. The idea behind this coupling is to have access to another mode of ionization than EI, while doing GCxGC. Negative chemical ionization (NCI) is an ionization process that uses a reagent gas to soften the process and reduce the probability of fragment ion formation, thus simplifying the mass spectra. Because less fragments are formed from the parent ion, higher intensity is recorded for the parent ion, improving the signal intensity. Halogenated POPs are high electron affinity molecules that are efficiently ionized in NCI and for which high sensitivity and high selectivity are observed for most halogenation levels. NCI has been known to be subject to reproducibility problems from the past[15], a fact responsible of the scarce use of NCI in dioxin and PCB analysis. Recent improvements in NCI hardware is supposed to significantly improve response factor stability and eventually make NCI a robust alternative to EI for GCxGC. Extremely good sensitivity can be expected for such a coupling. This can motivate the use of NCI despite the reduced information on fragmentation and the reduced flexibility in terms of quantification procedures.

**Discussion**

MS-based alternative to sector HRMS instruments do exist. Some of them offer comprehensive mass information useful for screening. If this is the responsibility of analysts to set the scene by reporting emerging analytes present in samples to regulation bodies, many efforts should thus be focussed on comprehensive analysis for screening rather than target analysis. Discrepancies reported when comparing bio-screening approach results to MS results motivate such an approach. But then comes the challenge to get financial funding for such extensive physico-chemical screening work… An option might be to redirect some of the large resources dedicated to heavy and sometimes redundant accreditation procedures for target analysis to finance comprehensive approaches. Next comes the question of validation and ‘accreditability’ of alternatives to HRMS… The later is for sure a gold standard, but no doubt that if regulation requirements were not tailor made for HRMS, alternatives exhibiting the same dimensionality could probably do (at least) the same job in many situations… One can sometimes have the feeling that HRMS has been there for so long that nobody can imagine doing something else for ‘dioxins’…

Whatever the method used, issues regarding LOQs and non-detect handling will remain. Not only in terms of 'How low can I measure?', but also, and mainly, 'How do I define how low I can measure?'. Monitoring of blank levels and incorporation of those levels in validation procedures is a really obscure area where analysts can nearly do what suits them the most, what can have big impact on the quality of the data and on the commercial competitiveness of the laboratory in the target analysis business. Additionally, method LOQs are often used to lay the foundations for new regulation (we actually can only regulate on levels we can measure with good confidence), so that this is of prime importance to define them properly.

The general trend to develop faster analytical methods is also present in the dioxin field. A perspective is the total integrated approach between sample preparation and the final measurement step. By coupling the extraction technique to the clean-up steps, and ultimately to GC-MS and/or biological detection, a multidimensional comprehensive approach is conceivable. In that case, efforts still have to be carried out to improve the data processing for comprehensive MS methods.

Finally, because it is the measurement of the global toxicity of all analytes present in a sample that is important, it is quite obvious that bioassays should always be the preferred tools for toxicity estimation. Recent advances in proteomic and metabolomic should allow the identification of specific biomarkers of exposure to be used for screening and identification of samples to be further analyzed by GC-MS for non-target molecule identification.

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