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Quantitative non-targeted analysis: Bridging the gap between contaminant discovery and risk characterization

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Abstract

Chemical risk assessments follow a long-standing paradigm that integrates hazard, dose–response, and exposure information to facilitate quantitative risk characterization. Targeted analytical measurement data directly support risk assessment activities, as well as downstream risk management and compliance monitoring efforts. Yet, targeted methods have struggled to keep pace with the demands for data regarding the vast, and growing, number of known chemicals. Many contemporary monitoring studies therefore utilize non-targeted analysis (NTA) methods to screen for known chemicals with limited risk information. Qualitative NTA data has enabled identification of previously unknown compounds and characterization of data-poor compounds in support of hazard identification and exposure assessment efforts. In spite of this, NTA data have seen limited use in risk-based decision making due to uncertainties surrounding their quantitative interpretation. Significant efforts have been made in recent years to bridge this quantitative gap. Based on these advancements, quantitative NTA data, when coupled with other high-throughput data streams and predictive models, are poised to directly support 21st-century risk-based decisions. This article highlights components of the chemical risk assessment process that are influenced by NTA data, surveys the existing literature for approaches to derive quantitative estimates of chemicals from NTA measurements, and presents a conceptual framework for incorporating NTA data into contemporary risk assessment frameworks.

Keywords

Non-targeted analysis; Risk characterization; Exposure modeling; Quantitation

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

1. Introduction

Advances in analytical mass spectrometry and high-throughput data processing capabilities have instigated a paradigm shift in molecular analysis over the past twenty years. At the turn of the millennium, biologically, environmentally, and pharmacologically relevant chemical assessments were based on targeted analyses of small panels of chemicals. Targeted methods are accurate and robust, but a strict focus on defined chemical panels leads only to an improved understanding of those pre-selected compounds. Modern chemical inventories have expanded drastically, with generic chemical registries like Pubchem and CAS containing hundreds of millions of registered chemical substances (CAS; Kim et al., 2020). Even a conservative chemical database, such as the EPA Comptox Chemicals Dashboard, lists over a million substances (McEachran et al., 2017). In contrast to these millions of chemical substances, only a fraction of known compounds have public monitoring and/or analysis methods in common use. In the United States, the EPA substance registry service indicates that ~ 80,000 chemical substances are tracked or regulated by the agency (USEPA, xxxx). Therefore, traditional monitoring methods are useful for, conservatively, only ~ 5% of known chemical species, with limited physicochemical, toxicity, or exposure data available for the many chemicals of emerging concern (Brack et al., 2012; Naidu et al., 2016; Weinberg et al., 2019; Egeghy et al., 2012). Recent years have ushered in new methods to fill the gap of targeted analyses. Burgeoning chemical “-omics” fields (e.g. exposomics, metabolomics) utilize holistic, non-targeted analytical approaches to interrogate compounds in complex environmental and biological systems (Rappaport and Smith, 2010; Wild, 2005; German et al., 2005).

Non-targeted analysis (NTA) methods have unique value in that they can garner informative chemical measurements from samples of interest *without* the need for predefined chemical targets. The workhorse techniques for NTA are nuclear magnetic resonance (NMR), which is well described in a recent review article, (Giraudeau, 2020) and high-resolution mass spectrometry (HRMS), which we discuss here. HRMS methods (often coupled with separation techniques) utilize ionization sources paired with high resolving power mass detectors to isolate and identify chemicals based on their observed accurate masses, isotopic fingerprints, and MS/MS fragments. Applications of HRMS-based NTA abound, with demonstrated capabilities in screening for disease (López-López et al., 2018; Trivedi et al., 2017) and exposure biomarkers, (Pourchet et al., 2020) detecting environmental contaminant sources, (Ruff et al., 2015; Brack et al., 2019; Focazio et al., 2008) discovering emerging environmental pollutants, (McMahen et al., 2016; Bletsou et al., 2015) and monitoring effectiveness of pollutant treatment technologies (Newton et al., 2018; McCord et al., 2020).

A unifying feature of NTA studies is a primarily qualitative focus on the unambiguous identification of individual species—and there are large concerted efforts to improve and formalize chemical identification processes (Schymanski and Neumann, 2013; Ulrich et al., 2019; Domingo-Almenara et al., 2018). Quantitative interpretations of NTA data to date have been largely based on *relative quantitation*, where measured chemical responses are compared across two or more sample groups (e.g. fold-change comparison) for prioritization of chemicals, without attempt to link them directly to fixed concentration values. Seldom has

there been *absolute quantitation*, where direct measurements of compounds are translated to estimated sample (e.g., drinking water, blood) concentrations. Ideally, there would be approaches to convert measurements of feature response in NTA experiments to informative sample concentrations (Fig. 1).

The lack of well-defined concentration estimates from NTA measurements is a fundamental challenge in using NTA data to support chemical safety evaluations. As strictly a screening and prioritization tool, NTA has proven useful for directing future research activities. For example, non-targeted screening occurrence is an explicit component of the EU funded NORMAN emerging contaminant prioritization scheme. (NORMAN, xxxx) However, existing risk assessment paradigms ultimately require quantitative estimates of chemical abundance (for example, within a consumer product, in an environmental matrix, or within a living organism) to support defensible decisions by regulatory bodies. In this article, we demonstrate the importance of quantitative predictions of chemical abundance, based on NTA data, for supporting risk-based decisions in context; we further survey existing literature on approaches for deriving quantitative estimates from NTA measurements; and finally, we present a conceptual framework for incorporating quantitative NTA (qNTA) estimates into contemporary risk assessment workflows.

2. The need for quantitative estimates of chemical concentration

The 1983 National Research Council (NRC) risk assessment paradigm provides a historical framework for evaluating the safety of chemical species using knowledge of the chemicals' inherent hazards and dose–response relationships, and the anticipated exposures within target populations (Fig. 2).(Council, 1983) This framework was designed around single-chemical evaluations, reflecting the available technology and views at the end of the 20th century. The early 21st century has witnessed a shift in scientific perspective and experimental capabilities, making way for an era of high-throughput (HT) testing and systems-level evaluations. New approach methodologies (NAMs) consisting of clear experimental (e.g., *in vitro* toxicity testing) and computational (e.g., *in silico* molecular modeling) advancements have the potential to improve the efficiency of chemical safety assessments; a number of agencies have developed NAM test systems and guidelines (USEPA List of Alternative Test). Nevertheless, the original NRC risk assessment paradigm is no less relevant today than in 1983; chemicals are still evaluated for safety one at a time, using information about the underlying hazard, dose–response relationship, and anticipated exposures. The fundamental advancement is in the way that hazard, dose–response, and exposure data are collected and interpreted. Moving forward, experimental data from NTA studies can enhance all aspects of the risk assessment paradigm, from hazard identification onward.

2.1. Hazard identification

Hazard identification is considered the initiating step for traditional risk assessment. Chemicals are identified as requiring formal evaluation and then examined for associations with environmental, ecological, or human health-related endpoints. For commercial chemicals, hazard identification is a component of review performed by regulatory bodies

(for example, the U.S. EPA under the Toxic Substances Control Act [TSCA] or the Federal Insecticide, Fungicide, and Rodenticide Act [FIFRA], or the European Chemicals Agency under the legislation on the Registration, Evaluation, and Authorization of Chemicals [REACH]). However, only a subset of chemicals with human and ecological exposure routes are identified via legal disclosure mandates. There are, for example, TSCA specific exceptions for low-volume chemicals (CFR 723) and byproducts serving no “separate commercial purpose” (CFR 720). Finally, there are naturally occurring products/extracts which may pose health risks. Thus, there remains a need for hazard identification beyond existing chemical safety legislation focused on narrow lists of chemicals of commerce.

Non-targeted analysis approaches are well suited for hazard identification because compound detection is often sufficient for initial steps. In wide-scale screening investigations, NTA can identify chemicals not included on registration lists, highlight occurrence of emerging contaminants, and support downstream hazard classification (NORMAN). Such screening efforts may seek to identify “known unknown” chemicals with limited occurrence data, or implement more in-depth analyses to identify novel “unknown unknown” substances (Schymanski and Williams, 2017). For example, NTA methods have identified novel halogenated transformation products from water disinfection, (Ding et al., 2013; Tao et al., 2020) minor products of perfluorinated chemical manufacturing, (McCord et al., 2020; McCord and Strynar, 2019) and generic chemical components of unknown/variable (but registered) complex mixtures (Salvito et al., 2020).

In addition to environmental screening, non-targeted methods can support effect-directed analyses (EDA), wherein complex samples/mixtures are first fractionated, and fractions then individually screened for bioactivity (primarily using *in vitro* assays) (Burgess et al., 2013; Brack et al., 2019). NTA enables follow-up evaluation of risk drivers within active fractions via compound identification. As a recent example, Tian et al. used EDA and examined sequential fractions of a tire rubber extract, using NTA methods, to identify a quinone transformation product that causes lethality in coho salmon (Tian et al., 2021). Other examples include the use of EDA/NTA to identify estrogenic and antiandrogenic compounds in water (Pochiraju et al., 2020; Muschket et al., 2018) and biological matrices (Dusza et al., 2022).

It is noteworthy that, when dealing with complex mixtures, analyte identification is limited to the form of the chemical substance that can be observed by mass spectrometry. This generally does not include counterion salts, adjuvants, dispersants, and other common mixture components of registered chemical substances. Therefore, it is not easy to pair observed analytes to hazard information for parent substances without a means to correlate the two. Overcoming this challenge requires that mappings exist between the chemicals identified in NTA experiments and the substances registered in public databases. The MS-Ready (McEachran et al., 2018) module of the EPA Comptox Dashboard is one such tool that provides these mapping and facilitates correlation between observable MS analytes and chemical substances with known or predicted hazard data.

Overall, compound identification in NTA experiments remains nontrivial, with annotation uncertainty propagating to downstream steps of risk evaluation (as we later discuss)

(Pourchet et al., 2020). Nevertheless, the growing body of research indicates that NTA is well suited to aid the identification of emerging stressors that may pose unacceptable health risks to humans and/or other ecological species.

2.2. Dose-response assessment

Having identified a hazard, the risk-assessment paradigm envisioned the determination of a dose–response relationship via *in vivo* treatment studies, primarily using whole animals. In this context, targeted endpoints would be selected by a risk assessor consistent with the initial hazard identification (e.g. endocrine disruptor potential). These endpoints are measured in model systems with the goal of defining a clear point-of-departure (POD) on a single dose–response curve for that endpoint. With the application of uncertainty factors (related to intra-/inter-species variability from *in vivo* models, etc.) the POD becomes the basis for a chemical-specific reference dose (RfD) or reference concentration (RfC). This approach is prohibitively resource intensive for the modern chemical landscape, which is replete with chemicals of emerging concern (Council, 1984). An updated vision of hazard identification and dose–response assessment relationship was therefore set forth by the National Research Council (NRC) in their 2007 report, “Toxicity Testing in the 21st Century”. (Council, 2007) Therein, the NRC proposes a vision of toxicity testing centered around biomolecular pathways and mechanistic *in vitro* assays suitable for high-throughput chemical screening. Such assays are still carried out in a concentration–response format, but potential *in vivo* relationships are extrapolated from *in vitro* results using toxicokinetic modeling strategies and uncertainty factors (Kavlock and Dix, 2010; Paul Friedman et al., 2020). The work of Friedman et al. indicates that the use of the *in vitro* to *in vivo* extrapolation (IVIVE) introduces a maximum of 10-fold error compared to traditional targeted approaches, and that the estimation error is protective (i.e. overestimation of response) (Paul Friedman et al., 2020).

Non-targeted analysis has limited application in dose–response assessments when consideration is given to only known target analytes. There are, however, multiple scenarios in which NTA can augment both *in vitro* and *in vivo* dose–response studies. First, when testing chemicals which elicit adverse response only after metabolic activation, NTA is well suited to rapidly identify both anticipated and unexpected metabolites (Catron et al., 2019; Weitekamp et al., 2019) (note: an increasing number of metabolically competent *in vitro* test systems (DeGroot et al., 2018; Deisenroth et al., 2020)) now allow tracking of parent depletion and product formation as a function of dose and time, and with respect to the measured assay response). Second, a large portion of registered chemical substances are of Unknown or Variable Composition, Complex Reaction Products and Biological Materials (UVCB Substance) (Agency USEP,xxxx). For these compounds, toxicological screening of mixture fractions can be employed in a concentration–response format alongside NTA approaches to characterize active chemical(s). As described for EDA above, this is bottlenecked by difficulties mapping detections to parent chemical substances, and the accuracy of NTA annotations (Schymanski and Williams, 2017; McEachran et al., 2018). Further, the ability to recover and detect relevant portions of chemical mixtures is dependent on sample preparation and the applicable analysis domain (e. g. LC-MS, GC-MS, solvent solubility, etc.). Recent work by Lowe et al. has attempted to model the

applicability of different analytical techniques to chemical subsets based on their structure and properties, (Lowe et al., 2021) but this is mostly useful for improving certainty in annotation, and defining the bias of your measurements. In simple cases where it is possible to assume good method applicability, quantitative NTA methods can theoretically inform dose–response curve measurements, experimental PODs, and (given appropriate uncertainty factors) eventual RfDs/RfCs. These thresholds are the critical point of comparison for quantitative measurements in exposure assessment.

2.3. Exposure assessment

Traditional exposure assessments couple quantitative measurements of chemicals in environmental media (e.g., drinking water, consumer products, blood) with activity (e.g., location, product use) and “exposure factor” information, (USEPA,xxxx) such as consumption rates, to yield final exposure estimates; these can be interpreted with respect to a RfD or RfC to characterize risk and establish “no-effect” concentrations for specific media. The task of exposure estimation has recently broadened in accordance with the rapid expansion of the known chemical universe. The NRC recommended emphasis on high-throughput exposure estimation strategies in their 2012 report “Exposure Science in the 21st Century: A Vision and A Strategy.”(Council, 2012) In accordance with NRC recommendations, efforts from organizations such as the NORMAN network have begun to incorporate non-targeted screening approaches to hazard prioritization and EPA’s ExpoCast project (Wambaugh et al., 2019) has developed HT exposure prediction models and frameworks for harmonizing exposure estimates across disparate existing models (Rager et al., 2016; Sobus et al., 2018; Wambaugh et al., 2013; Wambaugh et al., 2014; Ring et al., 2017; Ring et al., 2019). To date, exposure estimates have been generated for hundreds-of-thousands of known chemicals; for example the work of Ring et al. used machine learning and thirteen previously developed exposure models, built on the limited CDC NHANES exposure dataset, to calculate consensus predictions for the median intake rate of nearly 500,000 chemicals based on their chemical structure (Ring et al., 2019; Sobus et al., 2015; Control, 2006). Prediction uncertainty can be large for some chemicals due to a lack of available chemical measurement data for model parameterization, evaluation, and calibration. The study of Ring et al. exhibited credible intake intervals of up to eight orders of magnitude (Ring et al., 2019). A considerable increase in chemical monitoring data is therefore needed to reduce uncertainties in HT exposure predictions. Most existing environmental and biological monitoring studies focus on targeted chemical panels, yielding measurement data for only a few well-known compounds, (Egeghy et al., 2012; Sobus et al., 2015) with major data gaps for metabolites, degradants, and other unknowns. NTA studies can help fill these gaps, providing broad chemical measurements that inform presence and putative exposure sources, routes, and pathways and allowing for prioritization of chemicals of interest (Sobus et al., 2018).

2.4. Risk characterization

Risk characterization requires the full synthesis of available hazard, dose–response, and exposure information, and computational and high-throughput techniques, including NTA, are anticipated to be the backbone of future efforts in the field (Thomas et al., 2019; Academies and of Sciences, Medicine, 2017). Efficient quantitative data streams can

predict the likelihood and severity of potential health risks consistent with an exposure (Academies and of Sciences, E., Medicine, , 2017). For example, quantitative structure–activity relationship (QSAR) models can be used to predict environmental fate, (Mansouri et al., 2018) determine bioactive exposure thresholds, (Pearce et al., 2017) and estimate receptor activity (Mansouri et al., 2016). These types of models are built from reference data for thousands of chemical compounds, but accept simple chemical structures as input. The constructed models leverage existing chemical data to predict outputs for novel chemicals by structural comparisons; limited application of these approaches is beginning to be seen in regulatory decision making. For example, the EPA endocrine disruptor screening program allows high-throughput computational approaches for tier 1 evaluation (USEPA Use of High Throughput).

Implementation of NTA to support high-throughput risk assessment has been seen in a limited capacity, with most applications identifying structures of emerging contaminants in need of characterization. Specifically, results of NTA experiments have been used to prioritize chemicals requiring further study (via targeted analysis) based on perceived exposure and hazard potential (NORMAN; Rager et al., 2016). The toxicological prioritization index (ToxPi) (Reif et al., 2010) is one framework used to prioritize chemical detections based on the potential for health risks. While originally conceived to visually examine chemicals using targeted toxicity data, the ToxPi framework has been extended to consider NTA experimental data (i.e., the detection frequencies and measured intensities of identified chemicals) as quantitative surrogates of exposure (Newton et al., 2018; Rager et al., 2016; Sobus et al., 2018). These efforts have proven suitable for provisional chemical evaluation and ranking. Yet, a clear delineation remains between the type of data produced in NTA studies and the type needed to support eventual quantitative risk characterizations. Simply put, targeted analytical methods remain the source of quantitative experimental data used in formal risk calculations. Since targeted quantitative methods: 1) require the procurement/synthesis of standards; 2) are time/resource intensive; and 3) are not readily available for the majority of newly discovered compounds, scientific advances are needed that will allow quantitative NTA (qNTA) data to be directly utilized for rapid risk characterization. Achieving this end will require significant advances on current qNTA techniques.

3. Current approaches for quantitative predictions in non-targeted analysis

Targeted quantitative mass spectrometry is a gold-standard analytical technique for many chemicals, allowing absolute quantification and high precision/accuracy when incorporating external and/or internal standardization procedures. With sufficient resources, quantification of chemicals can be readily explored using extensive targeted analyses in tandem with, or as a follow up to NTA. For example, the work of Moschet et al. screened nearly every water soluble pesticide in Switzerland, with follow-up targeted quantitation, (Moschet et al., 2014) and a study by Gago-Ferrero et al. screened for thousands of emerging contaminants with similar follow-up (Gago-Ferrero et al., 2015). These exhaustive studies represent the best case scenario for combined analysis, with a well characterized chemical panel,

available standards, and significant resources to develop and apply quantitative methods for hundreds if not thousands of chemicals detected in an initial NTA screen. In general, this level of cost and time commitment to broad quantitative screening is infeasible. Further, screening experiments are most attractive for conditions where other methods do not currently exist, and for under-studied and/or novel compounds with limited chemical information where targeted analysis is not only difficult, but impossible. As a result, targeted quantitative approaches for chemicals of emerging concern require supplementation with NTA experiments.

While there is a strong impetus to carry out NTA screening, it lacks structured sets of reference materials, surrogate chemicals, and matched internal standards common in targeted assays, and therefore cannot match the quantitative accuracy or precision of targeted methods. At present, there is no universally accepted method for quantifying chemicals detected/identified in an NTA experiment. There are, however, several approaches for using NTA data in a quantitative context. The first approach is the common process of “relative quantitation” and involves the comparison of measured intensities or normalized response values rather than concentration values. This quantitative treatment does not yield concentration estimates but has been used to compare samples for chemical prioritization and follow-up work. True quantitative approaches yield concentration estimates either using calibration (mimicking traditional quantitative methods) or direct response modeling. Each quantitative approach has intrinsic levels of uncertainty due to the numerous factors that influence chemical response in mass spectrometry. The sections below describe important sources of quantitative uncertainties and discuss the extent to which they’ve been considered in previous qNTA applications.

3.1. Relative quantitation by compound response comparisons

Relative quantification is the default method of comparison in NTA experiments. While not an absolute concentration measure, instrument response is assumed to be proportional to concentration, allowing comparison of analyte response across samples. The naïve assumption of linear response relationships and minimal matrix effects are seldom accurate; data normalization approaches are therefore often required to help correct for instrument variability and non-linearity (Yi et al., 2016; Di Guida et al., 2016). Simple relative comparisons are nevertheless easy and useful; an investigator could examine the fold-change in observed response between an exposed and unexposed population (i.e., $\text{Response}_{\text{exposed}} / \text{Response}_{\text{unexposed}}$) to identify statistically significant chemicals for prioritization (Fig. 3). Indeed, binary comparisons of two sample sets (e.g., case vs. control, upstream vs. downstream, treated vs. untreated) have been used in environmental analyses to identify geographic origins of emerging contaminants (Nakayama et al., 2010; Lindstrom et al., 2011; Strynar et al., 2015) and to associate disinfection byproducts with specific treatment methods (Tang et al., 2016; Liberatore et al., 2020). With thousands of measurements conducted in a typical NTA experiment, traditional significance thresholds of statistical comparison would yield an unacceptably high false-positive rate. Controlling the so-called false discovery rate (FDR) can involve adjusting p-values for multiple hypothesis testing, as proposed by Benjamini-Hochberg (Benjamini and Hochberg, 1995) (Fig. 3), or using other significance criteria such as Storey *q*-value (Storey, 2003; Storey, 2002) or local FDRs

(Chong et al., 2015). Comparisons and statistical corrections are commonplace and enabled by data processing software (Xia et al., 2009; Misra, 2021).

Time-based comparisons (e.g., time 1 vs. time 2) have further helped identify environmental metabolites in wastewater, (Gago-Ferrero et al., 2015) assess real-time pollutant levels in the surface water, (Hollender et al., 2017) and characterize emerging contaminant levels in human blood (Plassmann et al., 2016; Plassmann et al., 2018). In these examples, observed changes in the relative abundance of detected chemicals was the basis for follow-up investigation, such as contacting point sources to confirm chemical releases (Hollender et al., 2017) or performing targeted analyses to confirm chemical identities and estimate sample concentrations (Plassmann et al., 2018).

There are clear limitations to the direct use of measured HRMS intensities and response factors for relative quantitation. First, numerous factors (e.g., instrument cleanliness and tuning, matrix effects) influence HRMS response measures for any given chemical, and analyte response can be non-linear over large concentration ranges. The most straightforward normalization approach is to correct all measured intensities using reference compounds or internal standards (i.e. applying a scalar correction factor to measured intensities from a sample so that reference responses are constant across samples); (Sobus et al., 2018) although additional correction may be necessary for concentrations that fall outside the linear response range (Yu et al., 2020). Measurements can also be compared relative to a reference sample or pooled QC sample to ensure inter comparability between analytical batches and methods (Liu et al., 2020; Go et al., 2015). This has the further benefit that relative measurements can be converted to absolute measurements *post hoc* if concentrations are determined in the reference sample (Liu et al., 2020). Comparative measurements can thus reduce the number of necessary targeted measurements for quantification in addition to allowing retroactive concentration determination. However, without complementary quantitative measurements, normalization can only improve intra-chemical comparisons between samples, but not cross-chemical comparisons. Furthermore, they do not yield specific concentration estimates in prepared sample extracts or the original sample medium (Fig. 1). For this reason, relative quantitation methods are viable for prioritizing future work, and for retaining valuable sample information for future interrogation, but alone cannot produce the data needed for other components of risk characterization. Ultimately, methods for absolute determination of concentration are needed.

3.2. Quantification by surrogate standard

Without a direct reference standard, a straightforward alternative for compound quantitation involves using a calibration curve (i.e. sample concentration vs. instrument response/internal standard correct response) from a chemical surrogate (Fig. 4). The goal in surrogate selection would be to identify a known chemical that is likely to mimic the analytical behaviors (e.g., chromatographic separation, ionization, and HRMS detection) of the compound in question. A single surrogate can be selected in a variety of ways. For example, a parent chemical may be chosen as a surrogate for a putative metabolite, or a well-known

compound from a specific chemical class (e.g., perfluorinated carboxylic acid) may act as a surrogate for an emerging chemical in that same class.

Significant challenges can exist when attempting to identify chemical surrogates for quantification. Specifically, a wide range of observed experimental inaccuracies indicate that the differences in estimated concentration when using a “closely matched” surrogate vs. a poorly matched surrogate can far outweigh the intrinsic error of any individual calibration curve (Fig. 4). Experimental examples using calibration curves of parent chemicals to quantify related metabolites have yielded inaccuracies of between four-fold for simple methyl- and hydroxylmetabolites (Dahal et al., 2011) and up to seventy-fold for more complex metabolic products (Hatsis et al., 2017). Using calibration curves of structurally similar surrogates, inaccuracies have been reported ranging from three-fold for small molecules in food, (Pieke et al., 2017) to an order-of-magnitude or more for perfluorinated contaminants in surface water, (McCord et al., 2018) and up to several hundred-fold for drugs in biological samples (Liigand et al., 2018). The measured inaccuracies derive from multiple sources, both the intrinsic variance of analytical measurement (i.e. curve preparation, instrument variability, internal standard addition etc.) and the inaccuracy introduced by applying calibration models between chemicals (i.e. deviations from predicted chemical similarity, inaccuracy of regression models etc.).

It is desirable for a closely matched surrogate to account for the intrinsic effects of the ionization, solvent composition, and matrix effects on analyte response. To date, the most accurate results from surrogate applications have been observed when considering chemicals within a defined class and with close retention times, (Pieke et al., 2017; Cech et al., 2001) or when extrapolating across bracketed homologous compounds (Kamga et al., 2014). In GC-MS experiments, observed quantitative error has been much smaller than in LC-MS experiments. In one study by Banerjee et al., an average calibration curve from a panel of surrogate compounds was able to estimate concentrations for several hundred pesticides with an average error of 10% from their true values, demonstrating significant similarity in response across the chemical space (Banerjee et al., 2012). Other work by Bu et al. observed maximum errors of only two-fold for select classes of organic contaminants when using an average curve (Bu et al., 2014). For the selected organic classes, the comparatively simpler chromatographic and ionization source interactions likely contribute to the more accurate quantitative predictions.

While the desire for closely matched surrogate is well known, methods for quantitatively describing surrogate fit are limited. A study by Aalizadeh (Aalizadeh et al., 2021) did use common substructural elements (Cao et al., 2008; Bajusz et al., 2015) to quantitatively define surrogate similarity and domain of applicability for surrogate usage with great success, as well as a mechanism for adjusting to future instrumental conditions, but only provided post hoc accuracy estimates of their fit, rather than a model for predicting estimate uncertainty. In fact, all the remaining referenced works provided only retrospective accuracy determination for tested compounds and lack a framework to bound future quantitative estimates or select appropriate surrogates beyond intuition. Crucially, the future utility of quantitative chemical estimates is intrinsically linked to the bounded accuracy of that

estimate. Thus, there is a need to develop predictive models of uncertainty to accompany methods for quantitative estimation.

3.3. Response modeling from chemical structure

Since the process of selecting appropriate surrogate calibrators for small molecules contains fundamental challenges, some researchers have opted to model the ionization response of compounds based on their molecular structure and chemical properties. This approach bases quantitative predictions on the physicochemical rationale for observed responses using models containing multiple factors that can influence compound response.

Mass spectrometry relies on the ionization of chemicals for analysis and is therefore inherently confounded by signal suppression due to matrix effects, (Taylor, 2005) as well as instrument-specific parameters that can influence the response factor of any given analyte. Selection of ionization type (e.g. electrospray [ESI], chemical ionization [CI], electron ionization [EI]) affects the end results due to interactions with physicochemical properties; chemical parameters such as hydrophobicity (logP), (Null et al., 2003; Henriksen et al., 2005; Cech and Enke, 2000) polar-/non-polar surface area, (Cech and Enke, 2000; Walker et al., 2011; Golubovi et al., 2016) molecular weight, (Mehta et al., 2016; Cox et al., 2003) and both liquid and gas-phase acidity (Sunner et al., 1988; Ehrmann et al., 2008; Richter and Schwarz, 1978) are known to contribute to ionization potential, along with structural parameters like carbon number and ionization cross section (Bergmann et al., 2018; Kim et al., 2014; Szulejko et al., 2013). Solvent effects from buffer pH, concentration of organic solvent, (Cech et al., 2001; Liigand et al., 2014) temperature, (Page et al., 2007) and even flow rate (Smith et al., 2004) can yield variable responses for individual chemicals from experiment-to-experiment. We direct interested readers to a recent review article by Kruve (Kruve, 2020) for an in-depth discussion of the physicochemical and instrumental influences on ionization behaviors.

The wide range of properties that contribute to different chemical responses in MS experiments is daunting. Indeed, sensitivity differences of several orders-of-magnitude have been observed across even fairly similar compounds (Liigand et al., 2018; Kruve, 2020). Yet, it's encouraging that robust model-based quantitative approaches have seen widespread application in related fields (e.g., proteomics) that utilize comparable MS platforms (Bantscheff et al., 2007; Shuford et al., 2017). Models that predict compound response can incorporate disparate chemical and instrumental factors and, most importantly from a quantitative perspective, provide estimated errors that can bound the accuracy of any individual concentration estimate. Further, cross-platform compatibility can be encouraged by transferring predicted response factors between platforms using a panel of reference compounds (Panagopoulos Abrahamsson et al., 2020). A key consideration for ionization modeling approaches is the reliance chemical structure information. Any quantitative prediction model that relies on chemical structure is therefore limited in the domain of applicability by the chemicals used to construct it. Furthermore, quantitative predictions can only be as accurate as the preceding NTA identification. Assigning risk to an unknown based on uncertain identification can be fraught, but for scenarios where multiple potential structures exist, the hazard classification can be estimated for multiple structures based on

worst-case scenarios of classification, or weighted based on annotation likelihood, if such values are available.

Multiple models have been constructed that use physicochemical properties or chemical descriptors to predict chemical ionization efficiency often normalized to a reference standard (Liigand et al., 2018; Leito et al., 2008; Oss et al., 2010; Kruve et al., 2014; Chalcraft et al., 2009). Some challenges have been reported with predicting chemical ionization behavior related to specific ionization sources. Work by Chalcraft et al. reported an average ratio between measured and predicted response of 1.08 ± 0.75 for chemicals that ionize via positive mode ESI (Chalcraft et al., 2009). But the same study could not establish a model for compounds that exhibit multiple ionization states, either multiple charges or adducts, or that ionize in negative mode ESI; the authors speculated that multiple models would be needed for different chemical subclasses. Another study by Leito et al. developed a regression model based on ten compounds and reported an RSD consistency of 0.16 log units (~1.5 fold), (Leito et al., 2008) but later studies using larger chemical datasets have since yielded higher prediction errors due to the larger complexity of the chemical space modeled (Oss et al., 2010; Kruve et al., 2014). Other modeling efforts have yielded errors of 26% for sugar metabolites, (Ghosh and Jones, 2015) up to 13% for lipids, (Cífková et al., 2012) 37% for steroids, (Alymatiri et al., 2015) and as little as 1% for ion-paired oligonucleotides. (Basiri et al., 2017) Built-for-purpose models therefore seem to exhibit lower prediction error than more general models due to the narrower chemical space. However, an exhaustive recent modeling study by Liigand et al. considered both positive and negative ionization mode, as well as a hundred LC conditions and several hundred chemicals in multiple chemical classes to train a random forest, machine learning model to obtain average prediction errors of approximately 2-fold (Liigand et al., 2020). This implies that a general ionization response model for an MS source is hindered by only lack of appropriate dataset(s) to capture the wide range of chemical space, rather than an incompatibility of the approach with quantitative prediction. Importantly, we note these prediction errors are considerably smaller than those observed with computational exposure modeling, and on par with those experienced by *in vitro* extrapolation efforts. Therefore, continued collection of ion efficiency data across large and diverse chemicals sets represents a path forward for qNTA.

4. Sample concentrations from quantitative predictions

Previously described approaches (section 3.2) for surrogate calibration can naturally incorporate experimental recovery by utilizing matrix-matched extracted calibration curves. This relies on the assumption that chemical surrogates accurately mimic preparation losses of the chemicals of interest. With limited exceptions that used matrix-matched surrogate calibration, (Aalizadeh et al., 2021; Alygizakis et al., 2021) the quantitative research efforts described above have focused on estimating in-solution concentrations for compounds in prepared standards or extracts. For risk characterization, quantitative estimates must extend back to the original sample matrix, rather than relying on extract concentrations alone (Fig. 1). Most analyses of environmental or biological matrices require some amount of sample preparation (including extraction and/or concentration steps) prior to MS analysis. While NTA experiments strive to minimize bias, sample preparation procedures can strongly

affect recoveries for individual compounds. Thus, the estimated concentration in a prepared extract does not directly translate to the environmental concentration. A naive baseline assumption is that the preparation procedure is well suited for the analyte(s) of interest, and that all chemicals are transferred into the final extract (i.e., recovery = 100%). In practice, adjustment is needed to estimate concentrations in both targeted and qNTA methods.

Solid phase extraction (SPE) is a common preparatory technique for concentration of dilute environmental samples, and there are hundreds of SPE methods, such as the dispersive SPE QuEChERS approach (Perestrelo et al., 2019). Extracts can also be prepared using direct solvent extraction, for example multi-solvent extraction of soil (Fisher et al., 1997) or small molecule solvent extractions from biological samples (Vuckovic et al., 2020). Recovery of chemicals of interest is dependent on the same chemical factors that influence MS response (e.g. pH, hydrophobicity, pKa) along with the choice of extraction solvents and SPE phases. Comparing common preparation techniques reveals that optimized methods can achieve near 100% recovery, but naïve applications of the same methods can have yields as low as 10%. (Chambers et al., 2007) This sample preparation variability adds another layer of uncertainty to qNTA predictions. Most NTA studies that have calculated recoveries did so for benchmarking purposes (Parry and Young, 2016; Crimmins et al., 2014; McCord and Strynar, 2019) with only limited examples of correction for predicted concentrations. A notable exception is the previously mentioned work of Aalizadeh et al., who applied chemical similarity measures to predict matrix effects and analyte recovery for chemicals based on surrogates (Aalizadeh et al., 2021). Importantly, robust recovery estimates are only known for chemicals with available standards. Therefore, there is significant uncertainty in chemical recovery for unknowns which compounds with other sources of error when determining quantitative estimates (note that recovery has a theoretical upper bound of 100%, but no definable lower bound [e. g., 10%, 1%, 0.1%...]). It is possible to account for the interaction of chemical structure with matrix, sample preparation, and analysis conditions using recovery surrogates. However, recovery surrogates from a lack of quantitative measures for surrogate suitability, just as was discussed for surrogate calibration (Aalizadeh et al., 2021; Alygizakis et al., 2021). Surrogate and purely computational models of recovery estimation will require expansive training sets to predict the recoveries for arbitrary chemicals and conditions. The development of these models is a final barrier to generating true concentrations needed to carry out a risk characterization from qNTA experiments.

5. Risk characterization as part of an NTA workflow

There are three anticipated scenarios when identifying chemical substances in qNTA studies, differentiated by the amount of existing guidance information available. In the simplest scenario, direct comparison can be made between estimated concentrations and an existing guidance or reference level, such as a drinking water maximum contaminant level. While straightforward, this scenario is seldom expected given: 1) the comparative paucity of chemicals with source-specific regulatory guidance/reference levels; and 2) the primary focus of NTA studies being data-poor and/or novel compounds.

In a second scenario (Fig. 5), the identified chemical would not have fixed source-specific guidance/reference levels, but instead have only provisional bioactivity or toxicity data and provisional risk thresholds. EPA's Toxicity Forecaster (ToxCast) project, for example, has generated *in vitro* bioactivity data for thousands of chemicals across hundreds of assays (Thomas et al., 2018). Using this readily available data, a lower-bound bioactivity threshold (e.g., 5th percentile) could be selected from a distribution of all existing potency measures (e.g., AC₅₀ values, which are concentrations associated with half-maximal assay activity) (Richard et al., 2016) to represent a surrogate POD in the absence of traditional *in vivo* dose–response data (Paul Friedman et al., 2020). Linking these measures to qNTA estimates would require several modeling efforts. First, toxicokinetic modeling (for example, using EPA's HTKK modeling platform) (Pearce et al., 2017) would be needed for *in vitro* to *in vivo* extrapolation, yielding a plausible distribution of dose-equivalent values that correspond to the lower-bound potency measure. This would answer the question of what dose levels (e.g., µg/kg/day) are expected to produce steady-state blood levels that are consistent with *in vitro* assay concentrations. Next, source-specific exposure modeling (e.g. EPA's SHEDS-HT modeling platform (Isaacs et al., 2014), or numerous QSAR models from the NORMAN ecotox database) would be needed to determine what matrix concentrations (e.g., ng/L of drinking water) would be protective given the lower bound dose-equivalent value. Finally, the acceptable matrix concentrations and qNTA estimated concentrations could be compared to enable provisional risk evaluation for particular single source or aggregate exposure (s).

The third scenario pertains to identified chemicals for which there is no existing bioactivity or toxicity data – this is likely the most common scenario for chemicals identified in NTA experiments. Here, there are no existing hazard-based measures and, instead, researchers must rely on chemical read-across techniques (Cohen Hubal et al., 2010; Rovida et al., 2020) or *in silico* toxicity predictions (US-EPA, User's Guide for TEST (version 4.2)(Toxicity Estimation Software Tool): A Program to Estimate Toxicity from Molecular Structure. Washington (USA): US-EPA, 2016; Raies and Bajic, 2016). Given hazard-based metrics derived from read-across and predictive models, the risk-based interpretation of qNTA data can use computational results as provisional estimates for exposure and/or toxicity. It is expected that most estimated upper-bound concentrations based on NTA data will be smaller than risk-limiting lower-bound concentrations based on bioactivity/toxicity data. This would suggest that environmental levels of individual chemicals are too low to elicit an adverse biological response. If this circumstance holds true, many observed compounds could be considered “low-risk” despite potentially large uncertainties in concentration estimates. For example, the credible intake ranges of Ring et al. spanned up to eight orders of magnitude, but of ~ 500,000 chemicals modeled only ~ 2,000 were expected to have exposures exceeding 0.1 mg/kg/day (Ring et al., 2019). Quantitative NTA estimates discussed in Section 3 broadly possess errors of only one or two orders of magnitude, which is similar to the uncertainty of the toxicokinetic modeling. Substantial overlap between concentration estimates and effects-based thresholds would indicate a need for more robust predictions of the anticipated exposure levels and/or toxicity thresholds due to increased risk potential. In the short term, it will still be necessary to acquire or synthesize chemical standards and to apply targeted analytical methods for chemical substances with a high degree of

risk. However, this focused use of resources on higher risk chemicals is more feasible than attempting to perform rigorous quantitation on all compounds detected in an NTA experiment.

6. The future of quantitative NTA

Our review has highlighted the motivating need for higher-throughput chemical safety assessments in the modern chemical landscape and explored the existing data/methodology gaps between current NTA implementation and its incorporation into a rapid risk characterization workflow as a quantitative data source. Moving forward, we can foresee qNTA experiments that contribute to every step of the risk assessment process, providing chemical identities to support components of existing frameworks (e.g., hazard identification) and sample concentration estimates to facilitate next generation dose, exposure, and risk assessments. The goal is to enable seamless linkage between outputs of qNTA experiments and computational toxicology studies that will yield rapid risk categorizations (e.g., high, medium, low) for emerging chemicals.

Current NTA approaches rely on data processing pipelines to yield large chemical feature lists and putative structures. Future developments should retain and improve the automated nature of data analysis to allow high-throughput processing while improving the accuracy of compound identifications and subsequent characterization. Further, the adoption of one or more general qNTA techniques should provide for bounded estimates of concentrations, with continuous improvements to prediction accuracy and domain of applicability. Existing methods function well for specific chemical sub-classes but may have limited utility outside of their defined chemical domain. Ideal quantitative methods should therefore strive to extend to any arbitrary chemical structure or possess the ability to flag chemicals (or chemicals sub-classes) that are likely to be not well estimated. Finally, while there is clearly a desire to maximize the accuracy of any predictive model, it must be universally recognized that quantitative estimates will possess intrinsic error and future methods must bound this error to facilitate protective decision making.

Integration of qNTA methods with other HT chemical evaluation tools offers a means to radically advance the utility of NTA data. In addition to enabling detection of chemicals, qNTA will allow the immediate prioritization of emerging contaminants based on anticipated risk; importantly, this utility extends to unexamined chemicals not covered by traditional chemical registration efforts. Quantitative NTA will further provide a pathway for efficient hazard screening in rapid-response scenarios, such as chemical spills or natural disasters, where chemical exposures may be heterogenous in scale and complexity. Finally, qNTA will provide a means to rapidly communicate chemical-related risks to potentially affected populations in all manner of environmental and biological monitoring studies. Provided that existing challenges can be addressed, it is likely that qNTA will support rapid and defensible risk-based decisions for hundreds to thousands of understudied chemical stressors.

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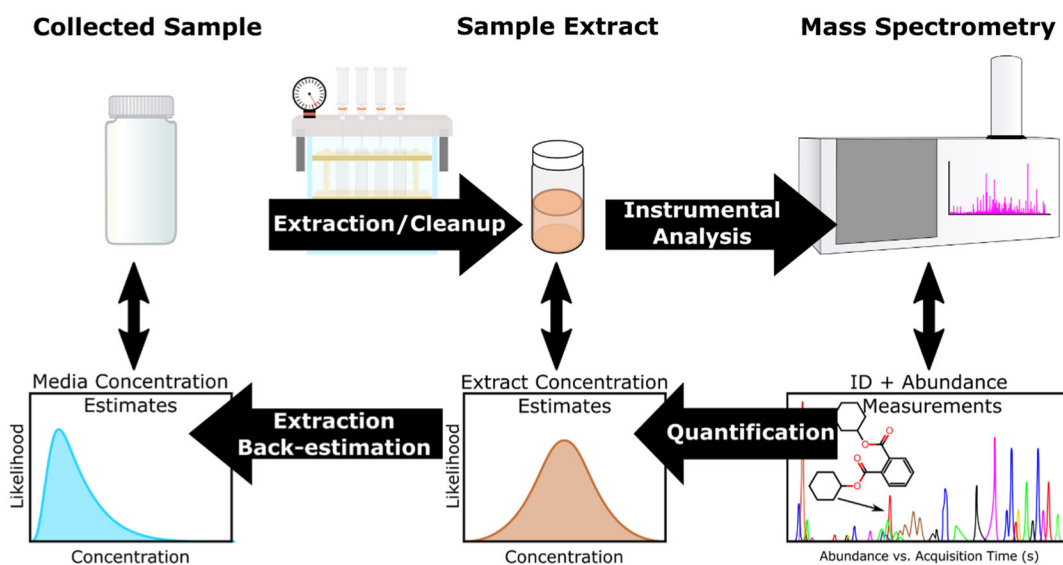


Fig. 1. Relationship between sample processing for MS analysis (top) and level of chemical information derived (bottom). NTA techniques primarily focus on the identification chemical species in MS analysis (bottom-right), but quantitative modeling approaches allow estimation of the likelihood of given concentrations in prepared sample extracts and parent samples (bottom-left).

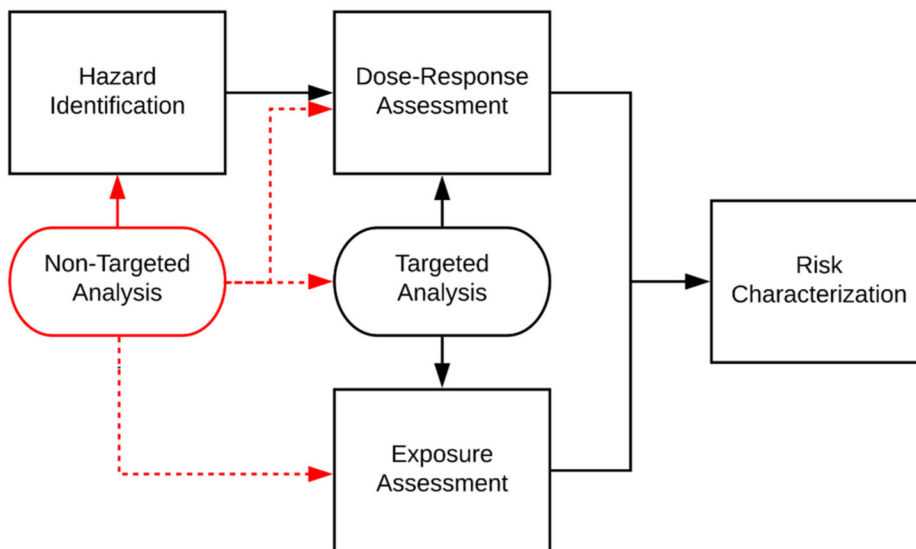


Fig. 2. Integration of targeted and non-targeted analysis data (ovals) into the traditional risk assessment paradigm (rectangles). Current non-targeted analysis provides primarily qualitative data for hazard identification (via compound discovery) and occurrence information (presence/absence) for exposure assessment; this influences the development of targeted analysis methods for follow-up quantitative examinations. Further connection of NTA to existing dose–response, exposure assessment, and risk-characterization tools, designed for compatibility with targeted analysis approaches, requires reformulation of NTA data to adhere to workflows designed for quantitative inputs.

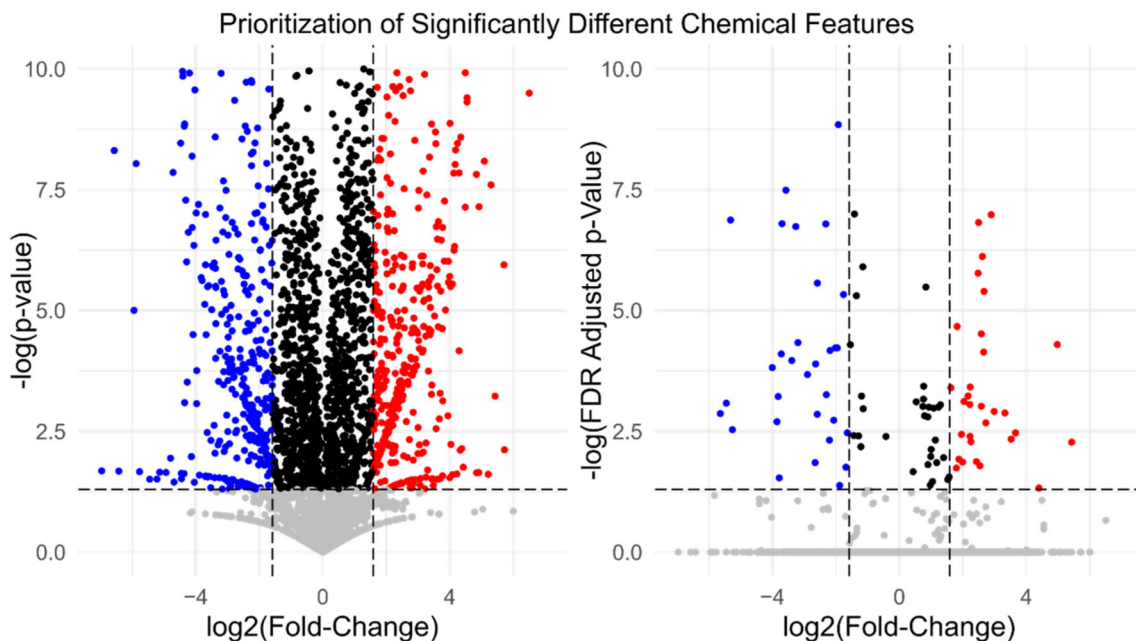


Fig. 3. Prioritizing chemical features by statistically significant fold change using volcano plots of individual chemical features compared between two study groups (data reprocessed from Rager et al.⁵⁶). The fold-change (FC) for each feature intensity (where $FC = \text{MeanIntensity}_{\text{Group1}} / \text{MeanIntensity}_{\text{Group2}}$) is plotted versus p-values for the unadjusted (left) and Benjamini–Hochberg corrected (right) group differences. Dashed lines show designated p-value (horizontal lines; $p = 0.05$) and FC (vertical lines; $FC = 3$) thresholds. Red chemical features are elevated (above designated thresholds) in study group 1 and blue chemical features are elevated in study group 2.

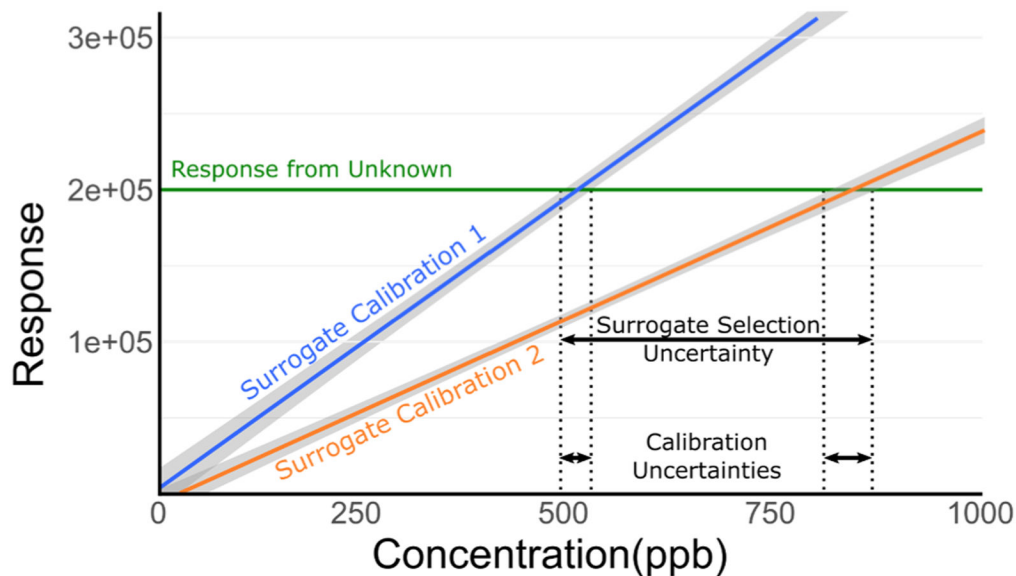


Fig. 4. Estimation of the concentration of an unknown compound from a measured intensity (horizontal green line) and multiple surrogate calibration curves (blue and orange lines) with 95% prediction bands (grey envelopes). In this theoretical example, the uncertainty incurred from surrogate selection (i.e., calibration curve 1 vs. 2) far outweighs the calibration estimate uncertainty associated with either individual curve.

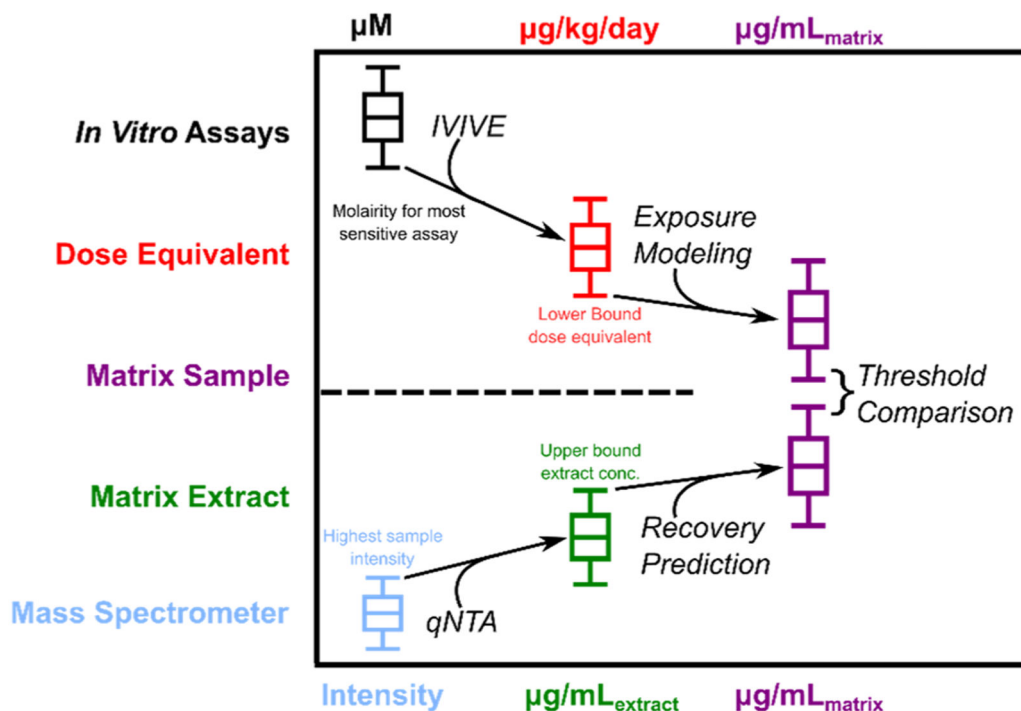


Fig. 5. Toxicity/bioactivity assay measures (e.g. ToxCast AC₅₀, top left) can be scaffolded to lower bound acceptable concentrations via *in vitro* to *in vivo* extrapolation (IVIVE) and exposure modeling. Chemical instrument response from NTA experiments (bottom left) can likewise be converted to protective upper-bound estimates via quantitative NTA modeling and estimation of sample recovery. Risk assessment decision making can then be based on the comparison between upper-bound estimated concentration and a lower-bound acceptable concentration. Even sizable estimation errors may be acceptable for risk-based prioritization if the degree of separation is large.