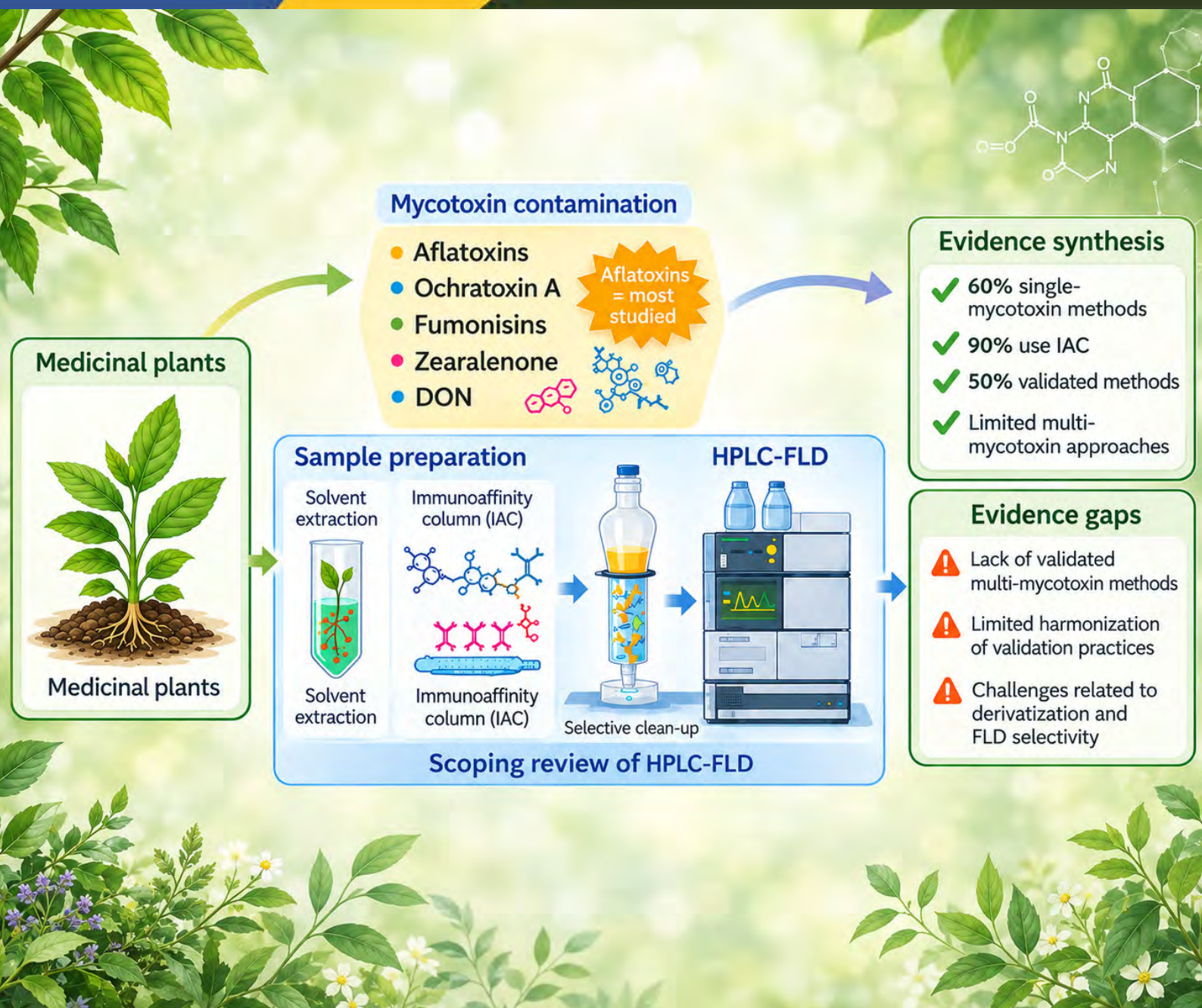
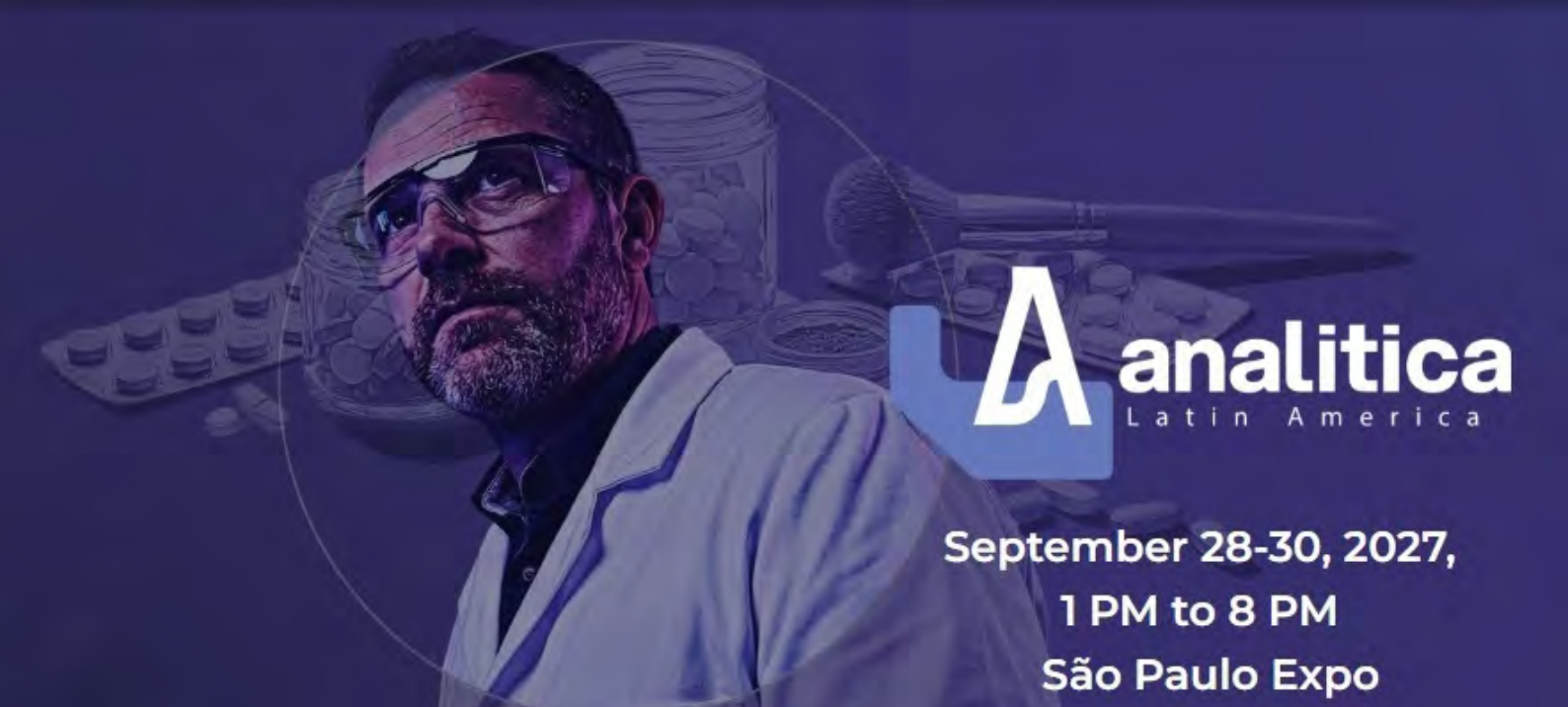




Cover illustration. Graphical abstract highlighting mycotoxin contamination in medicinal plants, sample preparation, immunoaffinity clean-up, and HPLC-FLD analysis. The graphical abstract and cover artwork were created with the assistance of ChatGPT (OpenAI), using its integrated image generation tool, and were reviewed and approved by the authors.

HPLC-Based Analytical Methods for the Determination of Mycotoxin Contaminants in Medicinal Plants: A Scoping Review

Marineli A. Gelinsk, Giovanna A. M. Trindade, Fabrício C. Fanhani,
Raul E. L. Lazo, Gustavo Bertol, Roberto Pontarolo, Luana M. Ferreira



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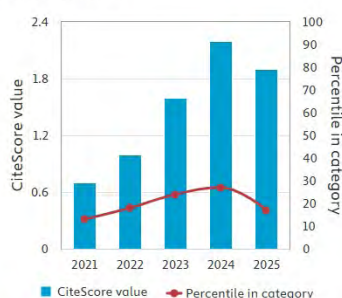
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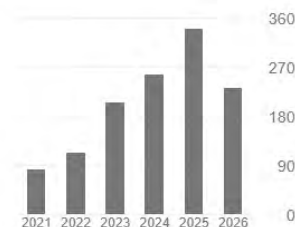
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EDITORIAL

Analytical Chemistry: Innovation, Reliability, and Impact

Pedro Vitoriano Oliveira  

Full Professor at the Department of Fundamental Chemistry, Institute of Chemistry
Universidade de São Paulo , São Paulo, SP, Brazil

Analytical chemistry plays a fundamental role in contemporary science by transforming chemical phenomena into reliable information that supports decision-making. Its contribution extends beyond the laboratory, reaching strategic areas such as healthcare, pharmaceutical industry, environmental science, agriculture, and as support for technological development. This edition of the Brazilian Journal of Analytical Chemistry brings studies that reflect the diversity of the field. The interview provides insights into the development of analytical chemistry and the challenges facing by the scientific research, while a point of view paper on the proteome and precision therapies highlights new possibilities at the interface of molecular analysis and personalized medicine. The reliability of analytical data is also a key focus of this issue. A metrological approach to performance assessment and measurement uncertainty in qualitative analysis is presented in the letter section. This concern is also evident in studies addressing the determination of mycotoxins in medicinal plants and the evaluation of extractables from packaging materials, both of which are directly related to safety and quality control. Innovation is demonstrated through the development of electrochemical sensors, the application of LC-MS for the determination of opioids in urine, and the integration of EDXRF and chemometrics for soil analysis. These studies illustrate how the combination of advanced technologies, instrumental strategies, and data analysis can lead to methods that are more efficient, selective, rapid, and potentially more sustainable. We hope that the work presented here will stimulate further research, foster connections across different areas of knowledge, and contribute to the continued advancement of the science of chemical measurement.

We hope you enjoy the contributions in this 53rd edition of BrJAC.



Pedro Vitoriano Oliveira is a Full Professor at the Institute of Chemistry, University of São Paulo (IQ-USP). His research is in Analytical Chemistry, with an emphasis on optical analytical methods (AAS, ICP-OES, ICP-MS), separation methods, sample preparation, and environmental chemistry, focusing on the development and application of methods for the determination of elemental species and chemical speciation in various materials for food, environmental, agrochemical, pharmaceutical, and technological purposes. He coordinates the Group of Analysis and Research in Spectrometry (GAPE) at IQ-USP. He has published more than one hundred articles in specialized journals and book chapters and has contributed to several research projects, coordinating others. He collaborates in various innovation activities and is currently the coordinator of the [EMBRAP II Unit – Synthetic Chemical and Biochemical Inputs – IQSint-USP](#).



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INTERVIEW



Professor Renato Zanella kindly granted an interview to BrJAC

Professor Renato Zanella earned his PhD in Analytical Chemistry from TU Dortmund Universität, Germany, in 1994, with postdoctoral training completed at the Free University of Amsterdam (the Netherlands). He served as an Adjunct Professor at the Universidade Federal de Santa Maria (UFSM) from 1994 to 2006, was an Associate Professor from 2007 to 2014, and has been a Full Professor since 2014.

He has coordinated the Pesticide Residue Analysis Laboratory (LARP) since 2001. The laboratory has been accredited by the General Coordination for Accreditation of the Instituto Nacional de Metrologia, Qualidade e Tecnologia (CGCRE/INMETRO) since 2012. He has also led the Research Center for Chromatography and Mass Spectrometry (CPCEM) since 2001, coordinating several research projects on residues and contaminants in food, environmental, biological, and industrial samples. He has held a research productivity fellowship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) since 2004 and currently holds a PQ 1B level.

He served as a member of the Chemistry Advisory Committee of the CNPq (2020–2023) and of the Superior Council of the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (2017–2022), serving as Vice President in 2021 and 2022. He served as Administrative Director (2020–2024) and has been President (2024–present) and a member of the Superior Council of the Fundação de Apoio à Tecnologia e Ciência (FATEC) (2020–present). He served as President of the Red de Análises de La Calidad Ambiental en América Latina (RACAL) (2013–2024) and has been a member of the Extended Executive Committee of the International Association of Environmental Analytical Chemistry (IAEAC) since 2013.

Professor Zanella served as Director (2006–2008) and Vice Director (2004–2006) of the Analytical Chemistry Division of the Sociedade Brasileira de Química (SBQ). He served as Coordinator of the Graduate Program in Chemistry (PPGQ) at UFSM and as a member of the University Council of UFSM (2008–2010). He also served as President of the Advisory Committee of the Pro-Rector of Graduate Studies and Research of UFSM (2009–2010).

He is Vice Coordinator of the Instituto Nacional de Ciência e Tecnologia (INCT) in Analytical Chemistry of Food: Food Safety and Characterization of Functional Foods (INCT-ALIM). He has been a member of the Scientific Committee of the Brazilian Society of Mass Spectrometry (BrMASS) since 2018.

He served as a member of the Coordination Center of the National Food Analysis Network (RENALI) of the Brazilian Technology System at the Ministério da Ciência, Tecnologia e Inovação. (SIBRATEC)/MCTI and as a member of the Scientific Council of the Residues and Contaminants Network of MCTI/MAPA. He is the author of more than 220 articles and 18 book chapters, with more than 5,200 citations and an h-index of 40 (Scopus). He has supervised 51 master's dissertations, 30 doctoral theses, 6 postdoctoral fellows, and 41 undergraduate research projects. Currently supervises 2 master's, 6 doctoral, 1 post-doctoral and 4 undergraduate research students.

Professor Zanella serves as Associate Editor of Food Analytical Methods and Scientia Chromatographica. He is a member of the Editorial Board of the International Journal of Environmental Analytical Chemistry (IJEAC) and Separations, and acts as an ad hoc reviewer for several scientific journals and the main Brazilian funding agencies. He coordinated the IX Latin American Symposium on Environmental and Sanitary Analytical Chemistry, in Salvador-BA (2011). He was a member of the Organizing Committee of the Latin American Congress on Chromatography and Related Techniques (COLACRO) in 2010 and 2012 and served on the Organizing Committee of the Latin American Symposium on Environmental Analytical Chemistry (LASEAC) in 2015 (Ecuador), 2017 (Colombia), 2018 (Chile), 2019 and 2024 (Brazil), and 2024 (Colombia).

He has delivered lectures at scientific events in Argentina, Chile, Colombia, Costa Rica, Ecuador, France, Germany, Greece, Italy, Mexico, Paraguay, Portugal, Spain, Uruguay, and the United States, as well as numerous events in Brazil. He has also taught courses in Argentina, Chile, Colombia, Uruguay, and Brazil. According to his latest CV the most frequent keywords associated with his work include pesticides, veterinary drugs, pharmaceuticals, water, soil, food, fruits, cereals, milk, meat, chromatography, GC-MS/MS, UHPLC-MS/MS, SPE, and QuEChERS.

BrJAC: How was your childhood?

Prof. Zanella: I grew up in the countryside of Encantado, in Rio Grande do Sul State, in a region of mountains and valleys. My childhood was truly very good. I had many adventures with total freedom and lots of friends. We knew all our neighbors and there were no safety concerns.

My family had a winery next to our house, and I enjoyed watching the entire winemaking process, from the arrival of the grapes to their storage in giant wooden vats. In winter, two distilleries were used to produce grappa from fermented grape pomace, and their fires were a great place to roast green corn and tell stories.

BrJAC: What early influences encouraged you to study chemistry? Did you have any influencers, such as a teacher?

Prof. Zanella: My curiosity about science in general, together with Professor Zanchettin's chemistry classes during the three years I lived in Dourados, Mato Grosso do Sul, played a decisive role in my decision to study chemistry. The coincidence of having a cousin, Jairo Pedrotti, studying Industrial Chemistry at UFSM was exactly what I needed to decide what and where to study.

He later completed his master's and doctoral degrees at the Universidade de São Paulo (USP) and currently works in São Paulo. He provided me with all the information and support I needed to start my undergraduate degree in chemistry.

BrJAC: How was the beginning of your career in chemistry?

Prof. Zanella: I graduated in Industrial Chemistry at UFSM at a time when there was a cooperation agreement between UFSM and the German Technical Cooperation Agency (GTZ), and we had classes with several German professors. When I finished my undergraduate degree, I began my master's degree in chemistry at UFSM. I developed a project related to the determination of metals in coal, ash, and environmental samples and became the second master's graduate in the program.

I applied to the German Academic Exchange Service (DAAD) and was selected for a scholarship to pursue a doctoral degree in Dortmund, Germany. My doctoral thesis, written in German, focused on the determination of organic compounds in air using chromatography. This international experience allowed me to be approved as a professor at UFSM.

BrJAC: What has changed in your profile, ambitions, and performance since the time you started your career?

Prof. Zanella: The experience I have accumulated, mainly in advising undergraduate and graduate students, as well as in extension activities, has guided my work in the pursuit of knowledge and producing results that meet the demands of society. I consider the training of highly qualified personnel to be my greatest motivation to continue working.

BrJAC: Could you comment briefly on the recent evolution of analytical chemistry, considering your contributions?

Prof. Zanella: Analytical chemistry, through the development of new methods of sample preparation and analysis, has contributed greatly to recent advances, especially in food quality control, broader clinical analyses, environmental control, and other fields.

In our research group, we have been working on the development of new methods of analysis, mainly of pesticides, aiming to assess the impacts on human health, animals, and the environment.

BrJAC: What are your lines of research? You have published many scientific papers. Would you highlight any?

Prof. Zanella: I work on the development of new methods for analyzing residues and organic contaminants, with an emphasis on pesticides, veterinary drugs, pharmaceuticals, polychlorinated biphenyls, and polyaromatic hydrocarbons, using liquid and gas chromatography coupled with mass spectrometry techniques. I have published more than 220 scientific articles in this field, with over 5,200 citations. However, although many projects developed in collaboration with public and private organizations have not resulted in scientific publications, I consider them to be just as important.

BrJAC: What is your opinion about the current progress of chemistry research in Brazil? What are the recent advances and challenges in scientific research in Brazil?

Prof. Zanella: We continue to train qualified professionals in the field of chemistry in Brazil. The challenge is to provide them with the conditions to work in the country in an adequate manner, since many have sought to work abroad, where they are valued. There are important initiatives to retain PhDs in companies, which are very important for the start of their professional careers.

BrJAC: For you, what have been the most important recent achievements in analytical chemistry research? What are the landmarks?

Prof. Zanella: The search for the development of analytical methods that meet the precepts of green chemistry has advanced significantly, and new low-cost materials and new procedures have enabled excellent results. Modern analytical instrumentation, developed with collaboration in the field, has enabled comprehensive, reliable analysis at levels appropriate to modern demands.

BrJAC: There are, in Brazil and in the world, several conferences on chemistry. To you, how important are these meetings to the chemistry scientific community? How do you see the development of national chemistry meetings in Brazil?

Prof. Zanella: I consider the participation of researchers and undergraduate and graduate students in scientific events to be of fundamental importance. These events provide opportunities to meet researchers from Brazil and abroad, learn about new topics, present and discuss the work developed, contributing to professional growth. Both general events in the field of analytical chemistry and more specialized meetings are recognized by the international community for their quality.

BrJAC: What is the importance of awards for the development of science and new technologies?

Prof. Zanella: Recognition of the careers of researchers who have made outstanding contributions to the progress of science and new technologies is highly relevant and serves as an incentive for those working in the field.

BrJAC: For you, what is the importance of the national funding agencies for the scientific development of Brazil?

Prof. Zanella: National funding agencies for scientific development play an important role in the development and consolidation of the country's progress. The fact that funding is not prioritized during certain periods hinders the continuity of research.

BrJAC: At the moment, the situation for scientific research in Brazil is one of decreasing investment. How do you see this situation, and what would you say to young researchers?

Prof. Zanella: Brazil has already experienced periods of severe investment shortages in this area. Currently, the country is experiencing a resumption of investment and a renewed appreciation for science. The work of researchers aimed at strengthening state and national funding agencies is of great importance. Raising awareness among young researchers about this importance is essential.

BrJAC: What advice would you give to a young scientist who wants to pursue a career in chemistry?

Prof. Zanella: I consider it a career that offers many possibilities for those who have a solid education and have specialized in their field of interest. It is a very broad field that allows you to work in very attractive areas and achieve professional fulfillment. Opportunities arise for those who are well prepared.

BrJAC: For what would you like to be remembered?

Prof. Zanella: I would like to be remembered for coordinating a research group that trained hundreds of professionals, providing enriching experiences in a well-structured laboratory, with many national and international collaborations and a strong presence in outreach work supporting public agencies, producers, companies, and the community at large.



Pesticide Residue Analysis Laboratory (LARP) team, 2025.

POINT OF VIEW

The Proteome Is the Prescription: From Static Stratification to Longitudinal Precision Therapeutics

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Precision medicine has invested two decades in genomic stratification, and that investment has been valuable: germline sequencing defines a landscape of biological potential, whereas serial tumor genotyping in oncology provides a partially dynamic record of clonal evolution. Yet stratification at diagnosis, or even periodic repeat genotyping, incompletely describes what a patient's cells are doing under a specific treatment at a particular time point. I argue that quantitative proteomics, enabled by advances in data-independent acquisition (DIA), dia-PASEF on trapped ion mobility instruments, and high-resolution Astral platforms, is positioned to become a dynamic functional layer within precision therapeutics, complementing rather than replacing genomics. I propose the Proteomic Pathway Response Index (PPRI), a patient-referenced metric summarizing serial proteomic change relative to a prespecified therapeutic target state and intended to report downstream pathway response rather than target occupancy or clinical benefit. The PPRI extends a simpler fixed-baseline pathway index that my colleagues and I previously built and piloted in bladder cancer, with molecular alerts that coincided with subsequent clinical assessments in six longitudinally monitored patients. I outline how high-frequency microsampling paired with DIA could support a proteomic N-of-1 framework for longitudinal treatment monitoring and, following prospective validation, contribute to dose adaptation or rational combination strategies. I also describe the analytical, clinical, regulatory, and implementation requirements that would need to be met before such measurements could inform prescribing. I close with a ten-year research agenda organized around testable milestones rather than a finished roadmap.

Keywords: quantitative proteomics, data-independent acquisition, dia-PASEF, precision medicine, prescriptomics, biomarker validation, sustainability by design

Genomics defines biological potential; it does not fully describe current therapeutic state

The genomic architecture of precision medicine is not wrong, in my view. Germline sequence defines a relatively stable landscape of biological potential: the protein variants a patient can produce, the metabolic enzymes whose function may be altered, and the pharmacogenomic variants that may influence drug disposition. In oncology, serial tumor genotyping and circulating tumor DNA extend this framework by tracking clonal evolution, copy-number alterations, and treatment-selected resistance mutations over time. The cancer genome is therefore not static in the way germline sequence is, and other molecular layers, including the epigenome and transcriptome, are also dynamic.

What genomic data, including serial genomic measurements, do not establish by themselves is the functional state of a pathway at a specific treatment time point. A resistance mutation detected in circulating tumor DNA indicates that a clone carrying that alteration has expanded; it does not tell us whether the

intended drug target remains detectable at the expected abundance, whether abundance changes consistent with bypass-pathway activation have emerged, or whether a dose modification made two weeks earlier has altered the relevant protein signature. These are different kinds of information, and I think proteomic measurements are particularly well positioned to contribute to them.

Quantitative proteomics is distinctive not because it is the only dynamic molecular modality. Transcriptomics, metabolomics, phosphoproteomics, epigenomics, and spatial omics can all report context-dependent biological change. Its particular value, I think, lies in measuring many of the molecular entities directly targeted by therapeutics while simultaneously capturing downstream alterations in cellular state that may lie mechanistically closer to treatment action than genomic variation alone.^{1,12} Conventional abundance proteomics does not provide a complete readout of pathway activity, which may also depend on phosphorylation, localization, protein complex formation, conformation, and other regulatory processes. Nevertheless, the combination of proximity to therapeutic targets and responsiveness to treatment is what I want to build the rest of this argument on.

A fundamental limitation must also be stated at the outset, because it does not disappear no matter how good the assay becomes: proteomics reports the molecular state of the sampled compartment, which may not correspond directly to the state of the disease-driving tissue. Plasma, urine, and dried-blood proteomes may reflect systemic inflammation, hepatic acute-phase responses, renal filtration and tubular handling, immune-cell composition, tissue injury, treatment toxicity, hydration, or vascular permeability. None of these is equivalent to measuring pathway activity within a solid tumor, a brain lesion, or another inaccessible site of disease.

Such specimens may nevertheless provide clinically valuable information at different biological levels. Some signals may reflect systemic treatment response, others may be organ-proximal, and only a subset may validly report processes occurring within the disease tissue itself. The relationship between a sampled biofluid and the relevant pathological compartment must therefore be established for each disease, specimen type, biomarker set, and intended use rather than assumed. A patient-referenced trajectory of the kind I propose below may reduce interindividual variability, but it does not, by itself, resolve the problem of tissue representativeness.

DDA and DIA: two acquisition strategies with different sampling properties

In data-dependent acquisition, the mass spectrometer selects the most abundant precursor ions in real time and fragments them one at a time. It is intuitive, well-validated, and remains the workhorse of discovery proteomics, but it is inherently stochastic. Which precursors get selected varies from run to run, particularly for lower-abundance species, producing the missing-value problem that has long complicated cross-sample comparisons and limited the quantitative completeness of conventional DDA in large, longitudinal clinical cohorts.

Data-independent acquisition inverts this logic: instead of selecting individual precursors, the instrument co-isolates and fragments populations of precursor ions within sequential or multiplexed m/z windows that collectively cover a predefined acquisition range. Recent head-to-head evaluations support what has become a working consensus: DIA generally provides substantially greater quantitative completeness and, in many workflows, improved sensitivity and quantitative precision relative to conventional DDA.² Quantitative completeness is the most secure of these advantages; precision differences are workflow-dependent.

A direct comparison of DDA and DIA on the same next-generation instrument found substantially greater peptide and protein coverage for DIA, with only moderate correlation between the two acquisition modes' quantitative outputs (~ 0.79), a reminder that depth and precision are related but distinct axes of comparison.⁷ This is a real and useful advance, but it should not be treated as a solved problem. Contemporary comparative studies and broader assessments of the field continue to identify acquisition strategy, preprocessing, spectral-library-based or library-free data-analysis workflows, false-discovery-rate control, normalization, and cross-study comparability as active sources of variability.^{2,7} DIA's advantage over DDA is well established within a single, well-controlled pipeline; whether that advantage survives intact

across different laboratories, platforms, specimen types, and multi-year longitudinal collection is a separate, and still open, empirical question.

dia-PASEF and Orbitrap Astral: converging capability gains, not a solved instrumentation problem

dia-PASEF, parallel accumulation–serial fragmentation combined with DIA on trapped ion mobility instruments, adds ion mobility as an additional gas-phase separation dimension and exploits the structured correlation between peptide m/z and mobility to coordinate precursor isolation with ion release from the trapped ion mobility device.¹ This coordination increases ion utilization by aligning quadrupole isolation with the mobility-dependent release of peptide precursors, and it is now being extended into demanding applications such as HLA immunopeptidomics, where it has been shown to improve peptidome coverage at lower sample amounts and enable quantification of less abundant peptide species, including neoantigens.³

That same underlying separation principle has continued to be engineered for greater sensitivity and throughput rather than superseded by a different one: higher-generation trapped ion mobility instruments have substantially increased the number of reproducibly quantified plasma precursors relative to earlier-generation ion-mobility instruments,¹⁴ and lower-input configurations of the same instrument family have pushed sensitivity toward sub-nanogram and single-cell inputs.¹⁵ In parallel, a second engineering trajectory, narrow-window DIA on Orbitrap platforms, and the Orbitrap Astral mass spectrometer, which pairs a quadrupole and Orbitrap analyzer with an asymmetric-track lossless analyzer for fast, high-resolution MS2 acquisition — has pursued the same goal of comprehensive, low-input proteome coverage through a different mass-analyzer design.^{4,5,6}

Reported depth figures in the range of ten thousand or more plasma proteins, or several thousand proteins from single cells, are frequently the product of depletion, immunoaffinity- or nanoparticle-based enrichment, increased sample input, or specific protein-inference and identification criteria.⁷ In optimized workflows, including depletion, enrichment, or other forms of plasma prefractionation, Astral- and ion-mobility-based DIA have produced markedly expanded proteome coverage, and that is genuinely useful technical progress. It should not be conflated with routine, direct, clinically validated analysis of neat plasma in a rapid workflow. Single-cell figures in particular need to distinguish proteins identified in at least one cell from median proteins per cell, from proteins quantified consistently across cells, and from carrier-assisted versus fully label-free approaches, distinctions the underlying literature is itself still working to standardize.¹⁵

Nor is it accurate to say instrumentation has stopped being a limiting factor. It is no longer the sole limiting factor. Plasma's enormous dynamic range, sample-preparation robustness, chromatographic stability and carryover, computational analysis, cross-platform harmonization, clinical interpretation, and regulatory validation are now, collectively, at least as consequential as instrument sensitivity for whether any of this becomes clinically deployable. Framed as engineering capability, ion mobility separation, ion-utilization efficiency, duty cycle, scan speed, quantitative completeness, and sample economy, rather than as a competition between named instrument families, the honest summary is this: two independently engineered platform lines are converging on the same target, which is a reasonable basis for optimism about where the technology is heading, and a poor basis for claiming the destination has already been reached.

From protein lists to a patient-referenced pathway metric: the Proteomic Pathway Response Index

Depth and reproducibility are necessary but not sufficient. Precision medicine requires translating a quantitative protein signature into an actionable therapeutic hypothesis, and the literature offers real, if still preliminary, evidence that proteomics can do this. Pathway-resolved quantitative proteomics of tumor cells under combination treatment has revealed proteome-level rewiring of apoptosis, metabolism, and antigen-presentation pathways not visible from genomic profiling alone.⁹ In pancreatic and neuroblastoma models, quantitative proteomic profiling of drug-treated cells has characterized synergistic combination effects at the pathway level, with functional assays confirming superior efficacy over monotherapy.^{9,10} Quantitative proteomics has been proposed as a predictive biomarker platform for antibody–drug conjugates, where target protein abundance and pathway context shape efficacy in ways genomic alterations alone do not

predict,⁸ and multi-omic integration of proteomic and transcriptomic data has been used to nominate drug-repurposing candidates matched to pathway-level disease signatures, candidates the original authors were explicit still require experimental and clinical validation.¹¹

This evidence establishes association and functional plausibility, not a route from measurement to prescription. Standard, global, abundance-based proteomics measures relative protein abundance; it does not, on its own, measure catalytic activity, receptor or target occupancy, phosphorylation state, proteolytic activation, conformational state, subcellular localization, complex formation, or flux through a pathway. An abundance-based pathway score cannot by itself demonstrate that a drug has engaged its target, and equivalence between protein abundance and pathway activity cannot be inferred from abundance measurements alone. A defensible framework has to keep at least five distinct quantities separate: target abundance; target engagement or molecular occupancy; proximal pharmacodynamic effect; downstream pathway response; and clinical response. These can, and often do, disagree with one another, and a useful proteomic metric has to say which of them it is actually reporting.

What quantitative proteomics may be particularly well positioned to report is the fourth of these, downstream pathway response, inferred from a prespecified multivariate pattern of patient-referenced abundance changes across selected pathway components, and that is worth formalizing, provided it is named and defined honestly. Contemporary DIA provides one promising measurement framework for this, given its quantitative completeness and retrospective data accessibility, though the underlying construct is not specific to any single acquisition method. I propose calling it the Proteomic Pathway Response Index (PPRI), deliberately avoiding the term ‘occupancy,’ which already has a specific pharmacological meaning, the fraction of a target bound by a ligand, that abundance-based proteomics does not measure and should not imply.

This is not an entirely untested idea. In earlier work, my colleagues and I introduced the differential Personal Pathway Index (dPPI), a patient-referenced, longitudinal pathway score computed from urine label-free quantitative proteomics in bladder cancer, built around seven hallmark-of-cancer pathways and referenced throughout to each patient’s own baseline sample.¹⁹ The underlying study compared urine proteomes from 30 patients, 13 with recurrence or progression and 17 without, and then followed six of these patients longitudinally, for as long as 62 months, computing a dPPI at each clinical time point. The resulting dPPI alerts were concordant with subsequently documented recurrence, progression, and, in one patient, a benign cystitis that did not require surgical intervention, at the evaluated time points across that six-patient series.¹⁹ We explicitly acknowledged that this was a small, non-independent longitudinal pilot, and that the approach needed replication in a larger population before any clinical claim could be made.¹⁹ What the study does establish is that a patient-referenced, fixed-baseline, pathway-level proteomic score is not merely a theoretical construct, it has already been built, and it has already produced clinically concordant alerts in a small, real longitudinal human series. PPRI extends that conceptual logic to drug- and combination-specific pathway modulation. Most importantly, PPRI retains the patient-specific pretreatment baseline while adding an independently defined therapeutic target state: a treatment-response index must ask not only how the patient has changed from where they started, but whether the observed trajectory is moving toward the state the intervention is intended to produce. PPRI and the proteomic N-of-1 framework described in the **Serial Microsampling and a Proteomic N-of-1 Framework** section are original proposals put forward in this article; they are conceptual starting points, not validated clinical or analytical endpoints, and require the validation sequence described in the **Validation Pathway this Would Actually Require** section before any clinical application.

Conceptually, for a given patient p , pathway, and treatment time t , PPRI compares the patient’s current pathway-level protein vector to two reference points: the patient’s own pretreatment baseline, and an independently defined therapeutic target state for that pathway and indication. These are not the same thing, since a pathway’s pretreatment state may represent severe, unwanted disease biology, and a drug may be intended to move the pathway toward a new state rather than simply back to where it started. Here, $\mathbf{p}_t$ denotes a prespecified normalized representation of the pathway proteins, for example, log-transformed

protein abundances expressed relative to a patient-specific pretreatment reference, rather than necessarily conventional population z-scores. One deliberately simplified candidate formulation is:

$$PPRI(p, t) = \frac{1 - d(z_{p,t} \mid z_{reference})}{[d(z_{p,0} \mid z_{reference}) + \epsilon]}$$

where $z_{p,0}$ is the patient's pretreatment pathway vector, $z_{p,t}$ is the vector at time t , $z_{reference}$ is the prespecified therapeutic target state, d is a weighted distance metric whose weights could incorporate analytical precision and biological relevance, and ϵ is a small regularization term. This expression is illustrative; it is not proposed as the final computational definition of PPRI. It exposes five requirements for method development. Denominator stability: $d(z_{p,0} \mid z_{reference})$ approaching zero makes an unregularized ratio unstable, so ϵ would need prespecification and sensitivity analysis rather than ad hoc insertion. Indication- and treatment-specific definition of the reference state: a single universal target is unlikely to exist, and the reference would instead need to be derived from mechanistic models, responding-cohort data, or experimentally defined perturbation states, each carrying its own causal assumptions. Preservation of directional and component-level information: a scalar distance can obscure which proteins are driving movement and in which direction, so directional pathway structure would require additional modeling, signed features, pathway topology, or a directional model, rather than weighting alone. Propagation of analytical and biological uncertainty: a clinically interpretable PPRI would need to be reported with a confidence or credible interval, not as a bare point estimate. And separation of temporal association from causal treatment effect: natural disease fluctuation, concomitant medication, cell-composition change, or toxicity could all produce an apparently favorable scalar movement, a distinction that matters especially for an N-of-1 framework with no concurrent control arm. None of this is solved; I am naming it as an open, tractable measurement-science problem, not presenting a finished instrument.

Serial Microsampling and a Proteomic N-of-1 Framework

The pilot study described in the ***From protein lists to a patient-referenced pathway metric: the Proteomic Pathway Response Index*** section supports the feasibility of using fixed-baseline, serial urine proteomes, collected during ordinary clinic visits, to produce alerts concordant with subsequent clinical assessment in a small, disease-surveillance context.¹⁹ It does not establish whether serial, patient-referenced proteomic monitoring can be extended to drug- or combination-specific titration decisions, to other specimen types, or from occasional clinic visits to something closer to routine, high-frequency collection, questions I want to leave open for the remainder of this section.

Everything argued so far still assumes proteomics happens the way it has for twenty years: a venous draw, a clinic visit, a batched run weeks later. Microsampling devices that collect dried plasma or blood from a fingerstick, designed for patient-centric, at-home, higher-frequency collection, have been validated against targeted proteomic panels.¹⁸ A 2025 study used frequent longitudinal dried-blood microsampling and a limited, targeted circulating-protein panel to track proteome dynamics in a mouse model of type 1 diabetes, identifying disease markers early enough, in principle, to support timely intervention.¹⁷ That is a genuine and useful proof of principle for the idea that frequent microsampling can capture longitudinal protein dynamics. It is not a demonstration that untargeted, high-depth DIA proteomics can deliver equivalent performance from serial human microsamples in routine clinical care, and it should not be read as one.

Clinical translation would also require control of microsampling-specific preanalytics, including hematocrit effects, collection volume and spot spreading, extraction recovery, drying and storage conditions, transport stability, hemolysis, and differences between capillary and venous blood composition, as well as temporal effects of food intake, exercise, and circadian rhythm. Higher sampling frequency does not automatically produce better biological information if these sources of variance are comparable in magnitude to the treatment-associated signal PPRI is meant to detect.

What I consider defensible is the following extrapolation, clearly labeled as an extrapolation: pairing a validated microsampling device with a high-sensitivity DIA workflow could, in principle, substantially reduce the sample-amount and access barriers that have kept proteomics confined to occasional, clinic-anchored venous draws. I will call the resulting concept, once, as a metaphor, not a technical description, a proteomic Holter monitor: not continuous sensing, but a structured cadence of low-volume, patient-collected microsamples, perhaps weekly or after each treatment cycle, feeding a DIA pipeline that returns a PPRI trajectory instead of a single snapshot. High-frequency longitudinal proteomic monitoring, or serial proteomic monitoring, is the accurate description of what this would be, and I use that description for the remainder of this argument. Continuous glucose monitoring changed diabetes management by shifting decisions from quarterly HbA1c checks to daily titration; serial, patient-referenced proteomic monitoring could plausibly do something analogous for combination oncology and immunology regimens, but the comparison is aspirational, not a claim that the enabling technology is already in place.

If such a system were analytically and clinically validated, the rationale is straightforward on at least three fronts: pharmacodynamic monitoring, assessing whether treatment is producing the expected proximal or downstream molecular response rather than merely achieving adequate systemic exposure; early resistance detection, where proteomic signatures of pathway reactivation or bypass signaling might precede radiographic or genomic evidence of relapse; and toxicity monitoring, tracking off-target proteome perturbation that correlates with adverse events before they become clinically manifest. None of these applications is conceptually new. What would be new is the measurement stability to make them routinely reliable, and that stability, as argued in the ***DDA and DIA: two acquisition strategies with different sampling properties*** section, is a real but not yet fully generalized advance.

The validation pathway this would actually require

None of the above becomes clinically usable through analytical performance alone, and it is worth spelling out the steps instead of gesturing at ‘standardization’ as a single undifferentiated task. Analytical validity comes first, and what must be harmonized is performance, reporting, and interpretation, not any single vendor’s software: harmonized specimen-handling procedures, reference materials, system-suitability criteria, acquisition controls, peptide-identification and protein-inference rules, normalization procedures, missing-data policies, model-locking procedures, reporting conventions, and external quality-assessment schemes, together with metrological traceability where achievable and demonstrated interlaboratory comparability using commutable reference materials, so that a PPRI computed on one pipeline means the same thing computed on another’s. ICH Q2 principles remain relevant to defined analytical procedures, but they do not by themselves address the full validation problem posed by an untargeted, multivariate proteomic pipeline coupled to a computational clinical score.

Clinical validity comes second: demonstrating that a PPRI trajectory actually correlates with a clinically meaningful state, response, resistance, or toxicity, across independent cohorts, specimen types, and treatment contexts, which is a substantially larger and slower undertaking than analytical harmonization. Clinical utility comes third, and is the highest bar: demonstrating, ideally in a prospective trial, that acting on a PPRI value changes a prescribing decision in a way that improves outcomes relative to not measuring it. Regulatory strategy and intended-use definition should nonetheless inform assay and software development from the outset, even though formal authorization, reimbursement, and laboratory-developed-test or in vitro diagnostic classification require the evidentiary package built across all three steps.

Because PPRI would be a multivariate computational output, not a single-analyte assay, validation would also have to separately address the underlying measurement itself, peptide identification and protein quantification, pathway membership and weighting, the locked score calculation, the clinical decision threshold, and the clinical interpretation, and would have to address feature locking, pathway-definition versioning, software updates, missing-feature handling, calibration drift, and the risk that changes in the analytical pipeline alter the score independently of any underlying biology.

Sustainability by design: from analytical efficiency to system-level value

Sustainability by design should accompany the validation pathway from its earliest stages, not be assessed only after a workflow has already been established. Analytical chemistry already has a mature vocabulary for this: green analytical chemistry codifies principles for reducing a method's environmental footprint, solvent and reagent consumption, waste generation, energy use, sample and reagent miniaturization, while white analytical chemistry evaluates analytical performance, environmental impact, and practical or economic feasibility as interconnected dimensions of method quality; greenness is not treated as a constraint that necessarily trades off against data quality.^{20,21} Although nano-flow chromatography limits mobile-phase consumption at the analytical column, the environmental footprint of large-scale proteomics also includes sample-preparation reagents, disposable plastics, enrichment consumables, instrument and gas-generator electricity, laboratory climate control, computational processing, data storage, and failed or repeated analyses. Solvent volume alone therefore provides an incomplete measure of analytical burden. This broader accounting also changes how chromatographic format should be evaluated. Nano-flow separations remain particularly well suited to very low sample inputs, including single-cell and near-single-cell workflows, where sensitivity is often the dominant constraint. For routine plasma, urine, and tissue-digest analysis at clinical scale, capillary- and microflow configurations may offer a more favourable compromise among sensitivity, chromatographic robustness, throughput, and operational complexity, although the optimal format remains dependent on sample amount, instrument sensitivity, and analytical objectives.^{22,23} Susceptibility to emitter blockage, the effectiveness of carryover-control procedures, and the degree of specialist intervention required may favour higher-flow formats in some settings; these are practical characteristics of a given chromatographic setup, not universal consequences of flow rate alone.

Solvent consumption per injection is therefore an insufficient primary sustainability metric for this architecture. The more relevant quantity is resource and energy use per analytically valid result meeting a prespecified intended-use performance standard. This is a proposed evaluation principle, not an already standardized metric with an agreed numerator, denominator, or system boundary. Such a metric should account for failed injections, repeat analyses, column and consumable lifetime, instrument utilization, computational processing and data-storage requirements, operator time, maintenance burden, and failure-associated resource use. Used in isolation, however, it can be improved without any genuine gain in clinical value, simply by reducing analytical scope, sensitivity, or clinical information content below what the intended use requires. Conversely, a narrower assay may represent a genuine efficiency gain if it preserves the analytical performance and clinical information required for the intended use. The metric is therefore only meaningful when interpreted alongside analytical scope, quantitative performance, and clinical information content. On this basis, a robust higher-flow method could plausibly outperform a more sensitive but less operationally stable nano-flow workflow, depending on the specimen, instrument, workflow configuration, and intended use.

The architecture proposed in this paper therefore presents a plausible sustainability hypothesis, but that hypothesis should be measured, not asserted. Microsampling could replace some venous collections with microlitre-scale sampling, potentially reducing biohazard waste and the logistical burden of repeated collection, although this benefit has not been established through formal life-cycle comparison. High-frequency monitoring could multiply the number of proteomic measurements performed per patient relative to episodic testing, and that added analytical burden must be evaluated against the procedures, visits, or ineffective treatments the monitoring system demonstrably changes. If prospectively shown to add sufficient clinical value, serial proteomic monitoring might eventually alter the timing or intensity of confirmatory procedures. In the bladder-cancer context described in the ***From protein lists to a patient-referenced pathway metric: the Proteomic Pathway Response Index*** section, this could include cystoscopy; however, the pilot study did not test whether dPPI could replace or reduce cystoscopy, so any such benefit remains hypothetical.

Similarly, proteome-informed treatment selection might reduce pharmaceutical and administration-related resource use if it were shown to prevent ineffective treatment exposure. Whether such savings would outweigh the analytical workflow's own material and energy requirements is not an analytical-performance question

alone; it requires formal life-cycle and health-system assessment. Any such assessment would need an explicit system boundary, for example, the analytical assay, laboratory workflow, patient-care episode, or full treatment pathway, because conclusions may change substantially depending on what is included. The net environmental balance could be favourable, neutral, or unfavourable depending on monitoring frequency, analytical configuration, clinical effectiveness, and what the assay actually displaces.

Sustainability by design should be built into analytical development, validation, and implementation from the outset and interpreted alongside analytical scope, quantitative performance, and clinical information content, not judged by resource consumption in isolation. Green and white analytical chemistry can provide the framework for defining assay-level metrics; assessing the complete architecture requires a broader, system-level evaluation involving life-cycle assessment specialists, clinicians, health economists, and health-system researchers.

Prescriptomics as the multidisciplinary implementation framework

In 2022, together with Capelo, Lodeiro, Dhir, and Kravchenko-Balasha, I proposed prescriptomics as a framework for integrating multi-omics data specifically to guide drug selection, dosing, effectiveness, safety, and rational combination therapy, not merely to integrate omics layers for diagnostic classification.¹³ Prescriptomics provides an appropriate implementation framework for the ideas developed above because none of them is adequately addressed as a single-omics problem. A PPRI will often be more interpretable when considered alongside genomic, transcriptomic, pharmacological, pathological, and clinical information that helps establish which pathways are relevant for a particular patient and treatment. The validation pathway described in the ***Validation Pathway this Would Actually Require*** section is likewise inherently multidisciplinary.

Prescriptomics will require institutional structure if it is to move from a conceptual framework to clinical practice. Training programs should begin defining and evaluating formal competencies in prescriptomic interpretation, spanning clinical pharmacology, molecular pathology, analytical proteomics, bioinformatics, biostatistics, and evidence-based therapeutic decision-making. These competencies are proposed here; they are not already established, and whether prescriptomics ultimately becomes a recognized subspecialty should depend on demonstrated clinical need and utility.

Hospitals conducting serial or combination-therapy protocols could evaluate prescriptomic boards structured analogously to molecular tumor boards. Such boards would review a patient's longitudinal proteomic trajectory, pharmacological context, and relevant multi-omic evidence at treatment decision points, not only at diagnosis. Their implementation would require defined membership, evidentiary standards, reporting formats, procedures for communicating uncertainty, and clear clinical accountability. This proposal builds on an emerging international scientific community reflected in initiatives such as the Caparica Conference series on Prescriptomics and Precision Medicine, although community formation should not be confused with implementation of a validated clinical service.

Computational studies reviewed in Reference 16 suggest that integrated multi-omic models can improve drug-response prediction in some benchmark settings, although performance depends strongly on cohort construction, data-integration strategy, comparator selection, and external validation. More defensible near-term applications of peptide- or fragment-level DIA representations include feature representation, cross-run and cross-platform alignment, quality control, anomaly detection, and pathway-level response modeling. These are distinct tasks, and success in one does not imply success in the others. Algorithmic denoising must also be approached cautiously because a model may remove weak biological signal together with technical variation.

Missing-value recovery is particularly sensitive. An imputed value may appear biologically plausible while suppressing genuine measurement uncertainty and creating a false impression of quantitative continuity. Any model-generated value should therefore be reported as an estimate with explicitly propagated uncertainty, not treated as a direct measurement.

Evaluation of these models would require patient-level and site-level separation, prospective temporal validation, uncertainty calibration, and explicit testing against batch, instrument, and workflow confounding. Many omics machine-learning studies overestimate performance through non-independent validation in which samples, patients, batches, or centers leak across training and test sets. Raw DIA data also encode substantial technical domain structure arising from sample preparation, chromatography, acquisition settings, instrument platform, laboratory practice, and data-processing version. A model that has not been challenged against these sources of domain shift is not ready to inform prescribing, regardless of its performance on randomly partitioned data from a single site.

External validation alone would not be sufficient. Any model intended for clinical decision support would need to demonstrate incremental value beyond standard clinical variables, conventional biomarkers, and appropriately chosen simpler models. It would also require auditability, controlled versioning, performance-drift monitoring, uncertainty reporting, and defined human oversight.

A ten-year research agenda with testable milestones

In 2022, together with Capelo, Lodeiro, Dhir, and Kravchenko-Balasha, I proposed prescriptomics as a framework for integrating genomic, transcriptomic, proteomic, metabolomic, and epigenomic data specifically to guide drug selection, dosing, effectiveness, safety, and rational combination therapy, not merely to integrate omics layers for diagnostic classification.¹³ Prescriptomics provides an appropriate implementation framework for the ideas developed above because none of them is adequately addressed as a single-omics problem. A PPRI will often be more interpretable when considered alongside genomic, transcriptomic, pharmacological, pathological, and clinical information that helps establish which pathways are relevant for a particular patient and treatment. The validation pathway described in the ***Validation Pathway this Would Actually Require*** section is likewise inherently multidisciplinary.

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A ten-year research agenda with testable milestones

I want to end with specific, checkable commitments, not a general call for more research. Seven problems need to be addressed, each through a measurable milestone with prespecified success and failure criteria; the targets below are illustrative research targets, not validated thresholds.

Cross-platform transferability: Within the next several years, the field should be able to show that a PPRI, or an equivalent pathway-response score, computed independently on ion-mobility and Orbitrap/Astral platforms, achieves concordance above a prespecified, use-case-specific acceptance threshold defined before analysis. This should be tested not only on split aliquots of the same longitudinal sample set, which establish analytical comparability alone, but also through repeat sampling, preanalytical replication, and independent execution in separate laboratories, which together are needed to establish longitudinal measurement reliability and to partition platform, laboratory, preanalytical, and within-person biological variance. Failing to meet that threshold would be an informative result showing the index is not yet transferable, not a reason to relax the criterion afterward.

Analytical and clinical validation: Analytical performance specifications and a locked candidate pipeline should be established before definitive clinical validation begins, including prespecified limits for longitudinal precision, missingness, batch effects, calibration drift, carryover, and score stability, consistent with the analytical-validity-first sequence described in the ***Validation Pathway this Would Actually Require*** section, although earlier-phase clinical data may still be needed during development to define the score's features, weights, and thresholds ahead of that lock. Once locked, a prospective cohort should demonstrate that PPRI trajectories, computed under that harmonized pipeline, predict an independently assessed clinical endpoint, treatment response, resistance emergence, or toxicity, using prespecified effect measures, calibration analyses, confidence intervals, and external validation. The strongest version of this milestone is a prospective, temporal design in which the score measured at time t predicts an outcome occurring after t , evaluated with decision-curve or net-benefit analysis.

Biological specificity: A pathway score should reflect the intended therapeutic perturbation when that effect is expected to be observable in the sampled compartment, while remaining distinguishable from variation caused by inflammation, cell-composition shifts, circadian effects, renal or hepatic dysfunction, concomitant medication changes, acute intercurrent illness, and unrelated treatment toxicity. Without this, a technically reproducible index might still be clinically nonspecific.

Clinically actionable turnaround: Serial monitoring is only useful if results return fast enough to inform the next clinical decision. The acceptable turnaround threshold is not fixed; it must be defined in relation to

the specific decision being supported, potentially hours for selected urgent applications, days for resistance monitoring, or completion before the next treatment cycle for dose adjustment. A workflow that cannot report a PPRI value within the threshold appropriate to its intended use should be described as a research tool, not a monitoring platform, until it can.

Regulatory and reimbursement qualification: No such index should be described as guideline-adjacent until it has moved through the analytical-validity, clinical-validity, and clinical-utility sequence described in the **Validation Pathway this Would Actually Require** section. Regulatory qualification and reimbursement will require overlapping but distinct evidence packages: regulatory bodies focus on intended use, safety, analytical validity, software control, and clinical performance, while payers focus more on comparative benefit, cost-effectiveness, utilization, and budget impact. Regulatory engagement, intended-use definition, and payer evidence planning should nonetheless begin much earlier and run in parallel with that sequence, not be treated as a final step attempted only once clinical utility is established.

Institutional implementation: Prescriptive review structures should be piloted and prospectively evaluated at selected centers over the coming years, generating publishable operational data on whether the prescriptive review structure is feasible, reproducible, operationally sustainable, and capable of influencing decisions appropriately. A pilot that instead surfaces governance failures or logistical barriers would still constitute progress, since knowing why the structure does not work is as informative as knowing that it does.

Causal attribution and intervention testing: A PPRI trajectory that merely correlates with outcome is not sufficient grounds for a treatment decision. Once the score, threshold, treatment algorithm, and safety constraints have been prospectively locked, the field needs a trial or quasi-experimental design in which acting on the score, adjusting, escalating, or de-escalating treatment based on a PPRI threshold, is shown to change outcomes relative to standard care. A randomized biomarker-strategy trial is likely the strongest primary design for this purpose, since stepped-wedge alternatives remain vulnerable to secular changes in treatment and analytical performance over the study period. This milestone is what would convert PPRI from a predictive marker into an actionable clinical tool.

Above all, PPRI would need to add predictive or decision value beyond baseline clinical variables, genomics, and conventional biomarkers, not merely correlate with outcome. Absent that, a reviewer would be right to conclude the index is an elaborate summary of already measurable disease burden, not a new source of clinical information. I consider substantial progress across all seven milestones achievable within a decade, but achievable and already achieved are different claims. The proteome is not yet the prescription. Current analytical capability is sufficiently advanced to justify prospective testing of that role in narrowly defined clinical use cases, and the analytical chemistry community is particularly well positioned to lead the measurement and standardization work needed to determine whether that role can be realized.

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LETTER

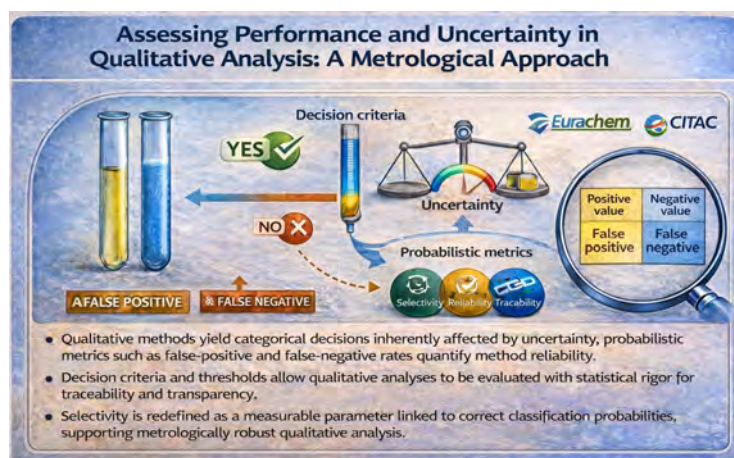
Assessment of Performance and Uncertainty in Qualitative Analytical Chemistry: A Metrological Approach based on the Eurachem/CITAC Guide

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While the aim of “Quantifying Uncertainty in Analytical Measurement” is to provide a standardized framework and methodology for estimating and expressing uncertainty in quantitative analytical measurements, the Eurachem/CITAC guide for qualitative analysis expands the concept of uncertainty to categorical decisions, allowing qualitative analyses to be evaluated with the same metrological and statistical rigor.

Published in 2021, the Eurachem/CITAC Guide on Assessment of Performance and Uncertainty in Qualitative Chemical Analysis is considered as a key methodological reference for assessing the reliability of qualitative

outcomes. Such qualitative tests yield categorical results, such as the presence or absence of a substance or the identification of a compound. Although such results do not yield a directly measured numerical value, they are not exempt from uncertainty. The guide proposes quantifying these uncertainties associated with the probability of false-positive or false-negative results to inform users of the analysis about the method’s reliability limits.¹

In this context, the document provides different methodological approaches that enable qualitative decisions to be treated with the same statistical rigor as quantitative analyses. It presents practical examples illustrating how these approaches can be implemented, recognizing that the outcome of a qualitative method, such as the identification of a compound or the confirmation of a substance’s presence, is subject to errors and uncertainties that must be systematically evaluated and communicated.²

Defining the types of criteria used in qualitative analysis involves distinguishing between quantitative criteria, which entail converting a numerical value into a category (e.g., compliant or non-compliant based

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on a threshold), and qualitative criteria, such as color change, visual observation, or other indications of presence or absence. Although the guide primarily focuses on binary nominal classification (e.g., yes or no, present or absent), it acknowledges that, in certain categorical cases, classification can be reduced to correct or incorrect to apply the same principles. By explicitly defining the decision criteria and the statistical or probabilistic thresholds that delineate the boundary between categories, the guide ensures metrological traceability and transparency in the interpretation of results.

As a central tool for characterizing the performance of qualitative methods, the guide introduces metrics based on false results. Laboratories are advised to collect samples with known or reference results, apply the method under evaluation to these cases, and estimate the frequency with which the process fails to correctly classify an item.

From these results, the proportions of true positives, true negatives, false positives, and false negatives are calculated. These proportions allow the estimation of error probabilities and the construction of confidence intervals, thereby expressing statistical uncertainty. Treated as direct measures of uncertainty, these error rates provide qualitative analyses with a quantitative dimension of reliability, which is important when analytical results affect technical, regulatory, or scientific decisions.^{3,4}

A crucial aspect mentioned by the guide is the representativeness and diversity of the cases used in performance estimation. If the test cases are too homogeneous or not representative of real conditions, the estimated error rates may underestimate or overestimate the actual uncertainty. Therefore, it is recommended to use test samples that cover the expected range of conditions, including different matrices, varying levels of interferents, and operational variations, to ensure the reliability estimate is robust.

This recommendation becomes especially relevant in methodologies based on spectroscopy and measurement instruments, where spectral variability and signal overlap among similar constituents pose practical challenges. By requiring sample sets that are representative of real analysis conditions, the guide promotes validation that reflects the natural variability of matrices and the presence of interferents, an essential element to realistically estimating the probability of incorrect classifications.⁵⁻⁷

In addition to representativeness, the number of samples used for performance estimation plays a critical role in the reliability and stability of validation results. Although no universally fixed minimum sample size can be defined since this number depends on the analytical objective, system complexity, and data variability, previous validation studies conducted under simple or simulated scenarios have shown that insufficient sample numbers can lead to unstable or overly optimistic estimates of error rates, particularly in qualitative and classification problems. As a general indication, validation studies require sample sizes on the order of several tens per class to achieve stable and convergent performance estimates, with larger sample sizes becoming necessary as model complexity increases.⁸

Therefore, representativeness and sample size should be considered jointly to ensure that estimated probabilities of incorrect classification realistically reflect the uncertainty associated with real analytical conditions.

When it comes to selectivity, the guide allows for a reinterpretation of this traditional concept. Instead of remaining a purely qualitative attribute, selectivity is viewed as a measurable parameter linked to the probability of correctly distinguishing between samples containing similar compounds or interferents. Thus, evaluating selectivity becomes an exercise in pattern recognition and probabilistic classification, where performance is expressed by the method's ability to assign each sample to its category correctly. This perspective broadens the understanding of selectivity, associating it with the statistical reliability of the response rather than solely the absence of visual or instrumental interference.⁹⁻¹³

The practical application of this methodology is especially relevant in non-targeted analyses, where the goal is to detect complex patterns and identify substances across diverse matrices without predefined targets. In such cases, the method must correctly differentiate signals corresponding to distinct compounds, even in the presence of instrumental noise and spectral overlap. Recent studies illustrate this by developing an automated approach for identifying microplastics using Raman spectroscopy, addressing the challenges of spectral variability and signal overlap among similar polymers. Instead of relying on an arbitrary similarity value, the method used a correlation distribution obtained via bootstrap sampling to determine the practical

acceptance threshold, aligning with the guide's recommendation to base qualitative decisions on probabilistic metrics and explicit performance assessments. This statistically controlled approach has been shown to significantly reduce classification errors, providing known confidence levels for each decision and bringing the analytical process closer to a metrologically traceable system where each decision is supported by uncertainty and performance estimates.¹⁴

When Raman spectroscopy is applied to quantify species in reactive mixtures, as in studies of urea and thiourea, adherence to the principles outlined in the guide is essential. Although the primary objective of such studies is to estimate concentrations from Raman signals quantitatively, a qualitative component remains inherent in spectral assignment, band identification, and the differentiation of interfering signals, steps that carry a risk of interpretative error. The guide emphasizes that any implicit qualitative decision, for instance, assigning a peak to a specific vibrational mode or determining whether a signal belongs to the analyte or to noise, is subject to uncertainty, and this uncertainty should be expressed in terms of error probabilities. In the urea/thiourea system, this involves evaluating the likelihood of misinterpretations—such as mistaking an interfering band for the analyte or overlooking weak peaks buried in noise—and designing validation experiments that account for varying compound ratios, noise levels, and instrumental conditions.¹⁵

Moreover, the guide recommends that qualitative conclusions, such as band assignments or confirmation of analyte presence, be accompanied by a confidence statement or an estimate of the local error probability derived from previously assessed spectral error rates, thereby enhancing transparency and discouraging absolute interpretations of spectroscopic signals. The systematic implementation of these guidelines also fosters a culture of continuous performance monitoring in laboratories. By acknowledging that even seemingly unambiguous spectral assignments may fail, researchers are encouraged to establish controls, retest protocols, and periodically review the criteria used to discriminate signals.^{16,17}

Beyond individual method performance, interlaboratory comparability of qualitative decisions is equally critical to ensure reproducibility across different analytical contexts. In this regard, the joint IUPAC/CITAC (2025) guide enhances metrological capacity by proposing a statistical framework specifically designed for analyzing agreement in categorical results obtained across laboratories, operators, or instruments. Tools such as CATANOVA (Categorical Analysis of Variance) and ORDANOVA (Ordinal Analysis of Variance) enable the treatment of nominal and ordinal variables, respectively, quantifying the degree of agreement among classifications and identifying sources of systematic variability between laboratories. This statistical evaluation is crucial for validating automated decision systems in qualitative methods. Consequently, it ensures that a method not only performs reliably within a single laboratory but also yields equivalent, traceable results across diverse analytical environments—an indispensable requirement for the international recognition of qualitative measurements.¹⁸

Furthermore, the consolidation of metrological approaches to qualitative methods has expanded across various domains, reflecting the need to transform descriptive judgments into traceable, comparable decisions. In environmental monitoring systems, the use of portable devices, assay kits, and continuous sensors highlights the importance of incorporating performance and uncertainty considerations into the interpretation of detection or non-detection results.¹⁹

In toxicological, forensic, and genetic sequencing contexts, harmonised procedures and the application of statistical metrics enable quantification of interlaboratory variability and reduction of classification errors.^{20,21} In the assessment of pharmaceutical equivalence and clinical diagnostic testing, risk models and sensitivity and specificity metrics reinforce the need to track the reliability of qualitative decisions.^{22,23}

Finally, studies involving bottom-up uncertainty estimation in complex matrices and interlaboratory comparisons demonstrate that selectivity and agreement among categorical results can be addressed with statistical rigor, thereby consolidating qualitative metrology as a structured discipline applicable across multiple analytical fields.²⁴⁻²⁶

The relevance of these principles and tools transcends specific domains, extending well beyond laboratory contexts. In sectors such as food and beverages, authentication and origin traceability depend strongly on spectroscopic and multivariate methods, in which trace elements and spectral profiles are used as markers

to distinguish regions or products. Despite the high performance of supervised classification models, the natural variability of elements and the overlap of compositional features can lead to false positives or negatives, which are precisely the central metrics discussed in the Eurachem and IUPAC/CITAC guides.

The explicit incorporation of performance and uncertainty measures could therefore enhance the validation of these methods, making results more comparable across laboratories and legally more defensible in cases of fraud or commercial dispute.²⁷

A comparison among these contexts generally reveals significant conceptual convergence, with all cases exhibiting a clear transition from descriptive approaches to quantitatively validated systems for qualitative decision-making. The central principle that emerges is that every qualitative decision is, in essence, a statistical inference and must therefore be accompanied by explicit measures of performance, uncertainty, and reproducibility. This perspective establishes a new paradigm for analytical chemistry, in which qualitative methods are no longer merely screening tools but rather integral components of the metrological domain, characterized by traceability, comparability, and transparency.

The scope of the guide is broad, reflecting a modern approach to the determination of neurotransmitters. The determination and correct interpretation of the limits of detection (LoD) and quantification (LoQ) are essential aspects for ensuring the reliability and sensitivity of the proposed analytical method. In the study, achieving extremely low LoD and LoQ values demonstrates not only the system's high instrumental performance but also its capability to detect and quantify analytes at trace levels, which is fundamental for biological and neurochemical applications.

Thus, defining LoD and LoQ within the context of this work not only confirms the procedure's sensitivity but also reinforces the method's metrological robustness and suitability for its analytical purpose, in accordance with internationally advocated principles of performance and uncertainty.²⁸

Finally, incorporating the recommendations from the guides promotes a culture of quality within laboratories: it does not blindly assume that a qualitative method is infallible. Instead, it acknowledges that it can and should be accompanied by reasonable estimates of reliability. This stance strengthens the credibility of results, supports analytical risk management, guides decisions on confirming or investigating borderline cases, and encourages continuous improvement of procedures, periodic method validation, and quality control strategies adapted to non-targeted analyses.

In this way, selectivity ceases to be merely a technical property and becomes a performance metric with direct implications for uncertainty assessment and the reliability of analytical decisions. This consolidation elevates qualitative metrology to the status of a mature scientific discipline, indispensable for ensuring confidence in analytical decisions at both local and international levels.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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REVIEW

HPLC-Based Analytical Methods for the Determination of Mycotoxin Contaminants in Medicinal Plants: A Scoping Review

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Mycotoxins are toxic substances produced by fungi and can occur in medicinal plants. Among them are aflatoxins, ochratoxins, fumonisins, zearalenone, and deoxynivalenol, which can be quantified by HPLC analysis. The objective of this scoping review was to evaluate HPLC-based analytical methods using fluorescence detection and diode-array detection (HPLC-FLD and HPLC-DAD) for the quantification of mycotoxins in medicinal plants. The study was conducted in accordance with the Joanna Briggs Institute

recommendations. 3,939 articles were identified, of which 30 were selected for review. Among the selected papers, 27.6% were published in China, where the use of medicinal plants is common due to Traditional Chinese Medicine. Of the 30 articles, 23 investigated aflatoxins, a group of mycotoxins with the highest health risk. Furthermore, 60% of the publications mention the development of an analytical method for only a single group of mycotoxins, highlighting the difficulty of developing multi-mycotoxin methods with the detector used. Of the articles included in the scoping review, 90% involve solvent extraction followed by immunoaffinity columns. Mobile phases consist mostly of a composition of water, methanol, and acetonitrile in varying proportions. Half of the articles reported performing analytical method validation, with the main parameters evaluated being limit of detection, limit of quantification, precision, and linearity.

Keywords: fluorescence detection, immunoaffinity columns, sample preparation, chromatographic analysis, method validation

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INTRODUCTION

Mycotoxins are toxic substances produced by certain fungi, mainly species of *Aspergillus*, *Penicillium*, and *Fusarium*.¹ In medicinal herbs, the most important mycotoxins are aflatoxins, ochratoxins, fumonisins, trichothecene (e.g. deoxynivalenol, HT-2 toxin, T-2 toxin), and zearalenone.¹ These substances are often found and are highly toxic. Aflatoxins, such as AFB1, AFB2, AFG1, and AFG2, are produced by certain *Aspergillus* strains and are among the most potent natural carcinogens and mutagens.² Ochratoxins, produced by *Aspergillus* and *Penicillium*, come in three types: A, B, and C, with ochratoxin A being the most common and toxic, causing cancer and weakening the immune system.² Fumonisins, mainly produced by *Fusarium verticillioides*, are considered potential human carcinogens, with fumonisin B1 being the most toxic.³ Zearalenone is a mycotoxin that acts like estrogen and is stable at high temperatures, showing fluorescence under UV light.⁴ Overall, aflatoxin B1, ochratoxin A, and fumonisins are considered the most hazardous to human and animal health, causing cancer, genetic mutations, liver damage, birth defects, and weakened immune systems.⁵

Herbal medicines include various plant parts such as flowers, fruits, leaves, seeds, stems, and roots, which can be used fresh or dried.⁶ However, these raw materials are highly vulnerable to fungal contamination during cultivation, harvesting, processing, and storage.⁷ Fungal growth not only reduces yield and quality but also leads to the accumulation of mycotoxins, posing serious risks to safety and therapeutic effectiveness. Therefore, effective measures for prevention, monitoring, and quantifying mycotoxins in medicinal plants are crucial for maintaining product quality and consumer safety.⁷

Liquid chromatography (LC) has become the analytical technique of choice for mycotoxin determination in complex matrices, due to its sensitivity and selectivity.⁸ Common detectors include diode array (LC-DAD), fluorescence (LC-FLD), and mass spectrometry (LC-MS/MS), the latter being the current gold standard. Although LC-MS/MS is currently considered the gold standard for mycotoxin determination due to its high sensitivity, selectivity, and suitability for multi-mycotoxin analysis, this technique was not included in the scope of the present review. The focus was intentionally restricted to HPLC-based methods using fluorescence detection and diode-array detection. This decision was based on the continued relevance of HPLC-FLD and HPLC-DAD in routine quality control laboratories, particularly because these techniques are generally less expensive, more widely available, and operationally simpler than LC-MS/MS.⁸ Therefore, this review does not aim to replace or compare mass spectrometry-based methods, but rather to map the evidence on the use of HPLC-FLD and HPLC-DAD for mycotoxin determination in medicinal plant matrices.

Critical to all LC-based methods is sample preparation, which involves extraction and clean-up to eliminate matrix interferences.⁸ Solid-phase extraction (SPE), especially with immunoaffinity columns (IAC), is the most frequently reported clean-up strategy due to its high selectivity and specificity.⁹ Because some mycotoxins, such as fumonisins, lack natural chromophores or intrinsic fluorescence, derivatization is required to enhance FLD detection.⁹ Strategies include pre-column derivatization with trifluoroacetic acid (TFA), post-column chemical derivatization with iodine or bromide, and post-column photochemical derivatization (PCD).^{10,11} Differences in fluorescence intensity between aflatoxins (B1/G1 versus B2/G2) can also be optimized through these approaches, improving the sensitivity and reliability of quantification.

The presence of mycotoxins in medicinal herbs poses a significant safety concern, and regulatory agencies and pharmacopeias recognize the need to control them in herbal materials. In Brazil, RDC No. 26/2014 establishes that, for the registration of herbal medicines or the notification of traditional herbal products, mycotoxin determination is required when contamination is reported for the species or cited in technical-scientific documentation.^{12,13} In addition, pharmacopeial requirements establish specific limits mainly for aflatoxins.¹³ The European Pharmacopeia sets limits for herbal drugs of not more than 2 µg/kg for aflatoxin B1 and 4 µg/kg for the sum of aflatoxins B1, B2, G1, and G2.¹⁴ The United States Pharmacopeia and the Brazilian Pharmacopeia establish limits of 5 µg/kg for aflatoxin B1 and not more than 20 µg/kg for the sum of aflatoxins.^{13,15}

These regulatory references highlight the need for analytical methods with adequate sensitivity and selectivity for complex plant matrices. Although established limits are predominantly focused on aflatoxins, other mycotoxins remain relevant due to possible co-occurrence and variations associated with geographical

and climatic conditions. In this context, this scoping review provides a comprehensive overview of HPLC-FLD and HPLC-DAD methodologies applied to mycotoxin determination in medicinal plants, highlighting their relevance as routine analytical tools for quantitative quality control.

Methodology

The study was conducted following the Joanna Briggs Institute (JBI) recommendations, and the results were reported according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses in a scoping review extension (PRISMA-ScR).^{16,17} The study protocol was registered in the OSF platform .

Research methodology

The search was conducted across PubMed, Scopus, and Web of Science databases using terms related to developing a method for quantitatively determining mycotoxins in herbal medicines. Each term was adapted to the selected databases using the Boolean operators “AND” and “OR” (Appendix 1). The research was conducted until August 2025.

Eligibility criteria

The eligibility criteria were based on the PCC (Product, Concept, and Context) framework, as recommended by the Joanna Briggs Institute for scoping reviews. This framework guided the formulation of the following research question: “Which HPLC-FLD and HPLC-DAD quantitative methods have been reported for the determination of mycotoxins (aflatoxin B1, B2, G1, G2, ochratoxin A, fumonisins B1 and B2, trichothecene, deoxynivalenol, and zearalenone) in medicinal plants?” In this review, the product considered was medicinal plants, including fresh or dried materials used in herbal medicines. The concept refers to quantitative analytical methods for determining mycotoxins — specifically aflatoxins, ochratoxin A, fumonisins, trichothecenes, deoxynivalenol, and zearalenone — using HPLC-based approaches with fluorescence or diode-array detection. The context comprised analytical studies focused on the safety and quality control of herbal drugs.

Specifically, we sought scientific articles describing quantitative analytical methods for these mycotoxins in medicinal plants. Articles published at any time frame and in Roman characters were included. Exclusion criteria comprised scientific articles published in non-Roman characters, review papers, studies involving foodstuffs, edible oils, or non-plant material, and studies employing non-quantitative or immunoassay-based methods. Studies using LC-MS/MS or other mass spectrometry techniques were excluded because they fall outside this review’s predefined scope. The focus was specifically on evaluating HPLC-based methods with fluorescence or diode-array detection, due to their suitability for routine quality control and labs lacking mass spectrometry equipment.

Study selection and data extraction

All references were added independently to the Rayyan web app (free version; Rayyan Systems, Inc.) based on the search results.¹⁴ In this platform, the duplicates were identified and excluded. After removing duplicates, each study was independently reviewed by the two authors of this study (MAG and LMF), who assessed the titles and abstracts for inclusion. Studies that met the eligibility criteria were also independently analyzed in full text by two reviewers (MAG and GAMT). The studies that show discrepancies in the reviewers’ decisions were resolved by consensus, and an additional reviewer was consulted if necessary. Unsuitable articles were excluded, and the identification and exclusion reasons were recorded in a table. The inclusion process was documented using an adapted PRISMA flow for scoping review (PRISMA ScR).

Data extraction was performed by the same reviewers who assessed study eligibility. The objective of this stage was to systematically collect all relevant information to address the aims of the present review.

The extracted data were organized in Microsoft Excel® and structured according to the following categories:

- **Publication details:** article title, authors, and year of publication;
- **Study details:** type of mycotoxin analyzed and the medicinal plant matrix or species investigated;
- **Methodological details:** HPLC analysis parameters, limits of detection (LOD), limits of quantification (LOQ), analytical range, and procedures for sample and standard preparation.

The extracted information was synthesized into summary tables and further illustrated through descriptive statistics, charts, and graphs to provide both qualitative and quantitative insights.

RESULTS AND DISCUSSION

After removing 1,264 duplicates, the remaining 2,675 articles were screened by title and abstract according to the predefined eligibility criteria described in the Methodology section. At this first screening stage, the inclusion and exclusion criteria were applied based on the information available in the title and abstract, considering whether the studies addressed quantitative HPLC-FLD or HPLC-DAD methods for the determination of mycotoxins in medicinal plants. Studies that clearly did not meet these criteria were excluded. As a result, 97 manuscripts were selected for full-text assessment.

During the full-text assessment stage, the same eligibility criteria were reapplied in greater detail to confirm whether each article met the scope of the review. After complete reading, 30 articles were included for further discussion, while 67 were excluded. Among the excluded full-text articles, 28 were excluded because they investigated matrices other than medicinal plants, and 39 were excluded because they employed analytical techniques or detection systems outside the scope of this review, namely, techniques other than HPLC-FLD or HPLC-DAD. The process of identification of the studies is depicted in Figure 1.

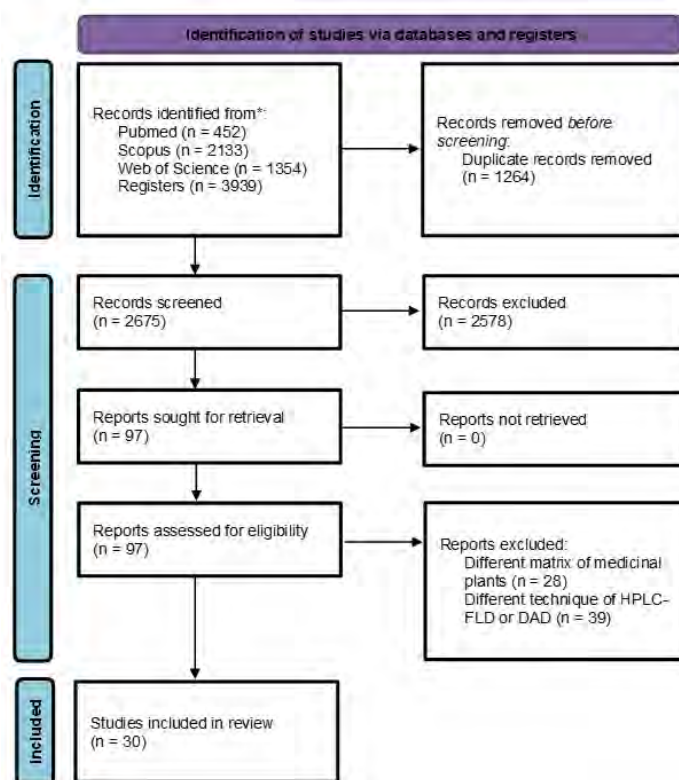


Figure 1. Flowchart of the selection process. Title and abstract screening were performed according to the predefined eligibility criteria described in the Methodology section. Full-text articles were subsequently assessed using the same criteria, and the specific reasons for exclusion were recorded.

The geographic distribution of studies shows a strong predominance of research originating in China (Figure 2A), accounting for nearly one-third of all included publications. This trend may be associated with the long-standing use of Traditional Chinese Medicine and the extensive scientific and regulatory interest in the quality control of traditional Chinese medicinal materials.¹⁹ A relevant contribution also came from European countries, such as Spain and Germany, as well as the United States, each of which contributed multiple studies to the field.

In contrast, fewer studies using HPLC-FLD or HPLC-DAD methods were identified from regions such as Brazil, Iran, and South Africa, despite their rich biodiversity and traditional use of medicinal plants. Within the scope of the present review, this imbalance suggests that fewer studies have reported these specific analytical approaches for the determination of mycotoxins in medicinal plants from these regions. Therefore, additional studies using validated HPLC-FLD and HPLC-DAD methods could help expand the evidence base for routine quality control, while studies employing other techniques, such as LC-MS/MS, may provide complementary information beyond the scope of this review.

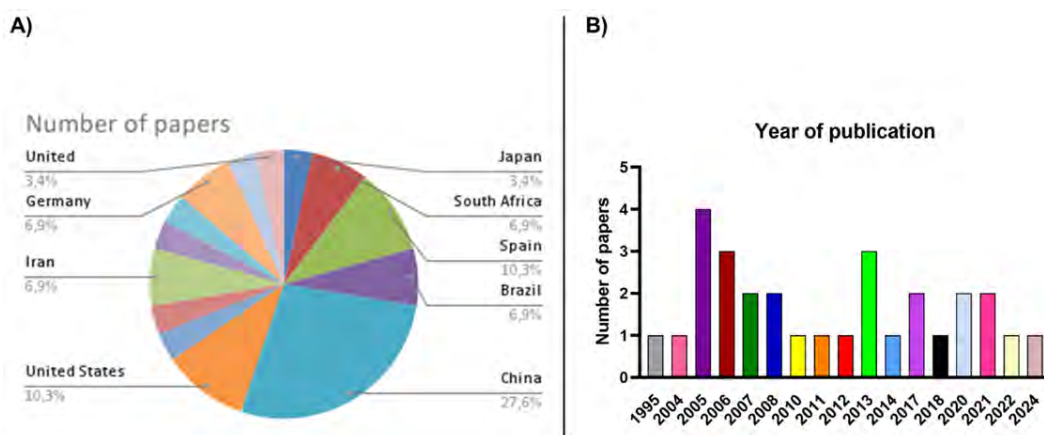


Figure 2. Distribution of the included studies by (A) country of origin and (B) year of publication.

The temporal distribution of publications reveals two distinct peaks of research activity (Figure 2B). The first peak occurred in the mid-2000s (2005–2007), coinciding with a period of increasing attention to mycotoxin control and the broader adoption of HPLC-based analytical methods. A second rise was observed in 2013–2014, which may reflect renewed methodological interest in improving analytical sensitivity and expanding the application of HPLC-FLD and HPLC-DAD methods to medicinal plant matrices.

Despite these peaks, the overall number of publications per year identified in this review remains low, indicating that studies employing HPLC-FLD and HPLC-DAD for mycotoxin determination in medicinal plants are still limited. After 2014, publications within this specific methodological scope became more sporadic, with only isolated contributions in the past five years. Therefore, although mycotoxin monitoring in medicinal plants remains analytically relevant, the evidence mapped in this review indicates that HPLC-FLD and HPLC-DAD approaches have not been consistently reported for this application, highlighting a specific methodological gap for future studies.

Mycotoxins and medicinal plants

Figure 3 shows the distribution of mycotoxins investigated in the studies included in this review. Aflatoxins clearly stand out as the most frequently analyzed group, followed by ochratoxin A and, to a lesser extent, zearalenone. This predominance reflects not only their widespread occurrence in herbal materials but also their well-established toxicological relevance, especially that of AFB1. These belong to Group I and are classified as potential carcinogens by the International Agency for Research on Cancer (2002). For health considerations, some countries and organizations have established reasonable regulatory limits.²⁰ According to the European Pharmacopoeia (2025), the limit for aflatoxin B1 in herbal drugs is not more than 2 µg/kg. A limit of 4 µg/kg is set for the sum of aflatoxins B1, B2, G1, and G2. The United States Pharmacopoeia (2025) has a limit of 5 µg/kg for aflatoxin B1 and a limit of not more than 20 µg/kg for the sum of aflatoxins.

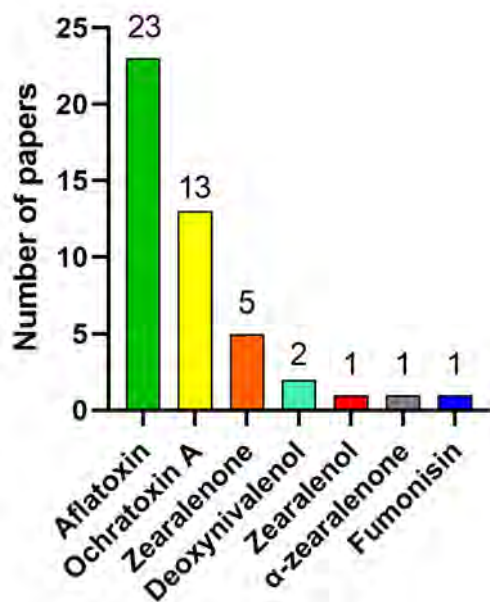


Figure 3. Number of studies reporting different groups of mycotoxins in medicinal plants.

The selected studies investigated between 1 and 4 mycotoxin groups; 60% (18 articles) focused on a single group, whereas 40% (12 articles) analyzed multiple mycotoxins. Table I summarizes the occurrence of different mycotoxins reported in medicinal plants, including contamination levels, plant species or matrix, and the specific plant parts analyzed when this information was available. It should be noted that 57% of the selected studies did not specify the plant part used, which limits a more detailed comparison among plant matrices.

Table I. Occurrence of mycotoxins in medicinal plants according to the included studies

Ref.	Mycotoxin	Contamination	Plant Species	Plant Part
21	AF	AFB1: 0.26 µg/kg AFB2: 0.07 µg/kg AFG1: 0.10 µg/kg	Medicinal plants	–
5	AF, OTA and ZEA	AF: 0.03 to 31.46 µg/kg OTA: 0.2 to 10.09 µg/kg ZEA: 0.1 to 23.35 µg/kg	Medicinal plants	Bark, root, leaves, stem, corm, bulbs, whole plants
22	OTA	1.4 to 252.8 ng/g	<i>Glycyrrhiza glabra</i> L.	Root
10	AF		<i>Monteverdia ilicifolia</i> (Mart. ex Reissek) Biral (sin. <i>Maytenus ilicifolia</i>)	
20	AF	0.26 a 27.52 µg/kg	Medicinal plants	
23	AF	16 ng/g	<i>Panax ginseng</i> C.A.Mey.	Root
24	AF	-	Medicinal plants	Aerial part and root
25	AF and OTA	-	<i>Cannabis sativa</i> L.	Flower
2	AF and OTA	AF: 205.1 to 4,704.94 ppb OTA: 0.11 to 0.53 ppb	Medicinal plants	–

(continued on next page)

Table I. Occurrence of mycotoxins in medicinal plants according to the included studies (continued)

Ref.	Mycotoxin	Contamination	Plant Species	Plant Part
26	AF	–	Medicinal plants	–
27	AF	0.2 to 57.5 µg/kg	Herbs and spices	–
4	ZEA	ZEA: 5.3 to 295.8 µg/kg	Medicinal plants	–
28	ZEA, ZOL, and DON	ZEA 0.52 to 14.51 µg/g ZOL 1.28 µg/g DON 5.60 to 14.45 µg/g	Medicinal plants	Rhizome and root tubers
29	OTA	Extracts: 26 to 141 µg/kg Root material: 8 to 52 µg/kg	<i>Glycyrrhiza glabra</i> L.	Root powder and extracts paste, and powder
30	AF	–	<i>Hibiscus sabdariffa</i> L.	Calyx and bract
31	AF and OTA	AF: 0.67–2.81 µg/kg OTA: 0.54-30.86 µg/kg	<i>Camellia sinensis</i> (L.) Kuntze / Black and green tea	–
32	AF, OTA, ZEA, and DON	AF, DON, and ZEA of 2.364 to 5.714 µg/kg	Medicinal plants	–
33	AFB1	< 1.0 µg/kg	Medicinal plants	–
34	OTA	2.82 – 208.70 µg/kg	<i>Glycyrrhiza glabra</i> L.	–
35	AF	B1: 1.780 ppb B2: 1.333 ppb G1: 1.444 ppb G2: 0.659 ppb	Medicinal plants	–
36	Fumonisin B1 and AFB1	AFB1: not contaminated Fumonisin B1: 8 to 1553 µg/kg	Medicinal plants	–
37	AF	1.7 to 14.3 ng/g	Medicinal plants	–
38	AF and OTA	AF: 0.25 to 16.0 µg/kg OTA: 0.25 to 8.0 µg/kg	<i>Panax ginseng</i> C.A.Mey. and <i>Zingiber officinale</i> Roscoe	–
39	AF and OTA	AF: 1 to 31 ng/g OTA: 1 to 10 ng/g	<i>Panax ginseng</i> C.A.Mey. and <i>Zingiber officinale</i> Roscoe	Root
11	AF and OTA	AF: 3.37 - 5.76 µg/kg OTA: 1.05 - 1.19 µg/kg	<i>Zingiber officinale</i> Roscoe	–
40	AF and OTA	1 to 10 µg/kg	<i>Cannabis sativa</i> L.	Leaves and flowers
41	AF	2.90 to 32.18 µg/kg	Medicinal plants	–
42	AF	6.75 µg/kg	Medicinal plants	Fructus, cortex, radix, and rhizome
43	ZEA	18.7 to 211.4 µg/kg	Medicinal plants	Mature seed

Notes: AF: Aflatoxins; DON: Deoxynivalenol; OTA: Ochratoxin A; ZEA: Zearalenone; ZOL: Zearalenol.

Among all the plants evaluated, contamination levels ranged from 0 to 4,704.94 µg/kg. The highest contamination level was reported by Haq et al. (2024)² for *Justicia adhatoda*. Furthermore, contamination rates varied among studies: Areo et al. (2020)⁵ reported that 86% of the plants examined were contaminated with aflatoxin B1; according to Trucksess et al. (2007),³⁹ 67% of the samples were contaminated; and Pakshir et al. (2020)³¹ reported that 40% of the samples contained aflatoxins. Of the medicinal plants evaluated, 74% of the samples tested positive for ochratoxin A, according to Trucksess et al. (2007).³⁹ The highest contamination level reported was 252.8 µg/kg by Ariño et al. (2007).²² The mycotoxin zearalenone was

detected in 55.62% of the plants surveyed by Zhang et al. (2011),⁴³ with the highest contamination level of 295.8 µg/kg reported by Kong et al. (2013).⁴ Based on the articles reviewed, fumonisins were detected in the tested samples at levels up to 1,553 µg/kg. Furthermore, deoxynivalenol was detected in 6.92% of the medicinal plants evaluated, with a maximum contamination level of 14.45 µg/g.

These findings highlight both the diversity of mycotoxins contaminating medicinal plants and the variability in their distribution across different plant parts. The frequently detected plant matrices include roots, leaves, flowers, bark, and whole plants. Roots and rhizomes are highly susceptible to fungal colonization due to their nutrient composition, which may explain the frequent detection of these toxins in such tissues.²⁸ Underground rhizomes and root tubers are susceptible to mycotoxin contamination during harvesting and storage. In these plant tissues, the production of these toxins can be activated by the high concentration of mineral salts and carbohydrates, including simple sugars.²⁸

The contamination of herbal drugs results from improper cultivation, harvesting, drying, and storage practices. The growth of fungi and the subsequent production of mycotoxins in medicinal plants are substantially affected by environmental factors. Elevated levels of humidity and temperature during both the cultivation and storage periods are particularly conducive to fungal proliferation and the resultant mycotoxin formation.²⁴

It is noteworthy that several studies did not provide quantitative levels of contamination, indicating variability in reporting practices. Nevertheless, the consistent detection of aflatoxins and ochratoxin A across different studies underscores their importance as major contaminants in herbal materials, reinforcing the need for reliable analytical methods for routine monitoring.

Extraction method

Plant samples, both solid and liquid, must undergo solvent extraction, followed by a clean-up step. In the reviewed articles, 10% reported using only Liquid Liquid Extraction (LLE), which employed immiscible liquid phases such as acetonitrile, iso-octane, and chloroform²⁸ or dichloromethane.⁵ In 90% of the articles reviewed, LLE and Solid Phase Extraction (SPE) were employed. The SPE typically involved an immunoaffinity column (IAC). This method is often chosen for aflatoxin analysis due to its operational simplicity and reduced solvent consumption.²⁰

Pre-concentration and sample cleanup are critical steps. They serve to remove matrix compounds and eliminate potential interference from co-extractives,²⁵ thereby ensuring the method's high specificity and efficiency.⁴² Immunoaffinity extraction is a highly selective cleanup method that uses columns packed with a gel matrix. This matrix has antibodies against mycotoxins immobilized on it. When a sample passes through the column, the mycotoxins bind to these antibodies, allowing for a very specific and effective separation process.¹⁰ According to Zhang et al. (2017),⁴² the use of IAC can result in low recoveries when applied to complex herbal matrices.

High acidity in an herbal extract can reduce the binding affinity of a column for aflatoxins, a problem reported by Ip and Che (2006).²⁶ However, this affinity can be improved through several sample preparation methods, including reducing the sample amount, diluting the sample, and adding additives such as PBS and Tween, as noted by Wen et al. (2013).¹¹

In conclusion, the extraction/clean-up strategies proposed above are intended to yield purified extracts with minimum matrix effects, thus facilitating the chromatographic analysis. The quality of the preparative step is a critical step, as it will have a direct effect on the results obtained in the analysis. In the above approach, the choice of chromatographic systems/conditions will be the next step in the approach, as discussed in the *Analytical method* section of this review.

Analytical method

Based on the articles reviewed and summarized in Table II, reversed-phase C18 columns were the most frequently employed stationary phases for mycotoxin determination in medicinal plants. This predominance is consistent with the physicochemical characteristics of several target mycotoxins, which generally present low to moderate polarity and can be retained under reversed-phase conditions. C18 columns provide hydrophobic interactions that allow the separation of structurally related compounds, such as aflatoxins

B1, B2, G1, and G2, while also being compatible with aqueous-organic mobile phases commonly used in routine HPLC-FLD and HPLC-DAD method.⁴⁴

The mobile phases reported in the reviewed studies were predominantly composed of water, methanol, and/or acetonitrile in different proportions.^{2,5,10,20,21,23,24,26,27,30,31,33,39,41,42,45} The selection of these organic solvents is not merely related to cost, but also to chromatographic selectivity, elution strength, viscosity, pressure, baseline stability, and detector compatibility.⁴⁶⁻⁴⁸ Acetonitrile generally provides stronger elution strength in reversed-phase chromatography, lower viscosity, and reduced system backpressure, which may favor sharper peaks and shorter analysis times.^{49,50} Methanol, in contrast, can provide different selectivity due to its protic character and hydrogen-bonding capacity, which may improve the resolution of some analytes depending on the stationary phase and mobile-phase composition.^{49,51} Therefore, the choice between methanol and acetonitrile should be understood as a chromatographic optimization variable rather than only as an economic consideration.

For aflatoxins, the most frequently reported mobile phases consisted of mixtures of water, methanol, and acetonitrile. This composition allows adequate retention and separation of aflatoxins with different fluorescence responses. In some methods, potassium bromide and nitric acid were added to the mobile phase to support post-column derivatization, improving the fluorescence response of aflatoxins B1 and G1.^{25,35,38,40} Such derivatization strategies are particularly relevant in HPLC-FLD methods because fluorescence intensity differs among aflatoxin analogs, and signal enhancement may be necessary to achieve adequate sensitivity at regulatory concentration levels.^{52,53}

Derivatization is therefore a critical analytical strategy in fluorescence-based mycotoxin determination.⁵³ Its main purpose is to enhance the fluorescence response, improve detectability at low concentration levels, and enable the quantification of analytes with weak or absent native fluorescence.⁵³ In the case of aflatoxins, post-column derivatization is commonly used to increase the fluorescence of AFB1 and AFG1, which generally show lower native fluorescence responses than AFB2 and AFG2.^{53,54} Chemical derivatization with bromide or iodine, as well as photochemical derivatization, can improve sensitivity and enable quantification at concentrations compatible with regulatory requirements.^{30,52-54}

For other mycotoxins, different chromatographic adjustments were required. In the case of ochratoxin A, acidic mobile phases containing acetic acid were commonly employed.^{11,32} This condition helps control the ionization of acidic functional groups, thereby improving retention, peak shape, and reproducibility.⁴⁴ Similarly, fumonisins, which contain multiple carboxylic groups and are more polar than aflatoxins, require specific chromatographic conditions and, frequently, derivatization to enable fluorescence detection.³⁶ For fumonisins, pre-column derivatization is usually required because these compounds do not present adequate native fluorescence. This approach allows the formation of fluorescent derivatives suitable for HPLC-FLD analysis, but it introduces additional analytical complexity, since reaction time, pH, reagent stability, and derivative stability may affect precision, accuracy, and robustness.

Although derivatization can substantially improve the sensitivity of HPLC-FLD methods, it also has limitations.^{55,56} Pre-column derivatization may be affected by incomplete reaction, derivative instability, matrix interference, and increased sample preparation time.^{55,57} Post-column derivatization reduces some sample-handling limitations but requires additional instrumentation, reagent control, and optimization of reaction conditions.^{57,58} Photochemical derivatization avoids the continuous use of chemical reagents but still requires specific equipment and validation of irradiation conditions.⁵⁶ Therefore, derivatization should be considered a critical method variable and should be adequately optimized and validated, especially when applied to complex medicinal plant matrices.^{56,57}

Zearalenone, due to its intrinsic fluorescence and more hydrophobic character, can be separated using aqueous-organic reversed-phase systems, although matrix interferences may still affect selectivity and quantification.^{4,5,28,32,43}

Table II. Summary of HPLC-based methods for the determination of mycotoxins in medicinal plants

Ref.	Mycotoxin	Column	Detector	Mobile Phase	Sample preparation	LOD	LOQ	Validation*	Parameters	Key Results
21	AF	Capcell Pak C18 UG120 and Shim-pack CLC-ODS	FLD	Water, methanol, and acetonitrile (70:20:10, v/v)	LE/SPE	0,01 ug/kg	–	0	–	Contamination is relatively low.
5	AF, OTA, and ZEA	Nova-Pak C18 Waters Symmetry C18	FLD	AFs: Water, methanol, and acetonitrile (60:20:20, v/v) OTA: Acetonitrile, water, and acetic acid (50:48:2, v/v) ZEA: Acetonitrile, water, and methanol (46:46:8, v/v)	LLE	AFB1: 0,03 µg/kg AFB2: 0,01 µg/kg AFG1: 0,05 µg/kg AFG2: 0,02 µg/kg OTA: 0,05 µg/kg ZEA: 0,03 µg/kg	AFB1: 0,1 µg/kg AFB2: 0,03 µg/kg AFG1: 0,2 µg/kg AFG2: 0,1 µg/kg OTA: 0,2 µg/kg ZEA: 0,1 µg/kg	1	Recovery, LOD, and LOQ	Of the 36 samples analyzed, 86% were contaminated with AF. OTA occurred in 61% of samples. 39% ($n=36$) of the samples contained ZEA.
22	OTA	Ace 5 C18 250 x 4.6 mm	FLD	Water, acetonitrile, and acetic acid (51:48:1, v/v)	LE/SPE	–	0.5 ng/g	0	–	The concentration was unaffected by sorting or washing, but it was much reduced by peeling (a 53% reduction).
22	OTA	Kromasil RP C18	FLD	Water, acetonitrile, and acetic acid (51:48:1, v/v)	LE/SPE	–	0.5 ng/g	0	–	
10	AF	Shim-pack CLC-ODS	FLD	Water, methanol, and acetonitrile (56:29:17, v/v)	LE/SPE	B1 and G1: 3,5 ng/g B2 and G2: 0,1 ng/g	B1, B2, and G2: 4 ng/g G1: 7 ng/g	1	Selectivity, recovery, accuracy, precision, linearity	No aflatoxins were detected for these samples.
20	AF	Capcell Pak C18	FLD	Methanol and water (50:50, v/v)	LE/SPE	AFB1: 0.08 ng/mL AFB2: 0.02 ng/mL AFG1: 0.06 ng/mL AFG2: 0.02 ng/mL	AFB1: 0.20 ng/mL AFB2: 0.05 ng/mL AFG1: 0.20 ng/mL AFG2: 0.08 ng/mL	1	Linearity, LOD, LOQ, precision, recovery, and repeatability	Ten out of 11 samples were contaminated by AFB1, and the levels of 3 samples exceeded 5 ug/kg.
23	AF	YMC ODS-AQ S-3 C18	FLD	Methanol, acetonitrile, and water (25:15:60, v/v)	LE/SPE	0.1 ng/g	–	0	–	–
24	AF	Scharlau Chemie C18	FLD	Methanol and water (52:48, v/v)	LE/SPE	–	0.05 - 0.1 ng/g	0	–	If the levels of aflatoxins are over 1-2 ng/g, can be allowed a reliable identification.
25	AF and OTA	Inertsil ODS-3V C18	FLD	A: Water and methanol (55:45, v/v) containing 90 mL/L of 4 N nitric acid and 119 mg/L of potassium bromide B: Water and methanol (80:20, v/v) containing 90 mL/L of 4 N nitric acid and 119 mg/L of potassium bromide	LE/SPE	aflatoxin B1: 0.2 ug/kg in all samples Total aflatoxins: 0.5 ug/kg to 0.7 ug/kg OTA: 1.8 ug/kg to 2.0 ug/kg	aflatoxin B1: 0.6 ug/kg to 0.7 ug/kg Total aflatoxins: 1.6 ug/kg to 2.4 ug/kg OTA: 5.9 ug/kg to 6.5 ug/kg	1	Repeatability, recoveries, LOQ	Recoveries were in the range 77 to 99% for aflatoxin B1. Similarly, ochratoxin A recoveries were from 64 to 94%.

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Table II. Summary of HPLC-based methods for the determination of mycotoxins in medicinal plants (continued)

Ref.	Mycotoxin	Column	Detector	Mobile Phase	Sample preparation	LOD	LOQ	Validation*	Parameters	Key Results
2	AF and OTA		FLD	Acetonitrile, methanol, and water (8:27:65, v/v)	LE/SPE	–	–	0	–	26.6% herbal plants were contaminated by various types of aflatoxins and 13.3% by ochratoxin A.
26	AF	Alltima C18	FLD	Water, methanol, and acetonitrile (3:1:1, v/v)	LE/SPE	0.06 ug/kg	0.3 ug/kg	1	linearity, recovery, precision, LOQ, LOD	–
27	AF	C18	FLD	Water, acetonitrile, and methanol (60:20:30, v/v)	LE/SPE	AFB1: 0.033 ppb	AFB1: 0.1ppb	0	–	The highest prevalence of aflatoxin was attributed to aflatoxin B1 (30.8%).
4	ZEA	Ultimate XB-C18	FLD	Acetonitrile and water (50:50, v/v)	LE/SPE	ZON: 4 ug/kg α -ZOL: 2.5 ug/kg	not included	1	linearity, LOD, precision, reproducibility, accuracy	12% of these tested samples were contaminated with ZON and, no samples were detected with α -zearalenone.
28	ZEA, ZOL and DON	ODS, RP-C18	FLD	Acetonitrile and water (55:45, v/v)	LLE	–	–	0	–	Contamination of ZEN was detected in 13.07% samples, DON was detected in 6.92% samples, and only one sample was contaminated with ZOL.
29	OTA	Zorbax SB-C18 XTerra RP18 Lichrospher C18 endcapped ZORBAX eclipse XDB-C18 Zorbax SB-C18 ODS2 Alltech Alltima C18 SphereClone ODS (2)	FLD	A: Methanol and water–acetic acid solution (70:30, v/v; water:acetic acid 3:1) B: Methanol (100%, v/v)	LE/SPE	–	–	0	–	–
30	AF	CAPCELL PAK C18	FLD	Methanol and acetonitrile (40:18, v/v) with water as the aqueous phase	LE/SPE	AFB1: 0.18 μ g/kg AFB2: 0.15 μ g/kg AFG1: 0.65 μ g/kg AFG2: 0.41 μ g/kg	AFB1: 0.60 μ g/kg AFB2: 0.53 μ g/kg AFG1: 2.18 μ g/kg AFG2: 1.22 μ g/kg	1	linearity, LOD, LOQ, accuracy, precision, stability, selectivity and robustness	–

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Table II. Summary of HPLC-based methods for the determination of mycotoxins in medicinal plants (continued)

Ref.	Mycotoxin	Column	Detector	Mobile Phase	Sample preparation	LOD	LOQ	Validation*	Parameters	Key Results
31	AF and OTA	ZORBAX Eclipse XDB C18	FLD	Water, methanol, and acetonitrile (55:15:30, v/v)	LE/SPE	AFB1: 0.1 µg/kg AFB2: 0.2 µg/kg AFG1: 0.3 µg/kg AFG2: 0.2 µg/kg OTA: 0.47 µg/kg	AFB1: 0.4 µg/kg AFB2: 0.7 µg/kg AFG1: 0.9 µg/kg AFG2: 0.6 µg/kg OTA: 1.23 µg/kg	1	linearity, LOD, LOQ, accuracy,	40% of samples were contaminated with AFs. All of the samples were contaminated with OTA.
32	AF, OTA, ZEA and DON	Phenomenex Gemini C18	DAD	A: Water containing 0.5% acetic acid B: Methanol and acetonitrile (50:50, v/v)	LLE	0.01563 – 0.5161 ng/mL	0.05210 – 1.720 ng/mL	1	Linearity, LOD, LOQ, precision and recovery	–
33	AFB1	Shim-Pack CLC – ODS	FLD	Water, acetonitrile, and methanol (60:20:20, v/v)	LE/SPE	0.6 µg/kg	1.0 µg/kg	0	–	Aflatoxin B1 was noticed in a single sample (< 1.0 µg/kg).
34	OTA	RP C-18 ODS	FLD	Water, acetonitrile, and acetic acid (26:70:4, v/v)	LE/SPE	–	–	1	Repeatability, Reproducibility and Recovery	–
35	AF	Radial-Pak cartridge, Type 8 NVC C-18	FLD	Water and methanol (58:42, v/v) containing 119 mg/L of potassium bromide and 100 µL of nitric acid (65%) per liter	LE/SPE	0.05 ppb	–	1	Specificity, precision, accuracy and linearity	–
36	Fumonisin B1 and AFB1	ODS Luna C18	FLD	Monopotassium phosphate buffer (0.01 M), acetonitrile, and methanol (690:220:75, v/v)	LE/SPE	–	–	0	–	None of the plant extracts contained detectable levels of aflatoxin B1; however, eight plants, four dietary and four medicinal, were positive for fumonisin B1.
45	AF	Licrospher 100 RP18	FLD	Water, methanol, and acetonitrile (620:220:160, v/v)	LE/SPE	0.04 ng/g	–	1	Linearity, LOD, precision, reproducibility, accuracy,	No samples used in study had levels above of 20 ng/g. The contaminated products were those in tablet and capsule dosage forms.
38	AF and OTA	Aflatoxins: YMC ODS-AQ S-3 Ochratoxin A: Ultrasphere	FLD	Aflatoxins: Water, methanol, and acetonitrile (600:250:150, v/v) containing 4 M nitric acid and potassium bromide Ochratoxin A: Acetonitrile, water, and acetic acid (500:500:10, v/v)	LE/SPE	–	–	0	–	–

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Table II. Summary of HPLC-based methods for the determination of mycotoxins in medicinal plants (continued)

Ref.	Mycotoxin	Column	Detector	Mobile Phase	Sample preparation	LOD	LOQ	Validation*	Parameters	Key Results
39	AF and OTA	Aflatoxins: YMC ODS-AQ S-3 Ochratoxin A: Ultrasphere	FLD	AF: Water, methanol, and acetonitrile (600:250:150, v/v) OTA: Acetonitrile, water, and acetic acid (50:50:1, v/v)	LE/SPE	AFB1: 0.1 ng/g OTA: 0.1 ng/g	AFB1: 1 ng/g OTA: 1 ng/g	0	–	39 commercially samples were analyzed (26 with AF 67%, 29 with OTA 74%, and 10 no AF or OTA 26%)
11	AF and OTA	ACCHROM Unitary C18	FLD	Acetic acid (0.5%) and methanol (48:52, v/v)	LE/SPE	0.03 - 0.3 ug/kg	0.1 - 0.9 ug/kg	1	Linearity, LOD, LOQ, recovery, reproducibility and precision	–
40	AF and OTA	Manual IAC: Inertsil ODS-3V In-line IAC: InertSustain AQ C18	FLD	A: Methanol and water (45:55, v/v) containing 350 µL of 4 N nitric acid and 119 mg/L of potassium bromide B: Methanol and water (80:20, v/v) containing 350 µL of 4 N nitric acid and 119 mg/L of potassium bromide	LE/SPE	Manual IAC method Aflatoxins: 0.2 to 0.5 µg/kg Ochratoxin A: 0.1 to 0.3 µg/kg In-line IAC method Aflatoxins: 0.01 to 0.2 µg/kg Ochratoxin A: 0.10 to 0.2 µg/kg	not included	0	–	–
41	AF	LiChrospher 100 RP-18	FLD	Acetonitrile, methanol, and water (1:1:4, v/v)	LE/SPE	B1: 0.72 ug/kg B2: 0.22 ug/kg G1: 0.75 ug/kg G2: 0.30 ug/kg	–	1	Linearity, LOD, precision, reproducibility and accuracy	Aflatoxins were detected in 15.78% of the samples
42	AF	Venusil MP C18	FLD	Methanol, acetonitrile, and water (30:18:52, v/v)	LE/SPE	0.04–0.2 ug/kg	0.25–1.0 ug/kg	1	selectivity, stability, linearity, LOD, LOQ, precision, accuracy, and robustness	–
43	ZEA	Eclipse XDB-C18	FLD	Water, acetonitrile, and methanol (46:46:8, v/v)	LE/SPE	9.5 ug/kg	–	0	–	55.6% of samples were highly contaminated with ZON (>60 ug/kg)

Note: AF: Aflatoxins; DON: Deoxynivalenol; OTA: Ochratoxin A; ZEA: Zearalenone; ZOL: Zearalenol; LOD: Limit of Detection; LOQ: Limit of Quantification; *Validation 0: no validation; *Validation 1: validation realized; LLE: Liquid Liquid Extraction; LE/SPE: Liquid Extraction/ Solid Phase Extraction

Based on the results for aflatoxins, the Limit of Quantification (LOQ) for roots—the most frequently cited part of the plant—ranged from 0.03 $\mu\text{g}/\text{kg}$ ⁵ to 1.0 $\mu\text{g}/\text{kg}$.^{39,42} For other, less commonly cited matrices, the LOQ varied between 0.03 $\mu\text{g}/\text{kg}$ ⁵ and 7.0 $\mu\text{g}/\text{kg}$.¹⁰ The Limit of Detection (LOD) values for aflatoxins in roots ranged from 0.04 to 0.2 $\mu\text{g}/\text{kg}$,⁴² while other matrices showed LODs from 0.01 $\mu\text{g}/\text{kg}$ (Ali et al., 2005)²¹ to 3.5 $\mu\text{g}/\text{kg}$.¹⁰

The LOQ for ochratoxin A in the studies ranged from 0.2 $\mu\text{g}/\text{kg}$ ⁵ to 6.3 $\mu\text{g}/\text{kg}$,²⁵ while the LOD ranged from 0.05 to 2.0 $\mu\text{g}/\text{kg}$, also reported by Areo et al. (2020)⁵ and Greaves et al. (2021),²⁵ respectively. For zearalenone, the LOD values across studies ranged from 0.03 $\mu\text{g}/\text{kg}$ to 9.5 $\mu\text{g}/\text{kg}$. The only LOQ cited was 0.03 $\mu\text{g}/\text{kg}$.

In addition, the reliability of the analytical results depends on proper method validation. Analytical validation is the evaluation of an analytical procedure through experimental testing, with the purpose of providing objective evidence that the procedure is suitable for its intended use. In an international context, the ICH Q2(R2)⁶⁰ guideline provides recommendations for the selection and evaluation of validation tests for analytical procedures, including parameters such as specificity/selectivity, linearity, range, accuracy, precision, detection limit, quantitation limit, and robustness.^{59,60} Among the reviewed articles, 50% reported analytical validation data (Figure 4A). However, this result should be interpreted in light of the different objectives of the included studies, since some articles focused mainly on contamination/occurrence assessment, while others were specifically designed for analytical method development and validation.

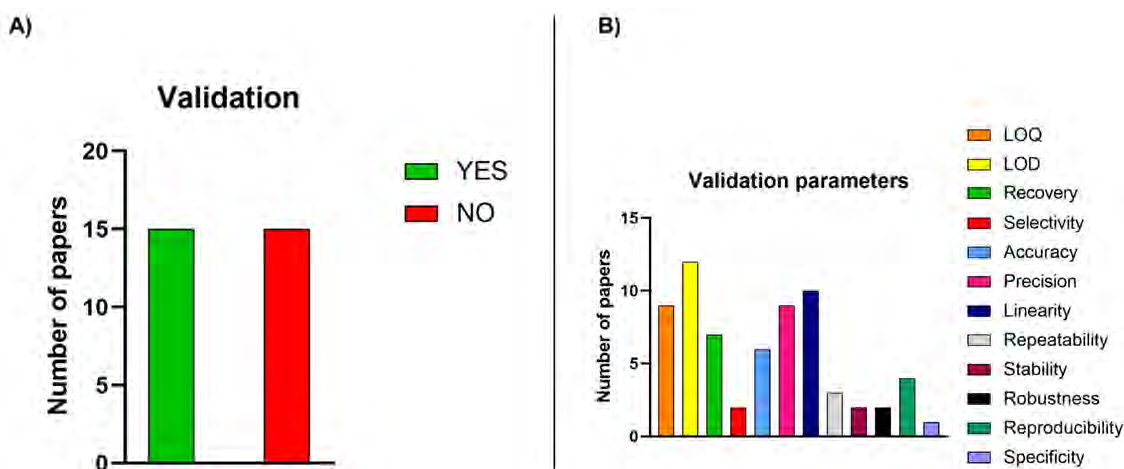


Figure 4. A) Number of papers that reported validation, and B) Validation parameters evaluated in the papers.

The main parameters evaluated were LOD, LOQ, linearity, and precision (Figure 4B). Selectivity was evaluated in 20% of the reviewed articles. It refers to a method's ability to identify an analyte in a matrix without interference from other substances, such as impurities, diluents, or matrix components.⁵⁹

Linearity was assessed in 80% of the reviewed articles, and this concerns the ability to obtain analytical responses that are directly proportional to the analyte concentration.⁵⁹ The range is the interval spanning from the lowest to the highest concentration values where the method remains precise, accurate, and linear.⁶¹

Precision was assessed in 93% of the reviewed articles and relates to the proximity between measured results. It is usually expressed as the variance, standard deviation, or coefficient of variation of a series of measurements. There are two types of precision: repeatability expresses precision under the same operating conditions over a short interval of time, and intermediate precision expresses variations within laboratories, such as those caused by different days, different analysts, different equipment, and so on.⁵⁹ The accuracy was assessed in 53% of the reviewed articles and refers to the degree of agreement between individual results of the method under study and a value accepted as the true value.⁵⁹

Articles reporting LOD and LOQ were assessed at 87% and 73%, respectively. The LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value. The LOQ is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy.⁶¹ Reporting these limits is essential in analytical methods for mycotoxin determination, as they are key parameters evaluated by regulatory agencies to ensure method suitability and compliance with established safety standards. Low LOD and LOQ values are particularly important for detecting trace levels of mycotoxins, which may pose significant health risks even at very low concentrations and are therefore required to meet current regulatory limits for contaminants in herbal and medicinal plant materials.

Robustness was assessed in 13% of the reviewed articles, typically during analytical method development. It refers to the method's ability to resist deliberate variations in parameters such as flow rate, column temperature, injection volume, mobile phase composition (in liquid chromatography), or any other variable inherent to the method of analysis (e.g., stability of analytical solutions, extraction time, etc.).⁶¹

Matrix effects are another important aspect to be considered in the validation and application of analytical methods for medicinal plants. Herbal matrices are chemically complex and may contain alkaloids, polyphenols, flavonoids, pigments, tannins, essential oils, sugars, organic acids, and other secondary metabolites.⁶²⁻⁶⁵ These compounds may interfere with extraction efficiency, clean-up selectivity, chromatographic separation, fluorescence response, and analyte recovery.^{62,64,65} In the studies included in this scoping review, matrix effects were not systematically evaluated as an independent parameter. This limited reporting should be considered a methodological gap, particularly because matrix composition may vary substantially according to plant species, plant part, cultivation conditions, processing, and storage.

Strategies to reduce or compensate for matrix effects include optimized extraction conditions, efficient clean-up procedures such as solid-phase extraction and immunoaffinity columns, dilution of the extract, adjustment of pH, reduction of sample mass, matrix-matched calibration, recovery studies at different concentration levels, and selectivity assessment in representative herbal matrices.^{64,66,68} For HPLC-FLD methods, these precautions are particularly relevant because co-extracted fluorescent compounds may affect the analytical response.^{30,67} For HPLC-DAD methods, spectral comparison and peak purity assessment may help detect co-elution, although these approaches do not provide the same confirmatory capability as mass spectrometry.^{67,69} Therefore, future studies using HPLC-FLD and HPLC-DAD for mycotoxin determination in medicinal plants should more explicitly evaluate and report matrix effects to improve method reliability and comparability.

The fact that 50% of the articles did not report analytical validation data should be interpreted with caution. The studies included in this scoping review had different objectives: while some were specifically focused on the development, optimization, and/or validation of analytical methods, others primarily investigated the occurrence or contamination levels of mycotoxins in medicinal plant samples, sometimes using previously established methods. Therefore, the absence of complete validation data does not necessarily indicate inadequate methodological quality but may reflect the study's aims, journal reporting practices, or the regulatory context at the time of publication. This aspect is particularly relevant because no publication period restriction was applied in the present review, and older studies may have been conducted under different validation requirements. Nevertheless, for current applications in routine quality control and regulatory assessment, analytical validation remains essential. In addition to national regulatory requirements, international guidelines such as ICH Q2(R2)⁶² underscore the importance of validation to demonstrate the reliability of analytical procedures. Thus, reporting parameters such as selectivity/specificity, linearity, range, accuracy, precision, detection limit, quantitation limit, and robustness is important to support comparability, regulatory acceptance, and confidence in analytical results obtained from complex matrices such as medicinal plants.^{60,61}

Taken together, the validation data reported in the included studies should be interpreted not only as isolated analytical parameters, but also in relation to the intended use of each method. For routine quality control and regulatory applications, the reliability of HPLC-FLD and HPLC-DAD methods depends on the combined evaluation of selectivity/specificity, linearity, range, accuracy, precision, recovery, detection and quantitation limits, robustness, and potential matrix effects.^{12,60,67,70} This is particularly relevant for medicinal

plant matrices, whose chemical complexity may affect extraction efficiency, chromatographic separation, detector response, and analyte recovery. Therefore, future studies should provide more complete validation reports aligned with international guidelines, such as ICH Q2(R2),⁶⁰ especially when methods are proposed for routine monitoring or regulatory decision-making.

Final considerations and evidence gaps

Based on the selected articles addressing analytical methods for mycotoxin determination in medicinal plants using HPLC with fluorescence detection, it is evident that aflatoxins are the most extensively investigated group, reflecting their well-established health risks and regulatory relevance. Despite this focus, the review highlights important methodological limitations, particularly regarding the development of multi-mycotoxin analytical methods. Most of the studies included evaluated only a single group of mycotoxins, indicating challenges associated with simultaneous determination when using fluorescence detection.

These limitations are likely related to differences in the physicochemical properties of mycotoxins, including molecular structure, polarity, and chromatographic behavior under varying mobile phase compositions. In addition, the requirement for specific derivatization steps, especially for aflatoxins and fumonisins, further complicates the development of robust multi-analyte methods using HPLC-FLD.

It should be emphasized that the exclusion of LC-MS/MS was a methodological choice based on the predefined scope of this scoping review. LC-MS/MS remains the gold standard for mycotoxin determination, especially for simultaneous multi-mycotoxin analysis, because of its superior sensitivity, selectivity, and confirmatory capability. However, HPLC-FLD and HPLC-DAD continue to play an important role in routine quality control due to their lower cost, broader availability, and established use for regulated mycotoxins such as aflatoxins and ochratoxin A. In this sense, the evidence gaps identified in the present review should be interpreted specifically within the context of HPLC-FLD and HPLC-DAD methods, and not as a limitation of the entire field of mycotoxin analysis in medicinal plants.

From an evidence gap perspective, there is a clear lack of standardized and validated multi-mycotoxin methods applicable to complex medicinal plant matrices using fluorescence detection. Furthermore, a considerable proportion of studies did not report full analytical validation, which limits the comparability and regulatory applicability of the reported methods. Future research should therefore focus on the development and validation of comprehensive multi-mycotoxin analytical approaches, as well as on harmonizing validation practices to meet regulatory requirements and improve the reliability of mycotoxin monitoring in medicinal plant materials.

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Conflicts of interest

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

Use of artificial intelligence tools

The authors declare that ChatGPT (OpenAI), through its integrated image-generation tool, was used solely to create the graphical abstract. No artificial intelligence tools were used in any other aspect of the preparation of this article.

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Appendix 1. Complete search strategies used in PubMed, Scopus, and Web of Science

Database	Search queries
Pubmed	(((((((mycotoxin[Title/Abstract]) OR (aflatoxin[Title/Abstract])) OR (ochratoxin[Title/Abstract])) OR (fumonisin[Title/Abstract])) OR (trichothecene[Title/Abstract])) OR (deoxynivalenol[Title/Abstract])) OR (zearalenone[Title/Abstract]))
	(((((((HPLC[Title/Abstract]) OR (“high performance liquid chromatography”[Title/Abstract])) OR (fluorescence[Title/Abstract])) OR (dad[Title/Abstract])) OR (“2-mercaptoethanol”[Title/Abstract])) OR (“o-phthaldialdehyde”[Title/Abstract]))
	(((“herbal drug”[Title/Abstract]) OR (“dried extract”[Title/Abstract])) OR (extract[Title/Abstract])) OR (oil[Title/Abstract]))
	(((((((((((mycotoxin[Title/Abstract]) OR (aflatoxin[Title/Abstract])) OR (ochratoxin[Title/Abstract])) OR (fumonisin[Title/Abstract])) OR (trichothecene[Title/Abstract])) OR (deoxynivalenol[Title/Abstract])) OR (zearalenone[Title/Abstract])) AND ((((((HPLC[Title/Abstract]) OR (“high performance liquid chromatography”[Title/Abstract])) OR (fluorescence[Title/Abstract])) OR (dad[Title/Abstract])) OR (“2-mercaptoethanol”[Title/Abstract])) OR (“o-phthaldialdehyde”[Title/Abstract])))) AND (((“herbal drug”[Title/Abstract]) OR (“dried extract”[Title/Abstract])) OR (extract[Title/Abstract])) OR (oil[Title/Abstract]))
Scopus	(TITLE-ABS-KEY (mycotoxin) OR TITLE-ABS-KEY (aflatoxin) OR TITLE-ABS-KEY (ochratoxin) OR TITLE-ABS-KEY (fumonisin) OR TITLE-ABS-KEY (trichothecene) OR TITLE-ABS-KEY (deoxynivalenol) OR TITLE-ABS-KEY (zearalenone))
	(TITLE-ABS-KEY (hplc) OR TITLE-ABS-KEY (“high performance liquid chromatography”) OR TITLE-ABS-KEY (fluorescence) OR TITLE-ABS-KEY (dad) OR TITLE-ABS-KEY (“2-mercaptoethanol”) OR TITLE-ABS-KEY (“o-phthaldialdehyde”))
	(TITLE-ABS-KEY (“herbal drug”) OR TITLE-ABS-KEY (“dried extract”) OR TITLE-ABS-KEY (extract) OR TITLE-ABS-KEY (oil))
	((TITLE-ABS-KEY (mycotoxin) OR TITLE-ABS-KEY (aflatoxin) OR TITLE-ABS-KEY (ochratoxin) OR TITLE-ABS-KEY (fumonisin) OR TITLE-ABS-KEY (trichothecene) OR TITLE-ABS-KEY (deoxynivalenol) OR TITLE-ABS-KEY (zearalenone))) AND ((TITLE-ABS-KEY (hplc) OR TITLE-ABS-KEY (“high performance liquid chromatography”) OR TITLE-ABS-KEY (fluorescence) OR TITLE-ABS-KEY (dad) OR TITLE-ABS-KEY (“2-mercaptoethanol”) OR TITLE-ABS-KEY (“o-phthaldialdehyde”))) AND ((TITLE-ABS-KEY (“herbal drug”) OR TITLE-ABS-KEY (“dried extract”) OR TITLE-ABS-KEY (extract) OR TITLE-ABS-KEY (oil)))

(continued on next page)

Appendix 1. Complete search strategies used in PubMed, Scopus, and Web of Science (continued)

Database	Search queries
Web of Science	mycotoxin (Topic) or aflatoxin (Topic) or ochratoxin (Topic) or fumonisin (Topic) or trichothecene (Topic) or deoxynivalenol (Topic) or zearalenone (Topic)
	HPLC (Topic) or “high performance liquid chromatography” (Topic) or fluorescence (Topic) or dad (Topic) or “2-mercaptoethanol” (Topic) or “o-phathaldialdehyde” (Topic)
	“herbal drug” (Topic) or “dried extract” (Topic) or extract (Topic) or oil (Topic)
	mycotoxin (Topic) or aflatoxin (Topic) or ochratoxin (Topic) or fumonisin (Topic) or trichothecene (Topic) or deoxynivalenol (Topic) or zearalenone (Topic) AND HPLC (Topic) or “high performance liquid chromatography” (Topic) or fluorescence (Topic) or dad (Topic) or “2-mercaptoethanol” (Topic) or “o-phathaldialdehyde” (Topic) AND “herbal drug” (Topic) or “dried extract” (Topic) or extract (Topic) or oil (Topic)

ARTICLE

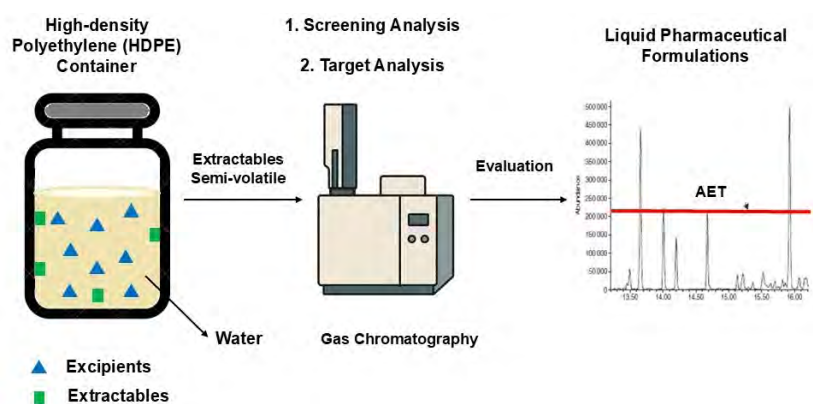
Study of Extractables in High-Density Polyethylene Packaging: Evaluation of the Impact of Excipients Propylene Glycol, Mint Flavor, and Benzalkonium Chloride on the Leaching Process of Semi-volatile Additives

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The study of extractables and leachables has become increasingly important in the pharmaceutical industry, with growing concerns about minimizing impurity risks in final products. Polymeric materials are widely used in pharmaceutical packaging due to their many advantages, but they can also introduce impurities. These materials often contain low molecular mass additives that may migrate into the product during storage and shelf life.

This issue is particularly critical in liquid pharmaceutical formulations, which are more prone to interactions with packaging materials. In this context, the present work evaluates the extractive potential of selected excipients - benzalkonium chloride, propylene glycol, and mint flavor - in aqueous formulations stored in high-density polyethylene (HDPE) containers. The main goal is to compare the physicochemical properties of these excipients in the formulation with the additives extracted from the packaging. In summary, benzalkonium chloride (the excipient with the highest log P value) demonstrated the greatest extraction power, through the extraction of 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, palmitic acid, and stearic acid. The mint aroma and propylene glycol contributed only to the extraction of 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (the additive

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with the lowest log P value). Therefore, the novelty of this study lies in its empirical approach to correlating the physicochemical parameters of excipients with leachables, providing a predictive framework for leaching behavior in the pharmaceutical industry.

Keywords: extractables, leachables, excipients, polymer, liquid pharmaceutical forms, partition coefficient, mass spectrometry

INTRODUCTION

Leachable compounds can be defined as “organic and inorganic chemical entities that migrate from the packaging/delivery system, packaging component, or packaging material of construction into the drug under normal storage and use conditions or during accelerated stability studies of the drug”.¹ Extractable compounds, on the other hand, can be defined as “organic and inorganic chemical entities released from a packaging system and/or packaging component through extraction under laboratory conditions”.² These compounds can be found in various products, including pharmaceuticals,^{3–7} cosmetics,⁸ and food,⁹ and may pose health risks due to their continuous ingestion. For example, additives such as Irganox® 1010 have been detected in ophthalmic drug products stored in plastic packaging.⁵ This process occurs due to the interaction between the plastic and the formulation, which can cause the migration of low molecular mass additives to the product. This phenomenon is dependent on the pharmaceutical form, being more relevant in liquid/aerosol formulations and lower in solid formulations.^{1,10}

Pharmaceutical products are formulated with excipients to provide a specific and definitive therapeutic benefit to the user. The function of these compounds is to protect and enhance the stability of the formulation (since some active pharmaceutical ingredients - API - do not maintain stability when isolated, either denaturing or adhering to the walls of the container), increase the volume of the formulation, aid in solving formulation problems such as dissolution and improve the consumer acceptance. Furthermore, they aid in the bioavailability of the API, as in many cases, these compounds are not easily absorbed by the human body. Thus, the mixture with an excipient can act as a solvent in this process.¹¹ In this context, the choice of excipients during formulation development can be crucial to the product success, potentially impacting the leaching of packaging components. The same properties of excipients that facilitate an increase in the solubility of the active ingredient may also facilitate the leaching of compounds in contact with various polymeric materials.¹²

Plastics are widely used in packaging and the production process within the pharmaceutical industry due to their excellent cost-effectiveness compared to other available materials, as well as their unique characteristics. The versatility of plastic can be attributed to the different types of monomers and additives added during the process to enhance the polymer performance and durability. Additives can be categorized into various classes, such as: plasticizers, flame retardants, antioxidants, acid removers, light and heat stabilizers, lubricants, pigments, antistatic agents, slip compounds, and thermal stabilizers.^{10,13}

In this article, the focus was on evaluating the extraction potential of different excipients present in liquid formulations stored in high-density polyethylene bottles in order to infer which excipients and concentrations might be critical for the product. This information can support the choice of excipients during formulation development and, subsequently, the risk analysis of extractables and leachables in the product. For this purpose, analytical methods were developed to evaluate the semi-volatile additives from the plastic bottle using gas chromatography coupled to mass spectrometry (GC-MS). In order to obtain realistic results, an accelerated study was conducted to simulate the interaction between the drug and the packaging material under storage conditions. The results were used to correlate the physicochemical characteristics of the excipients with the concentration of additives extracted from the packaging material.

MATERIALS AND METHODS

Extractables study

Potassium chloride, monosodium phosphate, disodium phosphate, sodium hydroxide, dichloromethane and sodium chloride were purchased from Merck (New Jersey, USA), isopropanol and acetonitrile were

purchased from J.T. Baker (Pennsylvania, USA), and hexane and hydrochloric acid were purchased from Biograde (North Carolina, USA). The high-density polyethylene packaging material purchased from Plastic Industrial Batic (São Paulo, Brazil).

A pH 2.0 solution ($0.01 \text{ mol L}^{-1} \text{ KCl}$ and $0.003 \text{ mol L}^{-1} \text{ HCl}$), a pH 10.0 buffer solution ($0.0045 \text{ mol L}^{-1} \text{ NaH}_2\text{PO}_4$ and $0.066 \text{ mol L}^{-1} \text{ Na}_2\text{HPO}_4$), and a water:isopropanol (1:1) solution were prepared. Isopropanol and hexane were used in their pure form. Both were used as extraction media. The packaging material was cut into pieces and submerged in the aqueous extraction media in an oven (at 70°C for 7 days) and ultrasound treatment (at 10°C for 2 h), and the organic extraction media were subjected to Soxhlet extraction for 24 h. The sample extracts were prepared as detailed in Figure 1.

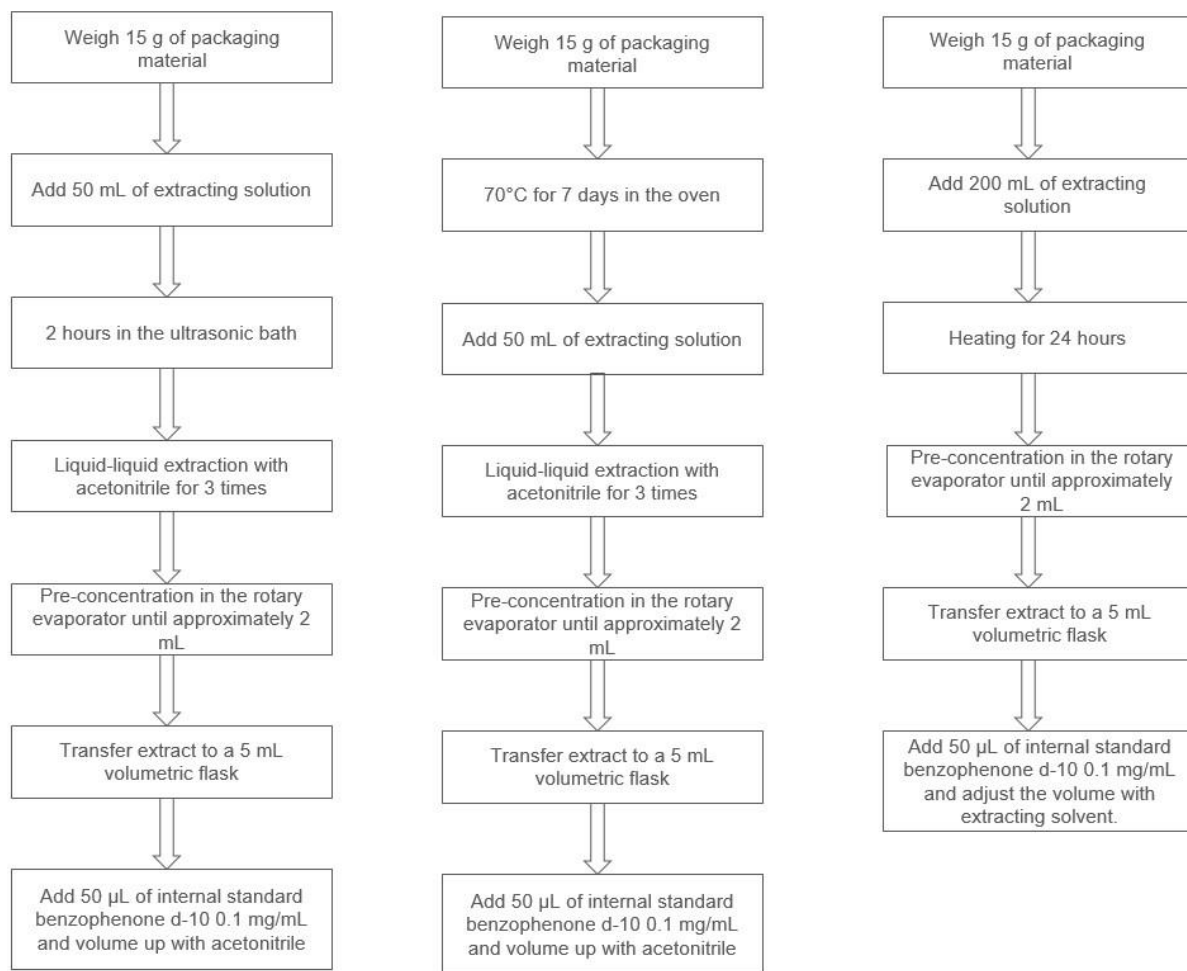


Figure 1. Flowchart of extract preparation using different extraction apparatuses in the extractables study.

Accelerated study

Propylene glycol was purchased from Dow Brasil Indústria e Comércio (São Paulo, Brazil), benzalkonium chloride from Novo Nordisk Pharmatech (Copenhagen, Denmark), and mint flavor powder from International Flavors & Fragrances (New York, USA) – composition: 90.8% d-carvone, 6.0% terpinen-4-ol, and 2-cyclopenten-1-one, and 3.2% 3-methyl-2-(2-pentenyl)-, (Z)-. Aqueous extraction media in concentration of 5% (m/v) mint flavor excipient, 30% (v/v) propylene glycol, and 0.02% (m/v) benzalkonium chloride were prepared. 50 mL of the solution were added to 15 g of the cut packaging material in a Teflon jar, and the set was exposed to the oven at 70°C for 7 days. Due to the large amount of packaging material required

and the limited availability of Teflon containers, the accelerated extraction experiments were performed in single replicate. A parallel study under more aggressive conditions was conducted to assess reproducibility, and the results confirmed the overall trends observed.

Analytical curves and sample preparation

Palmitic acid and stearic acid were purchased from Sigma Aldrich (Massachusetts, USA), linoleic acid was purchased from Pharmaffiliates (Haryana, India). For quantification of organic acids, an analytical curve was prepared at concentrations of 0.5, 1, 2.5, 5, and 7.5 $\mu\text{g mL}^{-1}$ palmitic and stearic acids, and 5 $\mu\text{g mL}^{-1}$ linoleic acid used as surrogate standard in dichloromethane. Both stock solutions were prepared at a concentration of 20 $\mu\text{g mL}^{-1}$. For the samples, 5 mL of the extract was pipetted, 1 spatula of sodium chloride was added, along with 2 drops of hydrochloric acid and 1.25 mL of the surrogate standard stock. Liquid-liquid extraction was performed using 10 mL of dichloromethane three times, with agitation for 1 min. After allowing the solution to rest and phase separation, the organic phase was isolated and the extract was concentrated to a final volume of 5 mL.

7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione was purchased from Simson Pharma Limited (Haryana, India) and benzophenone d-10 was purchased from Sigma Aldrich (Massachusetts, USA). For the quantification of 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, an analytical curve was prepared at concentrations of 5, 15, and 20 $\mu\text{g mL}^{-1}$ for and 5 $\mu\text{g mL}^{-1}$ for benzophenone d-10 as internal standard in dichloromethane. The 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione stock was prepared at a concentration of 80 $\mu\text{g mL}^{-1}$, and the benzophenone stock was prepared at 20 $\mu\text{g mL}^{-1}$. For the samples, 5 mL of the extract was pipetted, 1 spatula of sodium chloride was added, along with 2 drops of hydrochloric acid. Liquid-liquid extraction was performed using 10 mL of dichloromethane three times, with agitation for 1 min. After allowing the solution to rest and phase separation, the organic phase was isolated, and the extract was concentrated to a final volume of 5 mL. The sample was quantitatively transferred to a 5 mL volumetric flask, and 1.25 mL of the benzophenone d-10 stock standard was added. The flask was then filled to the mark with dichloromethane.

Instrumental analysis

The screening analyses were performed on a 7890B gas chromatograph coupled to a 5977B MSD single quadrupole mass spectrometer (Agilent, Santa Clara, CA). The column used was DB-XLB (60 m x 0.250 mm, and 0.25 μm) (Agilent, Santa Clara, CA). The temperature ramp was held at 40 $^{\circ}\text{C}$ for 4 min and then increased to 100 $^{\circ}\text{C}$ at a rate of 20 $^{\circ}\text{C min}^{-1}$. The temperature was then raised to 340 $^{\circ}\text{C}$ at a rate of 4 $^{\circ}\text{C min}^{-1}$ and held for 5 min, and further increased to 350 $^{\circ}\text{C}$ at a rate of 5 $^{\circ}\text{C min}^{-1}$. The injected volume was 0.5 μL . Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1} . The injector temperature was 340 $^{\circ}\text{C}$ in splitless mode. The solvent cut in the detector was at 9 min. Data acquisition was performed in scan mode from m/z 19 to 670, with ionization energy of 70 eV. The interface temperature was 350 $^{\circ}\text{C}$, and the ionization source temperature was 320 $^{\circ}\text{C}$. Total run time was 74 min. A mix of extractables and leachables GC 50 $\mu\text{g mL}^{-1}$, containing: 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate of octadecyl, ϵ -caprolactam, 2-mercaptobenzothiazole, bisphenol A, butylated hydroxytoluene (BHT), 1,3-di-tert-butylbenzene, oleamide, bis(2-ethylhexyl) phthalate, stearic acid, cis-13-docosenoamide, tris(2,4-di-tert-butylphenyl)phosphate, 2,4-di-tert-butylphenol, and 2,6-di-tert-butylphenol, purchased from Supelco (Pennsylvania, USA), was used as system suitability standard.

The quantification of organic acids was performed on a gas chromatograph (model 8890) coupled to a triple quadrupole mass spectrometer (model 7000E) (Agilent, Santa Clara, CA). The column used was HP 5-UI (30 m x 0.250 mm, and 0.25 μm) (Agilent, Santa Clara, CA). The temperature ramp started at 60 $^{\circ}\text{C}$ and was increased to 160 $^{\circ}\text{C}$ at a rate of 15 $^{\circ}\text{C min}^{-1}$. The temperature was then raised to 215 $^{\circ}\text{C}$ at a rate of 3 $^{\circ}\text{C min}^{-1}$, and finally, the temperature was increased to 340 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C min}^{-1}$ and held for 5 min. The injected volume was 3 μL . The injector temperature was 320 $^{\circ}\text{C}$ in split mode (1:3). Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1} . The solvent cut in the detector was at 14 min.

Data acquisition was performed in selected ion monitoring (SIM) mode, monitoring m/z 256, 213, and 129 (palmitic acid), 241, 185, and 129 (stearic acid), and 280, 124, and 110 (linoleic acid), ionized with 70 eV energy. The interface temperature was 350 °C, and the ionization source temperature was 320 °C. Total run time was 42.5 min.

The quantification of 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione analyses was performed on a gas chromatograph (model 8890) coupled to a triple quadrupole mass spectrometer (model 7000E) (Agilent, Santa Clara, CA). The column used was DB-XLB (60 m x 0.250 mm, and 0.25 μ m) (Agilent, Santa Clara, CA). The temperature ramp was held at 40 °C for 4 min and then increased to 100 °C at a rate of 20 °C min⁻¹. The temperature was then raised to 340 °C at a rate of 4 °C min⁻¹ and held for 5 min, followed by a temperature increase to 350 °C at a rate of 30 °C min⁻¹. The injected volume was 0.5 μ L. Helium was used as the carrier gas at a flow rate of 1.2 mL min⁻¹. The injector temperature was 340 °C in splitless mode. The solvent cut in the detector was at 9 min. Data acquisition was performed in SIM mode, monitoring m/z 192, 110 (benzophenone d-10), and 191, 206 (7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione), ionized with 70 eV energy. The interface temperature was 350 °C, and the ionization source temperature was 320 °C. Total run time was 74 min.

RESULTS AND DISCUSSION

Controlled extractables study

The controlled extraction study is characterized by employing aggressive extraction conditions, such as extended times, high temperatures, and a wide range of polarity and pH, aiming to prevent the decomposition of the material.¹⁴ The Product Quality Research Institute (PQRI) has defined a controlled extractables study protocol at parenteral and ophthalmic formulations, in which the study design depends on the composition of the product being evaluated, guiding the experimental procedure. For example, in products containing organic solvents as vehicles with continuous direct contact with the packaging material, the use of organic solvents with different polarities, such as isopropanol and n-hexane, is recommended.¹⁵⁻¹⁷ In this case, a simplified study was conducted to evaluate the extracts with different extraction techniques and extracting solutions, aiming to identify additives present in the packaging material at relevant concentrations that are likely to migrate into the aqueous formulation under the product storage conditions. The obtained data were compared with the National Institute of Standards and Technology (NIST) library, a database that contains various reference standard information, including mass spectra of these compounds. Based on this, the target compounds described in Table I were selected.

Table I. Selected extractables as targets and information related to identification

Extractables	Chemical Formula	CAS	Match Factor NIST	Reliability of Identification
7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	C ₁₇ H ₂₄ O ₃	82304-66-3	923	Confirmed
Palmitic acid	C ₁₆ H ₂₆ O ₂	57-10-3	856	Confirmed
Stearic acid	C ₁₈ H ₃₆ O ₂	57-11-4	889	Confirmed

The described compounds are of confirmed identification, meaning they are based on the comparison of the mass spectrum and retention time with the reference standard.² 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione is a associated compound of the Irganox® 1076.¹⁸

Accelerated extractables study

An accelerated extractables study produces an extractables profile similar to that of the leachables profile, but with a shorter extraction time, without affecting the extractable compounds. The leaching process

involves the diffusion of the additive through the polymer; thus, it is dependent on the diffusion coefficient and the thickness of the packaging material. To assess this effect, the factor of 10 rule is used, which is based on the energy range for compound migration from polymers, typically between 80 and 100 kJ mol⁻¹. In practice, this causes the diffusion coefficient to increase by one order of magnitude for every 20 °C rise in temperature, according to the accelerated aging factor, as described in Equation 1.¹⁸

$$AAF = Q_d^{\frac{TAA-TREF}{20}} \quad (1)$$

where:

AAF = Accelerated aging factor

Q_d = Aging factor, with the conventionally accepted value of 10.0

TAA = Accelerated contact temperature

TREF = Typical reference use temperature

The extraction time and temperature for the accelerated study were defined based on the factor of 10 rule, according to Equation 1, in order to simulate long-term stability conditions. Therefore, considering the accelerated contact temperature of 70 °C and the typical reference use temperature of 30 °C, the calculated accelerated aging factor is 100. This value is divided by the usual shelf life of a product (2 years, equivalent to 730 days), resulting in 7.3 days, which explains the proposed condition for the accelerated study. The concentrations of the target extractable compounds were determined in the excipient samples subjected to the accelerated study. The results are presented in Table II and Figure 2.

Table II. Concentration (µg g⁻¹) of the target compounds in the accelerated extractables study in the experiment conducted at unicata

Excipients	Palmitic Acid	Stearic Acid	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
Propylene Glycol 30%	< 1	< 1	5
Mint Flavor 5%	< 1	< 1	4
Benzalkonium Chloride 0.02%	8	12	25

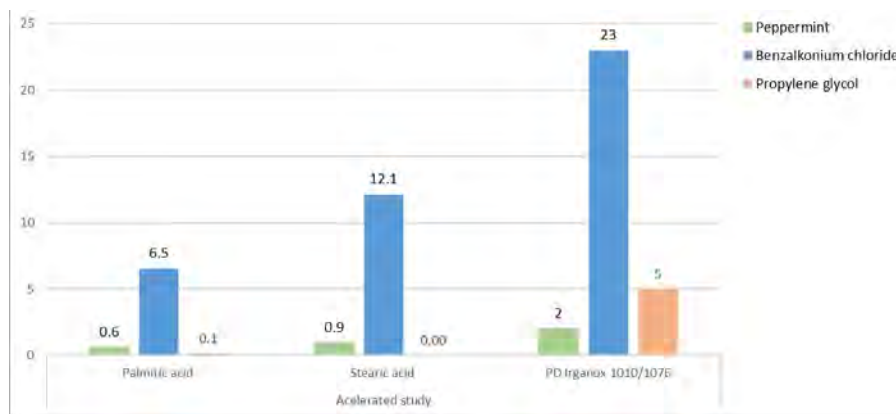


Figure 2. Comparison of the extraction power of different types of excipients in the experiment conducted at unicata.

In a quantitative assessment, the recovery of these compounds was evaluated across the different excipient matrices used, ensuring that the matrix effect was not significant and that no losses occurred during the liquid-liquid extraction. To this end, an accuracy test was conducted with all the excipients evaluated within the evaluated work range. Polyethylene glycol and benzalkonium chloride showed recoveries ranging from 75% to 113%. The mint flavoring exhibited recovery values between 36% and 87%. As a result, concentration values were corrected to enable a valid comparison.

A parallel study performed under more aggressive conditions revealed a variation of approximately 25% at higher concentrations and up to 100% at lower concentrations. Although such conditions do not represent realistic pharmaceutical scenarios, the results mirrored the trends of the accelerated extraction experiments, supporting the reliability of the conclusions despite the experimental limitations.

Based on these results, the influence of the physicochemical parameters on the leaching process of additives from the plastic material will be discussed in the topics of partition coefficient and pH.

Partition coefficient

There is an empirical approach that correlates the concept of partition coefficient within the scope of leachables. In this context, the solute is the leachable, the receiving phase is the simulant/product, and the donor phase is the packaging material. Based on this analogy, a mathematical relationship is established between the partition coefficient and $\log P_{o/w}$, as the polymer behaves like octanol and the liquid behaves like water, according to Equation 2.¹⁸

$$\log K_{\frac{P}{L}} = \text{slope} \times \log P_{\frac{o}{w}} + \text{intercept} \quad (2)$$

where:

$\log K_{\frac{P}{L}}$ = Partition coefficient between plastic and liquid

$\log P_{\frac{o}{w}}$ = Partition coefficient between octanol and water

The intercept and slope vary depending on the material, but the linear relationship remains consistent across all materials. The advantage of using this relationship is that $\log P_{o/w}$ is a well-established parameter with easily accessible values, relating characteristics such as solubility and bioavailability.¹⁸ The excipients propylene glycol, mint flavor, and benzalkonium chloride have partition coefficient values of -0.81, 2.27, and 9.98 - 82.5, respectively. The target compounds palmitic acid, stearic acid, and 7,9-di-terc-butyl-1-oxaspiro(4,5)dec-6,9-dieno-2,8-dione have partition coefficient values of 7.15, 8.22, and 3.08, respectively.¹⁹

The colloidal suspension of benzalkonium chloride presented the highest extraction potential, preferentially extracting compounds with lower $\log P_{o/w}$, i.e., compounds with greater solubility in aqueous media, in this case, 7,9-di-terc-butyl-1-oxaspiro(4,5)dec-6,9-dieno-2,8-dione. Benzalkonium chloride is a type of cationic surfactant,²⁰ composed of a mixture of benzyl dimethyl ammonium alkyl chlorides, which have various even alkyl chain lengths, totaling 24 structurally similar quaternary ammonium compounds characterized by a positively charged nitrogen covalently bonded to three alkyl group substituents and one benzyl substituent.²¹ This excipient stands out due to its surfactant nature, meaning that, in aqueous solution, at the critical micelle concentration (CMC), surfactant molecules aggregate around organic molecules in the medium, with the polar part facing the aqueous solution and the non-polar part facing the organic molecule, forming micelles, as illustrated in Figure 3 (the reverse procedure is also possible). This layer created is responsible for reducing surface tension, consequently increasing the solubility of organic molecules in aqueous media.²² In the context of extractables, this behavior causes the release of the packaging additive, typically organic, into the aqueous pharmaceutical product.

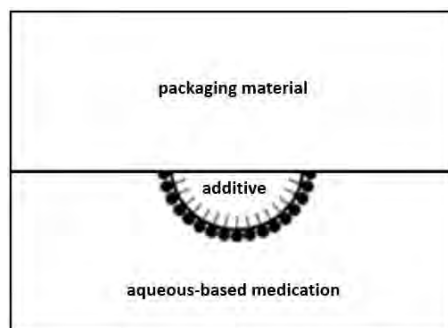


Figure 3. Interaction scheme between packaging material additives and surfactant excipients present in the formulation.

On the other hand, excipients with a low $\log P_{o/w}$ value and no surfactant properties, such as propylene glycol, have low extraction potential, as observed in previous studies that evaluated the impact of the formulation on the extraction of additives.²³ Despite this, these excipients contribute to the migration of compounds with low $\log P_{o/w}$, such as 7,9-di-*tert*-butyl-1-oxaspiro(4,5)dec-6,9-dieno-2,8-dione, which migrated in relevant concentrations.

Organic acids are in higher concentration in the packaging material, but due to their high $\log P_{o/w}$ value, which reflects their hydrophobic nature, only the excipient benzalkonium chloride was able to extract it in relevant amounts (higher than $1 \mu\text{g g}^{-1}$).

Conversely, 7,9-di-*tert*-butyl-1-oxaspiro(4,5)dec-6,9-dieno-2,8-dione is in lower concentration in the packaging, but due to its low $\log P_{o/w}$, i.e., hydrophilic nature, it migrated in all excipients. For this reason, an accelerated study was conducted with the packaging material in water to segregate the impact of the excipient's extraction power and its solubility in water. The experiment resulted in the extraction of only $1 \mu\text{g g}^{-1}$ of the additive; therefore, it can be concluded that the increase in additive concentration is due to the excipient present in the solution.

Thus, although the concept of “like dissolves like” is well-established, when it comes to excipients in aqueous formulations stored in plastic packaging, it can be inferred that the effect caused by the surfactant is dominant in the solubilization and extraction of additives from the packaging material. Although $\log P_{o/w}$ provides general insight into compound polarity, it does not quantitatively predict extractive behavior in this study. This is due in part to surfactants, which have a range of $\log P_{o/w}$ values, and because small variations in $\log P_{o/w}$ did not significantly influence leaching, preventing a linear correlation from being established.

pH

In aqueous formulations/simulants, the pH of the solution in contact with the packaging can directly alter the extractables profile of the study, as it modifies the partition coefficient, which is directly related to the solubility of an additive. This behavior is due to the fact that the solubility of the dissociated form of an acid or base is higher than the solubility of the undissociated molecules, so the solubility of an acidic or basic substance is pH-dependent.²⁴ Therefore, solutions with high pH values tend to favor the migration of acidic molecules, since under these conditions, these molecules are in their ionized form and consequently dissolve well in aqueous media. On the other hand, acidic solutions favor the migration of basic additives. Because of this, a correlation was made between the pH of aqueous excipient solutions and the pKa of the additives to investigate a possible link between the results. The pH of the propylene glycol, mint flavor, and benzalkonium chloride solutions are 5.6, 4.6, and 3.8, respectively. In practice, the behavior predicted by theoretical concepts was not observed in the results for palmitic acid (pKa: 4.75)¹⁹ and stearic acid (pKa: 5.50),¹⁹ which were extracted in higher proportions precisely in the more acidic medium (benzalkonium chloride surfactant mixture).

Although no pKa is available, the compound 7,9-di-*tert*-butyl-1-oxaspiro(4,5)dec-6,9-dieno-2,8-dione does not have acidic or basic groups. However, there is a chemical reaction occurring at high pH due to basic hydrolysis,²⁵ as demonstrated in Figure 4. Since both solutions are at slightly acidic pH, there is no significant impact from this variable. However, it is crucial to highlight that this compound was semi-quantified exclusively by gas chromatography coupled to mass spectrometry, a method that may have underestimated the results, thereby increasing the degree of criticality, given the finding of high concentrations.

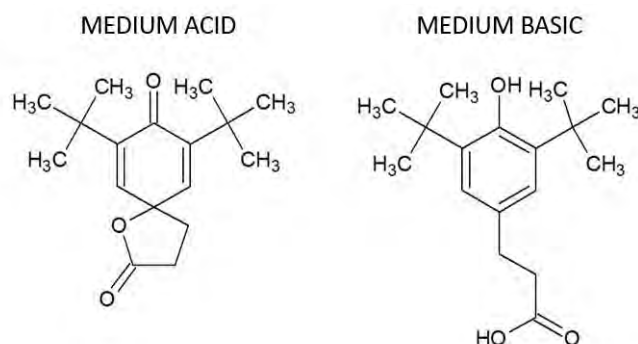


Figure 4. Comparison between the molecular structures of the two species of 7,9-di-*tert*-butyl-1-oxaspiro(4,5)dec-6,9-dieno-2,8-dione existing in aqueous medium at different pH values. Ref. No. 19: ACD/Labs Percepta Platform, PhysChem Module, version 14.00, accessed in April 2025, when it was available free of charge.

Therefore, these findings confirmed a lesser impact of extreme pH on the leaching of target compounds in relation to the partition coefficient of the excipient present in the aqueous solution.

Toxicity

In the toxicological context, organic acids are not carcinogenic, mutagenic, or genotoxic, and are classified as class I in the Cramer rule. Therefore, their maximum daily dose is 1,800 $\mu\text{g person}^{-1} \text{ day}^{-1}$, which is a relatively high limit. On the other hand, 7,9-di-*tert*-butyl-1-oxaspiro(4,5)dec-6,9-dieno-2,8-dione, although not carcinogenic, mutagenic, or genotoxic, is classified as class III in the Cramer rule, with a maximum daily dose of 90 $\mu\text{g person}^{-1} \text{ day}^{-1}$.²⁶ Available data indicate that both additives can be irritating to the skin, eyes, and respiratory tract. Therefore, depending on the concentration, this compound could be critical for eye drops, dermal solutions, and inhalation products.²⁷ Due to these warnings and the personalized assessment based on the route of administration and the maximum daily dose of the medication, a toxicological evaluation by a specialist is required, as the Cramer rule only covers general toxicity aspects, and a broader approach is essential for the safety of the medication.

The toxicological assessment presented herein is intended as a general framework, since patient exposure and safety thresholds (AET) depend on factors such as the maximum daily dose of the active pharmaceutical ingredient and the volume of the packaging material. Therefore, a product-specific evaluation is required for precise risk assessment.

CONCLUSIONS

Liquid pharmaceutical medications stored in plastic packaging are critical in terms of product safety due to the high interaction between the packaging material and the formulation, which increases the likelihood of additive migration from the polymer. This behavior can be exacerbated depending on the excipients used in the formulation, which can have a significant influence on the leaching process. In this context, the extraction potential of different excipients present in this type of pharmaceutical form in contact with high-density polyethylene was evaluated for some additives, aiming to establish correlations between the physicochemical properties of excipients and leachables during the leaching process.

Benzalkonium chloride, an excipient with the highest log $P_{o/w}$ value, proved to be an effective extractor, even at concentrations typically used in formulations (0.02%), demonstrating the high extraction capacity of additives from surfactant compounds. On the other hand, overall, mint flavor and propylene glycol exhibited low extraction potential under the different conditions evaluated, which can be attributed to the lower log $P_{o/w}$ values of these excipients. Therefore, the leaching process demonstrated a greater impact from the physicochemical properties of the excipients. Hence, this study demonstrates that it is possible to predict the leaching of compounds from the packaging into the aqueous product by correlating the log $P_{o/w}$ data of the additives identified in the polymer extractables study and the excipients of the aqueous formulation.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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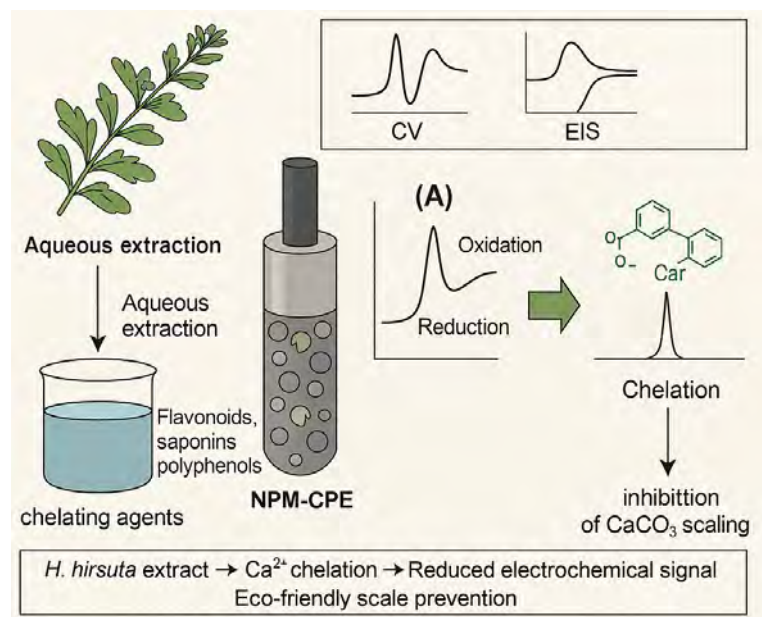
ARTICLE

Development of an Electrochemical Sensor for the Treatment of Scaling in the Presence of *Herniaria hirsuta*

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The formation of calcium carbonate (CaCO₃) scale presents a significant challenge in various industrial systems, impairing efficiency and increasing maintenance costs. In pursuit of environmentally friendly solutions, this study investigates the chelating potential of *Herniaria hirsuta* extract, a plant known for its rich phytochemical profile against Ca²⁺ ions, using an electrochemical approach. A natural phosphate-modified carbon paste electrode (NPM-CPE) was developed and characterized via cyclic voltammetry (CV), square wave voltammetry (SWV), and electrochemical impedance spectroscopy (EIS). The NPM-CPE sensor demonstrated enhanced electrochemical performance and clear detection of Ca²⁺ ions through defined redox peaks. Upon introduction of the *Herniaria*

hirsuta extract, these signals were significantly attenuated, indicating effective complexation and chelation of calcium ions. The EIS results further confirmed this interaction by showing increased electron transfer resistance in the presence of the plant extract. These findings highlight the potential of *Herniaria hirsuta* as a green, cost-effective agent for scale prevention and underscore the synergy between natural resources and electrochemical sensing technologies for sustainable industrial applications.

Keywords: *Herniaria hirsuta*, Ca²⁺ chelation, scale inhibition, electrochemical sensor, NPM-CPE, voltammetry, EIS, green chemistry

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INTRODUCTION

The formation of mineral deposits, particularly calcium carbonate (CaCO_3), represents a major problem in many industrial systems such as cooling circuits, boilers, and heat exchangers. This phenomenon, known as scaling, leads to reduced energy efficiency, increased maintenance costs, and a significant environmental impact.^{1–3} In response to these challenges, industries are increasingly turning toward sustainable and eco-friendly solutions capable of preventing the precipitation and accumulation of mineral salts.

Within this context, the use of natural materials and environmentally benign processes aligns with the principles of green chemistry. Medicinal plants, rich in bioactive compounds, offer great potential for developing low-cost, biodegradable scale inhibitors. Among these, *Herniaria hirsuta* L. traditionally used in North African medicine for the treatment of kidney stones, has attracted particular attention due to its abundance of polyphenols, flavonoids, and saponins, which are known for their strong metal-ion chelating abilities.^{4–8}

Several studies have demonstrated the effectiveness of *Herniaria hirsuta* in preventing or reducing calcium oxalate crystallization, thus confirming its complexing activity toward calcium ions.^{4–7} Meanwhile, recent works have employed voltammetry and electrochemical impedance spectroscopy to investigate metal–ligand interactions or to develop electrochemical sensors sensitive to various metal cations.^{9–15}

The use of modified electrodes especially carbon paste electrodes doped with natural materials such as natural phosphate has shown significant improvements in conductivity, active surface area, and selectivity toward metal ions.^{11,21}

These approaches have enhanced understanding of adsorption and complexation phenomena; however, they have mainly focused on analytical detection or metal corrosion, without integrating natural materials with an environmental purpose for scale prevention.

Despite these advances, several limitations remain. Most studies on *Herniaria hirsuta* have concentrated on its biomedical applications (kidney stone prevention) rather than its potential industrial use as a green antiscaling agent. Few investigations have attempted to couple the plant's chelating properties with electrochemical platforms to monitor, in real time, interactions between Ca^{2+} ions and bioactive compounds. Furthermore, existing studies on modified electrodes have not explored the incorporation of natural phosphate, a local, inexpensive, and eco-friendly mineral resource into the conductive matrix to enhance sensor sensitivity and durability.

Thus, the synergistic combination of *Herniaria hirsuta* and a natural phosphate–based electrochemical sensor remains unexplored, although it could provide an innovative and sustainable pathway for calcium scale prevention.

The present study aims to develop and characterize an electrochemical sensor based on a natural phosphate–modified carbon paste electrode (NPM-CPE) to evaluate, through voltammetry and electrochemical impedance spectroscopy, the complexation behavior of Ca^{2+} ions in the presence of an aqueous extract of *Herniaria hirsuta*.

The main objective is to demonstrate the plant's ability to trap calcium ions, thereby validating its potential as a natural antiscaling agent. This work introduces a dual innovation: the integration of a local mineral material (natural phosphate) to enhance electrochemical performance, and the use of a multifunctional plant extract for eco-friendly scale control.

Hence, this study proposes an original approach combining natural resources and advanced electrochemical technologies to develop green and economically viable solutions for industrial scale prevention.

Herniaria hirsuta

Herniaria hirsuta L., commonly known as Hairy Rupturewort in English, is referred to as Hashishat Al-Fatik, Marda, Noman Amrad, or Dizama in Arabic-speaking regions. It is also recognized by various vernacular names such as Herniaire velue (French), Behaartes Bruchkraut (German), and Luddknytling (Swedish).¹⁶

This species exhibits a broad geographical distribution, extending across multiple continents. In Africa, it is found in countries such as Morocco, Algeria, Egypt, and Ethiopia. In Asia, it is present in the Middle East

and Central Asia, including Iran, Iraq, Syria, Lebanon, Jordan, Palestine, Turkey, Armenia, Azerbaijan, the Russian Federation, Kuwait, and the Central Asian republics of Kyrgyzstan, Tajikistan, Turkmenistan, and Uzbekistan. Its range also spans large portions of Europe, with recorded occurrences in Austria, Belgium, Germany, Switzerland, Hungary, Slovakia, Albania, Bulgaria, Croatia, Greece, Italy, North Macedonia, Romania, Slovenia, France, Spain, and Portugal.¹⁷ Such widespread distribution underscores the ecological adaptability and ethnobotanical significance of *Herniaria hirsuta* across diverse climatic and cultural regions (Figure 1).



Figure 1. *Herniaria hirsuta*.

Chemical constituents

Phytochemical profiling of *Herniaria hirsuta* reveals a remarkable abundance of bioactive secondary metabolites, notably phenolic compounds, flavonoids, flavonols, and saponins. Quantitative analyses indicate that the plant possesses a total flavonoid content of approximately 4.51%, while the saponin content reaches 12.74%.¹⁸ In lyophilized aqueous infusions, high concentrations of specific phytoconstituents were recorded, including phenolics (90 ± 1 mg GAE^a/g), flavonoids (46 ± 3 mg CE^a/g), esters (38 ± 1 mg CAE^a/g), and flavonols (26 ± 1 mg QE^a/g).¹⁹ Moreover, both ethanolic and aqueous extracts of *H. hirsuta* exhibit substantial levels of these compounds. The ethanolic extract contained 28.2 mg GAE/g of phenolics, 4.6 mg CE/g of flavonoids, 12.1 mg GAE/g of tannins, 3.4 mg C₃GE^a/g of anthocyanins, and 16.2 mg QSE^a/g of saponins. The corresponding aqueous extract displayed slightly lower but still significant concentrations: 22.4 mg GAE/g (phenolics), 3.7 mg CE/g (flavonoids), 8.2 mg GAE/g (tannins), 3.8 mg C₃GE/g (anthocyanins), and 8.4 mg QSE^a/g (saponins).²⁰ These findings confirm the rich phytochemical composition of *Herniaria hirsuta*, highlighting its potential for diverse pharmacological and environmental applications.

MATERIALS AND METHODS

Electrochemical measurement

This section presents an in-depth analysis of the electrochemical investigations performed to elucidate the complexation behavior of *Herniaria hirsuta* extract toward Ca²⁺ ions, employing natural phosphate-modified carbon paste electrodes (NPM-CPE) as sensitive electrochemical sensors.

Reagents and chemicals

All reagents used in this investigation were of analytical grade and employed without further purification. Spectroscopic-grade graphite powder (RWB, Rings Dorff-Werke GmbH, Bonn-Bad Godesberg, Germany) was procured from Aldrich. Calcium nitrate (Ca(NO₃)₂) was supplied by Merck Chemicals. Deionized water

^aGAE: gallic acid equivalents; CE: catechin equivalents; CAE: citric acid equivalents; QE: quercetin equivalents; C₃GE: cyanidin-3-glucoside equivalents; QSE: Quillaja saponin equivalents.

was consistently used for the preparation of all solutions to ensure the highest purity standards. Additionally, a plant-based extract derived from *Herniaria hirsuta* was incorporated as a functional component in the experimental protocol.

Electrochemical apparatus

Electrochemical investigations were carried out using a Voltalab PGSTAT 100 potentiostat (Eco Chemie B.V., Utrecht, Netherlands), operated via the Voltalab Master 4 software suite for data acquisition and analysis. All measurements were performed in a conventional single-compartment electrochemical cell configured with a three-electrode system. The saturated calomel electrode (SCE) was employed as the reference electrode, while a platinum wire functioned as the counter electrode. All reported potentials are referenced against the SCE. The working electrode consisted of a carbon paste electrode (CPE) modified with the appropriate active material, tailored for enhanced electrochemical performance.

Electrode preparation

The fabrication of the carbon paste electrode (CPE) involved the thorough homogenization of graphite powder with paraffin oil to obtain a conductive and malleable matrix. For the preparation of the natural phosphate-modified carbon paste electrode (NPM-CPE), an equivalent proportion of graphite was systematically replaced by finely ground natural phosphate. Subsequently, paraffin oil was incorporated to achieve the desired paste consistency. The composite was meticulously blended using a mortar and pestle to ensure uniform dispersion of the components. The resulting paste was then tightly packed into the electrode cavity, and its surface was carefully polished to obtain a smooth and reproducible working interface.²¹

Electrochemical measurement and characterization

In order to investigate the electrochemical performance of the modified electrodes and elucidate the complexation capability of *Herniaria hirsuta* extract toward Ca^{2+} ions, a comprehensive suite of electrochemical techniques was employed, including cyclic voltammetry (CV), square wave voltammetry (SWV), and electrochemical impedance spectroscopy (EIS). These methods provided valuable insights into the interfacial charge transfer processes, enabling a detailed analysis of the redox behavior and the coordination interactions between Ca^{2+} ions, the phytochemical constituents of the *H. hirsuta* extract, and the surface of the functionalized electrodes.

Extraction of *Herniaria hirsuta*

The extraction of bioactive constituents from *Herniaria hirsuta* leaves was carried out using a solid–liquid extraction technique. Precisely 50 g of finely ground leaf material was introduced into an Erlenmeyer flask, followed by the addition of 500 mL of solvent, maintaining a 1:10 (w/v) ratio to ensure efficient mass transfer. The flask was hermetically sealed with aluminum foil to minimize solvent evaporation and protect light-sensitive compounds. Two extraction methods were employed: maceration at ambient temperature with continuous magnetic stirring for 24 hours, or reflux-assisted extraction at 60–70 °C for 2–3 hours to enhance compound solubilization. Upon completion, the extract was subjected to vacuum or gravity filtration through Whatman filter paper to separate the liquid phase from the plant residue. To ensure maximal yield, the remaining solid matrix was washed with a small volume of fresh solvent. The combined filtrates were subsequently concentrated under reduced pressure using a rotary evaporator at low temperature, thus preserving thermolabile phytochemicals. The resulting concentrated extract was collected in a sterile, amber glass container and stored at low temperature in the absence of light to maintain its chemical stability and bioactivity (Figure 2).



Figure 2. Schematic illustration of the extraction process of the *Herniaria hirsuta* plant.

RESULTS AND DISCUSSION

Cyclic voltammetry and square wave voltammetry experiments

Figure 3 shows a cyclic voltammogram (CV) recorded in a KCl solution using carbon paste electrodes (CPE) and natural phosphate-modified carbon paste electrodes (NPM-CPE) at a scan rate (SR) of 50 mV/s. The comparison of the CVs for the two types of electrodes (CPE and NPM-CPE) allows us to observe the differences in their electrochemical behavior. Typically, NPM-CPE electrodes are expected to demonstrate superior electrochemical performance due to their larger active surface area and enhanced conductivity compared to traditional CPE electrodes.

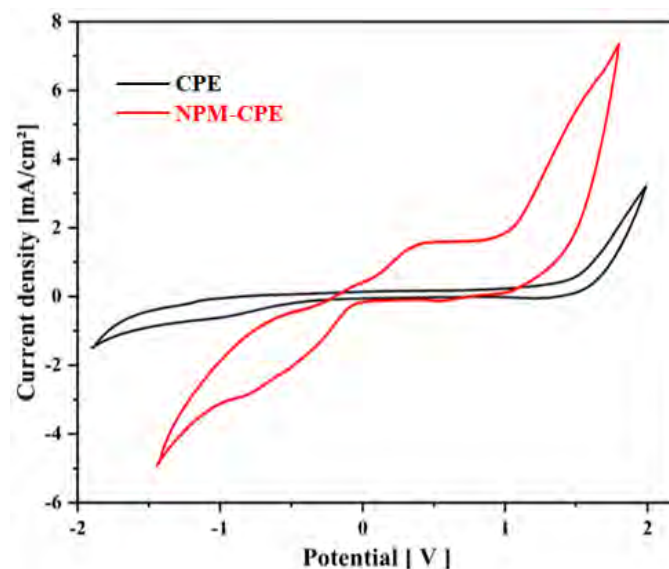


Figure 3. CV recorded in a KCl solution, at CPE and NPM-CPE, with a SR of 50 mV/s.

The positions and intensities of the oxidation and reduction peaks in the voltammograms can provide information about the electron transfer processes occurring at the electrode surface. Sharper and more defined peaks in the case of NPM-CPE would show better reaction kinetics. The analysis of peak currents (the maximum current values during oxidation and reduction) can give an indication of the electrode's ability to catalyze redox reactions. An NPM-CPE electrode would generally show higher peak currents due to its larger active surface area and greater catalytic efficiency. The shape of the curves in the CV can also

provide insights into the reaction mechanisms and diffusion phenomena occurring at the electrode surface. A more symmetrical shape and closer peak separation in the case of NPM-CPE may mention faster reaction kinetics and better electrochemical reversibility. These results are confirmed by square wave voltammetry in Figure 4.

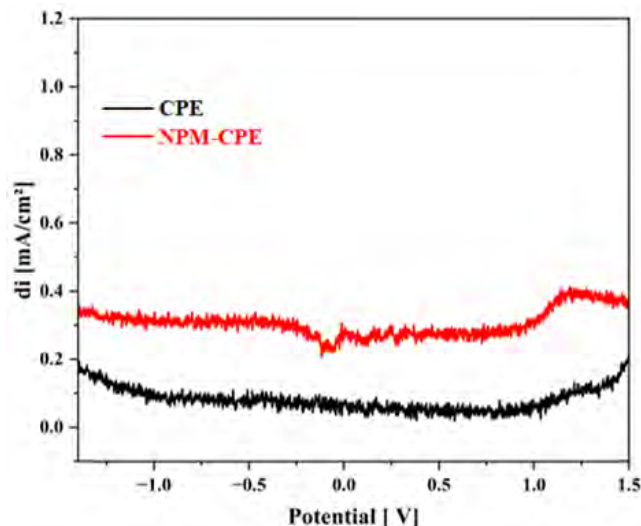


Figure 4. SWV recorded in a KCl solution, at CPE and NPM-CPE, with a SR of 50 mV/s.

Electrochemical response of the CPE electrode to Ca^{2+} ions

The cyclic voltammogram (CV) obtained at the surface of the unmodified carbon paste electrode (CPE) in an electrolyte medium containing Ca^{2+} ions (Figure 5) exhibits a characteristic passive electrochemical behavior toward calcium species. This is evidenced by the absence of any discernible redox peaks within the potential window explored, indicating that under the experimental conditions employed, calcium ions do not undergo electron transfer reactions at the bare CPE surface. The lack of electroactivity represent either the high thermodynamic stability of Ca^{2+} ions in solution, a sluggish electron transfer kinetics, or an insufficient affinity of the unmodified electrode surface for calcium ion complexation or adsorption. This passive response underlines the necessity of electrode surface modification to facilitate any possible electrocatalytic or sensing activity toward Ca^{2+} detection.

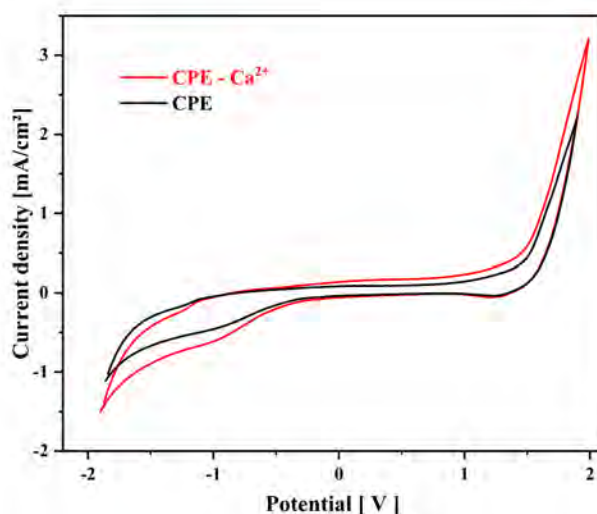


Figure 5. CV recorded in KCl, with and without Ca^{2+} ions, at CPE, at a scan rate of 50 mV/s.

Electrochemical response of the NPM-CPE electrode to Ca^{2+} ions

The electrochemical signature of Ca^{2+} ions in the electrolytic medium is clearly evidenced in the cyclic voltammogram (CV) recorded for the NPM-CPE electrode (Figure 6). Two well-defined redox processes are observed, highlighting the active participation of calcium species in the interfacial electrochemical phenomena.

The first redox feature appears at approximately -0.2 V during the cathodic scan, near the end of the potential window. This peak can be attributed to the reduction of Ca^{2+} ions to metallic calcium. The well-resolved nature of this cathodic signal as a result a relatively facile and localized electron transfer process involving Ca^{2+} at the electrode–electrolyte interface.

Upon reversing the scan direction, a broad anodic peak is recorded around 1.2 V. This anodic process corresponds to the oxidation of metallic calcium previously deposited during the cathodic scan. The breadth of this oxidation peak may reflect a distribution of calcium species in different chemical or physical environments, likely due to their partial entrapment within the natural phosphate matrix. The heterogeneous nature of the oxidation process is indicative of a complex solid-state dissolution mechanism rather than a simple surface-limited redox reaction.

Taken together, the presence of these two redox peaks strongly supports the reversible intercalation and deintercalation behavior of Ca^{2+} ions within the natural phosphate-modified carbon paste electrode (NPM-CPE), affirming the electrode's potential for calcium-ion electrochemistry applications.

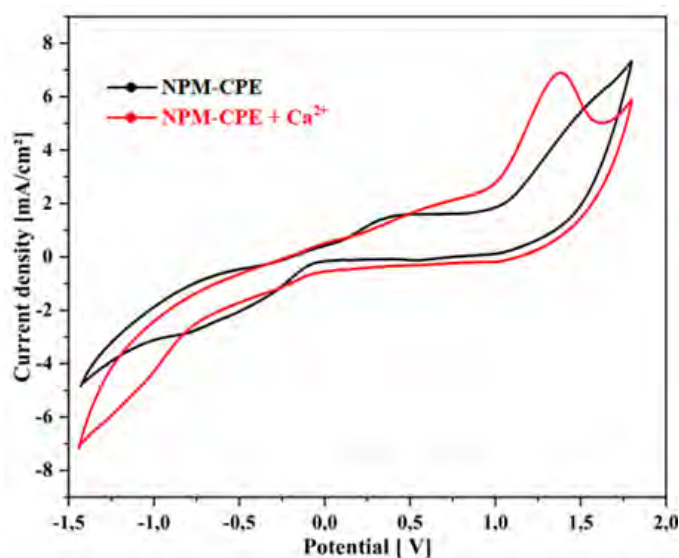


Figure 6. CV recorded in KCl, with and without Ca^{2+} ions, at NPM-CPE, at a scan rate of 50 mV/s.

Complexation effect of *Herniaria hirsuta* plant extract on Ca^{2+} ions

In this experiment, the extract of the plant *Herniaria hirsuta* was introduced into the KCl medium containing Ca^{2+} ions, and subsequently, CVs were recorded on the NPM-CPE surface. Notably, the addition of the *Herniaria hirsuta* plant extract to KCl with Ca^{2+} ions led to the disappearance of redox peaks in the CV. This observation strongly advises that the *Herniaria hirsuta* plant extract effectively chelated the Ca^{2+} ions.

Figure 7 represents the CV of the modified electrode with Ca^{2+} ions under similar conditions. The apparent differences in the shapes of these CVs may be due to variations in experimental conditions or specific characteristics of the studied systems. The differences observed express the possibility of strong complexation of Ca^{2+} ions upon the addition of the *Herniaria hirsuta* plant extract, although the oxidation potential mentioned in Figure 8 was around 1.2 V.

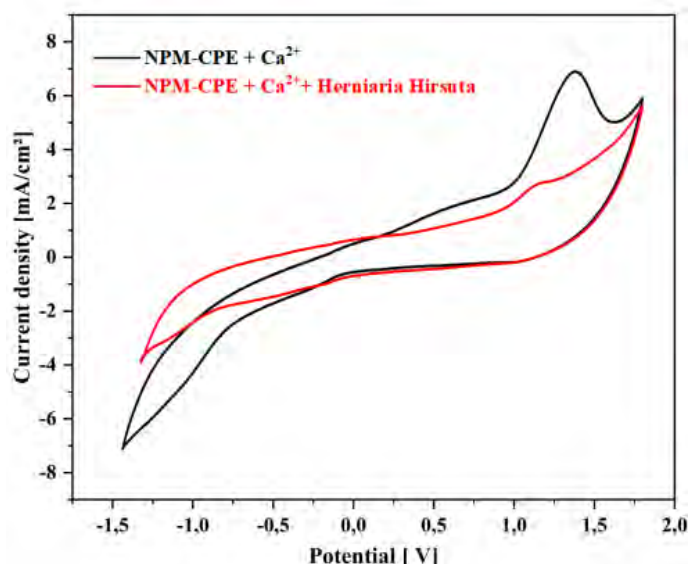


Figure 7. CV was recorded in KCl solutions, in the presence of Ca^{2+} , with and without *Herniaria hirsuta*, at a scan rate of 50 mV/s.

The square wave voltammetry (SWV) highlighted the influence of *Herniaria hirsuta* on Ca^{2+} ions, revealing a well-defined redox signal (Figure 8) that almost disappeared after the introduction of the plant extract. The attenuation of the peak around 1.2 V on the cyclic voltammetry (CV) curve in the presence of *Herniaria hirsuta* represents a significant finding. This alteration may be related to specific electrochemical interactions induced by the plant, suggesting the need for further studies to better understand the underlying mechanisms.

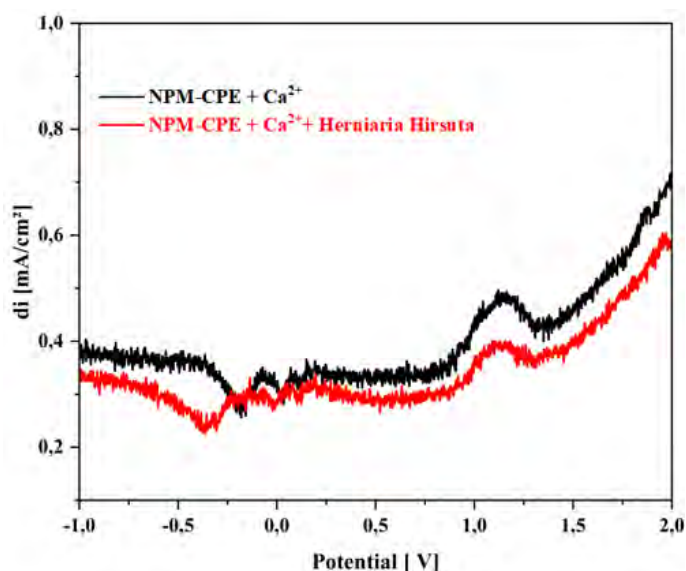


Figure 8. SWV was recorded in KCl solutions, in the presence of Ca^{2+} , with and without *Herniaria hirsuta*, at a scan rate of 50 mV/s.

Electrochemical Impedance Spectroscopy (EIS) analysis provided deeper insight into the chelation mechanism of Ca^{2+} ions by the *Herniaria hirsuta* extract (Figure 9). The Nyquist plots revealed a significant increase in the semicircle diameter upon the addition of the plant extract to the Ca^{2+} containing solution. This increase corresponds to a substantial elevation in the charge transfer resistance (R_{ct}), which is a

direct electrochemical manifestation of hindered ionic mobility and interfacial electron exchange processes at the electrode surface.

The observed augmentation in R_{ct} can be rigorously interpreted as a consequence of the complexation of Ca^{2+} ions by the bioactive phytochemicals present in the *Herniaria hirsuta* extract likely including flavonoids, saponins, tannins, and other polyphenolic compounds with known metal-chelating properties. The reduction in free, electrochemically active Ca^{2+} species in the bulk solution results in a diminished availability of these cations to participate in interfacial charge transfer reactions, thereby elevating the impedance response.

This chelation phenomenon aligns with the thermodynamic principles governing complex formation, where the stability constants of metal-ligand interactions favor the formation of stable organometallic complexes in solution. The EIS findings thus provide compelling electrochemical evidence supporting the sequestration of Ca^{2+} ions by the phytoconstituents of *Herniaria hirsuta*, reinforcing the extract's potential utility in modulating calcium-mediated processes, including but not limited to, the inhibition of calcium salt crystallization in biological or physicochemical systems.

In summary, the substantial increase in the semicircular domain of the Nyquist spectrum serves as a diagnostic electrochemical fingerprint for the efficient binding and immobilization of Ca^{2+} ions, corroborating the extract's role as a potent natural complexing agent.

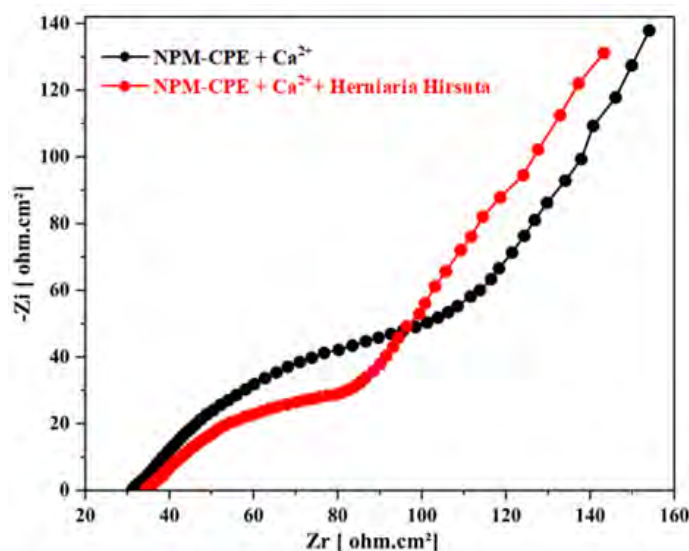


Figure 9. EIS was recorded in KCl solutions, in the presence of Ca^{2+} , with and without *Herniaria hirsuta*, at a scan rate of 50 mV/s.

Figure 10 shows the cyclic voltammetry (CV) curves obtained for the carbon paste electrode (CPE) in the presence of Ca^{2+} ions, with varying concentrations of the aqueous extract of *Herniaria hirsuta* leaves. As the concentration of the *Herniaria hirsuta* extract increases, the oxidation and reduction peaks of Ca^{2+} progressively decrease. At higher concentrations, the reduction peak of Ca^{2+} completely disappears.

This behavior is likely due to the high polyphenol content of the *Herniaria hirsuta* extract. Polyphenols are well-known for their strong metal-chelating properties.²²⁻²⁴ In particular, deprotonated phenolic groups exhibit high charge density, making them hard ligands capable of effectively binding Ca^{2+} ions.

The proposed electrochemical mechanism can be described in three steps:



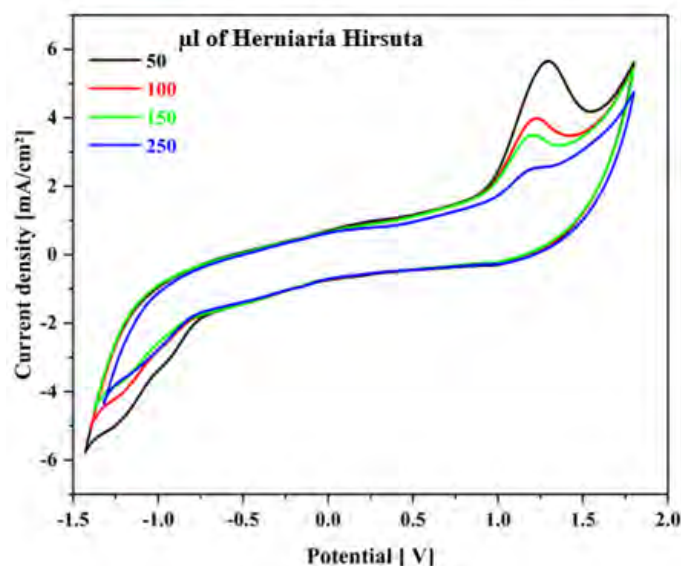


Figure 10. CV were recorded at the NPM-CPE electrode in a 1 M KCl solution containing *Herniaria hirsuta* extract, at a scan rate of 50 mV/s, effect of *Herniaria hirsuta* concentrations.

Figure 11 presents the electrochemical impedance spectroscopy (EIS) spectra recorded for various increasing concentrations of *Herniaria hirsuta* extract, designated as V_1 to V_4 . A detailed analysis of these spectra reveals a progressive increase in the diameter of the capacitive semicircle as the extract concentration increases.

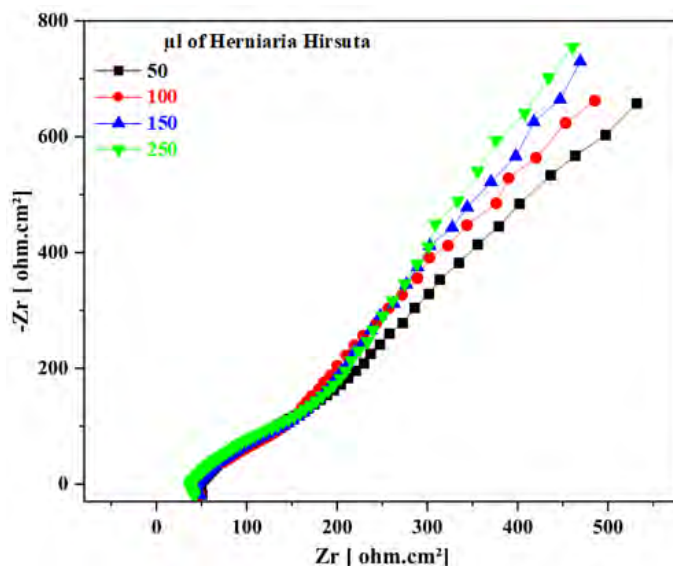


Figure 11. EIS were recorded at the NPM-CPE electrode in a 1 M KCl solution containing *Herniaria hirsuta* extract, at a scan rate of 50 mV/s, effect of *Herniaria hirsuta* concentrations.

This trend highlights a significant rise in the charge transfer resistance (R_{ct}), which is generally interpreted as an improvement in the passivation of the metal surface. This behavior offer that the bioactive compounds present in the extract interact more strongly with the electrode surface at higher concentrations, forming either a more compact protective layer or more stable surface complexes. This enhanced electrochemical

response thus reflects increased inhibitory efficiency, attributable to a more pronounced adsorption of the active constituents onto the metal surface, in accordance with the Langmuir model or a pseudo-linear chemisorptive adsorption behavior.

Table I provides a comprehensive summary of the electrochemical parameters derived from the electrochemical impedance spectroscopy (EIS) data. A pronounced and systematic increase in the double-layer capacitance (C_{dl}) is observed with the progressive addition of *Herniaria hirsuta* extract, indicating enhanced interfacial activity at the metal/solution interface. This trend strongly supports the hypothesis of effective adsorption of phytochemical constituents from the extract onto the metallic surface. From a physicochemical standpoint, the observed rise in C_{dl} reflects a substantial alteration of the interfacial structure, likely due to the formation of a more compact, homogenous, and dielectric-rich adsorption layer. The increased dielectric constant and the reduced effective thickness of the electrical double layer, as described by the Helmholtz model, point to the accumulation of organic molecules at the interface, thereby increasing the surface coverage and modifying the interfacial capacitance. This behavior is in strong agreement with the results previously obtained from polarization curves and weight loss measurements, both of which demonstrated improved corrosion inhibition efficiency with increasing extract concentration. Mechanistically, the enhancement in C_{dl} serves as an indirect yet reliable indicator of the adsorption of active inhibitor molecules, proposing their ability to form coordination bonds or π -d interactions with the metal surface. This leads to the development of a protective barrier that impedes charge transfer processes, thus mitigating corrosion reactions. Collectively, these electrochemical findings substantiate the effectiveness of *Herniaria hirsuta* extract as a potent green corrosion inhibitor and reinforce the conclusions drawn from complementary experimental techniques.

Table I. Electrochemical parameters from EIS

Drops	Diameters (Ohm.cm^2)	Correlation	R_1 (Ohm.cm^2)	R_2 (Ohm.cm^2)	$R_2 - R_1$ (Ohm.cm^2)	C ($\mu\text{F/cm}^2$)
1	368.4	0.957	50.01	337	286.99	336.2
2	626.3	0.992	43.64	530.2	486.56	213.7
3	642.9	0.997	42.54	551.5	508.96	205.4
4	666	0.998	36.73	574.1	537.37	197.3

CONCLUSION

This study rigorously demonstrates the effectiveness of an electrochemical sensor based on carbon paste modified with natural phosphate (NPM-CPE) for the detection of calcium ions (Ca^{2+}) in aqueous media. The sensor was developed through the synergistic integration of low-cost, naturally derived materials with advanced electroanalytical techniques, aiming to achieve high sensitivity and selectivity toward divalent cations.

Electrochemical characterization using cyclic voltammetry (CV), square wave voltammetry (SWV), and electrochemical impedance spectroscopy (EIS) revealed distinct redox peaks corresponding to the presence of Ca^{2+} ions. These responses demonstrate a strong electrochemical affinity of the NPM-CPE sensor for calcium ions, as well as efficient electron transfer kinetics at the electrode–solution interface key indicators of the sensor's analytical performance.

The addition of an aqueous extract of *Herniaria hirsuta*, a plant known for its anti-lithiasis properties, led to a marked decrease or disappearance of electrochemical signals from free Ca^{2+} , indicating chelation by phytochemicals such as flavonoids or saponins. This was further supported by EIS measurements showing increased charge transfer resistance (R_{ct}), consistent with reduced free calcium availability and the formation of electrochemically inactive complexes.

These findings position *Herniaria hirsuta* as a promising natural calcium chelator, useful for preventing scale in water systems. Its phytochemical interactions with metal ions support sustainable, eco-friendly solutions integrating local resources and modern electrochemical sensing, in line with green chemistry principles.

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Conflicts of interest

The authors declare that there are no conflicts of interest, whether personal or financial, that might have impacted the findings or publication of this study.

Use of artificial intelligence tools

The authors declare that they have used Grammarly for language editing during manuscript preparation. The authors take full responsibility for the accuracy, integrity, and originality of the article and confirm that artificial intelligence was used only for language editing.

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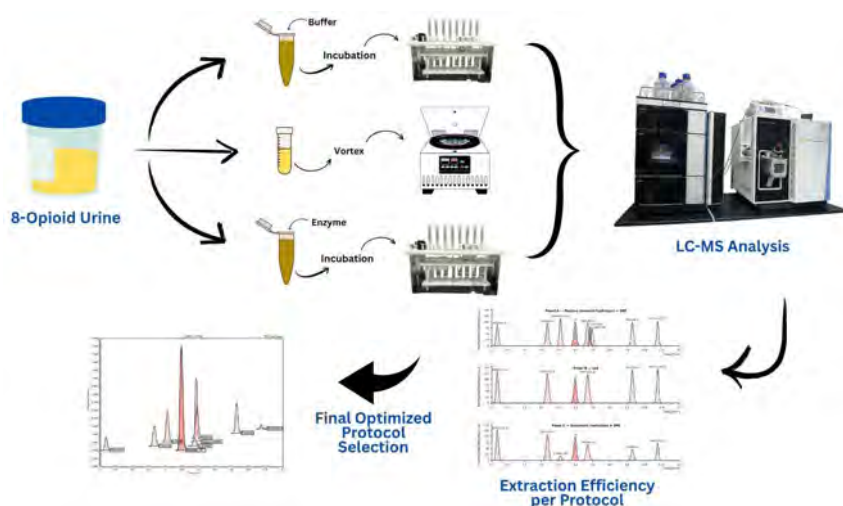
ARTICLE

Comprehensive Extraction Optimization and LC–MS Validation for Detection of Illicit and Prescription Opioids in Human Urine

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The misuse of opioids, including fentanyl, methadone, and heroin, has increased the demand for reliable and economical toxicological screening techniques. In resource-limited forensic settings, conventional approaches, such as enzymatic hydrolysis combined with tandem mass spectrometry (MS/MS), are often impractical. This study aimed to develop, optimize, and validate a reliable liquid chromatography–mass spectrometry (LC–MS) method for the quantification of eight opioids in urine using a single-quadrupole mass

spectrometer. Three sample preparation strategies were comparatively evaluated: enzymatic hydrolysis followed by solid-phase extraction (SPE), liquid–liquid extraction (LLE), and passive chemical hydrolysis followed by SPE. Analyte stability (up to 96 h), carryover, matrix effect (ME), linearity, precision, and accuracy were assessed during method validation in accordance with the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The most consistent recovery (RE) was obtained with passive chemical hydrolysis followed by SPE at 54 °C for 6 h, with RE values ranging from $82.0 \pm 2.1\%$ to $114.4 \pm 3.0\%$, whereas heroin showed a slightly lower RE ($78.0 \pm 3.4\%$) because of partial hydrolysis to 6-monoacetylmorphine and morphine. The method demonstrated excellent linearity, with the coefficient of determination (R^2) ranging from 0.991 to 0.998, good precision, expressed as relative standard deviation ($\%RSD$) $\leq 16.8\%$, and acceptable bias (-13.0%

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to 18.0%). All analytes exhibited ME values within 80–120%, indicating negligible ion suppression or enhancement. The applicability of the method to real-world samples was confirmed in urine casework: one sample tested positive for tramadol, whereas two were negative, supporting the method's forensic and clinical utility. Overall, the optimized single-quadrupole LC–MS method provides a reliable, cost-effective, and selective alternative for urinary opioid analysis, suitable for routine use in low-resource laboratories owing to its enzyme-free extraction and reliance on a single-quadrupole mass spectrometer.

Keywords: cost-effective analysis, method validation, single-quadrupole LC–MS, urine, opioid detection

INTRODUCTION

The International Narcotics Control Board (INCB) has reported a concerning increase in counterfeit opioid drugs, particularly in underdeveloped regions, where approximately 25–50% of dispensed prescription drugs may be fraudulent, thereby intensifying the risks associated with opioid use.^{1,2} The cultural, economic, and criminal ramifications of the opioid crisis highlight the pressing need for robust analytical frameworks. Opioid-related deaths are rising sharply, placing a significant burden on healthcare systems and law enforcement worldwide. Heroin and fentanyl, along with their analogs, are primary drivers of this trend.^{3–7} The spread of illicit synthetic opioids has increased drug trafficking and violent and organized crime, thereby worsening societal instability.^{6,7} Given the widespread availability and increased potency of synthetic opioids, the forensic community has an ongoing need to improve toxicological methods that accurately identify and differentiate illicit opioids, prescription opioids, and their metabolites.^{8–10}

In forensic investigations, identifying opioids in biological samples is crucial for evaluating drug use and intoxication, and for determining the cause of death, especially in cases involving overdoses, drug-facilitated crimes, and impaired-driving incidents.¹¹ The urinary marker 6-monoacetylmorphine provides definitive evidence of heroin use, distinguishing it from morphine use and ingestion of poppy seeds.^{12–14} 6-acetylcodeine, an impurity in illicit heroin production, also serves as a forensic marker for heroin abuse.^{14–16} Routine opioid screening in forensic and clinical toxicology is vital for assessing treatment adherence and identifying potential misuse, particularly for potent opioids such as fentanyl and methadone, which are frequently associated with fatal overdoses.^{3,17}

Advances in analytical methods have revolutionized opioid detection and quantification, with liquid chromatography–mass spectrometry (LC–MS) becoming the preferred technique over conventional gas chromatography–mass spectrometry (GC–MS).^{12,18} LC–MS offers increased sensitivity, selectivity, and flexibility for drug analysis in complex biological matrices, including urine and blood.^{19,20} Urine-based assays typically employ β -glucuronidase hydrolysis with multi-hour incubations,^{21–23} which can be inefficient and may cause loss of labile analytes.^{14,24} Current analytical workflows often rely on tandem mass spectrometry (MS/MS) with multiple reaction monitoring (MRM) for detection, a highly effective yet resource-intensive approach that can limit accessibility in low-resource settings.^{25,26} Rigorous validation is also required to ensure accuracy, precision, and specificity across matrices and conditions, as underscored by international analytical and forensic guidelines.^{27–29}

These gaps motivated the development of a thoroughly validated, economically viable, and optimized single-quadrupole LC–MS method for the reliable quantification of opioids in forensic and clinical urine samples.

The physicochemical properties of the natural opioids (morphine and codeine), the semi-synthetic opioids (6-monoacetylmorphine, 6-acetylcodeine, and heroin), and the synthetic opioids (fentanyl, methadone, and tramadol) guided the development and validation of the LC–MS method. These opioids are weak bases ($\text{pK}_a \approx 8.7\text{--}9.3$), with lipophilicity ranging from that of morphine ($\log K_{ow} \approx 0.9$) to those of methadone and fentanyl ($\log K_{ow} \approx 4.0\text{--}5.0$), and molecular weights spanning 263–369 g mol⁻¹.^{30–32} As shown in Figure 1, structural features that affect analytical behavior include free phenolic hydroxyls in morphine, codeine, 6-monoacetylmorphine, and tramadol (increasing polarity and causing earlier elution on C18); acetyl groups in heroin and 6-acetylcodeine (masking phenols, increasing lipophilicity, enabling retention,

introducing hydrolytically unstable esters); and the overall higher lipophilicity of fentanyl and methadone. All compounds contain basic nitrogen, which facilitates efficient ionization in positive electrospray ionization (ESI) mode. These properties determine the extraction recovery (RE) and ionization efficiency and thus inform the LC–MS method design and validation.^{31–34}

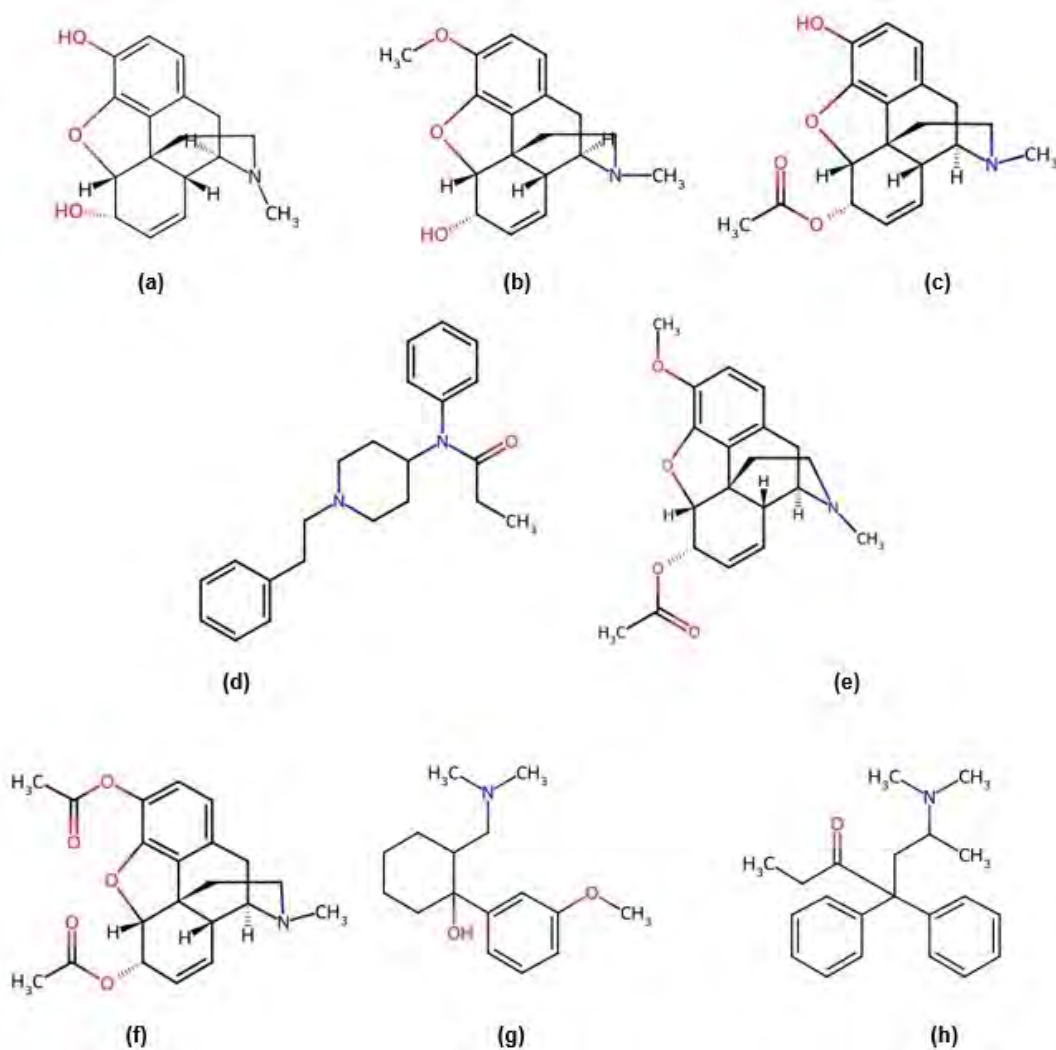


Figure 1. Chemical structures of the eight target opioids analyzed in this study: (a) morphine, (b) codeine, (c) 6-monoacetylmorphine, (d) fentanyl, (e) 6-acetylcodeine, (f) heroin, (g) tramadol, and (h) methadone.

This study presents an innovative and cost-effective single-quadrupole LC–MS assay for the simultaneous analysis of eight opioids in urine. It minimizes the need for expensive MS/MS instrumentation and eliminates the need for β -glucuronidase hydrolysis, offering a practical solution for resource-limited laboratories. Using simple procedures, the method employs passive chemical hydrolysis followed by solid-phase extraction (SPE), and through systematic assessment of incubation times, a 6 h incubation at 54 °C was identified as optimal, yielding higher RE efficiency than shorter or longer durations.^{21–23} Furthermore, the method avoids the routine use of stable isotope-labeled internal standards (SIL-IS), preserves labile opioids, simplifies sample preparation, and enables reliable, consistent, and legally defensible quantification in forensic and clinical contexts.

MATERIALS AND METHODS

Chemicals and equipment

LC–MS grade methanol ($\geq 99.9\%$), ethyl acetate, and acetonitrile were obtained from Thermo Fisher Scientific (Mumbai, India). Ultrapure water (Type I; $18.2 \text{ M}\Omega\cdot\text{cm}$ at 25°C) was produced in-house using the XTRAPURE Plus LabLink water purification system (Mumbai, India). Sigma-Aldrich (Karnataka, India) supplied LC–MS grade acids and buffers, including ammonium formate (LiChropur, $\geq 99\%$), formic acid (98–100%), acetic acid (LiChropur, 100%), and sodium acetate. Additional analytical grade reagents, including ammonia solution ($\geq 25\%$, EMSURE ISO), phosphate buffers, sodium chloride, magnesium sulfate, and sodium azide, were obtained from VWR Chemicals and RANKEM (Gurugram, India). Oasis HLB SPE cartridges (60 mg, 3 mL) were purchased from Waters Corporation (Bengaluru, India). β -glucuronidase (abalone-derived aqueous solution; $\geq 100,000 \text{ U mL}^{-1}$) was obtained from Sigma-Aldrich. Vadilal Chemical Ltd (Gujarat, India) supplied ultra-high purity nitrogen gas (grade 5.0). Cerilliant Corporation (Bangalore, India) provided certified reference standards for the target analytes (morphine, codeine, 6-monoacetylmorphine, tramadol, 6-acetylcodeine, heroin, fentanyl, and methadone). Because SIL-IS were temporarily unavailable, atropine was used as a substitute internal standard (IS). Equipment for sample preparation and storage included a Waters 20-position SPE manifold (Massachusetts, USA), a Labman LMUC25D ultrasonic cleaner (Tamil Nadu, India), a Neuation iTherm D150-4 dry heating block (Gujarat, India), and an ultra-low temperature freezer (MarkEn; -20 to -86°C ; Mumbai, India).

Biological specimen collection and handling

For method development and validation, urine samples were collected from ten healthy laboratory staff volunteers. The samples were screened for the target analytes. Negative specimens were pooled and centrifuged to eliminate particulates. Immediately after collection, sodium azide (0.1% w/v) was added to each urine sample as a preservative to limit microbial growth and endogenous enzymatic activity during storage and handling, thereby minimizing analyte degradation.^{35,36} The pooled blank urine was stored at -20°C until use in further method development and validation. To protect privacy, all demographic information was excluded, and donors provided samples at least two weeks after any medication use to prevent potential interference.

In addition, three urine specimens were obtained from Kanoria Hospital & Research Center, Ahmedabad, Gujarat, India, as part of a rehabilitation monitoring and de-addiction program, and were immediately analyzed using the validated assay. All procedures were approved by the Institutional Ethics Committee (Approval No. NFSU/SDSR/IEC/Certificate/1054/2024).

Preparation of calibration, quality control (QC), and IS solutions

Because of differences in solubility, 6-monoacetylmorphine, 6-acetylcodeine, and heroin were prepared in acetonitrile, whereas morphine, codeine, tramadol, fentanyl, and methadone were prepared in methanol; all stock solutions were prepared at 1.0 mg mL^{-1} . A mixed working solution was then prepared by combining the individual stocks to yield $100.0 \mu\text{g mL}^{-1}$ of each analyte. A working IS solution ($100.0 \mu\text{g mL}^{-1}$) was prepared in methanol by diluting the 1.0 mg mL^{-1} stock solution. Calibration standards (0.5 – $25.0 \mu\text{g mL}^{-1}$) were obtained by serially diluting the mixed working solution in a 90:10 (v/v) diluent consisting of 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid. QC levels at 0.5, 1.0, 10.0, and $20.0 \mu\text{g mL}^{-1}$ were prepared from the same mixed working solution by dilution to the target levels, followed by spiking into pooled blank urine. IS was added to all prepared samples (calibrators, QC samples, and case specimens) to a final concentration of $10.0 \mu\text{g mL}^{-1}$. All study samples were stored at -20°C when not in use.

LC–MS instrumentation and conditions

Chromatographic conditions

A C18 Acclaim 120 Å column ($150 \times 3 \text{ mm}$, $3 \mu\text{m}$) was used for chromatographic separation on a

Vanquish liquid chromatography (LC) system (Thermo Fisher Scientific, USA). The column temperature was maintained at 35 °C, with a 5 μ L injection volume and an autosampler temperature of 8 °C. The binary mobile phases consisted of 10 mM ammonium formate with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient was optimized to effectively separate analytes of differing polarity, starting at 90% A/10% B at 1.0 mL min⁻¹ from 0.00 min, changing to 70% A/30% B at 0.4 mL min⁻¹ at 2.00 min, and then to 60% A/40% B at 8.50 min. At 10.00 min, the flow rate was returned to 1.0 mL min⁻¹ and the mobile phase was changed to 20% A/80% B. From 12.50 to 14.50 min, the system was re-equilibrated to the initial conditions (90% A/10% B). Consistent retention time (RT) and smooth solvent transition were achieved using a gradient curve value of 5.

Mass spectrometric conditions

A single-quadrupole ISQ EC mass spectrometer (Thermo Fisher Scientific, USA) was used for detection in positive ESI mode. The source operated at a vaporizer temperature of 350 °C and an ion transfer tube temperature of 325 °C, a spray voltage of +3500 V, and sheath, auxiliary, and sweep gas pressures of 50, 7.5, and 1.5 psig, respectively. The mass spectrometry (MS) data were acquired in full-scan mode over the mass-to-charge ratio (m/z) range of 30–550 with a scan time of 1 s. Data were then processed as extracted ion chromatograms (EIC). The analytes were identified by RT and two in-source fragment ions (quantifier and qualifier) for each analyte, with further confirmation based on the qualifier-to-quantifier ion ratio.

MS acquisition and ion selection

In the positive ESI mode, the analytes formed protonated molecules $[M+H]^+$. The data were acquired in the full-scan mode and processed as EIC. Specific ions were selected as quantifiers and qualifiers to provide structural information and strong signal intensity under in-source fragmentation conditions. The monitored ions (m/z ; quantifier and qualifier) were: codeine (300.1 and 243.1); morphine (286.1 and 229.0); 6-monoacetylmorphine (328.1 and 211.1); 6-acetylcodeine (342.2 and 225.1); heroin (370.1 and 165.0); fentanyl (188.1 and 337.2); and methadone (265.1 and 105.1). A consistent qualifier ion was not established for tramadol under these conditions (Table II); therefore, identification relied on the quantifier ion together with RT and peak purity checks. Atropine, the IS, was monitored at m/z 290.1 and 124.1. All confirmation limits (RT repeatability and ion ratio tolerance) are detailed in the method development plan under the RT repeatability and ion ratio criteria section.

Optimization of extraction for analyte RE and matrix cleanup

We systematically evaluated three urine extraction methods to optimize analyte RE and matrix cleanup: liquid-liquid extraction (LLE), passive chemical hydrolysis followed by SPE, and enzymatic hydrolysis followed by SPE.

LLE

A 500 μ L aliquot of pooled blank urine was spiked with a QC mixed standard containing the eight analytes and 5 μ L of the IS (100.0 μ g mL⁻¹). The sample was first basified with three drops of 5 N potassium hydroxide, then diluted with 250 μ L of deionized water and mixed for 30 s. Salts were precipitated by adding 0.375 g of sodium chloride and 0.250 g of magnesium sulfate, and the mixture was vortexed for 5 min. Analytes were extracted with 1 mL of ethyl acetate, and the mixture was centrifuged at 3000 rpm for 15 min. The upper organic layer was collected and dried at ambient temperature under a gentle nitrogen stream. The residue was reconstituted in 50 μ L of mobile phase, a 90:10 (v/v) mixture of 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid, centrifuged, filtered, and injected into the LC–MS system.

Passive chemical hydrolysis followed by SPE

A 500 μ L aliquot of pooled blank urine was spiked with a QC mixed standard containing the eight analytes and 5 μ L of the IS (100.0 μ g mL⁻¹), then combined with 250 μ L of 0.1 M acetate buffer (pH 5.6) and incubated

at 54 °C for 6 h. After cooling to room temperature, an equal volume (1:1) of 0.1 M phosphate buffer (pH 6.0) was added. The samples were processed using HLB cartridges preconditioned with 1 mL of methanol and 2 mL of 2% aqueous ammonia. After sample loading, the cartridges were washed with 1.5 mL of 5% methanol, dried under vacuum for 5 min, and sequentially eluted with 0.5 mL of methanol and 0.5 mL of ethyl acetate. The eluate was dried under nitrogen at room temperature, reconstituted in 50 µL of mobile phase consisting of a 90:10 (v/v) mixture of 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid, centrifuged, filtered, and injected into the LC-MS system.

Enzymatic hydrolysis followed by SPE

A 500 µL aliquot of pooled blank urine was spiked with a QC mixed standard containing the eight analytes and 5 µL of the IS (100.0 µg mL⁻¹). Then, 40 µL of β-glucuronidase and 250 µL of 0.1 M acetate buffer (pH 5.6) were added, and the mixture was incubated at 54 °C for 6 h. The sample was cooled to ambient temperature and mixed with an equal volume (1:1) of 0.1 M phosphate buffer (pH 6.0) after hydrolysis. SPE was performed on HLB cartridges preconditioned with 1 mL of methanol and 2 mL of 2% aqueous ammonia. After sample loading, the cartridges were washed with 1.5 mL of 5% methanol, dried under vacuum for 5 min, and sequentially eluted with 0.5 mL of methanol and 0.5 mL of ethyl acetate. The eluate was dried under nitrogen at room temperature, reconstituted in 50 µL of a 90:10 (v/v) mixture of 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid, centrifuged, filtered, and injected into the LC-MS system.

Unless otherwise specified, all QC levels are based on nominal concentrations in the final extracts, corresponding to a 10-fold enrichment relative to the original urine according to the relationship $C_{\text{urine}} = C_{\text{extract}} / 10$, where C_{urine} is the concentration in the original urine and C_{extract} is the concentration in the final extract after 10-fold enrichment; this relationship was also applied to determine the back-calculated urine concentrations of analytes in casework specimens.

Assessment of analyte RE across three extraction methods

Three replicates ($n = 3$) of pooled blank urine samples were spiked and processed, resulting in a final extract concentration of 10.0 µg mL⁻¹ per analyte. To determine analyte-specific extraction efficiency, samples were processed separately for each method (LLE, passive chemical hydrolysis followed by SPE, and enzymatic hydrolysis followed by SPE). The RE for each method was reported as the mean ± standard deviation (SD).

Method development plan

Evaluation of incubation time on analyte RE

Passive chemical hydrolysis followed by SPE was performed at 54 °C to assess the effect of incubation time on analyte RE and to maximize RE, as described in the Optimization of extraction for analyte RE and matrix cleanup section. A 500 µL aliquot of pooled blank urine was spiked with a mixed standard solution containing the eight analytes and the IS, then processed to achieve a nominal concentration of 10.0 µg mL⁻¹ for each analyte and the IS in the final extract. Samples were incubated for 1, 6, 10, or 16 h before extraction. Three independent replicates were prepared at each time point ($n = 3$).

The extraction RE efficiency was calculated according to the Matuszewski protocol³⁷ by comparing the pre-extraction-spiked matrix with the post-extraction-spiked matrix, and the results were expressed as mean ± SD (%). The optimal incubation time was selected to preserve all analytes and provide consistent RE for subsequent validation.

RT repeatability and ion ratio criteria

The chromatographic precision and qualitative confirmation were evaluated under fixed LC-MS conditions, as described in the LC-MS instrumentation and conditions section, using the low QC (LQC) level (0.5 µg mL⁻¹ in the final extract). The data were acquired in full-scan mode and processed as EIC. For

RT repeatability, the same QC was injected ten times ($n = 10$) within a single batch, and RT was recorded and reported as mean $\pm$ SD and relative standard deviation (%RSD) for each analyte. The designated quantifier and qualifier ions for each analyte were extracted as EIC, as described in the MS acquisition and ion selection section. The target ion ratio was determined and compared with those in other QC and unknown samples, with a tolerance of $\pm 20\%$. The RT acceptance criterion required that each analyte's RT in a sample be within $\pm 2\%$ of the calibrator RT established from repeated injections. Tramadol confirmation under single-quadrupole in-source fragmentation, in the absence of a reliable qualifier ion, relied on the quantifier ion, the RT criterion, and chromatographic peak purity checks.

Validation and acceptance criteria

Eight opioids (morphine, codeine, 6-monoacetylmorphine, tramadol, 6-acetylcodeine, heroin, fentanyl, and methadone) were validated in human urine in accordance with standard practices in forensic toxicology and the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The validation assessed selectivity and specificity, the calibration model and linearity, the limits of detection (LOD) and quantification (LOQ), accuracy (bias, %), precision (repeatability and reproducibility), RE, matrix effect (ME), process efficiency (PE), carryover, and the stability of processed samples.^{27,28}

Evaluation of linearity and analytical range

Calibration standards were prepared at five concentrations (0.5, 1.0, 5.0, 10.0, and 25.0 $\mu\text{g mL}^{-1}$). Five replicates were analyzed at each level. The analyte-to-IS peak area ratio was used as the instrument response, and data were processed in Chromeleon Console 7 (version 7.3.1.6535). The linearity was assessed using linear regression with $1/x$ weighting. The analytical range was established based on two criteria: (i) a coefficient of determination (R^2) ≥ 0.990 and (ii) back-calculated concentrations within $\pm 20\%$ of the nominal values across all calibration levels.

Precision and accuracy: repeatability, reproducibility, and bias

Precision and accuracy were assessed in pooled blank urine samples at three QC levels, which corresponded to nominal concentrations of 1.0, 10.0, and 20.0 $\mu\text{g mL}^{-1}$ in the final extracts. Intra-day (repeatability) was evaluated using six replicates for each level ($n = 6$) within a single batch, and inter-day (reproducibility) was evaluated over three consecutive days with 18 replicates ($n = 18$). The response of the instrument was expressed as the ratio of the analyte-to-IS peak area. The accuracy (bias, %) was calculated as $[(\text{observed mean} - \text{nominal value}) / \text{nominal value}] \times 100$, and the precision (%RSD) was calculated as $(\text{SD} / \text{observed mean}) \times 100$. The acceptance criteria required accuracy (bias, %) within $\pm 20\%$ of the nominal value at each QC level and a precision (%RSD) $\leq 20\%$ for both intra-day and inter-day measurements.

Analytical sensitivity and carryover assessment

The analytical sensitivity was evaluated by determining the LOD and LOQ. The background noise at each analyte's RT was measured in ten individual blank urine samples on the MS quantification channel. The blank signal variability and the calibration curve slope were used to calculate the LOD and LOQ. The LOD was defined as $(3 \times \text{SD of blank urine signal}) / \text{slope of the calibration curve}$, and LOQ as $(10 \times \text{SD of blank urine signal}) / \text{slope of the calibration curve}$. Practical evaluation was conducted by spiking pooled blank urine samples and processing them to yield concentrations of 0.310–0.724 $\mu\text{g mL}^{-1}$ in the final extracts, after which five replicates ($n = 5$) were analyzed. The operational LOQ required a signal-to-noise ratio (S/N) ≥ 10 , accuracy (bias, %) within $\pm 20\%$, and precision (%RSD) $\leq 20\%$. Given the 10-fold enrichment from urine to the final extract, the LOQ was also expressed as an effective urine concentration in the Analytical sensitivity and carryover subsection within the Results and discussion section. Qualitative confirmation required that the RT be within $\pm 2\%$ of the calibrator value and that the qualifier-to-quantifier ion ratio be within $\pm 20\%$ of the calibrator's ratio.

Three replicates of pooled blank urine extracts were analyzed immediately after injection of the highest calibrator ($25.0 \mu\text{g mL}^{-1}$) to assess carryover. For each blank extract, the peak area of the quantifier ion was measured at the specified RT for each analyte. Carryover was considered absent if the mean blank peak area was $\leq 10\%$ of the LOQ response on the calibration curve.³⁸

Assessment of ME, RE, PE, and selectivity

ME, RE, and PE were assessed using the Matuszewski protocol.³⁷ Two concentration levels in the final extract were assessed: $1.0 \mu\text{g mL}^{-1}$ at the LQC level and $20.0 \mu\text{g mL}^{-1}$ at the high QC (HQC) level. Three sample sets were prepared: Set 1 (A) comprised neat standards in $50 \mu\text{L}$ of mobile phase (90:10, v/v; 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid); Set 2 (B) comprised post-extraction-spiked samples prepared by adding analytes to extracted pooled blank urine; and Set 3 (C) comprised pre-extraction-spiked pooled blank urine processed using the optimized extraction protocol. Each set at each level was prepared in triplicate ($n = 3$). ME (%) was calculated as $(B / A) \times 100$, RE (%) as $(C / B) \times 100$, and PE (%) as $(ME \times RE) / 100$. Alternatively, PE (%) was calculated as $(C / A) \times 100$. Values within 80–120% were considered acceptable for ME, RE, and PE.

Selectivity was evaluated by analyzing ten individual blank urine samples.^{39,40} Samples were extracted and injected without added analytes or IS to assess potential interferences at the RT of each analyte and at the IS RT by monitoring the relevant MS ions and target ion ratios. A pooled blank urine sample was also spiked with buprenorphine, hydrocodone, hydromorphone, meperidine, naloxone, naltrexone, oxycodone, and oxymorphone, then processed and analyzed. Interference was evaluated in the spiked pooled blank urine sample and blank donor samples. The acceptance criterion for selectivity was the absence of interfering peaks at each analyte's RT and at the IS RT.

Stability of processed urine samples

Stability was assessed using pooled blank urine samples at two QC levels (1.0 and $10.0 \mu\text{g mL}^{-1}$ in the final extracts). Three replicates ($n = 3$) were analyzed at each QC level immediately after extraction to establish the reference concentration at $t = 0$ h. The processed samples were stored at 4°C and analyzed in triplicate at 24, 48, 72, and 96 h. Quantification was performed using new calibration curves prepared at each time point. Stability was expressed as percent change (% change), calculated as $[(\text{mean } C_t - \text{mean } C_0) / \text{mean } C_0] \times 100$, where C_t is the mean concentration at each time point (24, 48, 72, and 96 h) and C_0 is the mean concentration at $t = 0$ h. The stability acceptance criterion was a % change within $\pm 20\%$ of the initial concentration.

RESULTS AND DISCUSSION

Results of method development

Chromatographic separation

The developed LC–MS method demonstrated high chromatographic efficiency and selectivity, achieving baseline separation of all analytes, ranging from moderately polar species (e.g., morphine and codeine) to highly lipophilic species (e.g., methadone and fentanyl), within 11 min (Figure 2).

An Acclaim C18 column maintained at 35°C provided baseline separation for all analytes, with sharp, symmetric peaks despite similarities in structure and retention behavior. A polarity-driven elution trend was observed: morphine (1.42 min) and codeine (4.35 min) eluted early owing to their hydrophilicity ($\log K_{ow} < 1.0$), whereas more lipophilic opioids, including fentanyl (9.31 min) and methadone (10.79 min), were retained longer, consistent with their higher $\log K_{ow}$ values (Figure 1, Figure 2).

The method also resolved closely related analytes, including tramadol (6.70 min), 6-acetylcodeine (6.83 min), and heroin (6.95 min), demonstrating the ability of the method to separate structurally similar opioids. The separation between heroin and its hydrolytic metabolite, 6-monoacetylmorphine, was distinct, enabling precise identification and quantification in urine samples (Figure 2).

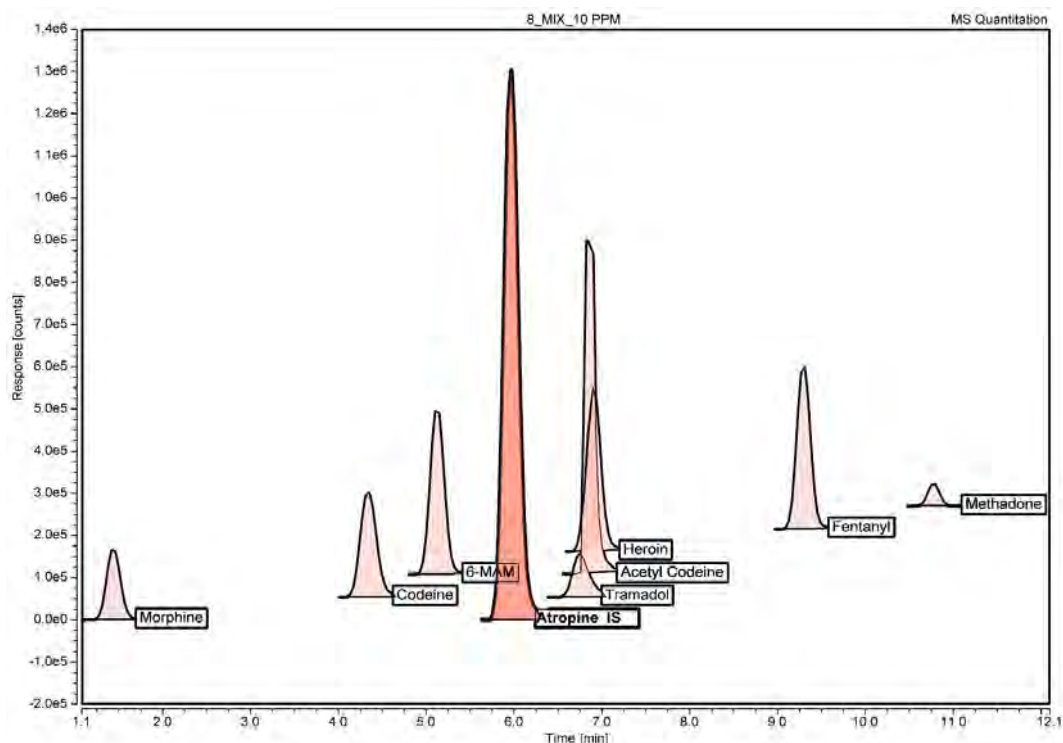


Figure 2. Representative LC–MS EIC showing the separation of eight target opioids and atropine IS at 10.0 $\mu\text{g mL}^{-1}$, prior to passive chemical hydrolysis followed by SPE. 6-monoacetylmorphine was labelled as 6-MAM.

Comparison of RE efficiency across three extraction methods

All analytes were successfully extracted with consistent RE and low variability using passive chemical hydrolysis followed by SPE. The RE (%) values (mean $\pm$ SD) were $87.5 \pm 3.1\%$ for morphine, $93.1 \pm 2.7\%$ for codeine, $114.4 \pm 3.0\%$ for 6-monoacetylmorphine, $95.5 \pm 2.6\%$ for tramadol, $82.0 \pm 2.1\%$ for 6-acetylcodeine, $78.0 \pm 3.4\%$ for heroin, $98.7 \pm 3.1\%$ for fentanyl, and $103.8 \pm 1.9\%$ for methadone. The chromatograms (Figure 3A) showed well-defined, intact peaks across the analyte panel, with %RSD values of approximately 2–3%, indicating good repeatability.

Enzymatic hydrolysis followed by SPE yielded variable results, with generally poor performance for ester-linked opioids. Heroin and 6-acetylcodeine were not detected, and 6-monoacetylmorphine was markedly low ($19.0 \pm 2.1\%$). Synthetic opioids, such as tramadol ($66.6 \pm 2.7\%$), fentanyl ($46.6 \pm 1.8\%$), and methadone ($72.2 \pm 2.2\%$), showed lower RE, whereas morphine ($128.7 \pm 2.0\%$) and codeine ($110.1 \pm 2.4\%$) showed elevated RE values (Figure 3C). This approach did not achieve comprehensive RE across the analyte panel, as indicated by the loss of ester-linked species in the chromatograms.

LLE favored lipophilic analytes and produced clear RE values exceeding 100% for several analytes, consistent with ESI ion enhancement. The observed RE values were $131.8 \pm 2.5\%$ for morphine, $122.9 \pm 2.7\%$ for codeine, $120.3 \pm 2.6\%$ for tramadol, $138.5 \pm 2.8\%$ for fentanyl, and $142.2 \pm 1.9\%$ for methadone. In contrast, heroin, 6-acetylcodeine, and 6-monoacetylmorphine were not detected, and their absence was confirmed in the chromatograms (Figure 3B).

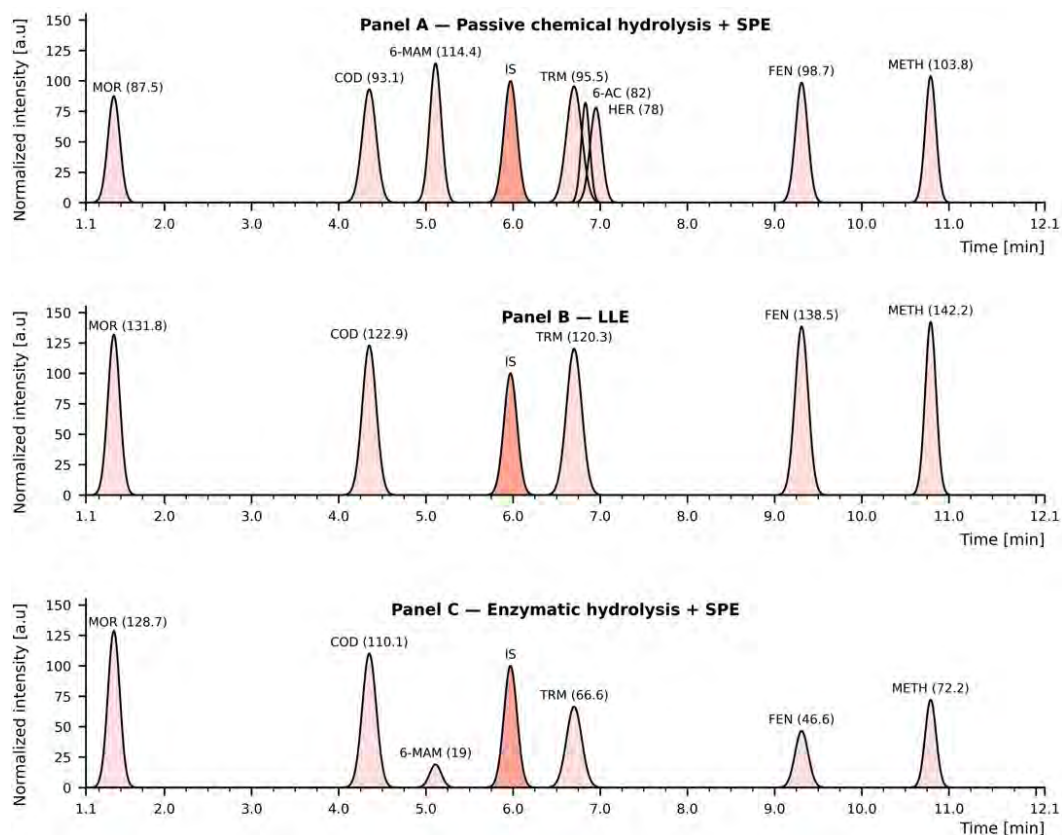


Figure 3. Chromatographic profiles of eight target opioids ($10.0 \mu\text{g mL}^{-1}$) obtained using three extraction approaches: (A) passive chemical hydrolysis followed by SPE, (B) LLE, and (C) enzymatic hydrolysis followed by SPE. Chromatograms are plotted as IS-normalized intensity. Values shown in parentheses above each analyte correspond to RE (%) for the respective method. Peak labels: MOR, morphine; COD, codeine; 6-MAM, 6-monoacetylmorphine; IS, atropine; TRM, tramadol; 6-AC, 6-monoacetylcodeine; HER, heroin; FEN, fentanyl; METH, methadone.

Collectively, these data indicate that passive chemical hydrolysis followed by SPE provides the most comprehensive and well-balanced RE profile across all analytes. Enzymatic hydrolysis resulted in the loss of labile ester derivatives, lowering the RE for several targets. In contrast, LLE preferentially extracted lipophilic analytes but failed to recover ester-linked analytes and often produced clear RE values $> 100\%$. Therefore, passive chemical hydrolysis followed by SPE was selected as the optimal extraction method for quantifying target urine opioids.

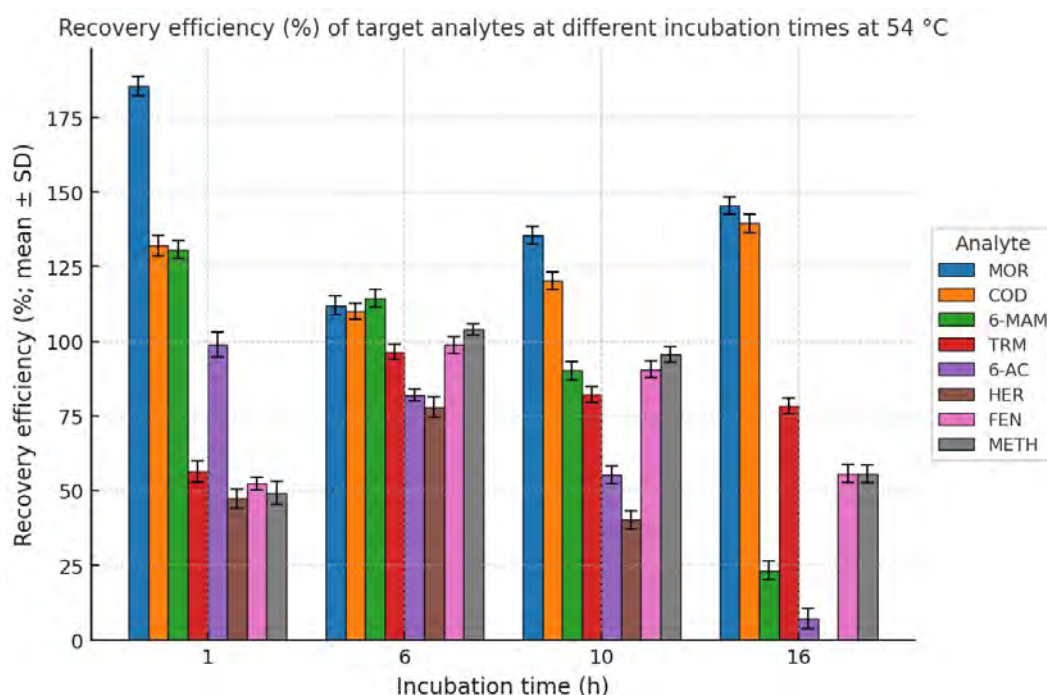
Effect of incubation time on RE

The RE values were inconsistent after 1 h at 54°C . RE (%) for morphine ($185.6 \pm 3.2\%$), codeine ($132.1 \pm 3.4\%$), and 6-monoacetylmorphine ($130.8 \pm 2.9\%$) was high, whereas the RE (%) for tramadol ($56.4 \pm 3.5\%$), fentanyl ($52.4 \pm 2.1\%$), and methadone ($49.3 \pm 3.8\%$) was low. The RE of 6-acetylcodeine ($98.9 \pm 4.1\%$) was approximately 100%, whereas that of heroin ($47.2 \pm 3.3\%$) was markedly low, indicating an imbalance during early hydrolysis (Table I, Figure 4).

Table I. RE efficiency (%; mean $\pm$ SD) for analytes at 1, 6, 10, and 16 h of passive chemical hydrolysis followed by SPE at 54 °C ($n = 3$)

Analyte	1 h (%)	6 h (%)	10 h (%)	16 h (%)
Morphine	185.6 $\pm$ 3.2	112.0 $\pm$ 3.1	135.5 $\pm$ 3.0	145.5 $\pm$ 2.9
Codeine	132.1 $\pm$ 3.4	110.0 $\pm$ 2.7	120.2 $\pm$ 2.9	139.5 $\pm$ 3.0
6-monoacetylmorphine	130.8 $\pm$ 2.9	114.4 $\pm$ 3.0	90.1 $\pm$ 3.1	23.3 $\pm$ 3.1
Tramadol	56.4 $\pm$ 3.5	96.5 $\pm$ 2.6	82.2 $\pm$ 2.7	78.5 $\pm$ 2.7
6-acetylcodeine	98.9 $\pm$ 4.1	82.0 $\pm$ 2.1	55.4 $\pm$ 3.0	7.1 $\pm$ 3.4
Heroin	47.2 $\pm$ 3.3	78.0 $\pm$ 3.4	40.2 $\pm$ 3.1	n.d*
Fentanyl	52.4 $\pm$ 2.1	98.7 $\pm$ 2.8	90.6 $\pm$ 2.9	55.7 $\pm$ 3.0
Methadone	49.3 $\pm$ 3.8	103.8 $\pm$ 1.9	95.5 $\pm$ 2.7	55.6 $\pm$ 2.9

*n.d.: not detected.

**Figure 4.** RE efficiency (%; mean $\pm$ SD) of target analytes at incubation times of 1, 6, 10, and 16 h during passive chemical hydrolysis at 54 °C followed by SPE ($n = 3$). Analytes are labeled as: MOR, morphine; COD, codeine; 6-MAM, 6-monoacetylmorphine; TRM, tramadol; 6-AC, 6-acetylcodeine; HER, heroin; FEN, fentanyl; METH, methadone.

Extending the incubation period to 6 h yielded a consistent, panel-wide RE profile with low variability ($\approx$ 80–115% across the panel). The RE values were 112.0 $\pm$ 3.1% for morphine, 110.0 $\pm$ 2.7% for codeine, 114.4 $\pm$ 3.0% for 6-monoacetylmorphine, 96.5 $\pm$ 2.6% for tramadol, 82.0 $\pm$ 2.1% for 6-acetylcodeine, 78.0 $\pm$ 3.4% for heroin, 98.7 $\pm$ 2.8% for fentanyl, and 103.8 $\pm$ 1.9% for methadone, confirming balanced hydrolysis and efficient extraction (Table I, Figure 4).

At 10 h, the RE values for ester-linked opioids decreased, whereas their deacetylated products increased: 6-monoacetylmorphine fell to $90.1 \pm 3.1\%$, 6-acetylcodeine to $55.4 \pm 3.0\%$, and heroin to $40.2 \pm 3.1\%$; morphine increased to $135.5 \pm 3.0\%$ and codeine to $120.2 \pm 2.9\%$. Tramadol ($82.2 \pm 2.7\%$), fentanyl ($90.6 \pm 2.9\%$), and methadone ($95.5 \pm 2.7\%$) remained within an intermediate RE range (Table I, Figure 4).

Degradation was evident by 16 h, as 6-monoacetylmorphine decreased to $23.3 \pm 3.1\%$, 6-acetylcodeine to $7.1 \pm 3.4\%$, and heroin was not detected. Further declines were observed for fentanyl ($55.7 \pm 3.0\%$) and methadone ($55.6 \pm 2.9\%$). Morphine and codeine continued to rise to $145.5 \pm 2.9\%$ and $139.5 \pm 3.0\%$, respectively (Table I, Figure 4).

Collectively, these data indicate that 6 h at 54 °C provides the most well-balanced and reproducible RE across the panel, achieving RE values of approximately 80–115% with low variability (low SD values), whereas 1 h produces an unbalanced profile, and durations of 10–16 h lead to progressive loss of ester-linked opioids with reciprocal increases in morphine and codeine (Figure 4, Figure 5).

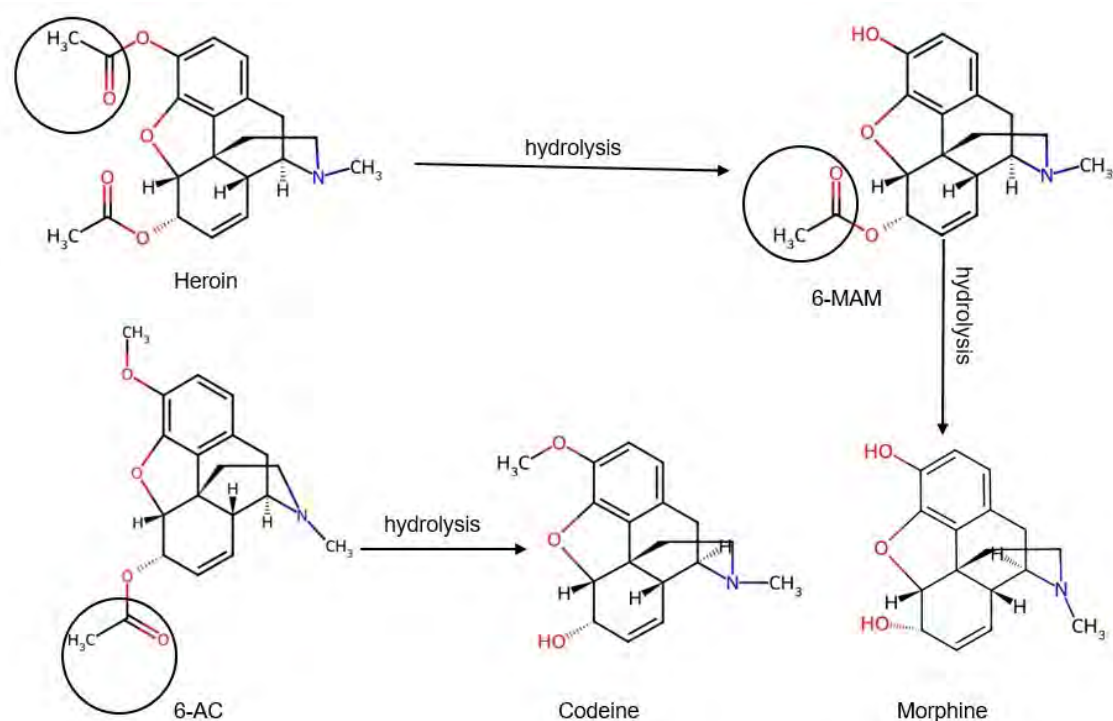


Figure 5. Hydrolytic instability of esterified opioids, illustrating ester cleavage and stepwise hydrolysis of heroin to 6-monoacetylmorphine (labeled as 6-MAM) and then to morphine, and of 6-acetylcodeine (labeled as 6-AC) to codeine.

RT repeatability and ion ratio performance

At the LQC level ($0.5 \mu\text{g mL}^{-1}$), RT was highly consistent across all analytes, with %RSD ranging from 0.000% to 0.563% (Table II). Tramadol showed the largest deviation (0.563%) but remained within acceptable limits; by comparison, fentanyl and methadone varied by 0.181% and 0.088%, respectively. The IS exhibited stable RT, supporting consistent normalization. Collectively, these observations demonstrate excellent chromatographic precision at the LQC level (Table II).

Table II. RT repeatability for analytes, including atropine as the IS, expressed as mean $\pm$ SD and %RSD, and MS transitions, with their m/z values and ion ratios, at the LQC level ($0.5 \mu\text{g mL}^{-1}$; $n = 10$)

Analyte	LQC ($\mu\text{g mL}^{-1}$)	RT (min)		MS transitions		Ion ratio evaluation			
		Mean $\pm$ SD	%RSD	Quantifier (m/z)	Qualifier (m/z)	Ion ratio (%)	Range ($\pm 20\%$)	Lower limit (%)	Upper limit (%)
Morphine	0.5	1.42 ± 0.000	0.000	286.1	229.0	7.31	1.46	5.84	8.77
Codeine	0.5	4.35 ± 0.000	0.000	300.1	243.1	8.75	1.75	7.00	10.50
6-monoacetylmorphine	0.5	5.11 ± 0.015	0.302	328.1	211.1	3.85	0.77	3.08	4.62
Tramadol	0.5	6.70 ± 0.038	0.563	58.2	—	—	—	—	—
6-acetylcodeine	0.5	6.83 ± 0.000	0.000	342.2	225.1	37.51	7.50	30.01	45.01
Heroin	0.5	6.95 ± 0.000	0.000	370.1	165.0	40.77	8.15	32.62	48.30
Fentanyl	0.5	9.31 ± 0.017	0.181	188.1	337.2	37.35	7.47	29.88	44.82
Methadone	0.5	10.79 ± 0.009	0.088	265.1	105.1	40.99	8.20	32.79	49.19
Atropine	10.0	5.97 ± 0.000	0.000	290.1	124.1	50.00	10.00	40.00	60.00

— indicates values that were not applicable to tramadol.

Identification was confirmed using in-source fragments monitored by EIC, and ion ratios were calculated from the corresponding EIC peak areas.

The qualifier-to-quantifier ion ratios for analytes with a qualifier ion fell within the $\pm 20\%$ tolerance range established from the calibrators (Table II), confirming specificity.⁴¹ For example, morphine and methadone had an ion ratio of 7.31 (range, 5.84–8.77) and 40.99 (range, 32.79–49.19), respectively. In this study, the single-quadrupole LC-MS method produced only weak, matrix-dependent in-source fragments for tramadol, which did not meet the predefined criteria for a reliable qualifier ion because of insufficient S/N and lack of an interference-free response across urine matrices. Therefore, the identification of tramadol relied on three criteria: quantifier ion, RT within $\pm 2\%$ of the calibrator value, and chromatographic peak purity checks. These criteria provide specificity without a qualifier ion. MRM qualifier transitions require tandem LC-MS. A qualifier ion was unavailable because this study used a single-quadrupole LC-MS system (Table II).

Therefore, sub-percent RT variability, together with compliant ion ratio checks at the LQC level, supports robust precision and reliable qualitative confirmation for single-quadrupole LC-MS and EIC-based analysis across the analyte panel (Table II).

Validation results

Linearity and range

Within the calibration range of 0.5–25.0 $\mu\text{g mL}^{-1}$, using five levels with 1/x weighting, all analytes demonstrated linear responses, with R^2 values ranging from 0.991 to 0.998 (Table III, Figure 6).^{22,25,26,28,39} The back-calculated concentrations were within $\pm 20\%$ of their nominal values across all levels, confirming a validated analytical range of 0.5–25.0 $\mu\text{g mL}^{-1}$ for routine quantification (Table III, Figure 6). The accuracy across the curve was within the acceptance limits for each analyte: morphine (94.2–116.9%); codeine (81.2–109.5%); 6-monoacetylmorphine (95.0–116.8%); 6-acetylcodeine (80.0–109.3%); heroin (81.2–110.4%); fentanyl (86.2–105.5%); methadone (89.2–106.3%); and tramadol (85.3–109.7%) (Table III).

Table III. Calibration parameters and accuracy assessments for analytes determined by LC-MS ($n = 5$ per calibration level)

Analyte	Calibration range ($\mu\text{g mL}^{-1}$)	Accuracy range*	R^2	Slope	Y-intercept	Calibration model
Morphine	0.5–25.0	94.2–116.9	0.991	1.0012	0.4788	Linear, 1/x weighting, Average
Codeine	0.5–25.0	81.2–109.5	0.995	1.7815	0.7866	Linear, 1/x weighting, Average
6-monoacetylmorphine	0.5–25.0	95.0–116.8	0.992	2.2494	1.3040	Linear, 1/x weighting, Average
6-acetylcodeine	0.5–25.0	80.0–109.3	0.995	3.3292	2.1199	Linear, 1/x weighting, Average
Heroin	0.5–25.0	81.2–110.4	0.996	2.1559	1.9800	Linear, 1/x weighting, Average
Fentanyl	0.5–25.0	86.2–105.5	0.998	2.5831	0.0615	Linear, 1/x weighting, Average
Methadone	0.5–25.0	89.2–106.3	0.997	0.3007	0.0219	Linear, 1/x weighting, Average
Tramadol	0.5–25.0	85.3–109.7	0.994	0.8128	0.2023	Linear, 1/x weighting, Average

* % of nominal value

The slopes reflected the relative detector response, with 6-acetylcodeine exhibiting the steepest slope (3.3292), followed by fentanyl (2.5831) and 6-monoacetylmorphine (2.2494), whereas methadone had the lowest slope (0.3007). Processing the data as analyte-to-IS peak area ratios in Chromeleon Console 7 (version 7.3.1.6535) confirmed the proportional signal response across the calibration range and supported the reliability of the calibration model for quantitative LC-MS measurements (Table III, Figure 6).

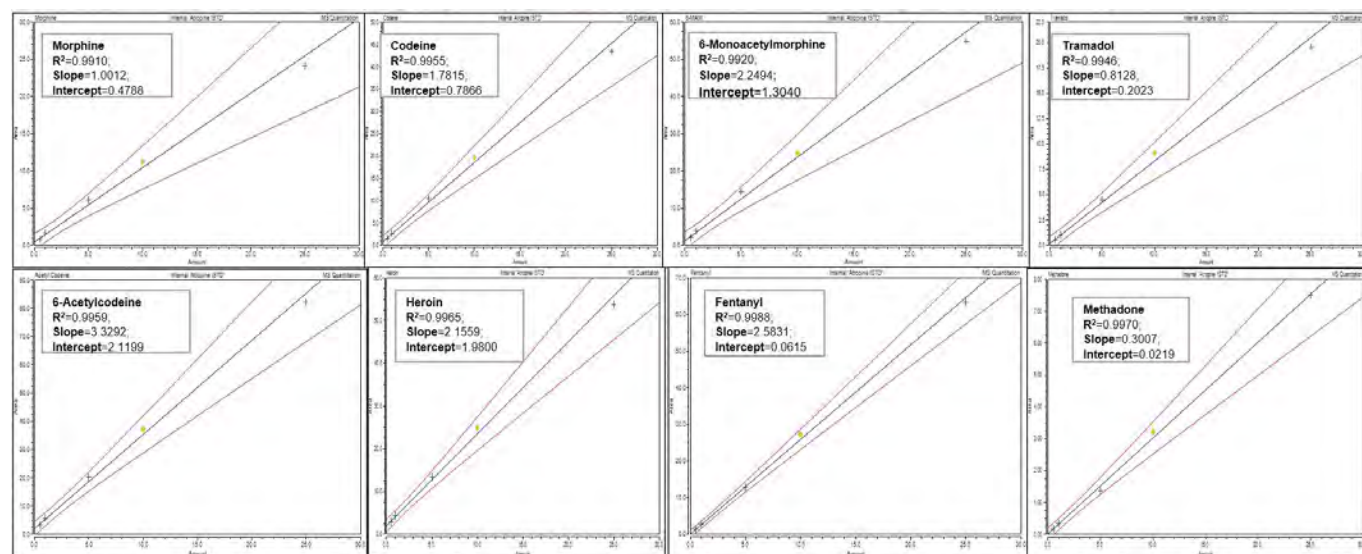


Figure 6. Calibration curves for analytes over the concentration range 0.5–25.0 $\mu\text{g mL}^{-1}$ using linear regression with $1/x$ weighting. R^2 values were ≥ 0.991 for all analytes. Peak area ratios were plotted against nominal concentrations.

Precision and accuracy

The developed LC-MS method demonstrated high accuracy and precision at three QC levels (1.0, 10.0, and 20.0 $\mu\text{g mL}^{-1}$) for all analytes (Table IV).

Accuracy (bias, %) ranged from -13.0% for 6-acetylcodeine at the LQC level to 18.0% for methadone at the same level, and all values, excluding heroin, remained within the $\pm 20\%$ acceptance limit.^{27,28} Heroin showed a substantial negative bias (-24.0% to -16.9%), consistent with its known instability and partial hydrolysis during sample processing.

The only analyte that fell outside the accepted limits for accuracy was heroin (Table IV). Bias exceeded $\pm 20\%$ at the LQC and middle QC (MQC) levels intra-day (-24.0% and -22.0%) and at the LQC level inter-day (-22.0%). The precision for heroin remained within the acceptable limits, indicating a systematic bias rather than random variability. This pattern is consistent with the reduced RE and ion suppression observed for heroin in the matrix study and is attributable to partial conversion to 6-monoacetylmorphine and morphine during processing. The bias decreased at higher concentrations (-17.0% intra-day; -18.0% inter-day), indicating a concentration-dependent effect.

The precision (%RSD) was excellent in both the intra-day (repeatability) and inter-day (reproducibility) assessments (Table IV). Intra-day %RSD ranged from 0.2% for heroin at the HQC level to 14.6% for morphine at the LQC level, whereas inter-day %RSD ranged from 1.3% for tramadol at the HQC level to 16.8% for methadone at the LQC level.

Table IV. Precision (%RSD) and accuracy (bias, %) for analytes at the LQC, MQC, and HQC levels ($n = 6$ intra-day; $n = 18$ inter-day)

Analyte	LQC/MQC/HQC ($\mu\text{g mL}^{-1}$)	Intra-day ($n = 6$)			Inter-day ($n = 18$)		
		Mean ($\mu\text{g mL}^{-1}$)	Bias (%)	Repeatability (%RSD)	Mean ($\mu\text{g mL}^{-1}$)	Bias (%)	Reproducibility (%RSD)
Morphine	1.0/10.0/20.0	1.04/11.36/22.72	4.0/13.6/13.6	14.6/0.6/6.0	1.1/11.5/22.8	6.8/15.0/14.1	5.1/1.6/3.2
Codeine	1.0/10.0/20.0	1.09/10.52/22.04	9.0/5.2/10.2	2.9/2.2/3.2	1.1/11.3/22.7	7.1/12.6/13.4	2.2/1.7/1.6
6-monoacetylmorphine	1.0/10.0/20.0	1.11/11.34/22.14	11.0/13.4/10.7	2.2/1.7/6.5	1.1/11.8/22.5	11.9/17.6/12.5	4.0/4.3/2.2
Tramadol	1.0/10.0/20.0	0.92/9.65/19.25	-8.0/-3.5/-3.8	5.3/3.0/0.3	1.0/9.8/18.8	-11.0/-2.0/-6.1	3.4/5.3/1.3
6-acetylcodeine	1.0/10.0/20.0	0.88/8.87/18.56	-12.0/-11.3/-7.2	13.3/2.6/1.8	0.9/9.0/18.7	-13.0/-10.9/-6.3	8.2/3.9/2.7
Heroin	1.0/10.0/20.0	0.76/7.80/16.60	-24.0*/-22.0*/-17.0	10.6/8.3/0.2	0.8/7.9/16.4	-22.0*/-16.9/-18.0	7.8/6.7/5.6
Fentanyl	1.0/10.0/20.0	0.96/9.62/19.42	-4.0/-3.8/-2.9	7.0/5.5/3.9	1.0/9.6/19.3	-6.0/-4.4/-3.8	4.1/2.1/3.9
Methadone	1.0/10.0/20.0	1.02/10.32/20.56	2.0/3.2/2.8	10.0/4.5/4.2	1.2/10.4/20.4	18.0/3.8/1.8	16.8/8.2/7.0

Validation limits: accuracy (bias, %) within $\pm 20\%$; precision (%RSD) $\leq 20\%$.

*Values exceeding the accuracy limit were observed for heroin at the LQC and MQC levels (intra-day bias: -24.0% and -22.0%) and at the LQC level inter-day (-22.0%).

All precision values (%RSD) were within acceptable limits.

Precision improved as the concentration increased, with the MQC and HQC levels showing lower %RSD values than the LQC level. Codeine, tramadol, and fentanyl demonstrated stable performance, with %RSD consistently below 7.0% across all QC levels. At the LQC level, heroin and 6-acetylcodeine exhibited modest variability, but %RSD values remained within acceptable limits, supporting the robustness of the method.

Except for the noted heroin bias, all analytes met the bioanalytical method validation criteria, with no evidence of systematic bias or loss of precision across the tested concentration range (Table IV).

Analytical sensitivity and carryover

The method achieved an LOQ of $0.5 \mu\text{g mL}^{-1}$ and an LOD of $0.3 \mu\text{g mL}^{-1}$ in pooled blank urine extracts. According to the relationship $C_{\text{urine}} = C_{\text{extract}} / 10$, the dried residues were reconstituted after extraction in 50 μL of mobile phase consisting of a 90:10 (v/v) mixture of 10 mM ammonium formate with 0.1% formic acid and acetonitrile with 0.1% formic acid, to enrich the analyte concentration ten-fold relative to the original urine sample and allow accurate quantification within the calibration range ($0.5\text{--}25.0 \mu\text{g mL}^{-1}$) of the method. Therefore, an LOQ of $0.5 \mu\text{g mL}^{-1}$ in the final extract corresponds to an effective LOQ of 50 ng mL^{-1} in the original urine sample. The effective LOQ is compatible with commonly applied confirmatory urine cut-off concentrations in forensic and clinical toxicology, as recommended by the European Workplace Drug Testing Society (EWDTS) for several opioids, such as 300 ng mL^{-1} for morphine, 300 ng mL^{-1} for codeine, 100 ng mL^{-1} for tramadol, and 250 ng mL^{-1} for methadone.⁴² However, analytes that require very low cut-off concentrations, such as 10 ng mL^{-1} for 6-monoacetylmorphine or sub-ng mL^{-1} for fentanyl, are still best suited to targeted tandem LC–MS methods with greater analytical sensitivity. At this LOQ, S/N, accuracy, and precision met the predetermined criteria; qualitative assessments of RT and ion ratio agreement were satisfactory, confirming sufficient sensitivity for routine quantification throughout the validation range.

Following injections of the highest calibrator ($25.0 \mu\text{g mL}^{-1}$), pooled blank urine extracts showed no analyte peaks at the target RT. The mean blank response was $\leq 10\%$ of the LOQ signal, which met the predefined carryover criterion. These results indicate that the autosampler, injection pathway, and LC system did not retain quantifiable residues from high-concentration injections, minimizing the risk of false positives or biased low-level results and supporting the suitability of the method for high-throughput workflows. No carryover was observed.

ME, RE, PE, and selectivity

At the LQC level ($1.0 \mu\text{g mL}^{-1}$), ME ranged from 85.8% for heroin to 109.1% for methadone, falling within the predefined acceptance range of 80–120%.^{43–45}

Table V. ME (%), RE (%), and PE (%) of analytes at the LQC and HQC levels in a urine matrix

Analyte	LQC and HQC ($\mu\text{g mL}^{-1}$)	ME (%)	ME (%RSD)	RE (%)	RE (%RSD)	PE (%)	Ionization effect
Morphine	1.0	103.9	6.8	112.8	8.2	117.2	Enhancement
	20.0	102.5	1.0	114.3	1.3	118.2	Enhancement
Codeine	1.0	103.1	2.8	111.0	4.5	114.4	Enhancement
	20.0	102.0	5.2	109.0	8.3	111.2	Enhancement
6-monoacetylmorphine	1.0	101.1	3.1	113.8	4.3	115.0	Enhancement
	20.0	100.8	1.8	114.0	2.1	115.9	Enhancement

(continued on next page)

Table V. ME (%), RE (%), and PE (%) of analytes at the LQC and HQC levels in a urine matrix (continued)

Analyte	LQC and HQC ($\mu\text{g mL}^{-1}$)	ME (%)	ME (%RSD)	RE (%)	RE (%RSD)	PE (%)	Ionization effect
Tramadol	1.0	108.2	2.1	91.5	4.1	99.0	Enhancement
	20.0	106.4	1.8	96.8	3.8	102.9	Enhancement
6-acetylcodeine	1.0	89.7	2.3	87.1	4.2	78.1*	Suppression
	20.0	98.6	2.0	88.7	4.0	87.4	Suppression
Heroin	1.0	85.8	3.6	75.8*	1.2	65.0*	Suppression
	20.0	89.2	2.4	77.2*	0.7	68.8*	Suppression
Fentanyl	1.0	96.9	4.0	89.6	6.9	86.8	Suppression
	20.0	98.5	2.7	98.8	5.2	97.3	Suppression
Methadone	1.0	109.1	1.9	101.8	3.3	114.3	Enhancement
	20.0	102.8	1.0	104.2	1.6	107.1	Enhancement

ME (%) values > 100% indicate ion enhancement, whereas values < 100% indicate ion suppression. *Values outside the predefined acceptance range (80–120%) were observed for 6-acetylcodeine (PE = 78.1% at the LQC level) and heroin (PE = 65.0% at the LQC level and 68.8% at the HQC level; RE = 75.8% at the LQC level and 77.2% at the HQC level).

Ionization enhancement was observed for morphine, codeine, 6-monoacetylmorphine, tramadol, and methadone. Ion suppression was exhibited by heroin, fentanyl, and 6-acetylcodeine, most notably at the LQC level for heroin (85.8%) and 6-acetylcodeine (89.7%) (Table V, Figure 7a).

The RE values were generally high, ranging from 87.1% for 6-acetylcodeine at the LQC level to 114.3% for morphine at the HQC level (Table V, Figure 7b). Methadone, tramadol, and fentanyl were close to 100%, whereas heroin was lower (75.8–77.2%), consistent with hydrolysis to 6-monoacetylmorphine and morphine during preparation (Figure 5).

The PE values varied from 86.8% for fentanyl at the LQC level to 118.2% for morphine at the HQC level; 6-acetylcodeine (78.1% at the LQC level) and heroin (65.0% at the LQC level and 68.8% at the HQC level) were the exceptions (Table V, Figure 7b).^{43,44} Morphine, 6-monoacetylmorphine, methadone, and codeine exceeded 110.0%, indicating ion enhancement, whereas tramadol and fentanyl were close to 100%, indicating minimal matrix loss (Table V, Figure 7a, c). The lower PE of heroin (65.0–68.8%) was associated with its chemical instability and ester cleavage susceptibility. The %RSD values for RE and ME were $\leq 8.3\%$ for all analytes and QC levels (Table V).

The method meets the matrix performance validation standards and is suitable for high-throughput forensic toxicology applications. The reduced RE values for heroin and 6-acetylcodeine were consistent with their susceptibility to ester cleavage during processing, and the precision remained within acceptable limits across all QC levels.

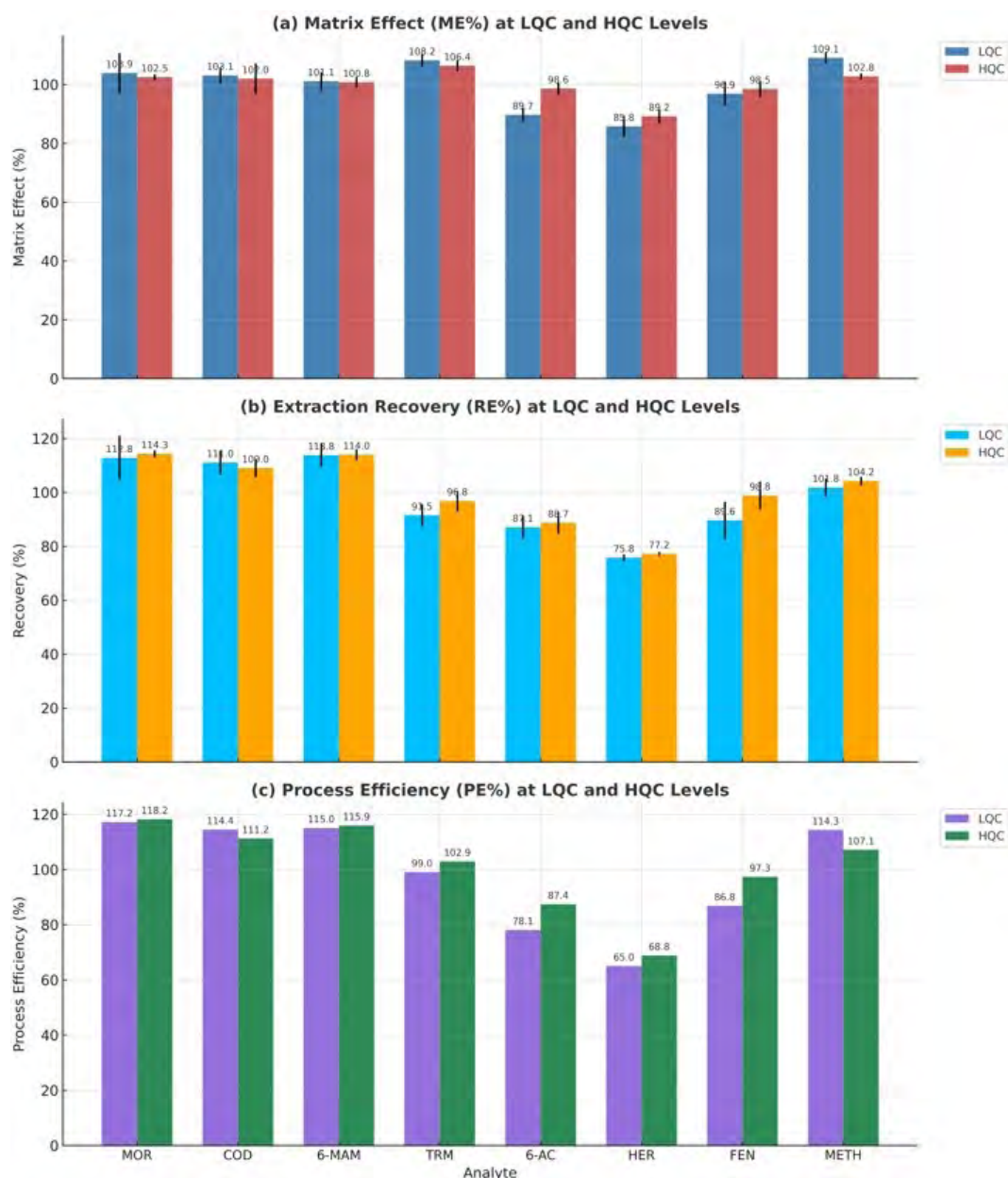


Figure 7. Panel (a) shows ME (%), panel (b) shows RE (%), and panel (c) shows PE (%) at the LQC ($1.0 \mu\text{g mL}^{-1}$) and HQC ($20.0 \mu\text{g mL}^{-1}$) levels. Bars in panels (a) and (b) represent mean $\pm$ SD ($n = 3$), whereas panel (c) shows PE (%) values at the same QC levels. Analytes are labeled as: MOR, morphine; COD, codeine; 6-MAM, 6-monoacetylmorphine; TRM, tramadol; 6-AC, 6-acetylcodeine; HER, heroin; FEN, fentanyl; METH, methadone.

Values for heroin and 6-acetylcodeine that fell outside the 80–120% acceptance range were attributable to the PE calculation, defined as $(\text{ME} \times \text{RE}) / 100$, and the hydrolytic instability of these esterified opioids (Figure 5). At the LQC level, 6-acetylcodeine exhibited mild ion suppression (89.7%) and reduced RE (87.1%), yielding a PE of 78.1%, which is consistent with the partial conversion to codeine (Table V, Figure 7c). Heroin showed the same trend at both QC levels, with PE values of 65.0% at the LQC level and 68.8% at the HQC level, driven by ion suppression (ME values of 85.8% and 89.2%, respectively) and reduced RE (75.8% and 77.2%, respectively). Low %RSD values indicate a systematic effect rather than a random

error. The results are consistent with the hydrolysis of heroin to 6-monoacetylmorphine and subsequently to morphine and the hydrolysis of 6-acetylcodeine to codeine (Figure 5). The other analytes were within the 80–120% acceptance range with low variability, reinforcing the overall method variability (Table V).

Selectivity was assessed using ten individual blank urine samples and a pooled blank urine sample spiked with additional non-target opioids (as described in the methods). Neither the individual blanks nor the spiked pooled sample showed interfering peaks at the RT of any target analyte or the IS. The ion ratios and RT values remained within $\pm 20\%$ and $\pm 2\%$ of the calibration values, respectively. These data confirm adequate selectivity and specificity under the tested conditions.

Stability of processed urine samples

The stability of the processed samples at 4 °C was assessed for each analyte at two QC levels (1.0 and 10.0 $\mu\text{g mL}^{-1}$) over 96 h, with results expressed as % change from the initial time point at $t = 0$ h. Across QC levels and time points, all analytes remained within the $\pm 20\%$ acceptance limit (Table VI, Figure 8).

Table VI. Time-dependent stability of analytes in processed urine samples stored at 4 °C for 0–96 h, expressed as % change, with %RSD values calculated for triplicate measurements ($n = 3$)

Analyte	QC levels ($\mu\text{g mL}^{-1}$)	*Mean Concentration at $t = 0$ h	% change from $t = 0$ h				%RSD at 96 h
			24 h	48 h	72 h	96 h	
Morphine	1.0	1.14	−1.4	−2.8	−5.8	−18.3	4.0
	10.0	11.32	−1.0	−2.2	−4.9	−15.6	3.5
Codeine	1.0	1.16	−11.0	−14.6	−16.5	−18.2	3.9
	10.0	10.81	−5.0	−7.7	−15.2	−16.2	3.5
6-monoacetylmorphine	1.0	1.13	−2.8	−8.4	−7.8	−14.1	3.9
	10.0	11.50	−1.3	−4.9	−9.7	−10.8	4.2
Tramadol	1.0	0.92	−2.1	−7.7	−10.1	−17.8	3.5
	10.0	9.60	−7.2	−6.3	−9.1	−15.4	2.4
6-acetylcodeine	1.0	0.85	−9.1	−12.0	−11.9	−19.5	4.8
	10.0	8.84	−6.5	−8.6	−14.2	−17.0	3.1
Heroin	1.0	0.76	−5.0	−8.3	−13.3	−18.1	3.5
	10.0	7.63	−0.7	−2.5	−4.6	−15.7	3.0
Fentanyl	1.0	0.90	−3.2	−6.5	−15.2	−18.4	2.7
	10.0	9.80	−3.8	−6.0	−10.5	−13.2	2.3
Methadone	1.0	1.02	−5.6	−14.6	−16.0	−19.8	4.8
	10.0	10.25	−1.9	−9.4	−15.1	−15.7	3.3

*Reference concentration at $t = 0$ h.

Analytes were considered stable when the % change was within $\pm 20\%$; values outside this range indicated instability.

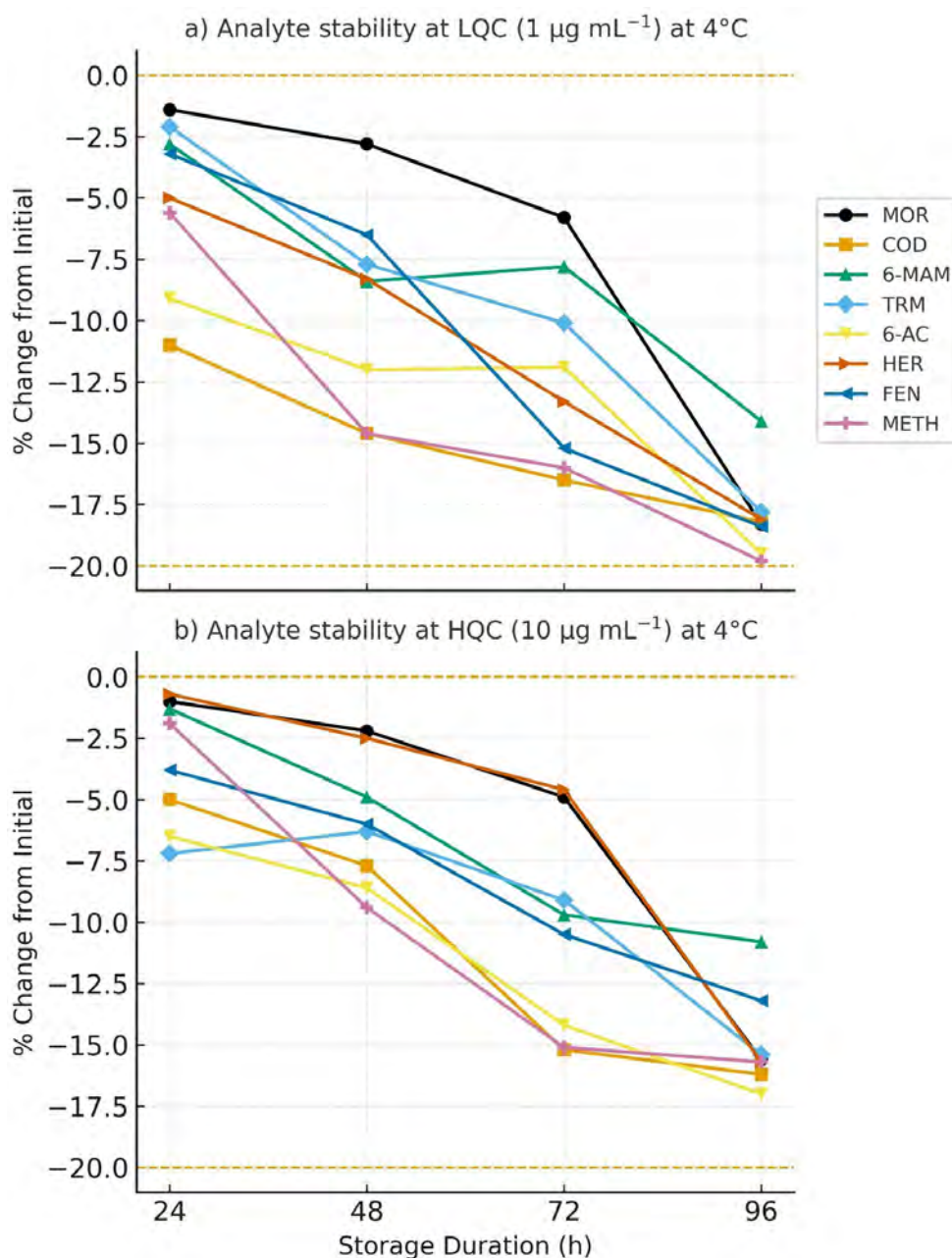


Figure 8. Stability profile of the analytes in processed urine samples stored at 4°C for up to 96 h: panel (a) LQC level ($1.0 \mu\text{g mL}^{-1}$); panel (b) HQC level ($10.0 \mu\text{g mL}^{-1}$). Analytes are labeled as: MOR, morphine; COD, codeine; 6-MAM, 6-monoacetylmorphine; TRM, tramadol; 6-AC, 6-acetylcodeine; HER, heroin; FEN, fentanyl; METH, methadone.

Concentrations generally decreased with storage duration, and this effect was more noticeable at the LQC level (Table VI, Figure 8a). At the LQC level after 96 h, the largest decreases were observed for methadone (-19.8%), 6-acetylcodeine (-19.5%), fentanyl (-18.4%), and both codeine and morphine ($\approx -18.0\%$ each) (Table VI, Figure 8a). At the HQC level, the greatest decline was -17.0% for 6-acetylcodeine, with smaller variations for the other analytes (Table VI, Figure 8b). Heroin and 6-monoacetylmorphine exhibited moderate time-dependent losses consistent with partial hydrolysis in an aqueous medium (Table VI, Figure 8).

High precision was maintained throughout the storage period, with %RSD $\leq 4.8\%$ at 96 h, confirming reproducible quantification (Table VI). Collectively, these findings indicate that processed urine extracts remain stable at 4 °C for up to 96 h, although some analytes at the LQC level approached the acceptance threshold near the end of the storage period (Table VI, Figure 8).

METHOD APPLICABILITY

Urine specimens from three anonymized cases were collected in August 2025 at Kanoria Hospital & Research Center. Immediately after collection, the specimens were analyzed using the validated assay as part of a rehabilitation monitoring and de-addiction program. Each sample was analyzed in duplicate alongside calibrators, three QC levels, and three blanks. QC performance was considered acceptable when the measured concentrations were within $\pm 20\%$ of the nominal values. Applying the relationship $C_{\text{urine}} = C_{\text{extract}} / 10$, one of the three specimens tested positive for tramadol, whereas the other two tested negative for the target opioids. Table VII summarizes the results.

The limited number of urine case specimens constrained the sample size available for analysis. These findings are preliminary but indicate the applicability of the proposed method in real-world contexts. Further large-scale case studies should be conducted to confirm and extend the findings of this study.

Table VII. Results for authentic urine samples analyzed using the validated single-quadrupole LC-MS method

Sample ID	Morphine	Codeine	6-monoacetylmorphine	Tramadol	6-acetylcodeine	Heroin	Fentanyl	Methadone
S1	—	—	—	—	—	—	—	—
S2	—	—	—	0.732	—	—	—	—
S3	—	—	—	—	—	—	—	—

— not detected; units: $\mu\text{g mL}^{-1}$.

CONCLUSIONS

This study developed and validated a cost-effective, sensitive single-quadrupole LC-MS assay for the simultaneous determination of eight opioids in human urine. The method used passive chemical hydrolysis optimized at 54 °C for 6 h and achieved high RE for most analytes, ranging from $82.0 \pm 2.1\%$ for 6-acetylcodeine to $114.4 \pm 3.0\%$ for 6-monoacetylmorphine, with slightly lower RE for heroin ($78.0 \pm 3.4\%$) because of its instability during sample processing. Passive chemical hydrolysis followed by SPE outperformed both enzymatic hydrolysis followed by SPE and LLE, and the validation met the following predefined criteria: $R^2 \geq 0.991$, precision (%RSD) $\leq 16.8\%$ for both intra-day and inter-day measurements, accuracy (bias, %) within $\pm 20\%$, acceptable ME, processed sample stability, and carryover. The applicability of the method was supported by casework: one of three case specimens tested positive for tramadol. Overall, the method provides a reliable, accessible option for clinical and forensic laboratories, particularly in resource-limited settings where access to MS/MS instrumentation and enzymatic hydrolysis is constrained.

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Conflicts of interest

The authors declare no conflicts of interest.

Use of artificial intelligence tools

The authors declare that no artificial intelligence tools were used in the preparation of this manuscript.

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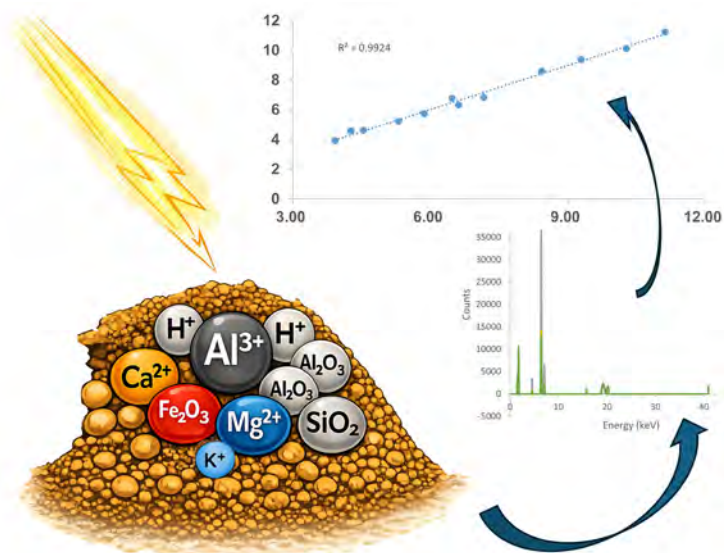
Integration of EDXRF and Chemometrics as a Fast and Green Alternative for Determining the Cation Exchange Capacity of Cerrado Soils Treated with Swine Manure

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Part of this figure was created with the assistance of Microsoft Copilot (ChatGPT), which was used to generate an initial conceptual description of the radiation incidence on the sample. The final artwork was produced and edited entirely by the authors. The authors confirm that the tool does not claim ownership over the generated content and that all creative decisions and responsibility for the figure rest with the authors.

Studies have shown that organic fertilizers derived from liquid swine waste (LSW) can improve several soil chemical properties, including cation exchange capacity (CEC). The determination of CEC is traditionally carried out using chemical methods based on cation extraction, followed by quantification through titrimetry, flame atomic absorption spectrometry (FAAS), or flame photometry. As an alternative to these classical methods, the present study aimed to evaluate energy-dispersive X-ray fluorescence (EDXRF) combined with chemometrics for the determination of CEC. For this purpose, multiple linear regression (MLR) was employed as the multivariate calibration method. The calibration model was built using X-ray intensities obtained by EDXRF and the CEC values determined by the chemical reference method (CEC at pH 7.0). The CEC values determined by the reference method ranged

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from 4.92 to 10.71 cmolc kg⁻¹. EDXRF analyses indicated a predominance of SiO₂ and Al₂O₃, reflecting the mineralogy of Cerrado soils, which are characterized by intense weathering and the presence of silicate and metal oxide minerals. The developed MLR model showed an adjusted R² of 0.97, indicating a strong correlation between the CEC values obtained by the reference method and those predicted by the model. The root mean square error (RMSE) of 0.40 cmolc kg⁻¹, approximately ten times lower than the lowest CEC value assessed, and the ratio of performance to deviation (RPD) of 5.97 demonstrate the high accuracy of the model. The characteristics of EDXRF, a rapid, non-destructive technique suitable for the analysis of solid samples, combined with MLR proved to be an efficient and environmentally friendly analytical strategy for determining CEC in Cerrado soils amended with LSW under different crops and application periods, in accordance with the principles of green chemistry.

Keywords: multiple linear regression, organic fertilizers, sustainable agriculture, tropical soils, green analytical chemistry

INTRODUCTION

Studies have shown that organic fertilization with animal manure improves several soil chemical attributes, including cation exchange capacity (CEC),¹⁻³ especially in the surface layers, due to the increase in soil organic matter (OM).³ The CEC represents the total amount of exchangeable cations that the soil can adsorb, which are retained by negatively charged clay and OM particles and can be readily exchanged and made available to plants.

The conventional method for determining CEC involves extracting cations using specific extractant solutions, KCl for Ca, Mg, and Al, and Mehlich-1 for Na and K, followed by the quantification of Al, Ca, and Mg by titration or flame atomic absorption spectrometry (FAAS), and of Na and K by flame photometry.⁴ Although widely employed, this procedure is time-consuming, requires chemical reagents, generates substantial post-analysis waste, and comprises labor-intensive laboratory steps.

As an alternative to conventional chemical methods, several spectrometric techniques have been investigated for the prediction of soil attributes, notably X-ray fluorescence (XRF),⁵⁻⁹ visible and near-infrared spectroscopy (Vis–NIR),¹⁰⁻¹⁴ gamma spectrometry (GR),¹⁵ diffuse reflectance spectroscopy (DRS),¹⁶ and laser-induced breakdown spectroscopy (LIBS).¹⁷ Additionally, data-fusion approaches combining spectral information, such as XRF with GR,¹⁸ XRF with Vis–NIR,¹⁹ and XRF, LIBS, and visible and near infrared diffuse reflectance spectroscopy (VNIR),²⁰ have shown promise for improving the accuracy of soil chemical property estimates. When integrated with chemometric methods, these techniques offer an efficient, rapid, and low-cost alternative, requiring minimal or no sample preparation.

However, to date, no studies have reported the use of XRF combined with chemometrics for determining CEC in soils amended with liquid swine waste (LSW). Considering that the addition of organic matter derived from this type of amendment can strongly influence CEC,²¹ investigating this relationship using advanced spectrometric and statistical methods becomes essential. In this context, the present study is the first to evaluate the application of EDXRF combined with chemometrics, through multiple linear regression (MLR), for determining the CEC of Cerrado soils amended with LSW.

MATERIALS AND METHODS

Sample collection and characteristics

The areas evaluated in this study encompassed six municipalities within the swine-producing region of the State of Mato Grosso, Brazil (Rondonópolis, Campo Verde, Lucas do Rio Verde, Sorriso, Tapurah, and Vera), with two sampling points in each municipality, totaling 12 samples. The sampled agricultural areas were cultivated with soybeans, corn, and pasture and had a history of fertilization with liquid swine manure for periods ranging from 5 to 10 years. Information on soil classes, sampling site locations, and the sampling procedures is provided in Silva et al.³ and Barros et al.²²

Briefly, within each property a 30-ha area was subdivided into two 15-ha plots. Soil sampling was performed following a zigzag pattern with five subsamples collected per plot using a Dutch auger at a depth of 0–0.20 m. The subsamples were combined and homogenized to form a composite sample for each plot. In the laboratory, the samples were oven-dried at 65 °C with forced air circulation, crushed, sieved through a 2 mm mesh, and manually ground using a mortar and pestle to ensure homogeneity.

Reference method

To obtain the reference CEC values required for constructing and validating the calibration model, the procedure described by Teixeira et al.⁴ was used. In summary, the extraction of Ca, Mg, and Al from soil samples amended with LSW was performed using 1 mol L⁻¹ KCl (Synth, Brazil) as the extracting solution, whereas the extraction of Na and K was carried out using the diluted acidic solution of HCl (Synth, Brazil) and H₂SO₄ (Éxodo, Brazil) (Mehlich-1 extracting solution: HCl 0.05 mol L⁻¹ and H₂SO₄ 0.0125 mol L⁻¹). Total acidity (H + Al) was extracted using a 0.5 mol L⁻¹ calcium acetate solution (pH 7.1–7.2; Synth-Brazil). The concentrations of Ca, Mg, Al, and H + Al were determined by complexometric and acid–base titration, whereas Na and K contents were quantified by flame photometry (ANALYSER 210).

EDXRF analysis

For the EDXRF chemical analysis, pressed pellets were prepared in triplicate (5–7 tons of pressure) were produced from the milled powder. A Shimadzu EDX-700HS spectrometer was employed, enabling quantitative measurements using the Fundamental Parameters (FP) approach implemented in the Qual-Quant FP[®] routine, including internal normalization and matrix correction procedures. A 10-mm X-ray beam and tube voltages of 15 and 50 kV were applied for detecting elements from sodium (Na) to scandium (Sc) and from titanium (Ti) to uranium (U), respectively. All spectra were collected under vacuum to enhance measurement accuracy for light elements, whose characteristic X-rays are strongly absorbed by matter, including air.

Data processing and model calibration (MLR)

To prevent systematic differences in the intensity levels of the spectra from affecting the performance of the multivariate calibration model, the data was autoscaled. Autoscaling consists of subtracting the mean of each variable from the dataset and dividing it by its corresponding standard deviation, resulting in variables with zero mean and unit variance.²³

After the data were preprocessed, multiple linear regression (MLR) was evaluated as the multivariate calibration model. Model performance was assessed using the root mean square error (RMSE), the adjusted coefficient of determination (adjusted R²), and the ratio of performance to deviation (RPD).²³ The RPD, defined as the ratio between the standard deviation of the reference values and the prediction error, is commonly used to evaluate the predictive ability of chemometric models.²⁴ In general, higher RPD values and lower prediction errors indicate better model performance. All calculations and modeling steps were performed using Microsoft Excel[®] and MATLAB[®] R2023a.

RESULTS AND DISCUSSION

CEC determination by the reference method and qualitative analysis by EDXRF

The CEC of the soils amended with LSW, determined by the reference method, is presented in Table I. The CEC values ranged from 4.92 to 10.71 cmolc kg⁻¹. The oxide contents obtained by EDXRF indicated a predominance of SiO₂ and Al₂O₃, ranging from 30.12% to 70.26% and from 25.86% to 51.02%, respectively, whereas Fe₂O₃ exhibited lower levels, between 3.20% and 14.07%.

Table I. Cation exchange capacity (CEC at pH 7.0) of Cerrado soils amended with liquid swine waste and oxide concentrations obtained by EDXRF

Parameters*	City					
	Rondonópolis	Campo Verde	Lucas do Rio Verde	Sorriso	Tapurah	Vera
CEC (cmolc kg ⁻¹)*	10.71	8.87	6.24	4.92	4.09	6.82
Al ₂ O ₃ (%)	42.14	51.02	28.83	31.45	25.86	45.52
SiO ₂ (%)	41.74	30.12	63.71	61.90	70.26	45.49
Fe ₂ O ₃ (%)	12.59	14.07	5.22	4.51	3.20	5.59

*CEC – Cation exchange capacity

Although EDXRF-derived oxide data suggest an indicative trend—Al₂O₃-rich soils tend to exhibit higher CEC, whereas SiO₂-dominated soils present lower values—the observed patterns should be interpreted as indicative rather than conclusive. More robust explanations would require information on clay mineralogy, oxide crystallinity, specific surface area, and organic matter fractionation. Even so, integrating CEC with the oxide composition obtained via EDXRF reinforces the potential of EDXRF not only as a rapid preliminary tool for assessing soil properties, but also as a complementary approach for the indirect estimation of CEC in heterogeneous Cerrado soils.

Spectral identification and data processing

EDXRF spectra were acquired for all soil samples amended with LSW from the evaluated cities. Figure 1 shows the EDXRF spectra for all sampling locations. Each energy peak corresponds to a specific chemical element present in the sample: the peak position reflects the energy of radiation characteristic of the element, while the peak intensity is associated with its relative concentration. This relationship enables both the qualitative identification and quantitative assessment of the detected elements (Table I). In Figure 1, characteristic peaks of Al, Fe, Si, Ti, and Zr are evident and were identified using the instrument's built-in software.

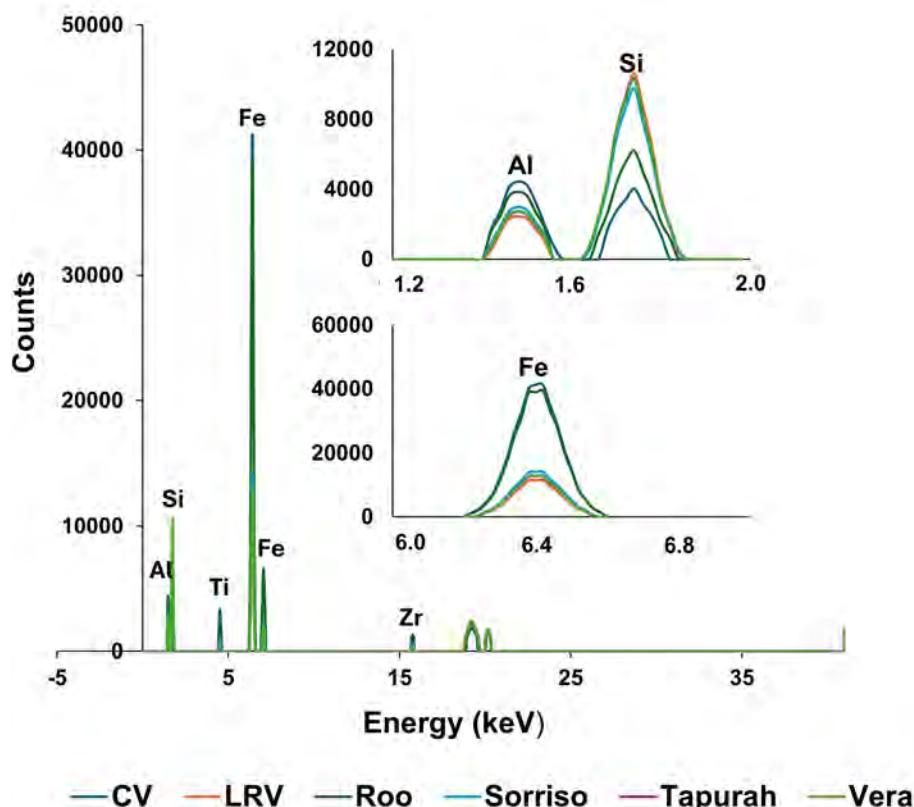


Figure 1. Energy-dispersive X-ray fluorescence (EDXRF) spectra obtained in the Ti–U channel for soil samples from the cities of Campo Verde (CV), Lucas do Rio Verde (LRV), Rondonópolis (Roo), Sorriso, Tapurah and Vera.

Figure 1 shows the raw spectra used for peak identification, without any preprocessing treatment. To prevent systematic differences in spectral intensity from affecting the performance of the multivariate calibration model, autoscaling was applied only to the variables used in the MLR model, as described in the Materials and Methods section. This preprocessing step ensures that differences in signal magnitude among the selected peaks do not bias the regression model.²³

Multivariate calibration model

The intensities obtained for TiK α , FeK β , ZrK α , AlK α , SiK α , SK α , KK α , and CaK α , together with the CEC values determined by the reference method, were used to construct the data matrices for the multivariate calibration model based on MLR. The performance of the developed model was evaluated using the adjusted coefficient of determination (R^2), root mean square error (RMSE), and the ratio of performance to deviation (RPD). The corresponding values obtained for the model are presented in Table II.

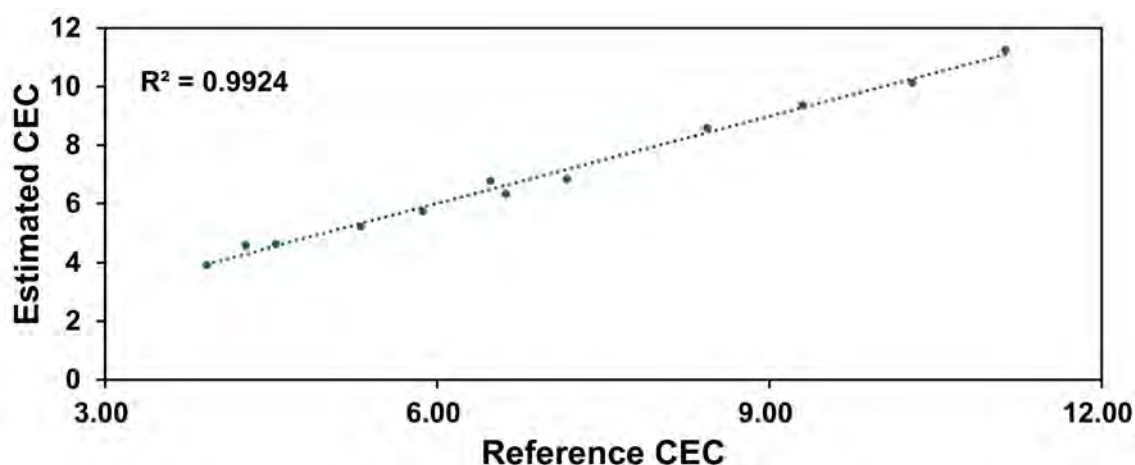
Table II. Multivariate multiple linear regression (MLR) calibration model for determining cation exchange capacity (CEC) using energy-dispersive X-ray fluorescence (EDXRF)

Parameters*	CEC
Sample concentration range (cmolc kg ⁻¹)	3.92 – 11.13
R ² _{adjusted}	0.97
RMSE (cmolc kg ⁻¹)	0.40
RPD	5.97

*Sample concentration range – concentration range of the samples used for model calibration; RMSE – root mean square error; RPD – ratio of performance to deviation.

An adjusted R² of 0.97 was obtained, indicating a strong correlation between the CEC values determined by the reference method and those estimated from the EDXRF-derived signal intensities using the MLR calibration model. The RMSE of 0.40 cmolc kg⁻¹ was well below the lowest CEC value observed in the samples, indicating good model performance within the studied concentration range. In soil science, models with RPD values > 2 are considered to exhibit excellent predictive ability.²⁴ In the proposed method, an RPD > 5 was obtained, indicating that CEC estimation using EDXRF spectral information presents high modeling capability.

Additionally, the CEC values estimated by the MLR calibration model showed strong agreement with the reference measurements, with a linear correlation coefficient (R²) greater than 0.99, as illustrated in Figure 2.

**Figure 2.** Relationship between the CEC values estimated by the EDXRF–MLR calibration model and the reference CEC measurements.

Comparing the results obtained in this study with those reported in the literature (Table III) is challenging due to several factors: (i) the type of instrumentation employed, since benchtop EDXRF systems generally provide higher energy resolution and lower noise than portable devices such as portable X-ray fluorescence (pXRF), (ii) the pedological heterogeneity of the sample sets, which reflects textural and mineralogical variability, (iii) differences in modeling approaches, including the use of linear or nonlinear models and the presence or absence of data fusion strategies, and (iv) the diversity of units used to express CEC (cmolc kg⁻¹ vs. cmolc dm⁻³) and the wide range of error metrics reported, such as RMSE, root mean square error of prediction (RMSEP), residual prediction deviation (RPD), ratio of performance to interquartile distance (RPIQ) and mean absolute error (MAE), which makes direct comparisons across studies difficult.

Table III. Comparison of models and methods for determining soil cation exchange capacity using X-ray fluorescence

Country – States*	Soil Type	Method**	Best Model***	R ² ****	Erro*****	Number of Sample (N)	Data Fusion	Ref.
Brazil – MT	Dystric and Rhodic Ferralsol Arenosol	EDXRF	MLR	0.97 R ² _{adj}	RMSE = 0.4 cmolc kg ⁻¹ RPD = 5.97	12	No	This work
United States – CA, NE	Hastings Filmore Butler Wasco	pXRF	MLR	0.908	RMSE = 2.498	360 – calibration 90 – validation	No	8
China – Yunnan	Agricultural and Forest Soils	pXRF + Vis-NIR	SVMR	0.82	RMSE = 3.02 RPIQ = 2.31	378 – calibration 194 – validation	Yes	19
Brazil – ES, BA, MG	Ultisols Oxisols Spodosols Entisols	pXRF	RF	0.75	RMSE = 0.89 cmolc dm ⁻³	197 – calibration 88 – validation	No	5
Brazil – SP, MS	Ultisol Inceptisol Entisol Oxisol Alfisol Entisol	XRF	XGB	0.78	RMSE = 12.12 MAE = 9.34 RPD = 1.94 RI = 0.00	~143 – calibration ~61 – validation	No	9
Brazil – PR	Rhodic Ferralsol Rhodic Nitisol	pXRF	PLS	0.9 - 1.0	RPD >2.0	~276 – calibration ~118 – validation	No	6
Brazil – PR (Cambé, Toledo)	Eutrophic Red Latosol	pXRF	PLS	Cambé – R ² = 0.68 Toledo – R ² = 0.69	RMSEP Cambé = 0.79 RPIQ Cambé = 2.95 RMSEP Toledo = 0.96 RPIQ Toledo = 2.28	calibration Cambé – 136 validation Cambé – 110 calibration Toledo – 100 validation Toledo – 65	No	7
Brazil – PR	Rhodic Ferrasol	XRF + GR	RF	0.70	RMSEP = 0.51 RPIQ = 2.83	64 – calibration 16 – validation	Yes	18
Brazil – MT, SP	Lixisol Ferralsol	VNIR + XRF + LIBS	GR	0.86	RPD = 2.63 RMSE = 9.95 mmolc dm ⁻³	68 – calibration 34 – validation	Yes	20

* MT – Mato Grosso; CA – California; NE – Nebraska; ES – Espírito Santo; BA – Bahia; MG – Minas Gerais; SP – São Paulo; MS – Mato Grosso do Sul; PR – Paraná. EDXRF – Energy-Dispersive X-ray Fluorescence Spectrometry; pXRF – Portable X-ray Fluorescence Spectrometry; GR – Gamma-Ray Spectrometry; VNIR – Visible and Near-Infrared Diffuse Reflectance; LIBS – Laser-Induced Breakdown Spectroscopy. ** MLR – Multiple Linear Regression; SVMR – Support Vector Machine Regression; RF – Random Forest; XGB – Extreme Gradient Boosting; PLS – Partial Least Squares Regression; GR – Granger and Ramanathan. ***R² – coefficient of determination; R²_{adj} – adjusted coefficient of determination. ****RMSEP – root mean square error of prediction; RPD – residual prediction deviation, RPIQ – ratio of prediction to interquartile range; RMSE – root mean squared error; MAE – mean absolute error.

Despite these limitations, the model developed in this study exhibited strong performance even with a reduced number of samples ($N = 12$). For comparison, Sharma et al.,⁸ using pXRF in soils from the United States ($N = 360$), reported $R^2 = 0.91$, whereas Andrade et al.,⁵ working with Brazilian soils, obtained $R^2 = 0.75$ with 197 samples. Silva et al.,⁹ using XRF and an Extreme Gradient Boosting (XGB) model ($N = 143$), reported $R^2 = 0.78$, and Ribeiro et al.,⁶ using 276 soil samples, reported R^2 values ranging from 0.90 to 1.00. More recent studies that incorporated spectral data fusion or advanced machine learning algorithms, such as Ribeiro et al.¹⁸ (XRF + GR, Random Forest (RF), $N = 64$, $R^2 = 0.70$), Wan et al.¹⁹ (pXRF + Vis-NIR, Support Vector Machine Regression (SVMR), $N = 378$, $R^2 = 0.82$), and Tavares et al.²⁰ (VNIR + XRF + LIBS, Granger and Ramanathan (GR), $N = 68$, $R^2 = 0.86$), reported results comparable to those observed in the present work. Overall, these findings highlight the potential of the proposed approach, particularly considering the limited sample size and the methodological simplicity adopted.

From the perspective of green analytical chemistry, the proposed EDXRF-based approach presents important advantages compared with conventional methods used for CEC determination. The reference method requires chemical extraction steps involving reagents and generates liquid waste during the analytical procedure. In contrast, the EDXRF technique allows the direct analysis of solid samples without the use of chemical reagents, minimizing waste generation and reducing the environmental impact of the analysis. In addition, the method is non-destructive, requires only simple sample preparation, and provides rapid measurements, which significantly reduces analysis time. These characteristics highlight the potential of EDXRF combined with multivariate calibration as a more sustainable analytical strategy for soil evaluation.

CONCLUSIONS

The association between EDXRF and MLR proved to be an efficient, rapid, and sustainable alternative for the indirect determination of CEC in Cerrado soils fertilized with LSW. The developed model exhibited excellent performance (adjusted $R^2 = 0.97$; RPD = 5.97; RMSE = $0.40 \text{ cmolc kg}^{-1}$), demonstrating a strong correlation with the reference method.

Even with a reduced number of samples ($N = 12$), the results were consistent, highlighting the potential of the proposed approach for exploratory applications. The samples analyzed were collected from agricultural areas cultivated with soybeans, corn, and pasture, where liquid swine manure has been applied as fertilizer for periods ranging from 5 to 10 years, reflecting real field conditions. The use of EDXRF combined with MLR proved to be a rapid and environmentally friendly strategy for estimating soil CEC. Furthermore, the method requires no chemical reagents, reduces analysis time, and preserves the samples, thereby aligning with the principles of green chemistry. Future studies including a larger number of samples and a broader range of soil conditions will be important to further validate and expand the applicability of the model.

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Conflicts of interest

The authors declare that there are no financial or personal conflicts of interest that could have influenced the work reported in this paper. This includes all affiliations, funding sources, and financial or management relationships.

Use of artificial intelligence tools

The authors declare that they have used ChatGPT for language editing and to improve the clarity of the manuscript. The authors take full responsibility for the accuracy, integrity, and originality of the article content, and confirm that no AI tool was used to generate novel scientific ideas, analyze data independently, or replace the critical role of the authors.

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IN MEMORIAM



Ramon M. Barnes (24.04.1940–06.07.2026)

Prof. Ramon Barnes finished his Ph.D. in analytical chemistry at the University of Illinois in 1966 and from 1968 to 1969 he was a postdoctoral research fellow at the Iowa State University. He then joined the University of Massachusetts (UMass, Amherst) where he taught analytical chemistry, with a focus on spectrochemical analysis from 1969 to 2000. After his retirement, he remained at UMass as Professor Emeritus of Chemistry.

To remember Prof. Barnes is to remember energy—inexhaustible energy to create and keep knowledge flowing and to help people learn and develop. His exceptional commitment to analytical chemistry was outstanding, reflecting a lifelong dedication to spectrochemical analysis, particularly plasma spectrochemistry. As he said in an interview with *The Analytical Scientist* in 2021: “I look at my career as one of public service – be it academic, industrial, or educational.”¹

In addition to his scientific contributions to this field, Prof. Barnes created the famous “Winter Conference on Plasma Spectrochemistry,” which has now been running for 40 years in the United States, followed by spin-off meetings in Europe and sometimes in Asia-Pacific countries. Anyone who has had the opportunity to participate in these meetings will testify to the hard work carried out by the professor and his team in bringing to all attendees a wonderful learning experience in a friendly atmosphere. How can one forget his beautiful (but not discrete!) Hawaiian shirts? Or the punctuality of the bell ringing to signal the restarting of sessions after coffee breaks? Hey, folks, let’s get back and get some work done!

Furthermore, it’s impossible not to recognize the professor’s dedication in founding the “ICP Information Newsletter” in 1975, which he went on to edit for decades. Over the years, I have had the wonderful opportunity to visit the Barnes research group in Amherst several times and I never ceased to be amazed by his commitment to bringing this updated compilation of plasmas in analytical chemistry to the ICP community. Absolutely admirable!

Later, Prof. Barnes founded and acted as the Director of the University Research Institute for Analytical Chemistry (URIAC) to foster education and research in spectrochemical analysis.

The professor also made major contributions in the area of sample preparation regarding inorganic analysis, with an emphasis on microwave-assisted sample preparation. In the 1990s he started the “Workshop on Sample Preparation,” held at UMass, and he was instrumental in bringing this workshop to Brazil, starting in 1996 at the Center for Nuclear Energy Agriculture (USP, Piracicaba) with Prof. Francisco José Krug as the chairman and reaching its XIV edition, 30th anniversary, in 2026 at the Federal University of Pelotas (RS). This sequence of workshops was essential to building a solid community in this field in Brazil with an appreciable international impact. The presence of Prof. Barnes at almost all of the workshops held in

several Brazilian universities was decisive in consolidating this event. He also played an important role in helping to bring specialists from abroad to collaborate in these workshops.

The fact that Prof. Barnes was always willing to return to Brazil and his readiness to share with us his huge experience in analytical chemistry was both impressive and unforgettable. His contributions to the development of spectrochemical analysis and sample preparation were recognized during the 17th Brazilian Meeting on Analytical Chemistry (Belo Horizonte, MG, 2013), where he presented a conference and received an award for his contributions to the development of analytical chemistry in Brazil. Recently, he was also honored in the XIV Workshop on Sample Preparation.

I could not conclude this tribute to Prof. Barnes without mentioning his adorable wife, Dorothy. I think it is better to allow the professor to speak for himself: “Yes, I’d like to mention my wife, Dorothy. We’ve been married for over 50 years, after meeting as graduate students at the University of Illinois. She’s much smarter than I am and certainly much more of a people-person! I’ve learned a great deal from her, and I appreciate all her encouragement and patience over the years. Without her, I think I’d not be doing what I’m doing. She deserves plenty of credit!”¹

What a great partners! We feel privileged to have known them. Thank you, Ramon; we so appreciate all that you achieved. It has been an honor to learn from you and is our mission to follow in your footsteps.

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By **Joaquim de Araújo Nóbrega** 

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FEATURE

Chemistry for National Sovereignty: 49th RASBQ Reaffirms the Strategic Role of Brazilian Science

From June 15 to 18, 2026, the city of Campinas hosted the 49th Annual Meeting of the Brazilian Chemical Society (49th RASBQ), once again establishing itself as the premier gathering of the national chemistry community. Over four days, researchers, professors, students, and professionals from different regions of the country engaged in an intensive scientific program addressing contemporary challenges in Chemistry, strengthening collaborative networks, and promoting Brazilian scientific production.



Participants arriving at the 49th RASBQ – Photo: SBQ 2026.

With the theme “Chemistry for National Sovereignty,” the 2026 edition of RASBQ reinforced the strategic role of science in the country’s development. Throughout the program, national sovereignty was addressed from different perspectives, highlighting the importance of scientific research, technological innovation, human resource development, and public policies capable of strengthening national autonomy in strategic areas.

Recognized as one of the largest chemistry events in Latin America, the 49th RASBQ brought together approximately 2,000 participants and offered a diverse program consisting of conferences, symposia, coordinated sessions, short courses, workshops, poster presentations, flash presentations, assemblies, and science communication activities. More than just a venue for presenting research results, the meeting has established itself as a prime setting for exchanging experiences, forging collaborations, and institutionally strengthening the Brazilian chemistry community.

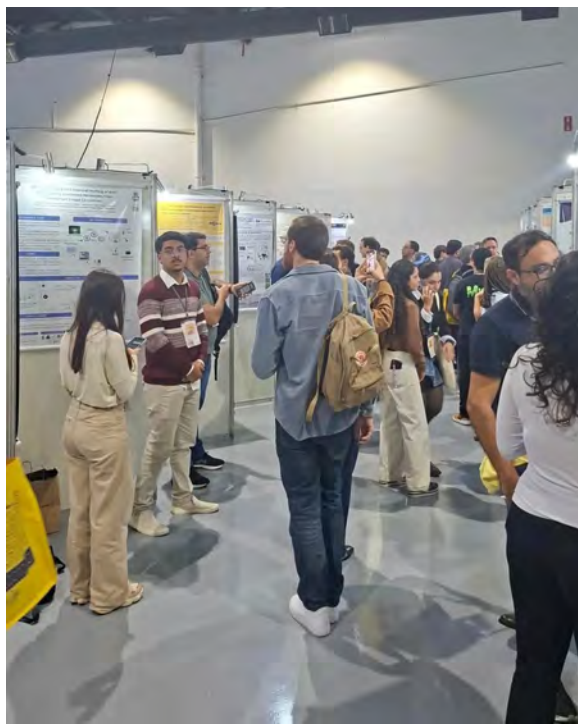


The 49th RASBQ brought together approximately 2,000 participants and offered a diverse scientific program – Photo: SBQ 2026.

The opening ceremony marked the beginning of the scientific activities and was enriched by a presentation by the [Projeto Canarinhos da Terra UNICAMP](#), a performing choir for children and young people that combines singing, theater, and body expression, with a focus on human and cultural development, symbolizing the integration of science, culture, and education. Also on the first day, technical visits to the National Center for Research in Energy and Materials (CNPEM) and specialized workshops provided opportunities for researchers to connect with major research centers in the country.

Among the scientific highlights was Symposium I, which promoted a broad reflection on the relationship between science, development, and national sovereignty. The lectures delivered by Renato Janine Ribeiro (University of São Paulo), who addressed science as an instrument for the good of everyone; Lucia Helena da Silva Maciel Xavier (Centre for Mineral Technology – CETEM), who highlighted Brazil's potential in critical and strategic minerals; and Carlos Graboio Gadelha (Fundação Oswaldo Cruz – Fiocruz), who discussed the relationship between sovereignty, development, and the right to life, showed that investing in science means investing in Brazil's future.

Another highly relevant moment was Symposium II, organized by the [Núcleo Mulheres SBQ](#), a group of women scientists established by SBQ in 2019, which addressed gender equity, institutional policies, and mentoring programs as fundamental instruments for transforming the academic environment. The lectures by Mônica Alonso Cotta (UNICAMP) and Valéria Neves Domingos Cavalcanti (UEM) brought reflections on how institutional policies, mentoring programs, and collective actions can transform the academic and scientific environment, strengthening gender equity in Chemistry and the sciences. The discussion highlighted that building a more diverse and inclusive science depends not only on recognizing existing inequalities, but also on implementing structural actions capable of expanding opportunities and strengthening the participation of groups historically underrepresented in scientific research.



Poster presentation session – Photo: SBQ 2026.

Poster presentation sessions remained among the busiest activities of the program. Much more than disseminating scientific results, these spaces fostered dialogue among research groups from different institutions, stimulated new collaborations, and expanded opportunities for exchange among researchers at different stages of their training. During the closing of the event, outstanding research in various areas of Chemistry was recognized with awards, highlighting the quality of the scientific production presented at the meeting.

The connection between universities and society also gained space through the traditional *SBQ na Escola* initiative, which brought together public-school high school students for experimental activities and interactive demonstrations. The initiative allowed young people to have direct contact with science, while also providing undergraduate and graduate students with an opportunity to develop scientific communication and knowledge dissemination skills for different audiences.



SBQ na Escola brought together public-school high school students for experimental activities and interactive demonstrations – Photo: SBQ 2026.

In addition to the scientific activities, the 49th RASBQ reaffirmed the role of the Brazilian Chemical Society as a coordinator of the national scientific community. Assemblies, meetings among scientific divisions, regional activities, and moments of integration strengthened the collective development of proposals aimed at advancing research, education, and innovation in the country. The event also highlighted the importance of cooperation among universities, research institutes, the productive sector, and funding agencies in addressing the scientific and technological challenges of the coming decades.

Science, Research, and the Productive Sector

The 49th RASBQ also featured a space dedicated to bringing together the scientific community, companies, and institutions involved in the development of technologies, products, and solutions for Chemistry. The exhibition pavilion enabled participants to learn about equipment, services, technologies, and different possibilities for research and development partnerships, expanding opportunities for interaction between academia and the productive sector.



The exhibition pavilion enabled participants to learn about equipment, services, and technologies – Photo: Lilian Freitas.

The event also received support from CAPES, CNPq, FAPESP, and OPCW, in addition to the participation of organizations and companies in different sponsorship categories. Diamond sponsors included ACS Publications, ACS Chemistry for Life, and CFQ/CRQs; Silver sponsors included HORIBA, NEON, and SHIMADZU; and Bronze sponsors included Anton Paar, analytikJena, BRUKER, CNPEM, flowacience, flowsupplyflowservice, General, LABORGLAS, Metrohm, Malvern Panalytical, Royal Society of Chemistry, Waters, TA Instruments, and Tns TENNESSINE. The exhibition pavilion also included Agilent, Nova Analítica, PerkinElmer, ALLCROM, PEAK, among other companies.

The presence of these organizations reinforced one of the central characteristics of the 49th RASBQ: the creation of an environment in which different actors in the scientific and technological ecosystem can share knowledge, present new solutions, and establish connections. For researchers and students, contact with companies and institutions during the event also represents an opportunity to learn about technologies available for their projects, identify new possibilities for collaboration, and bring academic research closer to the demands and challenges faced by the sector.

At the end of the meeting, the perception remained among participants that Brazilian Chemistry continues to play a central role in building solutions to strategic issues related to health, energy, sustainability, food security, industrial development, and environmental preservation. More than celebrating advances in national research, the 49th RASBQ reaffirmed that investing in science means strengthening Brazil's capacity to produce knowledge, train people, and build a future guided by innovation and scientific sovereignty.

Source: Sociedade Brasileira de Química (SBQ)

Text: Lilian Freitas – BrJAC Publisher

Disclosure

The feature was translated from Portuguese into English with the assistance of ChatGPT. BrJAC takes full responsibility for the final version.

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SPONSOR REPORT

Application note | 003883

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Comprehensive non-targeted workflow for confident identification of perfluoroalkyl substances (PFAS)

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Keywords

Perfluoroalkyl substances (PFAS), non-targeted analysis, Orbitrap Exploris 240 MS, HRAM, mass spectrometry, LC-MS, Compound Discoverer software

Application benefits

- High-resolution accurate-mass (HRAM) data acquired using the Thermo Scientific™ Orbitrap Exploris™ mass spectrometer platform enables confident identification of unknown perfluoroalkyl substances (PFAS).
- Thermo Scientific™ Compound Discoverer™ software (version 3.4) offers the most comprehensive commercially available workflow for PFAS analysis, covering over 40,000 PFAS.
- Compound Discover software enables complete and consistent application of the Schymanski¹ annotation confidence scale, including customizable prioritization of multiple spectral libraries and databases.
- Definition of annotation confidence scales using Compound Discoverer software is useful for other non-targeted applications in addition to PFAS analysis, including extractables and leachables, impurities analysis, food safety, toxicology, and others.

Goal

To demonstrate a comprehensive workflow for non-targeted PFAS analysis that combines HRAM sample analysis with the Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer and powerful automated data analysis and visualization using Compound Discoverer 3.4 software.

thermoscientific

Introduction

PFAS comprise a class of chemicals that, by definition, are fluorinated substances containing at least one fully fluorinated methyl or methylene carbon atom (without any H/Cl/Br/I atom attached to it), that is, with a few noted exceptions, any chemical with at least a perfluorinated methyl group ($-\text{CF}_3$) or a perfluorinated methylene group ($-\text{CF}_2-$).² Many PFAS are persistent, bioaccumulative, and toxic, making them contaminants of concern that are monitored by various regulatory agencies. However, regulatory methods typically use targeted quantitative analysis based on reference standards that do not address the thousands of novel PFAS and their transformation products for which there are no commercially available standards. Non-targeted methods that detect and identify compounds in complex mixtures with little to no prior knowledge about the PFAS present are therefore needed to understand the true extent of contamination and to facilitate source fingerprinting for remediation.

There are multiple fundamental challenges that limit the capability of non-targeted PFAS analysis. This has led to multiple working groups across the scientific community, such as the Best Practices for Non-Targeted Analysis (BP4NTA) group, that focus on non-targeted approaches.³ First and foremost, the general consensus is that identifications of unknown PFAS need to be made with as much confidence as possible. This requires a high level of selectivity, which is obtained through high-accuracy and high-resolution mass measurements of the monoisotopic masses of the intact molecule and the fragments generated using high energy collision-induced dissociation (HCD) or collision-induced dissociation (CID) or other fragmentation techniques. Second, with the limited availability of authentic standards to generate reference spectral libraries, scientists must rely on other tools and resources such as *in silico* generated spectral libraries, fragment databases, and exhaustive chemical databases.

One of the most important steps in a non-targeted PFAS workflow is to assign a level of confidence in the final PFAS annotation. Schymanski, et al.¹ and Charbonnet, et al. 2022⁴ provide a framework for coupling high-resolution accurate-mass analysis with a comprehensive set of libraries, databases, and other PFAS-specific tools to make such assignments. Because of differences in mass analyzer technologies that result in varied mass resolution and mass accuracy, and the assorted use of libraries, databases, and tools, there can be wide-spread inconsistencies in the assignment of PFAS annotation confidence levels. To evaluate these inconsistencies across

testing laboratories, NIST carried out the Per- and Polyfluoroalkyl Substances–Non-Targeted Analysis Interlaboratory Study (PFAS NTAILS).⁵ Thermo Scientific™ Orbitrap™ mass analyzer technology combined with Compound Discoverer^{3,4} software addresses annotation disparities by providing sub-1 ppm mass error and ultra-high mass resolution along with the most comprehensive collection of PFAS-specific libraries, databases, and tools and a simplified, automated, and accurate approach to consistently assigning annotation confidence levels.

This application note demonstrates a comprehensive non-targeted PFAS annotation workflow that combines Orbitrap Exploris 240 mass spectrometer sample analysis with powerful automated data analysis and visualization using Compound Discoverer 3.4 software. An analysis of “unknown” samples from the PFAS NTAILS⁵ is used to explain how the software leverages PFAS-specific databases, reference spectral libraries, and *in silico* generated spectral libraries covering more than 40,000 PFAS in a unified non-targeted data processing workflow. Software features that enable complete and consistent implementation of the Schymanski annotation confidence scale, including definition of the priority of spectral libraries and databases used for PFAS annotation, are presented. Data comparison and visualization tools that facilitate determination of PFAS sample composition are also covered, including principal component analysis (PCA) and differential analysis plots, mass defect plots for identification of homologous series of PFAS, and molecular networks to view structurally related PFAS.

Experimental Samples

Three samples from the NIST PFAS NTAILS⁵ representing unknowns were analyzed to demonstrate workflow performance. All samples were analyzed as received from NIST without further dilution. Sample A was a mixture of spiked PFAS reference standards in methanol. Each individual PFAS concentration was 0.1 µg/g of solution. The spiked PFAS in the sample are provided in reference 5. Sample B was a mixture of two aqueous film-forming foam (AFFF) formulations—an electrochemical fluorination product and a fluorotelomerization product—diluted in methanol to a total mass concentration of 1,000 µg of AFFF product per 1 g of solution. Sample C was a methanolic extract of AFFF-impacted soil, prepared by extracting approximately 400 g of soil via ultrasonication in 600 mL methanol. The final extract was concentrated 20-fold. The final extract was spiked with an analytical standard of perfluorohexane sulfonamido amine (N-AP-FHxSA) to a nominal concentration of 0.1 µg/g.

Instrument method

Ultra-high-performance liquid chromatography (UHPLC) was performed using a Thermo Scientific™ Vanquish™ Flex UHPLC system fitted with the PFAS Installation Kit that replaces all wetted materials containing fluoropolymers with PEEK, with the exception of the solvent degasser membrane. Furthermore, a delay column was installed between the solvent pump and the analytical column to shift any background contamination from the solvents to a later retention time in the chromatogram. All chromatographic conditions are listed in Table 1.

Table 1. Chromatographic conditions

Parameter	Value																										
Mobile phase A	Water + 20 mM ammonium acetate																										
Mobile phase B	Methanol + 20 mM ammonium acetate																										
Columns	Delay: 30 × 3 mm C18 column Analytical: 50 × 3 mm C18 column																										
Flow rate	0.400 mL/min																										
Column temperature	40 °C																										
Autosampler temperature	10 °C																										
Gradient	<table><tr><th>Time (min)</th><th>% B</th></tr><tr><td>0</td><td>10</td></tr><tr><td>0.3</td><td>55</td></tr><tr><td>4</td><td>65</td></tr><tr><td>7.4</td><td>75</td></tr><tr><td>9</td><td>80</td></tr><tr><td>9.5</td><td>85</td></tr><tr><td>11</td><td>90</td></tr><tr><td>12</td><td>95</td></tr><tr><td>13</td><td>99</td></tr><tr><td>13.5</td><td>99</td></tr><tr><td>15</td><td>10</td></tr><tr><td>17</td><td>10</td></tr></table>	Time (min)	% B	0	10	0.3	55	4	65	7.4	75	9	80	9.5	85	11	90	12	95	13	99	13.5	99	15	10	17	10
Time (min)	% B																										
0	10																										
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13.5	99																										
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Mass spectrometry analysis was performed using an Orbitrap Exploris 240 mass spectrometer equipped with the Thermo Scientific™ EASY-IC™ (internal calibration) source. The MS ion source parameters are listed in Table 2. The mass spectrometer was operated in the Full Scan (MS¹) plus data-dependent MS² (ddMS²) mode using the parameters shown in Table 3. The ddMS² mode acquires a full scan (survey scan) followed by a set of ddMS² scans during which HCD fragmentation is applied. Stepped normalized collision energies were used to ensure (1) consistent fragmentation of all precursors across the full scan mass range and (2) the collection of rich fragmentation patterns for all MS² spectra collected.

Table 2. MS ion source parameters

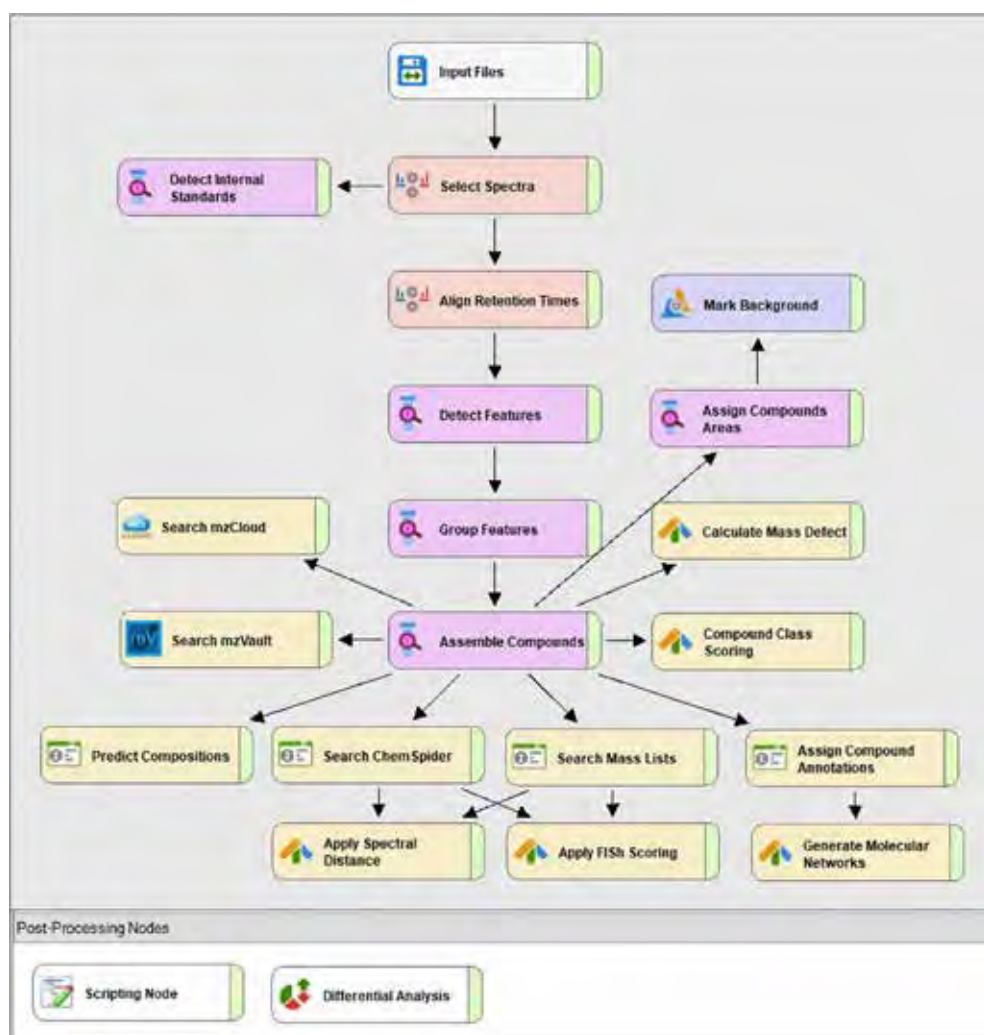
Parameter	Value
Spray voltage	-1,000 V
Vaporizer temperature	300 °C
Ion transfer tube temperature	300 °C
Sheath gas	56 a.u.
Aux gas	10 a.u.
Sweep gas	1 a.u.

Table 3. MS parameters for full MS-ddMS² experiments

MS parameter	Value
Full Scan	
AGC target	Standard (1e6)
Full Scan (MS ¹) resolution	240,000 (FWHM at <i>m/z</i> 200)
Full Scan (MS ¹) mass range	<i>m/z</i> 110–1,200
Lock mass correction	EASY-IC source, scan-to-scan, full scan only
Mild trapping mode	On
RF level (%)	55
ddMS² Scan	
Isolation window (<i>m/z</i>)	1.0
HCD collision energies (normalized, %)	5, 30, 60
MS ² resolution	30,000 (FWHM at <i>m/z</i> 200)
Maximum injection time	Auto
Intensity filter threshold	50,000 cps
Dynamic exclusion filter	<i>n</i> =3; 6 s exclusion; exclude isotopes

Data processing and analysis

LC-MS data were automatically processed in Compound Discoverer 3.4 software using the workflow shown in Figure 1. The workflow was built in the workflow editor starting with the “PFAS Unknown ID” pre-built template that is supplied with the software. The workflow uses multiple spectral libraries and other resources to automatically process data (Figure 1). A Scripting Node with the “PFAS.R” script is included to calculate values pertaining to the Orthogonal MS¹ data filtering approach developed by Kaufmann, et al.⁶ Additional information, including the PFAS.R script, can be downloaded from mycompounddiscoverer.com. Automatic data processing produces a results file that contains a list of compounds that have been detected through the various workflow nodes shown in Figure 1.



The initial compounds list generated by Compound Discoverer software includes all compounds it detected, including non-PFAS compounds. This list is filtered so only PFAS are included. Next, the list of PFAS must be evaluated to either approve the annotations automatically assigned or, in some cases, to assign alternative annotations from the resources used. At the same time, annotation confidence levels are assigned. All data reduction and organization steps are performed using the Data Filtering and Tagging features in Compound Discoverer software. As outlined in Figure 2, an annotation confidence level from one to four was manually assigned to each detected compound based on the resources (i.e., library, database, etc.) used to identify it per the approach described by Schymanski, et al.¹ and Charbonnet, et al.⁴ Level 1 annotations required all annotation criteria be met (except a match to the Duke University *in silico*-generated PFAS spectral library or the FluoroMatch™ PFAS fragment database), including a retention time match to a reference standard analyzed on the same instrumentation. The

reference standard retention times were in a custom Mass List built and stored in Compound Discoverer software. Level 2 annotations required a match to a reference spectral library, either the Thermo Scientific™ mzCloud™ advanced mass spectral database or 2023 NIST Tandem Mass Spectral Library. Level 4 was a suspect hit without a retention time or spectral library match but with a full match to a PFAS database (either the NIST Suspect List, EPA PFAS Structure List, or the Duke University *in silico* PFAS database) with a measured mass and isotope pattern match for the molecular formula of the suspect PFAS. If the Level 4 PFAS also contained MS² fragment information, then a match of at least one measured fragment to the Duke University *in silico* PFAS spectral library⁷ or the FluoroMatch PFAS fragment database⁸ moved its annotation confidence to Level 3. For complex samples like Samples B and C, applying additional resources such as the Duke University *in silico* PFAS spectral library and FluoroMatch PFAS fragment database greatly expands the scope of PFAS annotation.

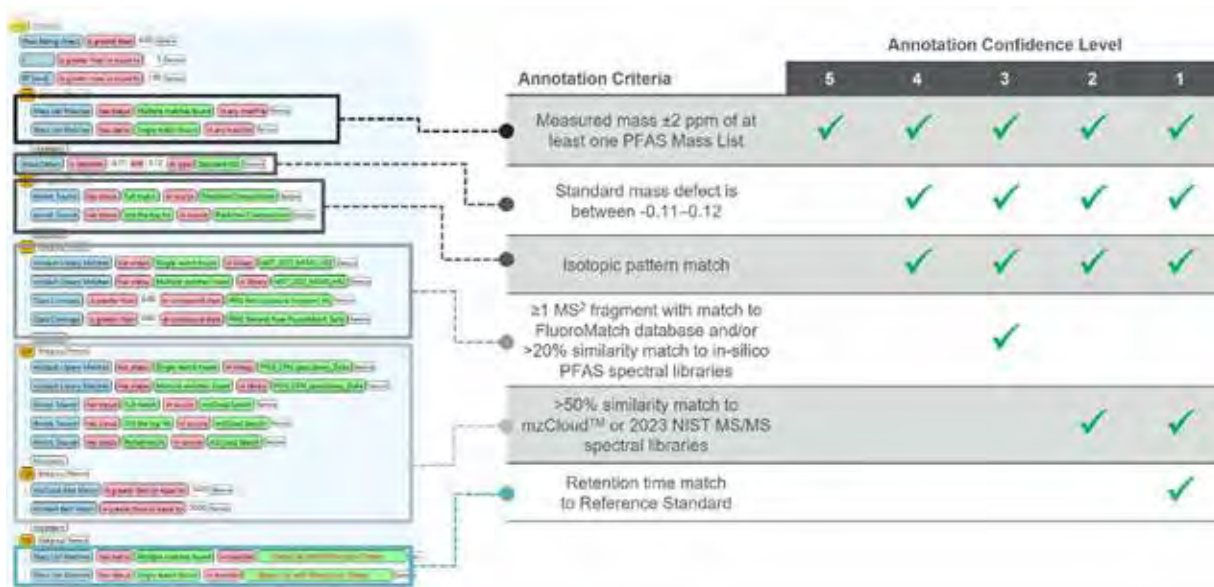


Figure 2. Detected compounds in the samples were filtered using the Data Filter tool in Compound Discoverer software (revision 3.4). Columns in the Compounds Table (Table 4 provides an example) can be used as variables by which the results can be filtered. Left: filters used to assign Level 1 annotation confidence. A filter set designed for Level 2 annotations would be the same except that the last two filter properties (highlighted in teal) would be removed. Right: relationship between the annotation criteria and confidence levels. The filter sets are available at mycompounddiscoverer.com.

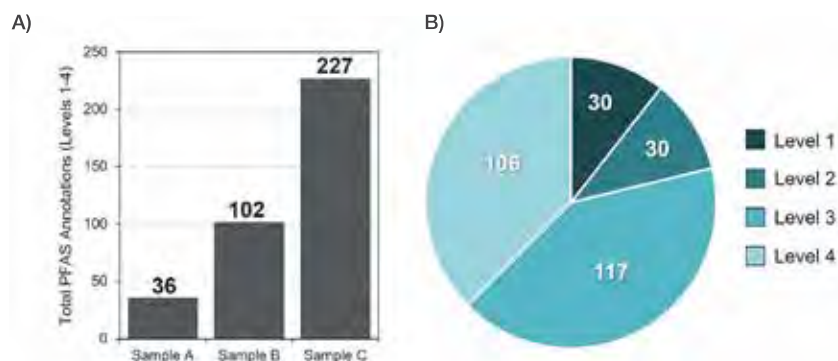


Figure 3. (A) Total number of PFAS detected and annotated at confidence Levels 1–4, per sample; (B) Distribution of all PFAS compounds, across all samples, per annotation confidence Level 1–4

Results and discussion

Automated PFAS annotation and assignment of confidence levels

The workflow described here combines the sub-1 ppm mass accuracy and ultra-high mass resolution of the Orbitrap Exploris 240 mass spectrometer with automated data processing and a comprehensive set of PFAS-specific databases, spectral libraries, and *in silico* fragmentation tools in Compound Discoverer 3.4 software to provide confident PFAS annotation. Compound Discoverer software processes the data files first to detect and group mass spectral features, and then assembles these features into individual compounds by combining the features that are adducts and isotopes of the same compound. In addition, Compound Discoverer 3.4 software leverages the mzCloud advanced mass spectral database to identify in-source

fragments that are then also grouped with the adducts and isotopes of the assembled compound. MS¹ and MS² spectra that were acquired closest to assigned retention times of the compounds are also linked to the assembled compound. Following detection and assembly, the compounds are annotated using libraries and databases the user specified in the workflow. This comprehensive, automated workflow enabled annotation of more than 250 PFAS across the three samples analyzed in this study. Figure 3 summarizes the total number of PFAS detected in each sample and the distribution of assigned annotation confidence levels. Due to the ultra-high mass accuracy and resolution of the Orbitrap Exploris 240 mass spectrometer, any detected feature with a monoisotopic mass not matching any of the three Mass Lists had an extremely low probability of being a PFAS. Therefore, only Levels 1–4 are shown.

As shown in Figure 2, Levels 1-4 annotations require confident assignment of elemental formula through both high mass accuracy and full match between the measured and theoretical isotope pattern. For PFAS with heteroatoms that contain multiple naturally occurring isotopes (e.g., N, S, O, etc.), the ability to fully resolve all isotopes is critical in confidently assigning the correct empirical formula. In this work we used the ultra-high mass resolution of the Orbitrap Exploris 240 mass spectrometer, collecting all MS¹ spectra at 240,000 resolution (FWHM at *m/z* 200), enabling fully resolved measurements of the ¹⁵N, ³³S, and ¹³C isotopes. With the Predict Composition node, all empirical formulas within 2 ppm mass error were determined. For each formula, the measured and theoretical isotope patterns are compared, providing pattern coverage scores and also annotating the MS¹ spectrum with the position of expected isotopes. Figure 4 shows an example of comparing the measured A+1 isotope pattern against the top three formulas based on Pattern Coverage scores. While all three formulas contain

nitrogen and sulfur, the measured relative intensities of the ¹⁵N and ³³S match to only one of the formulas. This fine isotope structure analysis of ¹⁵N and ³³S isotopes is not possible at lower mass resolutions.

The top 40 PFAS detected in each sample are shown in Tables 4, 5, and 6 with annotation Levels 1-4, with the exception of Sample A, which only contained 36 PFAS. These tables are screenshots of the Compounds table in Compound Discoverer 3.4 software, which offers a simple, easy-to-navigate interface that combines all the information from each resource for every detected feature and includes all the compound annotations that were automatically assigned. The confidence levels listed in the “Tags” column were determined using the criteria shown in Figure 2 and the “Comments” column shows the NIST Suspect ID for each PFAS, if available. Annotations with Level 4 confidence used the following mass list priority: NIST Suspect List, EPA PFAS Structure List, and Duke University *in silico* PFAS spectral library.

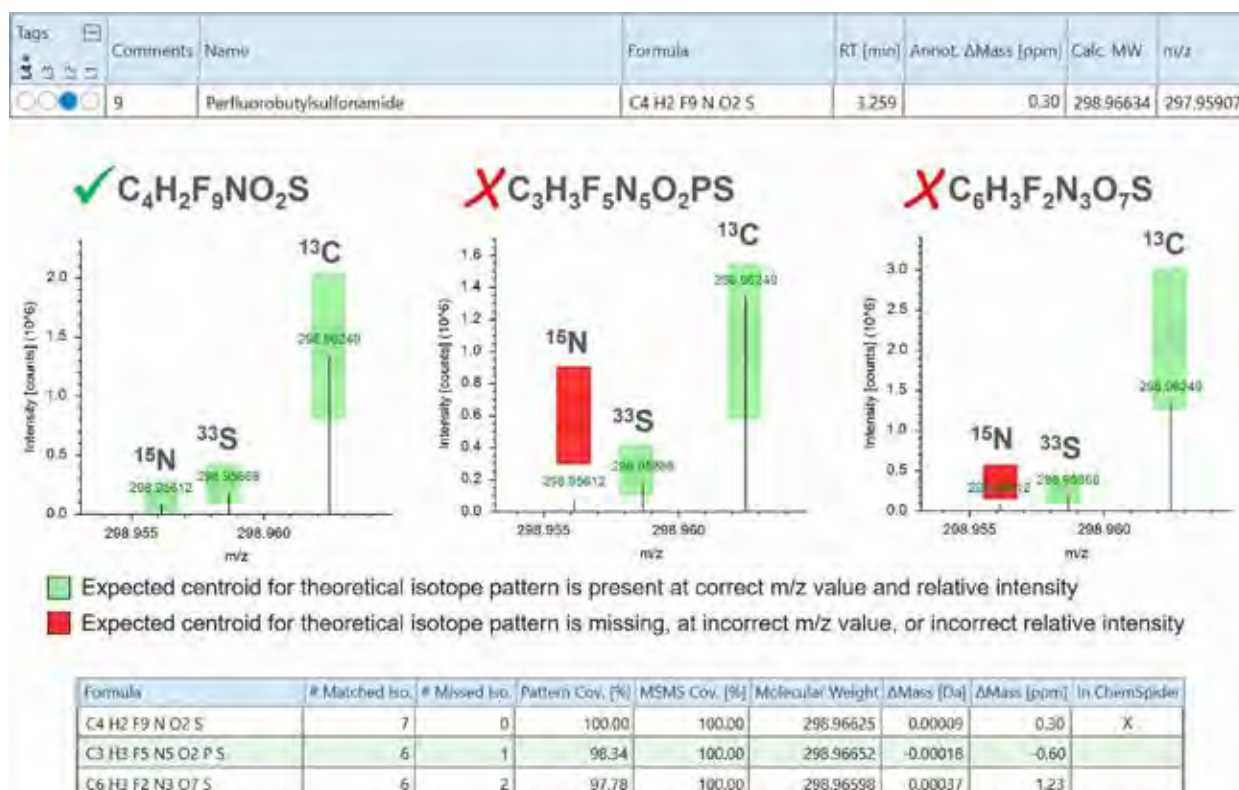


Figure 4. Example of using the measured A+1 fine isotope pattern to accurately predict the elemental formula for perfluorobutylsulfonamide. As the user selects a possible formula within the Predicted Compositions table, Compound Discoverer software annotates the correct position of the expected isotope pattern, providing a simple visualization of how well the measured and theoretical isotope patterns match. A minimum mass resolution of 240,000 (FWHM at *m/z* 200) is required to resolve each of the three ¹²C, ¹⁵N, and ³³S isotopes.

For complex samples like Samples B and C, applying additional resources such as the Duke University *in silico* PFAS spectral library and FluoroMatch PFAS fragment database significantly expanded the confidence and scope of PFAS annotation (Table 4). Using these resources, 35% and 44% of all PFAS

Table 4. List of PFAS detected (36) in Sample A with their annotation confidence level displayed in the Compounds table of the Compound Discoverer software interface. The confidence levels listed in the “Tags” column were determined using the criteria in Figure 2. The list is sorted based on the integrated peak areas of the sample. The “Comments” column shows the NIST Suspect ID for each PFAS, if available.

[illegible]

Table 5. List of top 40 PFAS detected in Sample B with their annotation confidence level displayed in the Compounds table of the Compound Discoverer software interface. The confidence levels listed in the “Tags” column were determined using the criteria in Figure 2. The list is sorted based on the integrated peak areas of the sample. The “Comments” column shows the NIST Suspect ID for each PFAS, if available.

Tag	Compound Name	Formula	RT (min)	Calc. MW	MS	Area, 2MMS (area)	MS, ms/Cloud (area)	Library Match	Class Coverage	Mass (of) Match	Mass (Def)	Match Score	Match Source	Library Name
351	N,N'-bis(2-hydroxy-2-propylhexafluoroethyl)sulfonamide	C18H27F13N2O4S	6.281	528.2780	527.08714	-1.19		100%	100%	100%	100%	100%	100%	100%
3043	Perfluorooctanesulfonic acid	C8H5F17O2S	7.044	498.0270	498.02572	-1.00		100%	100%	100%	100%	100%	100%	100%
3052	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	7.088	427.9187	425.90748	-0.97		100%	100%	100%	100%	100%	100%	100%
4390	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	8.000	511.0402	510.04001	-0.78		100%	100%	100%	100%	100%	100%	100%
3081	Perfluorooctanesulfonic acid	C8H5F17O2S	8.824	498.0271	498.02582	-0.79		100%	100%	100%	100%	100%	100%	100%
818	Perfluorooctanesulfonamide	C8H5F17N2O2S	9.718	484.0400	483.04042	-0.66		100%	100%	100%	100%	100%	100%	100%
3042	Perfluorooctanesulfonic acid	C8H5F17O2S	13.87	509.0452	509.04324	-0.81		100%	100%	100%	100%	100%	100%	100%
851	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	15.07	512.0870	511.08728	-0.97		100%	100%	100%	100%	100%	100%	100%
192	1,1'-bis(2-hydroxy-2-methylpropyl)-1,1'-bis(4,4,4-trifluorobutyl)	C24H38F9O4	16.07	584.2511	583.04791	-0.95		100%	100%	100%	100%	100%	100%	100%
827	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	17.08	503.0471	502.04848	-0.28		100%	100%	100%	100%	100%	100%	100%
879	Perfluorooctanesulfonamide	C8H5F17N2O2S	18.44	484.0401	483.04083	-0.21		100%	100%	100%	100%	100%	100%	100%
887	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	19.85	506.0920	505.09272	-0.26		100%	100%	100%	100%	100%	100%	100%
3043	Perfluorooctanesulfonic acid	C8H5F17O2S	21.38	509.0454	508.04627	-0.39		100%	100%	100%	100%	100%	100%	100%
3045	Perfluorooctanesulfonic acid	C8H5F17O2S	23.98	509.0453	508.04651	-0.29		100%	100%	100%	100%	100%	100%	100%
3053	Perfluorooctanesulfonamide	C8H5F17N2O2S	24.79	514.0581	513.05844	-0.34		100%	100%	100%	100%	100%	100%	100%
3044	Perfluorooctanesulfonic acid	C8H5F17O2S	25.60	518.0601	518.05958	-0.64		100%	100%	100%	100%	100%	100%	100%
2643	Perfluorooctanesulfonic acid	C8H5F17O2S	26.08	513.0604	512.06086	-0.17		100%	100%	100%	100%	100%	100%	100%
3062	Perfluorooctanesulfonic acid	C8H5F17O2S	28.80	518.0604	518.05818	-0.51		100%	100%	100%	100%	100%	100%	100%
2921	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	28.89	514.0582	513.05872	-0.13		100%	100%	100%	100%	100%	100%	100%
4489	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	29.89	483.0173	482.01899	-0.25		100%	100%	100%	100%	100%	100%	100%
4378	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	30.88	527.0435	526.04328	-0.08		100%	100%	100%	100%	100%	100%	100%
3402	Perfluorooctanesulfonic acid	C8H5F17O2S	32.00	518.0584	518.04824	-0.31		100%	100%	100%	100%	100%	100%	100%
2671	Perfluorooctanesulfonic acid	C8H5F17O2S	32.73	473.0183	472.01804	-0.90		100%	100%	100%	100%	100%	100%	100%
3041	Perfluorooctanesulfonic acid	C8H5F17O2S	33.19	489.0186	488.01829	-0.01		100%	100%	100%	100%	100%	100%	100%
3403	Perfluorooctanesulfonic acid	C8H5F17O2S	33.42	518.0581	518.04823	-0.19		100%	100%	100%	100%	100%	100%	100%
363	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	33.66	484.0187	483.01818	-0.17		100%	100%	100%	100%	100%	100%	100%
3238	Chloro-perfluorooctanesulfonic acid	C8H4ClF16O2S	33.97	515.0278	514.02809	-0.15		100%	100%	100%	100%	100%	100%	100%
3238	Chloro-perfluorooctanesulfonic acid	C8H4ClF16O2S	33.97	515.0278	514.02809	-0.14		100%	100%	100%	100%	100%	100%	100%
3401	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	34.03	484.0188	483.01877	-0.29		100%	100%	100%	100%	100%	100%	100%
861	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	34.14	506.0720	505.07083	-0.06		100%	100%	100%	100%	100%	100%	100%
2732	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	34.74	482.0180	481.01842	-0.18		100%	100%	100%	100%	100%	100%	100%
3042	Perfluorooctanesulfonic acid	C8H5F17O2S	34.84	489.0400	488.03828	-0.28		100%	100%	100%	100%	100%	100%	100%
3403	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	34.90	506.0721	505.06923	-0.24		100%	100%	100%	100%	100%	100%	100%
2654	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	35.13	484.0182	483.01884	-0.51		100%	100%	100%	100%	100%	100%	100%
2636	Perfluorooctanesulfonic acid	C8H5F17O2S	35.29	518.0603	517.05965	-0.19		100%	100%	100%	100%	100%	100%	100%
3401	6,6'-Difluorobiphenyl-2,2'-diol	C12H6F2O2	35.33	515.0278	514.02826	-0.36		100%	100%	100%	100%	100%	100%	100%
3401	Perfluorooctanesulfonic acid	C8H5F17O2S	35.33	515.0278	514.02826	-0.36		100%	100%	100%	100%	100%	100%	100%
2640	Perfluorooctanesulfonic acid	C8H5F17O2S	35.38	518.0603	517.05965	-0.35		100%	100%	100%	100%	100%	100%	100%
2640	Perfluorooctanesulfonic acid	C8H5F17O2S	35.38	518.0603	517.05965	-0.34		100%	100%	100%	100%	100%	100%	100%
176	Perfluorooctanesulfonamide	C8H5F17N2O2S	35.37	514.0423	513.04131	-0.87		100%	100%	100%	100%	100%	100%	100%
3027	Perfluorooctanesulfonic acid	C8H5F17O2S	35.96	413.0733	412.07025	-0.41		100%	100%	100%	100%	100%	100%	100%

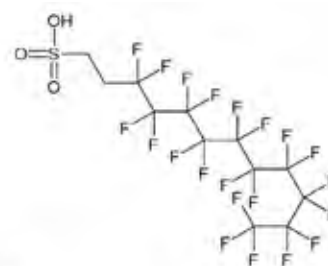
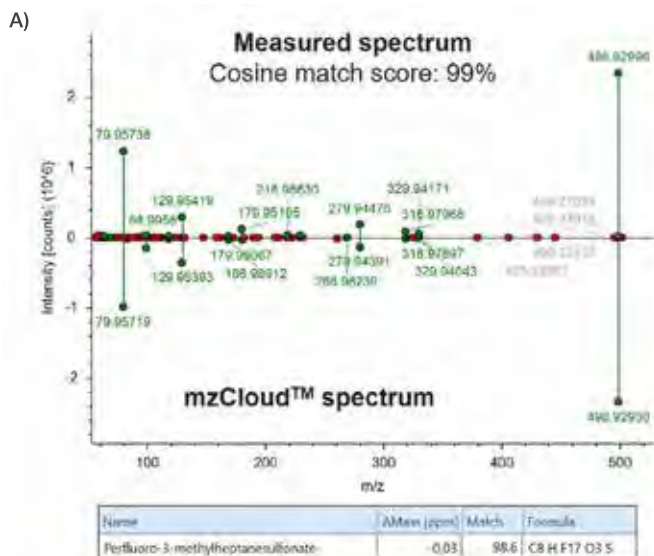
Table 6. List of top 40 PFAS detected in Sample C with their annotation confidence level displayed in the Compounds table of the Compound Discoverer software interface. The confidence levels listed in the “Tags” column were determined using the criteria in Figure 2. The list is sorted based on the integrated peak areas of the sample. The “Comments” column shows the NIST Suspect ID for each PFAS, if available.

Tag	Compound	Formula	FW	Cal. MW	Exp. MW	Mass Error (ppm)	Retention Time (min)	Abundance (cps)	Library Name	Library MW	Library Formula	Library Retention Time (min)	Library Abundance (cps)	Library Score	Library Tags	Library Comments
1	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.20	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
2	Perfluorooctanesulfonamide	C ₈ H ₁₅ F ₁₇ N ₂ O ₄ S	718.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonamide	718.0	C ₈ H ₁₅ F ₁₇ N ₂ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonamide
3	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
4	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
5	Perfluorooctanesulfonamide	C ₈ H ₁₅ F ₁₇ N ₂ O ₄ S	718.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonamide	718.0	C ₈ H ₁₅ F ₁₇ N ₂ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonamide
6	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
7	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
8	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
9	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
10	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
11	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
12	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
13	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
14	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
15	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
16	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
17	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
18	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
19	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
20	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
21	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
22	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
23	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
24	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
25	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
26	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
27	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
28	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
29	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
30	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
31	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
32	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
33	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
34	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
35	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
36	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
37	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
38	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
39	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid
40	Perfluorooctanesulfonic acid	C ₈ H ₁₇ F ₁₇ O ₄ S	704.0	489.02700	489.02512	-1.23	11.8	11.8	Perfluorooctanesulfonic acid	704.0	C ₈ H ₁₇ F ₁₇ O ₄ S	11.8	11.8	100%	100%	Perfluorooctanesulfonic acid

Spectral databases enhance annotation confidence

The Compound Discoverer 3.4 software workflow described here leverages the upgraded mzCloud advanced mass spectral database that currently contains MSⁿ spectra for more than 120 PFAS and the 2023 NIST Tandem Mass Spectral Library, enabling annotation of hundreds of PFAS compounds with Level 2 confidence or better. Figure 5 shows an example of using these libraries to identify a branched isomer of perfluorooctane sulfonate (with a 99% Cosine match score to a spectrum in the mzCloud advanced mass spectral database) and 6:2 fluorotelomer sulfonic acid (with an 87% NIST match

score to a spectrum in the 2023 NIST Tandem Mass Spectral Library). As previously mentioned, the Duke University *in silico* PFAS spectral library is also valuable for identifying less common PFAS that are not in other spectral libraries. In this case, more than fifteen PFAS were annotated with Level 3 confidence, including the 10:2 fluorotelomer sulfonate shown in Figure 6, using the Duke University library. Match scores for comparisons of measured MS² spectra to the Duke University *in silico* PFAS spectral library are also calculated using the NIST search algorithm.



10:2 fluorotelomer sulfonic acid
NIST Suspect ID: 3050

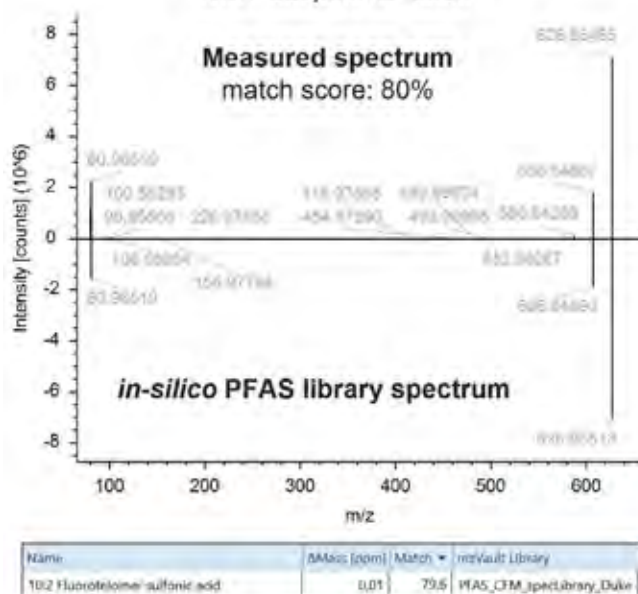


Figure 6. Example that applies the Duke University *in silico* PFAS spectral library to achieve Level 3 annotation confidence for 10:2 fluorotelomer sulfonic acid

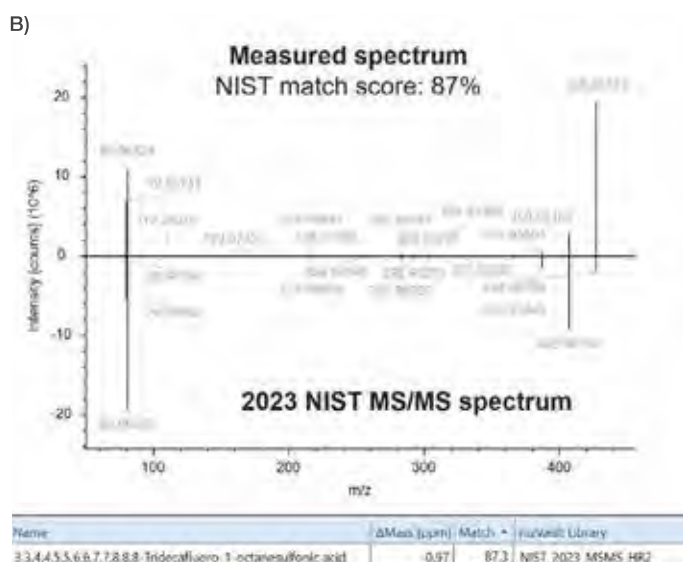


Figure 5. Example of using reference spectral libraries to achieve Level 2 annotation confidence. (A) Identification of perfluoro-3-methylheptanesulfonate (branched isomer of perfluorooctane sulfonate) using the m/zCloud advanced mass spectral database, and (B) identification of 6:2 fluorotelomer sulfonic acid using the 2023 NIST Tandem Mass Spectral Library.

Additional Level 3 annotations of less common PFAS not included in reference spectral libraries were possible using the FluoroMatch PFAS fragment database. The FluoroMatch PFAS fragment database is applied in the Compound Class Scoring processing node (Figure 1), which calculates the percentage of fragments in the measured MS² spectrum that match the fragments in the FluoroMatch database (within ± 5 ppm). Figure 7 shows an example of how the FluoroMatch PFAS fragment database was applied to annotate the PFAS in a homologous series with Level 3 confidence. The series in Figure 7 shows three PFAS, including two isomers of N-[3-(dimethylamino)propyl]-N-[(tridecafluorohexyl)sulfonyl]-beta-alanine.

Visualization of PFAS results adds insight

In addition to PFAS detection and annotation, Compound Discoverer 3.4 software includes multiple data visualization and statistical comparison options that provide insights about the PFAS composition of samples. For example, the mass defect plotting tool can facilitate the identification of homologous series of PFAS. Figure 8 shows the Kendrick mass defect plot for all PFAS detected and annotated in this study, with the Kendrick formula set to "CF₂." PFAS that are in the same homologous series exhibit the same Kendrick mass defect. An example of a homologous series of bis-perfluoro-N-alkyl sulfonamides

(bis-FASl) that is detected and identified in the AFFF-impacted soil is highlighted in red in Figure 8.

The Orthogonal MS¹ plot was developed by Kaufmann, et al⁶ as an alternative for identifying PFAS that contain heteroatoms with negative mass defects (i.e., S, Cl, and Br). In comparison to the standard mass defect plot, the Orthogonal MS¹ plot better distinguishes perfluorinated compounds from non-PFAS organic compounds. The PFAS NTA workflow in Compound Discoverer 3.4 software uses the Scripting mode (Figure 1) with the "PFAS.R" script to automatically calculate the values needed for the plot:

- Estimated number of carbons (eC; based on the observed A1/A0 ratio in the apex MS¹ spectrum of the feature)
- Molecular mass divided by eC (m/C)
- Mass defect divided by eC (md/C)

These values, and the number of fluorine atoms in the assigned formula, are recorded in the Compounds table associated with the results file. Using the Compound Discoverer software Result Chart tool, an Orthogonal MS¹ plot was generated from the calculated md/C and m/C values (Figure 9). Because the m/C and md/C values are calculated based on experimental data rather than the predicted formula, the Kaufmann Plot here slightly differs from those observed in previous studies.⁹

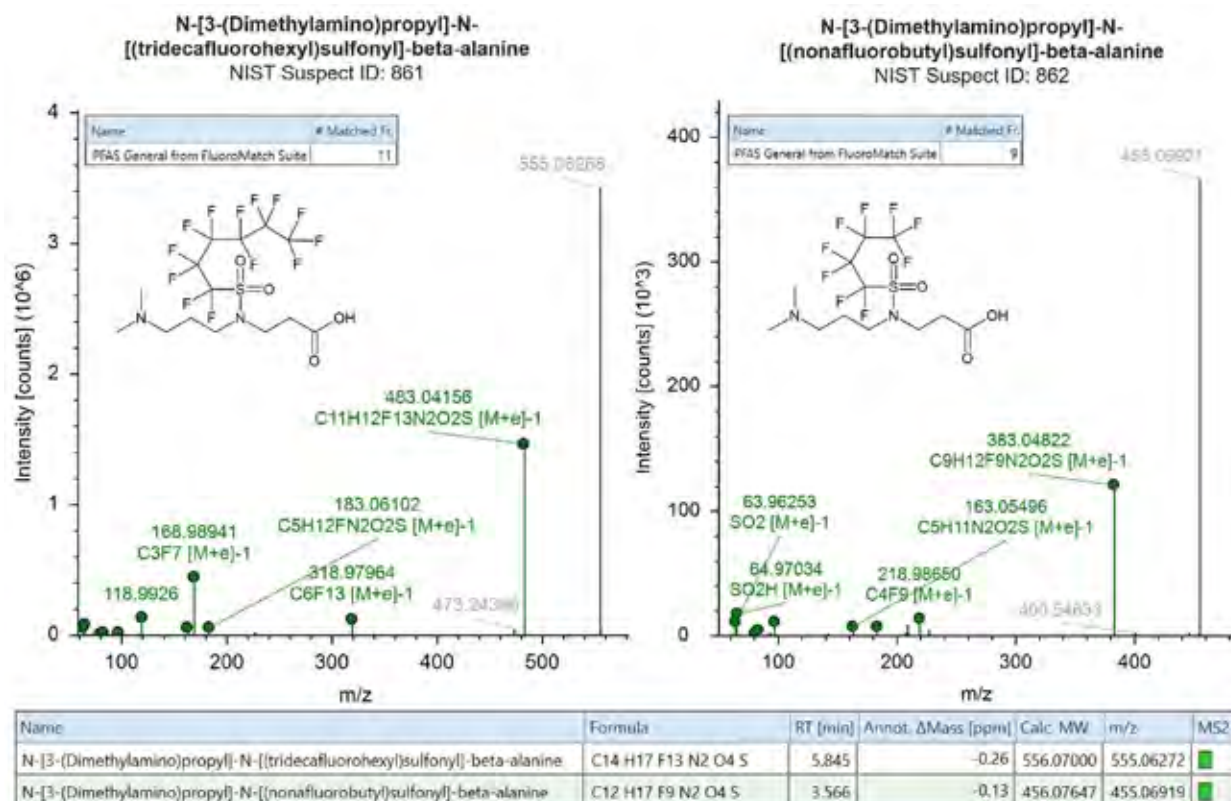


Figure 7. Example application of the FluoroMatch PFAS fragment database to identify compounds in a homologous series with Level 3 annotation confidence. Compound Discoverer software automatically highlights all fragment matches with those in the FluoroMatch PFAS fragment database (in green) and annotates the fragment's formula.

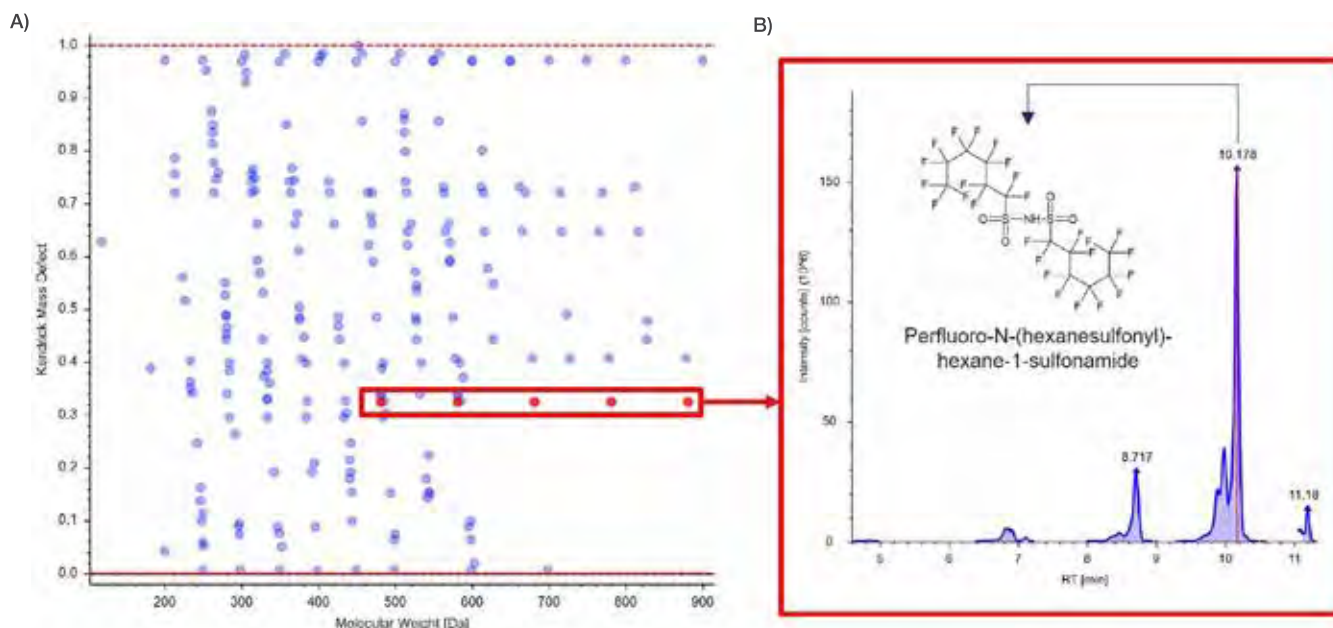


Figure 8. (A) Kendrick Mass Defect plot of study data created using the Compound Discoverer software mass defect plotting tool with the Kendrick formula set to “CF2”. Data points belonging to a specific homologous series of bis-perfluoro-N-alkyl sulfonamides (bis-FAS) are highlighted in red. (B) Overlaid extracted chromatograms of the series in the AFFF-impacted soil sample (Sample C).

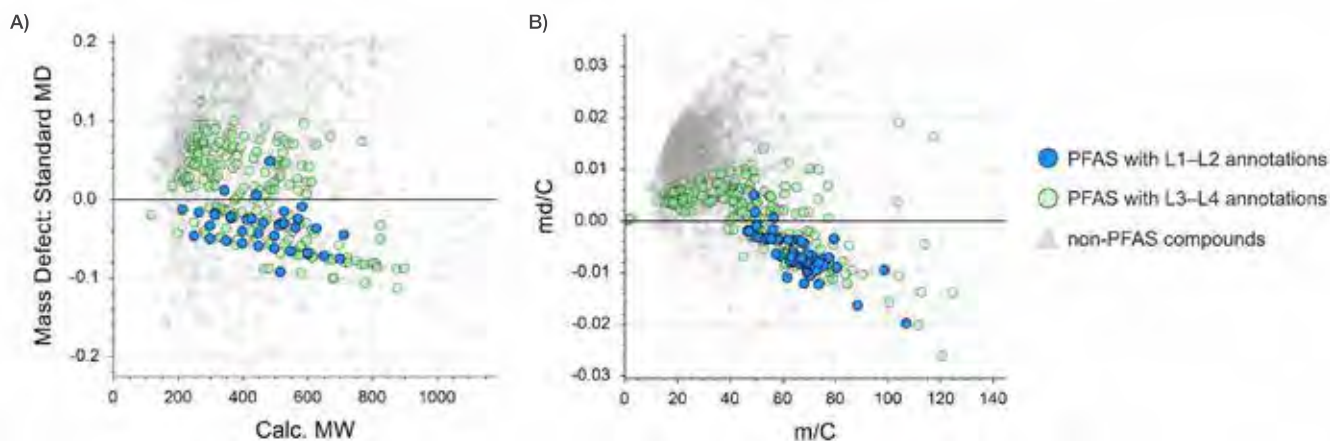


Figure 9. (A) Standard Mass Defect plot and (B) Orthogonal MS' plot created in Compound Discoverer software using the Result Chart tool. The Standard Mass Defect is calculated using the Calculate Mass Defect node and the “Calc. MW” is the calculated molecular weight for the neutral compound. The “md/C” and “m/C” values (B) were calculated using the PFAS.R script in the Scripting Node step of the processing workflow and are published in the Compounds table of the Results file. All PFAS annotations with confidence level between 1–2 are highlighted in blue, while those with confidence levels between 3–4 are highlighted in green. All other non-PFAS compounds are shown as gray triangles.

Compound Discoverer 3.4 software also provides statistical comparison tools to help users understand the differences between samples. As shown in Figure 10, the software’s principal component analysis (PCA) shows a statistically significant difference in the PFAS composition of all three samples. The differential analysis plot was applied to the AFFF mix and AFFF-impacted soil sample, with each data feature plotted by log fold change versus probability. Investigation of each data feature in the region of the plot with Log2 Fold change > 1 and -Log p-value > 1.3 revealed that the majority of the PFAS

that were more abundant in Sample C compared to Sample B are a series of C4–C8 perfluorocarboxylic acids, C6–C10 perfluorosulfonic acids, and perfluoroalkyl sulfonamides. Previously, these compounds had been observed in electrochemical fluorination AFFF products and found to persist in AFFF-impacted soils.¹⁰ More notably, the AFFF-impacted soil contained 125 PFAS that were not detected in the AFFF mixture, leading to the significant difference in overall composition between the two samples shown in the PCA plot (Figure 10A).

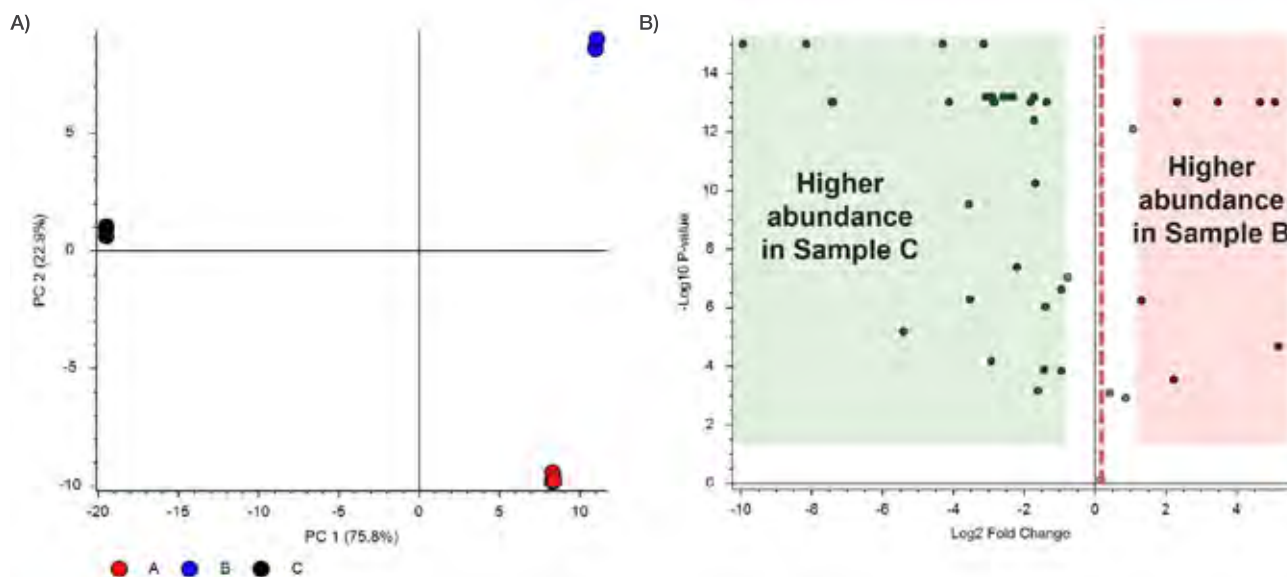


Figure 10. Statistical analysis plots were used to evaluate the PFAS composition of the three samples analyzed. The PCA plot (A) shows significant differences between the PFAS compositions of the samples. Differential analysis of Samples B and C (B) revealed more than 20 PFAS compounds at higher levels in Sample C. Further investigation determined that most of those compounds are part of a series of perfluorocarboxylic acids and perfluorosulfonic acids.

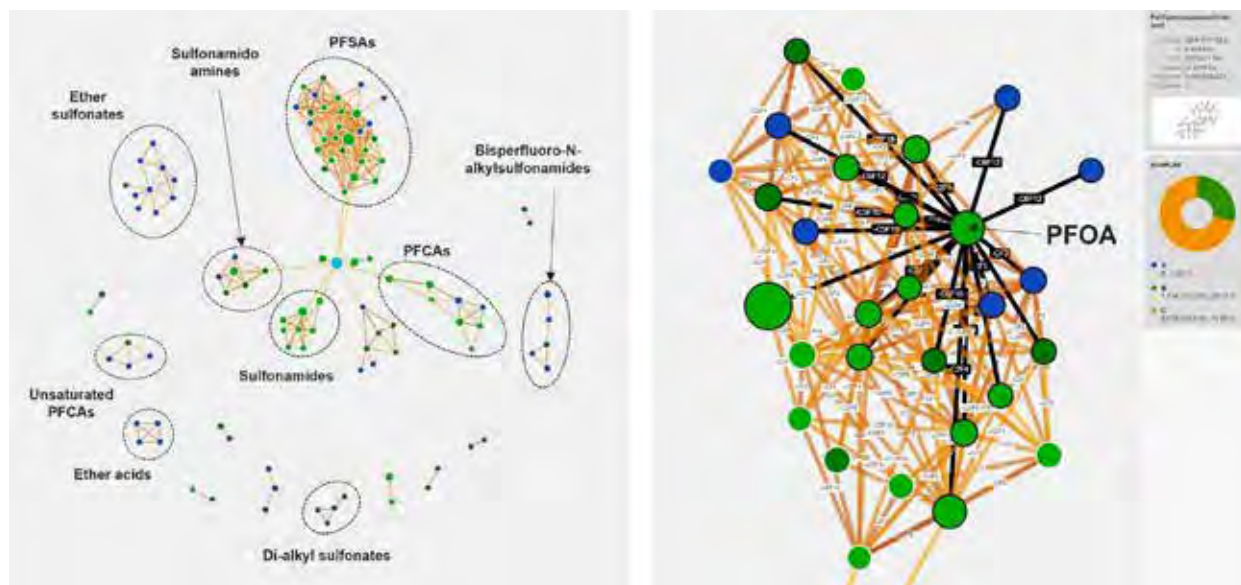


Figure 11. Comprehensive Molecular Network diagram created in Compound Discoverer software using the FluoroMatch PFAS fragment database and the general PFAS fragment compound class as fragment seeds to generate clusters of structurally related compounds. The connections between nodes were based on the thresholds: “Score” = 50, “Coverage” = 70, and “Matched Fragments” = 5. The general names of the various homologous series in the individual clusters are shown in the dashed circles. (Right) close-up view of the PFSA's cluster, highlighting PFOA and its link to multiple PFSA's along with the differences in the linked compounds' elemental formulas. The size of each node depicts the maximum observed peak areas across all samples. The network diagram is interactive for dynamic data visualization.

Identifying homologous series is an important step in fingerprinting the sources of PFAS in samples and determining the appropriate remediation strategies. The Molecular Networks node (Figure 1) enables the user to view structurally related PFAS by associating features that meet user-defined thresholds of matching fragments and spectral match score. The Molecular Network diagram (Figure 11) used the FluoroMatch PFAS

fragment database to cluster structurally related compounds using fragment seeds. The clustered homologous series are shown in Figure 11 by dashed circles. The close-up view of the “PFSA's” cluster (Figure 11, right side) highlights perfluorooctanesulfonic acid and its links to multiple PFSA's, along with the differences in the linked compounds' elemental formulas.

Conclusion

By accessing a comprehensive set of libraries, databases, and other PFAS-specific tools, Compound Discoverer 3.4 software provides a powerful framework that leverages the ultra-high mass accuracy and resolution of Orbitrap mass analyzer technology to consistently annotate over 40,000 PFAS with the highest confidence possible.

This application applied the PFAS NTA workflow to “unknown” samples from the NIST PFAS NTA database.⁵ The Schymanski¹ annotation confidence scale was easily implemented in Compound Discoverer software using a feature added to revision 3.4 that allows custom prioritization of spectral libraries and databases along with data filtering and compound tagging. Built-in statistical analysis and visualization tools, including PCA, differential analysis, mass defects, and molecular networks plots, facilitate comparison of PFAS sample composition.

While this application note described a workflow for environmental samples, it is as easily applied to other application areas, including metabolomics, lipidomics, clinical, pharmaceutical, and food safety analyses to name a few. For example, the PFAS NTA workflow (using Compound Discoverer revision 3.3 SP3 software) has been applied to identify unknown PFAS in food contact materials.¹¹

Resources

The resources used in the Compound Discoverer 3.4 software non-targeted PFAS workflow, including a step-by-step instruction guide, filter sets, tag set, PFAS.R script installation and user guide, and other relevant information, can be downloaded from mycompounddiscoverer.com at: <https://mycompounddiscoverer.com/pfas-analysis/>

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thermo scientific

Screening of PFAS compounds in wastewater using adsorbable organic fluorine with combustion ion chromatography (CIC)

Results from U.S. EPA draft Method 1621 Collaboration Study

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Keywords

EPA Method 1621, AOF, CIC, PFOA, PFHxS, GAC, Dionex IonPac AS24 column, PFAS (per- and polyfluoroalkyl substances), combustion ion chromatography (CIC), adsorbable organic fluorine (AOF), granular activated carbon (GAC)

Introduction

Per- and polyfluoroalkyl substances (PFAS) is the collective name for synthetic fluorinated compounds. The regulatory definition varies. The Organisation of Economic Co-operation and Development (OECD) defines a PFAS compound as one containing a fully fluorinated methyl group or methylene on an alkane functional group.¹ The more restrictive U.S. Environmental Protection Agency (EPA) definition, based on the degradation properties, states the fluorinated compound must have at least two adjacent carbons with one carbon fully fluorinated and the other at least partially fluorinated (*unit $R-CF_2-CF(R')(R'')$, where R , R' , and R'' do not equal "H" and the carbon-carbon bond is saturated*).² For example, the EPA classification excludes trifluoroacetic acid (CF_3-COOH) as a PFAS compound because of the absence of an adjoining fluorinated carbon. Since the 1940s, many products have been composed of or contain PFAS. It is found in many polymers, surfactants, fire-suppression chemicals, industrial products, and consumer products.³⁻⁶ In addition to their extensive presence, PFAS compounds are chemically stable due to their C-F bonds, and therefore, they are persistent and bioaccumulate. Consequently, PFAS compounds

are an environmental contamination concern. Extensive toxicity studies of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) indicate varying toxicity effects from acute to chronic, potentially impacting reproductive health and causing epigenetic effects.⁷⁻¹¹ Less is known about the thousands of other compounds grouped under the EPA PFAS classification.²

The EPA has defined PFAS analysis methods for select PFAS analytes using LC-MS/MS in drinking water (EPA Methods 533.0 and 537.1) and in aqueous, solid, biosolids, and tissue samples (EPA Method 1633, at the publishing date of this document in the 90-day comment phase prior to promulgation).^{12,13} Thermo Fisher Scientific has also previously demonstrated these methods.¹⁴⁻¹⁸ However, a screening method is needed to assess the extent of PFAS contamination in various matrices, including wastewater. Pyrohydrolytic combustion-ion chromatography (CIC) has been previously demonstrated to eliminate the sample matrices.^{19,20} More recently, CIC has been proposed as a screening method for PFAS by converting all organofluorine compounds (including PFAS) to HF, and the subsequent fluoride analyzed by ion chromatography with suppressed conductivity detection (IC).²¹⁻²⁷ The draft EPA Method 1621 has completed a single laboratory review and a round-robin collaborative study and was issued as an official method in the 90-day review and promulgation stage at the time of publication of this document.²⁸⁻³⁰ In this method, 100 mL of wastewater are adsorbed onto granular activated carbon (GAC) columns. Inorganic fluoride is rinsed from the GAC columns with 10 mM sodium nitrate followed by a deionized (DI) water rinse. The GAC is removed from the glass tube, combusted under pyrohydrolytic conditions, absorbed in DI water, and analyzed for fluoride by IC. In this application note, our results from the collaboration study are presented alongside practical advice that supports successful analyses. EPA draft Method 1621 includes method validation tests, calibration, method blanks, method detection limits, multiple process check standards, analysis of wastewater samples, and recovery results of four types of PFAS (PFHxS, PFOS, PFOA, and a blend described as a mixture of PFAS compounds).

Experimental

Equipment

Any Thermo Scientific™ Dionex™ RFIC systems with a conductivity detector (CD) combined with any combustion-absorption system and offline adsorption system can be used.

Recommended: Thermo Scientific™ Dionex™ Inuvion™ IC system with RFIC ([P/N 22185-60108](#)), Thermo Scientific™ Dionex™ Integrion™ RFIC system ([P/N 22153-60305](#)), and Thermo Scientific™ Dionex™ ICS-6000 HPIC™ system.

The Dionex ICS-6000 HPIC system was used in this study, including

- SP Single Pump, isocratic ([P/N 22181-60003](#))
- EG Eluent Generator ([P/N 22181-60019](#))
- DC Detector Column module ([P/N 22181-60043](#))
- CD Conductivity Detector ([P/N 079829](#))

In this study, Nittoseiko Analytech (Nittoseiko Analytech Co., Ltd) AQF-2100H combustion-absorption system with an autosampler for solid samples and an offline adsorption unit was used.

Software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.3.1 or higher, was used to control the IC. Contact your local Thermo Fisher Scientific specialist for the latest information on combustion unit control.

Consumables list for EPA Method 1621

- Thermo Scientific™ Dionex™ IC PEEK Viper™ fitting kits for:
 - Dionex Inuvion RFIC system ([P/N B51000232](#))
 - Dionex Integrion RFIC system with CD detection ([P/N 088798](#))
 - Dionex ICS-6000 HPIC system with CD detection ([P/N 302965](#))
- Thermo Scientific™ Dionex™ EGC 500 KOH eluent generator cartridge ([P/N 075778](#))
- Thermo Scientific™ Dionex™ CR-ATC 600 electrolytic trap column ([P/N 088662](#))
- Thermo Scientific™ Dionex™ HP EG degasser kit ([P/N 075522](#))
- Thermo Scientific™ Dionex™ ADRS 600 suppressor (2 mm) ([P/N 088667](#))
- Thermo Scientific™ Dionex™ IonPac™ AS24 analytical column ([P/N 064153](#))

- Thermo Scientific™ Dionex™ IonPac™ AG24 guard column (P/N 064151)
- Hamilton™ 100 µL microsyringe, metal plunger, Fisher Scientific (P/N 14-813-091)
- Fisherbrand™ Elite™ adjustable, 200 µL pipette and pipette tips, Fisher Scientific (P/N FBE00200 and P/N 02-707-409)
- HDPE 1.0 mL vials and caps, Fisher Scientific (P/N NC2038456, P/N 6ERC11PEC)
- Ceramic combustion boats for Nittoseiko Analytec ASC-270LS sample changer, Nittoseiko Analytec TX3SCY
- Granular activated carbon (GAC) columns used to adsorb samples in EPA Method 1621 multi-lab collaboration study with corresponding GAC column carriers, Analytik Jena 402-880.615, 402-880.013
- Quartz wool used in combustion tube and used to accumulate solids in high TSS samples, Nittoseiko Analytec TNQWL5K

Method

The method includes adsorption, combustion-absorption, and analysis by ion chromatography.

Adsorption conditions

All standards and samples are processed through the adsorption process discussed in section 11.3, EPA Method 1621. The empty boats, calibration standards, and calibration verification standards (CV) are the exceptions. They skip the adsorption process and enter the workflow at the combustion stage. For CV and calibration standards, pipette 200 µL of the individual µg/mL (mg/L) standard into a clean ceramic boat (resulting in “ng” amounts of fluoride). For example, 200 µL of 2.5 µg/mL fluoride = 500 ng.

Parameter	Setting
Adsorption	Pump 100 mL of each sample or standard onto the GAC columns.
Rinse off inorganic fluoride	Pump 25 mL 10 mM sodium nitrate for each sample, MB, Initial Precision Recover (IPR) standard, Ongoing Precision Recovery standard (OPR), or blank ceramic wool onto the GAC columns.
Rinse off nitrate	Pump 20 mL of DI water onto the GAC columns or blank ceramic wool.
Dry	Pump 5 mL of air onto the GAC columns or blank ceramic wool.

Combustion and absorption conditions

Parameter	Setting		
Transfer samples	Transfer GAC material from each GAC column or quartz wool from the GAC carrier into a separate ceramic boat. Combust according to the boat program.		
Boat program	<i>Position</i>	<i>Wait time (s)</i>	<i>Speed (mm/s)</i>
	90 mm	20	10
	End	600	10
	Cool	60	40
	Home	120	20
Furnace temp.	950 °C inlet, 1,000 °C outlet		
Combustion time	Ar: 10 s; O ₂ : 600 s; Ar replacement: 30 s		
Absorption sol.	3 mL DI water		
Final absorption sol.	10 mL, mixed		
Gas	Ar: 200 mL/min; O ₂ : 400 mL/min		
Hydration	Water/Ar: 100 mL/min		
Injection volume	100 µL		

IC conditions

Parameter	Setting
Columns	Dionex IonPac AG24 guard, Dionex IonPac AS24 analytical anion-exchange columns, 2 mm i.d. Note: Other hydroxide selective columns can be used instead (e.g., Dionex IonPac AS19, AS20, AS30 columns).
KOH gradient***	8 mM KOH (0 to 3.5 min, curve 5), 8–75 mM (3.5 to 9 min, curve 9), 75 mM (9 to 10.25 min, curve 5), 75–8 mM (10.25 to 15 min, curve 5), 8 mM (15 to 20 min, curve 5)
Eluent source	Dionex EGC 500 KOH eluent generator cartridge, Dionex CR-ATC 600 trap column, Dionex HP EG degas module
Injection vol.	100 µL
Flow rate	0.30 mL/min
Column temp.	30 °C
Detection temp.	35 °C
Suppressor temp.	20 °C
Detection	Suppressed conductivity, Dionex ADRS 600 suppressor, recycle mode, 56 mA
Conductance background	<0.5 µS/cm
System backpressure	≈2,600 psi (≈18,000 kPa)
Run time	25 min for the first sample, 20 min in overlap for the following samples

***Curve 5 is linear; Curve 9 is convex

Reagents

- DI water ASTM Type 1 (18 MΩ·cm resistivity, TOC < 50 ng/mL)³¹
- Ammonium hydroxide, 28–30 w/w%, Certified ACS, Fisher Scientific™ [P/N A669S-500](#) (CAS 1336-21-6)
- Methanol, UHPLC-MS grade, Fisher Scientific [P/N A458-1](#) (CAS 67-56-1)
- Sodium nitrate, ACS Certified, Fisher Scientific [P/N S343-3](#) (CAS 7631-99-4)
- 1,000 mg/L chloride standard for retention time determinations, TraceCERT™, MilliporeSigma™ Supelco™ [P/N 1.19897.0500](#), Fisher Scientific [P/N 11-101-6049](#)
- 1,000 mg/L certified fluoride standard for IC, SPEX™ CertiPrep™, Fisher Scientific [P/N AS-F9-2Y](#) or Dionex Sodium fluoride 1,000 mg/L, NIST traceable, [P/N 037158](#)
- 1,000 mg/L Fluoride solution, Ricca Chemical™, used as a second fluoride stock standard, Fisher Scientific [P/N 3173-6](#) or Dionex Sodium fluoride 1,000 mg/L, NIST traceable, [P/N 037158](#)
- 1,000 ng/mL 30-PFAS mixed spiking standard in methanol (Wellington Laboratories [P/N PFAC30 PAR](#), Lot PFAC300522), (Fluoride equivalence = 18.2 ng/mL)
- 50 µg/mL (in methanol) L-PFOS sodium perfluoro-1-octanesulfonate standard (Wellington Laboratories [P/N L-PFOS](#), Lot LPOS0922), used for spike-recovery experiments (Fluoride equivalence = 61.7%; 30.3 µg/mL fluoride)
- 50 µg/mL (in methanol) L-PFHxS sodium perfluoro-1-hexanesulfonate standard (Wellington Laboratories [P/N L-PFHxS](#), Lot LPFHxS1022), used for spike-recovery experiments (Fluoride equivalence = 64.6%; 29.26 µg/mL fluoride)
- 50 µg/mL (in methanol) L-PFBA perfluoro-n-butanoic acid standard (Wellington Laboratories [P/N PFBA](#), Lot PFBA1022), used for spike-recovery experiments (Fluoride equivalence = 62.1%; 30.45 µg/mL fluoride)

Preparation of solutions, standards, and samples

Use only ASTM Type I deionized water (DI water) for preparations.

Rinse all glassware, HDPE containers/bottles, and tools with methanol, followed by DI water, to eliminate potential fluoride contamination.

Adsorption solutions: 2 M sodium nitrate stock solution, 10 mM sodium nitrate wash solution, and 1% ammonium hydroxide in Fisher Chemical™ Optima™ LC-MS grade methanol cleaning solution, as described in section 7.1 of EPA Method 1621.³⁰

Standards, blanks, and samples: Follow EPA Method 1621 instructions to prepare inorganic fluoride and chloride standards. Prepare inorganic fluoride and chloride retention time standards, fluoride CV standards, and fluoride calibration standards (0.5, 1, 2.5, 5, 10, 12.5, 25, 37.5, and 50 µg/mL) from 1,000 mg/L stock standards. Refer to TN003056 for detailed processes to achieve low background levels and minimize cross-contamination needed for this application.³² Follow EPA Method 1621, section 11.3 to process the samples through the adsorption step.

PFAS standards in methanol: Use a microsyringe without Teflon™ or fluoropolymers-containing components to dose PFHxS aliquots or PFAS standards for MDL, IPR, OPR, and recovery samples. Clean the microsyringe multiple times with suitable high-purity methanol. Clean between sample- or standard-spiking. Store standards in fluorocarbon-free vials with lids, such as HDPE, at 20 °C. See EPA Method 1621, section 7.3.3 to calculate total F in the PFHxS standard.

Instrument setup and installation

Potential sources of contamination

To exploit the full potential of the trace analysis method, potential sources of contamination must be excluded in advance. A detailed discussion on achieving low contamination levels and tips for successful implementation of EPA Method 1621 are discussed in TN003056.³²

Adsorption standalone instrument

Set the dials of the instrument to the desired volume. Install empty GAC carriers. Clean the fluid flow with the GAC carriers in position using 1% ammonium hydroxide in methanol rinse followed by DI water rinse.

CIC system

Install and set up the Dionex HPIC system as described in Figure 1 and the instrument manuals.^{33–36} TTL triggers are effective ways to trigger the Dionex IC to start after the combustion-absorption is complete. Refer to the instrument manuals. Thermo Fisher Scientific Technical Note 000767 describes the configuration of CIC with a Dionex Integrion HPIC system using Chromeleon 7 CDS software, which is similar for other Dionex HPIC RFIC systems, such as the Dionex Inuvion RFIC system and the Dionex ICS-6000 HPIC system).³⁷ Hydrate and condition the IC consumables as described in TN000767.

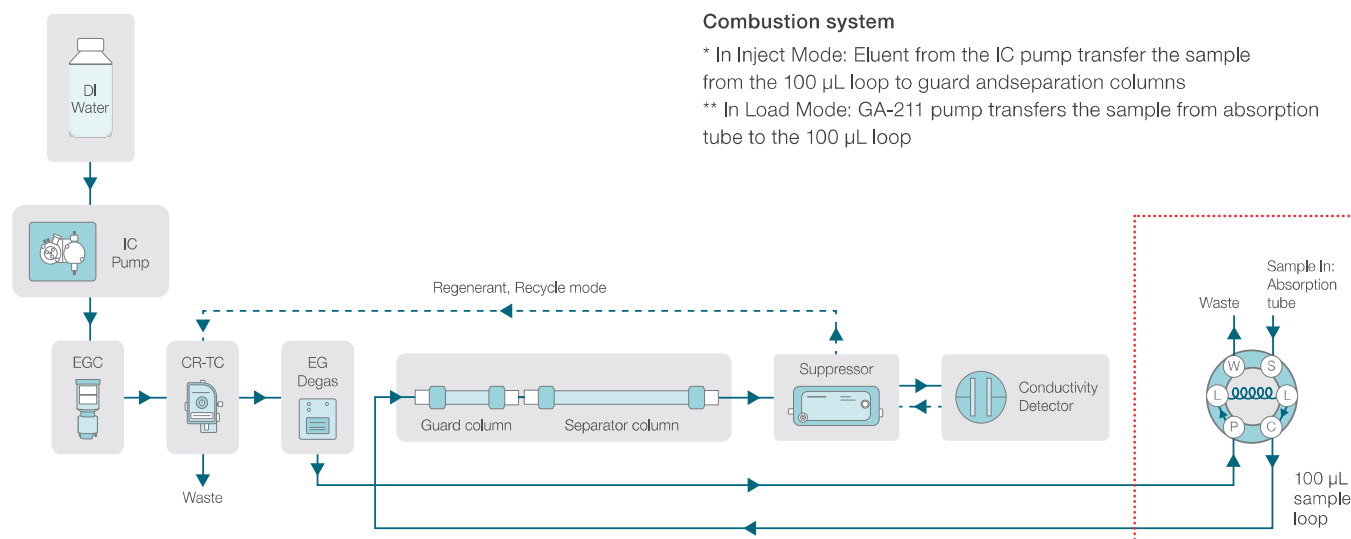


Figure 1. Flow diagram for CIC system

Results and discussion

This application follows EPA draft Method 1621 (similar to EPA Method 1621³⁰) statement of work and technical directives. The collaboration study was organized by the managing laboratory, General Dynamics Information Technology company, which provided GAC columns and GAC column carriers, and PFAS, chloride and fluoride stock standards.³⁸ Definitions are tabulated in the appendix.

Determining the fluoride retention time and window

Fluoride must be well-resolved from the water dip (void volume) and other peaks eluting near fluoride for accurate determinations. In hydroxide eluent separations with suppressed conductivity, as shown in Figure 2, fluoride is well resolved from the water dip, whereas carbonate eluents can be challenging. The results show that fluoride elutes well after (3.3 min) the water dip. The water dip height is also small ($<0.04 \mu\text{S}/\text{cm}$), characteristic of hydroxide eluents. Additionally, after being suppressed, electrolytically generated and purified hydroxide eluents provide higher signal-to-noise ratios, a measure for increased sensitivity and direct compatibility with gradient elution. Fully automated RFIC Eluent Generation (EG) is known for improved analytical and chromatographic performance. The precise and user-independent gradient formation produces superior separations when paired with high-performance Dionex IonPac columns. All the user has to provide is DI water.

Table 1. Determining fluoride retention time window

Peak	Retention time (min)	Peak window (min)	Separation (min)	Limit
Water dip	2.75 ± 0.008	--	--	--
1,250 ng Fluoride*	6.07 ± 0.014	5.98–6.14	3.32	>1 min
5,000 ng Chloride**	12.48 ± 0.005	12.45–12.51	6.42	>1 min
Performance	PASS			

*0.1 mL of 12.5 µg/mL fluoride, n = 3; **0.1 mL of 50 µg/mL chloride, n = 3

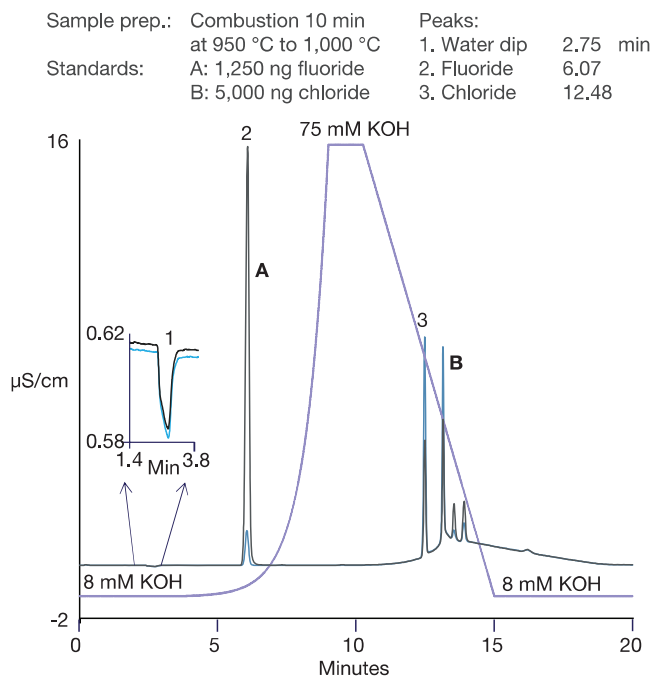


Figure 2. Determining fluoride retention time window

Calibration

Calibration curves were determined according to EPA Method 1621 Step 10.2.2, by the peak area response of fluoride versus concentration, in nanograms, after combusting 200 µL of each mg/L calibration standard in separate ceramic boats. The method requires that the best calibration fit is determined by using a relative standard error (RSE) defined in section 10.3.3 Instrument linearity, EPA Method 1621.³⁰

$$RSE = 100 \times \sqrt{\sum_{i=1}^n \frac{(x'_i - x_i)^2}{x_i^2}} \div n - p$$

with

x_i = Nominal concentration (true value) of each calibration standard

x'_i = Measured concentration of each calibration standard

n = Number of standard levels in the curve

p = Type of curve (2 = linear, 3 = quadratic)

Equation 1. Definition of the relative standard error to determine instrument linearity. The RSE must be $\leq 20\%$.

To determine the RSE, evaluate linear and quadratic fits by allowing offset from zero and calculating the curve fit without weighting and with weighting (1/A, 1/A²) by area. Select the calibration curve with the lowest RSE. In our experiments, we used a linear fit with 1/A² area weighting, providing the lowest RSE (0.774) and compliance with the RSE limit of 20% set by the EPA (Table 2). Figure 3 shows the resulting calibration plot.

Initial demonstration of capability

The collaboration study and Section 9.2, EPA Method 1621 require an initial capability demonstration (IDC) before analyzing wastewater samples. The IDC demonstrates that the instruments' and laboratory's performances suit this method. The accuracy (IDA) and precision (IDP) were determined by preparing and analyzing four replicates of DI water fortified with 15 µg/L fluorine (PFHxS).

Table 2. Comparison of relative standard error (RSE) and coefficient of determination (r^2)

	Linear			Quadratic			RSE limit
	No weighting	1/A	1/A ²	No weighting	1/A	1/A ²	
RSE ⁺	24.8	6.01	0.774	4.09	2.05	1.49	20
r^{2++}	0.9991	0.9993	0.9992	0.9996	0.9998	0.9997	

$n = 7$

⁺RSE = Relative Standard Error, weighting = 1/A or 1/A²

⁺⁺ r^2 = Coefficient of Determination

Type: linear with offset, 1/A² weighting

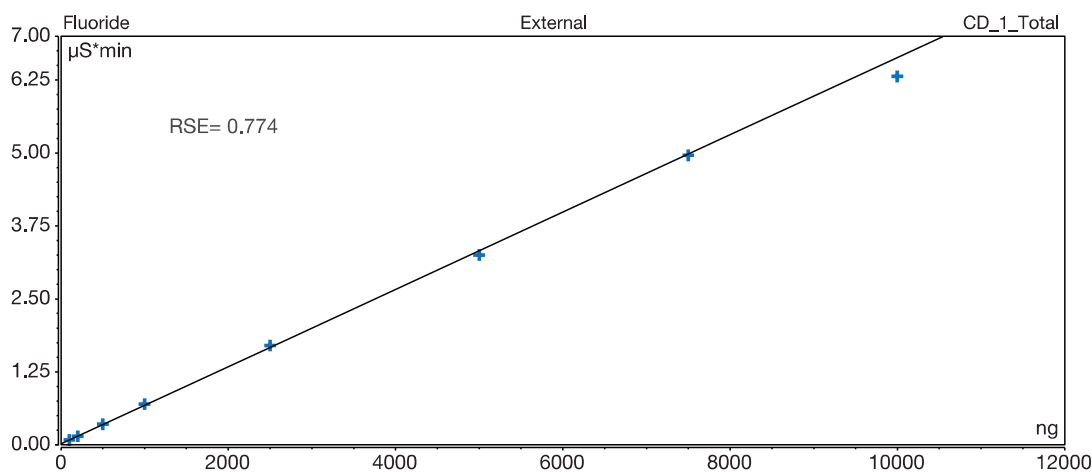


Figure 3. Calibration curve

In contrast to sections 9.2 and 10, the collaboration study required seven replicate method blanks (MB) and 5 µg/L method detection limit (MDL) standards to be prepared and analyzed over three days. The IDA and IDP results demonstrate that the method and this laboratory easily meet the IDC requirements (Table 3). Figure 4 shows the chromatography of the four replicate standards.

Table 3. Initial demonstration of capability

IDC Requirement (Section from EPA Method 1621)	Specification	Experimental value
IDA (9.2.1)	Recovery 80% to 120%	Recovery = 98.5%
IDP (9.2.1)	RSD < 20	RSD = 4.6

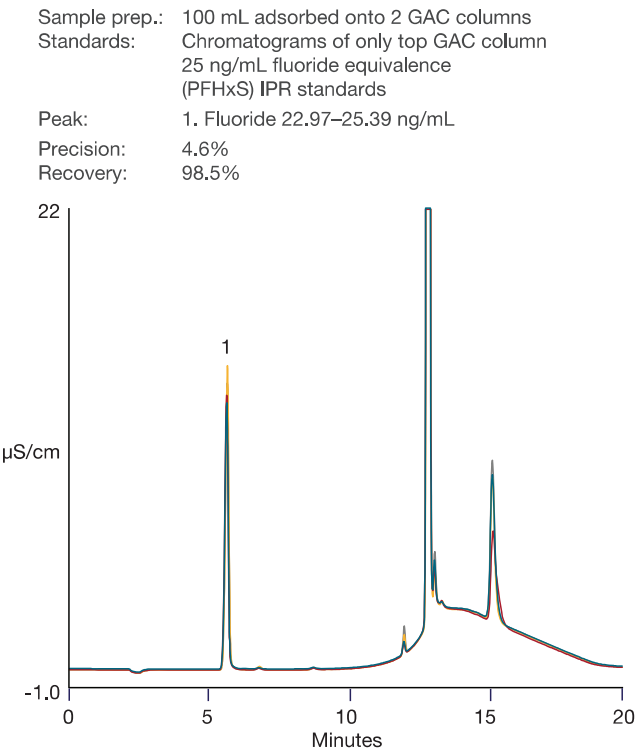


Figure 4. Determining initial precision and recovery

After three days of determining MBs and MDLs, the calculated MB was 1.23 ng/mL, and the calculated MDL was 2.5 ng/mL (Table 4). The chromatograms of MB and MDL are compared in Figure 5.

Table 4. Initial demonstration of determining method blanks and method detection limit standards

Day	Method blank Fluoride (ng/mL)	5 ng/mL MDL standard Fluoride (ng/mL)
1	0.51	3.99
1	0.89	4.35
2	0.64	5.24
2	0.67	4.39
3	0.43	5.77
3	0.85	3.88
3	0.81	5.70
Average, X	0.69	4.76
Standard Deviation (σ)	0.17	0.80
RSD	25.5	16.7
MDL	MDL _(b) [^] = 1.23	MDL _(s) ^{^^} = 2.50

$^{\wedge}MDL_{(b)} = X + (3.14 \cdot \sigma)$
Where $MDL_{(b)}$ is the MDL for MBs, X is average of MB, 3.14 is Student's *t*-test value for *n* = 7, 99 percentile, and σ is the standard deviation,³⁷

$^{\wedge\wedge}Section\ ii: MDL_{(s)} = 3.14 \cdot \sigma$
Where $MDL_{(s)}$ is the MDL for spiked MDL standards, 3.14 is Student's *t*-test value for *n* = 7, 99 percentile, and σ is the standard deviation,³⁹

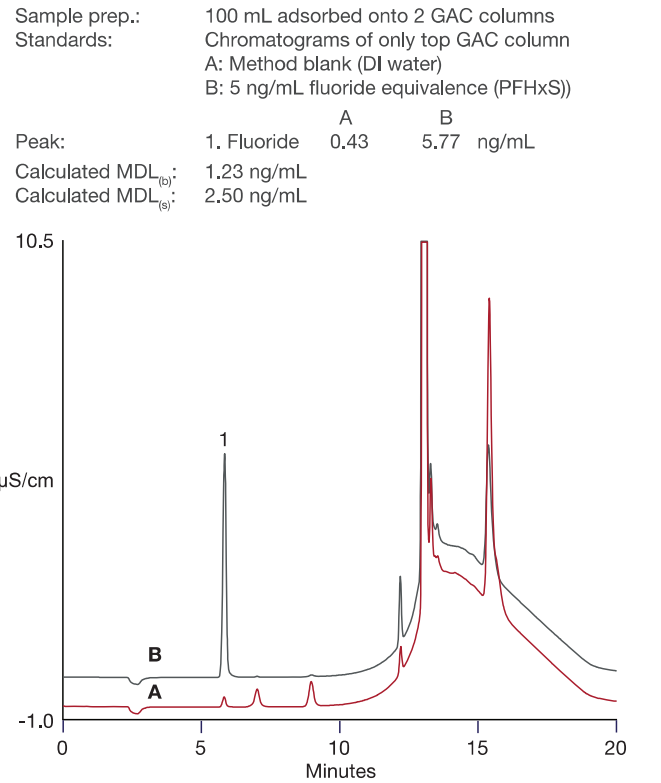


Figure 5. Determining method blank and MDL

Samples

The method was applied to wastewater samples. The results are shown in Table 5. Less than 15 ng/mL of fluoride are present in the samples, ranging from <1 (Sample 7) to 12 ng/mL (Sample 3). The method blanks and process checks show that the method is working well within the expected limits.

Table 5. Sample results and recovery results of added PFHxS standard

Sample	Total fluoride		Recovered (%)
	Measured (ng/mL)	Added (ng/mL)	
Sample 1	9.69	30.0	87.7
		30.0	87.1
		60.3	99.0
		60.3	95.9
Sample 2+	1.11	10.03	116
		10.03	117
		25.2	98.8
		25.2	90.4
Sample 3+	12.22	30.0	92.6
		30.0	91.2
		60.3	87.5
		60.3	88.7
Sample 4	9.69	30.1	108
		30.1	105
		60.3	90.2
		60.3	102
Sample 5	1.56	30.0	103
		30.0	102
		60.3	106
		60.3	104
Sample 6+	4.93	10.09	80.2
		10.09	81.9
		30.0	107
		30.0	105
Sample 7	0.53	10.03	104
		10.03	105
		30.1	102
		30.1	101
Sample 8	1.71	10.03	99.1
		10.03	103
		30.1	92.4
		30.1	96.8
Sample 9	3.23	30.1	98.3
		30.1	96.6
		60.2	85.5
		60.2	95.3

After MBs were subtracted

+ Required ceramic wool

Determining recoveries of added PFHxS standard

To understand method accuracy, recoveries of different volumes of added PFHxS standard were determined at two concentrations and duplicated for all nine samples, including those requiring ceramic wool (Table 5).

The method is accurate, as indicated by the recoveries of added PFHxS standards, 80 to 117%. The chromatogram of Sample 3 is compared with the replicate recovery samples (Figure 6).

Sample prep.: 100 mL adsorbed onto 2 GAC columns
Standards: Chromatograms of only top GAC column
A: Sample 3 wastewater (high TSS)
B: Sample A + 30 ng/mL fluoride equivalence from PFHxS
C: Sample B replicate

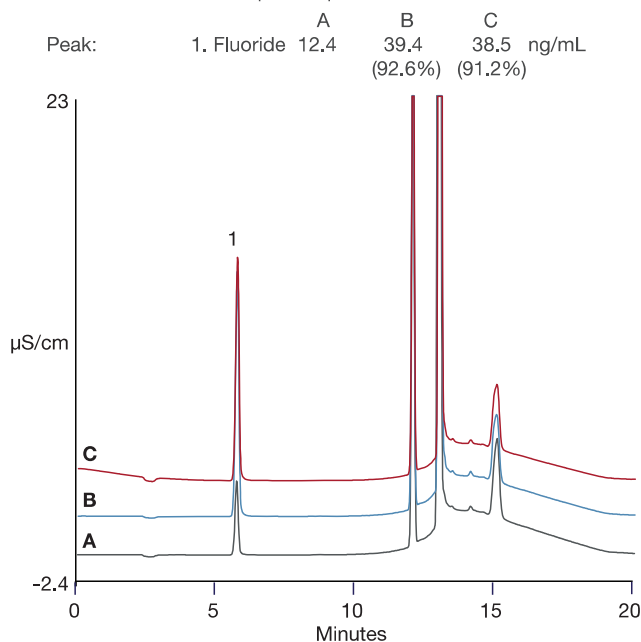


Figure 6. Determining recovery of PFHxS in Sample 3 wastewater

Determining recoveries of PFAS compounds in Sample 7

To evaluate the accuracy of determining other PFAS compounds, similar recovery experiments were conducted with added standards of PFBA, PFOS, and a PFAS mixture (Wellington Laboratories). Approximately 10 ng/mL and 30 ng/mL fluoride from the above standards were added to Sample 7 (defined by the managing laboratory). The results summarized in Table 6 show that the fluoride recoveries of PFBA, PFOS, and PFAS mixture ranged from 76 to 114% of added fluoride as perfluorocarbon. Figure 7 shows representative chromatograms of samples after the addition of PFOS, PFBA, and PFAS mixture to determine compound specific recovery of fluoride after combustion. No compound specific dependency was observed.

Table 6. Summary of recovery results from added PFBA, PFOS, and PFAS

Sample	Total fluorine		Recovered (%)
	Added (ng/mL)	Measured (ng/mL)	
Sample 7	–	0.53	–
Sample 7 with PFBA	10.05	10.86	103
	10.05	10.63	100
	30.4	33.31	108
	30.4	23.53	76.1
Sample 7 with PFOS	10.0	10.19	96.8
	10.0	9.08	86.2
	30.3	29.16	94.6
	30.3	35.2	114
Sample 7 with PFAS mix	9.99	11.05	105
	9.99	8.98	85.4
	30.0	25.60	83.9
	30.0	24.91	81.6

Sample prep.: 100 mL adsorbed onto 2 GAC columns
 Samples: Chromatograms of only top GAC column
 A: Sample 7 wastewater.
 Replicates: B) + 10.05 ng/mL fluoride equivalence (PFOS),
 C) + 30 ng/mL fluoride equivalence (PFAS mix),
 D) + 30 ng/mL fluoride equivalence (PFBA)

Peak: 1. Fluoride 0.53 9.08 25.6 33.3 ng/mL
 (86.2%) (83.9%) (108%)

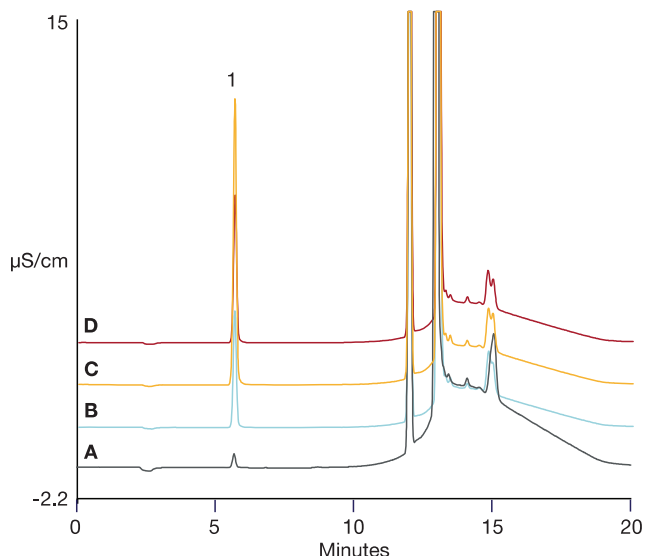


Figure 7. Determining recovery of different PFAS compounds. No compound specific dependency is observed.

Conclusion

This application note demonstrated our results from the multi-laboratory collaboration study of EPA draft Method 1621. This method shows that CIC is an excellent tool to measure AOF and screen for PFAS in wastewater. Along with its ease of use, RFIC provides more accurate results as fluoride is well separated from the water dip, ensuring optimized automated peak integration. The method is sensitive, with MBs of 1 ng/mL and MDLs of 2.5 ng/mL, and accurate, with recoveries of 80–120%. Together, these data highlight the power of CIC in eliminating the sample matrix and measuring only the adsorbable organically bound fluorine content in samples, successfully achieving the goals outlined in EPA draft Method 1621.

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Appendix

Table A1. Definitions

Term	Definition	Description
AOF	Adsorbable Organic Fluorine	Fluorocarbon and perfluorocarbon compounds in water or solvent that adhere to a sorbent like activated carbon or ion exchangers.
CV	Calibration Verification standard	Check standards prepared from an inorganic fluoride standard that are from a different (second) source than the calibration standards. Added directly to combustion cups. Run at the beginning and the end of each sequence.
GAC	Granular Activated Carbon	Carbon material used for adsorbing organic compounds.
IPR	Initial Precision Recovery standard	Used to determine the expected process check value (OPR). Prepared by adding PFHxS standard to DI water. Processed through adsorption, combustion, absorption, and IC analysis.
MB	Method blanks	DI water processed through adsorption, combustion, absorption, and IC analysis. Indicates the lowest actual measurements possible and the baseline contamination of the CIC and the Filtration module. Always run at the beginning and the end of each sequence.
MDL	Method detection limit	The lowest reliable measurement, typically 3× S/N. In this test, the standard is processed through the adsorption on carbon, combusted, absorbed, and analyzed by IC.
MDL_(b)	Method detection limit of blanks, n = 7	Defined as: (Mean of method blanks) plus (standard deviation × Student <i>t</i> -test factor).
MDL_(s)	Method detection limit of 5 ng/mL PFHxS standard, n = 7	Defined as: (standard deviation) × (Student <i>t</i> -test factor).
OPR	Ongoing Precision Recovery standard	Check standard run at the beginning of each sequence. Prepared by adding PFHxS standard to 100 mL of DI water. Processed through the adsorption on carbon, combusted, absorbed and analyzed by IC.
PFHxS	Perfluorinated sulfonate standard	Sodium perfluoro-1-hexanesulfonate standard. Used for MDL, and QC check standards.
RSE	Relative Standard Error	Error from calibration line. Defines the best fit of a calibration curve.
TSS	Total Suspended Solids	Includes any loose particulates or precipitated solids in the water sample.

 Learn more at thermofisher.com/combustionIC

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SPONSOR REPORT

APPLICATION REPORT
ETHOS X - PFAS - EPA Method 1633



OPTIMIZING EXTRACTION OF SOLIDS FOR PFAS - EPA 1633 ANALYTES

Extraction of PFAS compounds from solids is a common challenge for many laboratories. This application report demonstrates how using the Milestone ETHOS X platform with the XTR rotor simplifies sample preparation for extracting target PFAS compounds from soils, sediments and biosolids helps environmental laboratories address efficiency, regulatory, economic and quality challenges.

INTRODUCTION

Environmental laboratories face several types of challenges related to the determination of PFAS compounds in solid matrices such as soils, sediments and biosolids. Increasing industry competition for commercial labs puts analysis turn-around time at a premium. Labs face issues related to the availability of skilled labor, pressure on costs and budgets and limited lab space.

Sample preparation is a critical step in the entire analysis workflow as it is time-consuming, labor intensive and error prone. EPA method 1633 rev. A¹ describes extraction using a shaker table technique with multiple transfers between extractions taking a significant amount of time, labor and potential for losses due to repetitive

handling. However, EPA 1633 specifically allows improving method parameters such as extraction methodologies per section 9.1 of the method.

The use of microwave assisted extraction removes the obstacles of the shaker table approach, while providing consistent quality, lower cost and high efficiency in processing, with no regulatory obstacles and up to 70% reduction in extraction time. This Application Report describes how the Milestone ETHOS X platform provides superior efficiency and quality for the extraction of PFAS. Detailed independent laboratory data are presented showing PFAS Precision and Comparability along with Performance Evaluation data all using the Milestone ETHOS X with XTR extraction rotor.



EXPERIMENTAL

EQUIPMENT

- Milestone's ETHOS X equipped with XTR-44 extraction rotor or Milestone's ETHOS X LEAN equipped with XTR-30 extraction rotor
- 50-mL disposable PP vials
- Milestone's SFS-24 filtration system OR Centrifuge
- Nitrogen Evaporation system
- Automated SPE system
- LC/MS-MS system



Figure 1 – Milestone ETHOS X with XTR-44



Figure 2 – Milestone ETHOS X LEAN with XTR-30

SAMPLES

The study was conducted by preparing 3 different soil samples in triplicate to compare EPA 1633 shaker table vs. microwave extraction.

SAMPLE PREPARATION

In all cases, 5-gram samples (+ 0.01 g) were weighed on an as-received basis. Moisture content was determined per EPA 1633 guidance. For the blank spikes and QC samples, 5 g. of PFAS-free Ottawa sand was used. For the known soil (PT sample), 2.5 grams were utilized for extraction. Samples and QC were weighed into 50 mL disposable polypropylene centrifuge tubes and a NdFeB stir bar was introduced. An aliquot of extracted internal standard (EIS) was added to all samples/QC and allowed to sit 30 minutes per the method. 15 mL of 0.3% ammoniacal methanol was added to each sample/QC, cap tightened, and samples were placed into the Milestones ETHOS X- XTR-44 rotor. QC samples (LLOPR, OPR and MS/MSD) were spiked accordingly with PFAS targets. The samples were subjected to extraction using the program shown below:

Step	Time (min)	Power (W)	Temperature (°C)
1	5	up to 1000	65
2	10	up to 1000	65

Table 1 - Microwave Program

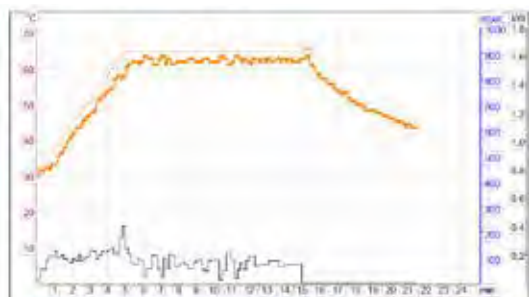


Figure 3 - Microwave run profile

EXTRACTION PROCESS AND CLEAN UP

At the end of cooling (< 5 mins), the vessels were placed into a centrifuge and centrifuged at 3000 rpm for 5-10 minutes. Alternatively, centrifugation can be eliminated by filtering through preconditioned, stacked 10um/1um on a Milestone SFS-24 vacuum filtration device

and filtered with rinsing into 50 mL PP tubes used for concentration.

Extracts were then concentrated using nitrogen evaporation to a volume of <10.0 mL. 40 mL PFAS-free water was then added. Extracts were then subjected to Solid Phase Extraction (SPE) resulting in a final volume of 5.0 mL. Non-Extracted internal standards (NIS) were then added to all samples/QC.

ANALYTICAL CONDITIONS

Extracts were then analyzed by EPA 1633A using an Infinity 1290 LC interfaced to an Agilent 6495 triple quadrupole Mass Spectrometer using Mass Hunter software. All preparation and analyses were performed by AEL².

RESULTS AND DISCUSSION

After sample preparation by Microwave extraction using the ETHOS X – XTR-44 system as described previously, the lab for this study provided all analytical data. Table 1 below summarizes the results of the shaker table extraction versus the triplicate microwave extraction data.

The data obtained by applying the same method to the reference material (PT soil) are reported in Table 2.

Table 1 Comparison of Sample Extractions using EPA 1633 Shaker Table vs. Microwave Extraction						
Data Reported to MDL (listed as < values) and J-Flagged ¹ between MDL and verified LOQ						
Target Compounds	Shaker Table Data	Microwave Data	Shaker Table Data	Microwave Data	Shaker Table Data	Microwave Data
	2509157004 Sample	2509157004 Sample	J2509157018 Sample	J2509157018 Sample	J2507676006 Sample	J2507676006 Sample
	µg/kg, dry	µg/kg, dry	µg/kg, dry	µg/kg, dry	µg/kg, dry	µg/kg, dry
11Cl-PF3OUdS	<0.49	<0.51	<0.36	<0.36	<0.62	<0.62
9Cl-PF3ONS	<0.49	<0.51	<0.36	<0.36	<0.62	<0.62
ADONA	<0.49	<0.51	<0.36	<0.36	<0.62	<0.62
4:2FTS	<0.49	<0.51	<0.36	<0.36	<0.62	<0.62
6:2FTS	<0.58	<0.51	<0.44	<0.44	<0.74	<0.74

APPLICATION REPORT

ETHOS X – PFAS – EPA Method 1633



8:2FTS	<0.49	<0.51	<0.36	<0.36	<0.62	<0.62
HFPO-DA	<0.49	<0.51	<0.36	<0.36	<0.62	<0.62
NFDHA	<0.49	<1.0	<0.73	<0.73	<1.2	<1.2
PFBS	<0.49	<0.51	<0.36	<0.36	<0.62	<0.62
PFBA	0.93 J*	0.768 J*	0.89 J*	0.880 J*	<0.62	<0.62
PFDA	0.80 J*	0.982 J*	0.41 J*	0.369 J*	<0.62	<0.62
PFDS	<0.49	<0.51	<0.36	<0.36	<0.62	<0.62
PFDaA	0.92 J*	1.03 J*	<0.36	<0.36	<0.62	<0.62
PFHpS	<0.68	<0.71	<0.51	<0.51	<0.87	<0.87
PFHpA	0.96 J*	0.747 J*	1.5	1.3	<0.62	<0.62
PFHxS	<0.49	<0.51		<0.36	9.1	11.3
PFHxA	0.67 J*	0.573 J*	1.6	1.5	1.10 J*	1.15 J*
PFMBA	<0.49	<0.51	<0.36	<0.36	<0.62	<0.62
PFMPA	<0.49	<0.51	<0.36	<0.36	<0.62	<0.62
PFNA	1.1 J*	0.815 J*	0.54 J*	0.419 J*	<0.62	<0.62
PFOS	21	20.7	38	34.5	53	57.7
PFOA	3.6	3.14	3.2	3.03	1.3	1.38
PFPeS	<0.49	<0.51	<0.36	<0.36	0.66 J*	0.799 J*
PFPeA	0.63 J*	0.596 J*	1.4	1.5	<0.62	<0.62
PFUnA	<0.49	1.15 J*	< 0.36	< 0.36	<0.62	<0.62
PFEESA	<0.49	<0.51	< 0.36	< 0.36	<0.62	<0.62

	Shaker Table Data	Microwave Data	Shaker Table Data	Microwave Data	Shaker Table Data	Microwave Data
	250915700 4 Sample	250915700 4 Sample	J250915701 8 Sample	J250915701 8 Sample	J250767600 6 Sample	J250767600 6 Sample
Target Compound s	µg/kg, dry	µg/kg, dry	µg/kg, dry	µg/kg, dry	µg/kg, dry	µg/kg, dry
PFNS	<0.78	<0.81	<0.58	<0.58	<0.99	<0.99
PFOSA	<0.49	<0.51	< 0.36	< 0.36	<0.62	<0.62
PFTeDA	<0.49	0.542 J*	< 0.36	< 0.36	<0.62	<0.62
PFTrDA	<0.49	0.666 J*	< 0.36	< 0.36	<0.62	<0.62
NetFOSAA	<0.49	<0.51	< 0.36	< 0.36	<0.62	<0.62
NMeFOSAA	<0.49	<0.51	< 0.36	< 0.36	<0.62	<0.62
PFDoS	<0.97	<1.0	1.1 J*	<0.73	<1.2	<1.2
NMeFOA	<0.49	<0.51	< 0.36	< 0.36	<0.62	<0.62
NetFOA	<0.49	<0.51	< 0.36	< 0.36	<0.62	<0.62
NMeFOSE	<4.9	<5.1	<3.6	<3.6	<6.2	<6.2
NetFOSE	<4.9	<5.1	<3.6	<3.6	<6.2	<6.2
3:3FTCA	<1.9	<2.0	>1.5	>1.5	<2.5	<2.5
5:3FTCA	<9.7	<10.0	<7.3	<7.3	<12	<12
7:3FTCA	<9.7	<10.0	<7.3	<7.3	<12	<12

APPLICATION REPORT

ETHOS X – PFAS – EPA Method 1633



"J" indicates the results greater than the MDL and less than the Reporting limit

Table 2 EPA 1633 Microwave Extraction Data on reference soil (PT sample)

PFAS Compound-EPA 1633 40 compound list	<i>Certified Value</i>	EPA 1633A Microwave	Acceptance Range ¹
	µg/kg	µg/kg	µg/kg
11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid (11Cl-PF3OUdS)	<2.50	<2.5	ND ²
9-chlorohexadecafluoro-3-oxanonane-1-sulfonic acid (9Cl-PF3ONS)	17.9	20.4	8.95 - 26.8
4,8-dioxa-3H-perfluorononanoic acid (ADONA)	24.9	30.4	12.4 - 37.3
N-Ethylperfluorooctanesulfonamide (NEtFOSA)	30.4	34.5	15.2 - 45.5
N-ethyl perfluorooctanesulfonamidoacetic acid (NEtFOSAA)	36.0	40.7	18.0 - 53.9
N-Ethylperfluorooctanesulfonamido ethanol (NEtFOSE)	32.8	34.8	16.4 - 49.1
Hexafluoropropylene oxide dimer acid (HFPO-DA)	37.6	39.9	18.8 - 56.2
N-Methylperfluorooctanesulfonamide (NMeFOSA)	32.8	45.4	16.4 - 49.1
N-methyl perfluorooctanesulfonamidoacetic acid (NMeFOSAA)	32.0	33.1	16.0 - 47.9

Table 2 EPA 1633 Microwave Extraction Data on reference soil (PT sample)

PFAS Compound-EPA 1633 40 compound list	<i>Certified Value</i>	EPA 1633A Microwave	Acceptance Range ¹
	µg/kg	µg/kg	µg/kg
N-Methylperfluorooctanesulfonamidoethanol (NMeFOSE)	33.6	39.6	16.8 - 50.3
Nonafluoro-3,6-dioxaheptanoic acid (NFDHA)	18.0	21.9	9.00 - 26.9
1H, 1H, 2H, 2H-Perfluorodecanesulfonic acid (8:2 FTS)	47.9	54.6	24.0 - 71.7
1H, 1H, 2H, 2H-Perfluorohexanesulfonic acid (4:2 FTS)	33.6	37.2	16.8 - 50.3
1H, 1H, 2H, 2H-Perfluorooctanesulfonic acid (6:2 FTS)	47.4	52.5	23.7 - 70.9
2H, 2H, 3H, 3H-perfluorodecanoic acid (7:3 FTCA)	48.0	33.8	24.0 - 71.8
2H, 2H, 3H, 3H-perfluorohexanoic acid (3:3 FTCA)	10.4	7.1	5.20 - 15.6
2H, 2H, 3H, 3H-perfluorooctanoic acid (5:3 FTCA)	<2.50	<2.5	ND
Perfluorobutanesulfonic acid (PFBS)	16.3	19.7	8.15 - 24.4
Perfluorobutanoic acid (PFBA)	21.6	24.3	10.8 - 32.3
Perfluorodecane sulfonic acid (PFDS)	9.45	10.4	4.72 - 14.1
Perfluorodecanoic acid (PFDA)	12.4	14.7	6.20 - 18.6
Perfluorododecanesulfonic acid (PFDoS)	19.4	19.0	9.70 - 29.0
Perfluorododecanoic acid (PFDoA)	<2.50	<2.5	ND

APPLICATION REPORT

ETHOS X – PFAS – EPA Method 1633



Perfluoro(2-ethoxyethane)sulfonic acid (PFEESA)	12.1	14.4	6.05 - 18.1
Perfluoroheptane sulfonic acid (PFHpS)	13.0	14.9	6.50 - 19.4
Perfluoroheptanoic acid (PFHpA)	39.2	45.5	19.6 - 58.6
Perfluorohexanesulfonic acid (PFHxS)	24.0	26.6	12.0 - 35.9
Perfluorohexanoic acid (PFHxA)	12.8	15.5	6.40 - 19.1
Perfluoro-4-methoxybutanoic acid (PFMBA)	25.6	33.1	12.8 - 38.3
Perfluoro-3-methoxypropanoic acid (PFMPA)	38.4	45.8	19.2 - 57.4
Perfluorononane sulfonic acid (PFNS)	16.2	18.0	8.10 - 24.2
Perfluorononanoic acid (PFNA)	26.4	34.0	13.2 - 39.5
Perfluorooctanesulfonamide (PFOSA)	14.4	20.2	7.20 - 21.5
Perfluorooctanesulfonic acid (PFOS)	21.5	21.6	10.8 - 32.2
Perfluorooctanoic acid (PFOA)	16.8	24.9	8.40 - 25.1
Perfluoropentane sulfonic acid (PFPeS)	45.1	53.7	22.6 - 67.5
Perfluoropentanoic acid (PFPeA)	<2.50	<2.5	ND
Perfluorotetradecanoic acid (PFTDA)	21.6	22.8	10.8 - 32.3

Table 2 EPA 1633 Microwave Extraction Data on reference oil (PT sample)

PFAS Compound-EPA 1633 40 compound list	<i>Certified Value</i>	EPA 1633A Microwave	Acceptance Range ¹	
	µg/kg	µg/kg	µg/kg	
Perfluorotridecanoic acid (PFTrDA)	40.0	49.7	20.0 - 59.8	
Perfluoroundecanoic acid (PFUnA)	8.40	10.1	4.20 - 12.6	
Ranges were determined by Pt provider – ERA- based upon shaker table extraction, not microwave ND = not detected down to RL-<PTRL				

APPLICATION REPORT

ETHOS X – PFAS – EPA Method 1633



CONCLUSION

The Milestone ETHOS X platform with XTR-rotor and integrated stirring successfully processed solid matrices with excellent comparability, precision, and PT performance. All data were well within method requirement, and the studies clearly demonstrated the superior performance of the ETHOS X platform for EPA 1633A target analytes.

Compared with shaker table extraction, the ETHOS X configuration offers laboratories a reliable path to improve the sample preparation steps for PFAS analysis saving significant time and error prone handling steps.

A summary of the key benefits the Milestone ETHOS X – XTR solution brings to the lab include:

- Increased Capacity and ability to execute rush soil work
- Less labor and elapsed time - doing more with less- 15 mins. vs. hours
- Ability to improve and better predict analysis turn-around times
- Improved data quality, consistency and confidence in the results
- More robust extraction given varying matrices received by the lab
- Easier workflow implementation and on-boarding of new staff members.

REFERENCES

- 1- EPA 1633 Revision A Analysis of Per- and Polyfluoroalkyl Substances (PFAS) in Aqueous, Solid, Biosolids, and Tissue Samples by LC-MS/MS
<https://www.epa.gov/system/files/documents/2024-12/method-1633a-december-5-2024-508-compliant.pdf>
- 2- Advanced Environmental Laboratories, Inc (AEL), Jacksonville, FL an independent laboratory approved for EPA 1633 PFAS under NELAC/TNI and DOD QSM programs.

ABOUT MILESTONE

At Milestone we help chemists by providing the most innovative technology for metals analysis, direct mercury analysis and the application of microwave technology to extraction, ashing and synthesis. Since 1988 Milestone has helped chemists in their work to enhance food, pharmaceutical and consumer product safety, and to improve our world by controlling pollutants in the environment.

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- Next-generation instrument design
- EASY-IC ion source: Exceptional mass accuracy as standard
- Resolve sample complexity for confident results
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- Sensitivity and quantitative excellence as standard
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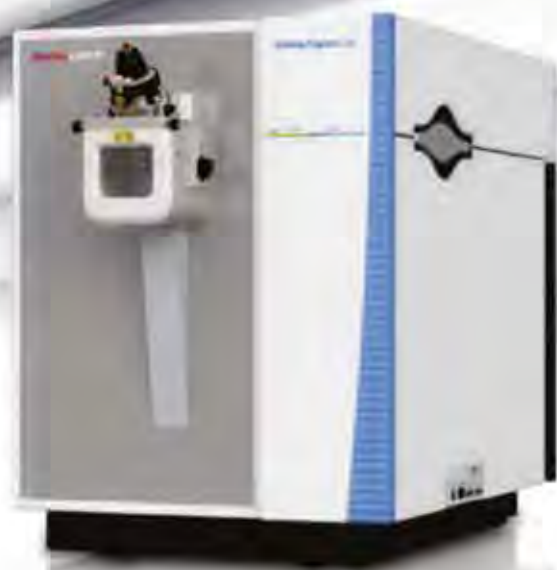
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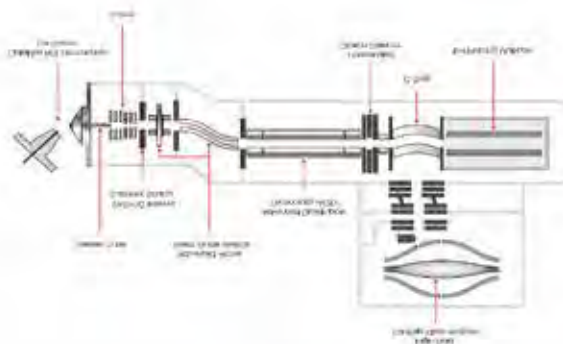
Leading performance

Orbitrap Exploris 240 mass spectrometer



Deliver the exceptional

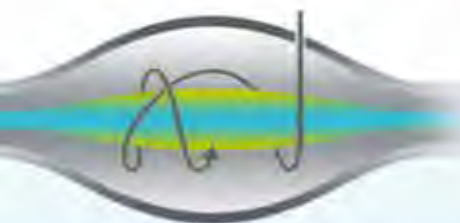
The Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer provides leading performance and application versatility to drive discovery and identification, with the quantitative precision and accuracy to confidently scale up to achieve impact. Operational simplicity, together with intelligent data acquisition, fast tracks you to confident results.



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containing components within the sample pathway, the Thermo Scientific™ Cindion™ Combustion Ion Chromatography (C-IC) System eliminates potential sources of PFAS during analysis to deliver the lowest achievable background levels enabling the most sensitive detection.

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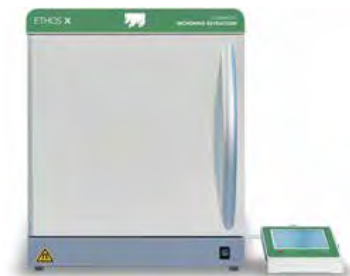
ETHOS™ X

Microwave Extraction of PFAS



EFFICIENT SAMPLE PREPARATION

Per- and polyfluoroalkyl substances (PFAS) are persistent contaminants found in soil, water, food, and air, requiring precise monitoring methods. Microwave-Assisted Extraction (MAE), widely used in sample preparation for organic pollutant analysis, now extends its capabilities to PFAS. The ETHOS X for PFAS analysis is specifically designed to deliver the highest throughput, processing up to 44 samples per batch. This ensures an efficient workflow and the highest productivity available on the market.



PFAS-FREE DISPOSABLE VESSELS
EPA 1633 COMPLIANT
NO CLEANING REQUIRED
HIGH THROUGHPUT
FASTER SAMPLE PREPARATION

MAE Features and Benefits for PFAS Analysis

HIGH THROUGHPUT

The XTR-44 Extraction Rotor accommodates up to 44 samples per batch, simplifying workflows with commercially available polypropylene centrifuge tubes in various volumes, such as 50 mL. By keeping samples in the same tube throughout the process, this approach minimizes contamination. Additionally, the dedicated microwave configuration meets the requirements of EPA Method 1633, while providing a 70% reduction in sample extraction time.

DESIGNED FOR PFAS-FREE PROCESSING

The rotor design, specifically developed for PFAS, ensures that all vessel components in contact with samples are PFAS-free. Using disposable vessels eliminates the need for cleaning and prevents carry-over between runs. This approach significantly reduces contamination risks and streamlines the sample preparation process, while providing excellent recoveries vs. shaker table extractions.

CONSISTENT PROCESS CONTROL

Advanced sensors in the ETHOS X system deliver precise temperature control throughout the extraction process. The easyTEMP sensors and easyCONTROL software ensure uniform conditions across all positions, guaranteeing consistent and reproducible results. The intuitive software of the ETHOS X provides effortless user experience for routine PFAS sample preparation.





MILESTONE
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ETHOS X



*Chlorinated pesticides Semivolatile organics
PAHs PCBs Chlorinated herbicides (phenoxyacid
herbicides) Phenols Organophosphorus pesticides
and chlorinated herbicides Dioxins and furans
Petroleum Hydrocarbons*

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DISPOSABLE VIALS

MINIMUM SOLVENT USAGE

SUITABLE FOR US EPA METHOD
3546 AND ASTM METHOD
D5765-05

ADVANCED MICROWAVE EXTRACTION SYSTEM FOR ENVIRONMENTAL APPLICATIONS

The overall concept of the ETHOS X for environmental applications has been developed to fully comply with the requirements of US EPA and ASTM methods.

This system has been developed by studying the working routine of several thousand contract laboratories around the world performing solvent extraction, with the aim of helping them, by offering an integrated solution able to render their activity easier, faster and safer.

A completely new rotor has been specifically developed by Milestone to fully accomplish the US EPA method 3546 requirements. This new rotor consists of a 24-position carousel, which holds large pressure vessels made of an innovative and unique inert polymer material. At the core of the vessel there is a disposable and inexpensive 100 mL glass vial. A self-regulating pressure cover assures safe operations of the system. Temperature and pressure are monitored and controlled in all vessels by non-contact sensors.

With an installed power of 1900 Watt, the ETHOS X is the most powerful microwave platform system available for extraction.

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WEBSITE

Is your Laboratory following US EPA 3546?
Learn more at: www.milestonesrl.com/environmental



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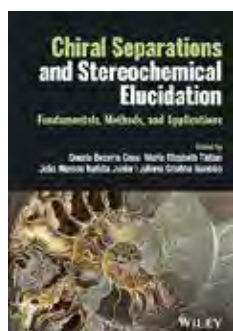


Introduction to Quality by Design in Pharmaceutical Manufacturing and Analytical Development

Márcia Cristina Breitkreitz, Hector Goicoechea (Eds.)

July 2024 Springer Cham

Written by twenty-five authors from academia, pharmaceutical industry and Pharmacopeias worldwide, this monograph covers the fundamentals and applications of Quality by Design (QbD) and Analytical Quality by Design (AQbD) in a practical and didactic manner. The book starts by describing the motivation and the urgent need for the implementation of the QbD framework in pharmaceutical development, along with the definition of its major elements. The concept of life cycle and regulatory perspectives are discussed. [doi](#)

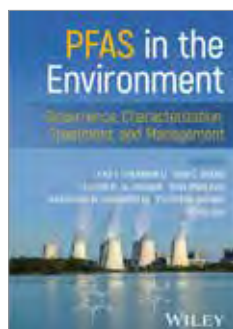


Chiral Separations and Stereochemical Elucidation: Fundamentals, Methods, and Application

Quezia B. Cass, Maria Elizabeth Tiritan, João Marcos Batista Junior, Juliana Cristina Barreiro (Eds.)

March 2023 John Wiley & Sons, Inc

An expert resource for chemists using stereochemical analysis methods. A team of distinguished researchers delivers a robust and authoritative discussion of the theoretical fundamentals of chiral separation, the most commonly used chiral selectors, and stereochemical elucidation methods. The book offers expert discussions of a variety of chiral separation methods by GC, SFC, CE, and LC. [doi](#)

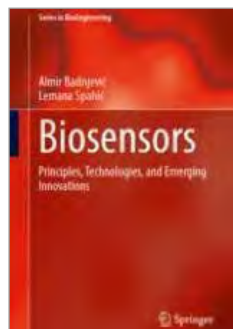


PFAS in the Environment: Occurrence, Characterization, Treatment, and Management

Surampalli, R. Y.; Zhang, T. C.; Al-Hashimi, B. M.; Kao, C.-M.; Ghangrekar, M. M.; Bhunia, P.; Das, S. (Eds.)

October 2025 John Wiley & Sons Ltd.

This book comprehensively summarizes the chemical and ecotoxicological properties of different types of per- and polyfluorinated alkanes (PFAS) as well as current and emerging detection methods, known and suspected health risks, and removal technologies from water and soil. It considers the recently enacted and much stricter regulations set by the US EPA and its European counterpart on the production and use of PFAS. A special focus is placed on how water treatment plants may be upgraded to reduce PFAS content in drinking water. [doi](#)



Biosensors: Principles, Technologies, and Emerging Innovations

Almir Badnjević, Lemana Spahić, Authors

February 2026 Springer Cham

This textbook provides a comprehensive introduction to biosensors, covering their fundamental principles, diverse applications, and the latest advancements in biosensing technologies. It explores various biosensor types, such as electrochemical biosensors for glucose monitoring and drug analysis, optical biosensors using fluorescence and surface plasmon resonance for medical diagnostics and food safety, and impedance biosensors for cellular analysis and cancer detection. Additional sections cover acoustic and piezoelectric biosensors, field-effect transistor-based biosensors, and cutting-edge nanotechnology-enhanced immunosensors. [doi](#)

Events on Analytical Chemistry in 2026 and 2027

October 4 – 9, 2026

XIII International Mass Spectrometry Conference on Petrochemistry, Environmental and Food Chemistry (Petromass 2026)

Yerevan, Armenia



October 13 – 16, 2026

20th International Symposium on Preparative and Industrial Chromatography and Allied Techniques (SPICA 2026)

Muttenz (Basel), Suíça



October 13 – 16, 2026

4th European Sample Preparation Conference (EuSP2026) & 3rd Green and Sustainable Analytical Chemistry Conference

Santiago de Compostela, Spain



November 23 – 24, 2026

13th International Conference and Exhibition on Chromatography and Analytical Techniques

Paris, France



November 23 – 25, 2026

PharmaLab Congress

Darmstadtium, Darmstadt (Germany)



January 08 – 09, 2027

International Conference on Analytical Chemistry and Applied Spectroscopy (ICACAS 2027)

Zurique, Suíça



April 24 – 28, 2027

PITTCON 2027 – Conference & Expo

David L. Lawrence Convention Center, Pittsburgh, Pennsylvania, USA



September 5 – 10, 2027

45th Colloquium Spectroscopicum Internationale (CSI 2027) & 14th Euro-Mediterranean Symposium on Laser-Induced Breakdown Spectroscopy

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Analítica Latin America 2027

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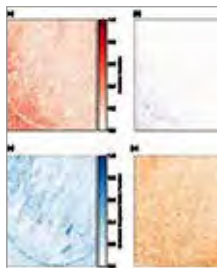
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Chromatographyonline delivers practical, nuts-and-bolts information to help scientists and lab managers become more proficient in the use of chromatographic techniques and instrumentation. **Article:** *Separation Science in 2025–2026*. The Products Shaping Analytical Workflows. A 2026 snapshot of chromatography, mass spectrometry, software, and laboratory tools advancing automation, compliance, sensitivity, and workflow efficiency. [Access here](#)



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Aims & Scope

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