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Technical Note

Heptafluorobutyl Chloroformate Derivatization Enables Molecular-Ion-Centric Metabolite Screening and Fluorine-Assisted Elemental-Composition Assignment by GC−APCI−HRMS

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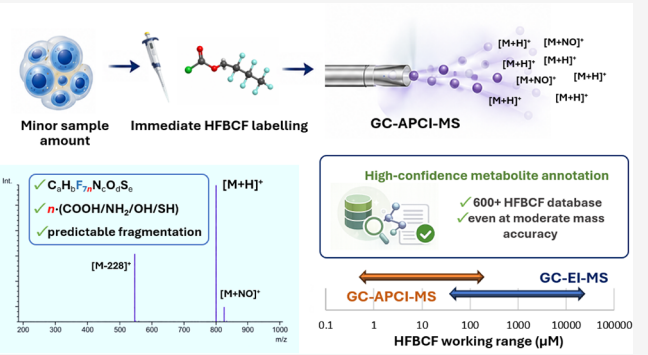
ABSTRACT: Metabolomics enables the simultaneous monitoring of hundreds to thousands of metabolites in complex biological matrices; however, confident identification of low-abundance and unknown compounds remains challenging. Gas chromatography−mass spectrometry (GC−MS) workflows based on electron ionization (EI) often provide limited molecular-ion information because of extensive fragmentation. Here, we present a novel rapid screening workflow combining 1 min heptafluorobutyl chloroformate (HFBCF) derivatization of protic metabolites and concurrent liquid−liquid microextraction with gas chromatography−atmospheric pressure chemical ionization mass spectrometry (GC−APCI−MS). The proven HFBCF−mediated reaction produces stable heptafluorobutyl derivatives that yield abundant protonated molecular ions in the APCI mass spectra, predictable class-specific fragmentation, and characteristic fluorine-specific mass signatures. Compared with conventional full-scan GC−EI−MS, full-scan GC−APCI−MS provided up to 10−1000-fold higher sensitivity, enabling metabolite screening from extremely limited sample amounts. In low-input HeLa cell extracts, at least 100 metabolites were consistently detected from as few as 15,000 cells. Specific heptafluorobutyl-derived mass shifts and fluorine-based elemental constraints facilitated determination of the number and, in selected cases, the type of derivatized functional groups and supported elemental-composition assignment of unknown metabolites, even at mass accuracies of several tens of ppm. To aid compound annotation, we established an openly accessible database comprising more than 620 derivatizable metabolites and experimentally characterized retention and mass-spectral data for 240 reference standards. Together, these results establish HFBCF−GC−APCI−MS as a sensitive molecular-ion-centric workflow for exploratory metabolomics, enabling annotation of unknown metabolites across defined confidence levels, from standard-confirmed identifications to database-supported candidate assignments, suitable for limited biological samples.



INTRODUCTION

Gas chromatography−mass spectrometry (GC−MS) remains a powerful analytical tool for the analysis of small and moderately polar molecules. In metabolomics, however, its broader application is often limited by the extensive fragmentation caused by widespread electron ionization (EI), which typically results in low molecular-ion signals. Although comprehensive EI spectral libraries such as NIST, Wiley, MassBank, and FiehnLib[1] enable reliable identification of many known metabolites, confident annotation of low-abundance or previously unreported compounds remains challenging. This limitation is particularly evident in environmental and metabolomic screening studies as well as in studies using stable isotope labeling, where preservation of molecular-ion information is essential.

Softer ionization techniques such as chemical ionization (CI) and atmospheric pressure chemical ionization (APCI) have been introduced to enhance molecular-ion preservation and sensitivity.[2] APCI has gained practical relevance over the past decade owing to substantial improvements in ion-source robustness and analytical performance.[3] It has been successfully applied to a wide spectrum of chemically diverse environmental analytes, especially in targeted analytical workflows,[4,5] and more recently to nontargeted screening and metabolomics applications.[3] In exploratory metabolomics of complex biological matrices, APCI−HRMS alone does not

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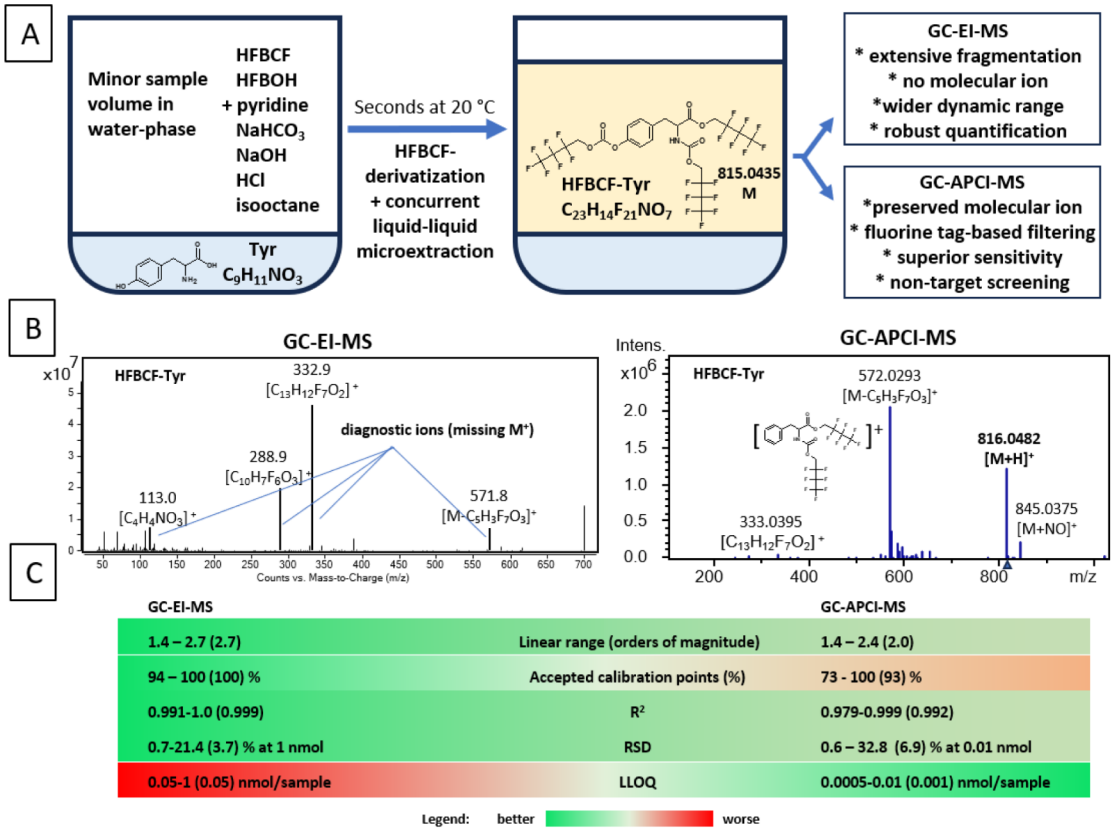

Figure 1. Rapid HFB derivatization and concurrent liquid–liquid microextraction of tyrosine (Tyr). B. Representative GC–EI–MS and GC–APCI–MS spectra of HFB-Tyr highlighting fragmentation-dominated EI spectra and preserved protonated molecular ion in APCI. C. Values represent the minimum and maximum observed across the 30 metabolites investigated, with the median shown in parentheses. Green shading indicates the relatively better performance for a given metric. Complete metabolite-specific data are provided in the Sheet S5.

fully resolve challenges related to analyte polarity, volatility, variable ionization efficiency, and confident elemental-composition assignment across chemically diverse metabolite classes.

In practice, chemical derivatization remains an important complementary strategy to extend GC–APCI–MS accessibility and analytical performance. Typically, protic functional groups (with active hydrogen) can thus be converted into more stable, volatile products possessing improved chromatographic behavior and ionization efficiency. Moreover, the derivatization may also introduce chemically informative features that support subsequent metabolite annotation.[6] Previously, the common methoximation–silylation (MO-TMS) workflow has mostly been applied.[7−9] However, pentafluorobenzyl bromide esterification of fatty acids (FAs)[10] and methyl chloroformate (MCF) derivatization have also been examined.[11,12] Previous studies using APCI sources from different vendors demonstrated detection limits in the nM–μM range, highlighting the potential of GC–APCI–MS for metabolite analysis in complex biological matrices.

Nevertheless, the potential of fluorinated alkyl chloroformates such as heptafluorobutyl chloroformate (HFBCF) for GC–APCI–MS metabolomics has not been systematically explored. Compared with shorter-chain alkyl chloroformates, the HFBCF reagent exhibits higher reactivity and rapidly and reproducibly derivatizes accessible protic functional groups, including carboxyl, hydroxyl, thiol, and amino groups in high yields.[13,14] The highly nonpolar heptafluorobutyl moiety simultaneously promotes partitioning of derivatives into neat nonpolar hydrocarbon solvents during simultaneous liquid–liquid microextraction process reducing coextraction of polar matrix constituents and providing highly clean extracts.[4,13−17] The physicochemical properties of HFBCF derivatives also make them attractive candidates for GC–APCI–MS analysis. The combination of liquid–liquid microextraction (LLME) with HFBCF derivatization in a single step has demonstrated broad applicability in metabolic profiling[14,18] and in both nonchiral and cost-effective chiral GC–MS analysis of a comprehensive range of primary and secondary amino acids.[19,20]

Although high mass accuracy is often considered essential for identifying unknown compounds, even subppm measurements do not necessarily yield unambiguous elemental-composition assignments.[21−23] Alternative strategies that improve elemental-composition assignment are needed, such as those using low-resolution data through spectral accuracy, postacquisition correction, or analyte labeling.[24] Current GC–MS workflows in particular still lack suitable strategies that would translate derivatization chemistry into explicit constraints for molecular-ion annotation. Compounds containing fluorine atoms occupy a particularly characteristic region of elemental-composition space due to the absence of related fluorine isotopes, distinct mass defects, and restricted stoichiometric patterns. In environmental HRMS workflows, especially in PFAS analysis, these fluorine-specific features are exploited through Kendrick mass defect filtering, homologous-series analysis, and fluorine-driven compositional constraints to

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facilitate annotation of unknown compounds.[25] However, similar concepts have remained largely unexplored in derivatization-based metabolomics despite the high fluorine content of certain derivatization reagents.[26]

In this work, we present a rapid and highly sensitive GC−APCI−MS metabolomics workflow based on heptafluorobutyl chloroformate (HFBCF) derivatization. Beyond improving volatility and chromatographic compatibility, HFBCF produces fluorine-rich derivatives that enable highly efficient APCI ionization and abundant molecular-ion formation, allowing sensitive metabolite screening from minimal sample amounts. The defined fluorine content, characteristic mass increments, and predictable fragmentation behavior of these derivatives provide derivatization-specific constraints that support elemental-composition assignment of unknown compounds. By combining analytical sensitivity with fluorine-assisted elemental-composition assignment, the proposed strategy substantially reduces formula ambiguity and enables prioritization and annotation of derivatizable metabolites across confidence levels ranging from unequivocal molecular-formula assignment (Level 2b-4) to reference-standard-confirmed identification (Level 1),[27] even at mass accuracies below 50 ppm.

EXPERIMENTAL SECTION

Chemicals and Standards

Heptafluorobutyl chloroformate (HFBCF) and heptafluorobutanol (HFBOH) were obtained from Pragolab (Prague, Czech Republic) or Apollo Scientific (Manchester, UK). Analytical standards, including ^{13}C-labeled internal standards, were obtained from commercial suppliers (Merck, Toronto Research Chemicals, and CIL). A complete list of chemicals and standards is provided in Table S2.

Sample Preparation

Sample LLME/derivatization with HFBCF was performed according to previously described procedures.[16,17] Briefly, aqueous samples (1−100 μL) were derivatized in the presence of pyridine and the corresponding HFBOH alcohol, resulting in the HFBCF-mediated derivative formation within seconds and simultaneous extraction into isooctane. The organic phase was directly analyzed by GC−MS (Figure 1A). The experimental details are summarized in Table S3.

GC−APCI−MS Analysis

Analyses were performed using a gas chromatograph (Bruker GC, Bruker Daltonics, Bremen, Germany) coupled to a high-resolution QTOF mass spectrometer (Compact HD, Bruker Daltonics) equipped with an APCI source. Samples were injected in a splitless mode at 280 °C. Separation was achieved on a DB-XLB capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) using helium at a constant flow rate of 1.1 mL min^{-1}. The oven temperature program was 45 °C (1.4 min), 25 °C min^{-1} to 150 °C, 15 °C min^{-1} to 280 °C (2 min), and 30 °C min^{-1} to 300 °C (2 min). The mass spectrometer was externally calibrated with heptacosafluorotributylamine. The GC−MS transfer line was maintained at 220 °C. APCI II source parameters were set as follows: end plate offset, 500 V; capillary voltage, 3500 V; corona discharge needle current, 2500 nA; nebulizer gas (nitrogen) pressure, 3.5 bar; and dry gas flow rate, 2.5 L/min. Data were acquired in positive ion mode over an m/z range of 250−1150 at a scan rate of 5 Hz.

MS data were processed by using DataAnalysis 4.2. For experimental APCI conditions, see Sheet S3.

GC−EI−MS Analysis

Comparative GC−EI−MS analyses were performed using a 7890B GC coupled to a 7010B triple-quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) operated in EI mode (70 eV). Chromatographic conditions were identical to those used for GC−APCI−MS. Data were processed using MassHunter 10.0 Quantitative Analysis software. For details, refer to Sheet S3.

Cell Culture Extraction

Applicability to biological samples was demonstrated using HeLa cell extracts prepared from triplicate cultures containing 1.5 × 10^4−2 × 10^6 cells per well in 12-well plates. After 6 h of attachment, cells were washed with water and extracted using precooled methanol/acetonitrile/water (2:2:1, $v/v/v$). Extracts were dried, reconstituted in water, and derivatized using the standard HFBCF protocol described above. Procedural blanks containing cell culture medium without cells were prepared in parallel. Metabolites were classified as confirmed when both accurate mass and retention time matched reference standards, while features matching only expected exact masses were considered tentative annotations. A metabolite was considered as detected when observed in at least 2 out of 3 biological replicates. These experiments were performed using an earlier GC−APCI−MS configuration and are included solely to demonstrate applicability of the workflow to biological samples.

RESULTS AND DISCUSSION

Comparison of GC−APCI−HRMS and GC−EI−MS Performance

For qualitative and quantitative benchmarking, the GC−APCI−MS workflow was directly compared with the routinely used conventional robust GC−EI−MS full-scan workflow using 30 representative protic metabolites derivatized according to the earlier elaborated HFBCF-based sample preparation protocol.[16] Only the GC oven program was shortened compared with the original HFBCF protocol to increase sample throughput while maintaining sufficient chromatographic resolution for untargeted metabolomics. For each experimentally characterized metabolite, the corresponding HFBCF relative retention index (HFBCF-RRI) was calculated, using HFBCF derivatives of saturated fatty acids as homologous retention anchors to improve interlaboratory transferability of retention data.

As reported previously,[14] EI mass spectra of HFBCF derivatives are dominated by several characteristic fragment ions, whereas APCI preserves abundant protonated molecular ions (as illustrated in Figure 1B). The $[M + H]^+$ signal is frequently accompanied by a nitrosonium adduct $[M+NO]^+$, most likely formed through reactions involving NO^+ reactant ions.[25] The MS sensitivity and abundance of the protonated molecular ions arising from the HFBCF derivatives in the APCI source were affected by the source conditions. In contrast to previous reports,[4,9,25] in which water or organic modifiers (mainly methanol and acetonitrile) were continuously infused into the APCI source, we found that such additives significantly reduced the intensity of protonated molecular-ion signals, particularly for poly(carboxylic acid)s. Consequently, all measurements in the present study were performed under simpler dry APCI source conditions (S4).

HFBCF derivatives analyzed by GC−APCI−HRMS showed markedly enhanced sensitivity compared with conventional GC−EI−MS. Calibration with appropriate ^{13}C-labeled internal standards revealed comparable working ranges of approximately 2 to 2.5 orders of magnitude for both ionization modes (Figure 1C; Sheet S5). While GC−EI−MS covered a slightly broader concentration interval, ranging from approximately 50−25,000 μM to 1,000−25,000 μM depending on the analyte, GC−APCI response window was shifted toward substantially lower concentrations, typically spanning 0.1−250 μM with LOQs of 0.5−2.5 μM. HFBCF derivatives generated

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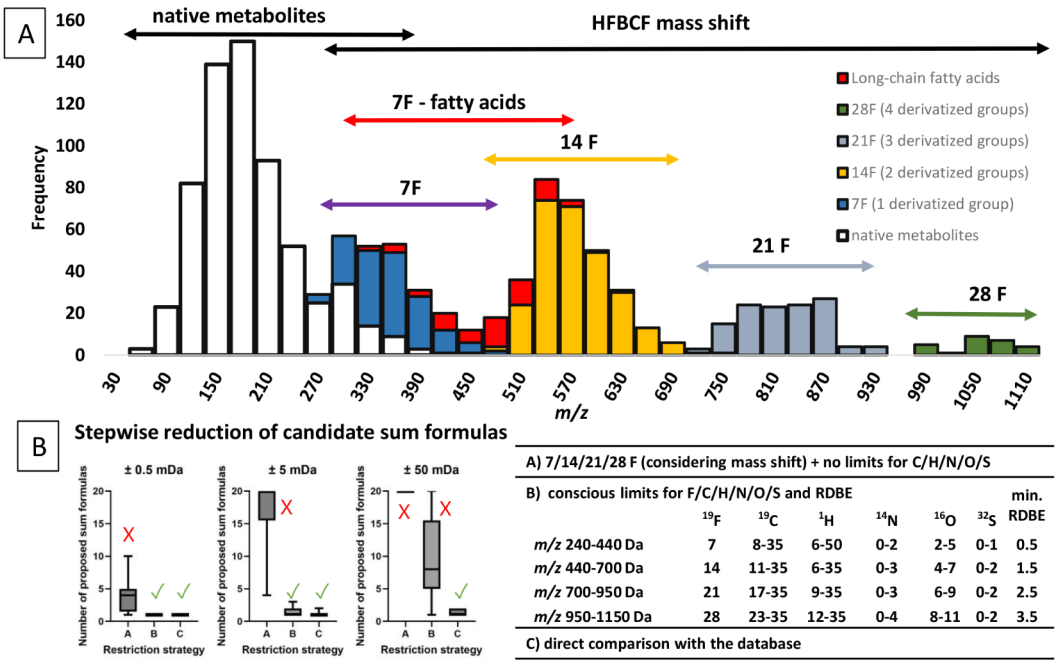

Figure 2. Mass distribution of 629 database-derived native metabolites and their HFBCF derivatives, showing discrete mass shifts corresponding to multiples of seven fluorine atoms per derivatized functional group according to the number of derivatized functional groups. B. Stepwise reduction of candidate sum formulas using fluorine-assisted elemental composition assignment, effective even at high mass error. The algorithm for back-calculating native metabolite formulas is described in the Supporting Information.

MS responses typically 10−1,000 times higher under APCI conditions than in EI mode, resulting in substantially lower limits of quantification. The upper limit of the APCI calibration range was governed by TOF detector saturation, while the lower limit was in many cases determined by background contamination from solvents, the laboratory environment, and trace impurities present in the HFBCF reagent. Common metabolites such as lactate, fatty acids, and basic amino acids were repeatedly detected in procedural blanks, indicating that background contamination may become the principal factor limiting quantification performance in ultratrace applications.

Importantly, the enhanced sensitivity of APCI was accompanied by the preservation of abundant protonated molecular ions, providing a substantial advantage for the molecular-ion-centric annotation of unknown compounds. In contrast, EI spectra relied predominantly on fragment ions and contained only weak or absent molecular ion signals.

Database of HFBCF-Mediated Protic Metabolites and the HFBCF Annotation Tool

The excellent APCI−MS response observed for HFB-labeled metabolites motivated evaluation of whether multiple HFB moieties could facilitate molecular-ion-centric annotation of unknown compounds. Appropriate reference GC−APCI−HRMS data were therefore collected to evaluate the efficiency of fluorine-assisted elemental composition assignment. We studied a set of 242 metabolites covering primary and secondary amino acids, their conjugates, dipeptides, organic acids, fatty acids, small biogenic amines, and related small protic metabolites of central metabolism sourced from the literature[14,28] (see Sheet S6). The metabolite coverage is limited by the extractability of HFBCF-treated metabolites into the highly nonpolar isooctane phase, which prevents efficient detection of highly polar compounds possessing multiple hydroxyls, untreatable guanidino or ureido functionalities, heterocyclic compounds with multiple nitrogen atoms, and nonvolatile metabolites with permanently charged phosphate, sulfate, or tetraalkylammonium ion groups. The collected data set demonstrated that the number and type of derivatizable functional groups can be reliably translated into predictable HFBCF-derived mass increments, enabling estimation of derivative composition for previously uncharacterized metabolites. The stoichiometry of HFBCF-mediated derivatives is straightforward: conversion of a carboxyl functional group to an HFB ester increases the molecular mass by 181.9965 Da ($+C_4F_7H$), while derivatization of amino, hydroxy, and thiol groups yields the corresponding carbamates, carbonates, or thiocarbonates, each introducing a characteristic mass increment of 226.9943 Da ($+C_5F_7HO_2$) per functional group. The acquired knowledge enabled us to expand the metabolite database with another 380 candidate primary metabolites extracted from HMDB 5.0.[28] Because comprehensive spectral libraries of HFBCF derivatives are currently unavailable, the resulting database provides a practical first-pass screening tool for rapid prioritization of candidate metabolites. For this data set, the expected $[M + H]^+$ ions in the molecular region were calculated. To facilitate further database expansion and annotation of previously uncharacterized compounds, the HFBCF Annotation Tool incorporates a conversion module that predicts the elemental composition and expected molecular ion signals of HFBCF derivatives from the native metabolite formula and the estimated number of derivatizable functional groups. Because the transformation rules are fully reversible, the same tool can also reconstruct the elemental composition of the native metabolite from an observed HFBCF derivative, enabling the interpretation of compounds not explicitly included in the database (Sheet S7).

Application of these derivatization rules revealed that protonated molecular ions can be readily differentiated

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according to the number of derivatized functional groups based on their m/z accurate masses (Figure 2A). Apart from monofunctional fatty acids, metabolites bearing one, two, three, and four derivatized functional groups exhibit distinct mass shifts and their molecular mass ranges remain below 440 Da, 680 Da, 900 Da, and above 900 Da, respectively, as documented in the HFBCF Annotation Tool (Sheet S6, publicly available at www.bclab.eu/projects/hfbcf-annotation-tool/). In summary, the established database enables rapid evaluation for the (non)presence of over 620 candidate protic metabolites, corresponding to 418 unique elemental compositions after exclusion of all positional and structural isomers.

Fluorine-Assisted Molecular-Ion Annotation

In complex biological or environmental matrices, reliable identification of protonated molecular ions may be challenging. In GC−APCI−MS, however, HFBCF-mediated protic metabolites exhibit abundant $[M + H]^+$ ions often accompanied by a characteristic nitrosonium adduct ($[M+NO]^+$), observed at a mass difference of 28.99744 Da against $[M + H]^+$.[29] The simultaneous occurrence of both signals provides a useful indicator for the recognition of molecular-ion-related species in the APCI spectra. The alternative cation radical ions $M^{+\cdot}$ were rarely observed and then typically represented minor signals relative to the dominant $[M + H]^+$.

Each derivatized protic functional group introduces one heptafluorobutyl moiety containing seven fluorine atoms. Because fluorine (18.9984 Da) is monoisotopic and has a negative mass defect relative to nominal mass (−1.6 mDa), incorporation of 7, 14, 21, or 28 fluorine atoms results in a characteristic cumulative downward shift (−11.2 mDa per derivatized functional group) of the monoisotopic mass. Consequently, HFBCF derivatives frequently display unusually low first decimal digits in their protonated molecular-ion masses. Within the HFBCF Annotation Tool database, approximately 76% of the HFBCF-mediated analytes exhibit a first decimal digit of zero (S6).

Together, the characteristic $[M + H]^+/[M+NO]^+$ ion pair and the fluorine-derived mass defect provide a rapid means of distinguishing HFBCF-labeled analytes from nonderivatized background ions and unrelated matrix components. Fatty acids with long alkyl chains belong to the minor "non-zero first decimal" group due to the relatively high hydrogen content. Nevertheless, their presence is frequently supported by characteristic HFBCF-derived fragment ions (Table 1).

In addition to fluorine-derived mass-defect information, HFBCF derivatives exhibit characteristic fragmentation behavior due to in-source fragmentation that reflects the nature of the derivatized functional groups (Table 1). Amino acids, dicarboxylic acids, and hydroxyl-containing metabolites display reproducible neutral losses associated with cleavage of derivatized functional groups. These class-specific fragmentation patterns provide an additional layer of structural information that can assist metabolite classification, support elemental-composition assignment, and support assignment of a probable or tentative structure (Level 2b−4) to unknown compounds in the absence of a matching reference standard. This contrasts with previously reported GC−APCI−HRMS analyses of MO−TMS derivatives, where accurate-mass measurements alone were insufficient for unique elemental-composition assignment and additional information regarding the number of derivatizing groups or CID fragmentation data was required.[9] In HFBCF derivatives, the number of incorporated fluorinated moieties, characteristic mass increments, and predictable fragmentation behavior provide direct derivatization-specific constraints that substantially reduce this ambiguity.

Table 1. Characteristic Fragment Ions Arising Most Probably as In-Source Neutral Losses from Pseudomolecular Ions of the HFBCF-Derivatized Protic Analytes during Full Scan GC−APCI−HRMS

Compound class	Diagnostic feature (Da)	Interpretation
Fatty acids (FA)	m/z 269.0413	HFBCF-derived fragment ion ($C_8H_8F_7O_5$)
	m/z 311.0882	HFBCF-derived fragment ion ($C_{11}H_{14}F_7O_5$),
Short-chain FA	m/z 283.0569	HFBCF-derived fragment ion ($C_9H_{10}F_7O_5$)
Monocarboxylic acids	Fragments ≤ 50 (max. 100)	only low-mass fragments originating from native skeleton
Dicarboxylic acids	$[M-200.0072]^+$	neutral loss ($-C_4H_3F_7O$), α-cleavage
	$[M-228.0021]^+$	neutral loss ($-C_5H_3F_7O_5$), α-cleavage + carbonyl loss
Amino acids	$[M-228.0021]^+$	neutral loss ($-C_5H_3F_7O_5$), α-cleavage + carbonyl loss, often the only major fragment
Hydroxylated compounds	$[M-243.9970]^+$	neutral loss ($-C_5H_3F_7O_3$), loss of carbonate moiety

Systematic Fluorine-Assisted Elemental-Composition Assignment of HFBCF Derivatives

The characteristic mass increments introduced by HFBCF derivatization, fluorine-specific mass signatures, and predictable fragmentation behavior collectively provide a set of compositional constraints that substantially reduce ambiguity during elemental-composition assignment of molecular ions. Additional restrictions arise from the known derivatization chemistry, including fluorine-to-oxygen stoichiometric relationships, the nitrogen rule, and RDBE (ring and double bond equivalents) filtering (Sheet S8).

To evaluate the practical impact of these constraints, a model set of HFBCF derivatives of 30 representative metabolites mediated covering a broad range of molecular masses and derivatizable functional groups was examined. For each analyte, the number of plausible elemental compositions was determined at mass-measurement errors ranging from ± 0.5 to ± 50 mDa, both with and without the application of the HFBCF-specific restrictive criteria (Figure 2B). Because only compounds extractable into the isooctane phase are detected, elemental-composition searches were limited to combinations of C, H, N, O, S, and F.

Application of the restrictive criteria resulted in a substantial reduction in the number of candidate elemental compositions across the entire mass-accuracy range. Even at a relatively large mass error of ± 50 mDa, the number of plausible compositions was reduced to only a few candidates in most cases. Subsequent comparisons against the HFBCF Annotation Tool database frequently enabled assignment of a unique elemental composition or, at minimum, a very small set of chemically plausible candidates.

Although fluorine-assisted composition assignment substantially reduces formula ambiguity, the complete reconstruction of the native metabolite is not always possible. For example, derivatives containing 14 fluorine atoms may originate from

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metabolites containing two carboxyl groups, two hydroxyl/amino groups, or a combination of these. Consequently, multiple native structures may correspond to the same derivative elemental composition. In such cases, additional information from retention behavior, characteristic fragmentation pathways, and database matching is required for a confident metabolite annotation.

Applicability to Cellular Metabolomics

The practical applicability of the proposed fluorine-assisted annotation strategy was evaluated using HFBCF-derivatized extracts prepared from HeLa cell samples containing approximately 15,000−250,000 cells (S9). Samples were analyzed on an independent GC−APCI−MS platform and processed using the annotation workflow described above.

Following feature detection, molecular-ion candidates were screened using accurate mass information, retention index filtering, and the HFBCF annotation tool database. To minimize false-positive detections, signals were corrected using procedural blanks, and only features reproducibly observed across replicate measurements were considered for further evaluation.

Across the investigated sample range, the workflow consistently yielded at least 100 metabolite candidates confirmed by a reference standard (level 1). Importantly, reducing the HeLa cell sample amount by approximately 1 order of magnitude did not markedly affect the performance of the fluorine-assisted elemental composition assignment procedure mainly due to the highly efficient LLME cleaning step.[13] The obtained results demonstrate that the proposed workflow remains applicable, even for highly mass-limited biological samples.

While comprehensive untargeted annotation of all detected features remains challenging and may require additional manual verification, the combination of fluorine-assisted compositional constraints, database matching, and characteristic fragmentation behavior substantially facilitates the prioritization of candidate metabolites in complex biological matrices.

CONCLUSION

In this Technical Note, we present a novel fluorine-assisted molecular-ion-centric GC−APCI−HRMS workflow based on HFBCF derivatization of accessible protic functional groups combined with efficient LLME, GC−APCI−HRMS and rule-based annotation to improve confident metabolite identification from limited biological samples.

The resulting HFBCF-mediated derivatives provide low-picomole sensitivity while preserving abundant protonated molecular ions. Molecular-ion assignment is further supported by characteristic nitrosonium adduct formation, limited and predictable fragmentation, and distinctive fluorine-driven monoisotopic mass signatures.

Exploitation of these derivatization-specific mass spectrometric features enables inference of both the number and, in selected cases, the type of derivatizable functional groups. Building on this information, a rule-based annotation framework was developed to constrain elemental-composition assignment of HFBCF derivatives and to enable reconstruction of the native metabolite composition.

Compound elemental composition assignment is further facilitated by a comprehensive, openly accessible in-house database comprising more than 620 potentially relevant metabolites, one-third of which are supported by experimentally determined retention data and APCI mass spectra. The applicability of the workflow was demonstrated on low-input HeLa cell samples, where at least 100 metabolites were consistently detected from as few as 15 000 cells.

The presented strategy is not inherently limited to GC−APCI−MS but may be extended to other GC−MS configurations capable of preserving protonated molecular ions, highlighting the broader potential of fluorine-based derivatization and constraint-driven annotation for molecular-ion-centric metabolomics. Nevertheless, it is important to note that metabolite coverage is restricted to metabolites containing protic functional groups accessible to HFBCF derivatization. The metabolite coverage is further narrowed by the LLME step, which removes highly polar and ionic metabolites together with partially derivatized compounds retaining untreated polar groups that are generally unsuitable for GC separation. The obtained data also show that cleaner reagents, solvents, and laboratory environments are required for future ultratrace applications of the new method.

Overall, this study demonstrates that coupling fluorinated derivatization chemistry with soft-ionization GC−MS and chemically constrained annotation provides a practical strategy for sensitive molecular-ion-centric metabolomics. More broadly, the findings indicate that soft-ionization techniques such as APCI−MS offer considerable yet still underexploited potential for confident detection and annotation of low-abundance and previously unknown metabolites in GC−MS-based metabolomics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.6c04134.

Additional experimental details, chemicals and reference standards, APCI source optimization, GC−APCI versus GC−EI performance comparison, HFBCF metabolite database including HFBCF relative retention indices (HFBCF-RRIs), HFBCF Annotation Tool and conversion module, elemental-composition prediction workflow, and HeLa cell metabolomics data (XLSX)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. I.K.: investigation, visualization, formal analysis, data curation, writing—original draft; P.Š.: conceptualization (HFBCF chemistry, metabolite analysis), funding acquisition, supervision, manuscript finalization; M.M.: conceptualization (molecular-ion-centric screening, annotation), writing—original draft, funding acquisition, supervision, administration, manuscript finalization; P.V., L.Ř.: methodology, GC−EI−MS analysis, investigation; S.O.: HFBCF chemistry, formal analysis, methodology; J.B., P.U., and P.H.: GC−APCI−HRMS analysis.

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Notes

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants involved in the study. During the preparation of this work, the authors used ChatGPT to improve language and support readability. After using this service, the authors reviewed and edited the content as needed and took full responsibility for the content of the published article.

The authors declare no competing financial interest.

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