Comparison of ESI-MS/MS and APCI-MS methods for the quantification of folic acid analogs in C. elegans

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Abstract

Folic acid (FA) plays a vital role in central metabolism, including the one carbon cycle, nucleotide, and amino acid biosynthesis. The development of sensitive, accurate analytical methods to measure FA intermediates in tissues is critical to understand their biological roles in diverse physiological and pathological contexts. Here, we developed a highly sensitive method for the simultaneous quantification of FA intermediates in the nematode Caenorhabditis elegans as a model to dissect metabolic networks. The method was further validated by analyzing the worm folate pool upon RNAi knockdown of the dihydrofolate reductase gene dhfr-1. Comparative mass spectrometry behavior of the FA analogs using two different ion sources, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), revealed ESI-MS/MS to be more sensitive, but APCI-MS provided more detailed structure inferences, which can elucidate chemical investigation and synthesis of FA analogs. Finally, we report on the use of in vitro oxidation coupled with high-resolution mass spectrometry as a tool to discover new endogenous FA derivatives in the nematode.

KEYWORDS

APCI, C. elegans, dhfr-1, ESI, folic acid

1 | INTRODUCTION

Folates play a major role in several physiological processes and biological reactions including methylation, DNA biosynthesis and amino acids, and purine and pyrimidine synthesis. Tetrahydrofolate, the active form of this vitamin, acts as one-carbon donor in S-adenosylmethionine and S-adenosylhomocysteine-dependent methylation reactions, creatine metabolism, and phosphatidylethanolamine methylation.¹⁻⁴ The folate cycle is not only essential to organismal metabolism but is also of medical relevance. Dysregulation of folate metabolism in humans can lead to cellular damage, including chromosome breakage and systemic disorders, such as hyperhomocysteinemia, cardiovascular, and neurodegenerative diseases.⁵⁻⁹ Moreover, several FA analogs are being developed as anti-cancer drugs.

Folic acid (FA) is a water-soluble vitamin synthesized primarily in bacteria and composed of three components, namely para-amino benzoic acid, a glutamate moiety, and a pteridinic ring.¹⁰ Several metazoan enzymes participate in the folate cycle and generate at least seven derivative intermediates, which differ by their oxidation states and substitutions of the pteridinic ring with formate, formaldehyde, or...
hydroxyl group at position 5 and 10. These species include dihydrofollic acid (DHF), tetrahydrofolic acid (THF), 5-methyl-tetrahydrofolic acid (5MTHF), formyl-tetrahydrofolic acid (FO), 5,10-methylene-tetrahydrofolic acid (ML), and 5,10-methenyl-tetrahydrofolic acid (MN). Given this complexity, it becomes challenging to simultaneously quantify various species in the folate pool. Moreover, to understand the network of the FA metabolism, it is useful to have an in vivo model system to unravel mechanisms and develop diagnostics.

Caenorhabditis elegans has recently emerged as a facile model organism for studying metabolic processes, fluxes, and disorders. Remarkable metabolic homology between C. elegans and mammals has already yielded key insights into the role of FA deficiency and FA supplementation on organismal physiology. Moreover, the investigation of FA in a simple metazoan having only 959 somatic cells may expedite the discovery of new folate molecules. An expanded FA pathway repertoire may, in fact, reveal new physiological processes, which can be further translated to higher organisms. In order to have a clear overview of the folate pools in the nematode, however, more sensitive methods are needed.

The measurement of the FA derivatives in different biological matrices is usually achieved by liquid chromatography (LC) coupled with mass spectrometry (MS) due to its high accuracy, precision, sensitivity, and reproducibility. Reverse phase chromatography (RPC) is the first choice for the separation of the modified pteridine species. Previous work from Wang et al described a LC-MS/MS method for the quantification of FA, 5MTHF, and FO in human plasma. Parallel research, reported in Owens et al and Zayed et al, presented alternative methods for deciphering the concentration of FA, 5MTHF, and FO in whole human blood.

Electrospray ionization (ESI) is the primary ionization technique for ionizing polar organic compounds. In an optimized ESI condition, ions are generated from the dissociation of a charged droplet, leaving a stream of ions. Using collision-induced dissociation (CID), the ions, which entered the mass spectrometer can collide with an inert gas, typically nitrogen, to obtain product ion spectra, and is commonly combined with ESI (ESI-CID or ESI-MS/MS). Atmospheric pressure chemical ionization (APCI) is an alternative to ESI, which produces a corona discharge whereby atmospheric gases, such as O2 and N2, are ionized and further react with analyte molecules. Notably, the use of different ion activation modes as well as different ionization sources is a powerful tool to elucidate metabolite and drug structures in complex chemical mixtures.

Understanding the fragmentation behavior and the product ions of FA under different MS conditions can help elucidate the structure of FA analogs, as well as facilitate identification of new candidate molecules.

In the present study, we developed two approaches, ESI-MS/MS and APCI-MS, for the simultaneous quantification of all six FA analogs in C. elegans upon RNAi knockdown of dihydrofolate reductase, dhfr-1, the first enzyme in the pathway. We then compared and interpreted the ionization and fragmentation behavior of the two newly designed methods. We additionally explored new possible FA-derived molecules using an in vitro oxidation reaction and quantified these species in the nematode. These studies should provide a useful platform for further investigation of FA metabolism in metazoans.

2 METHODS

2.1 Chemicals

UHPLC grade water, formic acid, and UHPLC grade acetonitrile were purchased from Biosolve Valkenswaard, Netherlands. Chloroform was purchased from Merck KGaA (Darmstadt, Germany). UHPLC grade methanol and pteroic acid were purchased from Sigma-Aldrich, GmbH. The following standards were obtained from Schircks Laboratories (Jona, Switzerland): folic acid, DHF, 5-methyltetrahydrofolic acid disodium salt, THF, (6R,S)-5-formyl-5,6,7,8-tetrahydrofolic acid, (6R,S)-5,10-methylene-5,6,7,8-tetrahydrofolic acid, and (6R,S)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid.

2.2 Worm strains and RNAi treatment

All strains were grown and maintained on NGM media seeded with E. coli (OP50) at 20°C. Standard procedures for culturing and maintaining strains were used. RNAi was conducted as described previously. Briefly, N2 wild-type worms were fed with HT115 (DE3) bacteria transformed with L4440 vector that expresses a double-stranded RNA against the targeted gene. Synchronized worms were obtained by performing an egg lay on corresponding RNAi plates containing isopropyl-β-D-thiogalactoside and ampicillin. Luciferase (L4440::Luc) RNAi vector was used as non-targeting control. C36B1.7 (dhfr-1) RNAi clone was obtained from the Vidal RNAi library. The gene targeted by RNAi is indicated with an "i" after the gene name.

2.3 Extraction of FA

Synchronized worms were collected at the young adult stage after RNAi treatment. Soluble metabolites were extracted from worms as previously described. Briefly, synchronized young adult worms were collected and homogenized by bead beating using the Qiagen tissue lyser for 30 minutes at 50 oscillations/s at 4°C. Folic acid intermediates were extracted from an homogenate volume, which corresponds to 300 µg protein, using the Bligh and Dyer extraction (chloroform:methanol, 2:1) for 1 hour at 4°C, followed by desiccation using a speed-vac. Samples were reconstituted in 90:10, water:acetonitrile (v/v), and 10 µL was injected into the LC-MS/MS system. Mass spectrometry experiments with worm extracts were performed in eight independent biological replicates.

2.4 LC-MS/MS instrumentation and conditions

Identification and relative quantification of folic acid intermediates were performed on a triple quadrupole mass spectrometer (QQQ-MS) (TSQ Altis, ThermoFisher Scientific GmbH, Bremen, Germany).
Folate standards including folic acid, DHF, 5-methyltetrahydrofolate acid, THF, FO, ML, and MN were dissolved in water and were transferred into amber Eppendorf tubes (for light protection) and stored at −20°C. We additionally added 0.1% w/v butylated hydroxytoluene (BHT) to all solutions to prevent any degradation and any autooxidation reaction. Folic acid standards and worm extracts were separated by reverse phase column (XSelect HSS T3 2.5 μm 2.1x100, Waters) using a binary pump system (Vanquish, ThermoFisher Scientific GmbH, Bremen, Germany) with solvent A as water with 0.1% v/v formic acid and eluent B as acetonitrile with 0.1% v/v formic acid; 2 μL of the reconstituted sample was injected and separated using a gradient modified from Gutzeit et al. The gradient started with 1% eluent B and increased up to 5% eluent B in 1 minute. At 5 minutes, the gradient increased up to 90% eluent B and was held for 3 minutes. The gradient then decreased down to 1% eluent B in 1 minute and was held for 2 minutes. The total time was therefore 11 minutes. The flow rate was 0.1 mL/min for the first 5 minutes and then increased to 0.150 mL/min thereafter. The column temperature was set at 30°C.

Data was analyzed using Xcalibur version 4.0 and Trace Finder version 4.1. The relative response for each folate species was calculated by dividing the peak area of the analyte to the internal standard peak area and further normalized to protein concentration.

2.5 ESI-MS/MS conditions

ESI ionization parameters were as follows: 3.5 kV, 25 a.u. sheath gas, 5 a.u. auxiliary, 350°C transfer ion capillary. All the spectra were acquired in positive ion mode. The product ions were obtained for each single precursor using a scan rate of 500 Da/s, using Q1 resolution of 0.7 m/z and Q3 resolution of 0.7 m/z. Tandem mass spectra were obtained using CID using Argon set at 2 mTorr with collision energy between 30 and 40 V. Multiple reaction monitoring (MRM) measurements using ESI-MS/MS were achieved using the following parameters: Q1 resolution 0.7 m/z, Q3 resolution 1.2 m/z, and 30-V collision energy. Unique combination of Q1 and Q3 mass ranges was used to quantify folic acid analogs. We used the following ions as qualifier for FA 442.22->176.04, DHF 444.28->177.07, THF 463.38->206.88, 5MTHF 460.57->337.23, ML 458.06->355.20, MN 456.46->310.14, FO 474.21->299.14. The following transitions were used as quantifier: FA 442.22->295.10; DHF 444.28->297.12; THF 463.38->342.02; 5MTHF 460.57->378.21; ML 458.06->376.23; MN 456.46->349.83; FO 474.21->327.15.

2.6 APCI-MS conditions

APCI ionization parameters were: 15 a.u. sheath gas, 5 a.u. auxiliary, 300°C transfer ion capillary, 2-μA current on the corona discharge needle, and 400°C vaporizer temperature. Full scans were acquired in positive ion mode from 110 to 550 m/z, with a scan rate of 1000 Da/s using a Q1 resolution of 0.7 m/z. Relative quantification using APCI-MS was performed using selected ion monitoring chromatogram mode (SIM) using a scan rate of 250 Da/s, Q1 resolution was set to 0.7 m/z, and calibrated RF lenses were used. The following ions were monitored: FA->424.29, DHF->416.94, THF->484.33, 5MTHF->460.39, ML->341.28, MN->343.27, FO->456.34.

2.7 Identification of new potential folic acid derivatives

In order to identify new potential folic acid derived molecules, we initially oxidized folic acid in vitro (0.5 mmol/L in water) with or without FeSO₄ (80 μmol/L) and H₂O₂ (50 mmol/L) at 37°C for 2 hours. The in vitro oxidation mixture was analyzed using the same LC system coupled with a high-resolution accurate mass (HRAM) mass spectrometer (Q-Exactive Plus, Thermo Fisher Scientific GmbH, Bremen, Germany). Full scan acquisition experiments were performed from 100 to 500 m/z using a resolution of 70,000; injection time was set to 100 ms, and AGC target was set to 1e6. Data-dependent acquisition was performed using a Top 3, resolution of 17,500, injection time of 50 ms, AGC target set at 5e4, isolation window of 1.0 m/z, and stepped normalized collision energy (NCE) of 20 and 40. The relative quantification of newly identified molecules in the C. elegans extract was achieved by designing a specific t-SIM mode for targeting these compounds using a resolution of 70,000, injection time of 200 ms, AGC target set at 5e4, and isolation window of 1.0 m/z.

2.8 Statistics

Statistical analyses were performed using GraphPad Prism software 5.04. P-values were calculated with t-test.

3 RESULTS

3.1 Method optimization and validation

We employed the UHPLC-MS method coupled with ESI-MS/MS or APCI-MS due to the sensitivity and reproducibility of these techniques. We initially separated folate standards using a C8 column (Xbridge BEH 2.5 μm 2.1x100, Waters) with different mobile and stationary phases, and finally we obtained a good separation using a C18 column (XSelect HSS T3 2.5 μm 2.1x100, Waters) (Scheme 1). 5MTHF eluted at 6.0 minutes, whereas ML and MN co-eluted at 6.21 minutes. DHF and THF were found at 6.50 and 6.52 minutes. FA was detected at 6.78 minutes and FO eluted at 6.95 minutes (Figure 1). Comparable retention times were also obtained using APCI-MS (data not shown).

The use of positive ESI and positive APCI resulted in good ionization of folates, and singly protonated ions were chosen for the analysis. We additionally selected the product ions, which produced the highest response to increase sensitivity and specificity of the methods. Detection and quantification limits, LOD and LOQ, respectively, were determined from serial dilutions of the seven standards from 100 nmol/L to 1 pmol/L (Tables 1 and 2). Both methodologies exhibited high sensitivity. However, we obtained a lower LOQ for all intermediates using the ESI compared with the APCI source. For
both methods, we observed that ML and MN had higher LOQs compared with the other standards, but all external calibration curves exhibited high levels of linearity (>0.90). The ion at $m/z$ 295.10, which correspond to the para-amino benzoic acid and the glutamate moiety, was selected for the MRM analysis of folic acid (442.14) and used to assess the precision of ESI-MS/MS and APCI-MS (Table 3). Both methods resulted in high intraday and interday precision, 6.26% and 8.32% for ESI-MS/MS and 11.5% and 11.29% for APCI-MS. These results are comparable with works from Striegel et al and Garratt et al.33,34

We additionally investigated the recovery and the matrix effects related to folates. For the analysis of recovery, we spiked pteroic acid (100 ng) into the C. elegans extracts ($n = 4$). We extracted folates by adding cold methanol and Folch extraction (data not shown), and using the Bligh and Dyer method, we obtained the highest recovery 89.47% for the ESI-MS/MS method and 84.83% for the APCI-MS, which are similar to previously published data.35

3.1.1 Comparison of ionization modes and product ion spectra between ESI-MS/MS and APCI-MS

In this study, we compared two ionization and fragmentation methodologies for studying the MS behavior of seven folic acid-like molecules. The first method uses the combination of ESI with CID to obtain product spectra (ESI-CID or ESI-MS/MS). The second method uses the APCI source, which ionizes atmospheric gases that further react with the analyte molecules. However, because of the initial in source fragmentation using APCI, we retrieved the mass spectra of the FA intermediates using only a full MS scan (APCI-MS).
Both methods delivered clear decomposition of FA analogs into para-amino benzoic acid and the glutamate moiety (Figures 2 and 3). In addition, they produced the proposed fragment structures shown in Supplementary Figures S1-S4.

Using the ESI source, we were able to detect the protonated ion \([M + H]^+\) for the folate analogs. Additionally, all spectra had ion clusters that indicated the pteridine ring is the main target for protonation and decomposition. The CID fragmentation often results in the cleavage between the para-amino benzoic acid and the glutamate moiety leading to the formation of product ions such as, \(m/z\) 295.10 \((C_{14}H_{13}N_6O^+\)) in FA, \(m/z\) 297.10 \((C_{14}H_{13}N_6O^+\)) in DHF, \(m/z\) 299.15 \((C_{14}H_{13}N_6O^+\)) in THF, and \(m/z\) 313.04 in 5MTHF, which were already described by Striegel et al.33 The daughter ion at \(m/z\) 335.20 \((C_{17}H_{15}N_6O^+\)) in ML, as well as the product ion at \(m/z\) 310.14 \((C_{15}H_{16}N_6O^+\)) in MN and the product ion at \(m/z\) 327.15 \((C_{15}H_{16}N_6O^+\)) in FO, belongs to the same ion series (Figures 2 and 3).

Using ESI-CID, folic acid also decomposes into two fragments at \(m/z\) 176.05 \((C_7H_6N_5O^+)\) and the ion at \(m/z\) 120.06 \((C_7H_6NO^+)\), which

### FIGURE 1
Extracted ion chromatograms (EIC) of folate standards at concentration 100 pmol/L analyzed by ESI-MS. [Colour figure can be viewed at wileyonlinelibrary.com]

### TABLE 1
Limits of detection and quantification and calibration curves of folate analogs using ESI-MS/MS

| ESI-MS/MS | Compound | \(m/z\) | \(t_R\) (min) | LOD (nmol) | LOQ (nmol) | Slope \(\times 10^5\) | Intercept \(\times 10^5\) | \(R^2\) |
|-----------|----------|--------|---------------|------------|------------|----------------|----------------|--------|
| Folic acid  | 442.226  | 6.77   | 0.010         | 0.033      | 6.32       | 1.60           | 0.91          |
| DHF       | 444.282  | 6.50   | 0.004         | 0.013      | 1.20       | 3.20           | 0.99          |
| THF       | 446.388  | 6.52   | 0.002         | 0.006      | 9.61       | 5.68           | 0.99          |
| 5MTHF     | 460.563  | 6.00   | 0.002         | 0.006      | 6.98       | 9.75           | 0.93          |
| ML        | 458.178  | 6.21   | 3.62          | 12.05      | 4.02       | 5.72           | 0.99          |
| MN        | 456.460  | 6.23   | 3.78          | 12.58      | 3.12       | 1.16           | 0.91          |
| FO        | 474.213  | 6.95   | 0.002         | 0.006      | 1.30       | 6.35           | 0.91          |

### TABLE 2
Limits of detection and quantification and calibration curves of folate analogs using APCI-MS

| APCI-MS | Compound | \(m/z\) | \(t_R\) (min) | LOD (nmol) | LOQ (nmol) | Slope \(\times 10^5\) | Intercept \(\times 10^5\) | \(R^2\) |
|---------|----------|--------|---------------|------------|------------|----------------|----------------|--------|
| Folic acid  | 442.226  | 6.77   | 17.90         | 59.60      | 2.60       | 1.40           | 0.98          |
| DHF       | 444.282  | 6.51   | 2.68          | 8.92       | 9.19       | 2.99           | 0.97          |
| THF       | 446.388  | 6.54   | 8.34          | 27.77      | 9.76       | 4.00           | 0.98          |
| 5MTHF     | 460.563  | 6.01   | 60.46         | 201.33     | 3.52       | 4.65           | 0.97          |
| ML        | 458.178  | 6.21   | 2.92          | 9.72       | 7.95       | 1.06           | 0.99          |
| MN        | 456.460  | 6.23   | 28.72         | 95.63      | 5.26       | 3.41           | 0.93          |
| FO        | 474.213  | 6.94   | 2.28          | 7.59       | 1.51       | 1.22           | 0.99          |
correspond to the pteridinic ring and the para- amino benzoic acid moiety, while the ion at m/z 267.11 is formed due to the loss of CHO from the product ion at m/z 295.10 as already reported by Hignite et al.36 (Figure 2A).

The spectrum of DHF is characterized by major product ions with a mass shift of +2 Da compared with FA, as exemplified by the ion at m/z 295.10. Upon fragmentation of DHF, several other daughter ions have been detected, such as, the ion at m/z 402.87

| Table 3 | Intraday and interday precision (%RSD) and recovery using ESI or APCI interface |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Ion Source | tR (min) ± SD (%RSD) | Ng ± SD (%RSD) | tR (min) ± SD (%RSD) | Ng ± SD (%RSD) | Recovery |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ESI | 6.77 ± 0.01 (0.14) | 20.42 ± 1.28 (6.26) | 6.77 ± 0.01 (0.12) | 20.66 ± 1.72 (8.32) | 89.47% |
| APCI | 6.77 ± 0.01 (0.1) | 21.76 ± 2.5 (11.5) | 6.77 ± 0.01 (0.18) | 19.7 ± 2.22 (11.29) | 84.93% |

FIGURE 2 Comparison of ESI-MS/MS vs APCI-MS. (A, B, C, D) Tandem mass spectra of FA, DHF, THF, and 5MTHF upon CID. (E, F, G, H) Full scan run of FA, DHF, THF, and 5MTHF ionized by APCI [Colour figure can be viewed at wileyonlinelibrary.com]
(C$_{18}$H$_{20}$N$_{5}$O$_{6}$)$^+$, which has been assigned to the full para-amino benzoic acid and the glutamate moiety with a cleavage at the pteridinic ring (Figure 2B).

Fragmentation of THF resulted in the formation of product ions at m/z 398.02 (C$_{19}$H$_{24}$N$_{7}$O$_{3}$)$^+$ and ion at m/z 342.02 (C$_{16}$H$_{20}$N$_{7}$O$_{2}$)$^+$, which can occur after an initial loss of ~48 Da and further cleavage of the glutamate moiety. The ion at m/z 206.88 (C$_{11}$H$_{12}$NO$_{3}$)$^+$ is proposed to be formed by cleavage between the pteridinic ring and the para-amino benzoic acid moiety, while the ion at m/z 165.00 (C$_{7}$H$_{9}$N$_{4}$O$^+$) is formed by an additional cleavage at the pteridinic ring, which leads to the opening of the ring structure. At the end, the ion at m/z 103.22 (C$_{4}$H$_{7}$O$_{3}$)$^+$ was tentatively assigned as a derivative of the glutamate moiety (Figure 2C).

The product ion spectra of 5MTHF is composed of ions at m/z 378.21 (C$_{18}$H$_{24}$N$_{3}$O$_{6}$)$^+$ and at m/z 177.07. Both belong to a specific ion series of the tetrahydrofolate containing molecules. Although we were not able to assign a structure to the ion at m/z 337.23, we propose the elemental composition for this ion as C$_{16}$H$_{21}$N$_{2}$O$_{5}$ (Figure 2D).

Product ion spectra of ML and MN were more difficult to interpret but nevertheless showed characteristic fragments. Product ion at m/z 417.30 (C$_{20}$H$_{25}$N$_{4}$O$_{6}$)$^+$ and ion at m/z 376.23 (C$_{18}$H$_{22}$N$_{2}$O$_{6}$)$^+$ were found in ML, whereas the fragmentation of MN led to the formation of ions at m/z 416.06 (C$_{19}$H$_{22}$N$_{5}$O$_{6}$)$^+$, and an ion at m/z 163.13 (C$_{9}$H$_{11}$N$_{2}$O$^+$). Both product ion spectra had fewer fragments compared with the other modified folic acid analogs. The presence of the imidazole-like ring in ML and MN, likely led to the formation of these ions being more resistant to decomposition (Figure 3A,B).

Upon ESI-CID, FO showed three main fragments: the ion at m/z 456.22 formed by water loss from the precursor ion, whereas the ion at m/z 178.13 (C$_{7}$H$_{8}$N$_{5}$O$^+$) formed by the loss of CO from the ion at m/z 327.15. The daughter ion at m/z 208.11 (C$_{8}$H$_{10}$N$_{5}$O$_{2}$)$^+$ was assigned to the formylated pteridinic ring (Figure 3C).

The APCI-MS analysis resulted in a more complicated spectrum due to the fragmentation caused by the collision of the analyte with the multitude of ions formed at the corona discharge needle. APCI ionization resulted in the formation of specific ions that were detected in all MS spectra, such as the ion at m/z 178.13 (C$_{7}$H$_{8}$N$_{5}$O$^+$), which was assigned to the pteridinic ring, and the ion at m/z 257.19 (C$_{13}$H$_{15}$N$_{2}$O$_{5}$)$^+$ was formed by decarbonylation of the ion at m/z 295.20 as already described by Holcapek et al. (Figures 2 and 3).
The APCI ionization of FA results in the ion at m/z 424.29 formed by water loss from the molecular ion. The ion at m/z 380.28 (C_{16}H_{16}N_{7}O_{3}) was formed by fragmentation of the glutamate moiety (Figure 2E). Upon APCI, DHF formed the ion at m/z 426.15 due to water loss and the ion at m/z 416.94 by loss of 28 Da from the protonated ion. We also detected the ion at m/z 338.44, and we tentatively assigned the elemental composition of C_{16}H_{15}N_{7}O_{3}⁴⁻ (Figure 2F).

THF ionization with APCI led to the formation of two main adducts. The first at m/z 502.35 corresponded to the hydrated potassium ion [MH + H₂O + K]⁺, and the second ion at m/z 484.33 corresponded to the potassium adduct. Based on the fragmentation spectra reported by Holcapek et al, we assigned the ion at m/z 373.27 (C_{17}H_{28}N_{7}O_{3}) as para-amino benzoic acid and glutamate moiety with several cleavages at the pteridinic ring (Figure 2G).

The APCI-MS spectra of SMTHF exhibited fewer fragments. Among them, we were able to assign the ion at m/z 460.39 as protonated SMTHF. The ion at m/z 442.38 was formed by water loss from the protonated ion, whereas the ion at m/z 164.14 (C_{6}H_{6}N_{5}O⁺) was assigned to a pteridinic ring derived fragment (Figure 2H).

ML and MN ionization by APCI resulted in very similar MS spectra. However, we found some specific fragments, which were used to differentiate the two species. The ion at m/z 341.28 (C_{17}H_{28}N_{7}O_{2}⁴⁻) was found during the analysis of ML, while the ion at m/z 343.27 (C_{17}H_{28}N_{7}O_{3}⁴⁻) was detected in the MN spectrum (Figure 3D,E).

The ionization of FO using APCI was already reported by Holcapek.³⁹ We confirmed the formation of the protonated adduct at m/z 474.35, as well as the ion at m/z 456.34 formed by water loss. Moreover, we detected the ion at m/z 412.32, which formed via the decarboxylation from the [M + H]⁺ ion. We additionally detected the ion at m/z 327.25 (C_{15}H_{28}N_{7}O_{3}⁴⁻), which corresponded to the loss of the glutamate moiety (Figure 3F).

### 3.1.2 In vivo quantification of the folate cycle intermediates

The ESI-MS/MS and the APCI-MS methods were used to evaluate the changes in folate pool sizes induced by RNAI against *dhfr*-1 in the worm. The ESI-MS/MS was initially used because of the higher sensitivity compared with the APCI-based method. The relative levels of each intermediate were normalized to the control RNAI (luciferase) and were plotted in Figure 4. As expected, *dhfr*-1 RNAI resulted in an increase of FA of about three-fold and a decrease of 5MTHF, ML, MN, and FO of about half compared with the control. Additionally, we observed a complete depletion of the THF pool upon *dhfr*-1 treatment. Furthermore, we were not able to detect DHF in the worm using this method.

We also attempted to quantify folates using APCI-MS in the worm (Supplement S5). Only few intermediates were observed due to the higher LOQ of the method. Nevertheless, the concentrations of FA and FO were comparable with the ESI-based method. In contrast to ESI, however, using the APCI-MS method, we were able to quantify DHF in the worm matrix.

### 3.1.3 In vitro oxidation and relative quantification of new potential FA intermediates

The question arises as to whether other FA derivatives can be found in vivo. In particular, oxidation products of biomolecules can often have bio-reactivity. To test this idea, we performed oxidation experiments with the FA standard, assessed resulting products, and then asked whether such products could also be found in worm extracts.

The in vitro oxidation of FA using the Fenton reaction revealed the presence of a single product at m/z 208.04652 (Supplement S6 A). Due to the high mass accuracy of the instrument (below 5 ppm), we were able to assess the elemental composition of the species as C_{12}H_{24}N_{3}O_{3}. The tandem mass spectra of this compound was characterized by the presence of the product ion at m/z 180.05 and product ion at m/z 164.06, which were formed by the loss of CO and by decarboxylation from the precursor ion, respectively. Moreover, the daughter ions at m/z 138.05 and m/z 122.04 were formed via fragmentation of the pteridinic ring (Figure 5A). The structure of this compound belongs to 6-carboxypterine, a derivative from folic acid.⁴⁰⁻⁴²

We further investigated the presence of this compound in our standard mixture, and we were not able to detect any peak, which corresponded to 6-carboxypterine (Supplement S6 B). We asked if this specific oxidative product was also present in vivo and therefore analyzed the worm samples with and without RNAI treatment. Interestingly, we detected 6-carboxypterine in the worm extracts and found that it increased by three-fold to four-fold upon knock down of *dhfr*-1 (Figure 5B).

### 4 DISCUSSION

In this work, we developed two methods for simultaneous quantification of FA analogs in the nematode *C. elegans* using a triple stage quadrupole. The quantification of the folate pool in biological samples remains a challenge due to the complexity of the sample matrix. Work of Chen et al required a solid phase extraction (SPE) step using cyclohexyl-based chemistry cartridges for the enrichment of FA derivatives in cultured human cells.⁴³ A study from Pawlosky et al used C18 Sep-Pak cartridges,⁴⁴ whereas Nandam et al used strong anion exchange cartridges for the enrichment in human whole blood.⁴⁵ Because of the simple matrix and tissue composition of *C. elegans*, no SPE or enrichment steps were required for the extraction of the six intermediates. Our methods resulted in lower LOD and LOQ for most of the FA species, and it can be used not only for the analysis of FA in the round worm, but it can be potentially translated for the analysis of folates in other organism models and human tissues.

The selection of unique and intense fragment ions in the ESI-MS/MS method resulted in a better LOQ ranging from 6 pmol to 12 nmol, which is 20 fold lower compared with previous published data.³¹,³⁵ The ESI-based method was comparable with that of Striegel et al;
however, we were able to detect four more analogs of FA. The new APCI-MS approach was less sensitive when compared with the ESI-MS/MS, but the LOQs for most of the intermediates were comparable to the work of Chen et al. Nevertheless using the APCI-based method, we retrieved the concentration of DHF, which was not possible using the ESI ion source. We speculate that APCI analyses can be less susceptible to matrix effects (including ion suppression) when compared with ESI, which may explain the better ionization of DHF. As reported by Nandania et al, DHF quantification was also challenging using whole blood samples with ESI.

APCI ionization allowed the detection of new FA-dependent product ions, including ions at m/z 178.13 and m/z 267.19. Moreover, we detected two unique fragments at m/z 341.28 and m/z 343.27, which help to distinguish between ML and MN species, and hence can provide valuable structural information. Structural information-tandem MS is currently used to identify specific product ions of selected analytes and helps the structure elucidation process for analogs and isomers. In this work, we reported a multitude of novel fragments, which derive from the opening of the pteridinic ring as well as the decarboxylation and cleavage of the glutamate moiety. As already shown by Backer et al, the functional groups of the pteridine ring as well as the glutamate side chain seem to have great potential to develop folate agents to treat cancer and FA deficiency. The chemical structure knowledge from this study can be combined with high throughput property assays for physicochemical properties in order to rapidly retrieve the structure-property relationships of new FA analogs.

FA and its derivatives have already been detected and quantified in the nematode using a variety of MS-based approaches and different volatile ion-pairing agents, such as, N,N-dimethylhexylamine (DMHA). Researchers have reported on the dietary role of FA using supplementation experiments or the contributions of the microbiome.
to FA metabolism and host physiology. For example, Cabreiro et al used ESI-MS to quantify the total folate pool in the *C. elegans/E. coli* system upon treatment with metformin. Additional research from Virk et al reported FA cycle dysregulation in *C. elegans* upon FA supplementation. Despite these findings, only few works have reported on changes in the folate pool upon host genetic intervention. The present quantification of FA analogs in the worm upon *dhfr*1i treatment indicates that the single knockdown of *dhfr*1 is sufficient to impair the whole FA cycle in the nematode, which is expected by targeting the first enzyme of the catabolic pathway. These results are in good agreement with those reported by Ortbauer et al, which indicate that the *dhfr*1i dysregulates the immediate FA cycle, but also perturbs the expression of other enzymes, such as methionine synthase, which is part of the network involved in the transfer of methyl groups.

Further studies should highlight the role of *dhfr*1 not only in the one carbon pool by folate but also investigate the cross talk between folate-dependent purine nucleotide biosynthesis, methionine synthesis, and NAPH production. DHFR-1 is also a pharmacological target of folic acid analogs, which are currently used against cancer and rheumatoid arthritis. We believe that such investigations on the alteration of folates by RNAi and genetic intervention on FA enzymes will allow a better understanding of the physiological role of the FA cycle and will enable the identification of new potential targets against cancer.

Finally, we took advantage of *C. elegans*’ simplicity for the discovery of new endogenous oxidized derivatives and/or side products of FA, and we succeeded in quantifying the levels of 6-carboxypterine in the nematode. The formation mechanism of this compound is currently under debate. Works from Maynard et al as well as Dantola et al suggested that 6-carboxypterine is formed by an autoxidation reaction caused by poor storage conditions and is found as an impurity in commercially available FA. On the other hand, Orsomando et al reported 6-carboxypterine as a part of a new biological process involved in the folate-salvage pathway. From an initial breakage of FA, dihydropetidin-6-carbaldehyde can be formed and further reduced to form pteridin-6-carbaldehyde. This last compound can be formed via an oxidation reaction of the 6-carboxypterine (Figure 5C).

In the present work, we were not able to detect the 6-carboxypterine in the folic acid standard solution, suggesting it is not an inherent impurity. Furthermore, we quantified its concentration in the nematode and observed increased levels upon *dhfr*1i knockdown. Taken together these observations, we suggest a possible endogenous origin of 6-carboxypterine. The combination of newly targeted methods with biochemical assays might help to elucidate the biological activity of the 6-carboxypterine and potential sources of synthesis of these compounds.

5 | CONCLUSION

We developed two complementary methods for the simultaneous quantitation in vivo of FA analogs in the nematode *C. elegans*. We suggest that these results not only will help to further elucidate the folate cycle in worms but could also be used to describe fragmentation patterns of analogs or FA-derived molecules in other systems. Further studies may shed light on possible new FA derivatives and expand their biological roles.
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