Food Chemistry Advances 10 (2026) 101231

Contents lists available at ScienceDirect

Food Chemistry Advances

journal homepage: www.elsevier.com/locate/focha

Advanced LC-MS/MS methodologies for the analysis of neuroactive compounds in tomato-based diets: Insights from 2D In Vitro digestion models

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ARTICLE INFO

Keywords:
Tomato
Target analysis
Neuroactive compound
Digestion
Fecal fermentation
Bioaccessibility

ABSTRACT

The digestive fate of neuroactive food compounds remains poorly understood despite growing interest in their role in brain health. This study developed advanced analytical methods to identify neuroprotective and neuro-disrupting compounds in tomatoes before and after digestion and following fecal fermentation. Using in vitro digestion and fermentation models, we characterized compound behavior during digestion. Most compounds were quantified in undigested tomatoes. Digestion revealed diverse outcomes: some compounds transformed (e.g., chlorogenic acids), others produced (e.g., linoleic acid), and several remained stable with potential to reach the colon (e.g., serotonin, rutin). Notably, neuro-disrupting compounds like bisphenols remained largely unaltered and bioaccessible in the colon. Fecal fermentation further showed dynamic changes, with some compounds decreasing (e.g., rutin) and others increasing (e.g., quercetin). These findings provide both methodological advances and mechanistic insights into neuroactive compound behavior during digestion, informing future research on gut-mediated dietary effects in the context of neurodegenerative disease prevention.

1. Introduction

Neurodegenerative diseases (NDs) are progressive disorders affecting millions globally, marked by neuronal loss and cognitive decline (Ceppa et al., 2020; Gao et al., 2025). While no definitive cures exist, growing evidence suggests that dietary components may influence their onset and progression (Lu et al., 2024). The gut microbiota, significantly influenced by dietary components, plays a critical role in regulating health, including neurochemical pathways and the central nervous system, positioning it as a promising therapeutic target (de Carvalho et al., 2021).

Tomato is one of the most widely produced vegetables worldwide, and its availability, taste, variety of products, low price, and health benefits make it a popular and widely consumed food among both adults and children, as well as a staple in many cultures and countries (Salehi et al., 2019). Tomatoes, rich in bioactive compounds such as phenolic compounds, carotenoids, and glycoalkaloids, have demonstrated potential neuroprotective properties (Shah et al., 2020). These compounds reduce oxidative stress and may offer prophylactic and therapeutic benefits for NDs (Guan et al., 2021). Nevertheless, the health benefits provided by dietary bioactive compounds depends on their bioavailability, as digestion and metabolism within the gastrointestinal tract and utilization by the gut microbiota significantly influence their effects (M. C. Coelho et al., 2021). Additionally, tomato-based products may carry neuro-disrupting contaminants like pesticides and bisphenols, raising safety concerns (Aloizou et al., 2020; Denuzière & Ghersi-Egea, 2022).

To evaluate both beneficial and harmful compounds, in vitro digestion and colonic fermentation models provide valuable insight into how digestion conditions affect compound release, transformation, and fate (Veintimilla-Gozalbo et al., 2021). While studies have explored neuroprotective compounds, they often focus on isolated compounds or specific groups, neglecting the complex interactions between neuroprotective and neuro-disrupting substances (Cárdenas-Castro et al., 2021a; Wu et al., 2023). Furthermore, existing studies frequently analyze in vitro digestion or fecal fermentation in isolation, despite their interdependence in shaping bioactive compound bioavailability and conversion once digested and fermented in colon (Reboredo-Rodríguez et al., 2021). Current knowledge of in vitro gastrointestinal digestion of

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https://doi.org/10.1016/j.focha.2026.101231
Received 16 November 2025; Received in revised form 5 January 2026; Accepted 20 January 2026
Available online 20 January 2026

tomatoes remains limited, as most studies have focused on carotenoid bioaccessibility, particularly lycopene, or on total polyphenolic content (Cárdenas-Castro et al., 2021a).

A key challenge is the lack of robust analytical methods to simultaneously quantify structurally diverse neuroactive compounds across complex food and biological matrices (Valdés et al., 2022). Addressing this gap is essential to better understand exposure, transformation, and potential biological activity of such compounds throughout digestion.

In this study, we developed and applied targeted LC-MS/MS methods to quantify neuroactive compounds, including neurotransmitters, phenolics, amino acids, vitamins, carotenoids, and contaminants, in tomato extracts before and after in vitro digestion and fecal fermentation. Using optimized LC-MS/MS analysis on samples from in vitro digestion and fecal models as a proof-of-concept approach, we provided a comprehensive assessment of compound behavior throughout the digestive process. By identifying both neuroprotective and neuro-disrupting substances, the findings offer valuable insights into the digestive fate of neuroactive food components and support the development of dietary strategies relevant to brain health. The methods established in this study contribute to the advancement of food analysis and nutrition research.

2. Materials and methods

2.1. Chemicals and standard preparation

General information about the investigated 36 neuroactive compounds (food additives, industrial chemicals, pesticides, amino acids, fatty acids, neurotransmitters, organic acids, phenolic compounds, and vitamins) and 5 carotenoid is presented in the Supplementary Material (SM, Table SM 1). All analytical standards, purity > 95 %, were purchased from Sigma-Aldrich or TransMIT and Dr Ehrenstorfer GmbH (Darmstadt, Germany). Isotopically labelled surrogate standards: carbamazepine (carbamazepine-d_{10}, 98 %) was purchased from Sigma-Aldrich (Steinheim, Germany) and bisphenol S ($^{13}C_{12}$-BPS) from CanSyn Chem. Corp. (Toronto, Canada). All analytical grade solvents were obtained from Sigma-Aldrich. Formic acid (HCOOH, > 98.0 %) and ammonium formate (NH_4CHO_2, > 99.0 %) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Ultrapure water (18.0 MΩ cm at 25 °C) was prepared using the Milli-Q water purification system. Enzymes for in vitro digestion, inulin, and chemicals for the batch culture basal nutrient medium were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Fischer (Waltham, MA, USA).

Stock solutions (≈ 1.0 – 10.0 mg/mL) of 36 neuroactive compounds were prepared by dissolving each compound in methanol or combination of methanol/water, and working standards (0.01, 0.1 and 1 μg/mL) were prepared by appropriate serial dilutions of the stock solutions. Similarly, an internal standard solution (1.0 μg/mL) was prepared from a solution (1.0 mg/mL) of isotopically labelled compounds (carbamazepine-d_{10} and $^{13}C_{12}$-Bisphenol S). All standards were prepared in methanol and stored in amber glass vials at −20 °C. Stock solutions of the 5 carotenoid standards were prepared in ethanol and stored at −80 °C. Samples were filtered through 0.2 or 0.45 μm regenerated cellulose membrane syringe filters (Phenomenex, Torrance, USA).

Fecal donor: The main aim of the experiment was to apply an analytical method and to demonstrate its applicability for studying the fate of specific compounds previously added to tomato, using an in vitro digestion model. Therefore, only one healthy volunteer was included as the fecal donor for the in vitro fermentation experiment. The donor had not received antibiotic treatment within three months prior to stool collection, had no history of bowel disorders, and had not consumed prebiotic or probiotic supplements before the experiment. The donor was fully informed about the aims and procedures of the study and provided written consent (August 2023) for the use of his/her fecal material in the experiments, in compliance with the ethical procedures required by Fondazione Edmund Mach and the APSS (Azienda Provinciale per i Servizi Sanitari), Trento (TN), Italy. No metagenomic or personal biological data were collected or analyzed. The privacy rights of the donor were fully protected, and no identifying personal information has been disclosed.

2.2. Sample preparation

2.2.1. Tomato prior and after digestion

Tomato extracts from fresh tomato or digested tomato pellet were, after lyophilization, homogenized and stored in desiccators before further steps. For determination of neuroactive compounds, 0.1 g of lyophilized and homogenized tomato sample was weighed into a 15 mL centrifuge tube with the addition of isotope-labelled internal standard mixture (ISTD = $^{13}C_{12}$-BPS, d_{10}-carbamazepine, V = 50 μL; c = 1 μg mL^{-1}). Methanol (2 mL) was added, and the sample was vortexed (30 s) and shaken (10 min). The mixture was sonicated (15 min, T_r) and centrifuged at 5000 rpm for 10 min. The supernatant was transferred to a 10 mL glass vial, and the residue was again suspended in 2 mL of methanol, and further vortexed, shaken, sonicated and centrifuged. The solvent from the combined extracts was removed using N_2 (40 °C). Once dry, the extracts were reconstituted in methanol:ultrapure water (0.4 mL, 0.1:0.9), filtered through 0.2 μm regenerated cellulose membrane filters, and then diluted 0, 100, or 1000 times depending on the saturation of some of the compounds before analysis.

Carotenoids required different sample preparation which was modified based on our previous study (https://www.mdpi.com/2304-8158/14/22/3927). Briefly, on the day of sample preparation 0.1 g of lyophilized tomato or digested pellet was inserted into 15 mL centrifuge tube, and 10 μL ISTD mixture (5 μg/ml), at final conc. 0.1 ppm, was added. Extraction was performed with 5.0 mL mixture methanol/acetone/hexane, 25/25/50 %, v/v/v, vortexed and mixed on an orbital shaker for 10 min. Next, samples were ultrasonicated for 5 min at 10 °C and 59 KHz, centrifuge (10 min; 1800 g; 4 °C), and the organic layer was transferred to a fresh 15 mL flasks. The extraction steps were repeated two times more and finally the organic extracts were combined and dried under nitrogen at $T \leq 35$ °C. The residues were saponified overnight at room temperature, using 4 mL of a solution of 15 % KOH in methanol (w/v). Following the saponification, 3.5 mL of NaCl solution (9 %, w/v), and 4.0 mL of hexane:diethyl ether (3:1, v/v) was added to the samples, mixed on orbital shaker for 10 min, vortexed and centrifuged (5 min; 1800 g; 4 °C). This step was repeated twice more by adding organic phase. The organic extracts were again combined and dried under nitrogen at $T \leq 35$ °C. The dried extracts were reconstituted in 0.4 mL MTBE/ethanol =1/2 with internal standard mixture, filtered through 0.22 μm RC membranes and analyzed.

2.2.2. Fecal solution

Samples were defrizzed at +4° C during night in dark, centrifuged (13,000 rpm for 10 min at +4° C) and filtered (0.45 μm regenerated cellulose membrane filters). In next step, different sample preparation was tested: a) direct analysis of sample b) direct analysis after 5 times sample dilution with ultrapure water, c) direct analysis after 5 times sample dilution with ultrapure water and added antioxidant, d) direct analysis after 5 times sample dilution with methanol and, e) solid phase extraction (Oasis Prime HLB 96-well plate SPE cartridges, 30 mg sorbent). The optimal sample preparation method was selected, and 40 μL of fecal sample was diluted 5× with methanol. The internal standard mixture (30 μL, 1 μg mL^{-1}) was added to achieve a final concentration of 0.05 μg mL^{-1}. Throughout the sample preparation process, samples were maintained at +4 °C.

2.3. Instrumental analysis

2.3.1. LC–MS/MS analysis of neuroactive compounds in tomato and fecal samples

Samples were analyzed using an UHPLC-Qtrap, an Exion LC system provided by AB Sciex LLC (Framingham, MA, USA) coupled to a linear

Table 1
Retention times (RT) and MS/MS optimized compound-dependent parameters of investigated neuroactive compounds, including quantification (Q) and qualification (q) MRM transitions, declustering potential (DP), collision energy (CE), collision exit potential (CXP), retention times and MS/MS optimized conditions.

Analyte	Ionization mode	Transition type	RT [min]	Precursor ion	Product ion	DP	EP	CE	CXE
Method for determination of 36 neuroactive compounds									
Glutamine	$[M+H]^+$	Q	0.85	147.0	130.2	40	3	13	7
Glutamine	$[M+H]^+$	q	0.85	147.0	84.2	40	3	21	7
Glutamic acid	$[M+H]^+$	Q	0.88	148.0	130.1	24	10	14	16
Glutamic acid	$[M+H]^+$	q	0.88	148.0	120.2	24	10	20	14
Guanine	$[M+H]^+$	Q	1.36	152.0	135.1	60	8	27	16
Guanine	$[M+H]^+$	q	1.36	152.0	110.1	60	8	28	13
L-Dopa	$[M+H]^+$	Q	1.6	198.0	152.0	50	10	20	10
L-Dopa	$[M+H]^+$	q	1.6	198.0	181.0	50	10	15	10
Tyrosine	$[M+H]^+$	Q	2.08	182.1	136.0	60	10	20	10
Tyrosine	$[M+H]^+$	q	2.08	182.1	165.2	60	10	20	10
Tyramine	$[M+H]^+$	Q	2.19	137.1	92.1	50	10	28	14
Tyramine	$[M+H]^+$	q	2.19	137.1	120.1	50	10	12	12
Tyramine	$[M+H]^+$	q	2.19	137.1	137.1	50	10	46	10
Serotonin	$[M+H]^+$	Q	3.31	177.1	160.2	40	10	14	13
Serotonin	$[M+H]^+$	q	3.31	177.1	115.0	40	10	37	17
Phenylalanine	$[M+H]^+$	Q	4.06	166.1	120.1	40	10	19	10
Phenylalanine	$[M+H]^+$	q	4.06	166.1	103.1	40	10	36	12
Acetaminophen	$[M+H]^+$	Q	4.59	152.1	110.0	40	13	21	13
Acetaminophen	$[M+H]^+$	q	4.59	152.1	93.0	40	13	31	10
Pantothenic Acid	$[M+H]^+$	Q	4.72	220.1	90.0	25	10	18	10
Pantothenic Acid	$[M+H]^+$	q	4.72	220.1	202.1	25	10	17	11
Tryptophan	$[M+H]^+$	Q	5.34	205	188.1	35	10	14	10
Tryptophan	$[M+H]^+$	q	5.34	205	145.9	35	10	24	8
Aspartame	$[M+H]^+$	Q	7.31	295.1	120.2	50	14	34	10
Aspartame	$[M+H]^+$	q	7.31	295.1	235.1	50	14	20	27
Naringin	$[M+H]^+$	Q	8.9	581.3	273.4	80	10	25	10
Naringin	$[M+H]^+$	q	8.9	581.3	419.3	80	10	20	10
Naringin	$[M+H]^+$	q	8.9	581.3	581.3	80	10	10	10
Dihydrokaempferol	$[M+H]^+$	Q	9	289.1	243.1	40	9	19	15
Dihydrokaempferol	$[M+H]^+$	q	9	289.1	215.1	40	9	32	18
10d-Carbamazepine	$[M+H]^+$	Q	10.96	247.2	204.2	80	10	27	20
10d-Carbamazepine	$[M+H]^+$	q	10.96	247.2	187.1	80	10	52	22
Tomatine	$[M+H]^+$	Q	11.08	1034.6	1016.5	100	14	77	50
Tomatine	$[M+H]^+$	q	11.08	1034.6	416.1	100	14	50	30
Difenoconazole	$[M+H]^+$	Q	14.45	406.1	251.1	60	10	33	15
Difenoconazole	$[M+H]^+$	q	14.45	406.1	337.1	60	10	23	20
Difenoconazole	$[M+H]^+$	q	14.45	406.1	188.1	60	10	58	12
Quinic acid	$[M-H]^-$	Q	0.96	191.0	172.8	−30	−11	−21	−20
Quinic acid	$[M-H]^-$	q	0.96	191.0	93.0	−30	−11	−31	−11
Citric acid	$[M-H]^-$	Q	1.63	191.0	111.0	−30	−8	−17	−13
Citric acid	$[M-H]^-$	q	1.63	191.0	86.9	−30	−8	−20	−13
4-pyridoxic acid	$[M-H]^-$	Q	3.27	182.0	137.9	−36	−9	−6	−16
4-pyridoxic acid	$[M-H]^-$	q	3.27	182.0	92.1	−36	−9	−26	−11
Neochlorogenic Acid	$[M-H]^-$	Q	5	353.1	191.0	−23	−12	−38	−17
Neochlorogenic Acid	$[M-H]^-$	q	5	353.1	85.0	−23	−12	−48	−10
Chlorogenic Acid	$[M-H]^-$	Q	6.2	353.1	191.0	−23	−12	−38	−17
Chlorogenic Acid	$[M-H]^-$	q	6.2	353.1	85.0	−23	−12	−48	−10
Crypto-Chlorogenic Acid	$[M-H]^-$	Q	6.36	353.1	191.0	−23	−12	−38	−17
Crypto-Chlorogenic Acid	$[M-H]^-$	q	6.36	353.1	85.0	−23	−12	−48	−10
Caffeic acid	$[M-H]^-$	Q	6.8	179.0	134.9	−27	−10	−20	−18
Caffeic acid	$[M-H]^-$	q	6.8	179.0	106.8	−27	−10	−29	−14
Caffeic aldehyde	$[M-H]^-$	Q	7.31	163.0	134.9	−48	−8	−23	−16
Caffeic aldehyde	$[M-H]^-$	q	7.31	163.0	106.7	−48	−8	−24	−17
p-Coumaric acid	$[M-H]^-$	Q	7.96	163.0	118.9	−21	−8	−23	−20
p-Coumaric acid	$[M-H]^-$	q	7.96	163.0	92.9	−21	−8	−40	−11
Bisphenol S	$[M-H]^-$	Q	8.41	249.0	108	−70	−8	−36	−10
Bisphenol S	$[M-H]^-$	q	8.41	249.2	155.8	−70	−8	−28	−10
13C12BPS	$[M-H]^-$	Q	8.41	261.1	113.9	−70	−8	−36	−13
13C12BPS	$[M-H]^-$	q	8.41	261.1	98	−70	−8	−40	−10
m-Coumaric acid	$[M-H]^-$	Q	8.59	163.0	118.9	−21	−8	−23	−20
m-Coumaric acid	$[M-H]^-$	q	8.59	163.0	92.9	−21	−8	−40	−11
Rutin	$[M-H]^-$	Q	8.97	609.1	300.0	−100	−10	−51	−36
Rutin	$[M-H]^-$	q	8.97	609.1	271.0	−100	−10	−69	−23
Resveratrol	$[M-H]^-$	Q	9.16	227.1	142.9	−40	−9	−37	−17
Resveratrol	$[M-H]^-$	q	9.16	227.1	184.8	−40	−9	−26	−32
o-Coumaric acid	$[M-H]^-$	Q	9.21	163.0	118.9	−21	−8	−23	−20
o-Coumaric acid	$[M-H]^-$	q	9.21	163.0	92.9	−21	−8	−40	−11
Eriodyctiol	$[M-H]^-$	Q	9.87	287.1	150.9	−41	−10	−19	−18
Eriodyctiol	$[M-H]^-$	q	9.87	287.1	134.9	−41	−10	−33	−17
Quercetin	$[M-H]^-$	Q	10.6	301.0	150.9	−60	−10	−30	−8
Quercetin	$[M-H]^-$	q	10.6	301.0	178.8	−60	−10	−24	−9
Naringenin	$[M-H]^-$	Q	10.7	271.1	151.0	−50	−10	−26	−17

(*continued on next page*)

Table 1 (*continued*)

Analyte	Ionization mode	Transition type	RT [min]	Precursor ion	Product ion	DP	EP	CE	CXE
Method for determination of 36 neuroactive compounds									
Naringenin	[M-H]$^-$	q	10.7	271.1	118.9	−50	−10	−31	−14
Hesperitin	[M-H]$^-$	Q	11	301.0	164.0	−99	−9	−33	−20
Hesperitin	[M-H]$^-$	q	11	301.0	286.0	−99	−9	−24	−22
Bisphenol A	[M-H]$^-$	Q	11.72	227.1	212.1	−60	−10	−25	−30
Bisphenol A	[M-H]$^-$	q	11.72	227.1	133.1	−60	−10	−36	−15
Linoleic acid	[M-H]$^-$	Q	17.14	279.3	279.3	−80	−10	−10	−10
Method for determination of 5 carotenoids									
Lutein	$[M+H]^+$	Q	6.02	569.4	551.3	120	12	21	40
Lutein	$[M+H]^+$	q	6.02	569.4	477.3	120	12	22	40
Lutein	$[M+H]^+$	q	6.02	568.4	476.3	120	12	21	28
trans-β-Apo-8′-carotenal	$[M+H]^+$	Q	7.8	417.3	325.3	90	9	10	21
trans-β-Apo-8′-carotenal	$[M+H]^+$	q	7.8	417.3	293.3	90	9	21	16
Lycopene	$[M+H]^+$	Q	13	537.4	444.5	60	12	25	30
Lycopene	$[M+H]^+$	q	13	537.4	445.5	60	12	25	30
Lycopene	$[M+H]^+$	q	13	536.4	444.5	60	12	25	30
b-Carotene	$[M+H]^+$	Q	13.55	537.44	445.4	100	9	22	35
b-Carotene	$[M+H]^+$	q	13.55	536.4	444.5	100	9	20	28
Phytoene	$[M+H]^+$	Q	13.64	545.4	80.4	50	10	25	30
Phytoene	$[M+H]^+$	q	13.64	544.4	80.4	50	10	25	30

ion trap quadrupole, An AB Sciex LLC QTRAP 6500+ (Framingham, MA, USA). The separation of 36 neuroactive compounds was achieved on a Waters Acquity HSS T3 column 1.8 µm, 100 mm × 2.1 mm (Milford, MA, USA), kept at 40 °C. Mobile phase A was ultrapure water containing 0.1 % formic acid and 1 mM NH_4COOH and mobile phase B was methanol with 0.1 % formic acid and 1 mM NH_4COOH at a flow rate of 0.3 mL min^{-1}. The mobile phase gradient was as follows: 0 min, 70 % B; 0–2 min, 80 % B; 2–4 min, 85 % B; 4–7 min, 100 % B; 7.50 min; 100 % B; 7.50–11.50 min; 70 % B. 0 – 1.0 min at 5 % B, 1.0 – 3.0 min increase to 20 % B, 3.0 – 12.0 min increase to 80 % B, 12.0 – 16.0 min increase to 100 % B, 16.0 – 18.00 min hold 100 % B, 18.0 – 18.1 min decrease to 5 % B, and 18.10 – 20.00 min hold 5 % B. The injection volume of both sample extract (tomato and fecal solution) was 2 µL. After each injection, the needle was rinsed with 500 µL of weak wash solution (water/methanol, 90:10). Samples were kept at 15 °C during the analysis.

An AB Sciex LLC QTRAP 6500+ (Framingham, MA, USA) was operated in positive and negative scheduled ion multiple reaction monitoring (MRM) mode using a Turbo V ion source with the following settings: Curtain Gas (CR) 35 °C, Collision Gas (CAD) Medium, IonSpray Voltage (IS) 5500 V/−4500 V, Temperature 500 °C, Ion Source Gas 1 (GS1) 30 psi, and Ion Source Gas 2 (GS2) 40 psi. Each period was scheduled with 4000 cycles of 0.3 s cycle time each, with 120 s MRM detection window. Transitions were optimized for the compound-dependent parameters (scheduled MRM), including declustering potential (DP), collision energy (CE), collision exit potential (CXP) and entrance potential (EP). Retention times and the detailed settings for the MS/MS method are summarized in Table 1. MultiQuant and Analyst from AB Sciex LLC (Framingham, MA, USA) were used for data acquisition and elaboration, respectively.

The method for determination of 5 carotenoids was transferred from UHPLC-MS-DAD, published in our previous study (https://www.mdpi.com/2304-8158/14/22/3927), to AB Sciex LLC QTRAP 6500+ (Framingham, MA, USA). The MRM transitions and MS parameters were optimized for the four most abundant carotenoids, based on our previous results, with addition of trans-β-Apo-8-carotenal, known to be formed during carotenoid digestion (Table 1).

2.3.1.1. Method validation. Methods for the determination of 36 neuroactive compounds, selected based on our previous non-target analysis of different types of tomatoes (https://www.mdpi.com/2304-8158/14/22/3927), in tomato and fecal extracts, were validated for linearity, working range, stability, precision, limit of quantification (LOQ), and extraction recovery and matrix effect for tomato matrix following Eurachem guidelines (The Fitness for Purpose of Analytical Methods, 2014). Solvent blanks, midpoint quality control sample and pure standard were included after every 10th sample to evaluate potential carry-over and instrumental signal stability. Method validation was performed based on the working range of neuroactive compounds. For fecal samples, validation was conducted at 0.005/1 ppm (low level, LL) and 1/20 ppm (high level, HL). For tomato extracts, at least three concentration levels were tested: 0.4 µg/g dry weight (LL), 2.0 µg/g dry weight (medium level, ML), and 40 µg/g dry weight (HL). Additionally, due to the broad concentration range of compounds present in tomato samples, method validation was performed on differently diluted samples (1:1000, 1:100, and undiluted). To assess the sensitivity of the method, the LOQ was defined as the lowest calibration standard and at least five times the analyte signal of a blank sample. The method for determining neuroactive compounds in tomato (both prior to and after digestion) was developed and validated using the tomato matrix prior to digestion, due to the similarity between the matrices before and after digestion. Based on the compound content in the matrix, method validation was conducted using the more complex matrix (tomato prior to digestion). Appropriate dilution was applied to obtain reliable results and ensure that concentrations added before and after extraction were sufficiently large, preventing misleading extraction efficiency results. In the case of fecal samples, due to the high matrix effect and the observed instability of compounds in the fecal sample solution, method validation parameters were optimized according to the final sample preparation. A 5-fold dilution of fecal samples using methanol was applied. The calibration curve for fecal samples was matrix-matched, where the fecal sample solution, after dilution, was spiked with both native compounds (at calibration levels) and isotopically labeled compounds at the same concentration levels. Representative chromatograms of a calibration point from a standard solution mixture (tomato sample) and matrix-matched calibration points in fecal samples (1 ppm in the vial) are shown in Figure SM 1. A solvent blank, digested tomato, and fecal sample are shown in Figure SM 2, demonstrating chromatographic separation and signal specificity.

For the analysis of five carotenoids, linearity was tested and incorporated into the already validated sample preparation from our previous study where different instrumentation was used. Statistical analysis and data visualization were performed using R software (version 4.4.0).

2.4. Method application and study design

2.4.1. In vitro digestion

Plump tomato was obtained from local supermarkets Trento, Italy in August 2023 and washed with tap water. Tomato fruit was cut into pieces of approximately 2 x 2 x 2 cm and then frozen with nitrogen, grinded and lyophilized. Freeze-dried tomato paste was pooled into one

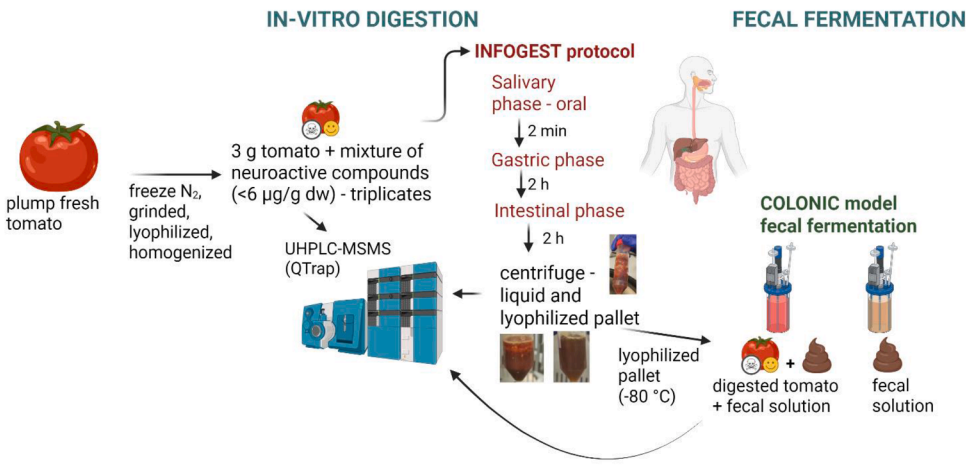

Fig. 1. Application of the Developed Method to an In Vitro Digestion and Fermentation Model – Study Design. Tomato extracts were analyzed prior to in vitro digestion, which was carried out following the INFOGEST protocol. Neuroactive compounds present at lower concentrations were mixed with lyophilized tomato before digestion. After digestion, separate samples were collected: the liquid phase and the undigested pellet. The undigested pellet was then included in the fecal fermentation model. In this model, one vessel contained the undigested tomato pellet, while another vessel contained only the fecal solution, without the addition of undigested tomato.

homogenized sample, packed in closed plastic bottles and stored in the presence of molecular sieves, at room temperature until further analysis (Fig. 1).

The *in vitro*-simulated gastrointestinal digestion was performed as described by Minekus et al. (2014) (Minekus et al., 2014) following a general standardised and practical static INFOGEST digestion method (Fig. 1). The protocol comprises three sequential steps: oral, gastric and small intestinal digestion. Initially, the tomato sample prior to digestion was analyzed to determine the concentration of 36 neuroactive compounds and 5 carotenoids. The experimental design included the digestion of tomato with the addition of neuroactive compounds (the existing amount of the compound + 30 µg/g dry weight) if their initial concentration was below 6 µg/g dry tomato before digestion. This adjustment ensured the ability to trace the fate of compounds present at lower concentrations. Each condition was performed in three replicates. Briefly, in the oral step, the needed amount of dried tomato, e.g., 3 g was mixed with 6 mL of Simulated Salivary Fluid (SSF) (alfa-amylase solution (1500 U/mL), $CaCl_2(H_2O)_2$ (0.3 M), H_2O) in centrifuge tube and in the case of adjusted tomato the mixture of neuroactive compounds was resuspended in SSF. The solution was incubated for 2 min at 37 °C on a rotary shaking plate (200 rpm). In the gastric digestion, 12 mL of Simulated Gastric Fluid (SGF) was added in the tube with SSF part, and the pH was adjusted to 3.0 using HCl (5 M). The bottle was incubated for 2 h at 37 °C on a rotatory shaking plate (200 rpm). Following gastric digestion, intestinal digestion was performed with the addition of 24 mL of simulated Intestinal Phase Fluid (SIF) (Pancreatin (800 U/mL), bile extract porcine (20 mg/ml), $CaCl_2(H_2O)_2$ (0.3 M), NaOH (5 M)) and the pH was adjusted to 7.0 using NaOH (5 M). The bottle was incubated for 2 h at 37 °C on a rotatory shaking plate (200 rpm). To simulate passive intestinal absorption of water and hydrolytic products from digestion in the small intestine, after digestion the centrifugation of the total digested material was performed using 5000 rpm, 30 min, 4 °C. Supernatant was transferred into 50 mL centrifuge tube and the remaining pellet was freeze-dried, grinded, homogenised and stored at room T until further analysis. The final ratio of the in vitro digestion was ~ 1:1.46 ± 3, obtaining ~1.5 gr of digested tomato. Finally, sample was stored in desiccator until use. Sampling involved collecting tomato samples before and after digestion, specifically 0.1 g of lyophilized tomato or digested tomato pellet, prepared according to the protocols described in "Sample Preparation". Additionally, 200 µL of the liquid phase after in-vitro digestion was immediately filtered. The same sampling procedure was applied for the separate analysis of carotenoids.

2.4.2. Fecal batch culture fermentation

After in vitro digestion of tomato, a pH-controlled anaerobic batch culture fermentation system was performed as described by Diotallevi et al. (Diotallevi et al., 2021) as a proof of concept of developed method of fecal samples (Fig. 1). Briefly, glass water-jacketed vessels (200 mL) were sterilized and filled aseptically with 180 mL of pre-sterilized basal nutrient medium, prepared as follows (per liter): 2 g Peptone, 2 g Yeast extract, 2 g NaHCO3, 2 mL tween 80, 0.5 g Bile Salts, 0.1 g NaCl, 0.04 g K2 HPO4, 0.04 g KH2 PO4, 0.01 g MgSO4 7H20, 0.01 g CaCl2 6H2O, 0.005 g Hemin dissolved in 1 mL of 1 M NaOH, 10 µL Vitamin K, 0.5 g L-Cysteine HCl, and 1 mL of Resazurin (0.1 g/100 mL). Anaerobic conditions were maintained through a O_2-free N_2 (15 mL/min) flow overnight. Temperature was set at 37 °C using a circulating water bath. Before the inoculation, both vessels were dosed with 1 mL of 10 % filtered, freshly prepared solution of L-cysteine chlorhydrate (Applichem) as oxygen reducing agent and 20 mL of fecal slurry (10 % w/v of fresh human feces) to a final concentration of 10 % (w/v), homogenised in pre-reduced phosphate buffered saline (PBS) (0.1 M, pH 7.0) within 1 h. To simulate the environmental conditions of the proximal colon the pH was adjusted to 5.5–6.1 and kept between this range throughout the experiment with the automatic addition of ammonium hydroxide or hydrochloric acid (0.5 M). When the basic conditions were achieved, one vessel remained as prepared, while in the second vessel it was added the digested tomato ($m = 2$ g) dosed prior digestion with the neuroactive compounds. Samples (2 mL) were taken at 0, 10 and 24 h, immediately centrifuged and prepared for the LC-MS/MS analysis.

3. Results and discussion

3.1. Method development and validation – LC-Qtrap-MS

3.1.1. Tomato extracts

3.1.1.1. Linearity. Given the high presence of certain compounds in the matrix (e.g., linoleic acid, glutamic acid, glutamine, citric acid), the calibration curves for all compounds in the tomato matrix, both prior to and after digestion, were constructed using standard mixtures with isotopically labeled dilutes. Additionally, the matrix effect after sample dilution for certain compounds met the criteria outlined in the method validation guidelines. The linearity of the method was obtained with preparation of 16 different calibration points across the working range using reconstitution solution and pure standard mixture. The

concentration of selected neuroactive compounds increased from < LOQ to 4, 20, 40, or 80 μg/g dry tomato, depending on the compound's working range, while the concentration of both internal standards remained fixed (0.1 ppm in the vial). According to the working range and the lowest calibration level, the LOQs were between 0.0004 – 20 μg/g dry tomato/digested pellet. Each calibrant was prepared in triplicate. The coefficient of determination (R^2) was > 0.82 for each neuroactive compound, and for the majority of them higher then 0.95, when using a weighted (1/x) linear calibration curve.

3.1.1.2. Accuracy and precision. Accuracy and precision were assessed by measuring sample extracts at low, medium and high calibration levels, within a single run. Also, instrument precision was obtained by repeated injections (n = 3) of one sample for each level. The accuracy

Table 2
The compound distribution of 36 neuroactive compounds and 5 carotenoids after in vitro digestion of tomato.

Compound	Digested liquid	Digested pellet	Tomato	Distribution after in vitro digestion [%]	Digested liquid	Digested pellet	Tomato	Distribution after in vitro digestion [%]
36 Neuroactive compounds	mass [μg]	RSD [%]	mass [μg]	RSD [%]	mass [μg]	RSD [%]	mass [μg]	RSD [%]
Bisphenol A	0.35±0.02	4.6	1.1 ± 0.1	11.2	1.4 ± 0.2	12.1	25	79
Bisphenol S	0.48±0.02	3.7	0.87 ±0.05	5.8	1.59±0.02	1.4	30	55
Acetaminophen	0.236 ±0.009	3.9	0.43 ±0.02	5.3	1.02±0.01	1.2	23	42
Aspartame	0.10±0.01	12	0.64 ±0.09	13.4	2.0 ± 0.1	0.5	5	32
Difenoconazole	0.0738 ±0.0002	0.3	0.025 ±0.04	14.7	1 ± 0.3	29.9	7	3
Phenylalanine	15±0.3	1.7	17±1	8.3	17.8 ± 0.8	4.3	84	96
Naringenin	0.071 ±0.007	10.2	0.80 ±0.05	39.8	0.44±0.06	15.5	16	159
Tomatine	5.5 ± 0.5	9.1	10±2	19	8.9 ± 0.9	11.2	62	112
Tryptophan	9.7 ± 0.2	2.3	12.4 ± 0.9	7.1	13.9 ± 0.8	6	70	89
Tyrosine	14.5 ± 0.1	0.9	16.6 ± 0.4	2.3	19±1	7.2	76	87
o-Coumaric acid	0.80±0.06	3.4	0.68 ±0.02	0.9	1.034 ±0.009	2.6	66	77
p-Coumaric acid	0.26±0.01	3.6	0.4 ± 0.01	3.7	0.5 ± 0.2	34.9	52	80
Hesperitin	3 ± 1	40	1.1 ± 0.4	24.6	2.9 ± 0.2	5.7	103	38
Quinic acid	0.62±0.02	3.6	0.88 ±0.08	8.6	1.13±0.09	7.8	55	78
Glutamic acid	2.95±0.09	2.1	4.8 ± 0.1	2.6	7.8 ± 0.5	6.2	38	62
Neochlorogenic Acid	13.3 ± 0.8	6.1	16±1	9	29±4	15.8	46	55
Resveratrol	0.04±0.03	78.6	4.3 ± 0.1	2.4	4.5 ± 0.4	9.5	1	96
Pantothenic Acid	0.28±0.07	23.7	0.44 ±0.09	21	0.9 ± 0.3	32	31	49
m-Coumaric acid	0.587 ±0.003	0.5	0.95 ±0.007	0.7	1.97±0.09	4.6	30	48
Naringin	0.52±0.03	5.9	1 ± 0.1	12	2.02±0.04	2	26	50
Glutamine	0.43±0.01	5	2.8 ± 0.1	3.4	5.8 ± 0.3	4.3	7	48
Citric acid	0.12±0.01	9.7	0.9 ± 0.3	2.9	2.2 ± 0.2	8.3	6	41
Rutin	0.41±0.05	11.4	60±10	17.7	120±10	11.2	0.3	50
Serotonin	0.006 ±0.003	37.8	6 ± 1	22.6	12±3	22.9	0.1	50
Eriodyctiol	0.0003 ±0.0001	46.2	0.224 ±0.005	2.3	0.46±0.04	8.8	0.1	49
Quercetin	4.4 ± 0.9	21.4	60±20	28.4	280±40	13.7	2	21
L-Dopa	0.072 ±0.007	9.5	0.33 ±0.06	16.5	2.0 ± 0.7	32.8	4	17
Dihydrokaempferol	0.05±0.01	23.5	0.28 ±0.03	12	1.78±0.04	2.2	3	16
Crypto-Chlorogenic Acid	0.14±0.004	10.1	0.8 ± 0.7	63.9	40±5	12.9	0.4	2
Chlorogenic Acid	0.031 ±0.008	26.6	0.7 ± 0.6	67.7	24±2	8.1	0.1	3
Caffeic aldehyde	0.001 ±0.0001	15.2	0.06 ±0.05	47.5	1.93±0.04	2.1	0.1	3
Caffeic acid	<loq		0.05 ±0.04	46.2	4 ± 1	28.6	0.0	1
Linoleic acid	134±8	6.2	170±30	15.9	18±7	38.6	717	909
p-Pyridoxic acid	36±1	3.9	17±76	12.5	7 ± 11	14.8	514	243
Trymaine	15±7	8.4	58±3	6	15±7	43.4	100	387
Guanine	0.42±0.02	4	0.22 ±0.01	5.2	0.17±0.02	13	247	129
5 Carotenoids	**mass [μg]**	**RSD [%]**	**mass [μg]**	**RSD [%]**	**mass [μg]**	**RSD [%]**	**mass [μg]**	**RSD [%]**
β-Carotene	2.3 ± 0.4	16	28.1 ± 0.9	3	21±4	19	11	134
Lutein	0.54±0.07	13	8 ± 2	28	5.8 ± 0.2	3	9	138
Lycopene	4.4 ± 0.9	20	68.1 ± 0.3	5	61±3	5	7	112
Phytoene	50±10	24	230±20	7	209±2	9	24	110
Trans-β-Apo-8-carotenal	0.015 ±0.005	27	0.28 ±0.03	11	0.213 ±0.008	4	7	131

was expressed as a percentage of the nominal value, and precision expressed as the relative standard deviation (RSD, %) of the target neuroactive compound in tomato extract. The obtained accuracy was for most compounds within required limits, i.e., 80.0–120.0 %, as well as method < 20 % and instrumental repeatability < 15 %.

3.1.1.3. Extraction recovery and matrix effect. The analyte recovery is reported as the ratio between peak areas of analyte/internal standard spiked before and after extraction at the least three concentration levels (LL, ML, HL) in triplicates. The recovery values reported in Table SM 2 reflect the well-known limitations associated with matrix effects and analyte instability in complex biological samples. In particular, the combined effects of extraction recovery and matrix-induced signal suppression or enhancement. The mean recoveries were 49 – 168 %, but

Fig. 2. Distribution of neuroactive compounds during in vitro digestion, presented as the percentage of each compound found in the pellet (orange) and liquid phase (green) after tomato digestion. The first row shows the distribution of neuro-disrupting compounds, followed by: neuro protective compounds with recovery >75 %, neuro protective compounds with recovery <75 %, neuro protective compounds with recovery >300 %, and carotenoids. The percentage of each compound per phase is displayed along with its relative standard deviation (RSD, %), calculated from three replicate (text color match the corresponding digested phase).

for most compounds between 80–120 %, for all tested levels. The matrix effect (± 0.5–96 %) was based on a comparison between the analyte peak area when the analyte mixture was added to tomato extract, compared to when added to the final reconstituted solvent (methanol: ultrapure water=1:9). The internal standards showed stable response RSD < 5 % within sample batch. All the method validation parameters are presented in Table SM 2.

3.1.2. Fecal extracts

3.1.2.1. Linearity. The linearity of the method was determined using matrix-matched samples at 17 different calibration points across the working range. The concentration of selected neuroactive compounds increased from < LOQ to 5, 25, 100, 200 ppm, whereas the concentration of both internal standards remained fixed (0.25 ppm). Each calibrant was prepared in triplicate. The coefficient of determination (R^2) was > 0.85 for each neuroactive compound when using a weighted (1/x) linear calibration curve, and for the majority of them, it was > 0.95, thus fulfilling the criteria of the Eurachem guidelines (The Fitness for Purpose of Analytical Methods, 2014). The LOQ, as the lowest point of calibration curve, were for all compounds between 0.0005 – 5 ppm according to the compound working range and linearity. All the method validation parameters are presented in Table SM 3.

3.1.2.2. Stability. Before diluting the samples with methanol, we observed degradation of certain compounds in undiluted samples stored at 15 °C before injection (data not shown). After 24 hours, the remaining levels of rutin, coumaric acid, and chlorogenic acid fell below 10 %. Diluting samples with water alone did not improve the stability of these compounds (data not shown), nor did additional clean-up using solid-phase extraction (SPE). Additionally, recovery using the Oasis HLB 96-well plate (30 mg sorbent) was particularly low for polar compounds (e.g., <10 %), such as phenylalanine, quinic acid, citric acid, and coumaric acid (Table SM 4). Dilution with methanol not only minimized ion suppression but also reduced compound degradation in the concentrated fecal matrix during instrumental analysis. To assess stability in final fecal extracts, we evaluated compound levels over multiple time intervals (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 24, 30, 36, 42, 48, 54, 72, and 96 hours) while samples were stored at 15 °C before injection (Table SM 5). Stability was also tested by diluting samples with pure water and adjusting the addition of an antioxidant (ascorbic acid) instead of methanol. Although similar stability was observed with a five-fold dilution using either a water-antioxidant combination or methanol (Table SM 5); however, for some compounds (e.g., Bisphenol A), chromatography was worse in samples diluted with water-antioxidant compared to those diluted with methanol. Further, stronger fecal dilutions with methanol (e.g., 10-, 20-, or 50-fold) did not improve stability, though greater dilution resulted in higher LOQs (data not shown). After 24 hours, the maximum duration of each individual batch analysis, most compounds remained above 80 %, except for quinic acid, chlorogenic acid, and its isomers, which remained above 70 % when fecal extracts were diluted fivefold with methanol. As a result, this dilution was selected as the final sample solvent.

3.1.2.3. Accuracy and precision. Following the procedure described above, the obtained accuracy, for most compounds, was within required limits, i.e., 80.0–120.0 % at both concentration levels, according to the neuroactive compound working range and precision < 15.4 %, at low and < 13.2 % at high concentration level.

3.1.3. Comparison with existing literature and analytical considerations

The analytical method developed in this study was validated in accordance with Eurachem guidelines (The Fitness for Purpose of Analytical Methods, 2014), for representative food matrices before/after digestion, and for fecal extracts. The validation results demonstrate the strong influence of matrix effects on method performance, highlighting the necessity of matrix-specific validation in analytical method development. Most available studies in food analysis focus on a single analyte (Hirabayashi et al., 2020) or a class of compounds (e.g., polyphenols, carotenoids, or amino acids) and report mainly total class content, without addressing the behavior of individual analyte (M. C. Coelho et al., 2021; Serena-Romero et al., 2023). As shown in our study, the chemical diversity within broad compound classes can lead to markedly different analytical responses and digestion-related fates. Furthermore, many studies rely on HPLC-DAD for quantification; although robust and reproducible, this technique generally offers lower sensitivity and selectivity than LC–MS/MS (M. Coelho et al., 2022; Wang et al., 2022). Importantly, the literature rarely reports separate validation and quantification procedures for digested food and fecal matrices, despite their fundamentally different compositions and analytical challenges. In addition, compound stability in complex matrices, particularly fecal extracts, is often overlooked. Our results indicate that commonly applied quenching procedures (e.g., low-temperature centrifugation and phase separation) may be insufficient to fully suppress microbial activity for all analytes, underscoring the importance of stability assessment during method validation and highlighting the need for future studies to take this aspect into consideration. Untargeted high-resolution mass spectrometry approaches (e.g., LC–QTOF–MS) provide valuable compositional information for undigested food matrices and, when combined with targeted quantitative methods, can substantially enhance understanding of bioactive compounds and their fate following ingestion (Ara et al., 2021). Compounds with potential neuro-disrupting activity are rarely reported in food digested or fecal matrices, while even trace concentrations may be biologically relevant; therefore, both beneficial and potentially harmful compounds should be considered when evaluating foods such as tomatoes. While some studies have examined individual compounds (e.g., bisphenol S, difenoconazole) in digestion or fecal matrices, they typically focus on single analytes (Craggs et al., 2020a; Lestido-Cardama et al., 2022). These limitations further support the need for the rigorous, multi-matrix validation strategy applied in the present study.

3.2. Application of developed methods

3.2.1. Characterization of neuroactive compound profile in tomato prior digestion

Out of 36 neuroactive compounds, all the neuroprotective compounds were detected in tomatoes before digestion, except for naringin. Among the five neuro-disrupting compounds, only two were found above the LOQ (Table SM 6). Aspartame, a food additive, was identified at 0.016 ± 0.002 μg/g dry weight, while difenoconazole, a pesticide commonly used in tomato production, was found at 0.72 ± 0.07 μg/g dry weight. The acceptable daily intake (ADI) for aspartame is 40 mg/kg bw/day (WHO | World Health Organization), and for difenoconazole, it is 0.01 mg/kg bw/day(Bellisai et al., 2023). With a serving size of 200 g of fresh tomato, neither of these levels exceeds the ADI. However, their presence, considering potential synergistic effects and daily consumption, should be taken into account. Additionally, their fate upon digestion should be further examined to assess their actual bioavailability and bioaccessibility once digested (Craggs et al., 2020a). Also, the presence of neurotransmitters such as serotonin (15 ± 2 μg/g dry weight) and tyramine (331 μg/g dry weight) was confirmed among the neuroprotective compounds. Linoleic acid was detected at 52 ± 2 μg/g dry weight, guanine at 0.61 ± 0.04 μg/g dry weight, and 4-pyridoxic acid (a vitamin B6 metabolite) at 8 ± 1 μg/g dry weight. Organic acids, such as quinic and citric acids, were present at concentrations above 1000 μg/g dry weight, and phenolic compounds, except for naringenin, were found in the range of 0.0012 – 57 μg/g dry weight. Tomatine, a common glycoalkaloid in tomatoes, was also detected at concentrations up to 71 ± 30 μg/g dry weight. Neuro-disrupting compounds present below the method LOQ, as well as other neuroactive compounds found at low

concentrations, were still included in our subsequent experiments on compound fate during digestion. Therefore, compounds detected at lower concentrations (< 6 μg/g dry weight) were added to tomatoes before digestion to investigate their fate during digestion and fecal fermentation. This decision was based on their identification in our previous non-target screening of neuroactive compounds in different types of tomatoes (https://www.mdpi.com/2304-8158/14/22/3927), as well as their reported presence in other studies, indicating their potential relevance in the development of neurodegenerative diseases (Ara et al., 2021; Bertuzzi et al., 2021; Elgueta et al., 2020; Rapa et al., 2021).

The separate analysis of carotenoids (Table SM 7) was necessary due to their chemical nature, in line with our previous observations. All target carotenoids, including apo-carotenal, were detected above the LOQ in tomato prior digestion. The highest concentrations were observed for phytoene (209 ± 9 μg/g dry weight) and lycopene (61 ± 3 μg/g dry weight), followed by β-carotene (21 ± 4 μg/g dry weight) and lutein (5.7 ± 0.2 μg/g dry weight), with trans-β-Apo-8-carotenal exhibiting the lowest concentration (0.212 ± 0.008 μg/g dry weight).

3.2.2. Distribution of neuroactive compounds during in vitro digestions

The in vitro digestion model involves three steps: oral, gastric, and intestinal digestion. The study was designed to evaluate the effect of tomato digestion on neuroactive compounds and, consequently, their potential bioaccessibility for the small intestine and colon. The distribution of compounds after digestion was measured in two phases: liquid and pellet. The results are presented in Table 2. The mass balance involved calculating the amount of each compound in these phases after digestion, compared to the amount present in the tomatoes before digestion. The in vitro model repeatability was confirmed with RSD < 20 % for most of the compound's distribution in liquid and pellet phase of digestion tomato performed in triplicates (Table 2). Fig. 2 and Table 2 presents the average distribution of 36 neuroactive compounds, and 5 carotenoids, respectively, after digestion, calculated as follows:

The gastrointestinal bioaccessibility of a compound (%, green) refers to the fraction of a compound released into gastrointestinal fluid (digested liquid) as a result of the digestion of tomato, representing the fraction of compounds potentially available for absorption by the intestine or may be excreted via urine. This is expressed relative to the total compound content in the original tomato (Eq. (1)). The non-bioaccessible compounds, i.e., those remaining in the intestinal digested fraction (i.e., digested pellet), are most likely transported to the colon with the potential to reach and become bioaccessible in the colon. This fraction of the compound (%, orange) is measured as the compound content in the intestinal digest (the pellet) compared to the original content in the tomato (Eq. (2)) (Lestido-Cardama et al., 2022; Reboredo-Rodríguez et al., 2021). It is also worth noting that not all compounds were recovered after intestinal digestion (i.e., (% in liquid + % in pellet) < 100 %), which may be attributed to transformations of compounds due to biochemical reactions (e.g., oxidative degradation, cleavage, conjugation, hydrolysis) (Guo et al., 2020) or because they were not released from the food matrix at all (Lestido-Cardama et al., 2022). On the other hand, the digestion of tomato led to the extensive production of certain compounds, with recoveries exceeding 300 %. This increase can be explained by upper gastrointestinal digestion, where enzymes, bile salts, and pH changes disrupt the tomato matrix, enhancing the release and bioaccessibility of compounds such as linoleic acid from complex lipids (Minekus et al., 2014). Digestion also liberates small polar metabolites and promotes biochemical transformations, making compounds such as tyramine and vitamin B6 derivatives (e.g., 4-pyridoxic acid) more detectable (Andac-Ozturk et al., 2022; Brodkorb et al., 2019). The matrix effect for some of these compounds was found to be minimal (<8 %), suggesting that an extensive increase observed in the measurable levels of certain compounds, are unlikely to be due to matrix interference. For others, a negative matrix effect was observed, indicating signal suppression rather than enhancement. This further supports the idea that the phenomenon can be attributed to the complex processes involved during digestion, such as enzymatic reactions and chemical transformations. For example, fatty acids can be formed from lipids, and neurotransmitters can be synthesized from amino acids(Mao et al., 2023).

$$\textit{intestinal bioaccesibility}\ (\%) = (\mu g\ \textit{compounds in liquid fraction} / \mu g\ \textit{compounds in tomato})\ x\ 100 \quad (1)$$

potential *bioaccesibility* in the colon (%)

$$= (\mu g\ \textit{compounds in pallet fraction} / \mu g\ \textit{compounds in tomato})\ x\ 100 \quad (2)$$

Among the disrupting compounds, bisphenols were almost completely recovered (> 80 %) and primarily remained in the digested pellet (> 50 %). Similarly, although with lower overall recovery, both acetaminophen and aspartame demonstrated potential to reach the colon. However, up to 30 % of these compounds also showed potential to be bioaccessible in the intestinal phase. The disrupting compound with the lowest recovery was difenoconazole (< 10 %), suggesting that probably it is largely transformed during digestion.

For the majority of neuroprotective compounds, belonging to various classes such as amino acids, alkaloids, phenolic compounds, vitamins, and fatty acids, the distribution between the liquid and pellet phases after digestion was similar, with high or nearly complete recovery (> 75 %). A portion of the recovered fraction of these compounds, such as phenylalanine, tomatine, tryptophan, tyrosine, hesperetin, coumaric acids, quinic acid, pantothenic acid, and naringin, was bioaccessible in the intestinal phase following digestion. Furthermore, these compounds also demonstrated potential to likely reach the colon. A higher fraction in the pellet was observed, especially for naringenin and resveratrol, as well as for protective compounds that were recovered in smaller amounts (18 - 56 %), such as glutamine, citric acid, rutin, serotonin, eriodictyol, quercetin, L-Dopa, and dihydroxykaempferol belonging to the class of organic acids, phenolic compounds, neurotransmitters, and amino acids. Additionally, compounds like chlorogenic acid isomers, caffeic acid, and its aldehyde derivative were found at concentrations close to or below LOQ after digestion, considering both the liquid and pellet phases. On the other hand, for some neuroprotective compounds, such as linoleic acid, pyridoxal acid, tyramine, and guanine, recovery exceeded 300 %. This means that compared to the initial amount of these compounds in tomatoes before digestion, their concentration increased after digestion. As a result, a significant portion of these compounds remained bioaccessible for the intestine while also having the potential to reach the colon.

The tested carotenoids exhibited similar behavior during digestion (Fig. 2). All five carotenoids, namely β-carotene, lutein, lycopene, phytoene, and trans-β-apo-8′-carotenal, proved to be robust to digestion and bypassed bioaccessibility in the small intestine. After digestion, they were primarily retained in the pellet fraction, indicating their potential to reach the colon. Additionally, the absence of apo-carotenal formation (non–vitamin A active metabolites) can be attributed to the stability of these carotenoids during digestion (Kopec et al., 2018)

Our findings reveal that neuro-disrupting compounds introduced

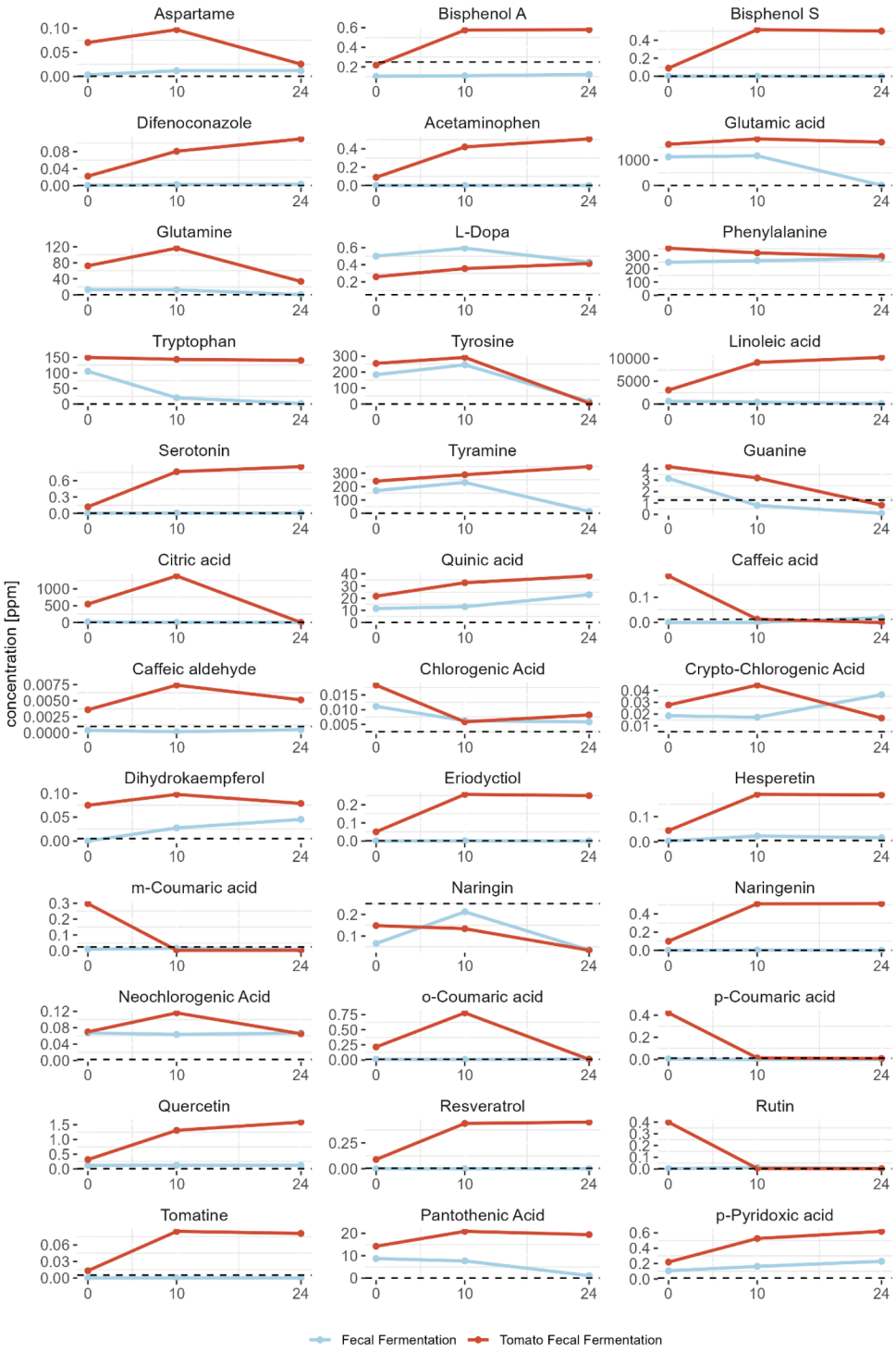

Fig. 3. Behavior of neuroactive compounds during the tomato fecal fermentation. Red lines represent the sample of fecal solution with digested tomato enriched with neuroactive compounds and blue lines correspond to the sample containing the fecal solution only. The dashed black lines represent the LOQ for each compound.

through food also have the potential for colonic bioaccessibility. For instance, bisphenols, undergo minimal transformation during digestion and appear more likely to be transported to the colon rather than bioaccessible in the intestinal phase. A similar pattern has been observed by Cardema et al. for bisphenol S, which was recovered in the range of 50–80 % at the end of digestion (Lestido-Cardama et al., 2022). The bioaccessibility following intestinal digestion reported for difenoconazole during rice digestion (Craggs et al., 2020b) was with a range from 13 % to 70.6 %, and in our case, this one was the lowest disrupting compound recovered (< 10 %), with a preference for the liquid phase, suggesting it is primarily transformed during digestion. However, this does not exclude the possibility that these disrupting compounds may still affect the brain, e.g., through its formed metabolites, possible influencing the other important metabolic pathways. The neuroprotective compounds monitored in this study represent various classes, including amino acids, fatty acids, nucleotides, neurotransmitters, organic acids, phenolic compounds, plant alkaloids, and carotenoids, many of which are also precursors to other neuroactive substances. These compounds are known to support brain health through multiple mechanisms: antioxidant activity, neurotransmitter synthesis,

mitigation of neuroinflammation, and the production of short-chain fatty acids (SCFAs) and bacterial metabolites (Anesi et al., 2022). For example, phenylalanine serves as a precursor to tyrosine, which is then converted into L-dopa and subsequently into neurotransmitters such as dopamine and norepinephrine, pathways that, when disrupted, are implicated in neurodegenerative diseases (Shebl et al., 2024). Consistent with previous research reporting an increase in amino acid content during digestion (Serena-Romero et al., 2023), we also observed elevated levels of certain protective compounds, including neurotransmitters. This further supports the idea that both neurotransmitters and their precursors can reach the colon, where they may be metabolized by gut microbiota and influence central nervous system function (Loh et al., 2024). Most studies investigating the digestion of various food types report a decrease in total phenolic content after digestion, with a preference for the liquid-digested phase (Reboredo-Rodríguez et al., 2021). In contrast, our study observed a near-complete transformation of certain phenolic compounds, such as chlorogenic acids and caffeic acids, while others, including resveratrol and naringenin, appeared more resistant to digestion, predominantly remaining in the pellet phase. Notably, the majority of neuroactive compounds, such as serotonin, rutin, and quercetin, also showed a preference for the pellet phase. Non-bioaccessible compounds, defined as those remaining in the intestinal digested fraction (digested pellet), are likely transported to the colon, where they may become bioaccessible. Once in the colon, these compounds can be absorbed by the colonic epithelium in their native form, metabolized by gut microbiota, or excreted without further metabolism. Through these processes, they can potentially influence brain function, either as native molecules or as metabolites formed during colonic transformation (Cárdenas-Castro et al., 2021a). Although reported bioaccessibility values for carotenoids such as β-carotene and lutein from fresh tomatoes vary in the literature, with some studies reporting up to 60 % bioaccessibility (Izzo et al., 2022) in the small intestine, the majority of reports indicate that over 90 % of β-carotene is not absorbed and proceeds to the large intestine. These findings align with our results (Eroglu et al., 2023; Izzo et al., 2022) and stress that due to the potential interaction of carotenoids with the gut microbiota, which is generally overlooked, it may be of relevance.

The relationship between a food contaminant and its presence in the human body is complex. Our results confirm that while many bioactive compounds in food are abundant, they are not necessarily bioaccessible to the colon (e.g., chlorogenic acids, caffeic acids, difenoconazole). Furthermore, this suggests the potential formation of transformation products or metabolites, whose biological activity may differ from that of the parent compounds (Barba-Ostria et al., 2022). While the exact biological impact of these transformation products remains speculative at this stage, their potential activity warrants further exploration to better understand their role in gut and brain health. Conversely, protective compounds, such as neurotransmitters, amino acids, polyphenols, carotenoids, and even harmful compounds like industrial chemicals and food additives, have the potential to reach the colon.

Furthermore, the methodology developed in this study offers a valuable tool for investigating the digestion of other neuroactive compounds and assessing factors influencing the digestion process, such as food processing, production methods, and storage conditions (Cárdenas-Castro et al., 2021a). Since bioactive compounds in tomatoes undergo decomposition and conversion, this research also emphasizes the importance of studying metabolic pathways and the metabolites formed during digestion.

3.2.3. *Neuroactive compounds during fecal fermentation*

The developed method for the determination of 36 neuroactive compounds in fecal solution was successfully applied to samples from a tomato fecal fermentation experiment. While only one fecal donor was used in this proof-of-concept study, we acknowledge this as a limitation. However, the primary objective was to validate the analytical method rather than assess biological variability, with the focus on demonstrating the method's applicability and robustness. We recognize that biological variability, particularly between individual donors, may influence the metabolic fate of neuroactive compounds. The absence of microbial characterization, also prevents the identification of specific microbial taxa responsible for the observed compound transformations. Future studies will address these limitations by incorporating multiple donors to better capture interindividual variability, and by employing metagenomic approaches to assess microbial composition and its influence on compound fate. Despite these limitations, this initial experiment provides a strong foundation for applying the method in more complex experimental designs, which have already been implemented in subsequent studies.

All fecal samples were prepared on the day of analysis and analyzed within a single batch, with randomized sample order to minimize analytical bias. An unexpected increase in compound concentrations between time zero and 10 hours of fermentation suggested that the initial sampling point immediately after inoculation may not reflect true baseline conditions. Therefore, in future studies, a short equilibration period (e.g., 15 min) will be introduced before collecting the initial sample. This adjusted sampling point will be designated as the new "time zero."

Fig. 3 highlights the dynamic nature of neuroactive compounds during tomato fecal fermentation. According to the in vitro digestion model, several neuro-disrupting compounds present in tomato remained in the digested pellet fraction, having the potential to reach the colon. While aspartame underwent more than 75 % transformation after 24 hours, other disrupting compounds, including bisphenol A, bisphenol S, difenoconazole, and acetaminophen, remained bioaccessible. Their persistence is concerning due to the potential for interaction with gut microbiota, systemic absorption, and interference with microbial metabolic pathways, particularly those involved in gut-brain signaling (Lu et al., 2024). Although compound bioaccessibility and bioavailability in the colon may vary depending on physiological and microbial factors, some substances may also be excreted via fecal matter, limiting their biological activity. Prior studies suggest that bisphenol degradation depends on microbial communities in environmental systems, but transformation by the human gut microbiota remains less understood (Mao et al., 2023). Xenobiotic exposure has been widely linked to shifts in gut microbiome composition (Defois et al., 2018). Interestingly, while Craggs et al. (Craggs et al., 2020a) reported degradation of difenoconazole during fecal fermentation, our findings indicate that this pesticide have the potential to persists, further emphasizing the variability of the food matrix and the complexity of microbial interactions.

While most amino acids remained stable during fecal fermentation, glutamine and tyrosine showed a decrease. Similar stability was observed for linoleic acid. Notably, neurotransmitters such as serotonin and tyramine, originating from tomatoes, were confirmed to reach the colon and even increased during fermentation. Likewise, the nucleotide guanine reached the colon but was progressively transformed (25 % after 10 hours, $>$ 80 % after 24 hours). Organic acids, namely citric acid was nearly undetectable ($<$ LOQ) after 24 hours, while quinic acid remained stable. All polyphenols except naringin reached the colon. Smaller polyphenols (e.g., caffeic acid, coumaric acid isomers) decreased significantly, while larger ones (e.g., eriodyctiol, hesperetin, naringenin) were more stable. Interestingly, quercetin concentration increased during fermentation, likely due to the breakdown of more complex polyphenols like rutin, which showed a clear decrease already after 10 h. The plant alkaloid tomatine and pyridoxic acid (a vitamin B6 catabolite) also increased after 10 hours and remained stable through 24 hours. Pantothenic acid followed a similar trend and was additionally detected in the sample containing only the fecal solution (in the absence of digested tomato), suggesting it is not exclusively tomato-derived. A similar pattern, indicating compounds not exclusively originating from tomato, was observed for other compounds, including L-dopa, phenylalanine, tryptophan, tyrosine, guanine, tyramine, and certain phenolics (e.g., chlorogenic acid isomers).

Gut microbiota communicate with other organs, including the nervous system, via three main mechanisms: secretion of active molecules, quorum sensing signals, and the production of microbial metabolites from dietary nutrients. The latter is considered the most influential route for host-microbiota communication (Lu et al., 2024). Understanding the metabolic fate of food-derived compounds is critical to evaluate their potential effects on the central nervous system. Xenobiotics, including those studied here, can alter microbial composition (Defois et al., 2018) and influence secondary metabolism, potentially affecting the biosynthesis of neuroprotective agents such as neurotransmitters and amino acids (Mao et al., 2023). These compounds, particularly during infancy, are vital for immune development, gut function, and neurological health function. They are regarded as "special dietary nutrients" with therapeutic potential in maintaining neurotransmitter balance and preventing neurodegenerative conditions (Qu et al., 2023). Our observations align with previous findings that gastrointestinal digestion promotes the release of amino acids and neurotransmitters (Serena-Romero et al., 2023). In fact, a significant proportion of neurotransmitters are produced in the gut and contribute to central nervous system functioning (Hirabayashi et al., 2020). Additionally, the reported general decline in polyphenol concentration during fermentation (Cárdenas-Castro et al., 2021b) aligns with our findings for smaller polyphenols. In contrast, the stability, or even increase of larger compounds, such as quercetin derived from rutin, highlights their potential resilience and the role of transformation pathways during fecal fermentation.

4. Conclusion

The advanced methodologies developed in this study enabled the quantification of both neuroprotective and neuro-disrupting compounds in tomatoes, before and after digestion, and in fecal samples through 24-hours of fecal fermentation. These methods showed strong analytical performance, with robust validation parameters and successful application across different stages. Using these techniques, we investigated the fate of neuroactive compounds throughout simulated digestion (oral, gastric, and intestinal phases), assessed their bioaccessibility to the colon, and examined their behavior over a 24-hour fecal fermentation. This comprehensive approach allows to generate an in-depth understanding of the transformations neuroactive compounds undergo along the gastrointestinal tract and during interaction with the gut microbiota. The in vitro digestion model allowed us to differentiate between bioaccessible and non-bioaccessible fractions, identifying compounds likely to reach the colon. Additionally, fecal fermentation experiments revealed how these compounds behave in the colon, highlighting complex interactions with the gut microbiome. Notably, some neuro-disrupting compounds remained bioaccessible and persistent throughout fermentation, while others showed a decline in concentration, likely due to microbial transformation.

Overall, this study presents valuable methodologies for investigating the fate of neuroactive compounds during digestion and fecal fermentation. The findings deepen our understanding of how both protective and disruptive compounds behave throughout digestion. Together with the developed methodological framework, these insights lay a strong foundation for future research into the post-digestive effects of neuroactive compounds on gut and brain health, emphasizing their complex dual role as both beneficial and potentially harmful bioactive compounds. In our next study, we will build upon these results by increasing the number of replicates and incorporating metagenomic analyses to more comprehensively examine the behavior of these compounds during fecal fermentation. This approach will provide a more detailed understanding of their transformation processes and the microbial interactions that influence their activity.

CRediT authorship contribution statement

Ana Kovačič: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Domenico Masuero:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Mar Garcia-Aloy:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Andrea Mancini:** Writing – review & editing, Methodology, Conceptualization. **Urška Vrhovšek:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Acknowledgments

The study was supported by the NeuroTOm project (Proposal No. 101062798), funded by the European Union under Horizon Europe (HORIZON) research and innovation program, MSCA Postdoctoral Fellowships 2021 (HORIZON-MSCA-2021-PF-01).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.focha.2026.101231.

Data availability

Data will be made available on request.

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