Supplementary material

Enhanced-Sensitivity Profiling of Natural Products from TLC Plates Using a Facile Graphite-Based LA-DART-MS Platform

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1 Materials and methods

1.1 Chemicals and reagents

The compounds 4',5,6,7-tetramethoxyflavone, 3,3',4',5,6,7,8-heptamethoxyflavone, 5-demethylnobiletin, hesperitin, naringin, synephrine, hesperidin, peiminine, daidzein, sophoranone, ferulic acid, ursolic acid, and asarone were purchased from Shanghai Standard Technology Co., Ltd. Chromatographic-grade methanol was obtained from Fisher Scientific (USA), and analytical-grade formic acid, toluene, and ethyl acetate were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HPTLC silica gel plates (60 F254, MS-grade; batch no. HX85515334) were purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared using a Millipore Milli-Q system, with a resistivity of >18 MΩ·cm at 25°C. The thin-layer chromatography (TLC) experiments were carried out using Merck HPTLC silica gel 60 F254 MS-grade plates (20 × 10 cm glass plates).

1.2 Sample Preparation

References including 4',5,6,7-tetramethoxyflavone, 3',4',5,6,7,8-heptamethoxyflavone, 5-demethylnobiletin, naringin, synephrine, hesperidin, peiminine, daidzein, sophoranone, ferulic acid, ursolic acid, and asarone were accurately weighed and dissolved in methanol to a final volume of 1 mL to obtain a stock solution at a concentration of 1 mg/mL. The solution was stored at −20 °C. A working solution (0.1 mg/mL) was prepared by a 10-fold dilution of the stock solution with methanol.

For sample preparation, 0.5 g of CP, QP, and GCP powders were accurately weighed and extracted with methanol to a final volume of 5 mL. The mixtures were sonicated for 20 min and subsequently filtered to obtain the final sample solutions.

1.3 TLC conditions

Reference solutions were applied onto the HPTLC plates as 1.5-cm-wide bands using a quantitative capillary, dispensing 2 μL per spot for direct analysis by graphite-assisted LA-TLC-DART-MS. For sample application, 8 μL of the citrus peel extract was applied as an 8-mm-wide band, positioned 8 mm above the lower edge of the plate, using an Automatic TLC Sampler 4 (CAMAG, Switzerland). Similarly, 8 μL of each herbal extract was applied as an 8-mm-wide band at the same distance from the bottom of the plate. Prior to development, the plates were equilibrated for 20 min in a 20 × 10 cm double-slot development chamber (CAMAG, Switzerland) saturated with the mobile phase in the opposite slot.

Chromatographic development was performed using ethyl acetate–methanol–water (100:17:13) as the first mobile phase, allowing the solvent front to ascend to approximately 3 cm. After removal and drying, a second mobile phase—toluene–ethyl acetate–formic acid–water (20:10:1:1)—was applied, and the plates were developed to a distance of approximately 8 cm. Following development, the plates were dried and documented under UV 366 nm using the TLC Visualizer Documentation System (CAMAG). HPTLC images and chromatographic profiles were processed using winCATS software (version 2.5.18072.1, CAMAG).

For subsequent LA-TLC-DART-MS analysis, the developed plates were cut into 1.5-

cm strips using a razor blade or a smartCUT plate cutter (CAMAG).

1.4 Analysis of Graphite-assisted LA-TLC-DART-MS

Graphite-assisted LA-TLC-DART-MS analyses were performed on an LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a DART-SVP ion source (IonSense Inc., Saugus, MA, USA). A continuous-wave diode laser (MTO Laser, Guangzhou, China; model M-33A450-4W-G) operating at 450 nm with an output power of approximately 4 W was used for laser ablation. The laser spot size on the sample surface was approximately 2 mm in diameter. The laser is classified as a Class 4 device, and appropriate protective eyewear was worn throughout operation.

A Vapur interface (IonSense), coupled to a vacuum pump, was positioned between the DART ion source and the MS capillary inlet to enhance ion transmission. The TLC plates were mounted on a custom-made plate retainer, which allowed adjustment along the x-axis. High-purity helium (99.999%) was used as the ionizing gas, and nitrogen (99.999%) was supplied as the auxiliary gas. The discharge needle potential was set to 1.5 kV, and the grid electrode voltage was maintained at 350 V.

After acquiring the full mass spectrometric data across each TLC plate, the datasets were processed using Xcalibur software to obtain averaged mass spectra and the mean response for each m/z value. The processed spectra were subsequently imported into SIMCA software for orthogonal partial least squares discriminant analysis (OPLS-DA).

2 Safety notes

The laser used in this study is classified as Laser Safety Class 4, a category that denotes high-power devices capable of causing severe and irreversible ocular injury through direct, diffuse, or indirect beam exposure. Class 4 lasers also pose risks of significant skin injury and may ignite combustible materials. Appropriate certified protective eyewear must therefore be worn by all personnel during laser operation. Furthermore, sample preparation and all TLC procedures were conducted within a chemical fume hood to ensure effective removal of volatile solvent vapors and to maintain laboratory safety.

Table S1 Different types of detected compounds by graphite-assisted LA-TLC-DART-MS technique.

Ionization mode	Samples	Concentration mg/mL	Molecular weight	Detected ion	S/N without graphite	S/N graphite-assisted
Positive mode	5-Demethylnobiletin	0.1	388	389	8	64
	Tangeretin	0.1	372	373	-	81
	3,3',4',5,6,7,8-heptamethoxyflavone	0.1	432	433	-	804
	Nobiletin	0.1	402	403	-	2484
	4',5,7,8-tetramethoxyflavone	0.1	342	343	-	300
	Sophoranone	0.1	460	461	-	152
	Luteolin	2.86	286	287	40	228
	Asarone	2.08	208	209	-	701
	Daidzein	0.1	254	255	-	17
	Ferulic acid	1.94	194	195	-	76
Negative mode	Ursolid acid	4.54	454	455	37	295
	Hesperetin	0.1	303	301	12	36
	Quercetin	3.02	302	301	-	25

- Not detected.

Table S2 The identification of compounds on TLC plates from citrus herbs was conducted using the graphite-assisted LA-TLC-DART-MS technique.

NO	m/z	MW	MS/MS	Proposed compound	Origin	Classification
1	343.2	342	328.4, 313.0, 285.4	4', 5, 7, 8-tetramethoxyflavone	QP,GCP	Flavonoids
2	343.4	342	328.1, 313.8, 285.0	Tetramethoxyflavone [8]	CP,QP,GCP	
3	373.3	372	358.3, 343.3, 315.0	Tangeretin、Sinensetin [8]	QP,CP,GCP	
4	373.3	372	357.2, 343.3, 299.1	Isosinensetin [8]	QP,CP,GCP	
5	403.4	402	388.1, 373.3, 343.1, 355.0	Nobiletin [8]	QP,CP,GCP	
6	303.0	302	285.1, 219.1, 177.0, 153.0	Hesperetin [8]	QP,CP,GCP	
7	433.3	432	418.3, 403.1, 345.2	Heptamethoxyflavone [8]	QP,CP,GCP	
8	389.2	388	374.2, 359.2	Demethylnobiletin [9]	GCP	
9	359.9	358	163.9, 147.1	Monohydroxytetramethoxyflavone [9]	QP,CP,GCP	
10	180.3	179	162.5, 144.8	Caffeic acid [9]	QP,CP,GCP	Organic acid
11	198.1	180	180, 138, 123,110	Hexose [9]	QP,CP,GCP	Polysaccharide
12	301.3	302	285.1, 219.1, 177.0, 153.0	Hesperedin* [8]	QP,CP,GCP	Flavonoids
13	271.1	272	153.9, 147.0	Naringin* [9]	QP,CP,GCP	
14	167.2	167	150.1, 135.8, 107.6	Synephrine* [2]	CP,QP,GCP	Alkaloids

*Detected in negative ion mode

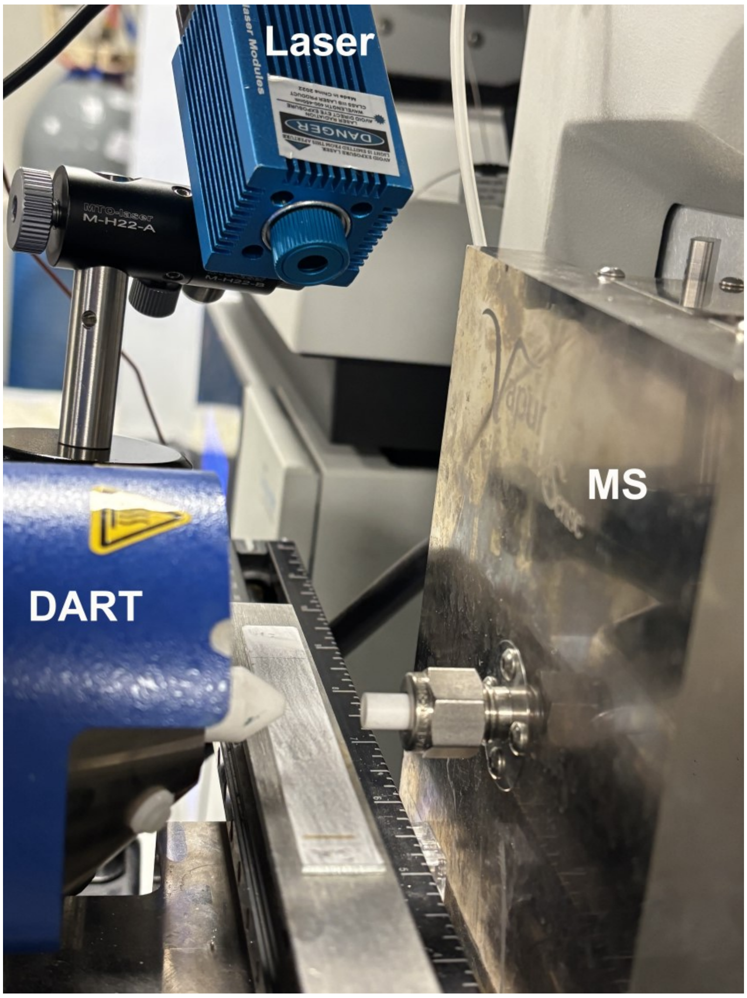

Fig. S1 Photograph of graphite-assisted TLC-LA-DART-MS setup.

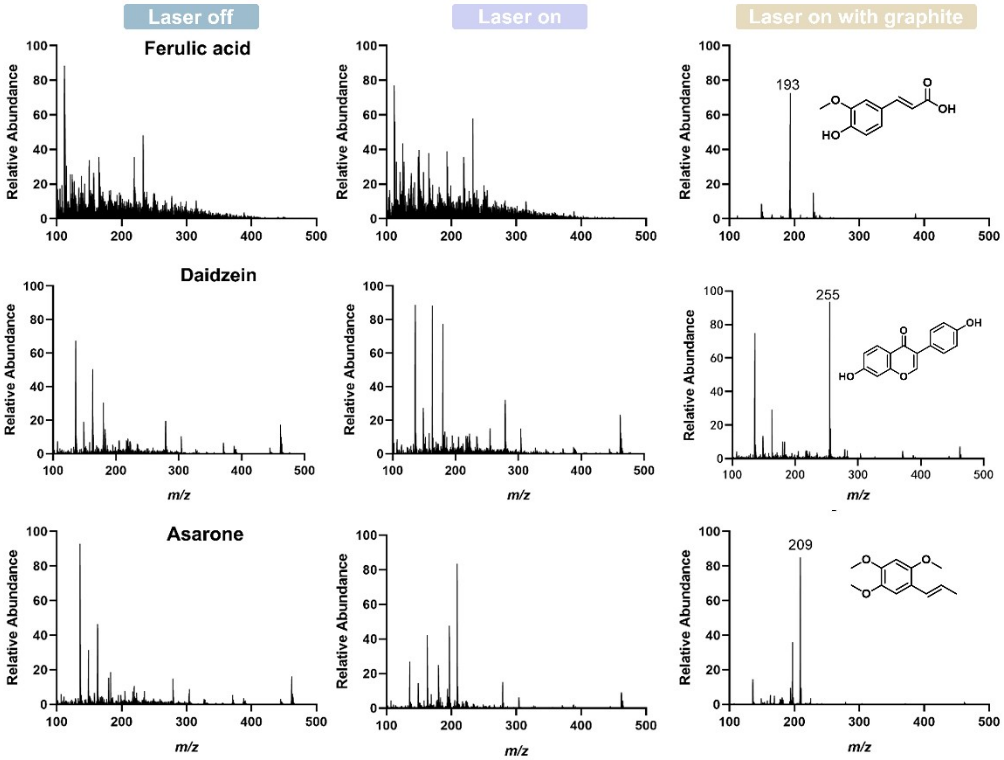

Fig. S2 Validation of the applicability of the graphite-assisted LA-TLC-DART MS technique using ferulic acid, daidzein, and asarone.

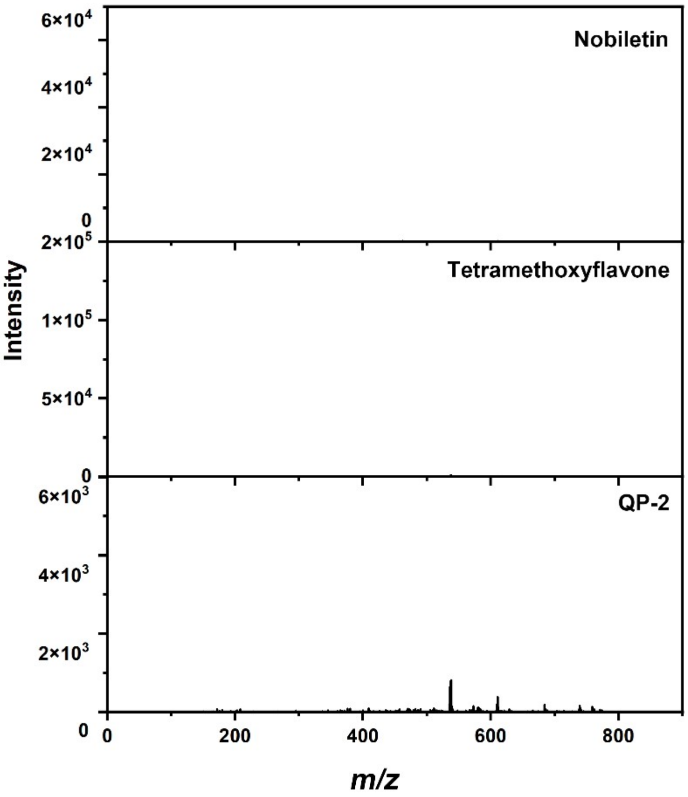

Fig. S3 TLC-LA-DART-MS spectrum without graphite assistance.

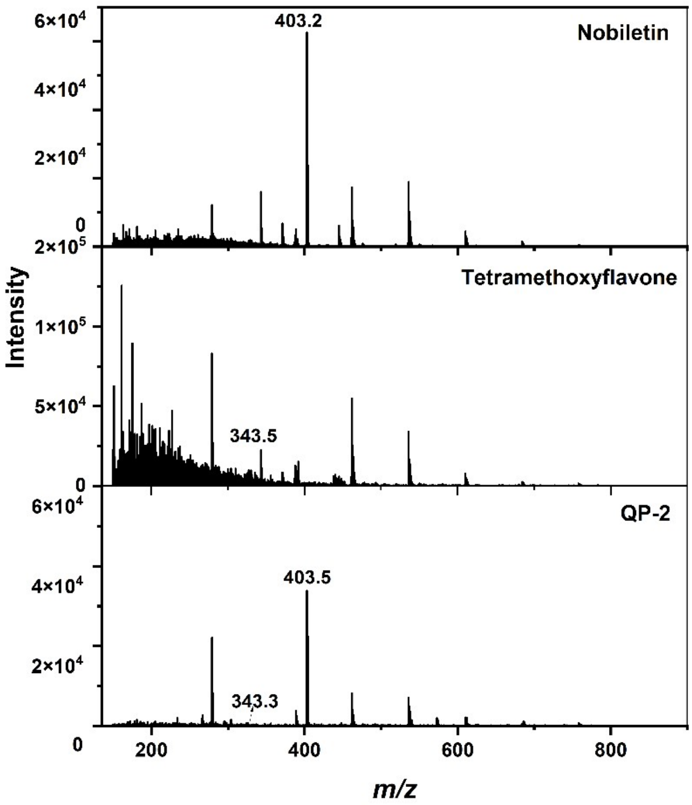

Fig. S4 TLC-LA-DART-MS spectrum with graphite assistance.

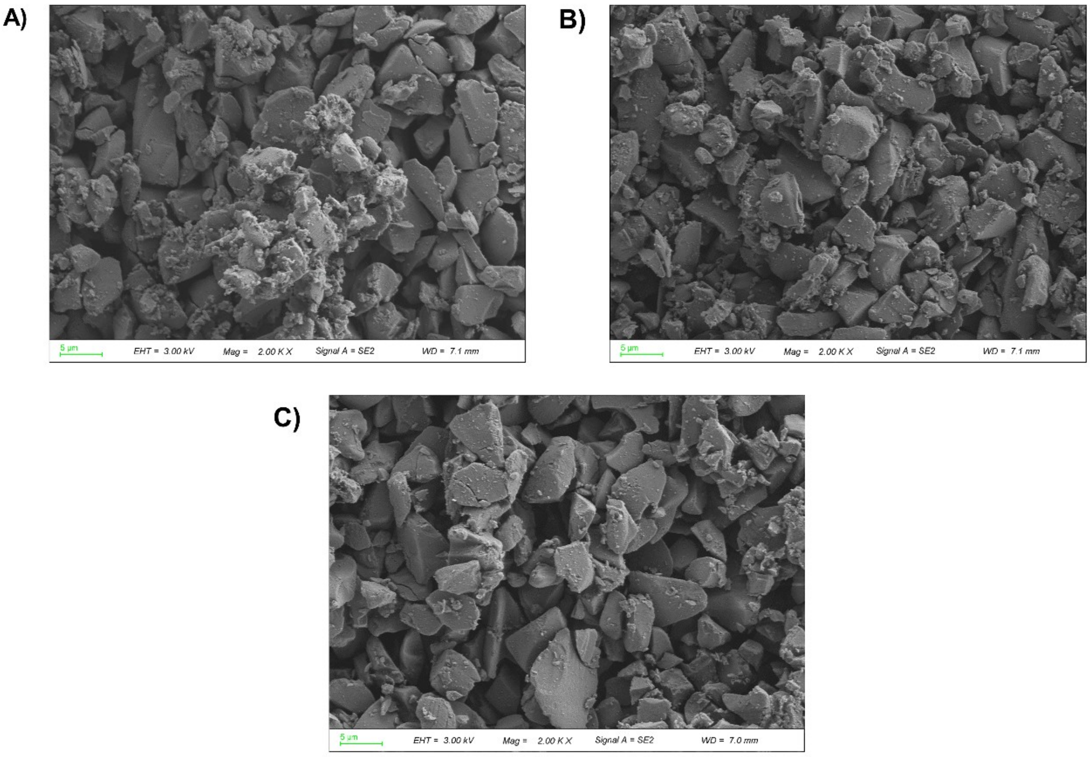

Fig. S5 SEM images of graphite-attached TLC plate after laser ablation, graphite-attached TLC plate, and blank thin layer plate

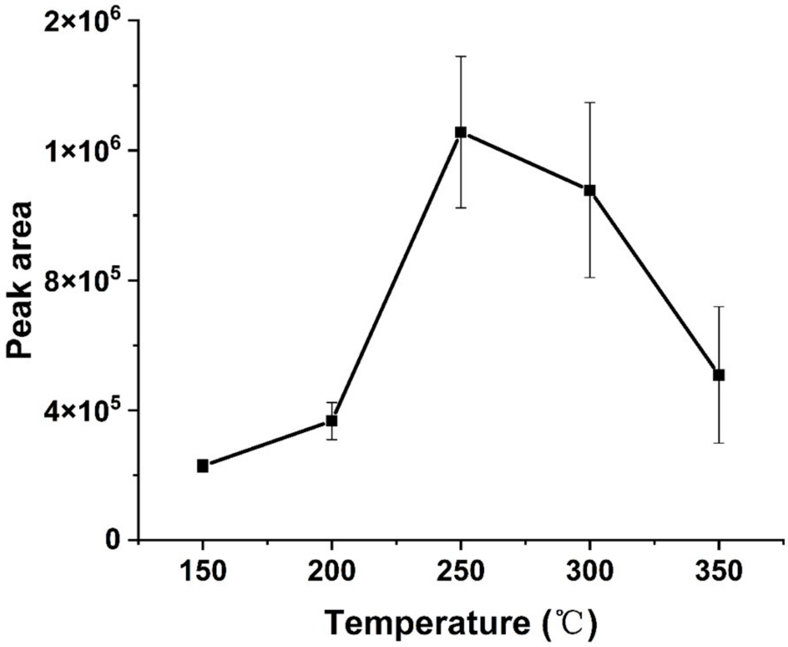

Fig. S6 Optimization of the gas heating temperature for the DART ion source.

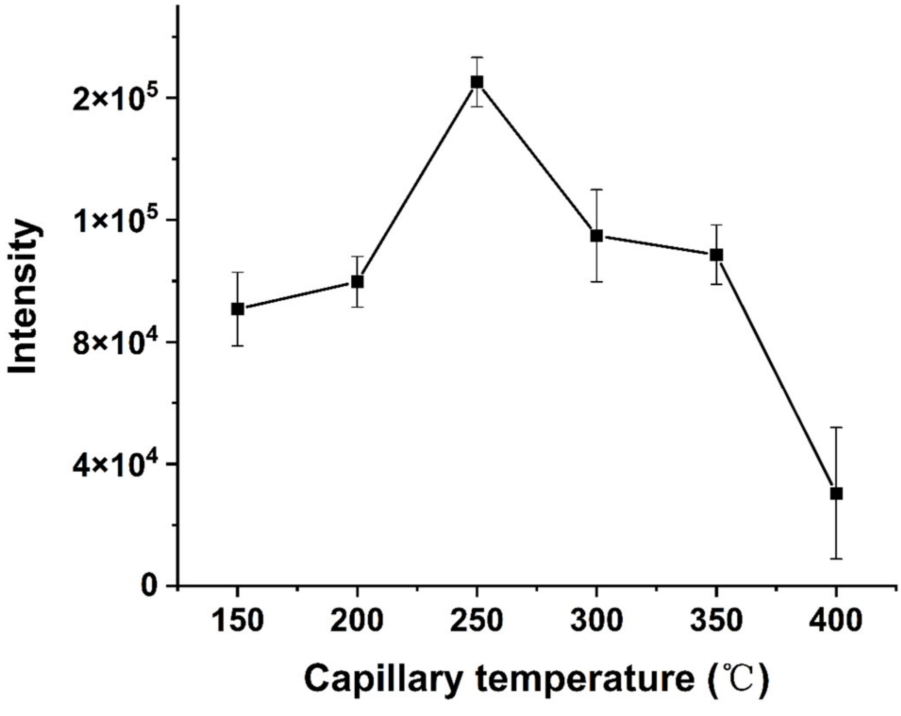

Fig. S7 Optimization of the capillary temperature in mass spectrometry.

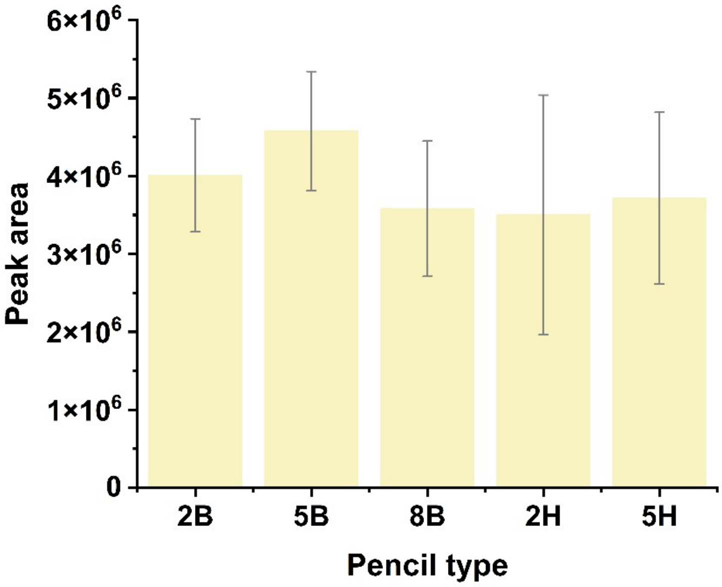

Fig. S8 The impact of different types of pencils on the experimental results.

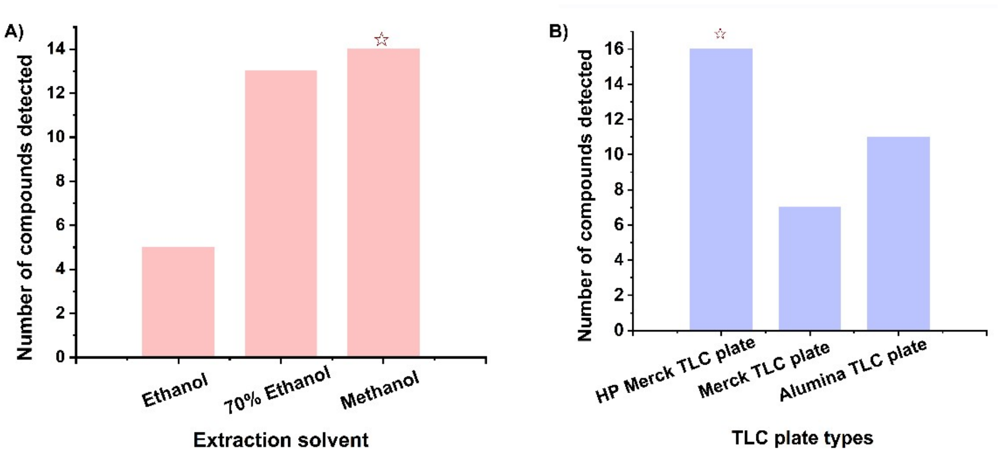

Fig. S9 The impact of different extraction methods and types of TLC plates on the experimental results.

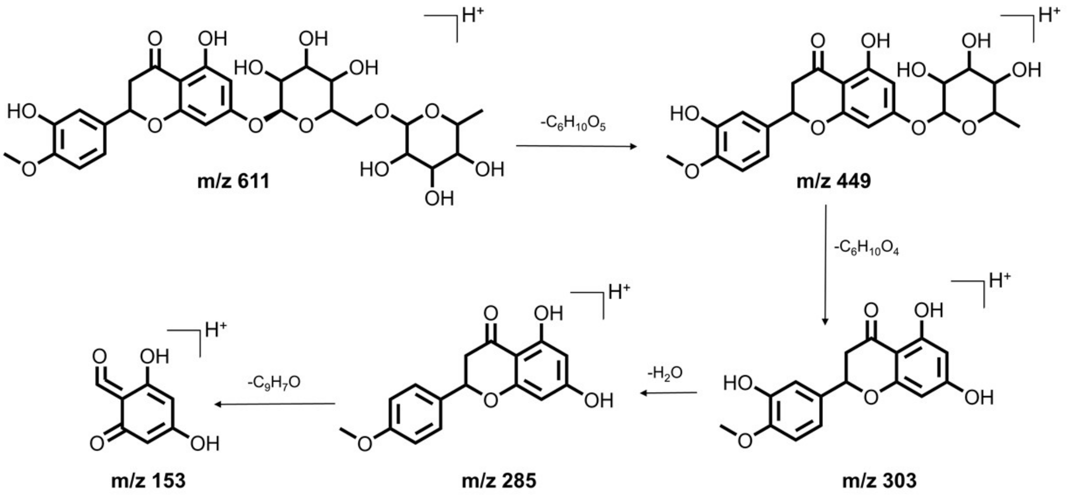

Figure S10 The fragmentation pathways of hesperidin.

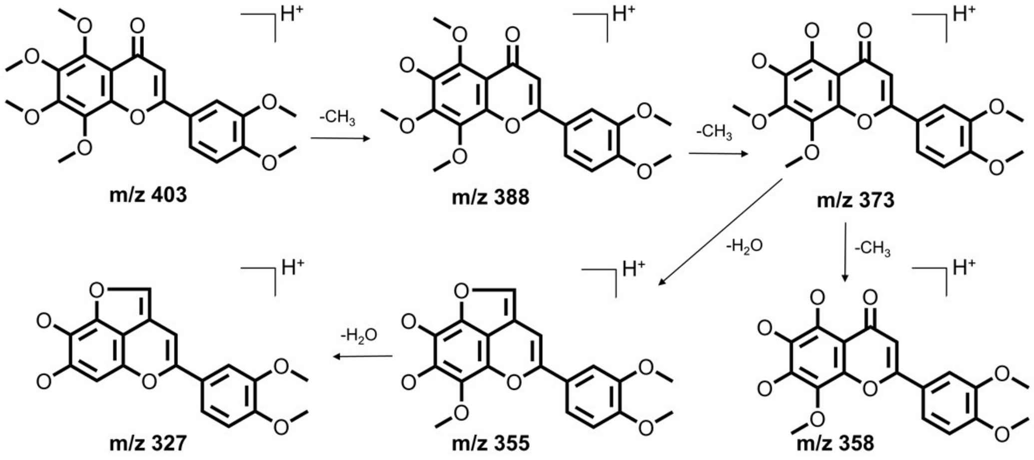

Figure S11 The fragmentation pathways of nobiletin.

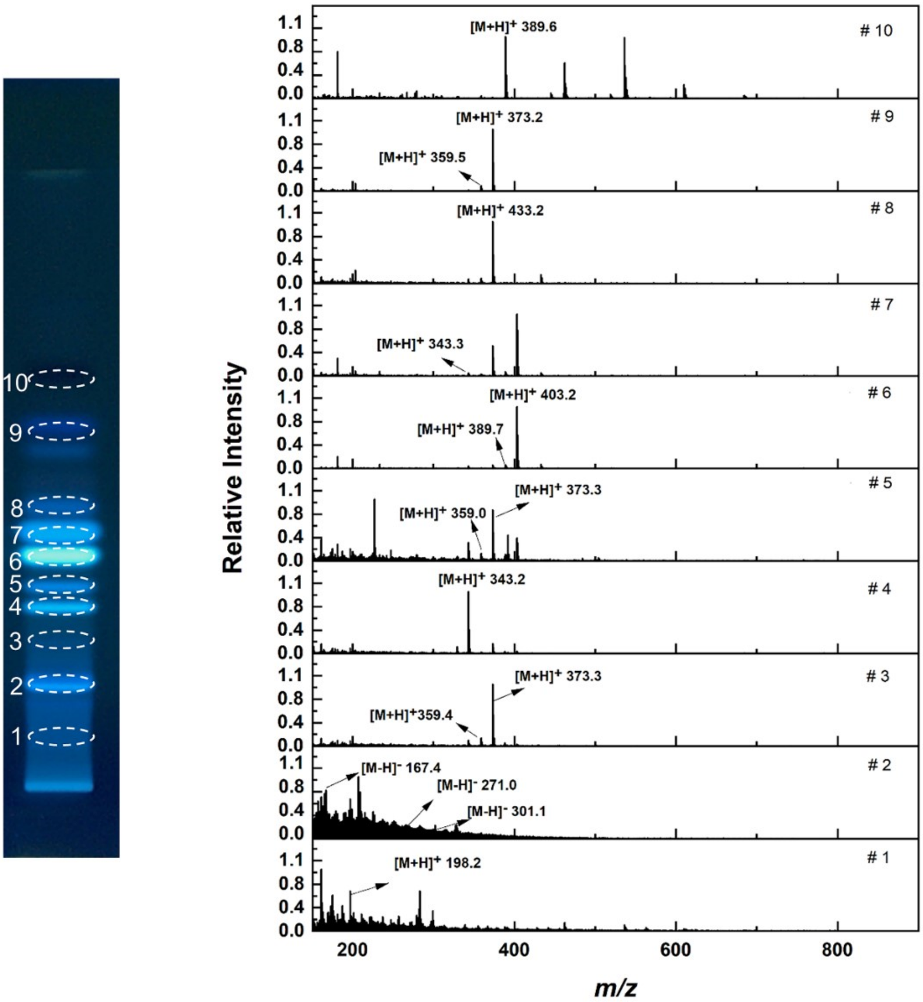

Figure S12 Identification of compounds on GCP thin layer chromatography using graphite-assisted LA-TLC-DART-MS.

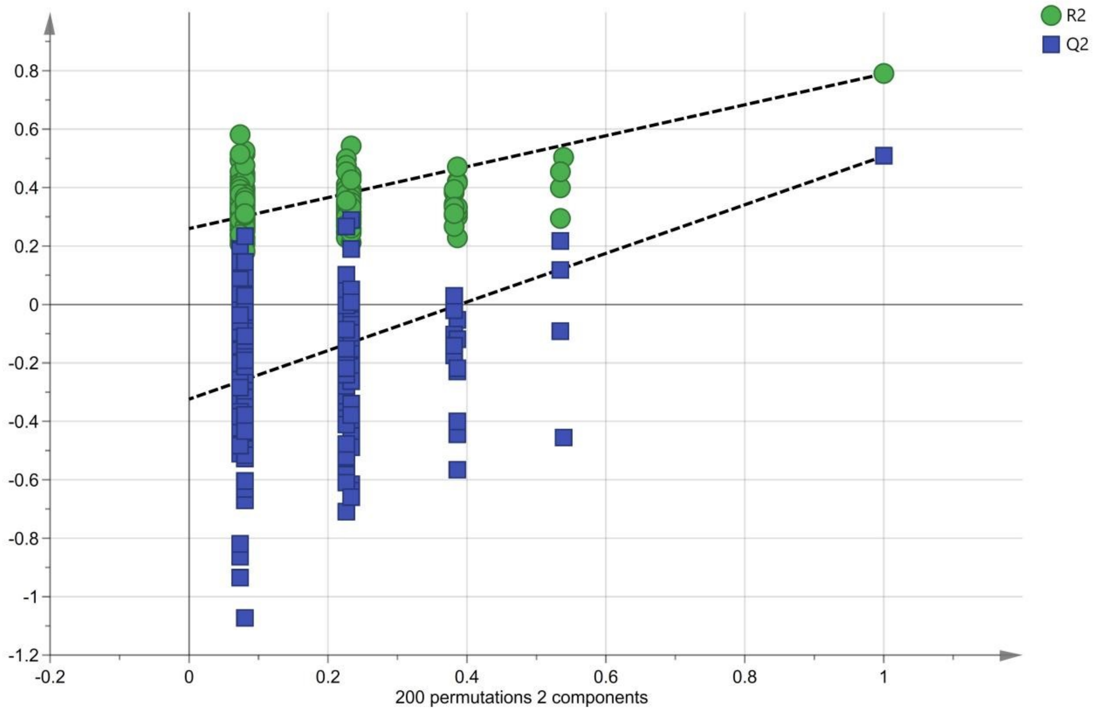

Figure S13 OPLS-DA permutation test results plot.