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HPLC-ESI-MS/MS for Concurrent Quantitation of Bioactivity Compounds in Fermented Mulberry

Chun-Mei Lu[1], Ting-Yuan Hsu[2], Ho-Shin Huang[3]
[1, 3]*Bio-Ray Biotech, INC, NO, 21-3, Shennong E. Rd., Changzhi, Pingtung, Taiwan*
[2]*Kao-Ho Hospital, Kaohsiung, Taiwan*

Abstract: ***In the present study, liquid chromatography tandem mass equipped method was developed for the separation and quantification of bioactive flavonoids and phenolic acid from fermented mulberry. This novel method was also validated for accuracy, precision and limit of quantification. A good recovery ranging from 80.1 to 92.1% and precision was achieved with seven compounds with the relative standard deviation of intraday ranging from 1.5-3.2 %. Consequently, the validated method for concurrent quantification of seven bioactive compounds in the fermented mulberry using LC-ESI-MS/MS analysis.***
Keywords: LC-MS/MS, fermented mulberry, flavonoid

I. INTRODUCTION

The Morus alba is widely distributed throughout Taiwan, Korea, China and Japan as medical herbs long time. As reported previously Morus alba mainly contains alkaloids, phenolic, flavonoids, tannin, and terpenes [1-2]. Flavonoids and phenolic acid are bioactive compounds be found in M. alba leaves including caffeic acid, rutin and chlorogenic acid [3]. Flavonoids and phenolic acid as natural compounds are found in many herb plant with bioavailability and safety for some indication. They were investigated for having beneficial to health, such as anti-inflammatory, anticancer, antioxidant, and antiviral activity [4-6].
Fermented fruits were proven to exhibit higher bioactivities such as antioxidant, anti-inflammation, etc. [7]. However few studies were reported about the effects of fermentation on the bioactive ingredients and bioactive properties of mulberry. [8-10].In the anti-virus plaque assay, we entrusted with the Taiwan Agricultural Research Institute for testing. We found that fermented mulberry shown the good antiviral activity and its EC50 was observed at 69.5μg/mL. The LC-MS/MS method is considered as a useful tool for qualitative and quantitative analysis of bioactive compounds in medical herbs. [13-14]. In this study, we developing an efficient and validated LC-MS/MS method for the quantification of the major flavonoids and phenolic acid in fermented mulberry.

II. MATERIAL AND METHOD

A. Reagents

Methanol and pure water (HPLC-grade) were purchased from Merck. Reference standards of Astragalin, Caffeic acid, Isochlorogenic acid, Isoquercetin, Kuwanon G, Chlorogenic acid and Rutin were purchased from Sigma-Aldrich (St Louis, MO., USA).

B. Plant Material and Fermentation

The tissues of Morus alba including the leaves, twig were harvested (Miaoli, Taiwan). Lactic acid bacteria used in this experiment was an isolated strain Lactobacillus plantarum PM-A0087 (BCRC910475). The Lactobacillus plantarum was grown in MRS broth (Oxoid, England) and were harvested after 24 h. About 10% (mass to volume) of mulberry were prepared in distilled water. Next, about 5% (mass to volume) of microbial culture and Morus alba was co-fermentation at 35 °C in an incubator for 24 hours. Fermentation was carried out in the stainless steel barrel. After co-fermentation finished, the production were drying by freeze dry machine until become powder.

C. HPLC-MS/MS analysis of the Fermented Mulberry

LC-MS/MS analysis was carried out on an XR-20A system (Shimadzu 8045, Kyoto, Japan) coupled to an triple quadrupole tandem mass spectrometer (API4000, Foster City, CA, USA). Chromatographic separation was performed using a Zorbzx C18 column (150×3.0 mm I.D, 5 μm; Agilent, USA). Mobile phase composition using gradient elution of solvent A (0.1% formic acid in pure water) and solvent B (0.1% formic acid in methanol) were used in the following gradient elution method: solution A, 90–60% (0–3 min), 60–40% (3–5 min), 40–0% (5–10 min), 0–40% (10–12 min) and 40–90% (12–15 min).

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The column temperature was fixed at 40 °C, the flow rate was set 0.3 mL/min, and injection volume was 2 µL. The electrospray negative mode was selected as an ion source for Astragalin, Caffeic acid, Isochlorogenic acid, Isoquercetin, Kuwanon G, Chlorogenic acid, Rutin. The quantification was performed in multiple reactions monitoring. The optimized ESI source parameters were as follows: ion spray voltage, -4500 V for negative mode; nitrogen nebulizer gas pressure, 51 psi; nitrogen curtain gas pressure, 12 psi; heater temperature, 500 °C; collisionally activated dissociation (CAD) gas, 11 psi. The precursor-to-product ion transitions were m/z 447/284, m/z 179/134, m/z 515/353, m/z 463/300, m/z 691/581, m/z, 353/191 and 609/300 for Astragalin, Caffeic acid, Isochlorogenic acid, Isoquercetin, Kuwanon G, Chlorogenic acid and Rutin. The optimization parameter as following: collision energies (CE), declustering potentials (DP) and collision cell exit potential (CXP) were shown in Table I. The Analyst 1.7.3 software (AB SCIEX, Concord, Canada) were used for result data processing.

D. Method Validaton of Phenlic Acid and Flavonoid Compounds

The linearity was estimated by the relationship between the concentration of the analytes and their signal obtained from LC-MS/MS analysis. The linearity of the proposed method was evaluated using five concentration in the concentration range of 0.5-5 (µg/mL). Linear regression equations were constructed by establishing calibration graph with the signal count (y), concentration (x, µg / mL). The signal of each component in the fermented mulberry was calculated from its corresponding calibration curve and the resulting data was represented as µg/g. The limit of quantification was evaluated according to the base line noise and determined at a signal-to-noise ratio of 10. The intraday precisions were observed by constantly injecting the sample solution for three replicates on the same day, and Relative standard deviation (RSD %) = (SD/mean) × 100%. The accuracy was evaluated as follows: Recovery (%) = detected concentration − initial concentration)/spiked concentration) × 100. The percentage recovery rate was calculated using the analytic signal and values provided by the calibration curves.

III. RESULTS AND DISCUSSION

A. Analysis of Seven Bioactive Compounds by LC-ESI-MS/MS

In this study, a LC-ESI-MS/MS method was achieved for the qualitative and quantitative determination of the phenolic and flavonoid compounds from fermented mulberry. The reversed-phase C18 column (Zorbzx, Agilent) was selected for HPLC -ESI-MS analysis based on its good resolution and efficiency.

With the ESI source of the mass spectrometer, Astragalin, Caffeic acid, Isochlorogenic acid, Isoquercetin, Kuwanon G, Chlorogenic acid, Rutin showed better sensitivity in the negative ion mode. The precursor-to-product ion transitions were m/z 447/284, m/z 179/134, m/z 515/353, m/z 463/300, m/z 691/581, m/z, 353/191 and 609/300 for Astragalin, Caffeic acid, Isochlorogenic acid, Isoquercetin, Kuwanon G, Chlorogenic acid, Rutin. The optimized parameter of LC-MS/MS including declustering potential (DP), collision energies (CE), and collision cell exit potentials (CXP) were shown in Table 1.

Seven compounds performed excellent linearity with coefficient of determination > 0.994 and results are shown in Table 2. The limits of quantification (LOQ) of seven compounds were 0.05, 0.01, 0.07, 0.1, 0.05, 0.02 and 0.1 µg/ml for Astragalin, Caffeic acid, Isochlorogenic acid, Isoquercetin, Kuwanon G, Chlorogenic acid, Rutin. A good recovery range from 80.1 to 92.1% and precision was acceptable that RSD values ranged among 1.5% to 3.2 % for intraday variation. This validated method was successfully applied to the quality control and concurrent analysis of fermented mulberry, which provided useful information for production and application. The quantitative analytical results were shown in Table 3 and indicated their contents distributed in these samples. The contents of Astragalin, Caffeic acid, Isochlorogenic acid, Isoquercetin, Kuwanon G, Chlorogenic acid, Rutin in fermented mullberry were in the range of 23-1570 µg/g. The phenolic acid compounds and flavanoids are well known with their pharmacological benefits for human health. In this results, isoquercetin with the highest abundance (1570 µg/g) among seven compounds were simultaneously observed.

IV. CONCLUSIONS

It is thought that the therapeutic efficacy of medicinal plant is determined by its bioactive compounds, but few studies have published about bioactive ingredients analysis of fermented herb.

In this study, we have successfully developed a simple and accurate LC-ESI-MS/MS method to determine the seven bioactive compounds in fermented mulberry.

According to validation results, the method can be useful for analytic seven bioactive compounds (Astragalin, Caffeic acid, Isochlorogenic acid, Isoquercetin, Kuwanon G, Chlorogenic acid, Rutin) under the specified LC-MS/MS parameter. This method was developed for the determination of phenolic acids and flavonoids in medicinal herb plant.

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Table I. MRM tansitions, declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) of seven bioactive compounds.

Compound	MRM transition (m/z)	DP(V)	CE(eV)	CXP(V)
Astragalin	447 >284	-97	-35	-12
Caffeic acid	179 > 134	-29	-24	-7
Isochlorogenic acid	515 > 353	-23	-22	-12
Isoquercetin	463 >300	-54	-32	-10
Kuwanon G	691>581	-105	-33	-9
Chlorogenic acid	353 > 191	-72	-19	-12
Rutin	609> 300	-110	-48	-15

Table II. Linear regression equations, coefficients, linear range, precisions and recovery yields, limit of detection and limit of quantification of seven bioactive compounds.

Compound	Regression equation	R^2	LOQ (μg/mL)	Precision (%)	Recovery (%)
Astragalin	y =1330000x+48100	0.997	0.05	1.5	91.3
Caffeic acid	y =2920000X+164000	0.996	0.01	1.7	82.3
Isochlorogenic acid	y= 2915.1x-3240	0.999	0.07	2.8	90.7
Isoquercetin	y = 174000x+11800	0.994	0.1	3.2	80.1
Kuwanon G	y = 124000 x + 191	0.999	0.05	2.7	86.9
Chlorogenic acid	y =	0.995	0.02	1.9	92.1
Rutin	y =	0.996	0.1	2.5	90.5

Table III. The contents (μg/g) of the seven bioactive compounds in fermented mulberry (n=3).

sample	Astragalin	Caffeic acid	Isochlorogenic acid	Isoquercetin	Kuwanon G	Chlorogenic acid	Rutin
fermented mulberry	80	23	35	1570	95	411	183

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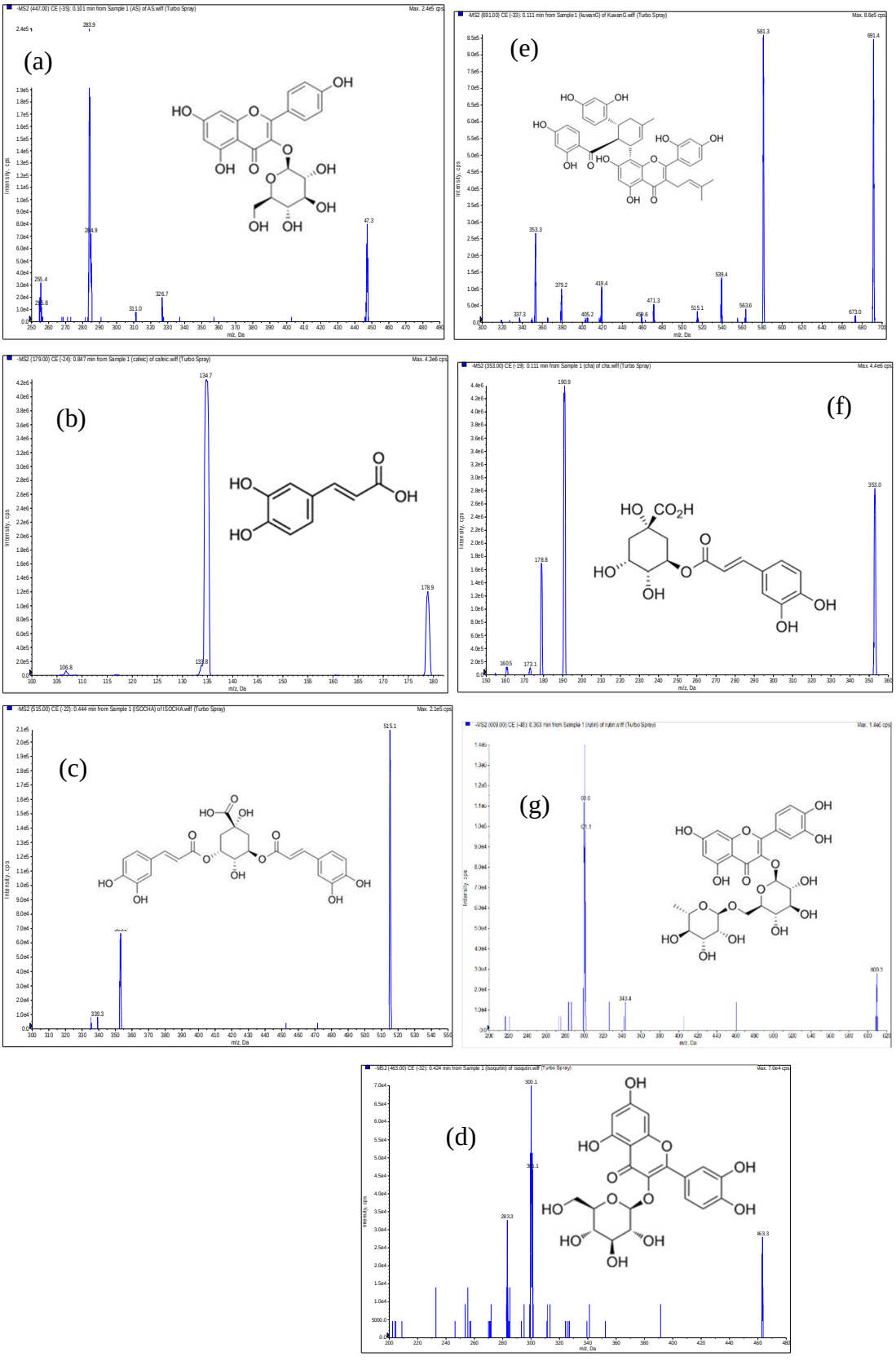
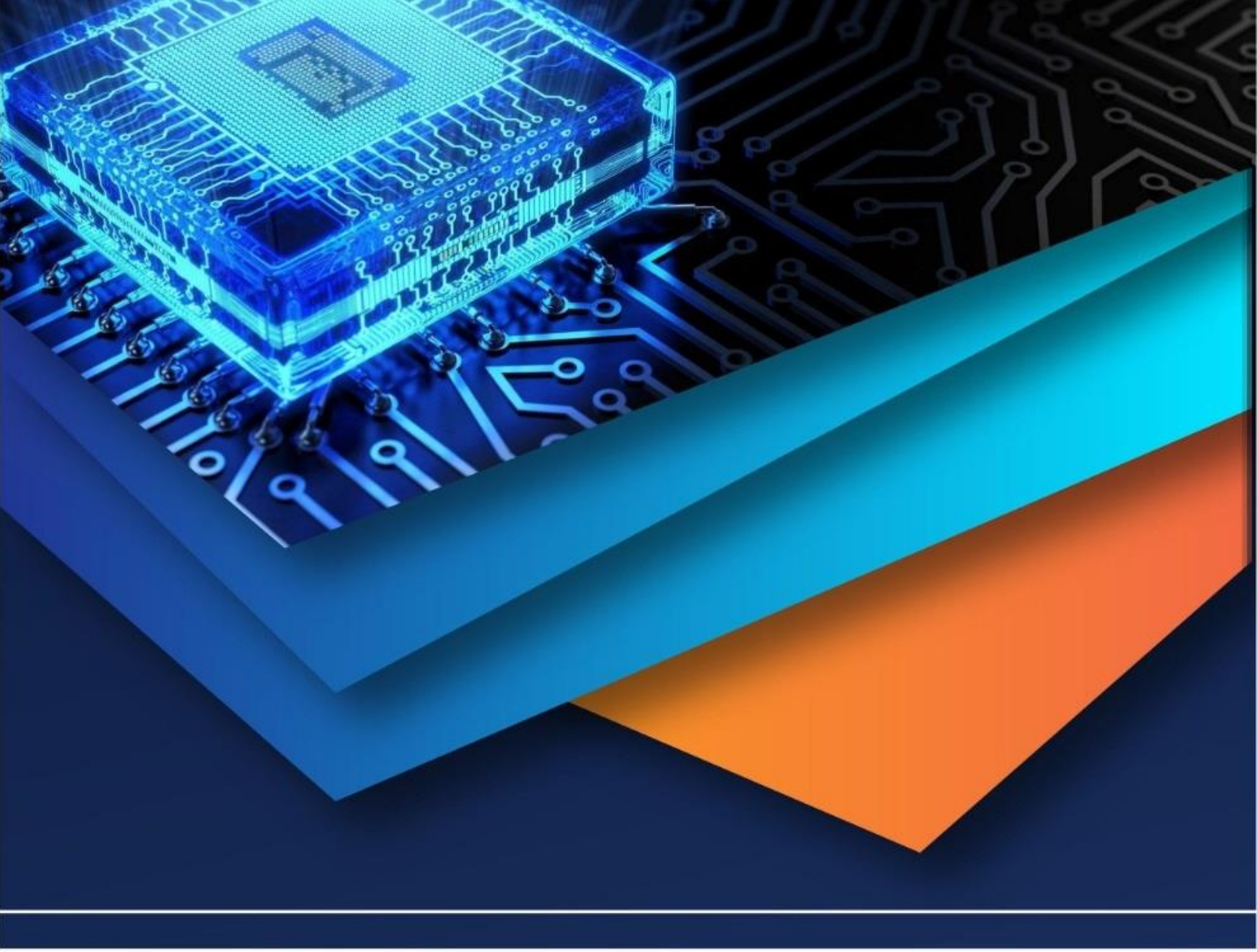


Fig. 2. The Structures and MS spectra of seven bioactive compounds in fermented mulberry. (a) Astragalin, (b) caffeic acid (c) Isochlorogenic acid (d) Isoquercetin (e) Kuwanon G, (f) Chlorogenic acid, (g) Rutin

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