Simultaneous determination of 49 amino acids, B vitamins, flavonoids and phenolic acids in vegetables by ultra-performance liquid chromatography–tandem mass spectrometry

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INTRODUCTION

Amino acids, vitamins, flavonoids, phenolic acids, and other natural plant active ingredients in vegetables have important nutritional and medicinal functions, and a protective potential for human health that has attracted considerable research interest. A sensitive and reliable analytical method was developed and validated for the simultaneous determination of 49 kinds of amino acids, B vitamins, flavonoids and phenolic acids in vegetables based on a rapid metabolomic extraction procedure combined with ultra-performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) in a single chromatographic run. Chromatographic and sample preparation conditions were thoroughly optimized, given the high diversity of the target analytes. Eight isotope-labeled standards were applied to validate the proposed method in terms of recovery, linearity, matrix effects, precision, and sensitivity.

MATERIALS AND METHODS

Sample preparation: Samples were ground to a fine powder in liquid nitrogen using an automatic tube mill (IKA, Germany), and 200 mg of the powder was weighed in a 1.5 mL centrifuge tube, then freeze-dried with a Christ Alpha 1-4 freeze dryer (Christ, Osterode, Germany). For the extraction process, 50 µL of isotope-labeled internal standard solution was added and allowed to stand for 30 min. Then, 1 mL of 50% methanol aqueous solution containing 0.2% vitamin C was added, and the tube was vortexed for 30 s. After extracting with a multi-tube vortexer for 10 min, the samples were centrifuged for 10 min at 15 000 rpm at 4ºC. Finally, 300 µL of supernatant was filtered through a PTFE filter (0.22 µm) and transferred into a sample vial for UPLC-MS/MS analysis.

HPLC conditions: The ACQUITY UPLC system was employed for analytical separation using a HSS T3 C18 column (2.1 × 100 mm, 1.8 µm, 100 Å, Waters). The mobile phases used were 0.02% formic acid in water (A) and 0.02% formic acid in methanol (B). The gradient was programmed as Table 1, the injection volume was 1 µL, the column temperature was set at 40ºC, and the flow rate was 0.2 mL/min.

Table 1. LOQ and linear range of detected mycotoxins.

Time (min)	A %	B %
0.5	99	1
2.5	92	8
15	50	50
18	5	95
18.1	99	1
20	99	1

MS/MS conditions: A QTRAP 4500 system (SCIEX) with electrospray ionization (ESI) probe was used. The MS source conditions were as follows: curtain gas (CUR), 35 psi; collision gas (CAD), medium; nebulizing gas (GS1), 50 psi; heater gas (GS2), 40 psi; ion spray voltage (IS), 5500V and -4500V in positive and negative modes, respectively; and source temperature, 550ºC.

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RESULTS

The parameters of the method were optimized, including chromatographic column, mobile phase, extraction solvent, and antioxidant addition. For the chromatographic column optimization, the experiment compared different brands and stationary phases. After examining the chromatographic peak shape, retention times and other aspects of evaluation, the final selection of the C18 chromatographic column ensured good separation and strong retention of the 49 individual nutrients in vegetables (Figure 1).

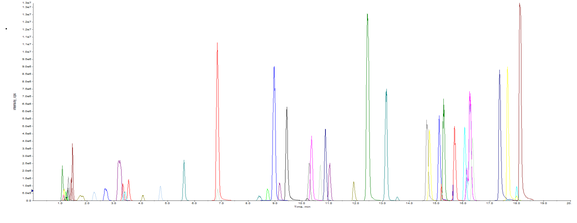

Figure 1. The extracted ion chromatogram (XIC) of the selected 49 standard compounds.

The mobile phase was also optimized. The responses and peak shapes of the selected analytes were affected by the addition of formic acid; thus, different concentrations of formic acid (0, 0.01%, 0.02%, and 0.05%) were tested. Stronger suppression was observed for higher formic acid concentrations for the majority of the compounds under both positive and negative conditions. Therefore, to balance the signal intensities and peak shapes, 0.02% formic acid was selected.

Methanol, ethanol, acetonitrile, and water can be used to extract plant metabolites. Here, these four extract solvents and mixtures were tested to extract the lyophilized powder of leeks. Except for quercetin-d3 in acetonitrile/water (1/1: v/v), the recoveries of the isotope internal standards were over 86.33%, within the acceptable range. It was found that the peak shape had an influence on the response, and the peak shape obtained using methanol/water (1/1: v/v) was the best.

The addition of the antioxidant vitamin C and its concentration were optimized. Increased recoveries and reduced RSDs were obtained for some compounds by the addition of vitamin C; in particular, the RSDs were reduced to less than 5% at a concentration of 0.2%. Therefore, 0.2% vitamin C solution was used in the extraction process.

Method validation was carried out in terms of recoveries, linearity, matrix effects, precision, and sensitivity. The application of isotope-labeled standards showed good method validation results. Most of the recoveries in the four vegetable matrices ranged from 65.0% to 105.3% with associated RSDs< 20% (Figure 2). Low LOQs were obtained, ranging from 0.06 to 17 µg/kg. Different ranges of linear calibration curves were established with R^2 >0.993. The relative standard deviations (RSDs) of intra-day precisions (n=5) for the analytes were ranged from 0.64% to 9.37%.(Table 2 and 3)

The proposed method has been successfully applied for accurate quantification of 49 compounds in 26 vegetables from different categories (Figure 3). The concentration of these compounds in vegetables was found to be highly species and variety dependent. Purple cabbage had the highest content of free amino acids and broccoli had higher levels of free B vitamins. Compositae and Apiaceae vegetables contained very high concentrations of chlorogenic acid, resulting in high total phenolic compound contents for these vegetables, especially red lettuce. This methodology was proved to be simple, reliable and highly efficient for determination of the selected 49 nutrients and antioxidants in vegetables, which could be used for nutritional and metabolomics studies.

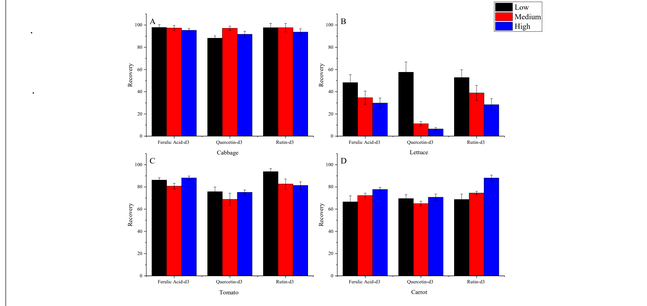

Figure 2. The recoveries of ferulic acid-d3, quercetin-d3, and rutin-d3 in four vegetables at different spiking levels.

Table 2. Linear regression, ME, LOD and LOQs of 8 isotope-labeled standards in lettuce.

Analyte (lettuce)	Linear range(µg/kg)	Regression equation	R^2	ME(%)	LOD (µg/kg)	LOQ (µg/kg)
L-Glutamic Acid-$^{13}C_5$	10-10000	y=1729.55621x+33623.4	0.9995	-55.63	0.22	0.74
L-Methionine-d3	100-10000	y=837.91433x+16745.43625	0.9996	2.77	1.70	5.62
L-Phenylalanine-d7	10-2000	y=13280.68722x+34370.6	0.9980	5.95	0.09	0.30
Pantothemic acid-$^{13}C_3$ ^{15}N	1-2000	y=2900.91092x+622.87375	0.9999	19.18	0.18	0.58
Pyridoxal-d3	1-500	y=9654.57849x+6433.70776	0.9968	11.21	0.15	0.50
Ferulic Acid-d3	10-2000	y=11431.91598x+17028.02787	0.9997	6.3	0.14	0.46
Quercetin-d3	1-2000	y=16208.78866x+50316.2	0.9963	4.48	0.09	0.31
Rutin-d3	1-10000	y=4435.43299x+5501.43207	0.9999	10.66	0.07	0.23

Table 3. Linear regression, ME, LOD and LOQs of 8 isotope-labeled standards in cabbage.

Analyte (cabbage)	Linear range(µg/kg)	Regression equation	R^2	ME (%)	LOD (µg/kg)	LOQ (µg/kg)
L-Glutamic Acid-$^{13}C_5$	10-10000	y=2020.95185x+17768	0.9997	-48.15	0.24	0.79
L-Methionine-d3	100-10000	y=862.97275x+4267.26	0.9987	5.84	2.5	8.3
L-Phenylalanine-d7	10-2000	y=13128.78236x+25419.9643	0.9988	4.74	0.10	0.32
Pantothemic-acid-$^{13}C_3$ ^{15}N	1-2000	y=2819.04048x+742.16426	0.9999	15.81	0.11	0.35
Pyridoxal-d3	1-500	y=9402.13662x+6544.84482	0.9970	8.31	0.17	0.56
Ferulic Acid-d3	10-2000	y=11105.05762x+20043.3755	0.9991	3.26	0.13	0.43
Quercetin-d3	1-2000	y=15676.74755x+54264.4	0.9967	1.05	0.06	0.19
Rutin-d3	1-10000	y=4105.75726x+6471.67083	0.9998	2.44	0.08	0.27

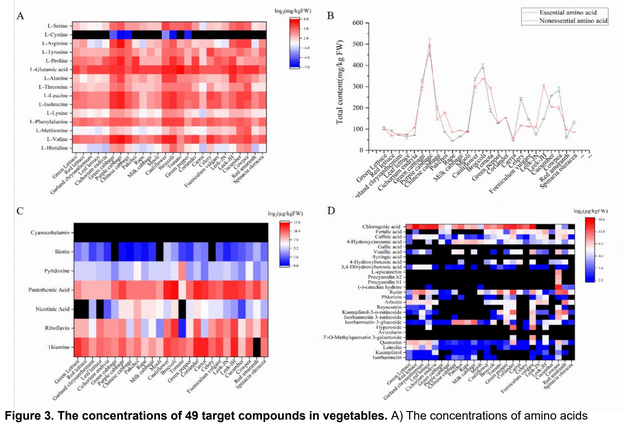

Figure 3. The concentrations of 49 target compounds in vegetables. A) The concentrations of amino acids [log2 (mg/kg FW)]. B) The total contents of 8 essential amino acids and 7 non-essential amino acids(mg/kg FW). C) The concentrations of B vitamins [log2 (µg/kg FW)]. D) The concentrations of phenolic compound [log2 (µg/kg FW)]. Black indicates undetected or lower than LOQ.

CONCLUSIONS

In this study, a sensitive and reliable UPLC-MS/MS method for the simultaneous rapid qualitative and quantitative analyses of 15 amino acids, 7 B vitamins, and 27 polyphenols in vegetables was developed, optimized, and validated. Method validation was carried out in terms of recoveries, linearity, matrix effects, precision, and sensitivity. The application of isotope-labeled standards ensured good method results. The proposed method has been successfully applied to the accurate quantification of 49 compounds in 26 vegetables from different categories.

The concentrations of these compounds in vegetables were found to be highly species- and variety-dependent. Purple cabbage had the highest content of free amino acids, and broccoli had higher levels of free B vitamins. Compositae and Apiaceae vegetables contained very high concentrations of chlorogenic acid, resulting in high total phenolic compound content for these vegetables, particularly red lettuce. This methodology was shown to be simple, reliable, and highly efficient for the determination of the selected 49 nutrients and antioxidants in vegetables and could be used for nutritional and metabolomics studies.

TRADEMARKS/LICENSING