

## Original Research Article

# A comparative study of HPLC-DAD and UPLC-UV methods for simultaneous determination of 11 polyphenols in *Moringa oleifera* leaves

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### Abstract

**Purpose:** To develop, validate and compare two chromatographic methods - high performance liquid chromatography with diode array detector (HPLC-DAD) and high performance liquid chromatography with ultraviolet detection (UPLC-UV) for the effective analysis of polyphenols in *Moringa oleifera* leaves.

**Methods:** HPLC-DAD and UPLC-UV methods were applied for the accurate determination of eleven major polyphenols in *Moringa oleifera* leaves. The chromatographic conditions of the eleven polyphenols was determined on two C18 column by gradient elution with 0.5 % phosphoric acid solution -acetonitrile as the eluate, and at a flow rate of 1.0 and 0.5 mL/min for HPLC-DAD and UPLC-UV methods, respectively. Detector parameter of UPLC-UV was fixed at 203 nm. The assay methods were validated systematically.

**Results:** The instrumental methods (HPLC-DAD and UPLC-UV) had good linearity, precision, repeatability and recovery. For both methods, quantification limits of UPLC-UV (0.057 - 0.363 µg/mL) were lower than those of UPLC-UV (0.094 - 1.532 µg/mL). The UPLC method with a shorter running time and more sensitive detection was applied in comparing to the HPLC method. After optimization and evaluation, the baseline of 11 compounds was separated effectively within 68 and 34 min, respectively.

**Conclusion:** The developed HPLC-DAD and UPLC-UV assays were successfully utilized for the simultaneous analysis of eleven major polyphenols and can readily be utilized as quality control tools for *Moringa oleifera* leaves in China, with UPLC-UV method showing better separation, lower organic solvent usage and shorter analytical period.

**Keywords:** *Moringa oleifera*, Polyphenols, HPLC-DAD, UPLC-UV, Validation

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## INTRODUCTION

*Moringa oleifera* is a multipurpose plant native to India and widely distributed in many tropical and sub-tropical countries and regions [1]. It is

regarded as one of the most valuable trees in the world, because almost all parts can be used as food and medicine [2]. Leaf powder has been used as a source of alternative medicines to combat malnutrition, especially in children and

infants [3]. Leaves of *Moringa oleifera* are popular as a dietary supplement to improve the malnutrition and related ailments [4].

Plant polyphenols are widespread in the important metabolites in plants. The secondary metabolites of these complex phenols include flavonoids, tannins, phenolic acids, and anthocyanins, etc., and they are the main active components with good antioxidant activity and bacteriostatic efficacy. *Moringa oleifera* polyphenol is a natural active substance. It can be used as the functional health care product. According to a large body of literature, the polyphenols assay of leaves was much higher than that in other parts, with the aqueous or alcohol extract of leaves containing polyphenols [5], that significantly inhibited bacteria [6-8], are antioxidants [5,6,9], prevented ulcerative colitis [6,9], immune regulation [6,7], inhibited human cancer cell proliferation [5] and liver damage [10], scavenging of free radicals harmful to human health [5] and hypoglycemic action [11-13].

Reported assay methods have been applied to determine polyphenols in *Moringa oleifera*, and seven phenolic compounds are discriminated by LC-MS from *Moringa oleifera* flowers and leaves [6]. A LC-MS method was exploited for the assay of twelve flavonoids constituents in the methanol fraction of leaf [14], while eleven phenolic compounds from *Moringa oleifera* leaves were determined by UPLC-ESI-MS/MS [15], although the overhead costs of such equipment are higher. HPLC was successfully used for quantitative determination of three active compounds in *Moringa oleifera* leaves [16]. However, this method still has some defects including high organic solvent usage, long analytical period and poor resolution.

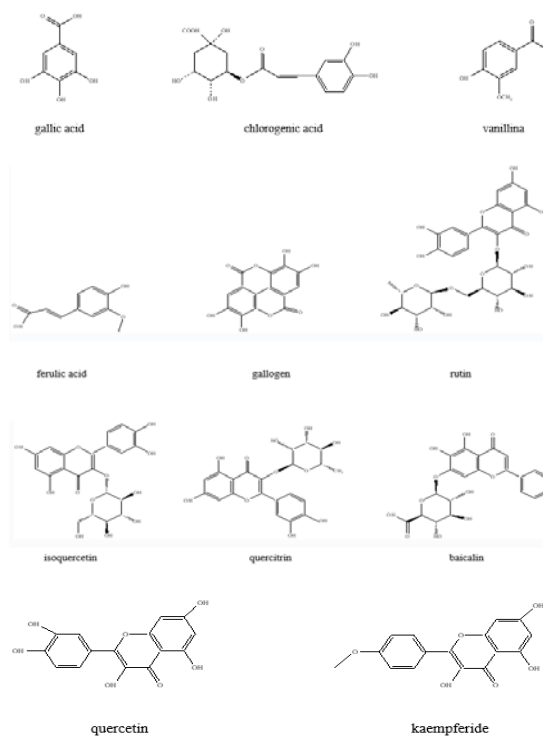
In this study, two chromatographic methods (HPLC-DAD and UPLC-UV) for effective components analysis in *Moringa oleifera* leaves. After optimization and evaluation, the two methods were established for determination the assay of 11 polyphenols in *Moringa oleifera* leaves from different habitats. The developed methods were efficiently validated and used in the quality research of *Moringa oleifera* leaves.

## EXPERIMENTAL

### Reagents and materials

Standards of 11 polyphenols were supplied by National Institute for Food and Drug Control (Beijing, China). The structures of eleven polyphenols are presented in Figure 1. Purified water was ultrapure (Milli-Q system, Millipore,

USA) and HPLC grade acetonitrile was imported from Germany (Merck Co., Darmstadt).



**Figure 1:** Chemical structures of the investigated eleven polyphenols

### Samples

*Moringa oleifera* leaves were gathered from various planting sites in Yunnan (Table 1). Each sample was homogenized, pulverized in a mill, and sifted through an aperture size of 0.15 millimeter before analysis.

### Sample preparation

1.0 g of *Moringa oleifera* leaves was precisely weighed, transferred into a brown measuring flask (10 mL), and then made to volume using added carbinol to scale. The mixture was subjected to ultrasonification on a water bath at 20 °C for 40 min and net loss in weight was replaced with methanol. All injected solutions should be filtered with a needle filter prior to analysis. Finally, the extracting solutions were automatically eluted by HPLC-DAD and UPLC-UV system for analysis, respectively.

### Standard preparation

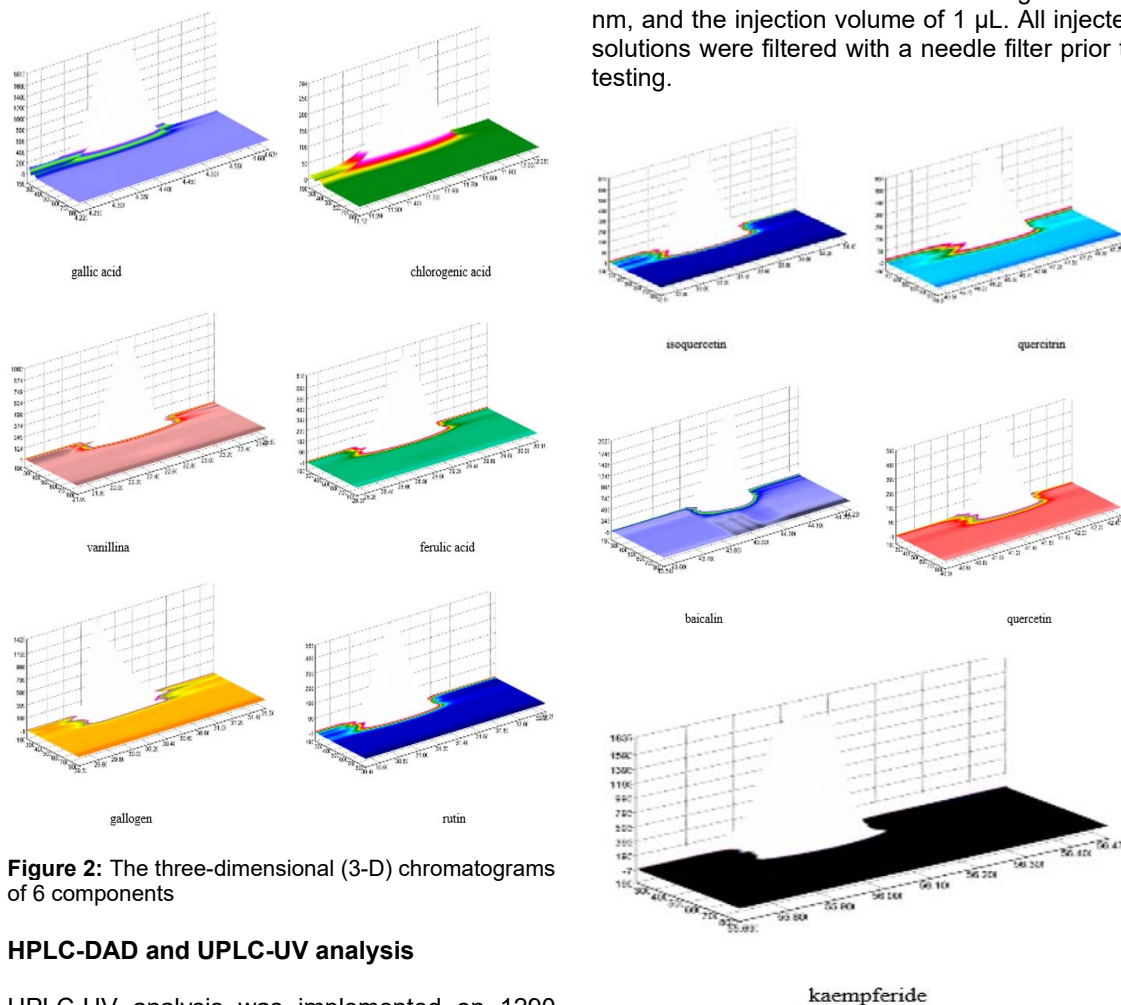
A standard stock solution for 11 polyphenols was prepared in methanol, respectively. The standard stock solutions were made up to different concentrations with methanol, and the mixed

**Table 1:** Sample information of *Moringa oleifera* leaves gathered from Yunnan Province

No.	Sample source	Collection time	Description
S1	Mili township, Yuanjiang county	2018-3-1	dried
S2	Sancun township, Honghe county	2018-3-2	dried
S3	Yangjie town, Yuanmou county	2018-3-3	dried
S4	Lianchi township, Yongren county	2018-3-3	dried
S5	Jinji township, Longyang district	2018-3-4	dried
S6	Mangshi jiangdong township	2018-3-6	dried
S7	Longtan township, Yuanjiang county	2018-3-1	dried
S8	Mangshi military division	2018-3-6	dried
S9	Pichang village, Binchuan county	2018-3-5	dried
S10	Yuanma town, Yuanmou county	2018-3-3	dried

working standard solution was prepared for the calibration curve. All standard solutions were placed in the refrigerated area of the refrigerator before analysis. The three-dimensional (3-D) chromatograms of 11 components were listed in Figures 2 and 3. Each peak corresponded to the different time and signal.

system, a 1290 variable wavelength scanning UV detector and an autosampler. The column used was an Agilent ZORBAX Eclipse Plus C18 (100 mm length, 1.8  $\mu$ m particle diameter). The elution was implemented on a gradient solvent system using acetonitrile-0.5%  $H_3PO_4$  solution as mobile phases. The linear gradient programs are as listed in Table 2. Other UPLC-UV parameters were set as follows: UV-vis wavelength of 203 nm, and the injection volume of 1  $\mu$ L. All injected solutions were filtered with a needle filter prior to testing.

**Figure 2:** The three-dimensional (3-D) chromatograms of 6 components

### HPLC-DAD and UPLC-UV analysis

UPLC-UV analysis was implemented on 1290 Infinity UPLC system (Agilent Technologies, Waldbroon, Germany), which was equipped with a four-dimensional pump solvent management

**Figure 3:** The three-dimensional (3-D) chromatograms of 5 components

**Table 2:** Gradients: UPLC-UV and HPLC-DAD

UPLC-UV				HPLC-DAD			
Time (min)	Flow (mL/min)	Acetonitrile (%)	0.5% H <sub>3</sub> PO <sub>4</sub> solution (%)	Time (min)	Flow (mL/min)	Acetonitrile (%)	0.5% H <sub>3</sub> PO <sub>4</sub> solution (%)
0.0	0.5	10.0	90.0	0.0	1.0	10.0	90.0
20.0	0.5	20.0	80.0	40.0	1.0	20.0	80.0
27.5	0.5	60.0	40.0	41.0	1.0	70.0	30.0
28.0	0.5	90.0	10.0	41.2	1.0	20.0	80.0
30.5	0.5	90.0	10.0	55.0	1.0	70.0	30.0
31.0	0.5	10.0	90.0	56.0	1.0	90.0	10.0
34.0	0.5	10.0	90.0	61.0	1.0	90.0	10.0
/	/	/	/	62.0	1.0	10.0	90.0
/	/	/	/	68.0	1.0	10.0	90.0

HPLC-DAD analysis was implemented on Dionex Ultimate 3000 HPLC system, which was equipped with a four-dimensional pump solvent management system, 3000 DAD detector and an autosampler. The column used was a Welchrom C18 (250 mm length, 5  $\mu$ m particle diameter). The gradient elution procedure was shown in Table 2. DAD detector was monitored to scan within the wavelength range of 200-800 nm and detected at 203nm for quantitative analysis.

As seen in Table 2, the UPLC-UV method had lower organic solvent usage and shorter analytical period.

#### Validation and verification procedures

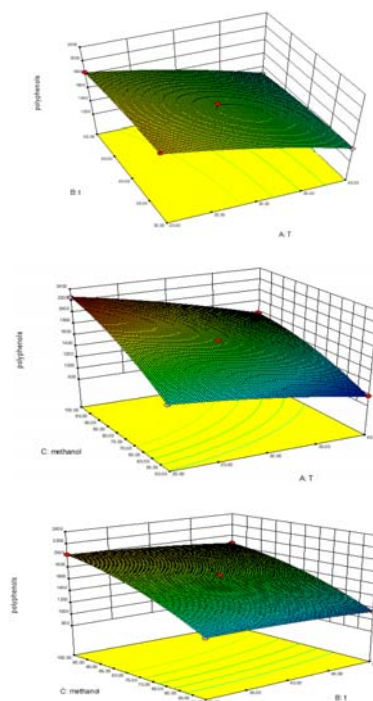
Linearity, LOD, LOQ, precision, repeatability and recovery were validated for the method.

Linearity was constructed on a series of 11 polyphenols standard solutions. The calibration curves were obtained by taking concentration as abscissa and peak area as ordinate. The correlation coefficients ( $R^2$ ) were determined on the basis of the regression analysis. LOD and LOQ of eleven polyphenols were determined by diluting the standard sample to a lower concentration. The intraday and interday precisions were assessed by 6 replicated injections of a mixture of standard solutions in a single day and once a day for 6 consecutive days. Repeatability was studied by injecting 6 parallel samples that were pretreated by the preparation procedure of the same batch sample. The recovery test was measured by spiking the same standard mixtures into six different powders of the same sample. The recovery rate was evaluated as follows: Recovery = (Detection content - original content) / added content  $\times$  100.

## RESULTS

### Extracting conditions

Response surface methodology is an optimization strategy used in the experimental analytical studies, which helps to understand the interactions between multiple variables. To obtain the optimum extraction conditions, extraction patterns (ultrasonication, reflux, and soxhlet extraction), extraction solvents (water, 30, 50, 70 and 100 % methanol), temperature of solution (20, 30, 40 and 50  $^{\circ}$ C) and leaching time (10, 30, 40 and 60 min) were researched. The optimal sample pretreatment parameters were modeled by response surface methodology according to Box-Behnken Design (Figure 4).



**Figure 4:** Response surface of extraction solvents, extraction temperature and extraction time

The extraction capacity was evaluated by the withdrawal rates and the total peak areas about eleven extracted polyphenols. The best extraction conditions were as follows: *Moringa oleifera* leaves were preprocessed for 40 min by ultrasonication using methanol solution at 20 °C. This method of sample preparation is simple, rapid, sensitive, and easy to apply in large scale production.

### Comparison of the validation results for UPLC-UV and HPLC-DAD methods

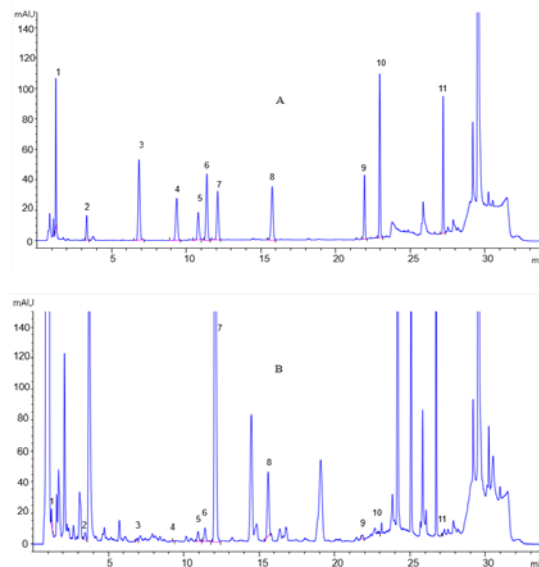
The contrastive research between the two methods of the assay was implemented for linearity, quantitation limit, precision, and accuracy. The eleven compounds in both methods showed a good linearity for the entire range ( $R > 0.999$ ). LOD and LOQ were measured in *Moringa oleifera* leaves by the UPLC-UV method and the HPLC-DAD method. RSDs of precision (intra-day and inter-day) and repeatability ( $n = 6$ ) were less than 2%. The recovery experiment was done with *Moringa oleifera* leaves. The recovery of 11 analyzed components ranged from 90.08 to 98.25%, and the resulting RSD values were all less than 3%. The validation data of eleven polyphenols are shown in Table 3 of UPLC-UV and Table 4 of HPLC-DAD.

### Quantitative analysis of samples by using UPLC-UV and HPLC-DAD

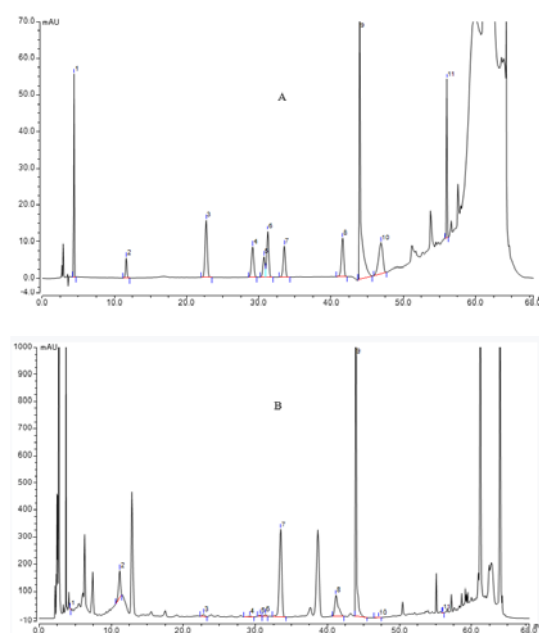
To test the analytical methods for practical application, the proposed methods were used for the measurement of eleven polyphenols in 10 samples of *Moringa oleifera* leaves sourced from different places. The representative chromatograms of 11 compounds and the sample (S7) as well as results for the contents of eleven components in *Moringa oleifera* determined using the UPLC-UV and HPLC-DAD methods are shown in Figures 5 and 6, Table 5 and 6, respectively. By comparing the standard chromatograms of Figure 5 and 6, it was observed that the UPLC-UV method had better separation and shorter analytical period. In addition, the concentration differed among eleven polyphenols in 10 samples from different places, with only two of the ten samples containing the 11 components tested.

Isoquercetin among all 11 components was the highest, and the isoquercetin content in S10 sample was the largest in all samples. The growth of *Moringa oleifera* leaves may be influenced by humidity, water, soil, elevation and climate [17], making the obtained result reasonable. These data suggest that plant source should lead to further study during

utilizing *Moringa oleifera* leaves as health care products.



**Figure 5:** UPLC-UV Chromatograms of (A) standards and (B) the S7 sample (1: gallic acid; 2: chlorogenic acid; 3: vanillina; 4: ferulic acid; 5: gallogen; 6: rutin; 7: isoquercetin; 8: quercitrin; 9: baicalin; 10: queretin; 11: kaempferide)



**Figure 6:** HPLC-DAD Chromatograms of (A) standards and (B) the S7 sample (the same peak marked in Figure 5)

## DISCUSSION

*Moringa oleifera* is considered one of the most useful trees in the world because almost all parts of this plant can be used as medicines.

**Table 3:** Validation results for UPLC-UV quantitative analysis for eleven polyphenols

Polyphenols	Gallic acid	Chlorogenic acid	Vanillina	Ferulic acid	Gallogen	Rutin	Isoquercetin	Quercitrin	Baicalin	Quercetin	Kaempferide
Linearity study											
Calibration curves	y=4908.85x	y=1894.59x	y=6254.19x	y=3667.83x	y=2772.89x	y=4504.22x	y=4188.93x	y=6337.53x	y=3652.60x	y=9221.25x	y=8000.53x
R <sup>2</sup>	0.99990	0.99991	0.99991	0.99994	0.99990	0.99995	0.99991	0.99996	0.99993	0.99995	1.00000
Linear range (µg/mL)	0.00-162.45	0.0-122.85	0.00-173.70	0.00-171.00	0.00-135.00	0.00-179.90	0.00-145.50	0.00-127.20	0.00-160.20	0.00-142.00	0.00-112.70
LOD (µg/mL)	0.019	0.091	0.043	0.085	0.109	0.059	0.070	0.050	0.054	0.019	0.017
LOQ (µg/mL)	0.063	0.304	0.144	0.282	0.363	0.195	0.235	0.168	0.179	0.063	0.057
Precision (n =6)											
RSD (%) (intraday)	0.252	1.132	0.323	0.951	1.158	1.304	1.668	1.013	0.848	1.097	0.872
RSD (%) (interday)	1.695	1.307	0.618	1.229	1.055	0.940	1.311	1.778	1.072	1.899	0.724
Repeatability (n =6)											
Mean (µg/g)	2.032	9.815	4.321	3.652	4.635	7.869	1304.671	5.418	1.635	4.192	1.653
RSD (%)	1.651	1.046	1.285	1.973	1.563	1.982	1.386	1.093	1.865	1.981	1.758
Recovery (n =6)											
Mean (%)	91.512	93.657	92.369	95.051	90.378	91.452	98.253	92.568	90.083	90.356	90.142
RSD (%)	2.057	2.965	2.124	2.158	2.893	2.596	1.145	2.628	2.142	2.971	2.265

**Table 4:** Validation results of HPLC-DAD quantitative analysis for eleven polyphenols

Polyphenols	Gallic acid	Chlorogenic acid	Vanillina	Ferulic acid	Gallogen	Rutin	Isoquercetin	Quercitrin	Baicalin	Quercetin	Kaempferide
Linearity study											
Calibration curves	y=769.98x	y=153.06x	y=783.12x	y= 458.55x	y= 326.62x	y= 346.75x	y= 443.08x	y= 792.59x	y= 792.59x	y=1094.03x	y=998.03x
R <sup>2</sup>	0.99992	0.99997	0.99993	0.99995	0.99998	0.99999	0.99993	0.99999	0.99999	0.99999	0.99998
Linear range (µg/mL)	0.00-64.98	0.00-49.14	0.00-69.48	0.00-68.40	0.00-54.00	0.00-71.96	0.00-58.20	0.00-50.88	0.00-64.08	0.00-56.80	0.00-45.08
LOD (µg/mL)	0.058	0.460	0.132	0.253	0.276	0.241	0.233	0.141	0.040	0.188	0.028
LOQ (µg/mL)	0.193	1.532	0.441	0.845	0.920	0.803	0.777	0.470	0.133	0.627	0.094
Precision (n =6)											
RSD (%) (intraday)	0.402	0.417	0.140	0.844	1.305	0.877	0.548	0.267	1.126	1.449	0.393
RSD (%) (interday)	1.497	1.086	1.122	1.702	1.210	1.898	1.052	1.644	1.920	1.866	0.541
Repeatability (n =6)											
Mean (µg/g)	2.015	9.837	4.306	3.638	4.681	7.825	1303.162	5.431	1.608	4.173	1.649
RSD (%)	1.205	1.158	1.072	1.869	1.756	1.095	1.462	1.251	1.743	1.934	1.859
Recovery (n =6)											
Mean (%)	92.313	90.025	91.351	90.802	93.625	91.846	92.384	90.408	92.071	90.536	91.863
RSD (%)	2.138	2.057	2.289	2.543	2.987	2.706	1.360	2.573	2.329	2.085	2.175

**Table 5:** Contents of eleven components in ten samples of *Moringa oleifera* leaves from the different places with UPLC-UV ( $\mu\text{g/g}$ )

No.	Gallic acid	Chlorogenic acid	Vanillina	Ferulic acid	Gallogen	Rutin	Isoquercetin	Quercitrin	Baicalin	Quercetin	Kaempferide
S1	2.905	80.172	3.680	1.032	1.889	104.562	1115.260	2.004	6.337	1.981	0.027
S2	2.059	97.541	5.760	1.286	5.641	120.582	1357.221	1.411	1.293	5.175	n.d
S3	1.451	97.049	13.456	1.340	6.306	0.597	1212.297	1.842	1.409	6.428	n.d
S4	1.661	99.410	6.077	n.d	2.748	1.308	1429.117	0.533	0.697	2.768	n.d
S5	2.425	48.933	6.866	0.724	3.915	2.182	1126.765	1.129	n.d	4.880	n.d
S6	4.665	96.439	16.712	1.868	8.619	n.d	1130.040	0.478	n.d	8.574	n.d
S7	2.032	9.815	4.321	3.652	4.635	7.869	1304.671	5.418	1.635	4.192	1.653
S8	6.574	107.419	14.263	1.548	8.794	1.420	1312.009	0.676	0.862	8.466	n.d
S9	5.119	58.818	13.975	0.894	7.164	720.619	1157.225	6.068	9.373	8.687	0.291
S10	1.835	135.346	2.051	0.877	1.997	20.663	1493.764	2.436	0.539	1.918	n.d

n.d: not detected

**Table 6:** Contents of eleven components in ten samples of *Moringa oleifera* leaves from the different places with HPLC-DAD ( $\mu\text{g/g}$ )

No.	Gallic acid	Chlorogenic acid	Vanillina	Ferulic acid	Gallogen	Rutin	Isoquercetin	Quercitrin	Baicalin	Quercetin	Kaempferide
S1	2.791	80.023	3.663	1.105	1.865	104.395	1114.956	1.859	6.579	1.975	n.d
S2	2.106	97.375	5.802	1.269	5.653	120.061	1356.984	1.291	1.316	5.408	n.d
S3	1.468	96.871	13.608	1.294	6.401	0.575	1212.153	1.764	1.391	6.394	n.d
S4	1.694	99.398	6.115	n.d	2.764	1.317	1430.002	0.549	0.715	2.705	n.d
S5	2.392	48.942	6.826	0.738	3.892	2.197	1126.981	1.093	n.d	4.790	n.d
S6	4.705	96.389	16.495	1.893	8.623	n.d	1131.056	0.496	n.d	8.703	n.d
S7	2.015	9.837	4.306	3.638	4.681	7.825	1303.162	5.431	1.608	4.173	1.649
S8	6.597	107.405	14.359	1.557	8.802	1.443	1311.929	0.628	0.882	8.621	n.d
S9	5.102	58.794	13.957	0.873	7.091	720.567	1156.837	6.307	9.043	8.915	0.316
S10	1.808	135.597	2.039	0.886	2.059	20.637	1492.897	2.698	0.497	1.879	n.d

n.d: not detected



Polyphenols have been reported to play an important role in *Moringa oleifera* leaves with good antioxidant activity and bacteriostatic efficacy. However, there has been no report on the simultaneous determination of eleven polyphenols contents from *Moringa oleifera* leaves in China by using HPLC-DAD and UPLC-UV. In this paper, according to optimized conditions, the two methods were validated and successfully applied to examine the contents of eleven polyphenols in *Moringa oleifera* leaves. These eleven compounds in both methods showed good linearity ( $R^2 \geq 0.9999$ ) and the low RSD values ( $< 2\%$ ) assured good precision. The recovery of the analyzed components ranged from 90.083 to 98.253%. As far as UPLC-UV and HPLC-DAD methods were concerned, the UPLC-UV method showed better resolution, speed and sensitivity for the quantification of eleven compounds in *Moringa oleifera* leaves.

In this work, two simple, reliable, and accurate UPLC-UV and HPLC-DAD method were used for the determination of eleven polyphenols in *Moringa oleifera* leaves from different locations in Yunnan. The proposed UPLC-UV and HPLC-DAD assay was optimized by the ratio of mobile phase and the shape of the gradient to obtain satisfactory separation results. It also compares the orthodox HPLC-UV method with the new fashioned UPLC-DAD method. Nevertheless, LOD and LOQ values of UPLC-UV method show the high sensibility. This made it more important not only for quality assurance and quality control researches but also for the determination of blood concentration and its pharmacokinetic study in the future. Generally, the analytical method that allowed the determination of eleven polyphenols using UPLC-UV and HPLC-DAD could be applied to an alternative in agriculture and food sector.

## CONCLUSION

The results show that the instrumental methods (HPLC-DAD and UPLC-UV) demonstrate good linearity, LOD, LOQ, precision, repeatability and recovery when applied in the quantitative assay of *Moringa oleifera* leaves. The proposed methods have been successfully used for the quantitative analysis of 11 main analytes in *Moringa oleifera* leaves simultaneously. At the same time, two methods could be directly applied for the quality assurance of *Moringa oleifera* leaves and related products. However, the UPLC-UV method has better separation, lower organic solvent usage and shorter analytical period.

## DECLARATIONS

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### Conflict of interest

No conflict of interest is associated with this study.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yanqin Zhu designed the study. Yanqin Zhu and Qinhong Yin did methodology and wrote this manuscript. Yanqin Zhu and Yaling Yang were responsible for data analysis. All authors read and approved the final manuscript for publication.

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