DEVELOPMENT OF AN LC/MS/MS METHOD TO SEPARATE AND ANALYZE CURCUMINOIDS, THEIR METABOLITES AND DEGRADATION PRODUCTS

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ABSTRACT

Turmeric, the powdered rhizome of *Curcuma longa*, has been widely used in Ayurvedic medicine to treat inflammation, tumors, skin wounds, infections and blistering diseases. It has also been used as a coloring agent and as a spice in many foods. The curcuminoids, namely curcumin, demethoxycurcumin, and bisdemethoxycurcumin, are the phenolic compounds responsible for the yellow color of turmeric. The biological activity of curcumin - as the major component of turmeric - has also been studied in recent years. It is not well known whether the curcumin, or the metabolites of the curcumin are responsible for the biological activity of turmeric. Also, curcumin and curcuminoids are not stable in blood and plasma, therefore the presence of degradation products might interfere with the analytical detection. The main goal of this study was to develop an LC/MS/MS method to simultaneously separate and identify the curcuminoids, their metabolites (tetrahydrocurcumin and hexahydrocurcumin) and degradation products (vanillin, vanillic acid, and ferulic acid) using a single analytical method. Reversed phase HPLC method with diode array detection was developed for the separation of the components. The eluent was monitored at 250nm, 280 nm and 425 nm for the presence of curcuminoids, their metabolites and degradation products. The segments of the negative electrospray ionization (ESI) mass spectrometric method were optimized for each individual component.

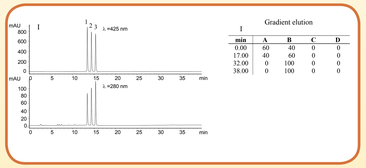
INTRODUCTION

Curcumin, demethoxycurcumin, and bisdemethoxycurcumin are the major yellow pigments obtained from turmeric, the rhizome of *Curcuma longa.* Turmeric has been used in different traditional systems in diverse Asian communities due its anti-inflammatory and chemopreventive effects. These effects have been attributed to curcumin, and because of its beneficial effects in human health, curcumin has become an interesting compound for researchers. Therefore, several studies have focused in understanding the metabolism of curcumin. A previous study in mice suggested that curcumin-glucuronidase, dihydrocurcumin-glucuronidase, tetrahydrocurcumin (THC), THC-glucuronidase are the major metabolites of curcumin in vivo [1]. Another study demonstrated that hexahydrocurcumin (HHC), hexahydrocurcuminol, and curcumin conjugates are the metabolites of curcumin in human and rat hepatocytes, and rats in vivo [2]. Vanillin, ferulic acid, and feruloyl methane were identified as minor degradation products when stability of curcumin was studied in different buffer solution [3]. Also, vanillin and ferulic acid was found as degradation products of curcumin when it was incubated with Ishikawa cells [4]. The analytical methods described in these studies were achieved to separate curcumin from its metabolites, from its degradation products, and from the other two curcuminoids. The main goal of this study was to separate and identify curcumin, demethoxycurcumin, bisdemethoxycurcumin, hexahydrocurcumin, tetrahydrocurcumin,vanillin, vanillic acid, and ferulic acid using a single analytical method.

EXPERIMENTAL PROCEDURE

►A reversed phase gradient HPLC and LC/MS/MS method were developed to separate and identify curcumin (C), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC), tetrahydrocurcumin (THC), hexahydrocurcumin (HHC), vanillin, vanillic acid and ferulic acid in a single analytical method.
►Agilent 1100 HPLC system (quaternary gradient pump, degasser, diode array detector, thermostatted column compartment and autosampler) with Phenomenex Synergi 4 µ Hydro-RP column (250 x 4.6 mm), and C18 guard cartridge (4.0 x 3.0 mm) was used to obtain the chromatograms.
►0.05% acetic acid in Nanopure water (A), 0.05% acetic acid in acetonitrile (B), 1% ammonium acetate (C), and 0.05% acetic acid in methanol (D) were used as mobile phases using the following profile: 0-4 min, 2% B-98%C; 4-10 min, 40%B-60%C; 10-11 min, 100%C; 11-13 min, 40%C-60%D; 13-14 min, 40%C-60%D; 14-15 min, 60%A-40%B; 15-32 min, 40%A-60%B; 32-47 min, 100%B.
►The flow rate was 1.0 mL/min, the signals were observed at 425 nm, 250 nm, 280 nm, 370 nm, and 340 nm.
►Agilent 1100 system tandem with Agilent MSD-Trap-SL ion trap mass spectrometer in negative electrospray ionization (ESI) mode was used to obtain de MS spectra. The parameters for each component were obtained performing optimization by infusing the compounds separately.The negative ESI mass spectrometric method was created with optimized segments for every compound.
►Nitrogen was used as a drying gas at a flow rate of 10 L/min, the nebulizer pressure was set at 60 psi, the drying temperature was maintained at 350 ˚C, and capillary voltage was 4000 V.
►Curcumin, demethoxycurcumin, and bisdemethoxycurcumin analytical standards were isolated and purified from turmeric rhizome. Hexahydrocurcumin was provided by Dr. Manfred Metzler, University of Kalrsruhe, Germany.Tetrahydrocurcumin, and Curcumin C[3] Complex® (74.77% C, 17.98% DMC, and 3.15% BDMC based on the official certificate of analysis) were obtained from Sabinsa Corporation, Piscataway, NJ, USA. Vanillin, vanillic acid, and ferulic acid were obtained from Fluka, Milwaukee, WI, USA. All standard solutions were prepared in acetonitrile.

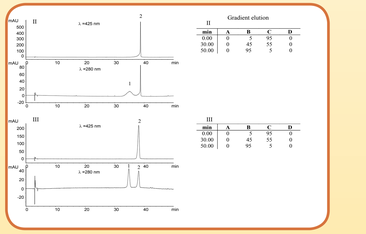
HPLC METHOD DEVELOPMENT



Gradient elution

I				
min	A	B	C	D
0.00	60	40	0	0
17.00	40	60	0	0
32.00	0	100	0	0
38.00	0	100	0	0

HPLC chromatograms of 100 µg/mL BDMC (1), 100 µg/mL DMC (2), and 100 µg/mL C (3) in methanol. Mobile phase: A = 0.05% acetic acid in Nanopure water; B = 0.05% acetic acid in acetonitrile. Original method for the separation of the three curcuminoids

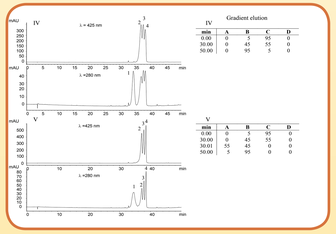


Gradient elution

II				
min	A	B	C	D
0.00	0	5	95	0
30.00	0	45	55	0
50.00	0	95	5	0

III				
min	A	B	C	D
0.00	0	5	95	0
30.00	0	45	55	0
50.00	0	95	5	0

HPLC chromatograms of 386 µg/mL THC (1), and 369 µg/mL C (2) in methanol. Mobile phase: B = 0.05% acetic acid in acetonitrile; C = 0.1% ammonium acetate (chromatograms II), 1% ammonium acetate (chromatograms III)

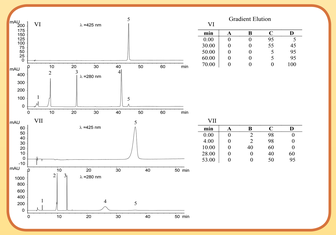


Gradient elution

IV				
min	A	B	C	D
0.00	0	5	95	0
30.00	0	45	55	0
50.00	0	95	5	0

V				
min	A	B	C	D
0.00	0	3	95	0
30.00	0	45	55	0
30.01	55	45	0	0
50.00	5	95	0	0

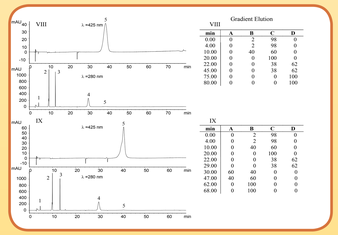
HPLC chromatograms of 502 µg/mL THC (1), 558 µg/mL BDMC (2), 522 µg/mL DMC (3), and 513 µg/mL C (4) standards in acetonitrile. Mobile phase: A = 0.05% acetic acid in Nanopure water; B = 0.05% acetic acid in acetonitrile; C = 1% ammonium acetate



Gradient Elution

VI				
min	A	B	C	D
0.00	0	0	95	5
30.00	0	0	55	45
50.00	0	0	5	95
60.00	0	0	5	95
70.00	0	0	0	100

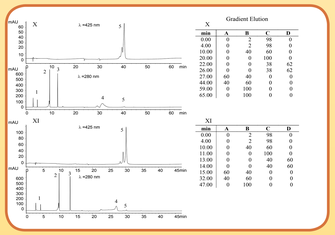
VII				
min	A	B	C	D
0.00	0	2	98	0
4.00	0	2	98	0
10.00	0	40	60	0
28.00	0	0	40	60
53.00	0	0	50	95



Gradient Elution

VIII				
min	A	B	C	D
0.00	0	2	98	0
4.00	0	2	98	0
10.00	0	40	60	0
22.00	0	0	100	0
45.00	0	0	38	62
75.00	0	0	0	100
80.00	0	0	0	100

IX				
min	A	B	C	D
0.00	0	2	98	0
4.00	0	2	98	0
10.00	0	40	60	0
22.00	0	0	100	0
45.00	0	0	38	62
29.00	0	0	38	62
30.00	60	40	0	0
47.00	40	60	0	0
62.00	0	100	0	0
68.00	0	100	0	0

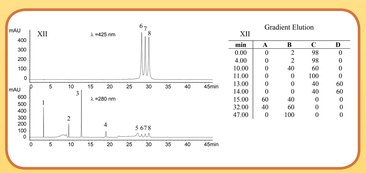


Gradient Elution

X				
min	A	B	C	D
0.00	0	2	98	0
4.00	0	2	98	0
10.00	0	40	60	0
22.00	0	0	38	62
26.00	0	0	38	62
27.00	60	40	0	0
32.00	40	60	0	0
59.00	0	100	0	0
65.00	0	100	0	0

XI				
min	A	B	C	D
0.00	0	2	98	0
4.00	0	2	98	0
10.00	0	40	60	0
22.00	0	0	100	0
11.00	0	0	40	60
14.00	0	0	40	60
15.00	60	40	0	0
32.00	40	60	0	0
47.00	0	100	0	0

HPLC chromatograms of 103 µg/mL vanillic acid (1), 104 µg/mL ferulic acid (2), 100 µg/mL vanillin (3), 386 µg/mL THC (4), and 40 µg/mL Curcumin C[3] (5) standards in methanol. Mobile phase: A = 0.05% acetic acid in Nanopure water; B = 0.05% acetic acid in acetonitrile; C = 1% ammonium acetate; D = 0.05% acetic acid in methanol



Gradient Elution

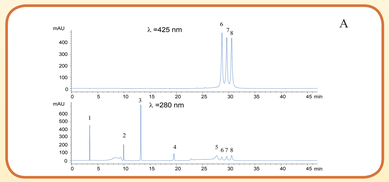
XII				
min	A	B	C	D
0.00	0	2	98	0
4.00	0	2	98	0
10.00	0	40	60	0
11.00	0	0	100	0
13.00	0	0	40	60
14.00	0	0	40	60
15.00	60	40	0	0
32.00	40	60	0	0
47.00	0	100	0	0

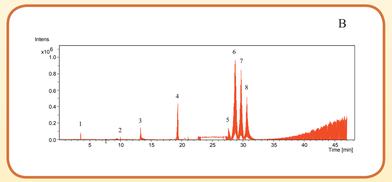
HPLC chromatograms of 101 µg/mL vanillic acid (1), 100 µg/mL ferulic acid (2), 100 µg/mL vanillin (3), 100 µg/mL HHC (4), 309 µg/mL THC (5), 102 µg/mL BDMC (6), 102 µg/mL DMC (7), and 102 µg/mL C (8) standards in acetonitrile. Mobile phase: A = 0.05% acetic acid in Nanopure water; B = 0.05% acetic acid in acetonitrile; C = 1% ammonium acetate; D = 0.05% acetic acid in methanol

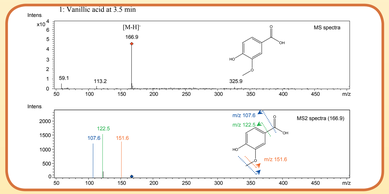
CONCLUSIONS

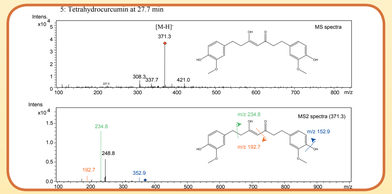
►Satisfactory HPLC separation was achieved for all the tested compounds by changing the composition of the mobile phase.
►The wavelength used to detect curcuminoids was 425 nm (maximum absorption). 280 nm was selected to detect vanillic acid, ferulic acid, vanillin, hexahydrocurcumin, and tetrahydrocurcumin at the same time.
►Mass spectrometric detection was more sensitive than ultraviolet detection in case of tetrahydrocurcumin and hexahydrocurcumin.
►The fragmentation patterns correlates with the chemical structures of the compounds.

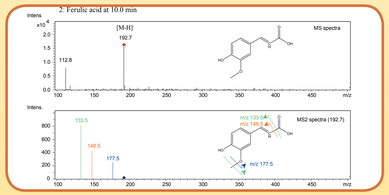
LC/MS/MS DATA

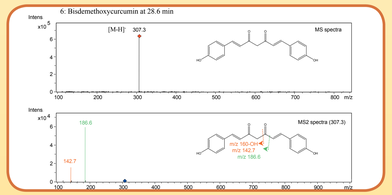


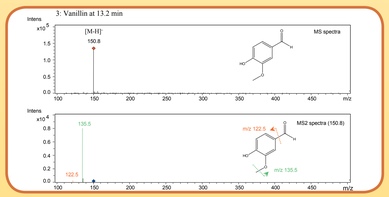


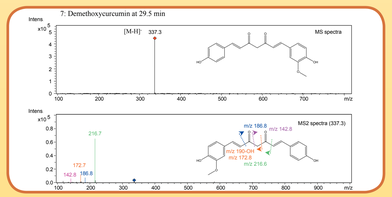


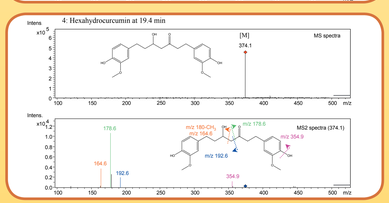


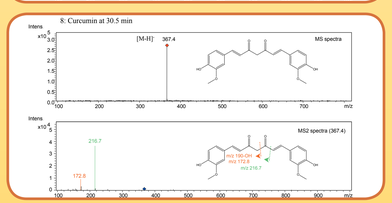












HPLC-UV chromatograms (A), base peak chromatogram (B), mass spectra, and fragmentation patterns of 101 µg/mL vanillic acid (1), 100 µg/mL ferulic acid (2), 100 µg/mL vanillin (3), 100 µg/mL HHC (4), 309 µg/mL THC (5), 102 µg/mL BDMC (6), 102 µg/mL DMC (7), and 102 µg/mL C (8) standards in acetonitrile.

REFERENCES

[1] Pan, M.H. *et al*. Drug Metabolism and Disposition, 27: 486-494 (1999)
[2] Ireson, C. *et al*. Cancer Research, 61: 1058-1064 (2001)
[3] Wang, Y.J. et al. Journal of Pharmaceutical and Biomedical Analysis, 15: 1867-1876 (1997)
[4] Pfeiffer, E. et al. Journal of Food Engineering, 56: 257-259 (2003)

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