Identification of Structural Isomers of Methylated Flavonoids by UHPLC Coupled with High Resolution Q-TOF Mass Spectrometry

Application Note

Authors

ChengYing Ma, Haipeng Lv, Xinzhong Zhang, Zongmao Chen, Jiang Shi, and Zhi Lin
Tea Research Institute, CAAS
Hangzhou, China

Meiling Lu, Shan Zhou
Agilent Technologies China Ltd.
Beijing, China

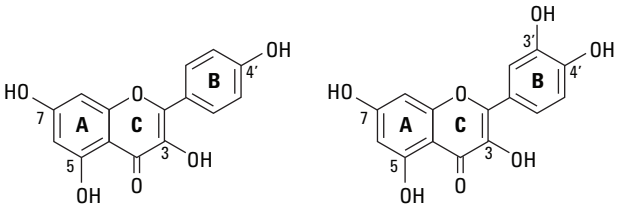
Abstract

This application note details the development of a method for identiying flavonoids after methylation based on the work published by Ma, *et al* [1]. The method uses the high performance Agilent 1290 Infinity LC System to achieve baseline separation of all the enzymatically-generated methylated flavonol isomers and further applies the Agilent 6530 Accurate-Mass Q-TOF LC/MS to determine the methylation status and methylation patterns. The characteristic fragmentation pattern analysis along with the retention behavior helped to identify ten compounds. Four of those compounds have not been reported in any plants using existing methods.

Agilent Technologies

Introduction

Flavonoids are a class of heterogeneous polyphenols that are present in foods ubiquitous in the daily diet such as tea, fruits, vegetables, and wine. They exhibit numerous biological and pharmaceutical effects, and contribute significantly to human health [2,3]. Methylated flavonoids can increase metabolic stability and oral bioavailability, enhance anti-allergic effects, and destroy cancer cells more effectively than their unmethylated analogs [4-6]. Such methylation is generally catalyzed by flavonoid *o*-methyltransferases (OMTs), which are widely present in plants. These OMTs often have distinguished specificities and notable substrate preferences. The methylated isomers produced by the OMT catalysis often differ in physical and chemical properties, and modulate the biological activities of the compounds. Therefore, identifying and characterizing the structural-related methylated flavonoid isomers are important. Structural variety and lack of standard compounds have been obstacles to conduct such studies. This application note describes the development of a strategy for identifying one subclass of methylated flavonoids, flavonols, based on high resolution MS/MS fragmentation patterns, using kaempferol and quercetin as examples.



A) Kaempferol B) Quercetin

Figure 1. Chemical structures of flavonols with free hydroxyls for methylation by flavonol OMTs. A) kaempferol; B) quercetin.

Experimental

Sample preparation

The methylated flavonol isomers were identified and characterized with limited standard compounds. Three flavonol OMT genes (CsFOMT-1, CsFOMT-2, and CsCOMT) were cloned from *C. sinensis* and expressed in a prokaryotic expression vector. The expressed enzymes were extracted and purified for further use. Kaempferol and quercetin in the amount of 750 µmol each were initially subjected to methylation by 0.3 mg of different flavonol OMTs in a buffer containing 100 mmol/L *tris*-HCl (pH 7.5), 0.2 mmol/L $MgCl_2$, 0.4 mmol/L S-adenosyl-L-methionine (SAM), and 2 mmol/L DTT. After methylation of the substrates for 1 hour at 35 °C, the resultant reaction mixtures were terminated by addition of 1 N HCl, and extracted using 25 mL ethyl acetate. Twenty microliters of organic phase was then transferred to a clean vial and evaporated under nitrogen atmosphere. The resultant residue was dissolved in 1.5 mL pure methanol, filtrated through a 0.22-µm membrane, and subjected to reversed phase LC separation followed with Q-TOF MS detection. A wide gradient elution using a binary mobile phase was developed for complete separation of the formed methylated isomers. Accurate mass extraction was then applied to examine the methylation status of the products. Collision induced dissociation (CID) MS/MS analysis of individual isomers was further conducted to differentiate these compounds based on the distinct fragmentation pathways.

Instrumentation

The method was developed using an Agilent high resolution LC/MS system. It consisted of a 1290 Infinity Binary Pump with built-in degasser, a 1290 Infinity Autosampler with thermostat, a 1290 Infinity Thermostatted Column Compartment, a 1290 Infinity Diode Array Detector, and an Agilent 6530 Accurate-Mass Q-TOF Mass Spectrometer with dual JetStream ESI source.

Instrument conditions

Column	Agilent ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm
Column temperature	30 °C
Mobile phase solvent A	0.5% formic acid/2 mM ammonium formate in water
Solvent B	acetonitrile
Flow rate	0.2 mL/min
Injection volume	5 µL
Post time	3 minutes
Gradient elution profile	0–5 minutes: % B increasing from 12% to 25% 5–20 minutes: % B increasing from 25% to 75% 20–25 minutes: % B decreasing from 75% to 12%
Drying gas temperature	300 °C
Drying gas flow	8 L/min
Sheath gas temperature	300 °C
Sheath gas flow	8 L/min
Nebulizer gas	35 psi
Capillary voltage	3,500 V
Fragmentor voltage	130 V
Reference ions	*m/z* 121.0509, *m/z* 922.0098

Results and Discussion

Methoxy position-specific methane elimination for discriminating regioisomers of methylated kaempferol analogs

Characteristic fragmentation of methylated kaempferol is mainly achieved through retro Diels-Alder (rDA) fragmentation pathway and neutral loss pathway (Figure 2) [2,7,8].

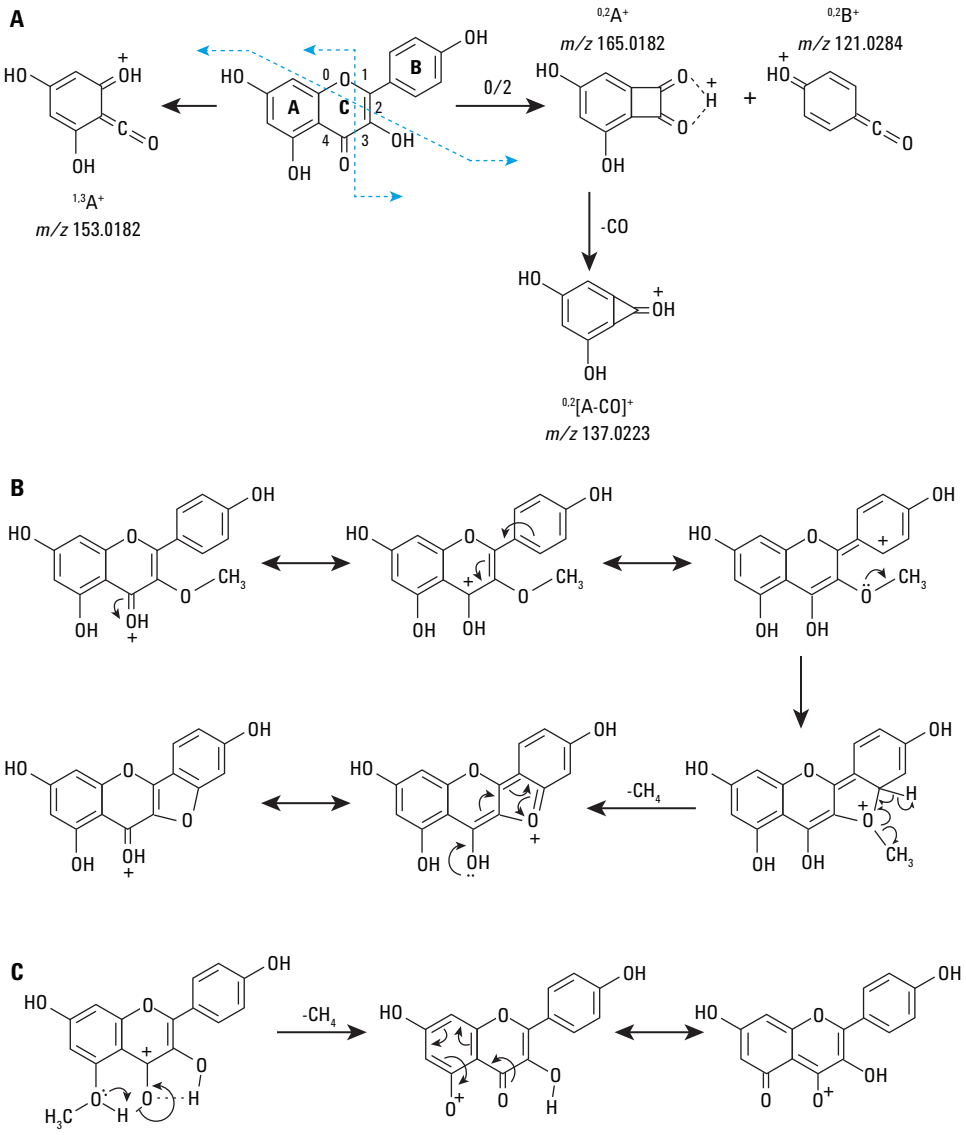


Figure 2. Fragmentation pathway for kaempferol and their methylated analogs. A) rDA fragmentation pathway; B,C) neutral loss of CH_4 pathway with methylation at C_3 and C_5 positions, respectively.

While fragments derived from rDA pathways can provide hints to the ring on which the methoxy group attaches, neutral loss of methane can be specific to the proximity of methylation positions. Methylation at the 4′ and 7 positions can generate intensive fragments with methyl radical loss $[M+H\text{-}CH_3\bullet]^+$ (Figure 3A,B), whereas fragmentation patterns for the 3,4′-DMK and 3,7-DMK yielded obvious $[M+H\text{-}CH_4]^+$ fragments (*m/z* 299.054) in addition to $[M+H\text{-}CH_3\bullet]^+$ (Figure 3C,D). It is suggested that proper orientation of C_3-OCH_3 with a C_2′ proton allows the C_2′ proton transfer to the C_3-OCH_3, leading to the elimination of a methane molecule through the formation of a stable furan ring (Figure 2B). Besides, the 4-keto group is easily protonated under ESI conditions, and the proton close to 5-methoxy, tends to lose methane during fragmentation (Figure 2C).

Application of the fragmentation pattern for identification of enzymatically synthesized methylated kaempferol analogs

Ultra high performance LC with optimized gradient elution was initially used to separate as many methylated kaempferol analogs as possible and identify the methylation status of the substrate. In the absence of any OMTs, only kaempferol (*m/z* 287.0547) was detected (Figure 4A). In comparison, three monomethyl kaempferols (MMK1, MMK2, MMK3) can be produced by the OMTs as evidenced by an *m/z* increase of 14.0158 from kaempferol (Figure 4B-D). In addition, *Cs*FOMT-1 and *Cs*COMT can produce one dimethyl kaempferol (DMK1) with an *m/z* increase of 28.0334 from kaempferol (Figure 4B,D).

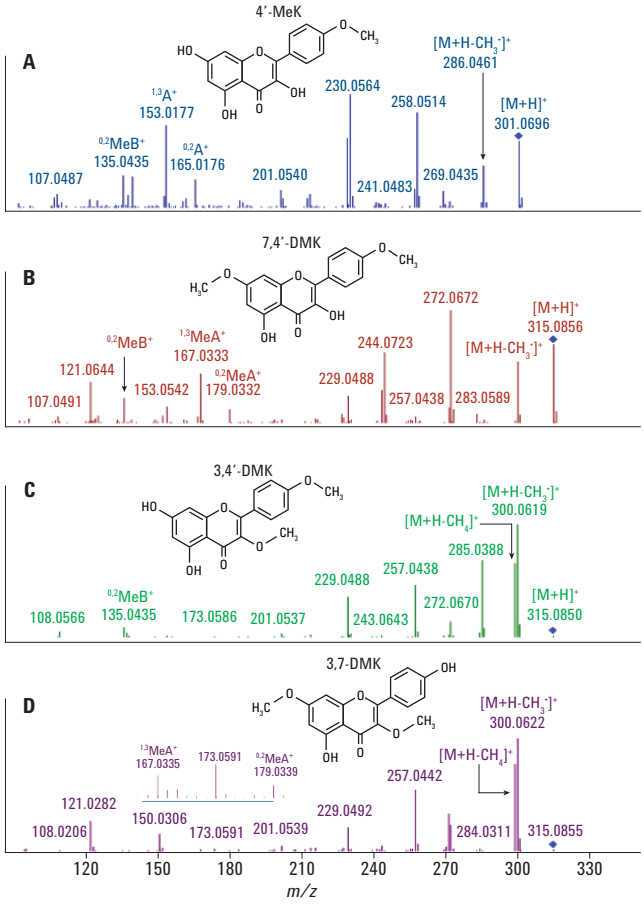


Figure 3. Characteristic MS/MS patterns for available standard compounds including rDA and neutral loss fragmentation pathways. (A) 4′-MeK, (B) 7,4′-DMK, (C) 3,4′-DMK, and (D) 3,7-DMK.

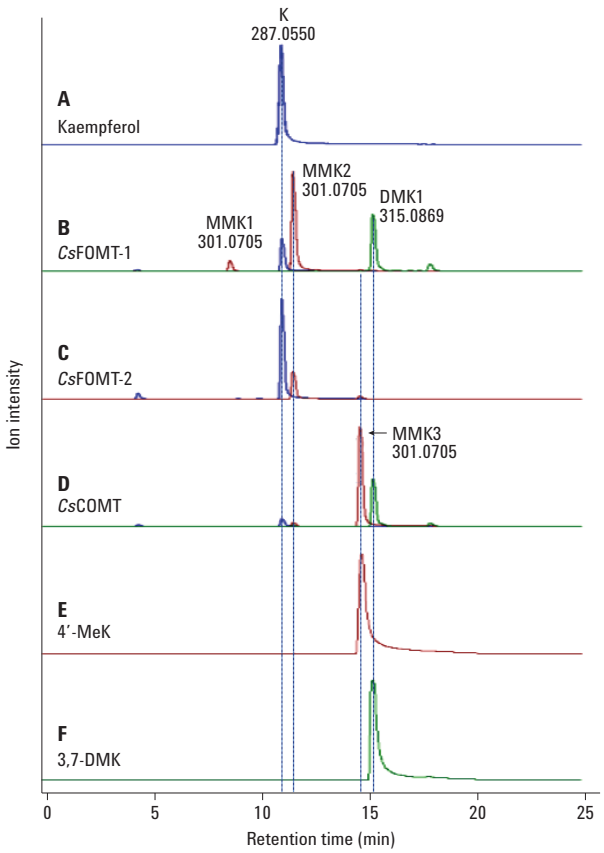


Figure 4. Extracted ion chromatograms demonstrating the formation of mono- and dimethylated kaempferol compounds catalyzed by cloned OMTs from tea. (A) without OMT; (B) CsFOMT-1, (C) CsFOMT-2, and (D) CsCOMT; (E) 4′-MeK standard, and (F) 3,7-DMK standard.

Identification of methylated products

Presence of methylated rDA ions related to the A-ring ($^{1,3}MeA^+$) and absence of fragments due to methane loss for MMK3 indicate that MMK3 correspond to 7-MeK (Figure 5C). MMK1 and MMK2 with fragments of $[M+H\text{-}CH_4]^+$ suggest that both are either 5-MeK or 3-MeK. The presence of visible methylated rDA ions related to the A-ring suggests that MMK1 should be 5-MeK, and MMK2 then correspond to 3-MeK (Figure 5-A,B). Presence of $^{1,3}MeA^+$ and $[M+H\text{-}CH_4]^+$ and absence of $^{0,2}MeB+$ suggest that DMK1 should be 3,7-DMK, which matched well with the standard compound (Figure 3D). The retention behavior under reversed phase elution of these four compounds is consistent with the tested result for the available standard compounds (Figure 4E,F), the hydrophobicity of the compounds by calculation (Table 1) [9], and those reported in the literatures [10].

Extension of the approach to characterize methylated quercetin analogs derived from OMT catalysis

Structural analysis of methylated quercetin analogs indicated that $C_4'\text{-}OCH_3$ with neighboring $C_3\text{-}OH$ can generate $[M+H\text{-}CH_4]^+$ during collision induced dissociation (CID) in addition to methylation at C_3 and C_5 positions. LC separation demonstrates that four monomethylated and two dimethylated quercetin anologs can be produced with the applied OMTs (Figure 6). Analysis of the Q-TOF MS/MS fragmentation patterns (Figure 7) suggests that MMQ3 and MMQ4 correspond to 3′-MeQ and 7-MeQ with the absence of $[M+H\text{-}CH_4]^+$. The presence of $^{1,3}MeA^+$ indicates that MMQ4 corresponds to 7-MeQ, therefore MMQ3 should be 3′-MeQ. The presence of $[M+H\text{-}CH_4]^+$ and absence of $^{1,3}MeA^+$ fragments indicate that MMQ1 and MMQ2 are 3-MeQ and 4′-MeQ, which can be further differentiated based on corresponding hydrophobicity (Table 1).

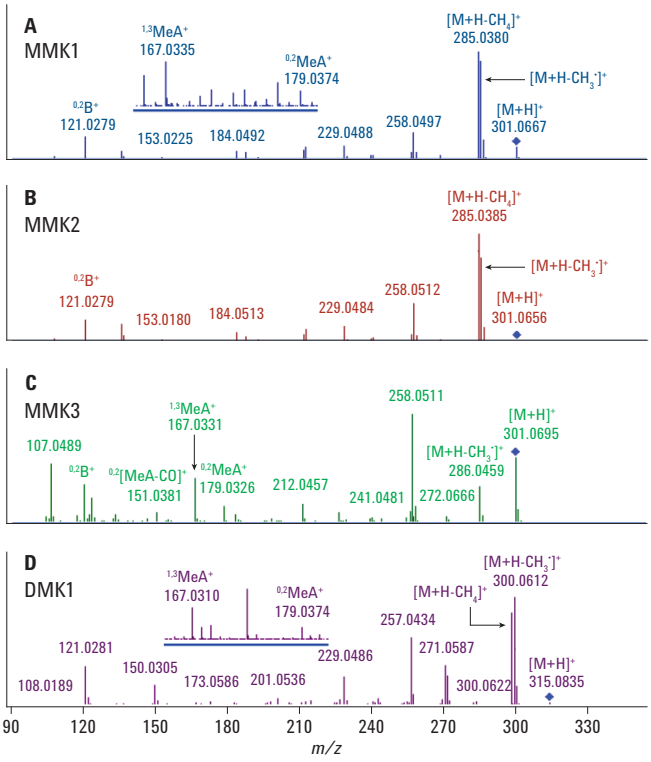


Figure 5. The characteristic MS/MS pattern for the monomethyl and dimethyl kaempferol compounds derived from OMTs. The identified compounds were 5-MeK for MMK1 (A); 3-MeK for MMK2 (B); 7-MeK for MMK3 (C); and 3,7-DMK for DMK1 (D), respectively.

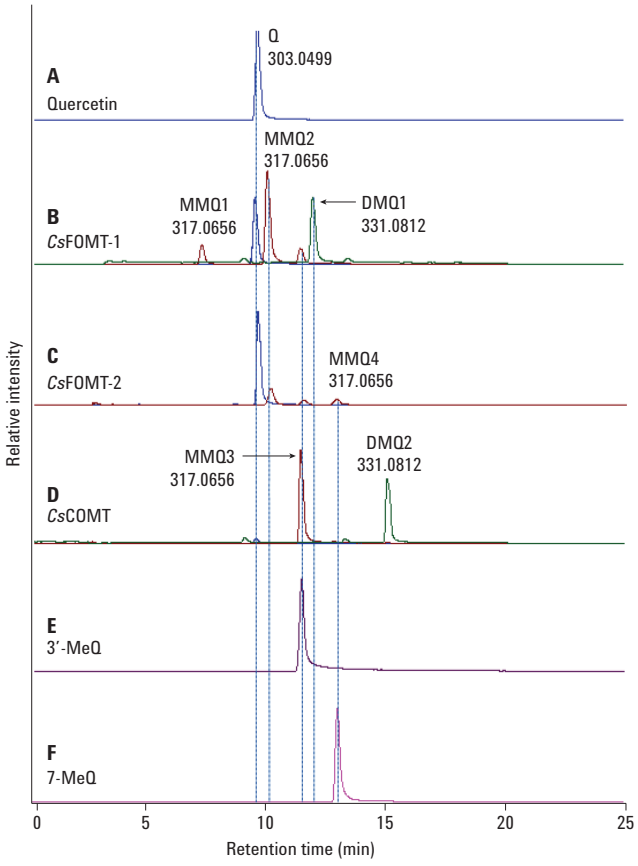


Figure 6. Extracted ion chromatograms demonstrating the formation of mono- and dimethylated quercetin compounds catalyzed by OMTs (A) without OMTs; (B) CsFOMT-1, (C) CsFOMT-2, (D) CsCOMT, (E) 3′-MeQ standard, and (F) 7-MeQ standard.

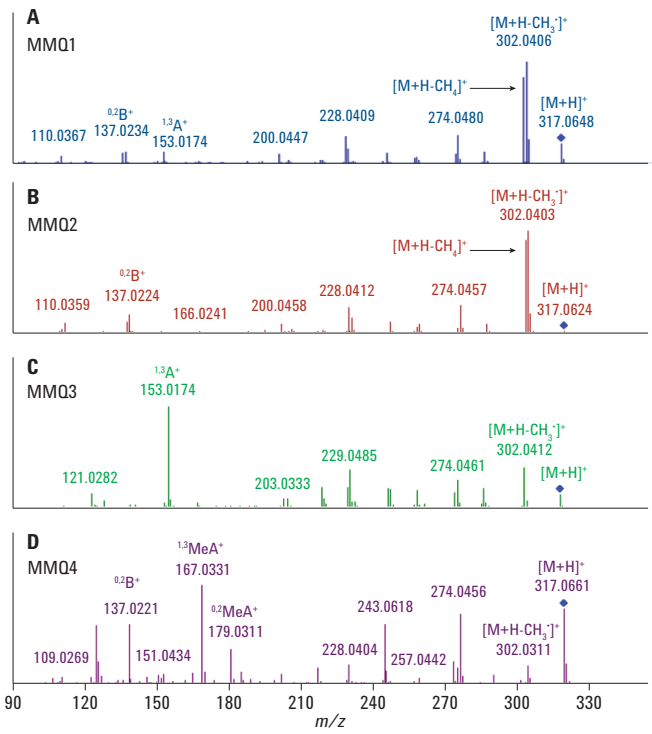


Figure 7. Characteristic MS/MS patterns for the four monomethylated quercetin isomers catalyzed by three OMTs and their identified structures. The identified compounds are 3-MeQ for MMQ1 (A), 4'-MeQ for MMQ2 (B); 3'-MeQ for MMQ3 (C); 7-MeQ for MMQ4 (D), respectively.

Table 1. Identified Methylated Flavonoids Compounds and their Retention Times

Observed compound		Theoretical candidates		Observed characteristic fragment ions				
ID	**t_R/min**	**Name**	**logP***	**$^{0,2}B^+$**	**$^{1,3}A^+$**	**$^{0,2}A^+$**	**$[MH\text{-}CH_4]^+$**	**$[MH\text{-}CH_3^{\bullet}]^+$**
MMK1	8.67	5-MeK	2.989	121.0276	153.0223	–	***285.0379***	286.0454
MMK2	11.59	3-MeK	3.008	121.0279	153.0182	–	***285.0385***	286.0453
MMK3	14.70	7-MeK	3.498	121.0281	***167.0331***	***179.0327***	–	286.0464
Std 1	14.70	4′-MeK	3.499	***135.0436***	153.0175	165.0178	–	286.0462
DMK1	15.29	3,7-DMK	4.011	121.0284	***167.0333***	–	***299.0541***	300.0612
Std 2	15.30	3,7-DMK	4.011	121.0282	***167.0336***	–	***299.0547***	300.0662
MMQ1	7.25	3-MeQ	1.765	–	153.0170	–	301.0327	302.0407
MMQ2	9.95	4′-MeQ	2.67	–	153.0174	–	301.0332	302.0403
MMQ3	11.40	3′-MeQ	2.787	–	153.0174	–	–	302.0413
MMQ4	12.72	7-MeQ	2.802	137.0222	***167.0337***	***179.0335***	–	302.0418
Std 3	11.38	3′-MeQ	2.787	–	153.0177	–	–	302.0413
Std 4	12.82	7-MeQ	2.802	137.0235	***167.0333***	***179.0338***	–	302.0406
DMQ1	11.83	3,3′-DMQ	2.681	–	153.0187	165.0184	***315.0492***	316.0562
DMQ2	15.00	7,3′-DMQ	3.600	–	***167.0332***	***179.0324***	–	316.0566

*logP values were obtained from www.chemspider.com (ACD/logP).

Similarly, DMQ1 and DMQ2 were identified as 3,3′-MeQ and 7,3′-MeQ respectively. Table 1 summarized the identified methylated flavonoid compounds with their retention times, corresponding hydrophobicity (logP), and the observed characteristic fragment ions. In total, ten compounds were identified, with four as new compounds that have not been previously reported in any plants.

Conclusions

Agilent 1290 Infinity LC System coupled with the high resolution and accuracy of the Agilent 6530 Accurate-Mass Q-TOF LC/MS provides sufficient separation and accurate determination of the methylation status of flavonol derivatives. This study details the development of a practical approach for identifying and characterizing isomers of individual methylated flavonol analogs based on accurate and specific MS/MS patterns. The developed method identifies methylated flavonols with very limited standards. It can potentially be applied to the identification of other methylated flavonoids and can be used for the rapid characterization of OMTs in plants.

References

1. C. Ma, H. Lv, X. Zhang, *et al*., *Analytica Chimica Acta* **795** (2013) 15.
2. E. Middleton, C. Kandaswami, T.C. Theoharides, *Pharmacol. Rev.* **52** (2000) 751.
3. C.S. Yang, X. Wang, G. Lu, S. C. Picinich, *Nat. Rev. Cancer* **9** (2009) 439.
4. X. Wen, T. Walle, *Drug Metab. Dispos*. **34** (2006) 1792.
5. W. Thomas, Intern. J. Mol. Sci. **10** (2008) 5019.
6. M. F. Guerrero, P. Puebla, R. Carron, M.L. Martin, L.S. Roman, *J. Pharm. Pharmocol*. **54** (2002) 1378.
7. Y. L. Ma, Q. M. Li, H. Van den Heuvel, M. Claeys, *Rapid Commun. Mass Spectrom*. **11** (1997) 1364.
8. E. D. Rijke, P. Out, W. M. A. Niessen, F. Ariese, C. Cooijer, U.A.T. Brinkman, *J. Chromatogr. A*. **1112** (2006) 63.
9. http://www.acdlabs.com/resources/freeware, last accessed on Mar. 20, 2013.
10. K. Arisumi, Y. Sakata, I. Miyajima, *Mem. Fac. Agr. Kagoshima Univ*. **21** (1985) 147.

www.agilent.com/chem

Printed in the USA
August 21, 2014
5991-3334EN

Agilent Technologies