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Isolation, identification and stability of acylated derivatives of apigenin 7-*O*-glucoside from chamomile (*Chamomilla recutita* [L.] Rauschert)

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Abstract

The major flavonoids in the white florets of chamomile (*Chamomilla recutita* [L.] Rauschert) were rapidly purified using a combination of polyamide solid-phase extraction and preparative HPLC. From the combined LC/MS, LC/MS/MS, and NMR data the apigenin glucosides were identified as apigenin 7-*O*-glucoside (Ap-7-Glc), Ap-7-(6″-malonyl-Glc), Ap-7-(6″-acetyl-Glc), Ap-7-(6″-caffeoyl-Glc), Ap-7-(4″-acetyl-Glc), Ap-7-(4″-acetyl,6″-malonyl-Glc), and a partially characterised apigenin-7-(mono-acetyl/mono-malonylglucoside) isomer. Malonyl and caffeoyl derivatives of Ap-7-Glc have not previously been identified in chamomile. The two mono-acetyl/mono-malonyl flavonoids have not previously been reported in any plant species. These acylated glucosides are unstable and degrade to form acetylated compounds or Ap-7-Glc. The degradation products formed are dependent on the extraction and storage conditions, i.e. temperature, pH and solvent.

Keywords: Asteraceae; *Chamomilla recutita*; LC/MS; NMR; Apigenin acyl-glucosides; Flavonoid stability

1. Introduction

Chamomile (*Chamomilla recutita* a.k.a. *Matricaria recutita*) is used for a variety of herbal remedies and several classes of biologically active compounds have been identified from the plant including coumarins and sesquiterpenes (Ceska et al., 1992; Adam and Zapp, 1998; Repčák and Eliášová, 1998, 2001a,b). There have also been many reports on the identification of flavonoids from chamomile; apigenin is quantitatively the most abundant flavonoid found in chamomile flowers and contributes to the observed pharmacological properties of the flower drug "*Chamomillae flos*" (Achterrath-Tuckermann et al., 1980; Della Logia et al., 1986). Developmental studies have been performed on the total apigenin content during the life cycle of both diploid and tetraploid forms of chamomile (Švehlíková and Repčák, 2000). Chamomile research continues because of the interest in its reported anti-inflammatory, antiviral and particularly anticarcinogenic properties (Caltagirone et al., 2000; Critchfield et al., 1996, 1997; Fuchs and Milbradt, 1993; Wang et al., 2000; Yin et al., 2001).

Apigenin accumulates in the white ligulate florets of the chamomile anthodium in a bound form as apigenin 7-*O*-glucoside (Ap-7-Glc) and various acylated derivatives. The first of the acylated derivatives of apigenin

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isolated and identified from chamomile was Ap-7-(6″-acetyl-Glc), also reported as being the most stable (Kunde and Isak, 1979; Redaelli et al., 1980). The other chamomile apigenin mono- and di-acetyl-glucosides are known to undergo rapid ester hydrolysis leading to the formation of Ap-7-Glc. The transformation and the resulting difficulties with purification made the identification of the *in planta* glucosides very difficult. Despite these problems six acetylated derivatives of Ap-7-Glc have been reported to date from chamomile; they were identified by mass spectrometry (Carle et al., 1993; Mulinacci et al., 2000) and by ^{13}C NMR analyses (Redaelli et al., 1982). Carle et al. (1993) identified mono-acetyl (**3**, **5**, **10**, **11**) and di-acetyl (**12**, **13**) forms of Ap-7-Glc in an extract of ligulate florets. However, a detailed NMR identification of the acylated derivatives has not been performed, and the identity of acyl substituents and their location on the glucose moiety have not been determined definitively.

The aim of the present study was to purify and determine the chemical structures of all the apigenin-derived flavonoids present in the anthodia of chamomile. Rapid extraction (SPE and preparative HPLC) methods were used, in combination with −20 °C storage of all fractions, in order to reduce degradation prior to LC/MS, LC/MS/MS and NMR analyses. In addition, the stability of the chamomile flavonoids was determined under different temperature, pH and solvent conditions in extracts from fresh and dried flowers, and with individual purified flavonoids.

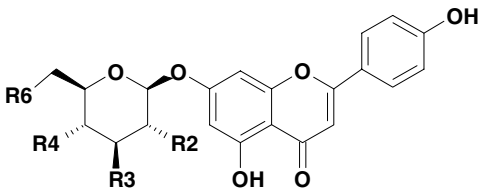
2. Results and discussion

There is considerable interest in the health-promoting effects of flavonoids from dietary sources e.g. from major crops, herbs and medicinal plants. Chamomile flowers have a long history of use as tea and for reputed medicinal properties. However, there has not been a comprehensive analysis of the flavonoids present in the flowers using a combination of contemporary extraction and LC/MS methods and NMR.

LC/MS and LC/MS/MS analyses of 70% v/v methanol (MeOH) petal extracts showed that they contained a mixture of one glucoside, one mono-caffeoyl glucoside, two mono-acetyl glucosides, one mono-malonyl glucoside, and two mono-acetyl/mono-malonyl glucosides (Table 1; Fig. 1). Based on previous studies it is known that polyamide can be used as a first step in the purification of flavonoid glycosides and flavonoid malonyl-glycosides (DuPont et al., 2000). This was used as the first step in the purification of the chamomile flavonoids, to separate the three malonylated flavonoids (**2**, **6**, **7**) from the Ap-7-Glc (**1**), other acylated flavonoids (**3**, **4**, **5**) and apigenin aglycone (**8**) (Fig. 2).

Table 1
Summary of LC/MS and LC/MS/MS data for apigenin-7-*O*-glucosides from *Chamomile* petals

	Chamomile flavonoid	ES + MS		ES + MS/MS			ES − MS		
		$[M+H]^+$	$[Aglycone+H]^+$	Product ions of …	$[Aglycone+H]^+$	Other ions	$[M-H]^-$	$[(M-CO2)-H]^-$	$[Aglycone\text{-}H]^-$
1	Ap-7-*O*-Glc	433 (100%)	271 (31)	433	271 (100%)	–	–		
2	Ap-7-*O*-(6″-malonyl-Glc)	519 (100%)	271 (0.06)	519	271 (100%)	433 (6.3, [(M-Malonyl) + H]$^+$)	517 (13%)	473 (44)	269 (100)
3	Ap-7-*O*-(4″-acetyl-Glc)	475 (100%)	271 (30)	475	271 (100%)	–	–	–	–
4	Ap-7-*O*-(6″-caffeoyl-Glc)	595 (100%)	271 (0.06)	595	271 (100%)	325 (3.1, [Caffeoyl-Glc + H]$^+$) 163 (51.3, [Caffeoyl + H]$^+$)	–	–	–
5	Ap-7-*O*-(6″-acetyl-Glc)	475 (100%)	271 (7)	475	271 (100%)	–	–	–	–
6	Ap-7-*O*-(6″-malonyl, 4″-acetyl-Glc)	561 (100%)	271 (0.12)	561	271 (100%)	475 (3.7, [M + H, loss of malonyl]$^+$)	559 (27%)	515 (100)	269 (55)
7	Ap-7-*O*-(X″-malonyl, Y″-acetyl-Glc)	561 (100%)	271 (0.06)	561	271 (100%)	475 (5.7, [M + H, loss of malonyl]$^+$)	559 (13%)	515 (23)	269 (100)



Reference Number	R2	R3	R4	R6	APIGENIN GLUCOSIDE
1	OH	OH	OH	OH	Ap-7-Glc
2	OH	OH	OH	Malonyl	Ap-7-(6″-malonyl-Glc)
3	OH	OH	Acetyl	OH	Ap-7-(4″-acetyl-Glc)
4	OH	OH	OH	Caffeoyl	Ap-7-(6″-caffeoyl-Glc)
5	OH	OH	OH	Acetyl	Ap-7-(6″-acetyl-Glc)
6	OH	OH	Acetyl	Malonyl	Ap-7-(4″-acetyl, 6″-malonyl-Glc)
7	?	?	?	?	Ap-7-(X″-acetyl, Y″-malonyl-Glc)
8	-	-	-	-	Apigenin (H replaces Glc on 7-position)
9	OH	OH	Acetyl	Acetyl	Ap-7-(4″, 6″-di-acetyl-Glc)
10	Acetyl	OH	OH	OH	Ap-7-(2″-acetyl-Glc)
11	OH	Acetyl	OH	OH	Ap-7-(3″-acetyl-Glc)
12	Acetyl	Acetyl	OH	OH	Ap-7-(2″, 3″-di-acetyl-Glc)
13	OH	Acetyl	Acetyl	OH	Ap-7-(3″, 4″-di-acetyl-Glc)

Fig. 1. Structures of apigenin glucosides in petals of chamomile (identified from the current and previous studies).

In the current study, using the improved extraction methods and rapid purification, the MeOH extracts of low temperature-dried ligulate florets were shown to contain: apigenin-7-*O*-β-D-glucoside (Ap-7-Glc) (**1**), Ap-7-(6″-malonyl-Glc) (**2**), Ap-7-(4″-acetyl-Glc) (**3**) (identification based on LC/MS and retention time), Ap-7-(6″-caffeoyl-Glc) (**4**), Ap-7-(6″-acetyl-Glc) (**5**), Ap-7-(4″-acetyl, 6″-malonyl-Glc) (**6**), Ap-7-(X″-acetyl, Y″-malonyl-Glc) (**7**) and apigenin aglycone (**8**). The flavonoid structures were confirmed by LC/MS and LC/MS/MS (Table 1) in addition to NMR of purified flavonoids (Tables 2 and 3). Malonyl (**2**, **6**, **7**) and caffeoyl (**4**) derivatives of Ap-7-Glc have not previously been reported for chamomile and this is also the first report of the occurrence of two apigenin mono-acetyl/mono-malonyl-glucosides (**6**, **7**). The current results clearly show a different flavonoid profile compared with previous studies in which acetylated compounds were predominant. Previous LC/MS (Carle et al., 1993; Mulinacci et al., 2000) and ^{13}C NMR (Redaelli et al., 1980, 1982) analyses of dried flowers had shown that the acylated derivatives of apigenin-7-*O*-glucoside (Ap-7-Glc) present in the chamomile ligulate florets were mono-acetylated (**3**, **5**, **10**, **11**) and di-acetylated (**12**, **13**).

Stability studies were performed on these newly identified chamomile flavonoids, with the aim of determining if they were precursors of the many acetylated compounds previously detected. Fresh petals extracted at low temperature contained a much higher proportion of the malonyl (**2**) and mono-acetyl/mono-malonyl glycosides (**6**, **7**) compared with other samples (Fig. 3). The 20 °C air-dried petals (Fig. 3) had a similar flavonoid profile compared with freeze-dried petals (data not shown), whereas the flavonoid content of the commercial tea was predominantly Ap-7-Glc (**1**) plus a complex mixture of mono- and di-acetylated Ap-7-Glc derivatives (data not shown). Mono-acylated derivatives (**2**, **3**, **4**, **5**) were degraded in solution forming Ap-7-Glc. Pure crystallised forms of both Ap-7-(6″-malonyl-Glc) (**2**) and Ap-7-(4″-acetyl, 6″-malonyl-Glc) (**6**) underwent rapid decarboxylation at room temperature forming Ap-7-(6″-acetyl-Glc) (**5**) and Ap-7-(4″, 6″-di-acetyl-Glc) (**9**), respectively. Ap-7-(4″-acetyl, 6″-malonyl-Glc) (**6**) produced a spectrum of different acylated compounds under different solvent conditions (Table 4). However, in all cases complete deacylation was the final step resulting in accumulation of Ap-7-Glc. Decarboxylation of the 6″-malonyl group was also observed in MeOH, together with accumulation of compound (**3**) – structure was not confirmed by NMR due to its extreme instability during purification, but based on the decomposition, MS and retention time data it is postulated that compound (**3**) is Ap-7-(4″-acetyl-Glc). High amounts of Ap-7-(6″-acetyl-Glc) (**5**) in the MeOH extract of dried chamomile anthodia and its greater stability explains the ease with which previous researchers isolated and identified this flavonoid, whereas malonylated compounds were not previously found. Ap-7-(6″-acetyl-Glc) (**5**) was the first apigenin-derived flavonoid isolated and identified in *Chamomilla recutita* (Kunde and Isak, 1979; Redaelli et al., 1980). It is the most stable of the acylated apigenin compounds (Carle et al., 1993; Tschiersch and Hölzl, 1992), due to increased stability of derivatives of the primary hydroxyl group on C6″ of the glucose moiety in comparison with the secondary hydroxyl groups. For the same reason, the 6″hydroxyl is that most often substituted in flavonoid glucosides. However, Ap-7-(6″-acetyl-Glc) (**5**) was present in much lower amounts in fresh tissues of *Chamomilla recutita*.

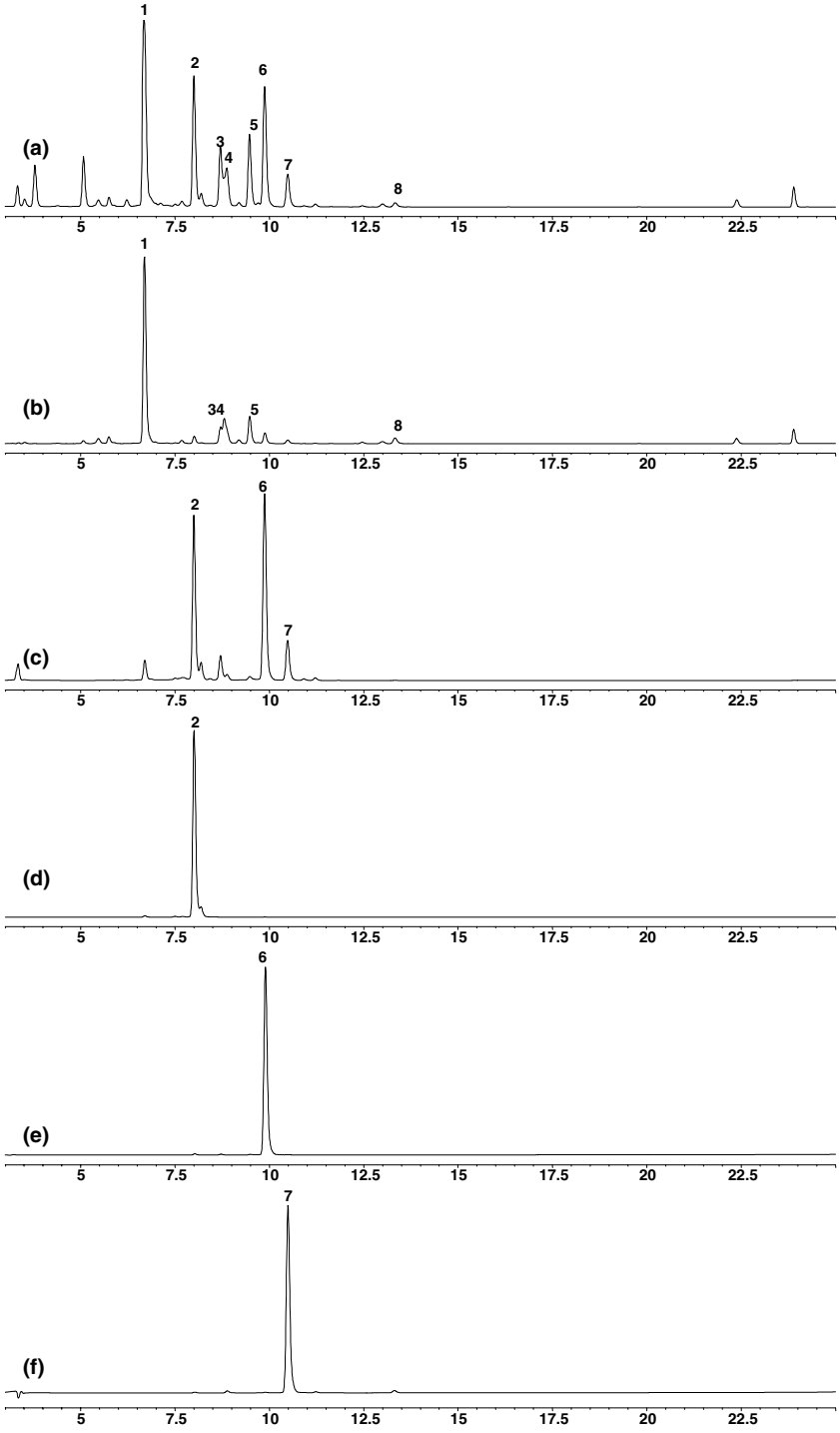


Fig. 2. HPLC chromatograms of the methanolic extract of 20 °C air-dried ligulate florets (a), MeOH fraction from polyamide SPE (b), MeOH/NH_4OH fraction from polyamide SPE (c), purified Ap-7-(6″-malonyl-Glc) (d), purified Ap-7-(4″-acetyl, 6″-malonyl-Glc) (e) and Ap-7-(X″-acetyl, Y″-malonyl-Glc) (f). Peak ID as for Fig. 1.

The stability of Ap-7-(6″-malonyl-Glc) (**2**) and Ap-7-(4″-acetyl, 6″-malonyl-Glc) (**6**), the major acylated flavonoids in the ligulate florets, were also studied using different temperatures, pH and solvents (Fig. 4). Light was found to have no significant effect on the degradation of these flavonoids, but increasing the temperature increased the rate of flavonoid degradation (Fig. 4). Ap-7-(6″-malonyl-Glc) (**2**) was more stable at neutral pH than the other compounds. It was also more stable at pH 9, conditions that caused rapid hydrolysis of acetylated, acetylated/malonylated and Ap-7-(6″-caffeoyl-Glc) (**4**) (Fig. 4).

Table 2
^{1}H NMR data of purified apigenin glucosides and the apigenin-(4″,6″-di-acetyl-Glc) degradation product[a]

Position	Flavonoid				
	Ap-7-(6″-malonyl-Glc) (**2**)	Ap-7-(6″-caffeoyl-Glc) (**4**)	Ap-7-(6″-acetyl-Glc) (**5**)	Ap-7-(4″-acetyl, 6″-malonyl-Glc) (**6**)	Ap-7-(4″,6″-di-acetyl-Glc) (**8**)
Aglycone					
H-3	6.86 *s*	6.57 *s*	6.69 *s*	6.87 *s*	6.56
H-6	6.44 *d* (2.3)	6.54 *s*, *d* (1.5)	6.53 *d* (1.5)	6.47 *d* (2.0)	6.43 *d* (1.5)
H-8	6.81 *d* (2.3)	6.77 *d* (1.5)	6.82 *d* (1.5)	6.82 *d* (2.0)	6.71 *d* (1.5)
H-2″	7.96 *d* (8.9)	7.80 *d* (8.0)	7.90 *d* (8.0)	7.96 *d* (8.9)	7.80 *d* (8.0)
H-3″	6.94 *d* (8.9)	6.89 *d* (8.0)	6.96 *d* (8.0)	6.94 *d* (8.9)	6.92 *d* (8.0)
H-5″	6.94 *d* (8.9)	6.89 *d* (8.0)	6.96 *d* (8.0)	6.94 *d* (8.9)	6.92 *d* (8.0)
H-6″	7.96 *d* (8.9)	7.80 *d* (8.0)	7.90 *d* (8.0)	7.96 *d* (8.9)	7.80 *d* (8.0)
Glucose					
H-1″	5.11 *d* (7.3)	5.12 *d* (7.5)	5.07 *d* (7.5)	5.24 *d* (7.9)	5.09 *d* (7.5)
H-2″	3.38 *m*	3.56 *m*	3.53 *m*	3.40 *dd* (7.9; 9.3)	3.63 *dd* (7.5; 9.0)
H-3″	3.38 *m*	3.56 *m*	3.53 *m*	3.57 *dd* (9.3; 9.3)	3.77 *dd* (9.0; 9.0)
H-4″	3.20 *dd* (8.9; 8.9)	3.44 *dd* (9.0; 9.0)	3.40 *dd* (9.0; 9.0)	4.68 *dd* (9.3; 9.3)	4.92 *dd* (9.0; 9.0)
H-5″	3.76 *ddd* (1.5; 6.9; 8.9)	3.87 *ddd* (2.5; 4.5; 9.0)	3.80 *ddd* (2.5; 4.5; 9.0)	4.11 *m*	3.99 *m*
H-6a″	4.16 *dd* (6.9; 11.8)	4.30 *dd* (4.5; 11.5)	4.27 *dd* (4.5; 11.5)	4.11 *m*	4.28 *m*
H-6b″	4.40 *dd* (1.5; 11.8)	4.68 *dd* (2.5; 11.5)	4.48 *dd* (2.5; 11.5)	4.11 *m*	4.28 *m*
Acetyl					
$COCH_3$-4	–	–	–	2.07 *s*	2.20 *s*
$COCH_3$-6	–	–	2.10 *s*	–	2.20 *s*
Malonyl					
CH_2	3.38 *s*	–	–	3.33 *s*	–
Caffeoyl					
H-2‴	–	6.84 *d* (1.5)	–	–	–
H-5‴	–	6.62 *d* (8.0)	–	–	–
H-6‴	–	6.71 *dd* (8.0; 1.5)	–	–	–
H-α	–	6.24 *d* (16.0)	–	–	–
H-β	–	7.46 *d* (16.0)	–	–	–

[a] Assignments confirmed by DQF-COSY and 1D-TOCSY experiments. *J* values in Hz presented in parentheses.

Table 3
^{13}C NMR data of chamomile flavonoids[a]

Moiety	C atom	Flavonoid				
		Ap-7-(6″-malonyl-Glc) (**2**)	Ap-7-(6″-caffeoyl-Glc) (**4**)	Ap-7-(6″-acetyl-Glc) (**5**)	Ap-7-(4″-acetyl, 6″-malonyl-Glc) (**6**)	Ap-7-(4″,6″-di-acetyl-Glc) (**8**)
Aglycone	C-2	164.5	166.9	166.9	166.8	166.8
	C-3	103.3	103.7	104.1	103.4	104.1
	C-4	182.2	184.2	184.2	182.2	184.2
	C-5	161.5	162.9	163.3	162.7	163.0
	C-6	99.8	101.0	100.6	99.6	101.1
	C-7	162.9	164.6	164.6	164.5	164.4
	C-8	95.0	95.6	96.3	95.0	96.0
	C-9	157.2	159.0	158.8	157.1	158.8
	C-10	105.6	106.7	106.7	105.7	106.8
	C-1″	121.2	122.2	122.9	121.2	122.8
	C-2″	128.8	129.5	129.8	128.8	130.0
	C-3″	116.2	117.2	117.2	116.2	117.4
	C-4″	161.3	163.8	163.3	161.5	163.0
	C-5″	116.2	117.2	117.2	116.2	117.4
	C-6″	128.8	129.5	129.8	128.8	130.0
Glucose	C-1″	99.8	101.1	101.9	99.5	101.1
	C-2″	73.2	74.3	74.5	73.1	74.4
	C-3″	76.3	77.7	77.6	75.5	75.4
	C-4″	69.7	72.0	71.3	70.8	71.9
	C-5″	74.0	75.5	75.4	71.2	73.2
	C-6″	64.3	64.4	64.9	63.3	63.9
Acetyl	*C*OCH$_3$-4	–	–	–	172.3	172.6
	CO*C*H$_3$-4	–	–	–	20.8	21.6
	*C*OCH$_3$-6	–	–	172.6	–	172.6
	CO*C*H$_3$-6	–	–	20.6	–	21.6
Malonyl	*C*OCH$_2$-COOH-6	167.0	–	–	167.7	–
	CO*C*H$_2$-COOH-6	41.5	–	–	41.4	–
	COCH$_2$-*C*OOH-6	167.9	–	–	169.9	–
Caffeoyl	C-1‴	–	126.9	–	–	–
	C-2‴	–	115.3	–	–	–
	C-3‴	–	147.2	–	–	–
	C-4‴	–	149.6	–	–	–
	C-5‴	–	116.6	–	–	–
	C-6‴	–	122.8	–	–	–
	C-α	–	114.6	–	–	–
	C-β	–	146.7	–	–	–
	C═O	–	169.0	–	–	–

[a] Assignments confirmed by HSQC and HMBC experiments.

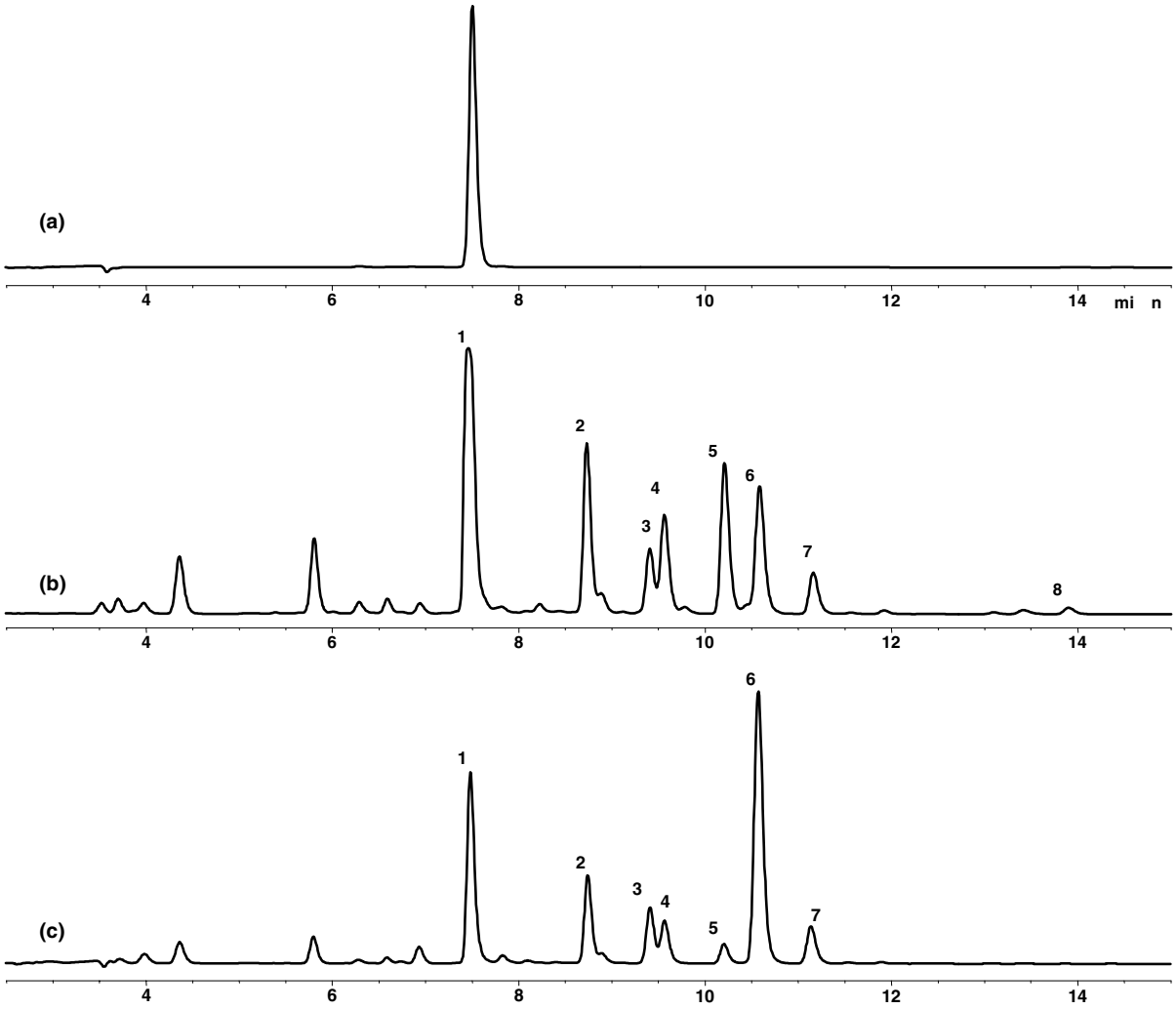


Fig. 3. Chromatograms showing Ap-7-Glc standard (a), flavonoids extracted from 20 °C air-dried petals with 70% v/v MeOH at 70 °C (b) and flavonoids extracted from fresh petals with 70% v/v MeOH at −20 °C (c). Chromatograms (b) and (c) are in the same scale. Peak ID as for Fig. 1.

Table 4
Formation of acetylated derivatives from apigenin 7-*O*-(4″-acetyl, 6″-malonyl)-glucoside in different solvents and pH condition

Solvent	Degradation products
Methanol	Ap-7-(4″-Acetyl-Glc) (**3**) and Ap-7-(6″-Acetyl-Glc) (**5**)
Ethanol	Ap-7-(4″-Acetyl-Glc) (**3**) and Ap-7-(6″-Acetyl-Glc) (**5**)
Acetonitrile:water (1:1)	Ap-7-(6″-Acetyl-Glc) (**5**) and Ap-7-(X″-Acetyl, Y″-Malonyl-Glc) (**7**)
Buffer solutions, pH 2–5	Ap-7-(6″-Malonyl-Glc) (**2**) and Ap-7-(4″-Acetyl-Glc) (**3**)
Buffer solutions, pH 5–7	Ap-7-(6″-Malonyl-Glc) (**2**) and Ap-7-(X″-Acetyl, Y″-Malonyl-Glc) (**7**)

Processing of the flowers, e.g. drying, could explain the presence of the diverse acetylated Ap-7-Glc derivatives found in previous studies. It has been reported that elevated temperature increases the cleavage of ester bonds (Tschiersch and Hölzl, 1992), and migration of acetyl groups was first reported for apigenin derivatives during a TLC experiment after short-term exposure to ammonia (Tschiersch and Hölzl, 1992).

The combined LC/MS, LC/MS/MS and NMR results from the present study clearly show that this important medicinal plant exhibits much more variability in both simple and complex acylated derivatives of Ap-7-Glc than previously reported. These rapid extraction and purification techniques could also be useful for studying flavonoids in other dietary herbs and medicinal plants.

3. Materials and methods

3.1. General

All solvents used were of HPLC grade and all water was ultra-pure (distilled, de-ionised, 18.2 MΩ). All other

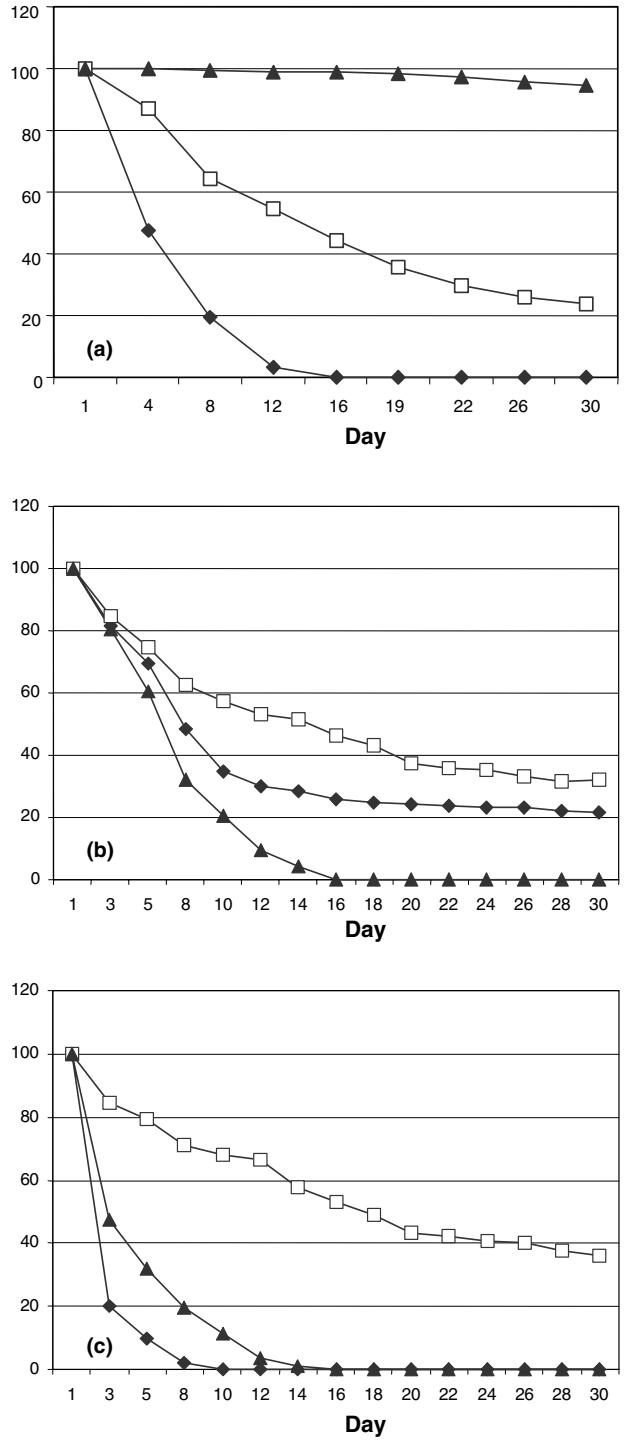


Fig. 4. Decrease of Ap-7-(4″-acetyl, 6″-malonyl-Glc) content in the methanolic extract of the ligulate florets of *Chamomilla recutita* due to its degradation during 30 days' experiment at the temperature of 25 °C (◆), 5 °C (□) and −18 °C (▲) (a); effect of pH 2 (□), pH 7 (◆) and methanol solvent (▲) on the stability of apigenin 7-*O*-(6″-malonyl)-glucoside in the methanolic extract of the ligulate florets of *Chamomilla recutita* during 30 day experiment at the temperature of 25 °C (b); effect of pH 2 (□), pH 7 (◆) and methanol solvent (▲) on the stability of apigenin 7-*O*-(4″-acetyl, 6″-malonyl)-glucoside in the methanolic extract of the ligulate florets of *Chamomilla recutita* during 30 day experiment at the temperature of 25 °C (c).

chemicals were of analytical grade and were obtained from commercial sources. Apigenin (aglycone) and apigenin 7-*O*-glucoside was obtained from Extrasynthese (Genay, France).

3.2. Plant material

Plants of diploid cultivar "Novbona" and tetraploid cultivar "Lutea" of *Chamomilla recutita* were cultivated in the experimental field of the Botanical Garden of P.J. Šafárik University in Košice (Slovakia) in the year 2000. Seeds of both diploid and tetraploid forms were collected from field plots in 2001 and grown in the Institute of Food Research in Norwich (UK) in the year 2002. Three sources of petals were used in the analyses. Air-dried flower samples from 2000, stored at −20 °C, dried petals removed from a commercial whole flower chamomile tea (Dr. Stuart's Botanical Teas, Only Natural Products Ltd., Horsham, West Sussex, RH12 5QW, UK) and also fresh and freeze-dried petals from the plants grown at the Institute of Food Research in 2002.

3.3. Extraction of floral tissues

Extractions were performed on fresh, freeze-dried, air-dried and commercially dried petals, but only selected data is presented in this paper. Sub-samples of the three types of dried petals (40 mg replicates of dry tissues) were homogenised and extracted with 1 mL 70% v/v MeOH at 70 °C for 20 min, in 2 mL screw-top tubes, with vortex mixing every 5 min to improve extraction. Fresh petals (12 mg replicates) were extracted with 1 mL 70% v/v MeOH at −20 °C for 20 min, in 2 mL screw-top tubes, with vortex mixing every 5 min to improve extraction. Post-extraction samples were centrifuged (17,000*g*, 4 °C, 20 min) and the supernatants filtered (0.2 μm PTFE filter, Chromos Express, Macclesfield, UK). These 70% v/v MeOH extracts were used for LC/MS and LC/MS/MS. Samples from the 70% MeOH extractions of 20 °C air-dried petals were used for further fractionation using polyamide. Unless processed immediately, all extracts at each stage in the analysis and subsequent purification steps were stored at −20 °C.

3.4. HPLC analyses

All samples and fractions from subsequent purification on the preparative HPLC were analysed using an Agilent HP1100 system with diode array detection in combination with a Phenomenex Luna C_{18} (2) reverse-phase column (250 × 4.6 mm, 5 μm) with a Security-guard® pre-column. Data were specifically collected at 220 nm (all UV-absorbing compounds), 335 nm (λ_{max} for the majority of the apigenin glucosides), 370 nm (flavonol glycosides) and overall from 200 to 600 nm.

Flow rate was 1 mL min^{-1}, solvent A = 81% ultra-pure water containing 19% HPLC grade acetonitrile (MeCN) and 0.1% trifluoroacetic acid (TFA), and solvent B = 100% MeCN. Gradient program: 0 min (95% A, 5% B), 5 min (80% A, 20% B), 10 min (70% A, 30% B), 15 min (65% A, 35% B), 20 min (40% A, 60% B), 22.5 min (100% B), 25 min (100% B), 27.5 min (95% A, 5% B), 35 min (95% A, 5% B).

3.5. LC/MS

All mass spectra were obtained using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK) coupled to a Jasco PU-1585 triple pump HPLC equipped with an AS-1559 cooled autoinjector, CO-1560 column oven and UV-1575 UV detector (Jasco (UK) Ltd., Great Dunmow, UK). The HPLC column temperature was maintained at 25 °C and the autoinjector at 4 °C. The 1 mL min^{-1} mobile phase flow exiting the HPLC column was split using an ASI 600 fixed ratio splitter valve (Presearch, Hitchin, UK) so that approximately 200 μL min^{-1} entered the mass spectrometer; the remainder of the flow was diverted to the UV detector (335 nm monitored). The flow split was monitored using a Humonics Optiflow 1000 flowmeter (Sigma–Aldrich, Dorset, UK) coupled to the outflow of the UV cell. HPLC conditions were otherwise as described above.

Mass spectra were obtained in both positive and negative ion electrospray mode using a Micromass Z-spray™ ion source. The electrospray probe was operated at 3.5 kV with a cone voltage of 22 V. The source and desolvation temperatures were 120 and 250 °C, respectively. The nitrogen nebulising and drying gas flow rate were optimized at 15 L h^{-1} and 500 h^{-1} respectively. Spectra were recorded (in centroid mode) between *m/z* 50–1000 with a scan duration of 2 s/scan and an inter-scan time of 0.1 s. MS1 was set to unit mass resolution or better (LM and HM resolution parameters both set to 15.0). Spectra were processed using MassLynx™ 3.4 software (Micromass, Manchester, UK).

3.6. LC/MS/MS

Conditions were the same as for LC/MS, above, with the following differences for the MS/MS experiment: argon gas was introduced into the collision cell of the mass spectrometer at a pressure of 10^{-1} Pa. Product ion spectra were obtained in positive ion electrospray mode at rates of 1 s per scan in raw data acquisition mode at a collision energy of 18 eV for the following masses: *m/z* 433 (retention window 5.5–7.7 min), 475 (retention window 8.5–11.0 min), 519 (retention window 7.7–8.5 min) and 595 (retention window 8.5–11.0 min). The MS/MS mass range scanned was from *m/z* 50 to 5% above the mass of the precursor ion. MS/MS spectra of each eluting component were averaged and smoothed using MassLynx™ 3.4 software.

3.7. Polyamide solid phase extraction (SPE) of flavonoids

Polyamide (MN Polyamide CC 6, Jones Chromatography) columns (1 g) were prepared: primed with 20 mL 100% MeOH followed by 60 mL ultra-pure water. To each column 1 mL of the 70% MeOH extracts of 20 °C air-dried petals, pre-diluted in 4 mL of ultra-pure water, was loaded. Sequential elution was done with 20 mL ultra-pure water (to elute unbound compounds), 40 mL 100% MeOH (elution of flavonoid aglycones and simple glycosides), and 40 mL 95.5% v/v MeOH/0.5% v/v NH_4OH (elution of malonylated and mono-acetylated/mono-malonylated flavonoids).

3.8. Preparative HPLC

The fractions from the polyamide separation were concentrated to a minimum volume and 1mL of the concentrate was diluted with 4mL ultra-pure water prior to loading. A Gilson preparative HPLC system was used in combination with a Prodigy C_{18} preparative column (250×21.2 mm, 5 μm) with a Prodigy guard column (60×21.2 mm, 5 μm). Data were specifically monitored at 280nm (general aromatic) and 335 nm (λ_{max} for the majority of the apigenin glucosides). Flow rate was 5 mL min^{-1}, solvent A = ultra-pure water containing 0.1% TFA, and solvent B = 100% MeCN. Gradient program: 0 min (80% A, 20% B), 15 min (70% A, 30% B), 60 min (27.5% A, 72.5% B), 75 min (80% A, 20% B), 90 min (80% A, 20% B). Multiple preparative HPLC separations were done and the fractions for each peak combined, once purity for each fraction had been confirmed using the analytical method. Solvent was removed from the samples at 20 °C with N_2gas and the residual aqueous phases were shell-frozen on dry ice prior to freeze-drying. The freeze-dried samples were stored at −20 °C until NMR analyses were performed.

3.9. Studies on the stability of apigenin glucosides

Stability of compounds was evaluated by means of HPLC over 30 days at 25 °C using the ligulate floret extracts (concentration of the extract was equivalent to 1 mg of ligulate florets mL^{-1} of MeOH) in different pH conditions: pH 2 (concentrated buffer solution FK21, Radelkis company), pH 4 (0.05 M solution of potassium phthalate), pH 5 (0.15 M solution of Na_2HPO_4), pH 5.6 (0.15 M solution of K_2HPO_4: 0.15 M solution of Na_2HPO_4[19:1]), pH 7 (concentrated buffer solution FK-71, Radelkis company), pH 9 (concentrated buffer solution FK-91, Radelkis company). Stability of the substances was also determined in aqueous MeCN (1:1 v/v) and

in 100% MeOH and 100% EtOH at 25 °C. For studies on temperature effects on stability the samples were monitored over 30 days at −18, 5 and 25 °C.

3.10. NMR identification of the compounds

Initial analyses were performed using a Bruker DRX-600 spectrometer operating at 599.19 MHz for ^{1}H and 150.86 MHz for ^{13}C using UXNMR software package for NMR measurements in CD_3OD solutions. 2D experiments: ^{1}H–^{1}H DQF-COSY (Bodenhausen et al., 1977), inverse-detected ^{1}H–^{13}C HSQC (Bodenhausen and Ruben, 1980) and HMBC (Martin and Crouch, 1991) were obtained by employing the conventional pulse sequences as described previously. The selective excitation spectra, 1D-TOCSY (Davis and Bax, 1985), were acquired using waveform generator-based GAUSS shaped pulses, mixing time ranging from 100 to 120 ms and a MLEV-17 spin-lock field of 10 kHz preceded by a 2.5 ms trim pulse. The rapid-purification samples were analysed in d_6-DMSO using a Jeol EX-270 spectrometer operating at 270.17 MHz for ^{1}H and 67.94 MHz for ^{13}C. Assignments were confirmed by a combination of ^{1}H–^{1}H COSY, Heteronuclear Shift Correlation (^{1}H–^{13}C COSY), and DEPT, and by comparison with the earlier measurements.

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