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Accumulation of coumarin-related compounds in leaves of *Matricaria chamomilla* related to sample processing

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ABSTRACT

This experiment was carried out to study the influence of sample processing on the quantitative changes of the main coumarin derivative in the leaves of *Matricaria chamomilla* – herniarin and its glucosidic precursors [(*Z*)- and (*E*)-2-β-D-glucopyranosyloxy-4-methoxycinnamic acids (GMCAs)]. Fresh and air-dried, as well as heat-dried samples (80, 100 and 120 °C), from four week-old plants were analysed. The content of *Z*-GMCA was the lowest in fresh samples while the content of herniarin was the highest in fresh samples. *E*-GMCA was not affected by sample processing. The *E*/*Z*-GMCA ratio decreased from fresh to heat-dried samples. The ratio of herniarin/*Z*-GMCA displayed the same trend. Probable mechanisms of these changes are also considered. The sum of GMCAs and herniarin, calculated as μmol g^{-1} DW, did not change significantly among all tested variants, to support our assumption that herniarin is not a volatile coumarin derivative.

1. Introduction

Coumarins are lactones derived from *o*-hydroxycinnamic acids by cyclization and ring closure between the *o*-hydroxy and carboxyl groups. These compounds are present mainly in the families Asteraceae, Apiaceae, Fabaceae and Poaceae. Coumarin occurs in plant tissue in bound form as the *trans-o*-glucosyloxycinnamic acid. After damage of tissue, it undergoes enzymatic loss of sugar, *trans-cis* isomerization, ring closure and release from the leaf surface. Some methoxycoumarins are present in plants bound in the same way (Harborne, 1980). Chamomile produces, besides other secondary metabolites, also coumarins (Schilcher, 1987). Herniarin (7-methoxycoumarin) is the main coumarin derivative in the leaves of chamomile present also in flowers (Repčák, Imrich, & Franeková, 2001a,b) and in related species (Ma, Winsor, & Daneshtalab, 2007). (*Z*)- and (*E*)-2-β-D-glucopyranosyloxy-4-methoxycinnamic acids (GMCAs), the glucosidic precursors of herniarin, were described as native compounds in chamomile (Kanamori, Terauchi, Fuse, & Sakamoto, 1993; Ohe et al., 1995; see Pastírová, Repčák, & Eliašová, 2004 for their chemical structure). The *Z*-form is unstable and will therefore tend to convert to the *E*-form. *E*–*Z* isomerization of the side-chain double bond occurs in the light. The *Z*-isomer, after hydrolysis by a specific enzyme β-glucosidase, spontaneously lactonizes to the coumarin derivative, herniarin. The *E*-forms of *o*-hydroxycinnamic acid derivatives are stable and cannot cyclize (Harborne, 1980). Similar change of GMCA to herniarin was described in lavender (Brown, 1963). Some minor coumarin derivatives were described in anthodia, e.g. aesculetin, scopoletin and isoscopoletin (Kotov, Khvorost, & Komissarenko, 1991; Redaelli, Formentini, & Santaniello, 1981). The contents of herniarin and the sum of GMCAs differ in diploid and tetraploid cultivars of chamomile and their contents are affected by $CuCl_2$ application on the leaves (Eliašová, Repčák, & Pastírová, 2004) or by their biosynthetic precursors, such as phenylalanine (Kováčik, Kron, Repčák, & Bačkor, 2007b). They also undergo quantitative changes during the development of the leaf rosettes (Pastírová, Repčák, & Eliašová, 2005) and nutritional deficit (Kováčik, Klejdus, Bačkor, & Repčák, 2007a).

Chamomile (*Matricaria chamomilla* L.) is a widely used medicinal plant. Phytotherapeutical effect is ascribed also to the amounts of coumarin-related compounds, which show antimicrobial and anti-inflammatory effects (Silván, Abad, Bermejo, Sollhuber, & Villar, 1996). Therefore, processing of harvested plants is important for the production of drug with a standard content of secondary metabolites. The aim of this experiment was (i) to study the quantitative changes of herniarin and *Z*/*E*-GMCA isomers related to varied sample processing and (ii) to find out whether herniarin can evaporate from samples during their processing.

2. Materials and methods

The 21 day-old plants of *M. chamomilla* (tetraploid 'Lutea') with first two-three true leaves (germinated in sand) were transplanted to slightly modified Hoagland solution (Kováčik et al., 2007a,b, 2008; Kováčik & Klejdus, 2008). Solution contained 4.03 mM

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$Ca(NO_3)_2 \cdot 6H_2O$, 0.522 mM $NH_4H_2PO_4$, 6.04 mM KNO_3, 1.99 mM $MgSO_4 \cdot 7H_2O$, 0.125 mM NaOH, 0.288 mM KOH, 89.2 μM EDTA, 89.6 μM $FeSO_4 \cdot 7H_2O$, 9.68 μM H_3BO_3, 2.03 μM $MnCl_2 \cdot 4H_2O$, 0.314 μM $ZnSO_4 \cdot 7H_2O$, 0.210 μM $CuSO_4 \cdot 5H_2O$, 0.139 μM Na_2MoO_4 and 0.0859 μM $CoCl_2 \cdot 6H_2O$. Five uniform plants per litre were cultivated in brown plastic 5l boxes (25 plants per box) with continual aeration of the solutions. The experiment was performed in a growth chamber under controlled conditions: 12 h day (6.00 am to 6.00 pm), the photon flux density was 210 μmol m^{-2} s^{-1} PAR at leaf level supplied by cool white fluorescent tubes TLD 36 W/33 (Philips, France), with a 25/20 °C day/night temperature and relative humidity 60%. Solutions were renewed weekly to prevent nutrient depletion. Plants, which had been cultivated in this solution for four weeks, were used for the quantification.

Fresh samples were immediately extracted (400 mg FW 5 ml^{-1} of 80% methanol) and analysed. The analyses of these extracts were repeated after 3 and 14 days to check their stability in the light. A second group of samples was air-dried at room temperature. The heat-dried samples were prepared at 80, 100 and 120 °C as follows: whole leaf rosettes were sliced to homogeneous fragments. After every hour of drying, a small part was used for HPLC estimation (40 mg DW 2 ml^{-1} of 80% methanol). After extraction at laboratory temperature (~20 °C), samples were centrifuged at 12,000*g* for 20 min (Boeco U-32R, Boeckel & Co, Germany) and filtered through membrane filters (0.5 μm pore size) prior to injection. Amounts of compounds in all variants were recalculated with the known tissue water content.

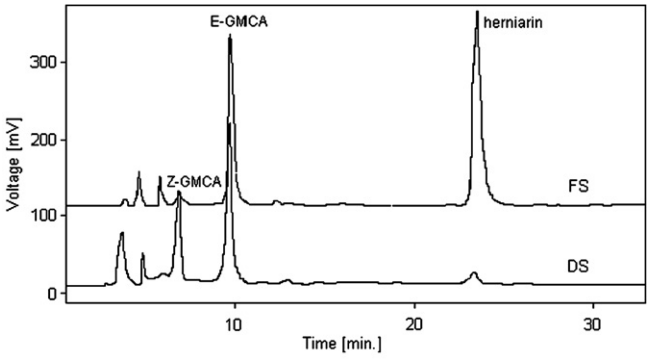

Fig. 1. HPLC chromatograms of methanol extracts from the leaves of chamomile with the peaks of analysed compounds. FS-fresh sample, DS-dried sample.

The HPLC system consisted of an Ecom pump (Prague, Czech Republic), a Rheodyne injector valve (20 μl), a Hewlett-Packard UV/Vis variable detector model 1050 (HP, Palo Alto, CA, USA), a SGX C 18 (7 μm) column (4 × 250 mm) and an Apex integrator (Prague, Czech Republic). The mobile phase was a mixture of A – acetonitrile:water:H_3PO_4 (19:80:1), B – 45% acetonitrile and C – 80% acetonitrile. The linear gradient elution programme was from 100% A to 100% B in 25 min, from 100% B to 100% C in 5 min, isocratic elution with 100% C for 5 min and from 100% C to 100% A in 10 min. All solvents were gradient grade (Merck, Germany). Detection was performed at 320 nm, flow rate 0.7 ml min^{-1}. Herniarin (Extrasynthese) standard compound was used for the quantification. Due to unavailability of standard compounds, (*E*)-GMCA was separated from methanol–water extract of the chamomile drug by silica gel column chromatography (for details see Repčák, Pastírová, Imrich, Švehlíková, & Mártonfi, 2001b). Due to the hygroscopic properties of (*E*)-GMCA, quantitative acid hydrolysis was done (10% HCl, 4 h) and the content of herniarin was estimated. (*Z*)-GMCA was prepared by UV-izomerization. The linearity of the method was determined using a known concentration of compounds by an external method of calibration. Recovery efficiency was determined by adding measured amounts of standards to the sample prior to extraction in order to evaluate the accuracy of the method. The recoveries were determined by subtracting the values obtained for the control matrix preparation from samples that were prepared with added standards. Three replicates at three concentration levels were performed. The identity of peaks was confirmed by UV–Vis spectra measured during HPLC analysis and by ^1H NMR, as described earlier (Repčák et al., 2001a,b).

3. Results and discussion

The typical chromatograms of 80% methanol extract from the leaves of chamomile are shown in Fig. 1. The recovery represented 94–105% for herniarin and 96–103% for GMCAs. The linearity of calibration was found in the range 1–12 and 0.4–15 μg ml^{-1} for herniarin and GMCAs, respectively. Higher content of *Z*-GMCA was observed in heat-dried and air-dried samples while its content in fresh samples was significantly lower. The content of *E*-GMCA remained significantly unchanged among all variants of sample processing (Table 1). The *E*/*Z*-GMCA ratio decreased from 11.1–6.5:1 for fresh samples to 1.6–1.4:1 among samples dried at 120 °C. The content of herniarin was higher within fresh samples

Table 1
The content of coumarin-related compounds in the leaves of chamomile related to the sample processing

			Z-GMCA (mg g^{-1} DW)	*E*-GMCA (mg g^{-1} DW)	Sum of GMCAs (mg g^{-1} DW)	Herniarin (mg g^{-1} DW)	GMCA + her (μmol g^{-1} DW)
NHDS	0D		0.40 ± 0.11 d	4.45 ± 1.32 a	4.85 ± 1.43 b	3.54 ± 0.56 a	44.5 ± 10.3 a
	3D		0.51 ± 0.17 d	3.90 ± 1.47 a	4.41 ± 1.64 b	3.59 ± 0.57 a	43.1 ± 9.77 a
	14D		0.56 ± 0.23 d	3.66 ± 1.23 a	4.22 ± 1.46 b	3.65 ± 0.56 a	42.4 ± 9.74 a
	AD		3.00 ± 1.19 a	4.68 ± 1.35 a	7.68 ± 2.54 ab	1.87 ± 0.65 b	50.2 ± 12.6 a
HDS	T	H	0.40 ± 0.11 d	4.45 ± 1.32 a	4.85 ± 1.43 b	3.54 ± 0.56 a	44.5 ± 10.3 a
	80	3	1.93 ± 0.50 c	6.06 ± 2.91 a	7.99 ± 3.41 ab	1.35 ± 0.41 bc	59.2 ± 12.2 a
		4	2.11 ± 0.56 bc	5.78 ± 3.11 a	7.89 ± 3.67 ab	1.52 ± 0.21 b	59.1 ± 18.1 a
	100	2	3.18 ± 0.72 abc	5.29 ± 1.41 a	8.47 ± 2.13 ab	0.49 ± 0.18 cd	46.5 ± 11.8 a
		3	3.33 ± 0.59 ab	5.42 ± 1.54 a	8.75 ± 2.13 ab	0.53 ± 0.15 cd	48.1 ± 11.01 a
		4	3.70 ± 1.05 a	5.68 ± 1.89 a	9.38 ± 2.94 a	0.55 ± 0.22 cd	51.5 ± 15.5 a
	120	1	3.12 ± 0.43 abc	4.52 ± 1.42 a	7.64 ± 1.85 ab	0.22 ± 0.07 d	40.6 ± 7.77 a
		2	2.94 ± 0.51 abc	4.66 ± 1.63 a	7.60 ± 2.14 ab	0.29 ± 0.09 d	40.9 ± 10.4 a
		3	3.35 ± 0.58 ab	4.65 ± 1.69 a	8.00 ± 2.27 ab	0.37 ± 0.11 d	43.3 ± 10.8 a
		4	3.59 ± 0.44 a	4.89 ± 1.37 a	8.48 ± 1.81 ab	0.42 ± 0.12 cd	46.1 ± 9.32 a
Results of ANOVA			$F = 19.54$	$F = 0.78$	$F = 2.96$	$F = 65.51$	$F = 1.09$
			$P < 0.001$	$P = 0.663$	$P = 0.003$	$P < 0.001$	$P = 0.393$

NHDS – non-heat-dried samples: 0D – immediately analysed fresh samples, 3D and 14D – the same fresh samples after 3 and 14 days of storage, AD – air-dried samples. HDS – heat-dried samples: T – temperature of drying (°C), H – duration of drying (hours). Data are means ± SD. Values in each vertical column followed by the same letter(s) do not differ significantly by Tukey's pairwise comparisons ($P < 0.05$, $n = 5$).

than within heat-dried samples. The ratio of herniarin/*Z*-GMCA decreased from 8.8–6.5:1 among fresh samples to 0.07–0.15:1 among samples dried at 100 and 120 °C. The sum of *Z*- and *E*-GMCA increased from fresh to heat-dried samples due to increase of *Z*-isomer. A similar course was recorded for the sum of GMCAs/herniarin ratio (1.4–1.1:1 among fresh samples and 34.7–20.2:1 for samples dried at 120 °C). No differences in the content of analysed compounds between sliced and non-sliced fresh leaf rosettes were observed (data not shown). Coumarin-related compounds are one group of phenolic metabolites. Chamomile has a relatively high total phenolic content (Yoo, Lee, Lee, Moon, & Lee, 2008). Additionally, in young fresh leaves of *Corchorus olitorius*, five coumarins were found to be produced as phytoalexins in responses to biotic and abiotic stress (Abou Zeid, 2002). One of the proposed mechanisms for increased accumulation of phenolics is enhanced activity of phenylalanine ammonia-lyase activity (Kováčik et al., 2007a). This pivotal enzyme of phenolic metabolism strongly reacts to environmental changes also in chamomile (Kováčik et al., 2007a; Kováčik & Klejdus, 2008). With respect to the coumarin-related pathway, a high content of herniarin in fresh samples may indicate enhanced activity of β-glucosidase, which converts the *Z*-isomer of GMCA to herniarin. This assumption can be supported by finding showing *E*/*Z*-GMCA and herniarin/*Z*-GMCA ratio of approximately 1:1 when samples were homogenized using liquid nitrogen (data not shown). When calculated, the sum of herniarin and GMCAs in μmol g^{-1} DW (note that GMCA were calculated without glucose on a molecular mass), non-significant differences among all tested variants were observed (Table 1). These results contradict the known fact that simple coumarin is released from the leaf surface after damage of tissue (Harborne, 1980).

The results obtained in this study can be summarized as follows: (i) the content of herniarin was the highest, while the content of *Z*-GMCA, a direct precursor of herniarin, was the lowest in fresh samples, (ii) the content of *E*-GMCA was not affected by sample processing, (iii) the content of the sum of GMCAs and herniarin, calculated as μmol g^{-1} DW, was not significantly affected by sample processing. These findings illustrate that, though quantitative changes of analysed compounds related to the sample processing were recorded, the total content of herniarin and its precursors remained unchanged, indicating that herniarin is not a volatile coumarin derivative.

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