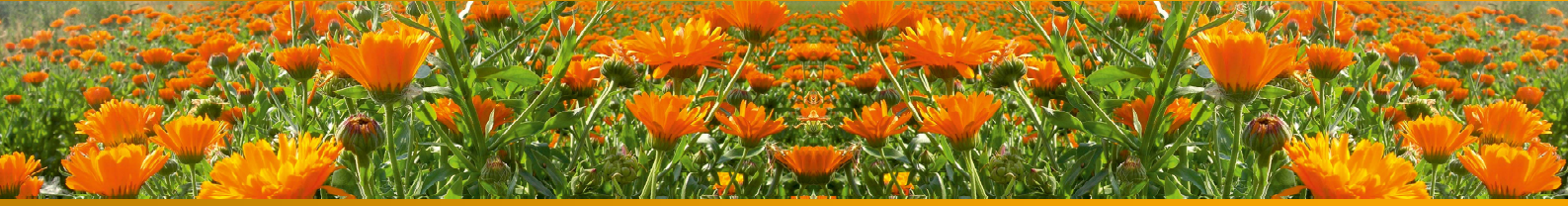

Characterization of flavonolglycosides in Calendula officinalis by orthogonal HPTLC and HPLC-MS

WELEDA

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Calendula officinalis has a long pharmacological tradition and especially the Calendula flowers have been the focus of investigations into its phytochemical constituents. In this work we compare the separation of flavonolglycosides in Calendula herba V.2a by normal phase and reversed phase HPTLC and structure characterization of the flavonoids by HPLC coupled with negative electrospray ionization (ESI) ion trap mass spectrometer employing an LTQ Orbitrap XL.

Sample preparation:

2 ml of the Calendula herba V.2a was diluted with 10 ml of water and applied to a previously activated (2 ml methanol followed by 2 ml water) Sep-Pak C_{18} cartridge. After washing with 5 ml water, the flavonoid fraction was eluted with 5 ml of 100 % Methanol. The eluate was evaporated to dryness and the residue was dissolved in 1 ml of methanol.

HPTLC Analysis:

Analytical HPTLC of the tinctures was performed on 20 cm x 10 cm glass plates precoated with Kieselgel 60 and Kieselgel 60 RP 18, W (Merck , Darmstadt, Germany). TLC of calendula tincture was performed with HCOOH/H_2O/AcOEt 15:15:70 (normal phase) and MeOH/HCOOH/H_2O 5,5:1:4,5 (reversed phase) as mobile phase. NP/PEG* at 365 nm was used for detection. The individual zones of underivatised HPTLC-plates were eluted with the CAMAG TLC-interface (CAMAG, Berlin, Germany).

**Natural products-polyethylene glycol reagent*

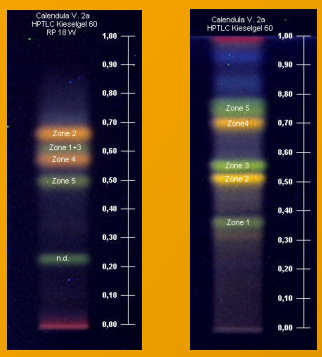

Figure 3: HPTLC (left : silica gel 60 RP 18 W, right: silica gel 60) of the calendula tincture according to the method used for the HPTLC analyses.

Instrumentation for HPLC-DAD Analysis

HPLC was performed with a Thermo Fisher Scientific Accela (Thermo Fisher Scientific, Dreieich, Germany) instrument with diode-array detector. The equipment was controlled by means of Xcalibur, Version 2.07. Compounds were separated on a 100 x 200 mm i.d., 1,9 µm particle, Hypersil Gold RP 18 column (Thermo Fisher Scientific; Dreieich; Germany) maintained at 25 °C.

Time	Percentage mobile phase B (%)	Remarks
0 → 32	12 → 20	Start of linear gradient 12-20 % B in 30 min
32 → 35	20 → 100	Washing step
35 → 40	100	
43 → 47	100 → 12	Reequilibration

Table 1: multi step linear gradient

The mobile phase was a multi step, 47 min, linear gradient prepared from 0,1 % HCOOH and CH_3CN (*Table 1*); the flow rate was 0,50 mL · min^{-1}. The volume of sample solution injected was 5 µL. Peaks were recorded in the range 250 – 450 nm, and chromatogramms were acquired at 280 and 370 nm.

Instrumentation for HPLC-MS Analysis:

The HPLC system described above was coupled with an LTQ Orbitrap XL (Thermo Fisher Scientific, Dreieich, Germany). Ionisation was performed with an electrospray interface equipped with a metal needle kit. The conditions used for mass spectrometry were as follows: capillary voltage -40 V, spray voltage 3.00 kV, tube lens -120 V. Nitrogen was used as drying gas. The capillary temperature was set at 300 °C. Collision-induced dissociation spectra (CID) were obtained with a collision energy of 35 and an activation time of 30 ms. Full scan spectra from m/z 150-1000 were obtained in negative mode and quercetin was used for the optimization of ionization parameters.

HPTLC-Zone	RF value normal phase	RF value reversed phase	Ret. time HPLC-MS (min)	m/z $[M-H]^-$	m/z of aglycon in MS^2 spectrum	Compound
1	0.36	0.61	7.8	769	314/315	Isorhamnetin-3-O-2^G-rhamnosylrutinosid
2	0.52	0.66	5.8	609	300/301	Quercetin-rutinosid
3	0.56		9.4 / 12.7	623	314/315	Isorhamnetin-rutinosid
		0.61	9.4	623	314/315	Isorhamnetin-rutinosid
4	0.70	0.57	7.5	463	300/301	Quercetin-3-O-glucosid
5	0.75	0.50	13.1	477	314/315	Isorhamnetin-3-O-glucosid
n.d.		0.23				not identified

Table 2: Identification of the typical flavonolglycosides in the zones extracted from the normal and the reversed phase silica plates according to the method used for the HPLC-MS analysis.

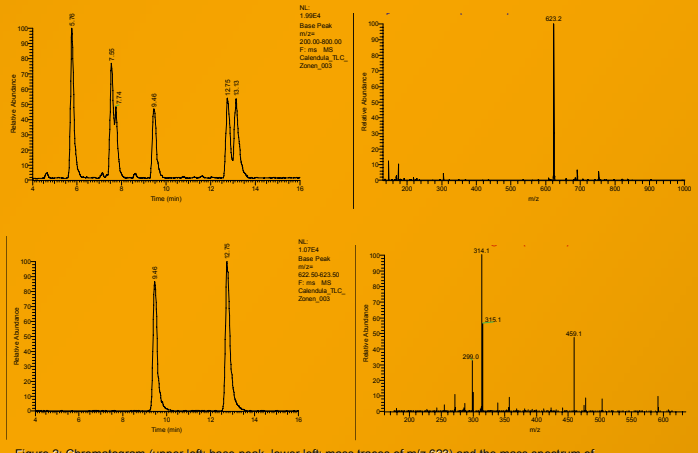

Figure 2: Chromatogram (upper left: base peak, lower left: mass traces of m/z 623) and the mass spectrum of the compound with m/z 623 (upper right: full mass spectrum, lower right: ms2 fragment spectrum of the peak at 9.5 min in the calendula tincture according to the method used for the HPLC-MS analysis.

Summary:

Figure 3 shows two yellow (quercetin) and three green (isorhamnetin) zones which, according to their observed m/z-ratio and the characteristical mass spectrometric fragmentation can be clearly identified as being flavonolglycosides. Due to the reduced interactions between the substance and the reversed phase silica plate, the compounds substituted with a disaccharide (rutinosid) reach a higher Rf-value compared to normal phase. The compounds substituted with a monosaccharide (glucose) reach a lower Rf-value compared to normal phase.

In the HPTLC-zone three, two compounds with the same m/z- ratio as well as a similar fragmentation pattern were detected, although they have different structural properties as is evident from their retention time. These compounds have thus been assigned a tentative structural identity.

The zone which is signed n.d. indicates no specific m/z ratio for flavonolglycosides.

Figure 2 shows an example for a typical base peak chromatogram and a fragmentation pattern from the m/z value of 623 which is identified as isorhamnetin-rutinosid by reference to published literature data [3].

Literature References

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