Identification of Radical Scavenging Compounds in *Rhaponticum carthamoides* by Means of LC-DAD-SPE-NMR

Giedrius Miliauskas,[†,‡] Teris A. van Beek,[*,†] Pieter de Waard,[§] Rimantas P. Venskutonis,[‡] and Ernst J. R. Sudhölter[†]

Laboratory of Organic Chemistry, Natural Products Chemistry Group, Wageningen University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands, Department of Food Technology, Kaunas University of Technology, Radvilenu pl. 19, Kaunas, Lithuania, and Wageningen NMR Centre, Laboratory of Biophysics, 6700 ET Wageningen, The Netherlands

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A hyphenated LC-DAD-SPE-NMR setup in combination with on-line radical scavenging detection has been applied for the identification of radical scavenging compounds in extracts of *Rhaponticum carthamoides*. After NMR measurements, the pure compounds were infused into a mass spectrometer. The technique enabled selective detection and identification of individual radical scavenging compounds without any prior off-line chromatographic steps. Seven compounds, namely, quercetagetin-7-β-glucopyranoside (**1**), quercetagetin-7-(6″-acetyl-β-glucopyranoside) (**3**), 6-hydroxykaempferol-7-β-glucopyranoside (**2**), 6-methoxykaempferol-3-β-glucopyranoside (**4**), 6-hydroxykaempferol-7-(6″-acetyl-β-glucopyranoside) (**5**), chlorogenic acid (**6**), and β-ecdysone (**7**), were identified in ethanol or aqueous extracts. Compound **5** is a new natural compound. Its radical scavenging activity was tested against DPPH radical and was found to be weaker than that of the reference antioxidants rosmarinic acid and Trolox.

Various degenerative diseases occurring in living cells are caused by so-called reactive oxygen species (ROS). Deterioration processes in food products, caused by ROS, are also associated with unfavorable effects. Significant changes can occur in product flavor, color, and texture and finally can lead to loss of nutritive value or complete spoilage. These facts stimulate the research on antioxidants, which are used to prevent these processes. The use of synthetic antioxidants in food products is under strict regulation due to uncertainty of their safety.[1,2] In recent years an increasing interest in "natural" food additives among consumers and consequently producers is observed.

The presence of antioxidant substances in plants depends on various factors, including the climatic or growing conditions. Studies of aromatic and medicinal plants grown in Lithuania by using various methods have provided information on the antioxidant properties of some less investigated plants[3−6] and led to the identification of new antioxidants.[3,4] These findings encouraged further investigations, and recently several new plants grown in Eastern and Central Europe were screened for their antioxidant properties.[7] The extracts of *Rhaponticum carthamoides* were shown to possess strong radical scavenging capacity.

Rhaponticum carthamoides (Asteraceae) is a widespread and often used medicinal plant. Originally an endemic plant of southern Siberia, now it is widely grown in Central and Eastern Europe.[8] The principal bioactive constituents of the whole plant are ecdysteroids, flavonoids, and phenolic acids. The aerial parts also contain sesquiterpene lactones of the guaianolide type, while the roots contain thiophene-based polyines.[8]

Our study was aimed at the identification of the major radical scavenging compounds of *R. carthamoides* extracts by using liquid chromatography setup coupled to a solid-phase extraction unit and NMR detector (LC-DAD-SPE-NMR).

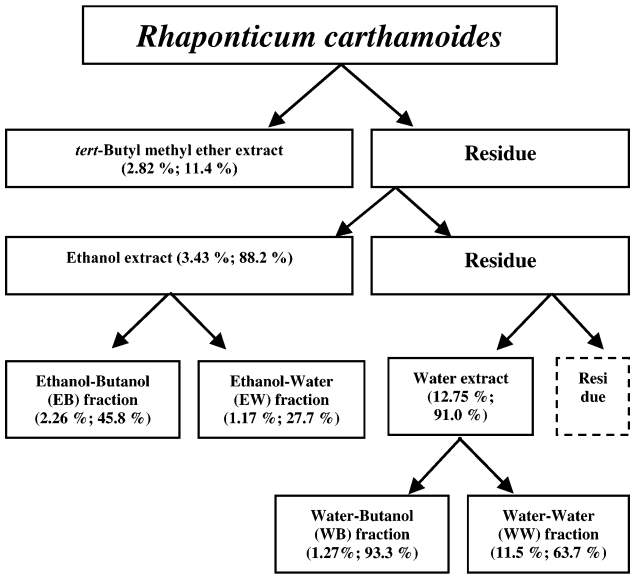

Figure 1. Extraction−fractionation scheme of the plant and the radical scavenging activity (RSA) evaluation of the obtained fractions. In parentheses: yield, %; decrease in absorbance (%) 30 min after initial mixing of DPPH• and extract solutions. Final mass ratio between them was 3:1 for *tert*-butyl methyl ether, ethanol, water extracts, and water−water fractions and 0.75:1 for others.

Results and Discussion

The scheme of the extraction−fractionation procedure of the plant material is shown in Figure 1. The plant was rich in polar substances, as the water fraction was the most abundant fraction. Off-line evaluation of 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging activity (RSA) of all obtained fractions provided data about the distribution of the active components in the plant. The ether extract was the least active against DPPH radicals. The water−water (WW) fraction also showed somewhat lower activity in comparison to the ethanol−butanol (EB), ethanol−water (EW), and water−butanol (WB) fractions (see Experimental Section for details). For further analysis all EtOH and H_2O fractions have been investigated by on-

* To whom correspondence should be addressed. Tel: + 31 317 482376. Fax: + 31 317 484914. E-mail: teris.vanbeek@wur.nl.
† Wageningen University.
‡ Kaunas University of Technology.
§ Wageningen NMR Centre.

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line LC-DPPH$^{\bullet}$ (ABTS$^{\bullet+}$) scavenging measurements. These methods are based on the post-column addition of stable radical (DPPH$^{\bullet}$ or ABTS$^{\bullet+}$ [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation]) solution to the HPLC eluent. When a radical scavenger elutes, the model radical is reduced, leading to a reduction in absorbance. This in turn gives a quick indication about the activity of individual peaks without prior isolation steps.[9,10]

On-line HPLC radical scavenging detection tests indicated that the WB and EB fractions of the plant possessed a similar profile of compounds, with slightly higher amounts of polar compounds in the WB fraction. Both fractions contained several major constituents (detected at 254 nm, see Figure 2b), showing radical scavenging activity. From the WB chromatogram one can also observe that minor compounds in that fraction, judging from the corresponding negative signals, possessed quite high radical scavenging activity, similar to that of major compounds. The HPLC profile of the WW fraction showed that it consisted of one major and a few minor active compounds (Figure 2a).

Next the HPLC setup was connected to a solid phase trapping unit that was in turn coupled to an NMR detector (see Experimental Section for scheme details). Earlier studies using the described LC-DAD-SPE-NMR setup have shown the potential of such a setup: a significant speed-up of the isolation−identification process, as time- and labor-intensive chromatographic steps can be avoided.[11] A limitation is the still relatively poor sensitivity of the NMR detector, especially for recording 2D NMR spectra like HMBC. Therefore, to obtain substantial amounts of compounds of interest, we have used a semipreparative HPLC column. More sample could be loaded on such a column, and consequently higher amounts of compounds were trapped. The larger injected volumes did not pose a problem because of the focusing effect of the SPE unit. Multiple peak trapping of the same analyte by repeated LC injections was implemented in some cases to obtain higher amounts of compounds.

Under optimized conditions of the LC-SPE-DAD-NMR setup (0.5 mL/min flow rate of HPLC pump and 1 mL/min of water makeup pump, proper cartridges) five relatively rare flavonoid glycosides, namely, quercetagetin-7-β-glucopyranoside (**1**), 6-hydroxykaempferol-7-β-glucopyranoside (**2**), quercetagetin-7-(6″-acetyl-β-glucopyranoside) (**3**), 6-methoxykaempferol-3-β-glucopyranoside (**4**), and 6-hydroxykaempferol-7-(6″-acetyl-β-glucopyranoside) (**5**), were isolated and identified from EB and WB fractions. Two of these glycosides (**1** and **2**) have been reported previously as constituents of *R. carthamoides*.[12] β-Ecdysone (**7**) was isolated and identified in the EB (WB) fraction. From the chromatographic profile (see Figure 2b), it appeared that β-ecdysone possesses radical scavenging properties; however this could be due to some minor phenolic compound coeluting at the same time. Chlorogenic acid was the major radical scavenger present in the water−water fraction.

Structures of the flavonoid glycosides **1**−**5** were elucidated by UV spectra, 1D and 2D ^{1}H and ^{13}C NMR techniques, and MS. Structures of chlorogenic acid and β-ecdysone were determined by ^{1}H NMR, UV, and MS data. 1D ^{1}H NMR spectra of all compounds were recorded directly (on-line) after drying cartridges and transferring compounds to the LC-NMR probe. Amounts of compounds trapped on one cartridge were insufficient for heteronuclear NMR experiments; therefore analyte trapping was continued for two or three additional runs on separate cartridges. Pure analytes obtained from these runs were combined and

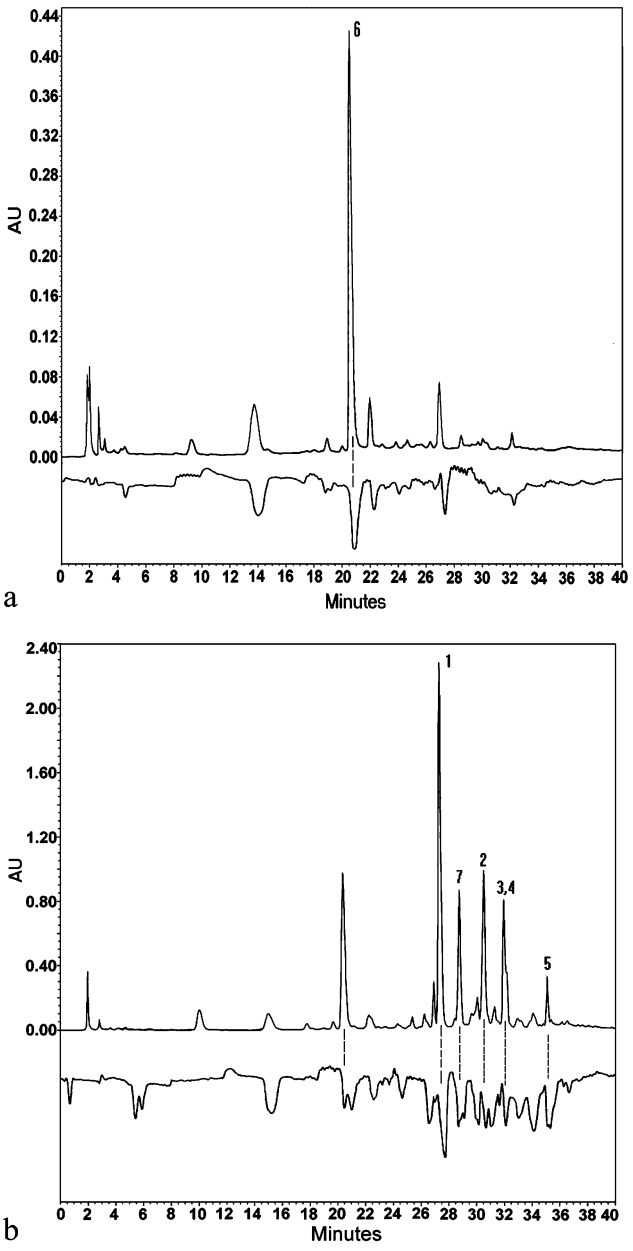


Figure 2. On-line RP-HPLC-UV-ABTS$^{\bullet+}$ scavenging assay profiles of *Rhaponticum carthamoides* WW fraction (a) and WB fraction (b). Upper profiles: UV signal at 254 nm. Lower profiles (negative peaks): ABTS$^{\bullet+}$ reduction signal. Numbers at the top of the peaks correspond to the compounds quercetagetin-7-β-glucopyranoside (**1**), 6-hydroxykaempferol-7-β-glucopyranoside (**2**), quercetagetin-7-(6″-acetyl-β-glucopyranoside) (**3**), 6-methoxykaempferol-3-β-glucopyranoside (**4**), 6-hydroxykaempferol-7-(6″-acetyl-β-glucopyranoside) (**5**), chlorogenic acid (**6**), and β-ecdysone (**7**).

redissolved, and ^{13}C or 2D (HMBC) spectra (off-line) were recorded in SHIGEMI tubes using a standard (5 mm i.d.) NMR probe.

The ^{1}H NMR spectrum of **5** (recorded on-line, see Figure 3) with 2H doublets at δ 8.0 and 6.9 suggested a kaempferol type of flavonoid. Multiple peaks between δ 3.2 and 4.0, a 1H singlet at δ 6.9, and 3H singlet at δ 2.00 in combination with the absence of other peaks in the low-field area suggested an acetylated 6-substituted kaempferol glycoside. APCI-MS gave a molecular weight of the compound of 506. A pronounced fragment at *m/z* 302 confirmed the presence of a hydroxy-substituted kaempferol structure (286 [kaempferol] + 16 [hydroxyl group] = 302), while the acetyl group was attached to the sugar. Exact mass measurements provided the elemental composition $C_{23}H_{22}O_{13}$.

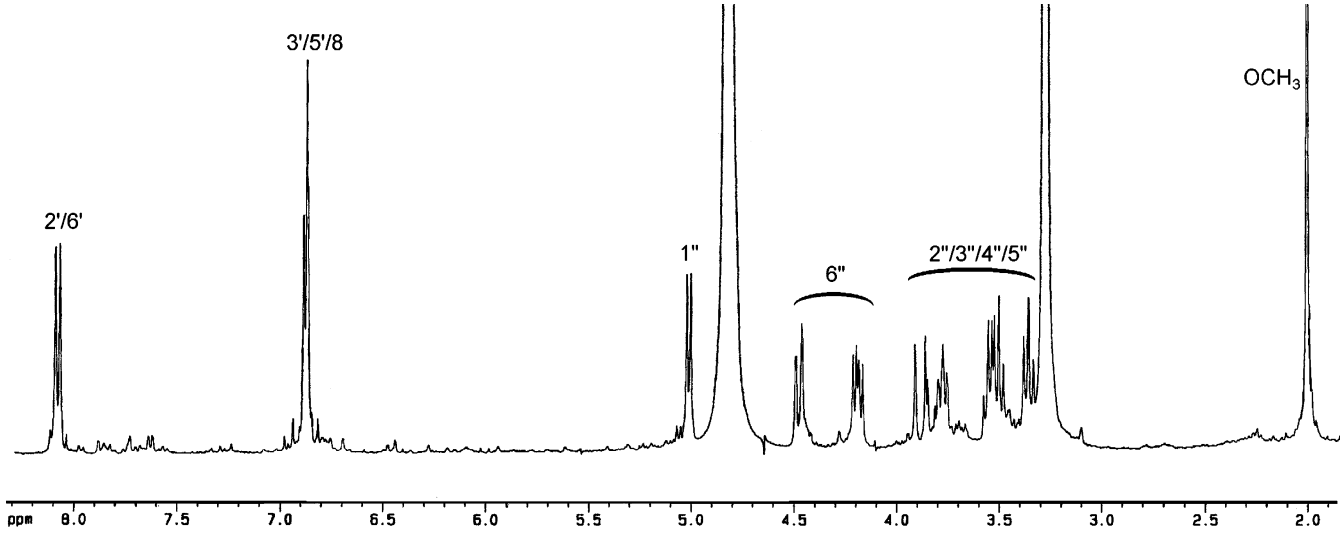
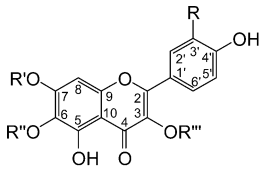


Figure 3. ^{1}H NMR spectrum (recorded on-line in CD_3OD) of 6-hydroxykaempferol-7-(6″-acetyl-β-glucopyranoside) (**5**).



	R	R′	R″	R‴
1	OH	-glucopyranoside	H	H
2	H	-glucopyranoside	H	H
3	OH	(6″-acetyl) glucopyranoside	H	H
4	H	H	CH_3	-glucopyranoside
5	H	(6″-acetyl) glucopyranoside	H	H

Figure 4. Radical scavenging compounds identified in *Rhaponticum carthamoides*.

The HMBC spectrum allowed total elucidation of the structure. The 6″-position of the glucopyranosyl moiety was acetylated, and the sugar was linked to position 7 of 6-hydroxykaempferol (see Figure 4). This compound has not been reported before as a natural product. Harborne et al. mentioned an acetylated glycoside of 6-hydroxykaempferol from *Chrysantinia mexicana*;[13] however the structure of the sugar moiety was not fully resolved.

The activity of **5** was tested off-line against the stable DPPH radical and compared to that of two reference antioxidants, rosmarinic acid and Trolox. Two molar concentration ratios of DPPH versus the tested compound were chosen (2 and 5). At a ratio of 2, 6-hydroxykaempferol-7-acetylglycoside (**5**) showed 75% inhibition of the absorbance of DPPH, while at the same concentration reference antioxidants rosmarinic acid or Trolox gave 98% inhibition. At a ratio of 5:1, **5** still showed good radical scavenging activity, although it remained lower than that of reference compounds: 36% versus 89% for rosmarinic acid and 79% for Trolox. For a complete evaluation of radical scavenging or antioxidant activity of *R. carthamoides* extracts or pure compounds, more studies by different methods, including real food systems, are needed.

This study shows that LC-DAD-SPE-NMR holds considerable promise for rapidly identifying secondary metabolites in plant extracts. All compounds were identified without prior off-line column chromatography or preparative HPLC by a combination of UV spectra, 1D and 2D NMR, and mass spectrometry. Mass spectra were simply recorded by infusion APCI-MS, as the compounds after LC-NMR were pure. In this study 2D NMR spectra were recorded off-line after multitrapping on the SPE unit because of the rather high volume and limited sensitivity of the 400 MHz LC-NMR probe used. However with smaller probes (<40 μL) and stronger fields (600−800 MHz) or with a cryoprobe (500−600 MHz), also 2D spectra can be recorded on-line, further speeding up the identification process.[11] It would also reduce the number of practical manipulations and the possible introduction of impurities. The SPE unit was very flexible in use and allowed analyte focusing, multitrapping, the use of nondeuterated solvents, and the measurement in 100% deuterated solvents. This is a considerable improvement over the more traditional LC-NMR with partially deuterated solvents. In the future combining LC-DAD-SPE-NMR with on-line bioassays, like an antioxidant screen in this study, and on-line MS will further speed up the identification and dereplication process, which is needed for high-throughput screening.

Experimental Section

General Experimental Procedures. All solvents used in HPLC analysis were of analytical grade. The melting points were measured on a Büchi 510 apparatus (Buchi, Flawil, Switzerland) and are uncorrected. The optical rotation was measured on a Perkin-Elmer 241 polarimeter at 589 nm in a 10 cm 1 mL cell. UV and IR spectra were recorded on Perkin-Elmer Lambda 18 UV/vis (Perkin-Elmer, Ueberlingen, Germany) and Vector 22 FT-IR (Bruker, Billerica, MA) spectrometers, respectively. Exact mass measurements were performed on a Micromass Q-Tof Ultima API mass spectrometer (Micromass, Manchester, UK). Mass spectra were recorded on a Finnigan LCQ spectrometer (Thermo Finnigan, San Jose, CA) in the ESI or APCI mode. All NMR spectra were recorded on a Bruker DPX 400 spectrometer (Bruker, Rheinstetten, Germany) except for the HMBC spectrum of **5**, which was recorded on a Bruker AMX 500 spectrometer. HMBC spectra of all compounds were recorded using SHIGEMI tubes (Campro Scientific, Veenendaal, The Netherlands).

Plant Material and Extraction Procedures. Aerial parts (leaves and stems) of *Rhaponticum carthamoides* were harvested in June 2003 in the experimental garden of medicinal plants of Kaunas Botanical Garden at the Vytautas Magnus University (Lithuania), and a voucher specimen (V01213) is deposited in the local herbarium. Leaves and stems were dried in a drying room with active ventilation at ambient temperature (<30 °C) and stored in paper bags for 3−4 months before use. Aerial parts (100 g) were ground in a Moulinex sample

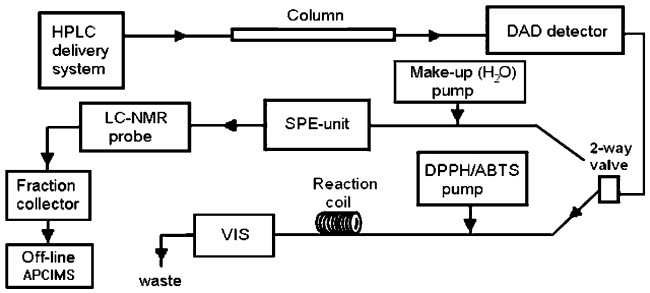


Figure 5. Scheme of experimental LC-DAD-SPE-NMR setup. Depending on the position of the 2-way valve, either radical scavenging detection or LC-NMR occurs.

mill (Erevete, France) and extracted with 2 × 250 mL of *tert*-butyl methyl ether at room temperature for 2 × 12 h under constant stirring with an Ikamag RTC magnetic stirrer (IKA Labortechnik, Staufen, Germany). The ether extract was filtered through medium-porosity filter paper, and the solvent was evaporated with a Büchi RE rotary evaporator connected to a Vacuubrand CVC2 vacuum pump and Büchi 461 water bath (Vacuubrand, Wertheim, Germany). The plant material remaining after extraction with ether was re-extracted with 2 × 250 mL of EtOH (2 × 12 h) and finally with 500 mL of hot H_2O (80−90 °C, 1 h). The EtOH and H_2O extracts were partitioned between H_2O and 1-butanol, resulting in EtOH−butanol (EB), EtOH−H_2O (EW), H_2O−butanol (WB), and H_2O−H_2O (WW) fractions (see Figure 1). EW and WW fractions were freeze-dried with a Christ Alpha 1-2 freeze-drier (Christ Gefriertrocknungsanlagen, Osterode, Germany), equipped with a Vacuubrand rotary vane vacuum pump.

Off-Line DPPH Radical Scavenging Assay. Radical scavenging abilities of different fractions (compounds) were determined by using an off-line DPPH• assay method.[14] Methanolic solutions (10^{-4} M) of DPPH• (95%, Sigma-Aldrich Chemie, Steinheim, Germany) and the fraction (compound) to be tested were mixed in a 1 cm path length disposable plastic half-micro cuvette (Greiner Labortech, Alphen aan den Rijn, The Netherlands). Samples were kept 30 min in the dark at room temperature, and the decrease in absorbance (515 nm) was measured using a Lambda 18 spectrophotometer (Perkin-Elmer, Ueberlingen, Germany). For RSA estimation various mass ratios between the fraction and DPPH• were chosen. For the compound activity comparison two reference antioxidants, rosmarinic acid (Extrasynthese, Genay, France) and Trolox (97%, Sigma-Aldrich Chemie, Steinheim, Germany), were used. All determinations were performed in triplicate. The RSA activity was expressed as percentage of inhibition in absorbance, as compared to the blank sample (methanol).

On-Line Radical Scavenging Assays. The most active fractions were further monitored by on-line RP-HPLC-DPPH• or ABTS•+ (Fluka Chemie, Buchs, Switzerland) assay methods, which were performed as described by Dapkevicius et al. and Koleva et al.[9,10] The equipment setup for on-line assessment of RSA was also identical to that described by Koleva et al. On-line assays were also carried out in combination with the LC-DAD-SPE-NMR setup (vide infra).

LC-DAD-SPE-NMR Instrumental Setup. The scheme of the experimental setup is presented in Figure 5. The HPLC consisted of the following parts: Bruker HPLC pump (LC 22), LC 225 gradient former, Bruker diode array detector (DAD). An Xterra semipreparative column (MS C18, 7.8 × 100 mm, 5 μm, Waters, Milford, MA) was used for the separation, and a binary gradient with 0.5 mL/flow rate was applied. Solvent A was 1% MeCN solution in H_2O acidified with 0.1% TFA; B, 100% MeCN. The gradient conditions for EB or WB fractions were as follows: 0−10 min B 12%, 10−50 min B increased to 35%, 50−55 min B reached 100% and kept constant until 60 min, 60−65 min B decreased until 12%. The gradient conditions for the WW fraction were almost the same as for EB and WB fractions, except that the first 10 min the percent of B was 5% instead of 12%. After the HPLC separation, compounds were detected with the diode array detector. The total flow was then directed to (i) the radical scavenging detector or (ii) the trapping (solid phase extraction, SPE) unit. Thus, two separate runs were carried out. In the first run radical scavenging activity of separate constituents in the extract mixture was determined by on-line methodology (see paragraph above and references noted there for details). After the HPLC separation an additional syringe pump was used (45 mL laboratory-made syringe pump, Free University, Amsterdam, The Netherlands), which added radical solution to the HPLC eluent. Radical reduction reaction took place during 30 s in the 15 m length PEEK tubing reaction coil. Any reduction of the radical was monitored with a visible light detector equipped with a tungsten lamp (759A, Applied Biosystems, Foster City, CA). In the second run peaks of interest were trapped with the SPE unit (Prospect 2, Spark Holland, Emmen, The Netherlands) according to the UV signal and their RSA determined in the first run. The additional so-called makeup pump was used to add extra H_2O (1 mL/min) to the eluent to reduce its elution power. Hysphere Resin SH cartridges (2 × 10 mm, packed with 10−12 μm particles, Spark Holland) were used for trapping. Afterward SPE cartridges were dried (with nitrogen flow) and active compounds transferred to a Bruker 4 mm inverse $^1H/^{13}C$ pulse-field gradient flow probe operating at 400 MHz (total volume 240 μL, actual detection volume 120 μL) using 100% deuterated solvents (CD_3OD or MeCN-d_3). The peaks were then collected for additional measurements, e.g., MS analysis.

Table 1. ^1H and ^{13}C Data for Compound **5** in DMSO-d_6 (δ = ppm)

position	^1H δ, mult; J (Hz)	^{13}C, δ
2		147.5
3		135.6
4		170.2
5		145.5
6		131.4
7		151.4
8	6.89, s	93.5
9		148.1
10		105.2
1′		121.7
2′	8.04, d; 8.9	129.6
3′	6.92, d; 8.9	115.4
4′		159.4
5′	6.92, d; 8.9	115.4
6′	8.04, d; 8.9	129.6
1"	5.03, d; 7.4	100.7
2"	3.39, m	73.1
3"	3.38, m	75.6
4"	3.20, m	69.9
5"	3.76, m	74.1
6"	4.06, dd; 7.6, 11.9	63.5
	4.39, dd; 1.4, 11.9	
O*CH*$_3$	2.00, s (3H)	
CO*C*H$_3$		20.6
*C*OCH$_3$		176.3

Quercetagetin-7-*O*-*β*-glucopyranoside (1): yellow powder; UV (MeOH) λ_{max} 258, 274, 355 nm; ^1H and ^{13}C NMR data are in agreement with those published;[15] APCIMS (positive ion mode) *m/z* 481 $[M + H]^+$.

6-Hydroxykaempferol-7-*O*-*β*-glucopyranoside (2): yellow powder; UV (MeOH) λ_{max} 255, 269, 350 nm; ^1H and ^{13}C NMR data are in agreement with those published;[16] APCIMS (positive ion mode) *m/z* 465 $[M + H]^+$.

Quercetagetin-7-*O*-(6″-*O*-acetyl-*β*-glucopyranoside) (3): yellow powder; UV (MeCN) λ_{max} 262, 346 nm; ^1H and ^{13}C NMR data are in agreement with those published;[15] APCIMS (positive ion mode) *m/z* 523 $[M + H]^+$.

6-Methoxykaempferol-3-*O*-*β*-glucopyranoside (4): yellow powder; UV (MeCN) λ_{max} 263, 350; ^1H and ^{13}C NMR data are in agreement with those published;[17] APCIMS (positive ion mode) *m/z* 479 $[M + H]^+$.

6-Hydroxykaempferol-7-*O*-(6″-*O*-acetyl-*β*-D-glucopyranoside) (5): yellow powder; mp 173−176 °C; $[\alpha]^{22}_D$ −87° (*c* 0.07, MeOH); UV (MeOH) λ_{max} 257, 274, 348 nm; IR (DMSO-

d_6) ν_{max} 3432, 3295, 2928, 1737, 1660, 1595, 1484, 1370, 1285, 1243, 1196 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6), see Table 1; APCIMS (positive ion mode) *m/z* 507 $[M + H]^+$, 303 [M − acetylglucose + H]$^+$; ESI-TOF MS (negative ion mode) *m/z* 505.0987 $[M - H]^-$ (calcd for $C_{23}H_{21}O_{13}$, 505.0982).

Chlorogenic acid (6): ESIMS (negative ion mode) *m/z* 353 $[M - H]^-$; UV and ^1H NMR (400 MHz) data are in agreement with those published.[18]

***β*-Ecdysone (7):** APCIMS (positive ion mode) *m/z* 481 $[M + H]^+$; ^1H NMR (400 MHz) data are in agreement with those published.[19]

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