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In vitro gastrointestinal biotransformation and characterization of a *Desmodium adscendens* decoction: the first step in unravelling its behavior in the human body

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† We dedicate this paper to our colleague, Prof Sandra Apers, who passed away much too early on February 5th, 2017.

Abstract

Objectives - The isolation and identification of the flavonoids present in a decoction of *D. adscendens* was performed. In view of the oral use of the decoction, this work focused on the stability in gastrointestinal conditions and biotransformation by intestinal microflora in the colon of D-pinitol, vitexin and the flavonoid fraction of the decoction, as a first step in unravelling its behavior in the human body.

Methods - The freeze-dried decoction was first subjected to column chromatography. Subsequently an enriched flavonoid fraction, was separated by repeated semi-preparative HPLC or by HPLC-SPE. The isolated compounds were elucidated by NMR. Biotransformation experiments were carried in an *in vitro* gastro-intestinal dialysis model.

Key Findings - The major flavonoids of a decoction of *D. adscendens* were characterised as vicenin-2, isoschaftoside, schaftoside, 2”-*O*-xylosylvitexin, 2”-*O*-pentosyl-*C*-hexosyl apigenin and a *O*-hexosyl-*C*-hexosyl apigenin, tentatively identified as 2”-*O*-glucosyl-vitexin. During their passage in the gastro-intestinal dialysis model, vitexin and *C*-glycosides thereof were found to be stable. Only the *O*-glycosidic bonds of *O*-glycosides of vitexin or isovitexin were hydrolyzed during the colonic phase.

Conclusion - A *D. adscendens* decoction was found to be rich in vitexin and isovitexin glycosides from which vitexin and the *C*-glycosides thereof were found to be stable in the simulated gastro-intestinal tract.

Keywords

Desmodium adscendens, Leguminosae, decoction, D-pinitol, flavonoid *C*-glycosides

Introduction

Desmodium adscendens (Sw.) DC. is a small herb belonging to the Leguminosae family. It occurs in Africa and South America, where it is used in traditional medicine for the management of anti-inflammatory disorders, hepatitis, and for the treatment of pain, fever, asthma, seizures, muscle spasms, and snake bites. For this purpose, a decoction or infusion is prepared from the leaves and/or stems of the plant [1, 2, 3]. Phytochemical investigations resulted in the identification of the triterpenoid saponins soyasaponin I, soyasaponin III, dehydrosoyasaponin I, sapogenol B and E; the nitrogen containing compounds salsoline, hordenine, tyramine, dimethoxy-phenylethylamine, indole-3-alkyl amines such as dimethyltryptamine; and flavonoids, such as vitexin, isovitexin, and rutin [1, 2, 4, 5]. Baiocchi et al. reported the presence of 22 flavonoid *C*-glycosides and 13 flavonoid *O*-glycosides by high resolution mass spectrometry (HRMS), the glycosides being apigenin, diosmetin or kaempferol derivatives [6]. In previous investigations we have reported D-pinitol as the major constituent of the decoction of *Desmodium adscendens*, being responsible at least in part for the hepatoprotective activity, more specifically the prevention of D-galactosamine-induced liver damage, although the results suggested a synergistic effect by other compounds [7]. In addition, commercially available food supplements are standardized on vitexin. Therefore, this work focused, after a general phytochemical screening, on the isolation and identification of the flavonoids present in *D. adscendens*, a class of natural products which are generally known to have anti-oxidative and free radical scavenging properties). In the present study further phytochemical analysis of the decoction was carried out, including semi-preparative high performance liquid chromatography (HPLC) and HPLC-solid phase

extraction (SPE)- nuclear magnetic resonance (NMR), and a series of flavonoid *C*-glycosides could be characterised. In view of the oral use of the decoction, the stability in gastrointestinal conditions and biotransformation by intestinal microflora in the colon of D-pinitol, vitexin and the flavonoid fraction of the decoction were investigated, as a first step in unravelling its behavior in the human body. This was the first time that the behavior of a decoction of *D. adscendens* in gastro-intestinal conditions was studied.

Materials and methods

Reagents and standards

Acetic acid (99.8%), ethyl acetate (HPLC grade) and formic acid (FA) (99+%) were obtained from Acros organics (Geel, Belgium). Benzoic acid, 3,4-dihydroxyphenyl propionic acid (98%), 4-hydroxybenzoic acid (99%), 4-hydroxyphenyl acetic acid (99%), vitexin (95%), bile salt (B-8631, porcine), bovine serum albumin (98%), L-cysteine, 4-(dimethylamino) cinnamaldehyde (≥98%), diphenylboric acid 2-aminoethyl ester (98%), disodium phosphate dihydrate, glycerol suitable for culture, hydrochloric acid (p.a. 25%), nitrotetrazolium blue chloride (thin layer chromatography (TLC) grade), pancreatin (76 190, from dog pancreas, 149 U.S. Pharmacopeia (USP) U/mg amylase), pepsin (P-7000, from porcine stomach mucosa, 800-2500 U/mg protein), resazurin sodium salt, sodium azide (>99,5%) sodium dihydrogen phosphate anhydrous, sodium thioglycolate broth, acetontitrile-d_3 (CD_3CN 99.8%) and dimethylsulfoxide (DMSO)-d_6 were obtained from Sigma Aldrich (St Louis, MI, USA). Acetonitrile (ACN) Far ultraviolet (UV) (HPLC grade), methanol (HPLC grade), *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) and Wilkins-Chalgren broth (WCB) were purchased from Fisher

scientific (Hampton, NH, USA). Helium, hydrogen and nitrogen, compressed air were provided by Air Liquide (Liège, Belgium), polyethylene glycol (PEG) 4000, sodium hydroxide, sodium bicarbonate by Merck (Darmstadt, Germany) and Tween 80 by Becton Dickinson (New Yrok, USA). Apigenin and vitexin (TLC quality) were obtained from Carl Roth (Karlsruhe, Germany), vitexin (≥ 98%) from Adipogen (Liestal, Switserland) and a *Passiflora incarnata* extract (Ph. Eur.) from ABC chemicals (Woutersbrakel, Belgium).

Plant material

Leaves and twigs from *Desmodium adscendens* (Sw.) DC. were collected in June 2010 in the central region of Ghana. The plant was identified by D.K. Donkor. Voucher specimens are kept at Biosources International, Inc. (Ghana) (no. BRI 03910), and at the University of Ghana (no. GC03910). The decoction of *Desmodium adscendens* was prepared as reported before [7].

Chromatographic methods

The freeze-dried decoction of *D. adscendens* was first subjected to column chromatogaphy using a Reveleris Flash Chromatography system (Grace, Deerfield, USA) equipped with a Reveleris silica column (80 g, 40 µm) from Grace. A gradient starting with 100% ethyl acetate going to 100% methanol in 14 min was used to elute the compounds. Detection was done by UV at 254 nm, 360 nm and by evaporative light scattering detection (ELSD). Every fraction contained 22 mL and was monitored by TLC. Normal phase TLC plates (20 x 20 cm, silica gel 60 F_{254}, Merck) were used, and subsequently the plate was developed with EtOAc-CH_3COOH-HCOOH-H_2O (100:11:11:26). For the detection of flavonoids, the developed TLC plates were sprayed with Neu-reagent (1%

diphenylboric acid 2-aminoethyl ester in methanol) and polyethyleneglycol (PEG) 400 solution, and heated to 105 °C. Based on the observed TLC pattern, similar fractions were combined.

An enriched flavonoid fraction, obtained by combining flavonoid containing subfractions from normal phase flash chromatography, was separated by repeated semi-preparative HPLC. An AutoPurification™ system from Waters (Milford, MA, USA) with diode array detector (DAD) and triple quadrupole detection mass spectrometry (TQD-MS) analyzer was used. Fractions were separated using a Luna C18 (2) column (250 x 10 mm, 5 µm, Phenomenex, Torrance, CA, USA). The following gradient was used: solvent A: 0.1% FA; solvent B: ACN, 0 min – 15% B, 5 min – 15% B, 39 min – 23% B, 43 min – 0% B, 45 min – 0% B, 47 min – 15% B, 55 min – 15% B. The flow rate was 3 mL/min. Detection was done by DAD at 210 nm, 254 nm, 330 nm and 360 nm and by MS. A full scan in the negative ion mode ranging from *m/z* 200 to 800 was performed and fraction collection was done for all peaks with $[M-H]^-$ *m/z* 593, 563, 431, and 327. The injection volume was 600 µL and the concentration of the flavonoid fraction was 20 mg/mL.

HPLC-SPE-NMR analysis

Four different fractions, obtained by normal phase flash chromatography, were separated by an Agilent 1200 HPLC using a Luna C18 (2) column (250 x 4.6 mm, 5 µm). UV-detection was done at 330 nm. Trapping of the analytes was performed with an online SPE system Prospekt 2 (Spark, Holland). HySphere™ Resin general phase (GP) cartridges (Spark, Holland) were used, after conditioning with methanol and equilibrating with water. To increase the concentration of the compound, the HPLC run was repeated and the same compounds were trapped several times on

the same cartridge (multi-trapping). After trapping, the cartridges were dried with N_2 and compounds were eluted with CD_3CN into an NMR tube (3 mm). ^1H, ^{13}C-NMR and 2D-NMR (^1H-^1H correlation spectroscopy (COSY), ^1H-^{13}C heteronuclear single quantum coherence (HSQC), ^1H-^{13}C heteronuclear multiple bond correlation (HMBC)) spectra of the isolated compounds were recorded on a Bruker DRX 400 instrument (Bruker, Rheinstetten, Germany) equipped with a z-gradient 5 mm dual probe and a 3 mm broadband inverse probe using standard Bruker pulse sequences. All samples were dissolved in either CD_3OD, 99.5% D or CD_3CN 99.8% D. Chemical shifts are given in δ and coupling constants (*J*) in Hz.

LC-MS analysis

LC-MS analysis of a solution of the decoction of *D. adscendens* (200 µg/mL; 50% methanol) and a solution of *Passiflora incarnata* dry extract (200 µg/mL; 50% methanol) was performed employing a Surveyor LC system coupled to an LXQ linear ion trap (Thermo Fisher Scientific, Waltham, MA, USA) and a Luna C18 column (250 x 4.6 mm, 5 µm). *P. incarnata* is known to contain a high amount of flavon *C*-glycosides and therefore it was used as a surrogate standard. The flow rate was 1 mL/min using a splitter, and the solvent program was as follows: solvent A: 0.1% FA; solvent B: ACN + 0.1% FA; 0 min – 5% B, 5 min. – 5% B, 30 min – 23% B, 35 min – 100% B, 45 min – 100% B, 47 min – 5% B, 55 min – 5% B. The injection volume was 10 µL. Spectra were recorded in both (-)-electrospray ionization (ESI) and (+)-ESI mode. Tune conditions were as follows: sheath gas flow: 50 arbitrary units; auxiliary gas flow: 5 arbitrary units; source voltage: 4.0 kV; ion transfer tube temperature: 350 °C; and capillary voltage: -34 V and 14 V, respectively. Mass spectral data were recorded in full scan mode in the mass range *m/z* 115 – 1000. For MS^n experiments an isolation

width of 2 Da was used and a normalised collision energy of 35% was applied. All data were acquired and processed using Xcalibur software, version 2.0.

An extract of *D. adscendens* was analyzed and compared with an extract of *Passiflora incarnata* dry extract by means of ultra-performance liquid chromatography (UPLC)-HRMS as described by De Paepe et al. and by means of LC-MS using a LXQ Linear Ion Trap and Luna C18 (2) column with the abovementioned parameters [8].

Gastro-intestinal dialysis model

Biotransformation experiments were carried out for D-pinitol (50 mg) and vitexin (5 mg) (pure standards) and on a flavonoid fraction of *Desmodium adscendens* (28 mg) in an *in vitro* gastro-intestinal dialysis model (GIDM) formerly developed and validated in our research group for the *in vitro* study of phenolic compounds [9]. An overview of the experiments can be found in scheme S1. For the *in vitro* experiments, a blank (no test compound) and three samples were used. The stomach was mimicked by adding 47 mL water to a given amount of sample. The pH was adjusted to 2 using 6 M HCl and to this solution, 3 mL pepsine solution (16% m/v in 0.1 M HCl) was added [9]. The samples were placed in a shaking water bath during 1h (120 strokes/min at 35-37 °C). The stomach phase was poured into the dialysis cell (Amicon stirred cells equipped with a dialysis membrane) and 50 mL water was added together with a tubular dialysis membrane (bag) filled with 985 µL, 1005 µL and 1265 µL $NaHCO_3$, respectively to obtain a pH of 7.5. The blank was prepared in the same way as the samples but without adding test compound. All four dialysis cells were attached in parallel to a system (GIDM) that feeds the cells with either water or gas (N_2) to mimic the transport mechanism from lumen to mucosa. The dialysate was collected in small

plastic jars. This part together with the colonic phase was executed in an anaerobic globe-box. After 30 min of dialysis (at pH 7.5), 15 mL of a pancreatine – bile solution (0.4% m/v pancreatine and 0.4% m/v bile in 0.1 M $NaHCO_3$) was added and dialysis was performed for one hour. The dialysate was collected. This phase mimicked the small intestine. The fluid that remained in the dialysis cells is called the retentate. It was stored at 4 °C overnight and a small sample (1 mL) was taken for further analysis. To the retentate of the of the blank and two sample replicates, 50 mL of a faecal suspension (10^8 colony forming units (CFU)/mL) was added. To the third cell containing sample only 50 mL of WCB medium was added, so no colonic bacteria were present to metabolise the compounds. The pH was adjusted to 5.8-6 with 1 M HCl and all the dialysis cells were connected to the dialysis system. Dialysis was performed during 2 hours at different time points (0, 5, 23 and 25 h for standard compounds and at 0, 3, 21, 27, 45, 51 and 69 h for the flavonoid rich fraction of *D. adscendens*). After every 2 hours of dialysis a sample from the retentate was taken and dialysis jars were renewed. The dialysate was freeze-dried, and the retentate stored at -80 °C and centrifuged (8 min, 17970 g, 14000 rpm) before analysis.

Vitexin and *D. adscendens* samples were analyzed by LC-UV and LC-MS, and D-pinitol samples were analyzed by means of gas chromatography with flame ionization detection (GC-FID) (Interscience) and GC-MS (Interscience) [10]. Before analysis with LC-MS samples were cleaned by the procedure described by Rechner et al [11]. The HPLC method as described above was used. Parameters for MS were: *Sheath gas flow* 65 arbitrary units; *auxiliary gas flow* 14 arbitrary units; *sweep gas flow* 3 arbitrairy units; the source voltage was 3,96 kV; the capillary temperature was 350 °C and the capillairy voltage was -3 V (negative mode). GC analysis was performed with the

analysis method described in [10]. For MS a total ion chromatogram (TIC) spectrum was recorded followed by single ion monitoring (SIM) at *m/z* 305, 318, 260, 217 and 147. Spectra of blank solutions were compared with spectra of solutions with either standard or flavonoid fraction. Samples taken at all time points for standards and at time point 0, 21, 45 and 69 h were analysed.

Results and discussion

Normal phase column chromatography of a decoction of *D. adscendens* resulted in a flavonoid-rich fraction 1A for semi-preparative isolation. The flash chromatography process was repeated and yielded fractions 2B (23 mg), 2C (43 mg), and 2D (322 mg), which were further processed by HPLC-SPE. Semi-preparative HPLC of fraction 1A resulted in compounds **1** (0.9 mg), **2** and **3** (1.9 mg), **4** (1.9 mg) and **5** (1.6 mg), **6** (5.6 mg), **7** (1.1 mg). HPLC-SPE-NMR resulted in the isolation of compound **7** from fraction 2B, compound **6** from fraction 2C and compounds, **1**, **2**, **3**, **4** and **5** from fraction 2D. ^{1}H-NMR analysis indicated that compounds **1** – **5** consisted of more than one flavonoid and/or they were too low in concentration to obtain a good signal to noise ratio.

Compound **6** was obtained as a yellow powder. The UV spectrum showed two absorbance maxima at 269 and 334 nm, typical for flavones [6, 12, 13]. Compound **6** showed a deprotonated molecular ion at *m/z* 563 $[M-H]^-$ and a protonated molecular ion at *m/z* 565 $[M+H]^+$. The MS^2 product ion spectra of *m/z* 563 and *m/z* 565 showed product ions characteristic for internal cleavage of a saccharidic unit suggesting the presence of a *C*-glycosidic bond. The presence of a small $^{0,2}X_0^-/^{0,2}X_0^+$ ion at *m/z* 443 $[M-120-H]^-$ and *m/z* 445 $[M-120+H]^+$, together with a Z_1^-/ Y_1^+ ion, an intense signal at *m/z* 413 $[M-150-H]^-$ and *m/z* 433 $[M-132+H]^+$, formed by the loss of a pentose moiety, is characteristic for a 1→2 interglycosidic bond [14]. This was confirmed by MS^3 analysis

of the most abundant ion in the MS^2 spectrum (Z_1^-), which resulted in a product ion at *m/z* 293 [Z_1^- - 120-H]$^-$. This product ion was also observed when MS^3 analysis was done of *m/z* 443 ($^{0,2}X_0^-$ ion), next to an ion at *m/z* 311 formed by the loss of the pentose moiety [14, 15]. MS^3 analysis of *m/z* 433 (Y_1^+ ion) led to a product ion at *m/z* 313 formed by a $^{0,2}X_0^+$ ion, whereas MS^3 analysis of the $^{0,2}X_0^+$- H_2O ion led to a Y_1^+ ion at *m/z* 295, formed by the loss of the pentose moiety. Product ions at *m/z* 293 [Aglycone + 41-18-H]$^-$, at *m/z* 311 [Aglycone + 41-H]$^-$, and at *m/z* 341 [Aglycone + 71-H]$^-$suggested apigenin as aglycone.

The ^{13}C-NMR spectrum showed the characteristic shift of δ 102.2, typical for C-3 of a flavone. Two doublets in the ^{1}H-NMR spectrum at δ 7.98 (*J* = 8.4 Hz) and δ 6.91 (*J* = 8.4 Hz) pointed to a para-disubstituted B ring. HMBC correlations between H-6 and C-5, C-7, C-8, and C-10 and between Glu H-1 and C-8, C-9 and C-10 indicated glycosylation at C-8. From these results, apigenin could be identified as the aglycon. Based on the typical chemical shifts of the sugar moieties, they could be identified as glucose and xylose. Glucose was directly attached to the C-8 of the aglycon as can be observed in the HMBC spectrum by the correlation between Glu H-1 and C-8. Correlations in the HMBC and COSY spectra showed that xylose was attached to glucose by a 1→2 glycosidic bond [16]. Based on these results, compound **6** was elucidated as 2”-*O*-xylosyl-vitexin (**6**) (Fig. 1). NMR assignments are listed in Table S1. This is the first report of 2”-*O*-xylosyl-vitexin from *D. adscendens* [17] and this finding was confirmed afterwards by Zielinska et al., who identified this compound in an ethanolic extract (60%) of leaves of *D. adscendens* [5]. Previously, it was also isolated from roots of *D. trifolium* [18].

MS^n analysis was applied for the identification of the minor flavonoids **1** – **5** and **7**. The crude extract of *D. adscendens* was compared with an extract of *Passiflora incarnata* known to contain *C*-glycosides of apigenin, such as schaftoside, isoschaftoside and vicenin-2. In the chromatogram shown in Fig. 2, it appears the signal at R_t 13.2 min consisted of two non-separated peaks due to two co-eluting compounds (**2** and **3**). This was also confirmed by examining the MS^2 spectrum of the first and last parts of the peak, which gave the same product ions but with totally different relative abundances. Compounds **2** and **3** showed a deprotonated molecular ion at *m/z* 563 $[M-H]^-$ and a protonated molecular ion at *m/z* 595 $[M+H]^+$. MS^2 of *m/z* 563 resulted in ions at *m/z* 503, 473, 443, 413, 383 and 353 [14, 19, 20]. A full overview is given in Table 1. After comparison with a surrogate standard, i.e. an extract of *Passiflora incarnata* and literature data, compounds **2** and **3** could be identified as schaftoside (**2**) and isoschaftoside (**3**), which were also isolated from other *Desmodium* species, such as *D. styracifolium* and *D. unicatum* [12, 13, 14, 18, 20, 21, 22, 23].

Compound **5** also showed the same deprotonated and protonated molecular ions as compound **6** at *m/z* 563 $[M-H]^-$ / 565 $[M+H]^+$; MS^2 and MS^3 analysis resulted in the same product ions. These findings suggested that compound **5** was a 2-*O*-pentosyl-x-*C*-hexosyl-apigenin isomer of compound **6**, possibly 2”-*O*-xylosyl-isovitexin (**5**) as described by Zielinska et al [5].

Compound **1** showed deprotonated and protonated molecular ions at *m/z* 593 $[M-H]^-$ and 595 $[M+H]^+$. An overview of MS^2 and MS^3 results is given in Table 1. These findings together with those in the negative ion mode confirmed the structure of a di-*C*-hexosyl-apigenin. Comparison with an

extract of *Passiflora incarnata* and literature data allowed to identify compound **1** as vicenin-2 (**1**), which had already been isolated from *D. styrachifolium* [12, 19, 20, 22, 23, 24, 25, 26, 27].

Compound **4** also showed deprotonated and protonated molecular ions at *m/z* 593 $[M-H]^-$ and 595 $[M+H]^+$. All MS^2 and MS^3 results are given in Table 1. This compound was characterised as a x-*O*-hexosyl-x-*C*-hexosyl-apigenin, and tentatively identified as 2"-*O*-glucosylvitexin (**4**), also isolated from the roots of *D. trifolium* before [18].

Compound **7** and **8** were identified as vitexin (**7**) and isovitexin (**8**), respectively, after spiking with a standard solution. MS experiments, showing deprotonated and protonated molecular ions at *m/z* 431 $[M-H]^-$ and 433 $[M+H]^+$, confirmed these findings. MS^2 and MS^3 findings (Table 1) were in agreement with these findings and literature data [2, 6].

Comparison of the chromatographic profiles of the flavonoid-rich fraction and the crude decoction showed they contained the same main flavonoids (Fig. 2). Two flavonoids showed a deprotonated molecular ion at *m/z* 593 $[M-H]^-$ and were identified as a di-*C*-hexosyl-apigenin, vicenin-2, (**1**) (R_t = 8.42 min) and an x-*O*-hexosyl-x-*C*-hexosyl-apigenin, tentatively identified as 2"-O-glucosyl-vitexin (**4**) (R_t = 17.64 min). Four flavonoids showed a deprotonated molecular ion at *m/z* 563 $[M-H]^-$ and were identified as as schaftoside (**2**) and isoschaftoside (**3**) (R_t = 13.27 min), the 2-*O*-pentosyl-x-*C*-hexosyl-apigenin isomer of compound **6**, possibly 2"-*O*-xylosyl-isovitexin (**5**) (R_t = 19.31 min), and 2"-*O*-xylosyl-vitexin (**6**) (R_t = 20.10 min). One flavonoid showed a deprotonated molecular ion at *m/z* 431 $[M-H]^-$, and was identified as vitexin (**7**) (R_t = 21.04 min).

Isovitexin (**8**) was present as a minor peak in the decoction (Rt = 21.72 min) but could not be detected in the flavonoid-rich fraction.

Following the phytochemical analysis, the stability and biotransformation of vitexin, the flavonoid fraction and D-pinitol was investigated in a gastro-intestinal dialysis model with colon-phase. The results of these experiments, as described below, are summarized in table S2. Vitexin, when administered as a pure compound, could be detected during the whole experiment in the dialysate. The chromatograms and spectra of the blank, containing only the reagents to mimic the gastro-intestinal tract but not vitexin, and samples were compared. At none of the time points, any difference between the blank or the samples was detected other than the absence/presence of vitexin for the blank and samples, respectively. In addition, potential metabolites such as 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid and 3-(4-hydroxyphenyl)-propionic acid, were not detected. This implies that vitexin, a *C*-glycoside of apigenin, is quite stable during its passage in the GIDM with colon phase under the conditions applied.

With regard to the flavonoid-rich fraction, after 2 h of incubation in the colon compartment and dialysis none of the flavonoid compounds had diminished, i.e. the intensities of all peaks from the compartment with colon bacteria and flavonoid fraction were similar to those of the compartment with only flavonoid fraction and no bacteria. This shows that no biotransformations had occurred yet. After 21 h of incubation the peak corresponding to compound **4** was not visible anymore, whereas the peak corresponding to compound **1** was still present (Fig. 3). The ratio of the peaks corresponding to compounds **2**, **3**, **5**, and **6** had changed at this time point, more specifically the peak corresponding to compound **5** had decreased. This effect was even more

pronounced after 45 h and at the end of the experiment it had almost disappeared. At the fourth time point, i.e. after 69 h, also the peak corresponding to compound **6** had decreased, indicating that it was metabolized by the colon bacteria. However, the intensity of the peaks of compounds **1**, **2**, and **3** was still the same in the cells with and without bacteria at the end of the experiment, and these compounds were considered metabolically stable.

These findings indicate that most probably only the *O*-glycosidic bonds of vitexin-*O*-glycosides were metabolized by the colon bacteria. These findings were as expected, since the *O*-deglycosylation by intestinal bacteria was described in literature [28]. Some of those compounds seemed to be more susceptible to biotransformation since they were almost completely metabolised after a few hours (the peak corresponding to compound **4**) or at the end of the experiment, as observed for the peak of 2”-*O*-xylosyl-isovitexin (**5**). This could also be concentration dependent. Apart from the disappearing peaks, also some peaks emerged during the experiment, being vitexin (**7**) and another peak with a deprotonated molecular ion at *m/z* 431 $[M-H]^-$, which was identified as isovitexin (**8**). This confirms that in addition to vitexin derivatives, some isovitexin glycosides may be present as well.

A specific search for potential metabolites such as apigenin, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid and 3-(4-hydroxyphenyl)-propionic acid was performed, but none of those metabolites was detected [29]. This indicates that vitexin, initially present and originating from the conversion of vitexin-*O*-glycosides, is quite stable during the passage in the gastro-intestinal dialysis model. In our experimental conditions, deglycosylation of *C*-glycosides and further breakdown of the flavonoid nucleus have not been observed. These findings were not in accordance to those in literature, were the both the deglycosylation into the aglycon and the

metabolization of vitexin into metabolites such as 3-(4-hydroxyphenyl)-propionic acid were described. The difference in bacterial species in the colon between the experiments could probably be an explanation for by the different results found [28].

With regard to D-pinitol, GC-FID quantification of the lyophilised dialysate showed that 27% D-pinitol was present and therefore 73% was available for the colonic phase of the experiment. Qualitative analysis of all dialysis samples, both from pure D-pinitol and from the decoction, by GC-MS showed that D-pinitol was still present at the end of the experiment and no D-*chiro*-inositol (its demethylated derivative) was formed. Thus, D-pinitol appeared to be stable in the gastric phase and small intestine as well as the colonic phase. This in agreement with literature data, where D-pinitol was found in blood of humans after oral intake of D-pinitol [7, 30].

In conclusion, the major flavonoids of a decoction of *D. adscendens* were characterised as vicenin-2, isoschaftoside, schaftoside, 2”-*O*-xylosylvitexin, 2”-*O*-pentosyl-*C*-hexosyl apigenin and a *O*-hexosyl-*C*-hexosyl apigenin, tentatively identified as 2”-*O*-glucosyl-vitexin. It should be noted that passive diffusion through a dialysis membrane of polar compounds may not reflect passive diffusion in the gastro-intestinal tract, which requires more lipophilic properties. On the other hand, since the model only mimics passive diffusion, no information about absorption by active transport or enzymatic phenomena taking place in the brush borders of the small intestine can be obtained. During the colonic phase, vitexin and *C*-glycosides thereof were found to be stable throughout their passage in the simulated gastro-intestinal tract. Only the *O*-glycosidic bonds of *O*-glycosides of vitexin or isovitexin were hydrolyzed. This was confirmed by the absence of

phenolic acids or *C*-deglycosylated aglycones, potential products of the colonic biotransformation of flavonoids.

Conflicts of Interest

The authors declare they have no conflicts of interest. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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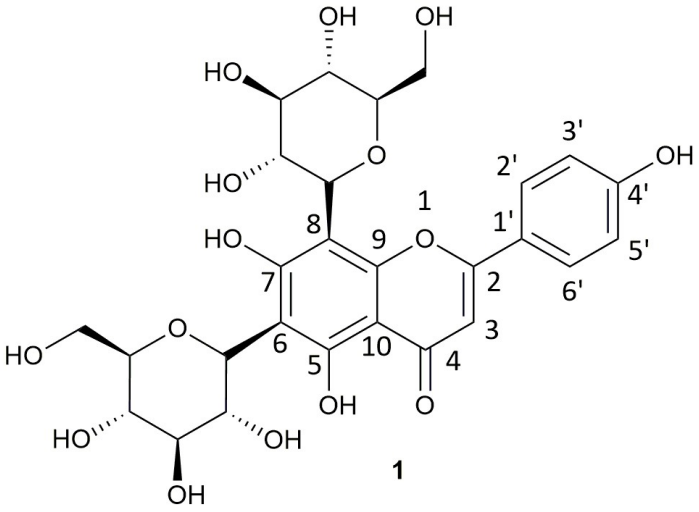
Table 1. Overview of MS, MS^2 and MS^3 experiments.

Compound	$[M-H]^-/[M+H]^+$	MS^2	MS^3	elucidation
1	593/595	575;503;473;383;353/577;559;541;529;475;457;499;489	503→485;413;383 473→455;383;353 457→439;421;409;379;337;325;307	Vicenin-2
2	563/565	545;503;485;473;455;443;425;413;395;383;365;353/547;529;511;493;499;481;475;457;469;451;445;427;409;415;397;379	443→425;383;353 473→455;413;383;353 503→485;473;443;413;383 427→409;391;379;349;337;325;295	Schaftoside
3	563/565	545;503;485;473;455;443;425;413;395;383;365;353/547;529;511;493;499;481;475;457;469;451;445;427;409;415;397;379	443→425;383;353 473→455;413;383;353 503→485;473;443;413;383 427→409;391;379;349;337;325;295	Isoschaftoside
4	593/595	475;473;413;293/433;415;337;313;271	431→413;395;387;341;323;311;293 413→293 433→415;397;379;367;337;313;295;283	2”-O-hexosyl-x-C-hexosyl-apigenin derivative
5	563/565	443;413;341;311;293/547;445;433;415;397;367;337;313	413→293 443→311;293 433→415;397;379;367;337;313;295;283 427→409;391;379;349;325;307;295	2”-O-pentosyl-x-C-hexosyl-apigenin derivative
6	563/565	443;413;341;311;293/547;445;433;415;397;367;337;313	413→293 443→425;311;293 433→415;397;379;367;337;313;295;283 427→409;379;349;337;325;313;307;295;283	2”-*O*-xylosyl-vitexin
7	431/433	341;311/415;397;379;367;337;313	341→323;311;283;281;269;247;179 311→283	Vitexin
8	431/433	341;311/415;397;379;367;337;313	341→323;311;283;281;269;247;179 311→283	Isovitexin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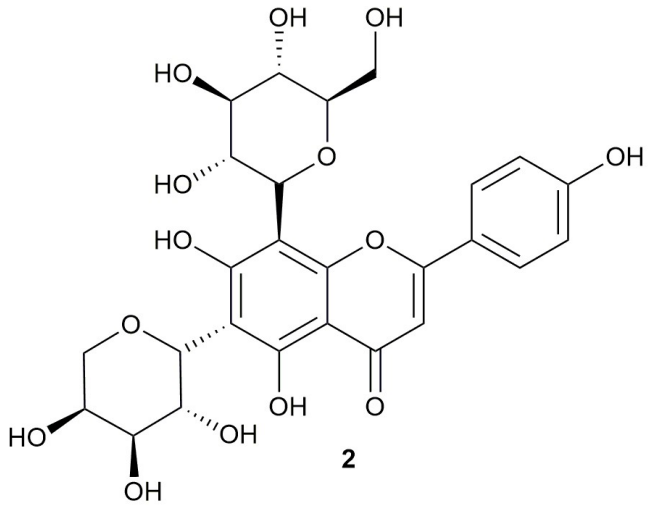
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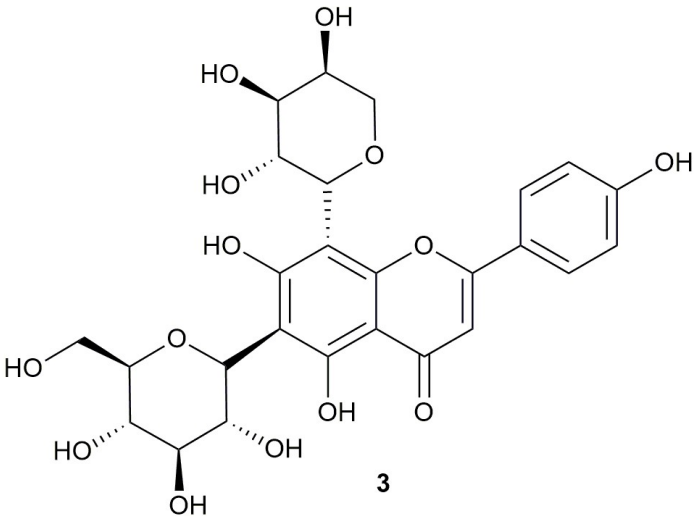
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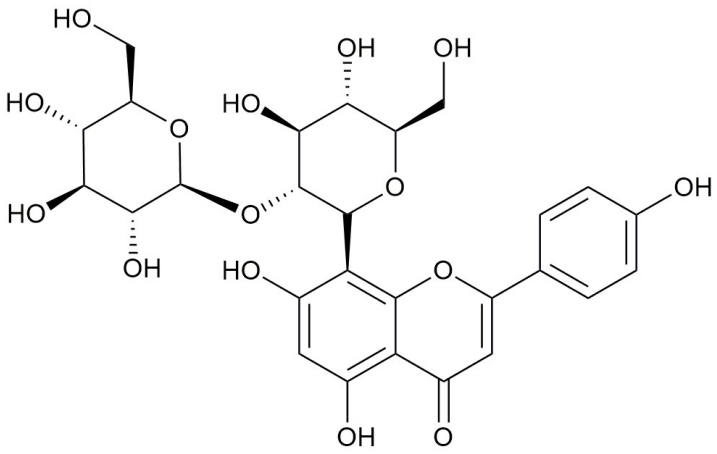
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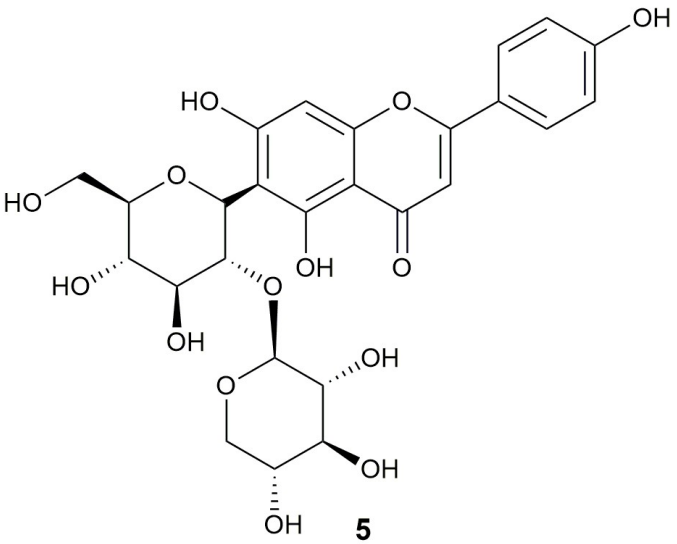
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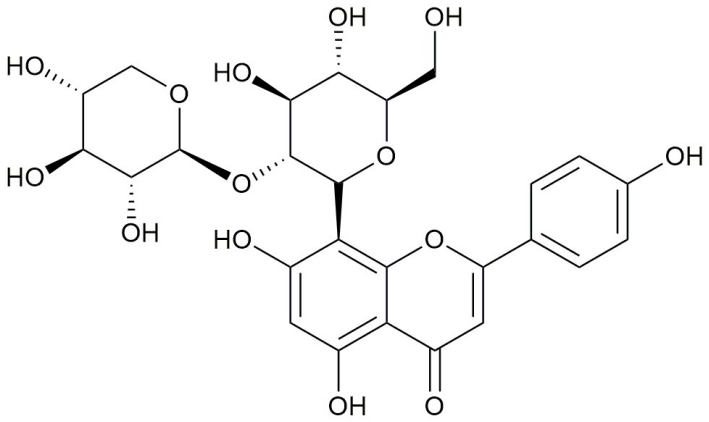
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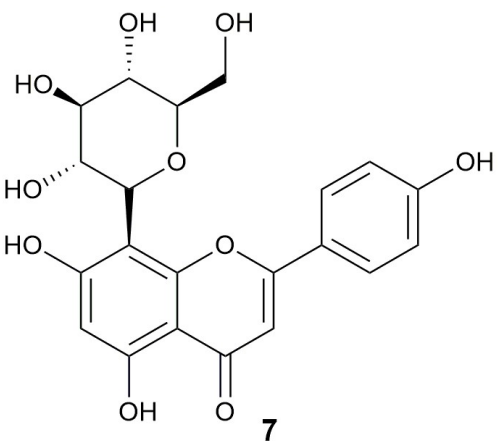
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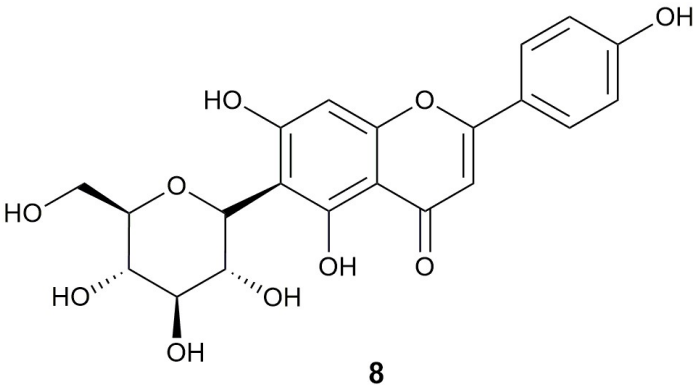
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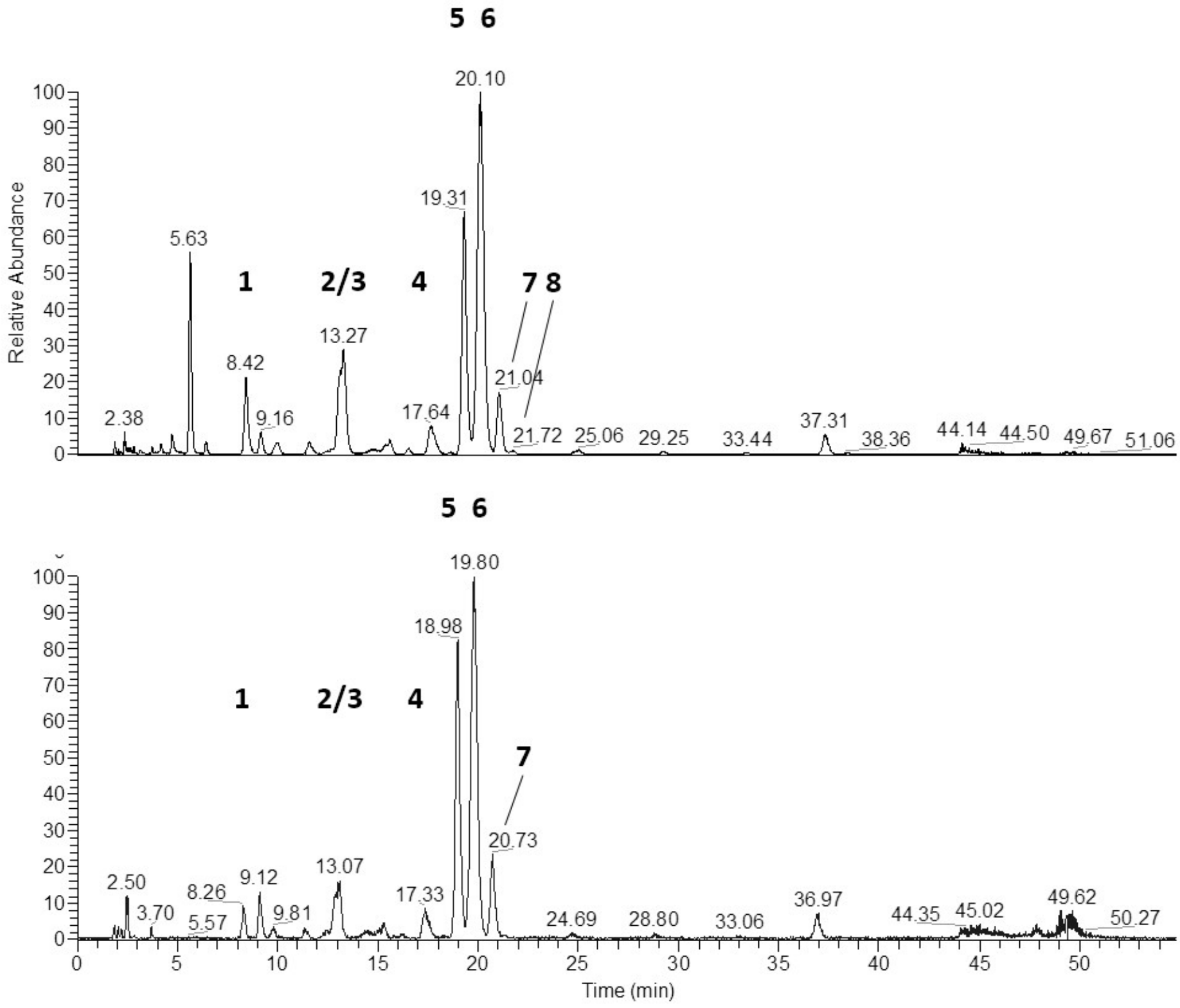
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Relative Abundance
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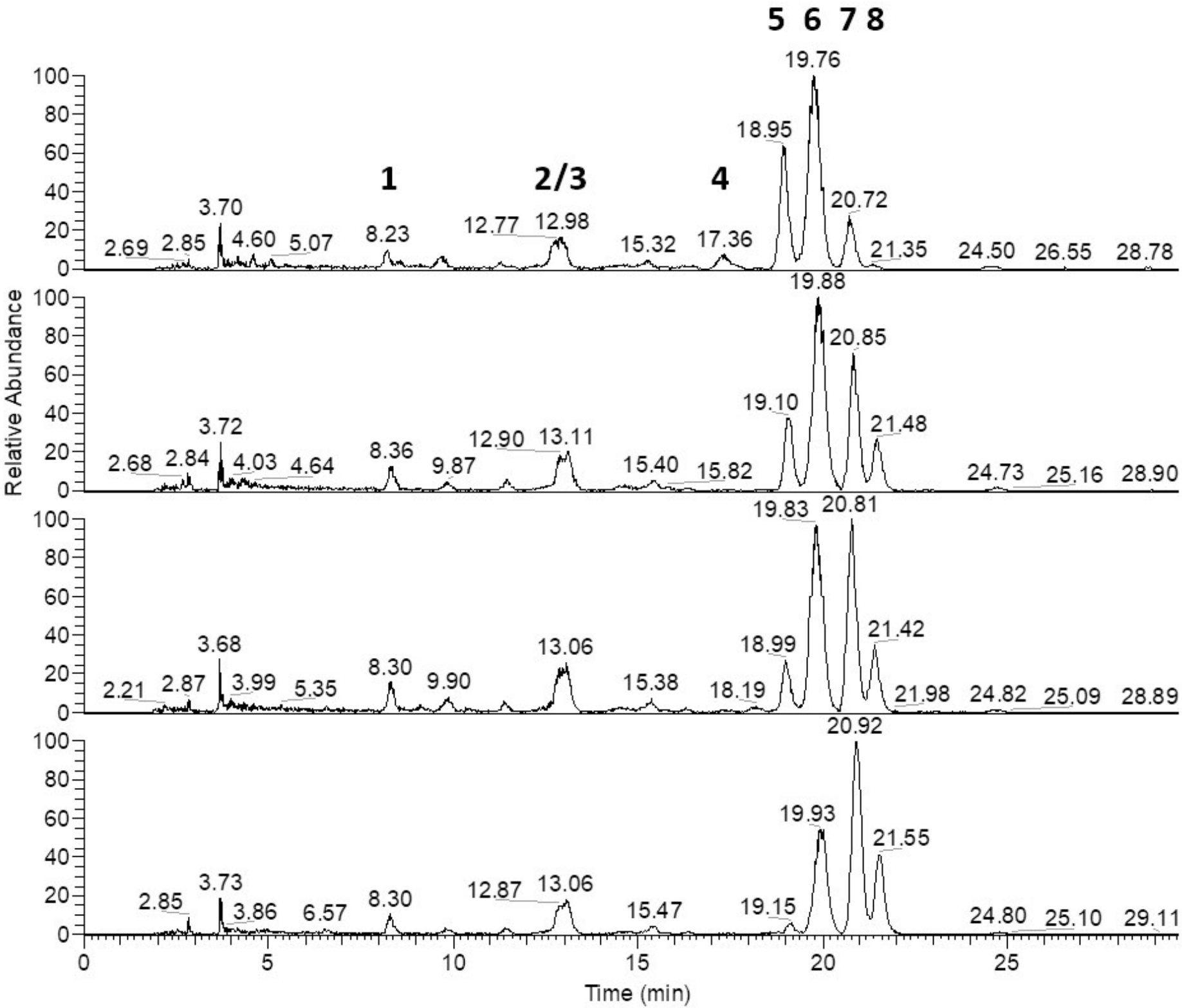
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