JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Identification of 8 O 4/8 5(cyclic)- and 8 8(cyclic)/5 5 Coupled Dehydrotriferulic Acids, Naturally Occurring in Cell Walls of Mono- and Dicotyledonous Plants

Martin Waterstraat, Diana Bunzel, and Mirko Bunzel

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.6b02720 • Publication Date (Web): 19 Aug 2016

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Identification of 8-O-4/8-5(cyclic)- and 8-8(cyclic)/5-5-Coupled Dehydrotriferulic Acids, Naturally Occurring in Cell Walls of Mono- and Dicotyledonous Plants

Martin Waterstraat[†], Diana Bunzel[#], and Mirko Bunzel[†,*]

[†]Department of Food Chemistry and Phytochemistry, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, 76131 Karlsruhe, Germany

[#]Department of Safety and Quality of Fruits and Vegetables, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Haid-und-Neu-Straße 9, 76131 Karlsruhe, Germany.

*Corresponding author. Post address: Karlsruhe Institute of Technology (KIT), Institute of Applied Biosciences, Department of Food Chemistry and Phytochemistry, Adenauerring 20a, 76131 Karlsruhe, Germany; Telephone: (+49) 721 608 42936; Fax: (+49) 721 608 47255; Email: mirko.bunzel@kit.edu.

ABSTRACT

Besides ferulate dimers, higher oligomers of ferulic acid such as trimers and tetramers were previously demonstrated to occur in plant cell walls. Here, we report the identification of two new triferulic acids. 8-O-4/8-5(cyclic)-triferulic acid was synthesized from ethyl ferulate under oxidative conditions using copper(II)-tetramethylethylenediamine [CuCl(OH)-TMEDA] as a catalyst, whereas 8-8(cyclic)/5-5-triferulic acid was isolated (preparative size exclusion chromatography, reversed-phase HPLC) from saponified insoluble maize fiber. Structures of both trimers were unambiguously elucidated by high resolution LC-ToF-MS/MS and one- (^{1}H) and two-dimensional (HSQC, HMBC, COSY, NOESY) NMR spectroscopy. The newly described trimers were identified by LC-MS/MS in alkaline hydrolysates of insoluble fibers from maize, wheat, and sugar beet, indicating that ferulic acid cross-links between cell wall polymers are more diverse than previously recognized. Saponification experiments also suggest that the newly identified 8-O-4/8-5(cyclic)-triferulic acid is the naturally occurring precursor of the previously identified 8-O-4/8-5(non-cyclic)-triferulic acid in plant cell walls.

KEYWORDS: ferulic acid, ferulate trimers, radical coupling, phenolic cross-links, plant cell walls

INTRODUCTION

Cell walls are important components of plant-based food products contributing to the texture of plant foods and being the major source of dietary fiber in most diets. Plant cell wall stability is affected by numerous structural components such as the cellulose-hemicellulose network, structural proteins, and, depending on the cell type, lignin. In cereals and in certain plants belonging to the order Caryophyllales, the cellulose-hemicellulose network and/or pectins are influenced by polymer bound ferulic acid (4-hydroxy-3-methoxycinnamic acid), which can be found in amounts up to 3.1 % in maize bran and in lower amounts (up to 0.6 %) in dicotyledonous plants such as sugar-beet pulp.[1-3] In monocotyledonous plants, ferulic acid predominantly acylates the arabinose residues of arabinoxylans, whereas it is mostly attached to the pectic arabinan and galactan side-chains of rhamnogalacturonan-I in dicotyledonous plants.[4]

Oxidative coupling of ferulate monomers by peroxidases and H_2O_2 results in ferulate dimers, trimers, and tetramers.[5] Formation of these cross-links between polysaccharides, polysaccharides and lignin, and, potentially, polysaccharides and proteins[6-9] increases cell wall stability against mechanical (and thermal) stress and fermentability.[10,11] Five types of linkages between the ferulate monomers resulting from oxidative coupling (8-5-, 8-O-4-, 8-8-, 5-5-, and 4-O-5-coupling) have been described to date. However, alkaline hydrolysis results in nine different ferulic acid dimers, which were found in the alkaline hydrolyzates of cell walls of several plants: three 8-8-coupled forms (cyclic, non-cyclic, and tetrahydrofuran), three 8-5-coupled forms (cyclic, non-cyclic, and decarboxylated), and the 5-5-, 8-O-4-, and 4-O-5-coupled structures.[5] It is assumed that all three 8-8-coupled dimers naturally occur in the plant whereas only the 8-5(cyclic) dimer appears to be native with the other dimers being artifacts of the alkaline hydrolysis.[12]

The ferulate units of dehydrotriferulic acids (TriFA) and dehydrotetraferulic acids (TetraFA) are bound through the same types of linkages as found in ferulate dimers, although an additional 8-O-4(H_2O)-type has been described.[13] The first trimer (5-5/8-O-4-TriFA) was isolated and structurally characterized from maize bran in 2003.[14,15] Since then, six additional regioisomers of ferulic acid trimers and two regioisomers of ferulic acid tetramers have been isolated (all from maize

bran) and characterized: 8-O-4/8-O-4-TriFA, 8-8(cyclic)/8-O-4-TriFA, 5-5/8-O-4(H_2O)-TriFA, 8-O-4/8-5(non-cyclic)-TriFA, 5-5/8-5(non-cyclic)-TriFA, 5-5/8-8(tetrahydrofuran)-TriFA, 4-O-8/5-5/8-O-4-TetraFA, and 4-O-8/5-5/8-5(non-cyclic)-TetraFA.[13,16,17] Here, we report the structural identification of two novel ferulic acid trimers (Figure 1), occurring in cell walls of both monocotyledonous and dicotyledonous plants.

Materials and Methods

Chemicals. Cu(I)Cl, tetramethylethylenediamine (TMEDA), HCl, NaOH, ethyl acetate, and ethanol were purchased from Carl Roth GmbH (Karlsruhe, Germany). Acetonitrile, methanol, acetone, petroleum ether, tetrahydrofuran, and Na_2SO_4 were from VWR International (Radnor, Pennsylvania, USA), acetone-d_6 and ferulic acid from Sigma Aldrich (St. Louis, Missouri, USA), and D_2O from deutero GmbH (Kastellaun, Germany). Acetyl chloride was purchased from Fluka (Buchs, Switzerland), formic acid from Merck KGaA (Darmstadt, Germany), $NaHCO_3$ from Riedel-de Haën AG (Seelze, Germany), and carbogen (5 % CO_2) from Air Liquide S.A. (Düsseldorf, Germany).

Enzymes. The thermostable α-amylase MATS L Classic (from *Bacillus licheniformis*, 8150 TAU/g), the peptidase Maxazyme NNP DS (from Bacillus amyloliquefaciens, 184,000 PCU/g), and the amyloglucosidase Amigase Mega L (from *Aspergillus niger*) were kindly donated by DSM Food Specialties (Heerlen, Netherlands) and the thermostable α-amylase Termamyl 120 L (from *Bacillus licheniformis*, 120 KNU/g), the peptidase Alcalase 2.5 L (from *Bacillus licheniformis*, 2.5 AU/g), and the amyloglucosidase AMG 300 L (from *Aspergillus niger*, 300 AGU/g) were kindly donated by Novozymes A/S (Bagsvaerd, Denmark). The carbohydrase mixture Driselase (from Basidomycetes, cellulase activity ≥ 100 U/g, laminarinase activity ≥ 10 U/g, Xylanase activity ≥ 3 U/g) was purchased from Sigma Aldrich, and the feruloyl esterases E-FAERU (from rumen microorganism, 600 U/mL) and E-FAEZCT (from *Clostridium thermocellum*, 7 U/mL) were from Megazyme (Chicago, Illinois, USA).

Plant samples. Popcorn maize (*Zea mays* L. var. *everta.*) and wheat grain (*Triticum aestivum* L.) was obtained from a local grocery store in Karlsruhe, Germany. Amaranth (*Amaranthus hypochondriacus* L.) was field grown, and seeds were harvested in 2012 in Moersingen, Germany. Sugar beet pulp (*Beta vulgaris* L. var. *vulgaris*) was kindly donated from Südzucker AG (Mannheim, Germany) in 2013.

Synthesis of trimer 1 (5-[(*Z*)-2-carboxy-2-(4-((*E*)-2-carboxyvinyl)-2-methoxyphenoxy)vinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid).

Ethyl ferulate was synthesized by adding 5 g (25.8 mmol) of ferulic acid and 2.5 mL (2.75 g, 35 mmol) of acetyl chloride to 50 mL of ethanol and stirring at room temperature overnight. After evaporation of the solvents, the reaction was repeated by a second addition of acetyl chloride and ethanol resulting in quantitative conversion. Following evaporation of the solvents, the remaining HCl was removed by washing with 3 x 10 mL of ethanol.[18]

Oxidative coupling of ethyl ferulate was carried out by using a catalytic procedure reported by Lu et al. (2012).[19] Briefly, 40 mg (0.4 mmol) of CuCl and 60 µL (0.4 mmol) of TMEDA were added to 60 mL of acetonitrile in a 200 mL two-neck flask. After stirring for 5 min, 888 mg (4 mmol) of ethyl ferulate were added, and the gas atmosphere was changed by attaching a balloon filled with carbogen (5 % CO_2 in O_2) to the two-neck flask. After stirring for 4 h at room temperature, the reaction was stopped by adding 24 mL of 1 M HCl. The organic solvents were evaporated under reduced pressure at 40 °C, and the crude product was extracted into ethyl acetate. The ethyl acetate volume was reduced by rotary evaporation to 2.5 mL.

Initial fractionation was carried out by flash chromatography (Biotage Horizon flash chromatograph, Charlottesville, VA, USA) using a prepacked silica column (Götec-Labortechnik GmbH, Bickenbach, Germany, 30 g Si60, 15-40 µm particle size). Elution solvents were petroleum ether (A) and ethyl acetate (B) with the following binary elution gradient: initially 30 % B, linear increase to 60 % B within 1065 mL, followed by an elution with 550 mL of 100 % B. The flow rate was approximately 70 mL/min, and UV-detection was performed at 254 nm. The first fraction (201 mL) was discarded;

subsequently, 48 fractions of 18 mL each were collected automatically. Based on UV-data, fractions 20-48 were combined.

After evaporation under reduced pressure, the combined fractions were saponified by adding 20 mL of 2 M NaOH under N_2 and protected from light for 18 h at room temperature. The alkaline hydrolyzate was acidified with concentrated HCl to pH < 2, and the precipitate was extracted three times into 5 mL of ethyl acetate. The organic solvent was evaporated under reduced pressure yielding 156 mg of solids.

Purification by preparative HPLC was carried out using a Luna C18(2) reversed-phase column (Phenomenex LTD, Aschaffenburg, Germany, 250 x 15 mm, 5 µm particle size, 10 nm pore size) and UV-detection at 320 nm. A gradient system consisting of 0.01 % formic acid in water (A) and 0.01 % formic acid in acetonitrile (B) was used for the separation of the fraction (flow rate: 8 mL/min): initially 15 % B, linear increase to 27.5 % B within 25 min, linear increase to 45 % B within 20 min, followed by rinsing and equilibration steps. Trimer **1** (1.6 mg) eluted after 45.5 min with sufficient purity (> 75 %) allowing for its structural characterization.

Isolation of trimer 2 ((*E*)-1-[5'-(2-carboxyvinyl)-2',6-dihydroxy-3',5-dimethoxy-(1,1'-biphenyl)-3-yl]-7-hydroxy-6-methoxy-1,2-dihydronaphthalene-2,3-dicarboxylic acid).

Insoluble maize fiber was isolated according to a previously published method.[20] Popcorn maize was milled (particle size < 0.5 mm) and defatted with acetone. Aliquots (20 or 40 g) of defatted flour (435 g) were suspended in phosphate buffer (pH 6.2, 0.08 M, 200 or 400 mL), and thermostable α-amylase (0.5 or 1 mL, DSM Food Specialties) was added. After incubation with occasional shaking at 92 °C for 30 min, flasks were cooled to room temperature, and the pH was adjusted to 5.2 with 0.325 M HCl. Partial protein removal was performed by adding a peptidase (100 or 200 µL, DSM Food Specialties and incubation of the suspension in a shaking water bath at 58 °C for 30 min. After heating the mixture to 92 °C for 5 min and cooling down to room temperature, the pH was adjusted to 4.2 with 0.325 M HCl. Samples were incubated with amyloglucosidase (50 or 100 µL, DSM Food Specialties) in a shaking water bath at 58 °C for 45 min. Following filtration, the residues were washed three times with 40 or 80 mL of water (60 °C), twice with 50 or 100 mL of 95 % ethanol and

twice with 50 or 100 mL of acetone. In total, 90 g (20.6 % yield) of insoluble maize fiber were recovered after drying overnight at 60 °C.

Alkaline hydrolysis and extraction was carried out as published previously.[20] In brief, insoluble maize fiber was saponified using 2 M NaOH (3 x 8 g of fiber in 200 mL; 10 x 5 g of fiber in 200 mL; 1 x 4 g of fiber in 200 mL; and 2 x 6 g of fiber in 100 mL) under protection from light and O_2 for 18 h at room temperature. The suspensions were acidified (pH < 2) with concentrated HCl. Liberated compounds were extracted into ethyl acetate (100 mL, 3 times), and the combined extracts were concentrated to 200 mL by rotary evaporation. Phenolic acids were extracted into 5 % (w/v) $NaHCO_3$ (200 mL, 3 times), and, following acidification (pH < 2), re-extracted into ethyl acetate (200 mL, 3 times). The combined ethyl acetate extracts were dried over Na_2SO_4 and evaporated. In total, approximately 5 g of a dark, sticky oil was recovered, which was dissolved in 70 mL of THF.

Semi-preparative size exclusion chromatography was performed on an HPLC-system consisting of a gradient pump (System Gold programmable solvent module, Beckmann Coulter, Brea, California), a column oven (LaChrom column oven L-7360, Merck), and a UV-detector (System Gold detector module 166, Beckman Coulter). The solution of phenolic acids in THF was injected manually in aliquots of 500 µL and separated on a TSK 1000_{HXL} column (Tosoh, Minato, Tokyo, 300 x 21.5 mm, 5 µm particle size, 100 – 1000 Da separation range) at 35 °C with a flow rate of 3.5 mL/min. Detection was carried out at 355 nm to avoid saturation of the semi-preparative flow cell (2 mm path length). The fractions containing ferulate trimers (elution between 9.8 and 10.5 min, Figure 2) were combined and dried by rotary evaporation.

A two-step fractionation of ferulate trimers and purification of trimer **2** was carried out using semi-preparative HPLC on a Luna phenyl hexyl column (Phenomenex, 250 x 10 mm, 5 µm particle size). The HPLC system consisted of a gradient pump (System Gold 126 solvent module, Beckman Coulter), a column oven (Beckman Coulter) operated at 35 °C, and a UV detector (System Gold 166 detector module, Beckman Coulter). Eluents were 1 mM aqueous trifluoroacetic acid (A) and methanol:A (9:1, v/v) (B). First, samples were dissolved in 1.25 mL of THF:H_2O (3:2, v/v) and injected manually in aliquots of 80 µL. The following gradient was used: isocratic elution with 50 % B for 10 min, linear

increase to 60 % B within 15 min; isocratic with 60 % B for 10 min, linear increase to 80 % B within 15 min, isocratic with 80 % B for 5 min. Detection was carried out at 270 nm. The fractions eluting between 16.5 and 20.0 min (Figure 3A) contained trimer **2** with impurities and were combined followed by evaporation. In the final clean-up step, residues were dissolved in 2 mL of THF:H_2O (1:1, v/v) and injected in aliquots of 200 µL. The binary gradient started with 13 % B that was increased to 27 % B within 25 min, followed by rinsing and equilibrating steps. Detection was carried out at 260 nm. Trimer **2** eluted after 37 min (Figure 3B); its purity (> 90 %) allowed for unambiguous structural elucidation.

NMR Spectroscopy. Both trimers were dissolved in 600 µL of acetone-d_6:D_2O (10:1, v/v) and were analyzed at 24.85 °C on a Bruker Ascend 500 MHz NMR spectrometer equipped with a Prodigy cryoprobe (Bruker, Rheinstetten, Germany). ^{1}H, phase-sensitive ^{1}H–^{13}C-HSQC with gradient selection, ^{1}H–^{13}C-HMBC with gradient selection, phase-sensitive ^{1}H–^{1}H-COSY, and phase sensitive ^{1}H-^{1}H-NOESY experiments (standard Bruker implementations) were performed. Data were analyzed using MestReNova software, and spectra were calibrated against the central acetone residual peak (methyl proton, 2.05 ppm; methyl carbon, 29.84 ppm).

High-resolution mass spectrometry. Determination of exact masses was performed on an LC-Time of Flight (ToF)-MS/MS system consisting of a 1290 Infinity HPLC system (1290 Bin Pump G4220A with degasser, 1290 Sampler G4226A autosampler, and 1290 DAD G4212A DAD detector, Agilent Technologies, SantaClara, California, USA) coupled to a Triple ToF 5600 mass spectrometer (AB Sciex Instruments, Framingham, Massachusetts, USA). The eluents were H_2O with 0.01 % formic acid (A) and acetonitrile with 0.01 % formic acid (B), and the flow rate was 0.5 mL/min at room temperature. Injection volume was 20 µL and the elution gradient was as follows: initially 10 % B, linear to 27 % B within 10 min, linear to 45 % B within 20 min, linear to 60 % B in 2 min, followed by rinsing and equilibration steps. The Duo Spray ion source was used in electrospray ionization (ESI) negative mode at 650 °C with curtain gas, 45 psi; ion source gas-1, 70 psi; ion source gas-2, 60 psi; and declustering potential, −100 V. ToF-MS experiments were completed over a scan range of *m/z* 100 − 1000 using an accumulation time of 200 ms and a collision energy voltage of −10 V.

ToF-MS/MS experiments (product ion scans of *m/z* 50 − 1000 for *m/z* 577.1) were performed with the following parameters, using nitrogen as collision gas: collision energy voltage, −45 V; collision energy spread, 25 V; and accumulation time, 80 ms.

Analysis of maize, wheat, sugar beet, and amaranth. Preparative isolation of insoluble dietary fiber from maize, wheat, sugar beet, and amaranth was carried out as described for the isolation of trimer **2**, but using 20 g of freeze-dried, milled plant material and enzymes from Novozymes A/S (1.5 mL of α-amylase, 600 µL of peptidase, 700 µL of amyloglucosidase) with optimized pH-values for these enzymes (pH 6.2 for α-amylase, pH 7.5 for peptidase, pH 4.5 for amyloglucoidase). Insoluble dietary fiber (100 mg) was saponified protected from light and O_2 using 5 mL of 2 M NaOH for 18 h. After acidification (pH < 2) the mixtures were extracted three times with 4 mL of diethyl ether. The extracts were dried under N_2, and the residues were dissolved in 0.5 mL of THF:H_2O (1:1, v/v). Because initial solutions of the extracts from wheat and amaranth formed two layers, they were dried and redissolved in methanol:H_2O (1:1, v/v).

For the enzymatic approaches, 100 mg of insoluble dietary fiber was incubated in duplicate with 6.5 mL of H_2O, 10 mg of Driselase, 100 µL of feruloyl esterase from *Clostridium thermocellum* (E-FAEZCT) and 100 µL of feruloyl esterase from rumen microorganism (E-FAERU) at 40 °C for 24 h under protection from light. To one replicate, 2.16 mL of 4 M NaOH was added (final concentration: 1 M NaOH), and the suspension was incubated at room temperature for 10 min. All samples were acidified with concentrated HCl (pH < 2) and extracted three times with 4 mL of diethyl ether. The organic solvent was evaporated, and the residues were redissolved in 0.5 mL of THF:H_2O (1:1, v/v).

The samples were analyzed with LC-MS/MS, equipped with a 2690 separations module (pumps, degasser, autosampler and a 996 PDA detector, Waters Corporation, Milford, Massachusetts, USA) coupled to a Micromass Quattro Micro triple quadrupol mass spectrometer (Waters Corporation) and a column oven (Jetstream Plus, Beckman Coulter). The sample (20 µL) was injected on a Kinetex phenyl hexyl column (Phenomenex, 150 x 4.5 mm; 2.6 µm particle size; 10 nm pore size). Gradient elution was performed with H_2O with 0.01 % formic acid (A), methanol with 0.01 % formic acid (B),

and acetonitrile with 0.01 % formic acid (C) as eluents. Flow rate was 0.5 mL/min, and oven temperature was maintained at 23 °C. Two different gradients were applied to detect the newly discovered triferulic acids. Ternary gradient: 86 % A, 14 % C, linear within 7 min to 81 % A, 19 % C, linear within 25 min to 69 % A, 9 % B, 22 % C, linear within 1 min to 74 % A, 26 % C, linear within 16 min to 64 % A, 36 % C, followed by rinsing and equilibration steps. Binary gradient: initially 30 % B, linear within 59 min to 80 % B, followed by rinsing and equilibration steps. MS parameters were: ionization, ESI negative mode; capillary, 3.0 kV; cone, 30 V; extractor, 2 V; RF lens, 0.1 V; source temperature, 100 °C; desolvation gas flow, 700 L/h; cone gas flow, 60 L/h, collision energy, 20 eV.

RESULTS AND DISCUSSION

Isolation/synthesis of trimers 1 and 2. Ferulate oligomers can be obtained from biomimetic synthesis,[21,22] chemical synthesis,[12,19] or from ferulate rich plant materials.[20] Whereas trimer **1** was synthesized using a chemical oxidative coupling approach, trimer **2** was isolated from ferulate-rich plant material, popcorn maize, after saponification. Although popcorn insoluble fiber contains comparably large amounts of ester-linked ferulates, diferulates, and higher oligomers, the alkaline hydrolyzate contains a variety of matrix components, which need to be separated from the target molecule. Also, the separation of ferulate dimers and tetramers from individual trimers is a challenging task, which requires the application of different chromatographic principles. After liberation of saponifiable components from maize insoluble dietary fiber, partition between ethyl acetate and 5 % $NaHCO_3$ is an easy and convenient step to roughly separate large quantities of phenolic acids and other carboxylic acids from phenols and other less acidic compounds. To further separate phenolic acids and other carboxylic acids, high-performance size exclusion chromatography was chosen. Because small molecules such as ferulic acid, *p*-coumaric acid, benzoic acid, and vanillic acid are the main phenolic acids in plant cell walls,[23] size exclusion chromatography removes a dominant fraction of phenolic acids. As shown in Figure 2, these small molecules can easily be separated from carboxylic acids with higher hydrodynamic volumes. These are mainly ferulic acid oligomers, which were partially separated into fractions 2-5, with fraction 3 containing most of the

ferulic acid trimers. This fraction was further separated using two subsequent procedures based on reversed-phase chromatography (Figure 3).

Different from trimer **2**, trimer **1** was synthesized chemically using ethyl ferulate with the copper(II)-TMEDA complex in an oxygen-rich atmosphere.[19] Therefore, plant-based matrix compounds do not interfere; however, because this reaction produces a plethora of radical coupling products of ferulates, basic chromatographic separation procedures are still required. A single silica flash chromatography purification step was sufficient to isolate a fraction containing trimer **1** besides two other ferulic acid trimers (8-O-4/8-5(non-cyclic)-TriFA,[13] and 8-O-4/8-O-4-TriFA[16]). These trimers were isolated by preparative reversed-phase chromatography (Figure 4). However, the fraction obtained by rinsing the flash chromatography column with a more polar solvent, ethyl acetate, was more complex, containing known (5-5/8-O-4-TriFA,[11] 5-5/8-5(non-cyclic)-TriFA[17]) and unknown ferulic acid trimers and tetramers. Compared to isolation procedures[20] and targeted synthesis[24], synthesis using the copper(II)-TMEDA complex is a fast and easy alternative to get ferulic acid trimers as standard compounds.

Structural elucidation of trimers 1 and 2. The UV-spectra of both isolated compounds show maxima at 323 – 326 nm and shoulders at 290 – 300 nm suggesting ferulic acid-containing structures (Figure 5). A broad shoulder as found for trimer **1** has been described earlier as an indicator for the involvement of an 8-O-4-linkage.[25] HPLC-ESI-MS (negative ion mode) analysis produced a high intensity of *m/z* 577 $[M-H]^-$ for both compounds, indicating the occurrence of a dehydrotriferulic acid. The sum formula of $C_{30}H_{25}O_{12}$ for the $[M-H]^-$ ion of both compounds was confirmed by high-resolution mass spectrometry with determined masses of 577.1342 $[M-H]^-$ for trimer **1** and 577.1348 $[M-H]^-$ for trimer **2**. This represents an error of -1.7 ppm and -0.7 ppm, respectively, in MS-mode. Interpretation of MS/MS-data resulted in errors of 2.7 ppm (46 fragments considered) and 3.8 ppm (22 fragments), respectively.

Unambiguous structural elucidation was achieved by interpreting spectra from various 1D- and 2D-NMR experiments including ^{1}H-, HSQC-, HMBC-, COSY- and NOESY-experiments. As determined for trimer **1**, two proton signals at 6.00 ppm and 4.37 ppm with coupling constants of 8.0 Hz suggest an 8-5(cyclic)-linkage within the trimer.[12] Because only two doublets with coupling constants of

16.0 Hz (indicative for unsubstituted *trans*-propenyl side chains) were found it was suggested that the second linkage also involves the 8-position. Two doublet of doublets with coupling constants of about 8 and 2 Hz (representing protons in position 6 of ferulate units) and two doublets with coupling constants of about 8 Hz (representing protons in position 5 of ferulate units) indicate that two ferulate units are not linked in their 5-position, hinting that the third ferulic acid is coupled via an 8-O-4-linkage. Theoretically, two triferulate regioisomers that contain 8-O-4- and 8-5(cyclic)-linkages exist. The proton at B7, which was unambiguously identified by HMBC- and HSQC-experiments, is represented by a singlet in the proton spectrum thus showing no correlations in the COSY-experiment. Therefore, it was concluded that proton B7 has no vicinal neighbor demonstrating that the propenylic side chain of the B unit is involved in the 8-O-4 linkage (Figure 1). All other signals from the different NMR-experiments are in good agreement with the proposed structure and are comparable to NMR data of the previously published 8-O-4/8-5(cyclic)-dehydrotriferulic acid triethyl ester.[21] Because the ^{1}H-shifts of the protons B2 and B6 overlapped, the corresponding ^{13}C-shifts at 115.5 and 121.1 ppm were not independently assigned but based on literature.[21] Also, the ^{13}C-shift of B1 was not determined, because long range-coupling was not observed using HMBC parameters as applied here.

The ^{1}H-spectrum of trimer **2** showed signals typical for 8-8(cyclic), i.e. two doublets at 4.66 ppm (B7) and 4.04 ppm (B8) with coupling constants of 2.0 Hz and a singlet at 7.70 ppm (A7) (Table 1). Because the proton spectrum did not show any doublets of doublets or 8 Hz doublets, it was suggested that the third ferulic acid is coupled via a 5-5-linkage. This hypothesis was confirmed by interpretation of the 2D NMR spectra including the NOESY-experiment, which shows through space interactions of the protons B6 and C6. Thus, the structure shown in Figure 1 is in agreement with all experimental data.

Natural occurrence of trimers 1 and 2. The existence of trimer **1** and trimer **2** (in other plant materials than popcorn maize) in plant cell walls was demonstrated by saponifying insoluble dietary fiber from maize, wheat, sugar beet, and amaranth for 18 h in 2 M NaOH and subsequent LC-MS/MS analysis. Because it was assumed that both newly identified trimers exist in comparably small amounts in plant cell walls, larger amounts of insoluble dietary fiber (100 mg each) were saponified and run

through the clean-up procedure. Based on fragmentation patterns obtained from LC-MS/MS-analysis (Table 2), daughter ions with *m/z* 193, *m/z* 311, and *m/z* 489 for trimer **1** and daughter ions with *m/z* 445 and *m/z* 489 for trimer **2** were chosen (with *m/z* 577 being the parent ion for both trimers) for MS/MS-analysis. Comparison of retention times with those of the isolated trimers **1** and **2** as standard compounds demonstrated the existence of trimer **2** in maize, wheat, and sugar beet pulp, but not in amaranth.

Trimer **1** was not detected in any plant materials after saponification for 18 h. This could be due to the absence or only very low levels of this trimer in plant materials or due to structural modifications during saponification and/or the clean-up procedure as suggested for ferulate oligomers containing 8-5(cyclic)-linkages.[5] It was demonstrated earlier that 8-5(cyclic)-dehydrodiferulic acid (DFA) is converted to 8-5(non-cyclic)-DFA and 8-5(decarboxylated)-DFA under alkaline conditions.[12] More recently, it was also shown that 8-5(decarboxylated)-DFA is partially formed from 8-5(cyclic)-DFA in aqueous buffers with pH values around 7.[26] Therefore, less severe methods of hydrolysis were tested. First, a combination of the carbohydrase mixture Driselase with two different feruloyl esterases was tested. The carbohydrases of the multi enzyme preparation Driselase were supposed to support the feruloyl esterases by partially cleaving the (feruloylated) polysaccharides to oligosaccharides. However, neither of the trimers were detected in the enzymatic hydrolyzates. Whereas some ferulate dimers were partially liberated from the plant cell wall polysaccharides (data not shown), enzyme accessibility of areas cross-linked by ferulate trimers may be restricted. However, when the enzymatic procedure was followed by a brief alkaline hydrolysis (10 min) using less concentrated NaOH (1 M) trimer **1** was detected in the hydrolyzates from maize, wheat, and sugar beet pulp by using the MS/MS-transitions described above. As for trimer **2**, trimer **1** was also not detected from amaranth.

The newly identified trimers add to the complexity of ferulate cross-links identified in plant materials. The analysis of ferulate based cross-links has been a challenging task so far[25,27] and will be more complex by adding these new trimers to the spectrum of ferulate oligomers to be analyzed. A major challenge in developing new protocols for the analysis of ferulate oligomers is the limited availability of standard compounds. Oxidation in organic solvents, as originally developed by Lu et al.[19] and

311 expanded to trimers in this study, is a promising path to synthesize ferulic acid trimers. Although our
312 studies were of qualitative nature only and do not allow definite conclusions on the quantities of these
313 newly identified trimers in plant materials, it needs to be kept in mind that even small amounts of
314 trimers and higher oligomers may have a major impact on the cell wall structure as they potentially
315 crosslink three or more polymers.

316

317 **ABBREVATIONS**

318 ESI, electrospray ionization; DFA, dehydrodiferulic acid; TriFA, dehydrotriferulic acid; TMEDA,
319 tetramethylethylenediamin; ToF, time-of-flight

320

321 **ACKNOWLEDGEMENTS**

322 The authors thank Falco Beer (Max Rubner-Institut) and Pepe Schrimpf (KIT) for technical assistance.

323

324

REFERENCES

(1) Saulnier, L.; Marot, C.; Chanliaud, E.; Thibault, J.-F. Cell wall polysaccharide interactions in maize bran. *Carbohydr. Polym.* **1995,** *26*, 279-287.

(2) Rombouts, F. M.; Thibault, J.-F. Feruloylated pectic substances from sugar-beet pulp. *Carbohydr. Res.* **1986,** *154*, 177-187.

(3) Hartley, R. D.; Harris, P. J. Phenolic constituents of the cell walls of dicotyledons. *Biochem. Syst. Ecol.* **1981,** *9*, 189-203.

(4) Colquhoun, I. J.; Ralet, M. C.; Thibault, J.-F.; Faulds, C. B.; Williamson, G. Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. *Carbohydr. Res.* **1994,** *263*, 243-256.

(5) Bunzel, M. Chemistry and occurrence of hydroxycinnamate oligomers. *Phytochem. Rev.* **2010,** *9*, 47-64.

(6) Ralph, J.; Grabber, J. H.; Hatfield, R. D. Lignin-ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr. Res.* **1995,** *275*, 167-178.

(7) Saulnier, L.; Crépeau, M.-J.; Lahaye, M.; Thibault, J.-F.; Garcia-Conesa, M. T.; Kroon, P. A.; Williamson, G. Isolation and structural determination of two 5, 5′-diferuloyl oligosaccharides indicate that maize heteroxylans are covalently cross-linked by oxidatively coupled ferulates. *Carbohydr. Res.* **1999,** *320*, 82-92.

(8) Piber, M.; Koehler, P. Identification of dehydro-ferulic acid-tyrosine in rye and wheat: evidence for a covalent cross-link between arabinoxylans and proteins. *J. Agric. Food Chem.* **2005,** *53*, 5276-5284.

(9) Bunzel, M.; Allerdings, E.; Ralph, J.; Steinhart, H. Cross-linking of arabinoxylans via 8-8-coupled diferulates as demonstrated by isolation and identification of diarabinosyl 8-8 (cyclic)-dehydrodiferulate from maize bran. *J. Cereal Sci.* **2008,** *47*, 29-40.

(10) Saulnier, L.; Thibault, J.-F. Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. *J. Sci. Food Agric.* **1999,** *79*, 396-402.

(11) Grabber, J. H.; Mertens, D. R.; Kim, H.; Funk, C.; Lu, F.; Ralph, J. Cell wall fermentation kinetics are impacted more by lignin content and ferulate cross-linking than by lignin composition. *J. Sci. Food Agric.* **2009,** *89*, 122-129.

(12) Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D. Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *J. Chem. Soc., Perkin Trans. 1* **1994**, 3485-3498.

(13) Bunzel, M.; Ralph, J.; Funk, C.; Steinhart, H. Structural elucidation of new ferulic acid-containing phenolic dimers and trimers isolated from maize bran. *Tetrahedron Lett.* **2005,** *46*, 5845-5850.

(14) Bunzel, M.; Ralph, J.; Funk, C.; Steinhart, H. Isolation and identification of a ferulic acid dehydrotrimer from saponified maize bran insoluble fiber. *Eur. Food Res. Technol.* **2003,** *217*, 128-133.

(15) Rouau, X.; Cheynier, V.; Surget, A.; Gloux, D.; Barron, C.; Meudec, E.; Louis-Montero, J.; Criton, M. A dehydrotrimer of ferulic acid from maize bran. *Phytochemistry* **2003,** *63*, 899-903.

(16) Funk, C.; Ralph, J.; Steinhart, H.; Bunzel, M. Isolation and structural characterisation of 8–O–4/8–O–4- and 8–8/8–O–4-coupled dehydrotriferulic acids from maize bran. *Phytochemistry* **2005,** *66*, 363-371.

(17) Bunzel, M.; Ralph, J.; Brüning, P.; Steinhart, H. Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize bran fiber. *J. Agric. Food Chem.* **2006,** *54*, 6409-6418.

(18) Fieser, M., *Fiesers' reagents for organic synthesis*. John Wiley & Sons: New York, 1967; Vol. 1.

(19) Lu, F.; Wei, L.; Azarpira, A.; Ralph, J. Rapid syntheses of dehydrodiferulates via biomimetic radical coupling reactions of ethyl ferulate. *J. Agric. Food Chem.* **2012,** *60*, 8272-8277.

(20) Bunzel, M.; Funk, C.; Steinhart, H. Semipreparative isolation of dehydrodiferulic and dehydrotriferulic acids as standard substances from maize bran. *J. Sep. Sci.* **2004,** *27*, 1080-1086.

(21) Bunzel, M.; Heuermann, B.; Kim, H.; Ralph, J. Peroxidase-catalyzed oligomerization of ferulic acid esters. *J. Agric. Food Chem.* **2008,** *56*, 10368-10375.

(22) Ralph, J.; Garcia-Conesa, M. T.; Williamson, G. Simple preparation of 8-5-coupled diferulate. *J. Agric. Food Chem.* **1998,** *46*, 2531-2532.

(23) Eraso, F.; Hartley, R. D. Monomeric and dimeric phenolic constituents of plant cell walls — possible factors influencing wall biodegradability. *J. Sci. Food Agric.* **1990,** *51*, 163-170.

(24) Mouterde, L. M.; Flourat, A. L.; Cannet, M. M.; Ducrot, P. H.; Allais, F. Chemoenzymatic total synthesis of a naturally occurring (5-5′)/(8′-O-4″) dehydrotrimer of ferulic acid. *Eur. J. Org. Chem.* **2013,** *2013*, 173-179.

(25) Dobberstein, D.; Bunzel, M. Separation and detection of cell wall-bound ferulic acid dehydrodimers and dehydrotrimers in cereals and other plant materials by reversed phase high-performance liquid chromatography with ultraviolet detection. *J. Agric. Food Chem.* **2010,** *58*, 8927-8935.

(26) Schendel, R. R.; Karrer, C.; Bunzel, D.; Huch, M.; Hildebrand, A. A.; Kulling, S. E.; Bunzel, M. Structural transformation of 8–5-coupled dehydrodiferulates by human intestinal microbiota. *J. Agric. Food Chem.* **2015,** *63*, 7975-7985.

(27) Jilek, M. L.; Bunzel, M. Dehydrotriferulic and dehydrodiferulic acid profiles of cereal and pseudocereal flours. *Cereal Chem.* **2013,** *90*, 507-514.

FIGURE CAPTIONS

Figure 1: Structures of the newly identified dehydrotriferulic acids (TriFA) 8-O-4/8-5(cyclic)-TriFA (trimer **1**) and 8-8(cyclic)/5-5-TriFA (trimer **2**).

Figure 2: Separation of ferulates (and other acids) from saponified maize fiber with size exclusion chromatography and UV-detection at 355 nm. Peak 1, ferulic acid and other hydroxycinnamic and benzoic acids; 2, ferulic acid dimers; 3, ferulic acid trimers; 4-5, higher ferulic acid oligomers.

Figure 3: Chromatograms of the first (A) and second (B) purification steps of trimer **2** (8-8(cyclic)/5-5-dehydrotriferulic acid) using semi-preparative phenyl hexyl-HPLC with UV-detection at 270 nm (A) and 260 nm (B). Asterisks indicate the fractions that were collected and contained trimer **2**.

Figure 4: Chromatogram of the purification of trimer **1** (8-O-4/8-5(cyclic)-TriFA, peak 3) using preparative C_{18}-HPLC with UV-detection at 320 nm. Peak 1, 8-O-4/8-5(non-cyclic)-TriFA; peak 2, 8-O-4/8-O-4-TriFA. TriFA – dehydrotriferulic acid.

Figure 5: UV-spectra of trimer **1** (8-O-4/8-5(cyclic)-dehydrotriferulic acid), and trimer **2** (8-8(cyclic)/5-5-dehydrotriferulic acid). The spikes at 363 nm were caused by a detector aberration.

TABLES

Table 1: NMR data for trimer **1** (8-O-4/8-5(cyclic)-dehydrotriferulic acid), and trimer **2** (8-8(cyclic)/5-5-dehydrotriferulic acid) measured in acetone-d_6:D_2O (10:1, v/v). Coupling constants are given in Hz.

Trimer 1			**Trimer 2**		
trimer unit	^{1}H [ppm]	^{13}C [ppm]	trimer unit	^{1}H [ppm]	^{13}C [ppm]
A1	-	130.1	A1	-	124.6
A2	7.43 (1H; d; 1.9)	112.2	A2	7.08 (1H; s)	113.0
A3	-	150.3	A3	-	147.5
A4	-	148.6	A4	-	149.6
A5	6.83 (1H; d; 8.4)	114.3	A5	6.79 (1H; s)	117.0
A6	7.13 (1H; dd; 8.4, 1.9)	122.8	A6	-	132.5
A7	7.59 (1H; d; 16.0)	145.2	A7	7.70 (1H; s)	138.2
A8	6.43 (1H; d; 16.0)	117.5	A8	-	124.1
A9	-	168.0	A9	-	168.5
A3-OMe	4.00 (3H; s)	56.3	A3-OMe	3.87 (3H; s)	56.2
B1	-	nd	B1	-	135.1
B2	7.50 (1H; br s)	115.5[a]	B2	6.81 (1H; d; 2.0)	110.7
B3	-	145.3	B3	-	148.6
B4	-	150.2	B4	-	143.6
B5	-	127.4	B5	-	125.4
B6	7.50 (1H; br s)	121.1[a]	B6	6.61 (1H; d; 2.0)	122.9
B7	7.46 (1H; s)	129.9	B7	4.66 (1H; d; 2.0)	46.1
B8	-	138.8	B8	4.04 (1H; d; 2.0)	47.4
B9	-	164.7	B9	-	173.5
B3-OMe	3.77 (3H; s)	55.9	B3-OMe	3.80 (3H; s)	56.2
C1	-	131.9	C1	-	127.1
C2	7.06 (1H; d; 1.9)	110.5	C2	7.30 (1H; d; 1.9)	109.6
C3	-	148.5	C3	-	149.2
C4	-	147.8	C4	-	147.2
C5	6.82 (1H; d; 8.2)	115.7	C5	-	126.8
C6	6.88 (1H; dd; 8.2, 1.9)	120.0	C6	7.05 (1H; d; 1.9)	126.1
C7	6.00 (1H; d; 8.0)	88.3	C7	7.59 (1H; d; 15.9)	145.9
C8	4.37 (1H; d; 8.0)	55.8	C8	6.38 (1H;z d; 15.9)	116.1
C9	-	171.9	C9	-	168.3
C3-OMe	3.81 (3H; s)	56.1	C3-OMe	3.94 (3H; s)	56.4

[a] - see text; nd - not determined

Table 2: Daughter ions of trimer **1** (8-5(cyclic)/8-O-4-dehydrotriferulic acid), and trimer **2** (8-8(cyclic)/5-5-dehydrotriferulic acid) measured with triple quadrupol mass spectrometry after fragmentation of *m/z* 577 as parent ion. MS parameters are given in Materials and Methods.

Trimer 1		**Trimer 2**	
m/z	Intensity [%]	m/z	Intensity [%]
193	100	489	100
489	10.1	445	29.2
311	9.6	533	5.5
417	2.3	271	4.2
544	2.1	430	2.8
		515	2.3
		256	1.8

FIGURE GRAPHICS

Figure 1

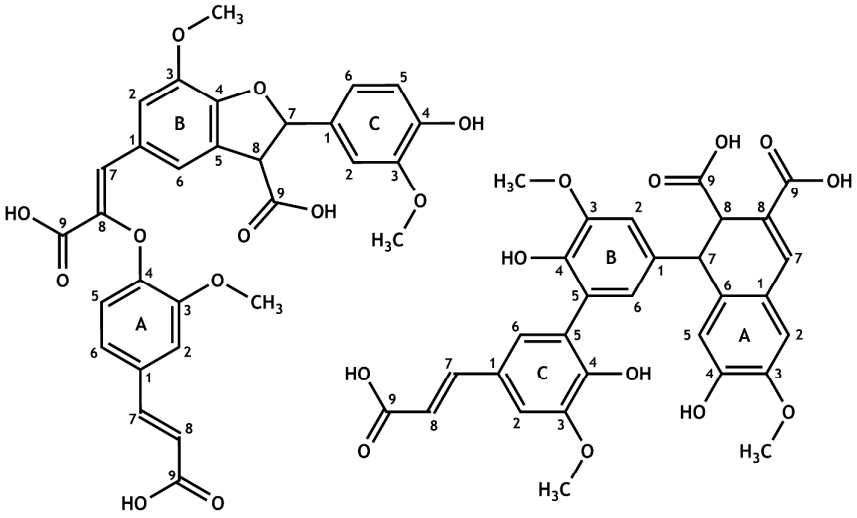


Figure 2

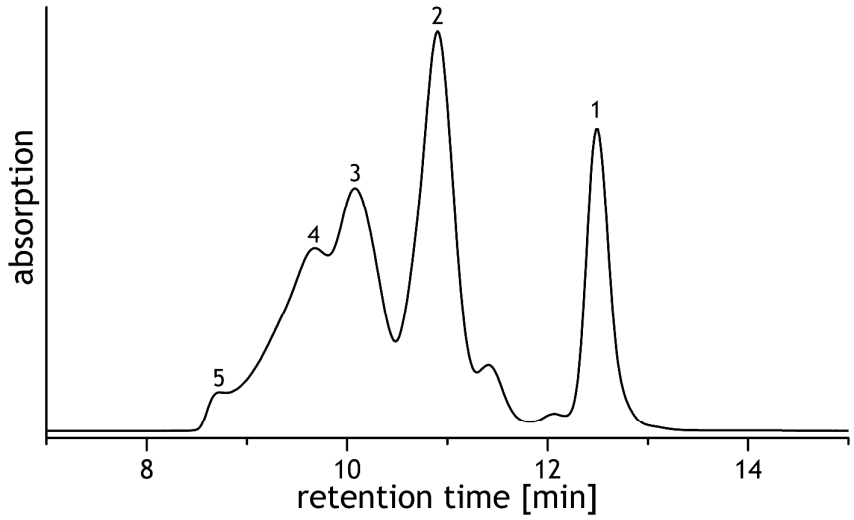


Figure 3

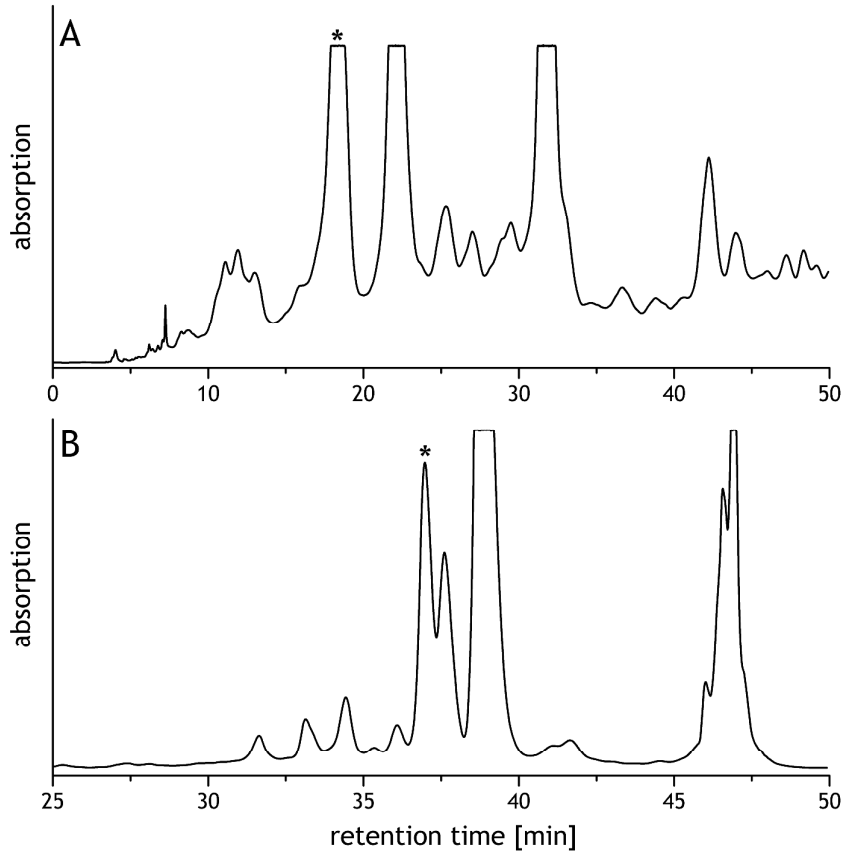

A
*
absorption
0
10
20
30
40
50
B
*
absorption
25
30
35
40
45
50
retention time [min]

Figure 4

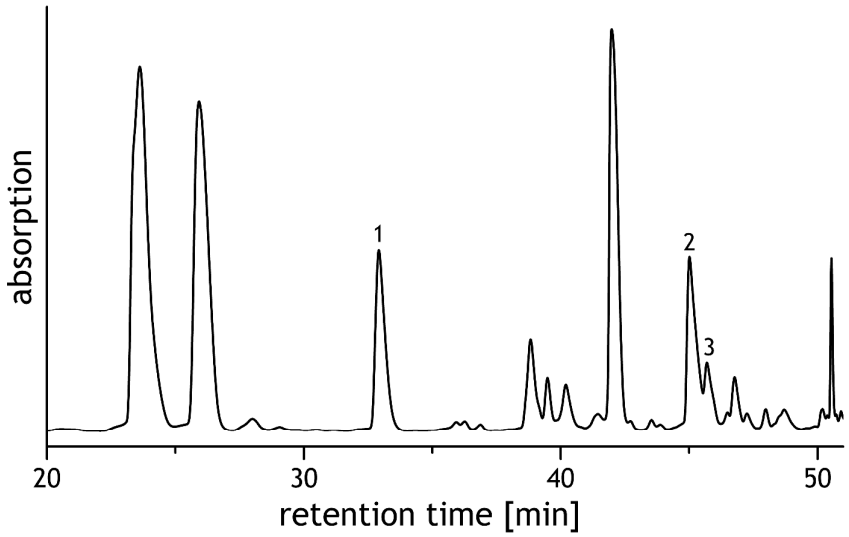
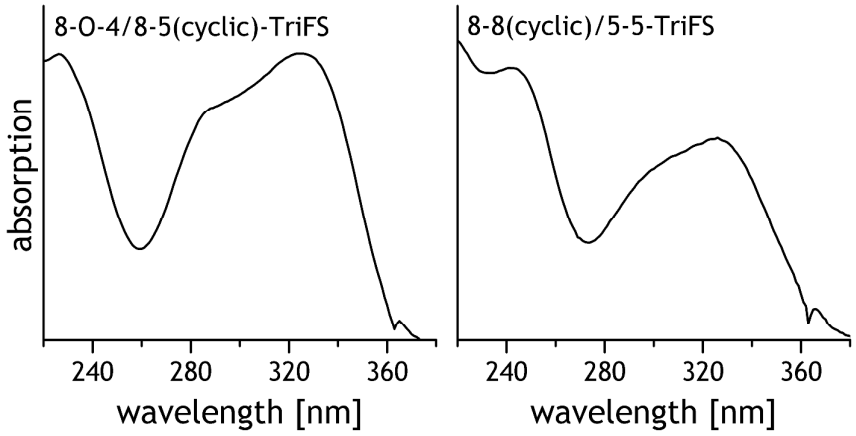


Figure 5


8-O-4/8-5(cyclic)-TriFS
8-8(cyclic)/5-5-TriFS
absorption
240
280
320
360
wavelength [nm]
240
280
320
360
wavelength [nm]

TOC graphic