Research Article

Phytochemical Analysis

Received: 7 November 2011; Revised: 23 December 2011; Accepted: 24 January 2012 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/pca.2359

Conversion of Phenolic Constituents in Aqueous *Hamamelis virginiana* Leaf Extracts During Fermentation

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Introduction – *Hamamelis virginiana*, known for its high level of tannins and other phenolics is widely used for treatment of dermatological disorders. Although reports on hydroalcoholic and aqueous extracts from *Hamamelis* leaf and bark exist, knowledge on fermented leaf preparations and the underlying conversion processes are still scant.

Objective – Aqueous *Hamamelis* leaf extracts were monitored during fermentation and maturation in order to obtain an insight into the bioconversion of tannins and other phenolics.

Methodology – Aliquots taken during the production period were investigated by HPLC-DAD-MS/MS as well as GC-MS after derivatisation into the corresponding trimethylsilyl compounds.

Results – In *Hamamelis* leaf extracts, the main constituents exhibited changes during the observational period of 6 months. By successive depside bond cleavage, the gallotannins were completely transformed into gallic acid after 1 month. Although not completely, kaempferol and quercetin glycosides were also converted during 6 months to yield their corresponding aglycones. Following C-ring fission, phloroglucinol was formed from the A-ring of both flavonols. The B-ring afforded 3-hydroxybenzoic acid from quercetin and 3,4-dihydroxybenzoic acid as well as 2-(4-hydroxyphenyl)-ethanol from kaempferol. Interestingly, hydroxycinnamic acids remained almost stable in the same time range.

Conclusion – The present study broadens the knowledge on conversion processes in aqueous fermented extracts containing tannins, flavonol glycosides and hydroxycinnamic acids. In particular, the analogy between the microbial metabolism of phenolics from fermented *Hamamelis* extracts, fermented sourdough by heterofermentative lactic acid bacteria or conversion of phenolics by the human microbial flora is indicated.

Keywords: HPLC-DAD-MS/MS; GC-MS; fermentation; flavonol glycosides; gallotannins; hydroxycinnamic acids; witch hazel; *Hamamelis virginiana*

INTRODUCTION

Hamamelis virginiana L. (Hamamelidaceae) is a traditional medicinal plant with a long history in pharmaceutical therapy. Originally used by the Native Americans for treatment of burns and injuries (Laux and Oschmann, 1993), witch hazel has lost nothing of its importance today. More recently, several studies were carried out to confirm the vast spectrum of application possibilities (Laux and Oschmann, 1993; Reuter *et al*., 2010). Because of its astringent, anti-phlogistic and anti-inflammatory effects, *Hamamelis* preparations are classically applied for treatment of dermatological disorders such as atopic dermatitis and neurodermitis, but also in wound management preventing intrusion of pathogenic germs (Laux and Oschmann, 1993; Reuter *et al*., 2010; Schilcher *et al*., 2010). Other indications reported are the treatment of anorectal disorders, diarrhoea and venous diseases. Additionally, *Hamamelis* is considered as a non-toxic and therefore safe ingredient for use in pharmaceutical applications (Laux and Oschmann, 1993).

The preparation of fermented extracts from *Hamamelis* leaves for pharmaceutical use is described in an official regulation (*German Homoeopathic Pharmacopoeia*; GHP, 2003). Special treatment of cut fresh plant material initiates a spontaneous fermentation and a pH reduction below 4.5. After 7 days, the batch is stored at 15°C in the dark for further maturation (GHP, 2003). According to current conception, these processes are typically catalysed by lactic acid bacteria, especially during the first few days (Millet, 2010; Schwarzenberger *et al*., 2012). As a consequence, the batch undergoes acidification which leads to growth inhibition of the accompanying microbial flora (Schwarzenberger *et al*., 2012). The fermentation and maturation period is defined as 6 months (GHP, 2003), whereas the decompartmentation and leaching of hydrophilic plant constituents proceeds within the first few days followed by their transformation over the remaining time period, resulting in a preserved extract (GHP, 2003; Schwarzenberger *et al*., 2012).

Fermentation processes by action of lactic acid bacteria are well implemented in classic and traditional food production such as sauerkraut (Kessler *et al*., 2010), wine (Hernández *et al*., 2007) and sourdough bread (Moroni *et al*., 2009). Objectives for fermentation are not only to bring about desirable changes in taste and smell, but also refinement, preservation or even detoxification of the product (Steinkraus, 1983; Caplice and Fitzgerald, 1999). Since fermented foodstuff plays an important role in human nutrition, only recently, the scientific focus was directed towards compositional and bioactivity changes as well as possible health promoting effects of the resulting products (Gonthier *et al*., 2003; Rodríguez *et al*., 2009; Van Hylckama

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Vlieg *et al.*, 2011). Phenolic constituents present in microbial fermentation systems play a special role: they may serve as substrates being transformed into bioactive low-molecular weight structures (Rodríguez *et al.*, 2009; Selma *et al.*, 2009). Some of these, including tannins, influence growth and therefore the fermentative action of lactic acid bacteria and other microorganisms (Scalbert, 1991). Generally, the microbial flora is able to metabolise hydroxycinnamic acids (Rodríguez *et al.*, 2009; Selma *et al.*, 2009) and flavonoids, as well as hydrolysable and condensed tannins (Selma *et al.*, 2009). In particular, the bioconversion pathways of several hydroxycinnamic acids and flavonoids of red sorghum sourdough fermented by lactic acid bacteria were studied recently (Svensson *et al.*, 2010). The transformation of dietary phenolics in the complex human gut microbiota by use of *in vitro* colonic model systems is another typical example (Justesen *et al.*, 2000; Selma *et al.*, 2009).

Aqueous fermented *Hamamelis* leaf extracts represent a preparation that is rich in tannins as well as flavonols (Duckstein and Stintzing, 2011). However, only scattered data on the fermentation of plant extracts by their own natural microflora exist (Bilia *et al.*, 2007; Millet, 2010; Millet *et al.*, 2010; Schwarzenberger *et al.*, 2012). Therefore, the objective of this study was to monitor aqueous *Hamamelis* leaf extracts during a fermentation and maturation period of 6 months to expand the picture of fermentative conversion processes on phenolics, especially of hydrolysable tannins and flavonol glycosides.

EXPERIMENTAL

Chemicals

For chromatographic analyses acetonitrile (LC-MS grade) and formic acid (98%, eluent additive for LC-MS) were obtained from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (HPLC grade) was purchased from J.T. Baker (Deventer, Netherlands). Purified water (0.055 μS/cm) from a Purelab Option-Q system (Elga Berkefeld GmbH, Celle, Germany) was used throughout. Analytical grade formic acid (98–100%), sodium sulphate, ethyl acetate, toluol and chloroform were obtained from Merck (Darmstadt, Germany). The silylation reagent Fluka I according to Sweeley was obtained from Fluka (Buchs, Switzerland). The following chemicals served as reference standards: gallic acid monohydrate (Roth GmbH & Co. KG, Karlsruhe, Germany), ellagic acid (Fluka, Buchs, Switzerland), quercetin (USP reference standard, Rockville, USA), kaempferol (Fluka, Buchs, Switzerland), 2-(4-hydroxyphenyl)-ethanol (ABCR GmbH & Co. KG, Karlsruhe, Germany), 4-hydroxybenzoic acid (Roth GmbH & Co. KG, Karlsruhe, Germany), 3,4-dihydroxybenzoic acid (protocatechuic acid; Sigma-Aldrich, Steinheim, Germany), glycerol (anhydrous; Merck, Darmstadt, Germany), phloroglucinol (anhydrous; Ph. Eur. Reference standard, France), (−)-shikimic acid (Merck, Darmstadt, Germany), succinic acid (Merck, Darmstadt, Germany) and quinic acid (Sigma-Aldrich, Steinheim, Germany).

Fermented *H. virginiana* leaf extract preparation

All fermented extracts from *H. virginiana* were produced at WALA Heilmittel GmbH (Bad Boll/Eckwälden, Germany). For preparation of the extracts, fresh leaves were collected in the medicinal herb garden of WALA Heilmittel GmbH (Bad Boll/Eckwälden, Germany). Further working steps were carried out according to an official production protocol 33d (GHP, 2003), where the preparation of cut leaves, honey, water and lactose monohydrate in the ratio of 100:0.75:200:0.75 (w/w/w/w) results in the maceration and spontaneous fermentation of the mixture by the natural microbial flora from *Hamamelis* leaves. After 3.5 days at room temperature, the solid leaf material was removed and the resulting turbid extract kept at room temperature for further fermentation. After 7 days, the batch was stored at 15°C protected from light for subsequent fermentation and maturation until 6 months. For each of the three batches (production years 2006, 2008 and 2010) allocated for investigation, aliquots were taken after 24 h, 3.5, 7 and 14 days as well as 1, 2, 3 and 6 months and stored immediately at −25°C. Prior to HPLC-DAD and LC-MS/MS analyses samples were thawed and centrifuged at 19,064 × *g*. For GC-MS analyses, samples were thawed and derivatised as described below.

Additionally, a representative aqueous non-fermented *Hamamelis* leaf extract from a previous study (Duckstein and Stintzing, 2011) served as an unaltered reference sample.

Derivatisation of the fermented extracts

For qualitative GC-MS analyses, the low-molecular weight constituents in the non-fermented and fermented *Hamamelis* leaf preparations were extracted and converted into their corresponding trimethylsilyl (TMS) derivatives (Lorenz *et al.*, 2008). In brief, 2.5–4 mL of the samples were extracted twice with 4 mL of ethyl acetate. The combined ethyl acetate extracts were dried with sodium sulphate, the desiccant filtered off and the solvent removed *in vacuo* by rotovaporation (T = 38°C). Afterwards, 10 mL of toluol were added and again reduced to dryness to remove acetic acid residues. The silylation of the dry extracts was performed by adding 0.5 mL chloroform and 0.2 mL silylating mixture Fluka I according to Sweeley (Lorenz *et al.*, 2008). The reaction mixture was transferred into a headspace vial (20 mL), subsequently sealed with a silicon/PTFE cap and incubated at 105 °C for 45 min. Then, the samples were diluted by adding 4.3–9.3 mL chloroform depending on the initial volume of the extracted material. After centrifugation (3,327 × *g*, 10 min), 1 μL of the silylated extracts was injected into the GC-MS system.

The silylation of the reference compounds (1–2 mg each) was conducted as described above. The following standards were used: 2-(4-hydroxyphenyl)-ethanol, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), glycerol, phloroglucinol, succinic and shikimic acids.

Chromatographic analyses

HPLC-DAD analyses. HPLC-DAD analyses were performed on a Dionex Ultimate 3000 system, consisting of a vacuum degasser SRD-3400, a binary pump HPG-3400 A, an autosampler WPS-3000 TSL, a thermostatted column compartment TCC-3000 SD and a diode array-detector DAD-3000 (Dionex GmbH, Idstein, Germany). A gradient system (eluent A: 1% formic acid (v/v); eluent B: acetonitrile:water (9:1; v/v)) was run on a SunFire C_{18} reversed-phase analytical column (150 × 2.1 mm i.d.; 5 μm particle size; Waters GmbH, Eschborn, Germany) at a flow rate of 0.21 mL/min (25°C) using the following working steps: starting at 100% A for 5 min, a linear gradient to 35% B at 105 min and a further increase to 100% B at 110 min was performed, staying isocratically for 5 min and return to initial conditions within a 5-min re-equilibration step at 100% A. The detection wavelengths were 280 and 360 nm, the injection volume was 10 μL. Data acquisition and processing

was performed with Chromeleon V 6.8 software (Dionex GmbH, Idstein, Germany).

LC-ESI/MS/MS analyses. Mass spectrometric analyses were carried out on an Agilent 1200 HPLC-system equipped with a degasser G1322A, a binary pump G1312A, an autosampler G1329A, a thermostatted column compartment G1316A, and a diode array-detector G1315B (Agilent, Waldbronn, Germany) connected to a HCTultra ion trap MS detector fitted with an ESI ion source (Bruker Daltonik GmbH, Bremen, Germany). Liquid chromotography separation was performed under the same conditions with the same eluents and HPLC-column as described in the HPLC-DAD section. Mass spectrometric parameters were chosen as follows: operation mode, negative; capillary voltage, 4000 V; dry gas flow (N_2), 9 L/min; nebuliser pressure, 35 psi; capillary temperature, 365°C. Mass spectra were recorded between *m/z* 50 and 2000 in the full scan option. The MS^n experiments were carried out with a compound stability and trap drive level at 100% in the automatic MS/MS mode. For data acquisition and processing Agilent ChemStation B.01.03 (Agilent, Waldbronn, Germany) and EsquireControl V 6.1 (Bruker Daltonik GmbH, Bremen, Germany) software were used. Constituents were identified via their specific UV and mass spectra as well as retention times in comparison with literature data and reference compounds.

GC-MS analyses. Gas chromatographic analyses were carried out according to Lorenz *et al.* (2008) using a PerkinElmer Clarus 500 gas chromatograph (PerkinElmer Inc., Massachusetts, USA), a Zebron ZB-5 ms capillary column (60 m × 0.25 mm i.d.; 0.25 μm film thickness, 5% phenylpolysiloxane and 95% dimethylpolysiloxane coating; Phenomenex, Torrance, USA) and an injection volume of 1 μL. Injection was performed by a PSS (programmed-temperature split/splitless injector; temperature: 250°C; split ratio: 30:1). Helium was used as carrier gas at a flow rate of 1 mL/min. The temperature gradient was 100 to 320°C following a ramp of 4.0°C/min and a final holding period of 30 min. Electron ionisation of the mass detector was set at 70 eV. The software Turbomass V 5.4.2 (PerkinElmer Inc., Massachusetts, USA) was used for data acquisition and processing. Constituents were identified in accordance to their specific MS data as well as retention times in comparison with the NIST MS database (NIST Mass Spectral Library, NIST2005, V 2.1, Perkin Elmer Inc., Massachusetts, USA) and commercially available reference compounds.

RESULTS AND DISCUSSION

Composition of *H. virginiana* leaf extracts

Fermented plant extracts consist of a complex mixture of components. The quality of the starting material and the manufacturing procedure play crucial roles (Biber *et al.*, 2009). Prior to the investigation of such a multifaceted preparation, comprehensive information about the constituents contained in the original plant material is needed. For this reason, in a preparatory step the composition of *H. virginiana* L. leaves was extensively investigated (Duckstein and Stintzing, 2011). Contrary to earlier reports (Vennat *et al.*, 1988; Wang *et al.*, 2003), it turned out that galloyl hexoses with 6 to 11 galloyl units represent the main fraction followed by several types of kaempferol and quercetin glycosides as well as the 3-, 4- and 5-caffeoylquinic acid isomers (chlorogenic acids; Fig. 1A). Also, minor proportions of a procyanidin dimer and trimer as well as some hydroxycinnamic acid esters (coumaroylquinic acid isomers, caffeoylshikimic acid) were present.

Figs 1A and 2A depict fingerprints of a representative unaltered leaf extract using a gentle extraction procedure preventing any degradation. Most of its constituents had already been identified by LC-MS/MS (Duckstein and Stintzing, 2011). In the current study, only a selection of significant phenolic constituents was addressed (Fig. 1A). To obtain an insight into the presence of low-molecular weight components that may have escaped HPLC-DAD detection, the same freshly prepared extract was investigated by GC-MS analyses after compound extraction with ethyl acetate and their derivatisation into TMS compounds. The total ion current (TIC) shows the freshly prepared extract to contain no ethyl acetate extractable compounds with the exception of traces of gallic acid (**Ga**) and an unknown compound (**Z**; Fig. 2A).

Biotransformation of phenolic constituents during fermentation

Three batches of fermented extracts (production years 2006, 2008, and 2010) were prepared according to the official production protocol 33d (GHP, 2003). After 24 h, 3.5, 7 and 14 days as well as 1, 2, 3 and 6 months, aliquots were taken under laminar flow and subsequently stored at −25°C before analyses. Sample treatment before HPLC analyses included only centrifugation. Prior to GC-MS analyses, a two-stage extraction step and subsequent derivatisation of the extracted low-molecular weight compounds was conducted.

A representative overview of the phenolic compound profile changes during *Hamamelis* leaf fermentation after 24 h, 1 month and 6 months is depicted in Fig. 1B–D (HPLC) and Fig. 2B–D (GC). A summary of all conversion products is given in Table 1.

In addition, *H. virginiana* aqueous leaf extracts also contain catechin and procyanidins, which, however, were not considered further because of their minute quantities (Duckstein and Stintzing, 2011). Moreover, typical conversion products from flavan-3-ol structures such as hydroxyphenyl-valerolactones or hydroxyphenyl-valeric acids (Selma *et al.*, 2009; Roowi *et al.*, 2010) were not detected.

Gallotannins. Hydrolysable tannins with galloyl hexoses 6 to 11 gallic acid units (**14–17**, **19**, **20**, Fig. 1A) constitute the most important phenolic fraction in *H. virginiana* leaf extracts (Duckstein and Stintzing, 2011). As shown in Fig. 1, a strong transformation of gallotannins took place during the observation period. After 24 h, the conversion to penta- (**G6**) and tetragalloyl hexose (**G5**) started with further break-up into tri- (**G4**) and digalloyl hexose units (**G3**). After 1 month, all of the higher molecular weight gallotannins changed mainly into lower galloylated tetra- (**G5**) and trigalloyl hexoses (**G4**) while the monogalloyl hexose peak (**G1**) rose considerably in the same time range.

Most interestingly, gallic acid (**G2**) was detected after 24 h (GC-MS; Fig. 2B), which alludes to the conversion of the gallotannins (HPLC; Fig. 1B). On the same line, the predominant gallic acid peak (**G2**) increased after 1 month and remained stable until 6 months (Fig. 1C and D). With the exception of the monogalloyl hexose no gallotannins were left. At this point, the initial character of the extract changed remarkably. Inversely, gallic acid, the biosynthetic building block of gallotannins and barely detectable in the genuine

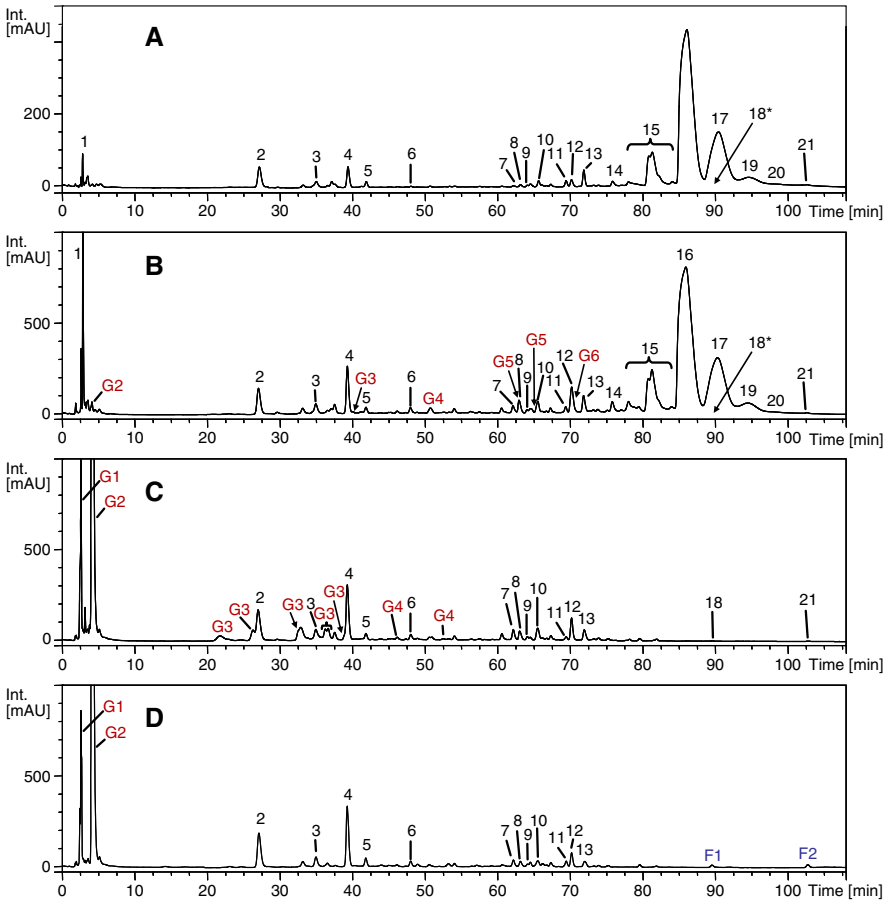


Figure 1. Conversion of phenolic constituents from aqueous *Hamamelis virginiana* leaf extracts depicted as representative HPLC-DAD fingerprints recorded at 280 nm of (A) a freshly prepared leaf extract and fermented leaf extracts after (B) 24 h, (C) 1 month and (D) 6 months. Coeluting compounds are marked with arrows; (*) detected at 360 nm. (**1**) Monogalloyl hexose, (**2**) 3-caffeoylquinic acid, (**3**) 3-coumaroylquinic acid, (**4**) 5-caffeoylquinic acid, (**5**) 4-caffeoylquinic acid, (**6**) 5-coumaroylquinic acid, (**7**) quercetin-galloyl hexoside, (**8**) ellagic acid, (**9**) quercetin hexoside-deoxyhexoside, (**10**) kaempferol-galloyl hexoside I/quercetin hexoside, (**11**) kaempferol hexoside I, (**12**) kaempferol hexoside-deoxyhexoside, (**13**) kaempferol hexoside II, (**14**) hexagalloyl hexose, (**15**) heptagalloyl hexose, (**16**) octagalloyl hexose, (**17**) nonagalloyl hexose, (**18**) quercetin, (**19**) decagalloyl hexose, (**20**) undecagalloyl hexose, (**21**) kaempferol; compounds identified by LC-MS/MS in a previous study (Duckstein and Stintzing, 2011). Conversion products from galloyl hexoses: (**G1**) monogalloyl hexose, (**G2**) gallic acid, (**G3**) digalloyl hexose isomers, (**G4**) trigalloyl hexose isomers, (**G5**) tetragalloyl hexose isomers, (**G6**) pentagalloyl hexose; conversion products from flavonol glycosides: (**F1**) quercetin, (**F2**) kaempferol. Further data, see Table 1.

extract, prevailed in the fermented preparation. A detailed summary of gallotannin conversion products and their characteristic chromatographic data is given in Table 1.

Gallotannin alteration in plant extracts may be catalysed in the presence of tannase, a special enzyme cocktail of microbial or plant origin, as has earlier been shown for oak (Mingshu *et al*., 2006). Another possibility of gallotannin cleavage is a depside bond scission between the gallic acid units, due to the slightly acidic pH of the *Hamamelis* leaf extracts. Since in a former study with similar external conditions (room temperature, light protection) the phenolic constituents of the *Hamamelis* leaf extracts were stable for 4 months (Duckstein and Stintzing, 2011), pH-driven autodegradation or degradation of the gallotannins by plant-derived tannases can be excluded. As shown in Fig. 1B and C, already after 1 month the galloyl hexoses with more than six galloyl moieties were completely transformed into their corresponding lower galloylated representatives. This finding led to the assumption that the galloyl hexoses were converted by microbial action.

Tannase, the key enzyme for gallotannin degradation, is able to hydrolyse depside and ester bonds to release gallic acid and sugar moieties (Bhat *et al*., 1998). The enzyme may be produced by bacteria, yeasts or fungi and, source dependently, exhibits differing activities against diverse groups of tannins (Mingshu *et al*., 2006). During processing of the extracts according to GHP (2003), no starter culture was added. Therefore it can be concluded that the natural microbial flora of *Hamamelis* leaves starts and upholds the fermentation process. It should also be mentioned that gallotannins themselves exhibit anti-microbial properties against some type of bacteria, especially by binding free iron from the medium, whereas Gram-positive were reported to be more sensitive than Gram-negative bacteria (Engels *et al*., 2011). Notably, the same authors found that lactic acid bacteria, although being Gram-positive were not inhibited by the structurally related gallotannins from mango. This observation was related to their ability to grow iron-independently (Engels *et al*., 2011).

Unlike tannin degradation by yeasts, several ways of gallotannin decomposition by bacteria can be found in the literature (Bhat *et al*., 1998). The first step is the cleavage of gallic acid from the higher galloylated representatives forming intermediate di- and trigalloyl structures, ending up with gallic acid and the

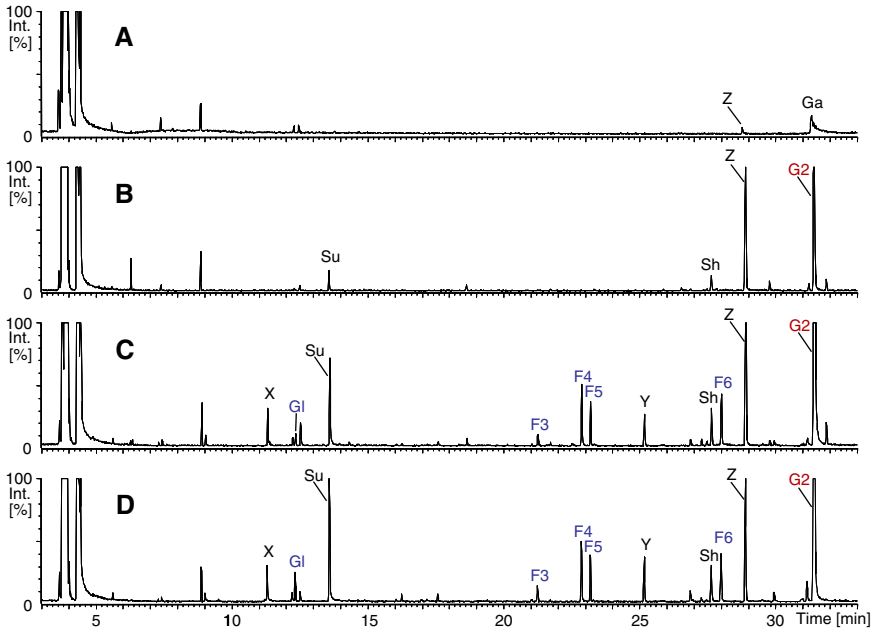


Figure 2. Conversion of phenolic constituents from aqueous *Hamamelis virginiana* leaf extracts depicted as representative GC-MS TIC EI+ fingerprints of (A) a freshly prepared leaf extract and fermented leaf extracts after (B) 24 h, (C) 1 month and (D) 6 months; (**Ga**) gallic acid, (**Su**) succinic acid, (**Sh**) shikimic acid, (**Gl**) glycerol, (**X**) saccharide structure, (**Y**) unknown compound, (**Z**) unknown compound. Conversion product from galloyl hexoses: (**G2**) gallic acid; conversion products from the flavonol glycosides: (**F3**) 2-(4-hydroxyphenyl)-ethanol, (**F4**) 4-hydroxybenzoic acid, (**F5**) phloroglucinol, (**F6**) 3,4-dihydroxybenzoic acid. Further data, see Table 1.

respective sugar moieties as carbon source. This way of tannin processing was also observed for *Lactobacillus plantarum* converting higher polymerised tannic acid into shorter chain derivatives, finally yielding polar monomers such as gallic acid and pyrogallol (Rodríguez *et al*., 2008a). This previously reported stepwise cleavage of gallotannins (Bhat *et al*., 1998; Mingshu *et al*., 2006; Rodríguez *et al*., 2008a) to yield their lower galloylated representatives and finally gallic acid was corroborated in the present study. Moreover, in a former report on fermented birch leaf extracts, lactobacteria were registered (Millet *et al*., 2010). A very recent study on aqueous fermented *Atropa belladonna* leaves characterised the microbial flora to consist of both hetero- and homofermentative lactic acid bacteria, the main components of which were characterised as *Lactobacillus brevis* and *L. plantarum* (Schwarzenberger *et al*., 2012). Together with previous literature the present findings support the assumption that lactic acid bacteria are part of the natural microbial flora on *Hamamelis* leaves.

Finally, a possible ellagic acid conversion into gallic acid via C–C cleavage of the hexahydroxydiphenoyl unit by phenoloxidase activity present in some microorganisms (Mingshu *et al*., 2006) is mentioned. Due to the small quantities of ellagic acid detected by HPLC in the genuine and the 24-h fermented extract, this way of conversion remains elusive. A schematic overview of the gallotannin conversion pathway in *Hamamelis* leaf extracts is given in Fig. 3, wherein the main tannin octagalloyl hexose (**16**, Fig. 1) was chosen as a representative component.

Flavonol glycosides. Another important phenolic fraction is represented by flavonol glycosides (Fig. 1A), namely quercetin-galloyl hexoside (**7**), quercetin hexoside-deoxyhexoside (rutin, **9**), kaempferol-galloyl hexoside I/quercetin hexoside (**10**), kampferol hexoside I (**11**), kaempferol hexoside-deoxyhexoside (**12**) and kaempferol hexoside II (**13**). Also, minute amounts of quercetin (**18**) and kaempferol (**21**) were detected in the non-fermented aqueous leaf extract (Duckstein and Stintzing, 2011). After 1 day of fermentation (24 h; Fig. 1B), several galloyl hexose conversion products coeluted with the flavonol glycosides. The flavonol aglycones quercetin and kaempferol were also detectable in minute amounts (Fig. 1B). Four weeks later (Fig. 1C), the peak spectrum of the flavonols was faintly changed, thereby concluding a certain degree of stability. After 6 months, the flavonol glycosides diminished whereas the quercetin and kaempferol peaks rose slightly (Fig. 1D). Further conversion products could not be detected by HPLC-DAD-MS. This observation indicated that flavonol glycosides are preferentially converted into their aglycones quercetin and kaempferol. This flavonol glycoside deconjugation has previously been described for fermented birch leaf extracts (Millet *et al*., 2010) as well as several other microbiological systems, e.g. deconjugation by colonic microorganisms (Jaganath *et al*., 2009; Selma *et al*., 2009), by lactic acid bacteria in sourdough (Svensson *et al*., 2010) or wine (Hernández *et al*., 2007). The deconjugation of quercetin-galloyl hexoside (**7**, Fig. 1) may also be assumed since LC-MS/MS investigations showed the galloyl unit to be released first followed by the hexose moiety. This indicates the gallic acid to constitute an outer part of the structure (Duckstein and Stintzing, 2011) thus allowing enzymatic attack. This way of cleavage yields both the conversion product of the gallotannins (gallic acid) and the flavonol glycosides (flavonol aglycon and sugar moiety).

These findings based on HPLC-DAD-MS data were complemented by GC-MS analyses. The major strengths of the latter analytical strategy are the selective extraction and therefore enrichment of low-molecular weight compounds on the one hand, the higher sensitivity of the GC-MS system to detect minute amounts of conversion products on the other. Inversely, high-molecular weight compounds such as gallotannins are not detectable by regular GC-MS, even after silylation. In conclusion,

Table 1. Conversion products of phenolic constituents in fermented aqueous *Hamamelis* leaf extracts and their chromatographic and mass spectrometric characteristics

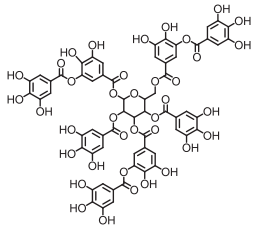
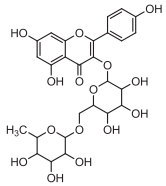
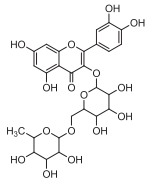
Genuine compound class representatives	Peak	Method of analyses		t_r (min)	Peak assignment	λ_{max} (nm)	MS data		Reference	Detection after		
							Pseudomolecular ion (BPI [%])	Fragments (BPI [%])				
		LC	GC				*m/z*	*m/z*		24 h	1 month	6 months
Galloyl hexoses	G1	x		2.6	Monogalloyl hexose[a]	274	331 (100)[b]	MS^2: 271 (82), 211 (47), 169 (100), 125 (21), MS^3: 125 (100)		+	+	+
 Octagalloyl hexose[c]	G2	x		4.2	Gallic acid	272	169 (96)[b]	125 (100), MS^2: 125 (100), MS^3: –	st	tr	+	+
	G3	x		21.8, 26.3, 32.9, 36.2, 36.7, 38.9, 50.3	Digalloyl hexose isomers[a]	275	483 (100)[b]	MS^2: 313 (25), 271 (100), 211 (17), MS^3: 211 (100), 169 (12)		tr	+	–
	G4	x		45.7, 50.8, 52.6	Trigalloyl hexose isomers	277	635 (100)[b]	MS^2: 465 (100), 313 (5), MS^3: 447 (3), 313 (100), 235 (8), 169 (34)	L1	tr	tr	–
	G5	x		63.1, 64.9	Tetragalloyl hexose isomers	278	787 (100)[b]	MS^2: 617 (100), 465 (3), MS^3: 465 (100), 313 (13), 175 (2)	L2	tr	–	–
	G6	x		70.5	Pentagalloyl hexose	–[d]	939 (100)[b]	MS^2: 769 (100), 617 (17), 431 (4), MS^3: 617 (100), 447 (28), 277 (7)	L2	tr	–	–
Flavonol glycosides	F1	x		89.5	Quercetin	256, 370	301 (100)[b]	MS^2: 273 (20), 257 (18), 179 (100), 151 (84), MS^3: 151 (100)	st	tr	tr	+
 Kaempferol hexoside-deoxy-hexoside[c]  Quercetin hexoside-deoxy-hexoside[c]	F2	x		102.7	Kaempferol	266, 366	285 (100)[b]	MS^2: 257 (99), 213 (68), 197 (61), 151 (100), 107 (89), MS^3: 107 (100)	st	tr	tr	+
	F3		x	21.1	2-(4-Hydroxyphenyl)-ethanol[e]	–[f]	282 (27)[g]	267 (18), 193 (12), 179 (100), 103 (10), 73 (40)	st	–	+	+
	F4		x	22.8	4-Hydroxybenzoic acid[e]	–[f]	282 (35)[g]	267 (100), 223 (75), 193 (43), 126 (17), 73 (51)	st	–	+	+
	F5		x	23.1	Phloroglucinol[e]	–[f]	342 (100)[g]	327 (80), 268 (15), 253 (8), 147 (15), 133 (7), 73 (54)	st	–	+	+

Table 1. *(Continued)*

Genuine compound class representatives	Peak	Method of analyses		t_r (min)	Peak assignment	λ_{max} (nm)	MS data		Reference	Detection after		
							Pseudomolecular ion (BPI [%])	Fragments (BPI [%])				
		LC	GC				*m/z*	*m/z*		24 h	1 month	6 months
	F6		x	27.9	3,4-Dihydroxybenzoic acid (protocatechuic acid)[e]	–[f]	370 (89)[g]	355 (47), 311 (22), 271 (11), 223 (11), 193 (100), 73 (64)	st	–	+	+
Miscellaneous compound classes	Gl		x	12.3	Glycerol[e]	–[f]	293 (1)[h]	218 (33), 205 (73), 147 (83), 133 (18), 117 (26), 103 (29), 73 (100)	st	–	+	+
	Su		x	13.6	Succinic acid[e]	–[f]	247 (16)[h]	147 (100), 75 (22), 73 (41)	st	+	+	+
	Sh		x	35.7	Shikimic acid [e]	–[f]	462 (0.4) [g]	204 (100), 147 (18), 75 (9), 73 (80)	st	+	+	+
	X		x	11.3	Saccharide structure[a]	–[f]	219 (91)	147 (89), 145 (74), 103 (100), 73 (81)	N	–	+	+
	Y		x	25.2	Unknown[e]	–[f]	310 (6)	295 (6), 220 (100), 103 (10), 73 (32)	N	–	+	+
	Z		x	28.9	Unknown[e]	–[f]	435 (14)	345 (100), 255 (32), 147 (20), 73 (39)	N	+	+	+

+, Detected; –, not detected; st, reference standard used to verify retention time and/or UV spectrum and/or MS fragmentation pattern; tr, trace amounts detected; L1, identified according to Gonzalez *et al.* (2010); L2, identified according to Engels *et al.* (2010); N, tentative structure assignments proposed by NIST MS database (NIST2005, V 2.1, PerkinElmer Inc., Massachusetts, USA).

[a]Tentative assignment

[b]$[M-H]^-$ species.

[c]Exemplary structure representative of the main constituent(s).

[d]Unambiguous DAD signal because of coelution.

[e]Compounds are trimethylsilyl (TMS) ethers but not designated more closely.

[f]Not available (GC-MS analyses).

[g]$[M]^+$ species of the TMS derivatives.

[h]$[M-CH_3]^+$ species of the TMS derivatives.

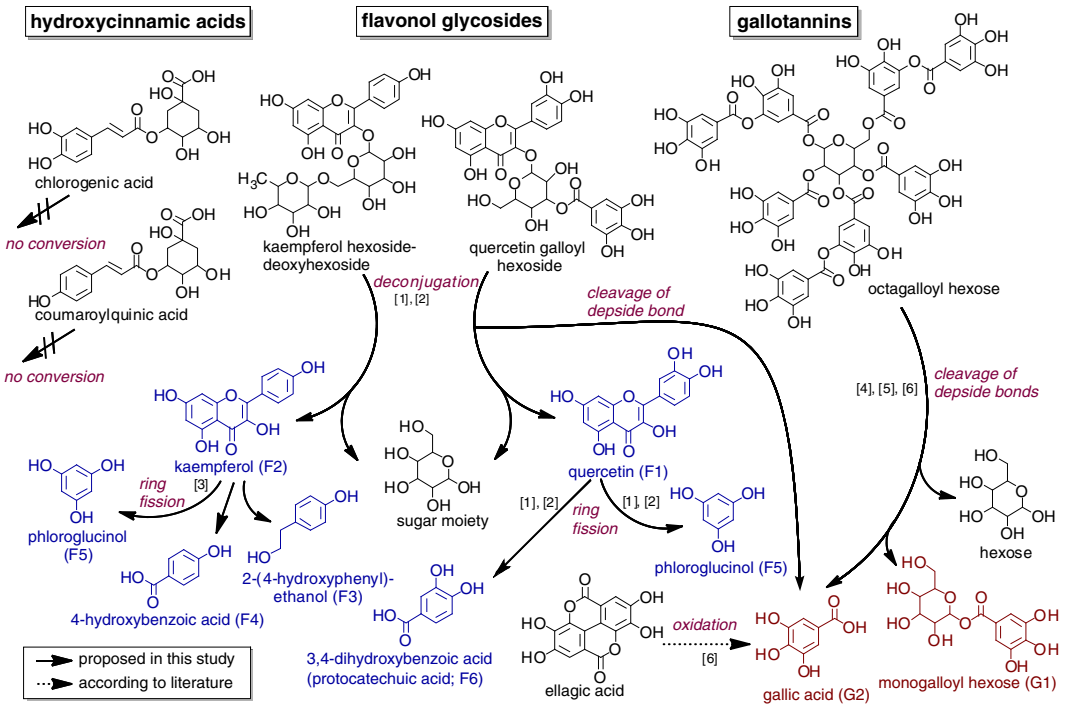


Figure 3. Structural transformation of phenolic constituents in aqueous fermented *Hamamelis* leaf extracts; structures of flavonol glycosides and gallotannins are representative structures of the main constituents from each compound class; (**F1–F5**) conversion products from the flavonol glycosides, (**G1**, **G2**) conversion products from the gallotannins, further data see Table 1; numbers in brackets refer to previous literature reporting similar reactions in other scientific consensus: [1] Jaganath *et al.* (2009), [2] Selma *et al.* (2009), [3] Blaut *et al.* (2003), [4] Bhat *et al.* (1998), [5] Rodríguez *et al.* (2008a), [6] Mingshu *et al.* (2006).

only the combination of HPLC and GC methods permits a comprehensive view on fermented *Hamamelis* leaf preparations.

Figure 2B–D depicts the TIC of silylated low-molecular weight conversion products extracted from the fermented preparation. After 1 and 6 months, several peaks potentially originating from the flavonols and neither present after 24 h nor in the non-fermented extract (Fig. 2A and B) were detected: 2-(4-hydroxyphenyl)-ethanol (**F4**), 4-hydroxybenzoic acid (**F5**), phloroglucinol (**F6**) and 3,4-dihydroxybenzoic acid (**F7**, protocatechuic acid). All these compounds were assigned by comparing chromatographic and mass spectrometric data with those from reference compounds and the NIST MS database.

The observation that most of the conversion products detected by GC-MS were present after 1 month, but not after 24 h, is in accordance with the HPLC stability assessment of the flavonol glycosides discussed above. Because quercetin and kaempferol as well as their glycosides were present in the non-fermented extract, conversion products of both flavonols need to be considered. The two compounds differ only in their 3′-hydroxyl group at the B-ring, which is lacking in kaempferol (Fig. 4). Originating from an identical A-ring, phloroglucinol (**F6**) was presumed as a shared C-ring fission product of both representatives. Similar flavonol conversions were observed for the degradation of quercetin (Blaut *et al.*, 2003; Labib *et al.*, 2004; Jaganath *et al.*, 2009; Selma *et al.*, 2009) and kaempferol (Blaut *et al.*, 2003). A further C-ring fission product derived from the B-ring of quercetin was 3,4-dihydroxybenzoic acid (**F7**, protocatechuic acid), formed via multistep conversion of 3-(3,4-dihydroxyphenyl)-propionic acid, as described earlier (Jaganath *et al.*, 2009; Selma *et al.*, 2009). In the present study, the conversion products 2-(4-hydroxyphenyl)-ethanol (**F4**) and 4-hydroxybenzoic acid (**F5**) derived from the B-ring

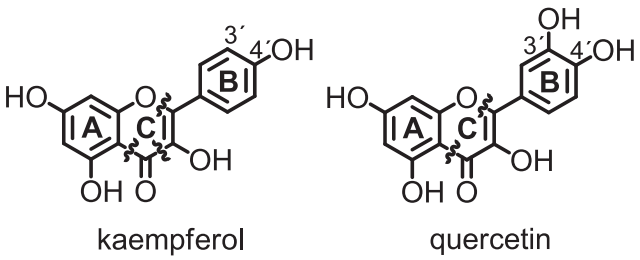


Figure 4. Structures of quercetin and kaempferol and positions of their C-ring fission marked with wavy lines.

of kaempferol were also detected. 4-Hydroxybenzoic acid may be formed analogously to 3,4-dihydroxybenzoic acid (**F6**) from quercetin (**F1**). Because of lacking literature data on fermentative kaempferol B-ring conversion products, this assumption is based on the strong structural similarities between both flavonols (Fig. 4). In the case of 2-(4-hydroxyphenyl)-ethanol (**F4**), it is assumed to be a B-ring conversion product from kaempferol, since the structurally related 2-(3,4-dihydroxyphenyl)-ethanol has been described earlier as a C-ring fission product from quercetin (Justesen and Arrigoni, 2001). A possible 2-(4-hydroxyphenyl)-ethanol release upon tyrosine metabolism, as described for yeasts (Narayanan and Rao, 1976), was excluded since no tyrosine was detected in the non-fermented and the 24-h fermented extracts (data not shown). An overview of the flavonol glycosides conversion products is given in Table 1 and a detailed conversion scheme depicted in Fig. 3.

Hydroxycinnamic acid derivatives. In the time period inspected, no scission of the hydroxycinnamic acid derivatives

(**2–6**, Fig. 1) could be observed. Typical microbial fermentation products of these hydroxycinnamic acid derivatives described in the literature are vinyl or ethyl catechol, 3-hydroxyphenylpropionic acid and benzoic acid (Gonthier *et al*., 2006; Rodríguez *et al*., 2008b, 2009). Also, reports on the structural transformation of hydroxycinnamic acids in several bacterial systems exist (Rechner *et al*., 2004; Selma *et al*., 2009; Curiel *et al*., 2010). In contrast, although Rodríguez *et al*. (2008b) observed the metabolisation of caffeic acid by *L. plantarum*, its depside chlorogenic acid remained unchanged. In the present study, none of these previously discussed fermentation products were detected, assuming that these structural changes are dependent on the specific microflora with their respective enzymatic activities.

Additional compounds. The GC-chromatograms of the fermented *Hamamelis* leaf extract (Fig. 2B–D) revealed some additional peaks, which at first sight cannot be directly connected with the gallotannin or flavonol glycoside conversion. Succinic acid (**Su**), shikimic acid (**Sh**) and glycerol (**Gl**) were detected in the fermented *Hamamelis* samples, but not in the genuine extract indicating that these compounds are formed during fermentation. Literature data on the fermentation of sourdough by lactic acid bacteria report glycerol to be a metabolite built up by lactic acid bacteria via an alternative pathway upon heterofermentative conversion of sugars. In the same manner, succinic acid is metabolised from fumarate in the citrate cycle (Gänzle *et al*., 2007). Both metabolites seem to arise from saccharide structures that may originate from the sugar moieties leading to the notion that heterofermentative microorganisms are prevalent.

Free shikimic acid (**Sh**, Fig. 2 and Table 1) detected upon fermentation may originate from sugar moieties as detected earlier for bacterial strains (Krämer *et al*., 2003), but its occurrence in the course of the fermentation of *Hamamelis* plant material by its natural microflora mostly remains unclear.

Three peaks (**X**, **Y**, **Z**, Fig. 2 and Table 1) were detected only in the fermented (**X**, **Y**) or in both the non-fermented and fermented extracts (**Z**). While the structure of these peaks remains unknown, propositions may be made according to the NIST MS database. Peak **X** was suggested to be a saccharide-type structure, potentially formed upon fermentation of sugar derivatives or related compounds, whereas no proposition on the structures of the unknown peaks **Y** and **Z** is possible.

It is worth noting that in addition to a previous study on the phenolic composition of *Hamamelis* leaves (Duckstein and Stintzing, 2011), quinic acid ($t_R = 2.0$ min, $[M-H]^-$ precursor ion: 191, fragments: *m/z* 127 → 109) was detected both in the genuine and the fermented extracts by LC-MS/MS (data not shown, verified by comparison with a reference compound). Also, galloylquinic acid ($t_R = 4.2$ min, $\lambda_{max} = 272$ nm, $[M-H]^-$ precursor ion: 343, fragments: *m/z* 169 → 125) was assigned according to literature data (Romani *et al*., 2012) and was detectable in all samples (data not shown). These findings complement former investigations and, in addition, underpin a certain degree of stability of these polar constituents towards fermentative actions.

In conclusion, the results obtained contribute to a better understanding of the alteration processes of tannins, flavonols and hydroxycinnamic acids initiated by the natural microbial flora from *Hamamelis* leaves. The structural conversions revealed may be transferred to other fermented tannin containing extracts analogous to sourdough fermentation (Gänzle *et al*., 2007; Svensson *et al*., 2010) or human metabolism of dietary phenolics by the intestinal flora (Justesen *et al*., 2000; Selma *et al*., 2009).

Furthermore, the present study corroborates the apprehension that data referring to genuine plant extracts cannot be transferred to fermented preparations derived therefrom (Biber *et al*., 2009). Moreover, these findings may serve as a basis to define marker compounds for quality control issues in the future.

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