

Different approaches to evaluate tannin content and structure of selected plant extracts – review and new aspects

M. Kardel^{1*}, F. Taube¹, H. Schulz², W. Schütze², M. Gierus¹

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Summary

Tannins occur in many field herbs and legumes, providing an immense variability in structure and molecular weight. This leads to complications when measuring tannin content; comparability of different methods is problematic. The present investigations aimed at characterizing four different tannin extracts: quebracho (*Schinopsis lorentzii*), mimosa (*Acacia mearnsii*), tara (*Caesalpinia spinosa*), and gambier (*Uncaria gambir*) and impact of storage conditions. Using photometrical methods as well as HPLC-ESI-MS, fundamental differences could be determined. Quebracho, mimosa, and gambier contained 164.3, 108.2, and 169.3 g kg⁻¹ of tannin (calculated as procyanidin C1); tara reached 647.5 g kg⁻¹ (calculated as epigallocatechin gallate). Alongside with compounds already described in the literature, several tannin molecules were found that have not been observed before in the analyzed sources. Extraction with hot water provided clear advantage over treatment with acetone or methanol; the organic solvents resulted in 9.2 to 15.3 % less tannin isolation. Tannin content decreased by a maximum of 1 % per year stored at room temperature compared to 4 °C, but proportions of some compounds slightly shifted. Oven drying of material should be avoided. In general, the tannin extracts proved to have very diverse structures, making application of an overall standard method difficult.

Introduction

Plant tannins are polyphenols, distributed into condensed and hydrolyzable tannins with an immense structural variability, reaching high degrees of polymerization (see reviews DIXON et al., 2005; MUELLER-HARVEY, 2006). This heterogeneity causes complications regarding a standardized tannin measurement (MOLE and WATERMAN, 1987a, b). Their role in the plants organism seems to be in the defensive system (AYRES et al., 1997; NICHOLS-ORIAN, 1991). Nutrient utilization of feeds can be derogated by high levels of tannins and other phenols (MARTINEZ et al., 2004; RUBANZA et al., 2005). Some of them can be toxic to ruminal microbes (MAKKAR et al., 1995; CLAUSEN et al., 1990) and even the animal itself (HERVAS et al., 2003). But there are beneficial aspects for herbivores, especially ruminants, in consuming small amounts of tannins, which cause increasing interest in tannins as a feed compound. Tannin supplementation in ruminant feeding is aimed at increasing nitrogen use efficiency by using the tannins ability to form complexes with proteins (BARRY and MCNABB, 1999; CASSIDA et al., 2000). Many legumes and field herbs contain tannins, but only in low (JACKSON et al., 1996) and varying amounts (AZUHNWI et al., 2011; THEODORIDOU et al., 2011), causing the need to measure the present concentration with every harvest to ensure the correct tannin amount in the feed.

Plant tannin extracts on the other hand, are used worldwide for the industrial production of leather (for example mimosa or quebracho; ROMER et al., 2011) and to a lesser extend in wine making (GARCIA-RUIZ et al., 2012). An amount of 33.728,465 kg of vegetable tanning

extracts, tannins, salts and derivates was imported to the EU in the year 2011, mainly from Argentina, with a trade value of nearly 63.5 mio. US Dollar (UN-COMTRADE, 2013). These extracts are produced industrially on a large scale. They could therefore maintain a relatively constant quality and thus provide an advantage in ruminant feeding.

Tannins from different sources can react very diverse regarding protein affinity (MCNABB et al., 1998; BUENO et al., 2008). Differences in tannin structure can occur even between similar plant species (OSBORNE and MCNEILL, 2001; HATTAS et al., 2011) and a higher degree of polymerization is not inevitably an indicator for better protein binding ability (HUANG et al., 2010; THEODORIDOU et al., 2011). Therefore, when considering tannins as possible feed additives, it is essential to describe and characterize the material as comprehensively as possible to gain information about their amount and source to be added in animal feeding.

This study aimed to evaluate different methods to characterize the material and possibly detect variability in tannin structures. For that purpose, different preparation methods for HPLC-MS analysis were applied using four selected tannin extracts (quebracho, mimosa, tara, gambier). The aim was to isolate as much of the tannin as possible while keeping matrix effects to a minimum, as they may interfere with the measurement (CIRIC et al., 2012; GHOSH et al., 2012). For comparison, a well established photometrical method was applied. The influence of different storage conditions was also accessed.

Material and methods

Description of extracts

Extracts of four tannin-rich plants were analyzed. Being an important part of leather manufacturing, there are several companies providing industrially produced tannin extracts of plants. For the purpose of this study, extracts of *Schinopsis lorentzii* (quebracho), *Acacia mearnsii* (mimosa), *Caesalpinia spinosa* (tara), and *Uncaria gambir* (gambier) were chosen.

Otto Dille® (Norderstedt; brand of Baeck & Co. Hamburg, Germany) provided quebracho, mimosa, and tara extracts (as well as a small portion of gambier); there was no information given regarding production method or storage. The mainly used gambier extract was kindly provided by Christian D. Markmann GmbH (Hamburg, Germany). Gambier leaves have been cooked in water, the tannin solution has then been spray dried and stored dry at room temperature.

Schinopsis lorentzii (Griseb.) Engler (synonym: *Schinopsis quebracho-colorado* Schltdl.) is a tree inhabiting Argentina, Paraguay, Brazil, and Bolivia. Quebracho tannin extract is already an authorized additive for feedstuffs in the European Union (see EU Community Register of Feed Additives). The mainly condensed tannin is extracted from the tree's heartwood. It has been subject of many feedstuff studies, though normally a characterization of the tannin structure is not included in the investigation. In the course of the present analyses, a small piece of natural quebracho heartwood was acquired, to produce own tannin extract in the laboratory.

* Corresponding author

Acacia mearnsii De Willd. (synonym: *Acacia mollissima* auct. non Willd.) is a leguminous tree known as mimosa or black wattle and occurs mainly in Australia, Brazil, South and East Africa. The condensed tannins are extracted from the bark. It is not part of the list of authorized additives for feedings yet, but there has been some scientific research regarding its qualification for feeding already (for example CARULLA et al., 2005; GRAINGER et al., 2009).

Caesalpinia spinosa (Molina) Kuntze, also a leguminous tree, occurs in South America, especially in Peru. Tara was chosen as a representative for hydrolyzable tannins, while the other extracts are supposed sources of mainly condensed tannins. There are fewer studies to be found regarding tara in the context of feeding (DRIEDGER and HATFIELD, 1972; PELLIKAAN et al., 2011). The tannin is extracted from the tara pods.

Uncaria gambir (Hunter) Roxb. is a shrub from Asia (mainly China, India, Indonesia, and Malaysia). It is used in the leather industry, where gambier condensed tannin is extracted mainly from leaves and small branches. In Asia, it has a traditional place as a natural remedy (ANGGRAINI et al., 2011). It has been investigated as a biosorbent in waste water (TONG et al., 2011), but it is largely unknown when it comes to animal feeding. No actual studies were to be found on this subject, even sole methodical approaches to the tannin structure were quite rare (TANIGUCHI et al., 2007a, b; 2008).

Photometrical analyses

Preparations for photometrical measurements of condensed tannins (CT) and total phenols (TP) were based on the methods of TERRILL et al. (1992) that can be regarded as a standard in tannin measurement. 10 ml of acetone:water (70:30) were added to 100 mg of extract in a test tube. Tubes were carefully vortexed and then sonicated in ice water three times for 5 min with 5 min breaks in between. The solution was then centrifuged at 4 °C with 3632 rcf (4800 rpm) for 15 min. The supernatant was collected and stored in dark bottles in the refrigerator for a maximum of two days until analysis. Analyses were carried out three times. All solutions were stored at 4 °C in the refrigerator and handled cold.

CT were determined modulating the butanol/HCl method (TERRILL et al., 1992). In tubes placed on ice, 50 µl sample solution and 200 µl of acetone:water (250 µl of acetone:water for blank value) were added to 1.5 ml of butanol:HCl (95:5; HCl concentration 37 %), the solution was then vortexed. After that, 50 µl of ferric:HCl (2 g of (NH₄)Fe(SO₄)₂, filled up to 100 ml with 2 N HCl) were added, the solution was vortexed again and then placed in a hot water bath (97 - 100 °C). After exactly 1 hour, the samples were removed and immediately cooled in ice to stop the reaction. The cooled down samples were vortexed again before measuring the extinction at 550 nm with a Biochrom Libra (S32PC UV, Biochrom Ltd., Cambridge, UK). CT content was calculated, using the following equation:

$$CT = ext * 156.5 * df / w * 100 / DM \quad (1)$$

with following representation:

CT: condensed tannin content in % of dry matter

ext: extinction measured at 550 nm

156.5: constant, resulting of measurements using leucocyanidin as a standard

df: dilution factor (in this case 250 / 50 = 5)

w: original sample weight in mg used for disintegration

DM: dry matter content of the original sample in %

TP were determined by modulating the Folin-Ciocalteu method (SINGLETON and ROSSI, 1965). The amount of sample solution had to be reduced to only 5 µl added to corresponding 995 µl deionized water, due to the high concentration of phenols. For blank value,

1 ml deionized water was used. 500 µl of folin solution (1:1 deionized water and Folin-Ciocalteu's phenol reagent, purchased from Merck KGaA, Darmstadt, Germany) were added to each sample tube, which was immediately vortexed. After exactly 3 min, 2.5 ml of sodium carbonate solution (88.5 g Na₂CO₃ x H₂O, filled up to 500 ml with deionized water) were added and the samples were vortexed again. After exactly 1 hour of reaction time, resting dark at room temperature, extinction was measured at 725 nm against a standard curve of tannic acid (purchased from Fluka, Sigma-Aldrich Chemie GmbH, München, Germany). TP content was calculated, using the following equation:

$$TP = conc * 10 / w / DM * 1000 \quad (2)$$

with following representation:

conc: concentration in the sample according to standard curve

10: dilution factor to the original amount of sample solution, 50 µl

w: original sample weight in mg used for disintegration

DM: dry matter content of the original sample in %

HPLC-MS analyses

Extraction methods, quebracho wood, charges

All analyses were performed twice. The tannin extracts were purified for analysis via HPLC-MS by different extraction methods: water, water:methanol (1:1; v/v), and acetone:water:formic acid (70:29.5:0.5; v/v/v). In the first two cases, 5 ml of the respective solvent were added to 100 mg of powder, placed in an ultrasonic bath for 15 min and then held at 90 °C for 2 hours; the solution was shaken every 30 min. The sample was then centrifuged at 7 °C with 3939 rcf (4500 rpm) for 10 min. The supernatant was filtered (0.2 µm pore size) to eliminate impurities and the sample was stored dark at 5 °C.

In the acetone treatment, 5 ml of acetone:water:formic acid were added to 100 mg of powder, the flask was thoroughly shaken and placed in an ultrasonic bath for 15 min. The solution was then centrifuged as described above. Each supernatant was pipetted into a test tube and acetone was evacuated with a vacuum concentrator (RVC 2-18; Martin Christ GmbH, Osterode am Harz, Germany) for 50 min at 40 °C. The residual solution was readjusted to 5 ml using water:formic acid (99.9:0.1; v/v) and placed in ultrasonic bath again for 5 min to redissolve some flakes that occurred during concentration. An aliquot was taken and filtered as described above.

Due to unexpected results of quebracho extract, a small piece of natural quebracho heartwood was acquired and a laboratory extract was produced manually. The wood was rasped by hand with a metal file and then milled in a ball mill twice for 5 min.

The wood powder was treated using the different methods with the same amounts of material and chemicals as described above. An aliquot was analyzed directly, with the injection volume increased to 20 µl. The remaining solutions were instantly frozen at -28 °C and then freeze-dried in a Gamma 1-16 LSC unit (Martin Christ GmbH, Osterode am Harz, Germany) for 70 hours. For the water:methanol method there was not enough dried wood extract available to run two analyses, so there is only one measurement to rely on. The freeze-dried extracts were treated according to hot water method before analysis.

After evaluation of the different extraction methods, following analyses were carried out using only the hot water method, which has resulted in the highest tannin measurements.

Aliquots were taken from each industrial tannin extract to examine if storage conditions have an impact on the tannin content. For this purpose, reasonable amounts of extracts were stored at 4 °C in the refrigerator as well as at room temperature with normal daylight on

a board in the laboratory for overall 1.5 years. Additionally, sub-samples were stored for 5 days at 60 °C in an incubator.

Device settings

For Reversed-Phase-Liquid-Chromatography/ESI-MS, a 1200 series device with diode array detector (DAD) was used (Agilent Technologies Deutschland GmbH, Böblingen, Germany). The DAD was set to detect signals at 280, 320, and 340 nm. An Accucore RP-MS (150x3.0 mm 2.6 µm Solid Core HPLC Column; Thermo SCIENTIFIC, Bellefonte PA, USA) with pre-column was used containing silica gel, followed by analyses with an ion-trap mass spectrometer (Esquire3000; Bruker Daltonics GmbH, Bremen, Germany). The mass spectrometer was equipped with electrospray ionization (ESI), running in negative ionization mode. The settings were: nebulizer (nitrogen): 40.0 psi, dry gas: 10.0 L/min, dry temperature: 320 °C; maximum accumulation time: 200.00 ms, scan: 100 to 1800 m/z (averages 5). All parameters were optimized by a standard tuning procedure.

The scanning area was set in compromise of covering as many potential oligomers as possible while on the other hand maintaining adequate sensitivity to detect small substance amounts.

Water:formic acid (99.9:0.1; A) and methanol:formic acid (99.9:0.1; B) were chosen as eluents with a maximum pressure of 450 bar, fitting the following timetable: 0-5 min = 5 % B isocratic, 5-25 min = 5-80 % B linear gradient, 25-27 min = 80-100 % B linear gradient, 27-32 min = 100 % B isocratic, 32-35 min = 100-5 % B linear gradient, 35-42 min = 5 % B isocratic. All chromatographic solvents were of HPLC grade.

The samples were held in a rack at 10 °C during analysis. The injection volume was 10 µl for quebracho, mimosa, and gambier as well as for the laboratory quebracho wood extract. For tara samples, the injection volume was 3 µl.

Quantification

HPLC peaks of external standards were used to quantify the amount of tannins present in the extracts. Beforehand, 11 different tannin standards were dissolved at 1 mg in 10 ml water:formic acid (99.9:0.1). Quantification data were obtained by HPLC-MS, injecting different amounts of the individual standard, resulting in a regression line for each standard. Analyzed standards were: (+)catechin, (-)epicatechin, (-)gallo catechin, (-)epigallocatechin, (-)epicatechin gallate, (-)epigallocatechin gallate (all purchased from Carl Roth GmbH & Co. KG, Karlsruhe, Germany); (-)catechin gallate, (-)gallo catechin gal-

late, procyanidin B1, B2, and C1 (purchased from Sigma-Aldrich Chemie GmbH, München, Germany); see Tab. 1.

The analyzed tannins express their highest intensity in UV/VIS spectra at 280 nm; therefore this wavelength was chosen for quantification. For each sample, the UV/VIS-spectrum of each peak was compared to the standards available. Additionally, molecular masses recorded by the MS were checked. If the respective peak was identified as a tannin substance, it was integrated manually. Using the regression equation, the peak area was converted into µg tannins. Summing up all tannin peaks and setting the result off with the injected volume and the original sample weight, the total tannin concentration was calculated.

For quebracho, mimosa, and gambier, procyanidin C1 was chosen as reference standard, because it fitted their mainly oligomeric condensed tannin structure best. For tara, a sufficient gallic acid derivative could not be acquired. Tannic acid would have fit the molecular structure. Yet because of its high molecular weight of 1701, it did not represent the majority of compounds found in the tara extract adequately. Therefore, epigallocatechin gallate was considered the better reference, because here galloyl groups are the major structural component.

Statistical evaluation and data processing

Statistical evaluations were carried out using the statistical software R (R, 2012). Simultaneous comparisons of means were performed using multiple contrast tests with corresponding custom contrast matrices. Differences between treatments were declared significant at $P < 0.05$ using the Tukey correction for multiple comparisons.

HPLC data were processed with Agilent Technologies ChemStation software for LC 3D systems, all data from mass spectrometry were processed and displayed with Bruker Daltonics DataAnalysis software Version 3.3. Molecular masses are described as $[M-H]^-$ (m/z), unless stated otherwise.

Results and discussion

Photometrical analyses

In accordance with the data communicated by the producer, tara showed a very small amount of measurable CT content, indicating its main tannin was of hydrolyzable nature. In Tab. 2, the content of CT and TP is listed for each extract. Due to very high TP contents, the solution had to be diluted several times. The resulting high dilution factor caused a slight overestimation.

Tab. 1: Tannin standards with their respective molecular weight (mw), chemical formula, CAS number and purity.

tannin standard	mw ¹	chemical formula	CAS number	purity (%) ²
(+)catechin	290.08	C ₁₅ H ₁₄ O ₆	154-23-4	≥ 98
(-)epicatechin	290.08	C ₁₅ H ₁₄ O ₆	490-46-0	> 99
(-)gallo catechin	306.07	C ₁₅ H ₁₄ O ₇	3371-27-5	> 99
(-)epigallocatechin	306.07	C ₁₅ H ₁₄ O ₇	970-74-1	≥ 98
(-)catechin gallate	442.09	C ₂₂ H ₁₈ O ₁₀	130405-40-2	≥ 98
(-)epicatechin gallate	442.09	C ₂₂ H ₁₈ O ₁₀	1257-08-5	≥ 98
(-)gallo catechin gallate	458.08	C ₂₂ H ₁₈ O ₁₁	4233-96-9	≥ 98
(-)epigallocatechin gallate	458.08	C ₂₂ H ₁₈ O ₁₁	989-51-5	≥ 98
procyanidin B1	578.14	C ₃₀ H ₂₆ O ₁₂	20315-25-3	≥ 90
procyanidin B2	578.14	C ₃₀ H ₂₆ O ₁₂	29106-49-8	≥ 90
procyanidin C1	866.21	C ₄₅ H ₃₈ O ₁₈	37064-30-5	≥ 75

¹ monoisotopic mass in Dalton (Da)

² as communicated by producer

Tab. 2: Content of condensed tannins and total phenols for the plant extracts in g kg⁻¹.

extract	condensed tannins ¹	total phenols ²
quebracho (<i>Schinopsis lorentzii</i>)	122.7	~1000 ³
mimosa (<i>Acacia mearnsii</i>)	235.4	~1000 ³
tara (<i>Caesalpinia spinosa</i>)	4.6	878.6
gambier (<i>Uncaria gambir</i>)	43.1	675.1

¹ determined by butanol/HCL method (TERRILL et al., 1992)

² determined by Folin-Ciocalteu method (SINGLETON and ROSSI, 1965)

³ Amounts exceeding 1000 g kg⁻¹, resulting from the extrapolation, were rounded down.

Accounting for the existing literature, especially the CT measurement of quebracho turned out very low (ROBBINS et al., 1991; BENCHAR and CHOUINARD, 2009). But it seems that in most studies, the CT content of incorporated extracts was not actually measured, but instead taken from other literature or communicated by the providing industrial company (for example EL-WAZIRY et al., 2007; VASTA et al., 2009). Even with analyses within the given study, the numbers range widely, most likely depending on the method (BEAUCHEMIN et al., 2007; BUENO et al., 2008); in the latter study, quebracho extract resulted in similar CT values as the present experiment.

Several studies suggest a reduced color development of quebracho tannin with butanol/HCl method, because of a resistance of its bonds against acid cleavage (LI et al., 2010; HATTAS and JULKUNEN-TIITTO, 2012). Measuring quebracho tannin content with a general standard like catechin would therefore cause underestimation (VIVAS et al., 2004). Therefore, it would cause overestimation of other tannin sources, if used as a standard itself.

For mimosa, higher amounts of CT than in the present experiment (CARULLA et al., 2005; GRAINGER et al., 2009) as well as lower values (BUENO et al., 2008) were found in previous studies. Again, there are experiments relying only on comparatively high data provided by the supplier (MAX, 2010; BELTRAN-HEREDIA et al., 2011). One measurement of total phenolics of steam-extracted mimosa was found, which was 253 g kg⁻¹ under optimized conditions (DUAN et al., 2005) and equal to only condensed tannin content in the present study.

As expected of a source of hydrolyzable rather than condensed tannins, measurements of tara resulted in quite low numbers. There are not many studies to be found on gambier, but measurements of total phenols are roughly comparable to previous findings (KASSIM et al., 2011; TONG et al., 2011).

Even though the butanol/HCl assay can be regarded as standard procedure in tannin measurement, it is known that specificity of different tannins to this method can vary (HEDQVIST et al., 2000; HATTAS and JULKUNEN-TIITTO, 2012). Apparently, even the inventors of the butanol/HCl method (SWAIN and HILLIS, 1959) noticed variations in the results when measuring different tannins. Logically, certain tannins are over- or underestimated and therefore results not overall comparable. SWAIN and HILLIS (1959) assumed the degeneration of the sample to be accelerated by light; therefore reaction tubes should be kept dark. No evidence on that could be found by PORTER et al. (1986), but they observed changes with temperature, age of the preparation, oxygen and water content. The best solution would be to standardize the conditions as much as possible as was tried in the present study. After optimization, the results were stable and repeatable for all standards and extracts.

The four selected extracts contain varying amounts of condensed tannins and total phenols. Photometrical analysis is a fast and cheap

way to determine phenolic content, but the method has restrictions. A major problem consists in the lack of structural information, which makes the choice of a suitable standard impossible.

HPLC-MS analyses

Standards

All standards showed high detection sensitivity and were distinguishable in their respective retention time. An adequate separation was accomplished (Fig. 1).

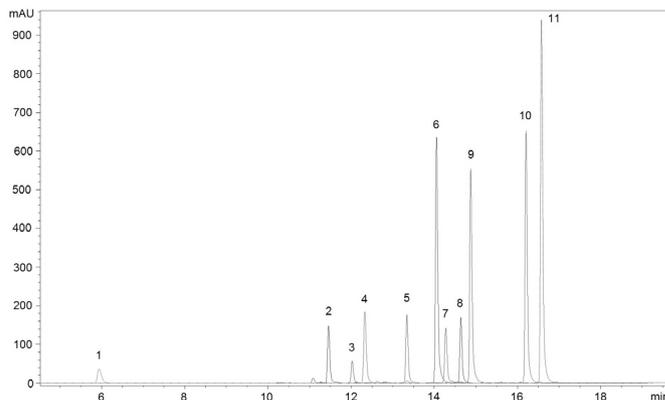


Fig. 1: HPLC separation of all standards detected at 280 nm (regression curves achieve a coefficient of variance of $R^2 = 0.9995-0.9999$; 1 gallic acid, 2 procyanidin B1, 3 epigallocatechin, 4 catechin, 5 procyanidin B2, 6 epigallocatechin gallate, 7 procyanidin C1, 8 epicatechin, 9 gallic acid gallate, 10 epicatechin gallate, 11 catechin gallate).

All purchased standards had very similar UV/VIS spectra with a maximum at 280 nm. This similarity among tannin UV spectra was already observed before (PUTMAN and BUTLER, 1989; REDEUIL et al., 2009). Therefore, these compounds could only be reliably identified through their retention time and MS spectrum.

Procyanidin B1, B2, and C1 were quite unstable in the ion trap spectrometer. Therefore, adjustments were performed before starting the sample analyses to optimize the sensitivity. In order to reduce fragmentation, the voltage settings were optimized by using the automated function of the MS program.

Tab. 3 lists the typical masses for each standard as they occurred during normal analysis and in fragmentation of the base ion.

Method development

HPLC-MS settings

Negative ionization mode was selected for mass spectrometry, because prior tests revealed a higher signal intensity and more discrete peaks. CIRIC et al. (2012) suggested, this is caused by hydroxyl groups which are predetermined to lose a proton. Formation of complex adducts (SOONG and BARLOW, 2005), reduced sensitivity (SIMIRGIOTIS et al., 2012), and higher fragmentation (VENTER et al., 2012a) in positive mode were also reported. REDEUIL et al. (2009) found advantages to the negative mode as well as to the use of ESI instead of atmospheric pressure chemical ionization (APCI) source. They also suggested to minimize peak tailing by acidifying the methanol eluent with 0.1 % formic acid, which was also found useful in the present experiment. There are some studies preferring positive mode, reportedly due to sensitivity (LIU et al., 2010) and for adduct formation with cations (JANKOVIC et al., 2010), respectively. In general, positive mode is used primarily to detect anthocyanins and

Tab. 3: Tannin standards with their respective ions as they occurred in MS in positive $[M-H]^+$ and negative mode $[M-H]^-$ as well as in $-MS^2$ mode of the main ion; most prominent peaks are bold (for mw see Tab. 1).

tannin standard ^x	positive mode	negative mode	$-MS^2$
(+)catechin	139.3, 291.1	289.0	N/A
(-)epicatechin	139.3, 291.1 , 313.0 , 602.9	288.9 , 578.8	137.2, 179.0, 202.9, 205.0, 244.9, 288.9 , 1120.6
(-)gallocatechin	139.3, 307.0	305.0 , 610.9	125.2, 137.1, 139.2, 161.1, 164.0, 165.1, 1179.1, 204.0, 219.0, 221.0, 261.0, 304.9
(-)epigallocatechin	139.3, 307.1 , 329.0, 634.9	305.0 , 610.9	N/A
(-)catechin gallate	123.4, 273.1, 443.0 , 465.0	441.0	N/A
(-)epicatechin gallate	123.3, 273.1, 443.0 , 465.0	441.0	N/A
(-)gallocatechin gallate	139.3, 289.1, 459.0 , 481.0	457.0 , 571.0	N/A
(-)epigallocatechin gallate	139.3, 289.1, 459.0 , 481.0	457.0 , 570.9	N/A
procyanidin B1	127.3, 163.2, 289.1, 579.0 , 601.1, 1179.2 , 1195.0	289.0, 425.0, 577.1 , 599.0	125.2, 161.1, 245.0, 287.0, 288.0, 289.0, 290.0, 299.0, 407.0, 408.0, 425.0, 426.0, 427.0, 451.0, 451.9, 533.1, 559.0, 560.0, 561.0, 577.0, 577.8
procyanidin B2	139.3, 289.1, 291.1 , 579.0	289.0, 425.0, 577.1	125.2, 245.0, 287.0, 289.0, 299.0, 407.0, 425.0 , 426.0, 451.0, 559.0, 560.0, 577.0
procyanidin C1	123.4, 127.3, 139.3 , 163.2, 271.1, 289.1, 291.1 , 579.0	865.2	286.9, 413.0, 422.9, 450.9, 533.0, 575.1, 576.1, 577.0, 578.0, 695.0, 713.1, 714.0, 739.0, 847.0, 864.9

^x solution of 1 mg in 10 ml; 5 µl injected twice; except for fragmentation, a threshold of 20 abundances was decided

N/A not available

negative mode to detect flavonols, phenolic acids, and flavan-3-ols (GAVRILOVA et al., 2011).

Although there are experiments using normal-phase for HPLC analyses (WHITE et al., 2010; WALLACE and GIUSTI, 2010), most research is done by reversed-phase method (e.g. VIVAS et al., 2004; REDEUIL et al., 2009; CAM and AABY, 2010). In comparison, normal-phase was found to give a better separation ordered by degree of polymerization (RIGAUD et al., 1993). But applying reversed-phase, increased elution time is also needed with higher molecular weight (PUTMAN and BUTLER, 1989).

Complete peak separation could not always be achieved with the tested extracts, because of the high number of tannin peaks. Especially with quebracho and mimosa, an elevation in the HPLC baseline was observed, as already reported before (KARONEN et al., 2004; FRANCISCO and RESURRECCION, 2009). Peak broadening may also be caused by rotational isomerism, were the units slowly rotate around the interflavonoid bond at room temperature (ESATBEYOGLU et al., 2010).

Determination of degree of polymerization was not possible with the available equipment, since it was not possible to assign a peak with several polymer masses to a specific polymer size. It is likely that oligomers were formed during handling due to oxidative polymerization or on-column reactions (PUTMAN and BUTLER, 1989) as well as higher polymers may have been changed in their structure during the process (ESATBEYOGLU et al., 2010). A determination would be possible using matrix-assisted laser desorption/ionization (PASCH et al., 2001) or nuclear magnetic resonance spectroscopy (THOMPSON and PIZZI, 1995), for example.

Extraction methods

The methods selected for the present study to find the best approach for tannin extraction from the given material were based partly on literature. The extraction process was performed only once, because even though repeated extraction does increase tannin isolation (FRANCISCO and RESURRECCION, 2009; ESATBEYOGLU et al., 2010),

at the same time this dilutes the solution and requires a further step of concentration for adequate analysis (TOMAS-BARBERAN et al., 2001).

Extraction experiments showed that temperature, time, and solvent to sample ratio had much more influence on the tannin isolation efficiency than the applied technique (ultrasonic treatment, stirring, shaking, soaking) when extracting with hot water (CAM and AABY, 2010). They found that extraction for longer than 120 min did not increase tannin isolation.

Acetone seems to be quite established in terms of tannin research (SINGH et al., 2009; GAVRILOVA et al., 2011), regularly as acetone:water (7:3) (KARONEN et al., 2004; SIVAKUMARAN et al., 2006; ESATBEYOGLU et al., 2010) with different kinds of further purification. Some researchers added acids as well (HOSSEINIAN et al., 2007; WHITE et al., 2010). The second most regularly used tannin solvent is methanol, either with (XU et al., 2012) or without acid addition (SANZ et al., 2010). It is possible that methanol extraction results mainly in mono- and dimers while acetone extraction yields higher polymers by trend. Acetone extracts can have a lower antioxidant activity (HOSSEINIAN et al., 2007). But there are also contrary results, for example SARNOSKI et al. (2012) hardly found any influence when comparing tannin extraction of different solvents. As known from the domestic use of tea, phenols are resolvable in hot water, and there are studies that established the suitability for laboratory use (OMAR et al., 2011; SIMIRGIOTIS et al., 2012). So the use of water for tannin extraction could provide an easy and environmentally friendly way to investigate tannin rich plants.

Tab. 4 shows the tannin content as quantified after three different extraction methods for the four plant species. Hot water extraction provided higher values than extraction with methanol or acetone ($P < 0.005$). For mimosa and gambier, no differences were observed between extraction with methanol and acetone, while for quebracho and tara acetone extraction yielded in smaller values ($P < 0.0001$).

The adoption of methanol or acetone in the preparation process resulted in noticeable increase of interferences in the UV/VIS spectra, rendering tannin identification more difficult. The utilization of ace-

Tab. 4: Tannin content of the extracts in g kg^{-1} by HPLC-MS analysis with different extraction methods, quantification based on procyanidin C1 (C1) and epigallocatechin gallate (EG), respectively (standard error = 1.3).

plant species	hot water	water:methanol ¹	acetone:water:formic acid ²
quebracho (C1)	164.3 ^{aB}	152.4 ^{bB}	139.1 ^{cC}
mimosa (C1)	108.2 ^{aC}	101.9 ^{bC}	98.2 ^{bD}
tara (EG)	647.5 ^{aA}	633.6 ^{bA}	572.9 ^{cA}
gambier (C1)	169.3 ^{aB}	154.3 ^{bB}	152.9 ^{bB}

¹ water:methanol (1:1)

² acetone:water:formic acid (70:29.5:0.5)

a,b different small letters indicate significant differences between methods within one extract ($P < 0.005$)

A,B different capital letters indicate significant differences between extracts within one method ($P < 0.005$)

tone also caused a disturbing peak at 2.5 min, overlapping several others and therefore contributing to an underestimation of the tannin content.

Ultrasonic treatment was found to be a valuable part of the preparation process, suspending the weighed extract in the provided solution optimally and probably releasing those tannins bound to cellulose (which is often used as a carrier in spray drying). The advantage of ultrasound was also proven by SIVAKUMAR et al. (2009).

In the process of method development, test samples of quebracho and tara solutions and residue from the acetone extraction were freeze-dried and weighed to see, how much of the sample would actually dissolve. The fraction dissolvable in acetone:water:formic acid was around 85 % for quebracho samples and 74 to 80 % for

tara. Overall recovery of the weighed sample was 97 to 100 %. The freeze-dried centrifugation residue was sticky, most likely because of sugars (GALVEZ et al., 1997).

Fig. 2 presents the MS spectra of all investigated extracts obtained by hot water extraction. Differences to methanol and acetone extraction as well as tannin structures are discussed below separately for the respective tannin sources. Generally, hot water method proved to possess advantages over the use of organic solvents. The industrial extracts were not completely soluble, most likely because of carrier substances like cellulose.

Quebracho

Tannin structure

Contrary to previous reports (VIVAS et al., 2004), quebracho does not contain robinetinidol, but ent-fisetinidol-4 β -ol (negat. mod., m/z 289), which has been identified as monomer quebracho tannin (VENTER et al., 2012a). There are great variations regarding the average molecular weight of quebracho tannin from 939 (VENTER et al., 2012a) and 1846 (THOMPSON and PIZZI, 1995) to 2800 (MAKINO et al., 2011). With scans up to m/z 1800, only quebracho tannins from monomer to hexamer were detectable in the present study. VENTER et al. (2012a) suggested that the majority of compounds would be in this range. They calculated a mean degree of polymerization (mDP) of 4.8 in conformation with previous results obtained by time-of-flight mass spectrometry and thioacidolysis (VIVAS et al., 2004).

There are only few studies measuring the tannin content of quebracho with HPLC, as this normally seems to be a structural or preparative approach. Tannin content as reported in the literature can roughly be compared to the current findings in quebracho (MAKINO et al., 2011; VENTER et al., 2012a).

Quebracho extract was found to contain previously reported monomers as well as oligomers and polymers (detectable up to hexamer)

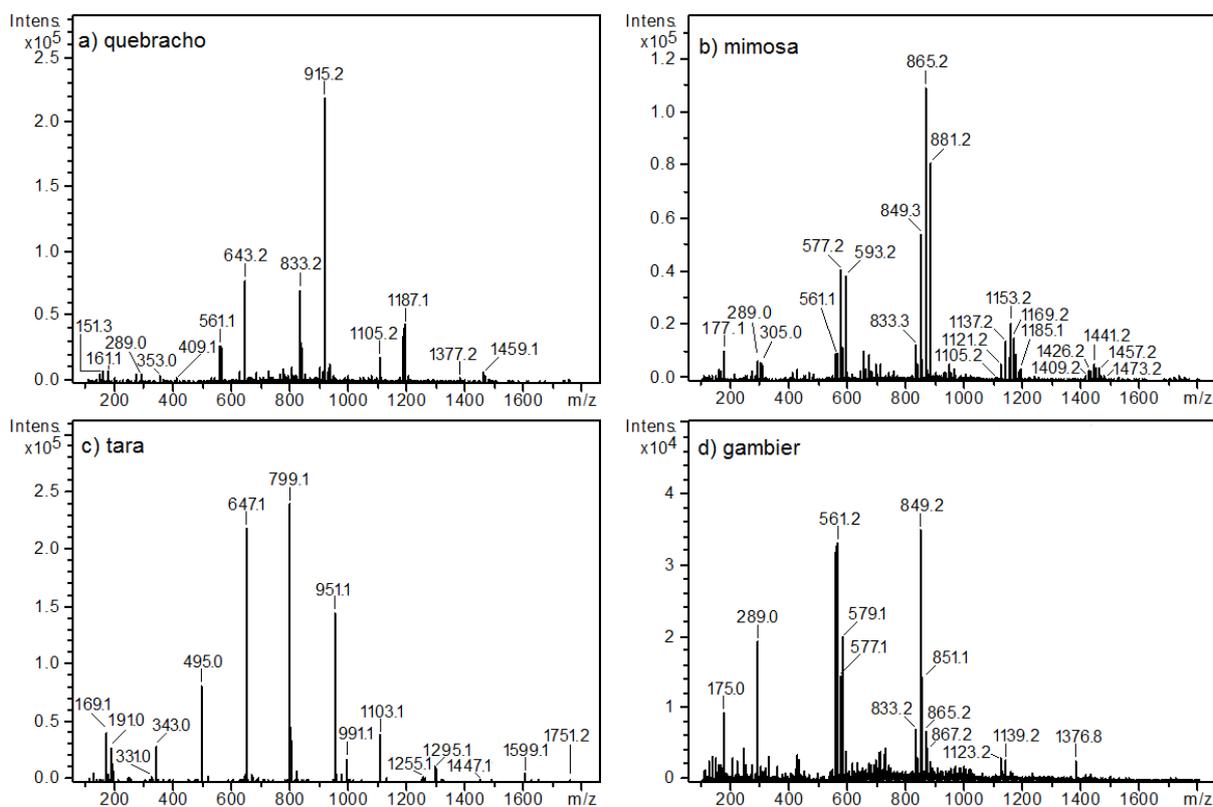


Fig. 2: Mass spectra of quebracho, mimosa, tara, and gambier extracts (hot water method) in negative mode ($[M - H]^-$).

(VENTER et al., 2012a). Even though in comparatively low intensities, all of them were detectable in several peaks in the present analyses; this might indicate them to be fragments of greater polymers. The monomer appeared only in very low intensity, while dimer (m/z 561) and trimer masses (m/z 833) resulted in considerably higher mass peaks. Masses of tetramer (m/z 1105) and pentamer (m/z 1377) appeared in intensities equal to the monomer, but further on in the analysis run (see Tab. 5). The hexamer mass (m/z 1649) was difficult to find. According to VENTER et al. (2012a), m/z 1667 and 1668 may indicate the hexamer via its ^{12}C and ^{13}C water adduct, respectively. At close inspection of a magnified MS spectrum, a peak at m/z 1667 is always detectable going along with m/z 1668 showing almost the same intensity.

In contrast, a prominent m/z value at 915 appeared from about 2 min up to nearly 17 min in the chromatogram with very high intensity. The UV/VIS signal shows the characteristic spectrum of a tannin (see Fig. 3).

Fragmentation of several quebracho tannin masses (see Fig. 2) was performed in negative mode with direct injection (without any HPLC separation) using a Cole Parmer 74900 series single syringe pump (Novodirect GmbH, Kehl/Rhein, Germany). With quebracho hot water extract MS^2 of m/z 561 was performed and the signals were

Tab. 5: Retention times of UV/VIS peaks of quebracho (hot water extraction) at 280 nm, representing over 1 % of the tannin content, and corresponding masses in $[M - H]^-$ (most prominent masses are bold).

retention time in min	prominent masses $[M - H]^-$	% of total tannin amount
1.3	161 ^a	6.99
1.6	561	1.37
1.7	181 ^x	2.20
2.1	161 ^a , 201 ^b , 353	2.70
3.2	775 ^x	17.58
6.8	289, 643 , 915	1.02
10.5	643 , 915	1.63
12.0	561, 643 , 915 , 1187	1.88
12.8	409, 561, 643	3.29
13.3	561, 643 , 915 , 1187	1.28
13.5	561, 643 , 915 , 1187	3.06
13.9	561, 643 , 915	1.68
14.1	561, 643 , 915 , 1187	1.51
14.3	561, 643 , 833, 915 , 1105, 1187	1.40
14.4	561, 643 , 833, 915 , 1105, 1187	1.47
14.7	643 , 833, 915 , 1105, 1187	2.66
14.9	561, 833, 915 , 1105, 1187	7.50
15.3	561, 643 , 833, 915 , 1105, 1187	6.10
15.8	289, 561, 833, 915 , 1105, 1187	1.13
16.3	833 , 1667/8 ^y	4.88
16.5	289, 561, 833, 915 , 1105, 1187, 1667/8 ^y	1.58
17.2	561, 833 , 915, 1105, 1187, 1377	3.31
17.5	833 , 915, 1105	3.04
17.7	833 , 1105, 1187, 1377, 1649, 1667/8 ^y	1.22
17.9	833 , 915, 1105, 1187, 1649	1.01
18.0	833 , 915, 1105, 1377, 1667/8 ^y	2.03

^a m/z described by VENTER et al. (2012a) and VENTER et al. (2012b)

^b m/z described by VENTER et al. (2012b)

^x undescribed m/z value

^y m/z 1667 and 1668 always appeared together in similar intensities

quite stable; 557, 409, and 289 appeared as fragments. After optimization of parameters, MS^2 of m/z 833 resulted in the following fragments with decreasing intensity: 681, 561, 529, 409, 289; m/z 915 was also present. With m/z 1105, fragmentation at normal scan range was extremely low in intensity and very unstable. With a scan range of m/z 800 to 1150, at least the masses 833 and 915 appeared as fragments.

MS^2 of m/z 915 appeared to be very unstable, which could be slightly improved by doubling the accumulation time to 400 ms. Fragments included the masses 833, 681, 561, 409, 289 and therefore confirmed this compound to be a derivate of quebracho tannin. This result was verified by analyses of VENTER et al. (2012b), identifying this compound as sulfited trimer. Sulfitation is usually performed by the industry to make quebracho extract soluble in cold water and also to enhance its performance in leather tanning.

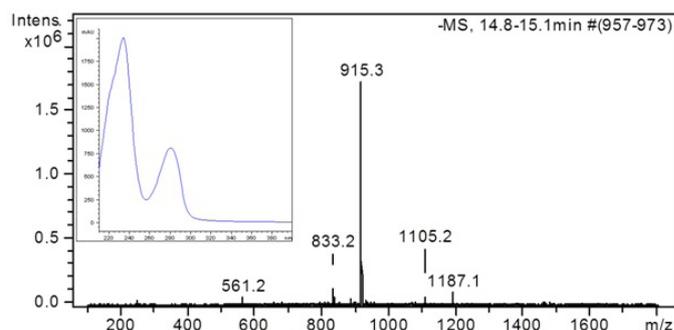


Fig. 3: MS spectrum of industrial quebracho extract (hot water extraction) at 15 min with corresponding UV spectrum.

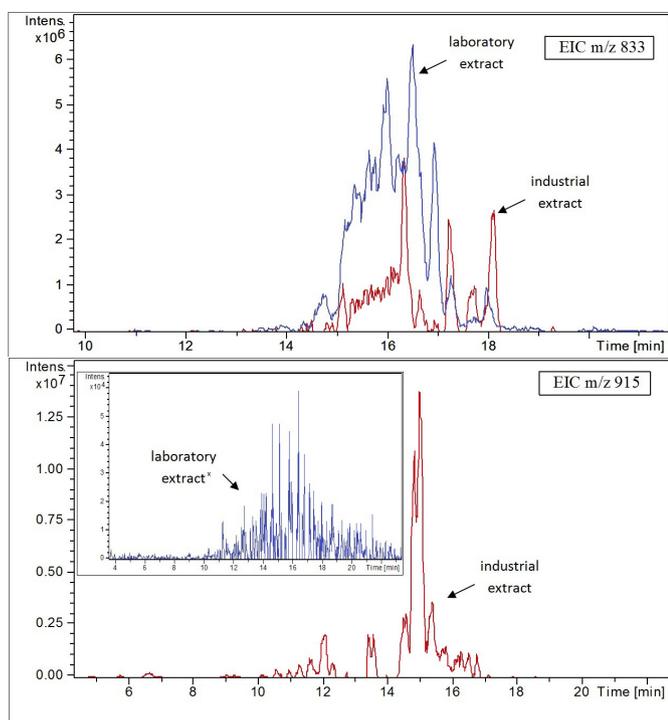
In the context of sulfitation products, m/z 643 (sulfited dimer) could be found in the present quebracho spectra; the mass mainly appeared together with m/z 561. The intensity in which the sulfited tetramer mass (m/z 1187) occurred was very low and comparable to the pure tetramer mass in retention times and intensity. While these compounds were sulfited by C-2 sulfitation, m/z 353 also appeared as C-4 sulfited monomer. The double-sulfited byproduct with m/z 435 suggested by VENTER et al. (2012b) could not be detected in the present study.

The laboratory quebracho hot water extract displayed very distinctive peaks in UV/VIS and MS (Fig. 4), portraying quebracho tannins from monomer up to hexamer with increased intensity compared to the industrial extract. Furthermore, the background in the MS seemed to be reduced in the laboratory extract. As expected, m/z 353, 643, 915, and 1187 did not rise remarkably above the normal background. Neither in the industrial nor the laboratory quebracho extract, fisetinidol (m/z 273) or sulfited robinetinidol (m/z 369) could be identified.

Several tannin fragments mentioned by Venter and coworkers (VENTER et al., 2012a, b) could be detected both in industrial as well as laboratory quebracho extract, including m/z 151, which was described as undetectable before. Intensities were relatively low, but the industrial extract generally showed higher signals (except for m/z 409), probably indicating increased fragmentation because of the acid treatment.

Extraction methods

Quebracho tannin extraction from the industrial quebracho with hot water resulted in higher intensities for oligomers and polymers compared to methanol or acetone treatment in manually integrated MS spectra. This could be indicating hot water treatment as a more gentle method. With the laboratory wood extract, a comparable pat-



* no distinct peaks for m/z 915 in laboratory extract

Fig. 4: Extracted ion chromatogram (EIC) of unsulfited (m/z 833) and sulfited trimer (m/z 915) for industrial (red) and laboratory (blue) quebracho extract.

tern was obtained after freeze-drying.

Before freeze-drying, acetone treatment resulted in the highest recovery of tannins with 7.12 % tannin in quebracho wood compared to hot water (5.71 %) and methanol extraction (5.16 %). After the extracts were freeze-dried and the powder was redissolved for analysis, different results were obtained. Freeze-dried hot water wood extract resulted in 19.11 % tannin, which was remarkably higher than results of methanol (15.37 %) or acetone treatment (14.82 %).

Mimosa

Tannin structure

Fewer studies than for quebracho were found as a useful basis for interpretation of the other extracts. Several *Acacia* species were accessed with HPLC-MS resulting in tannin contents comparable to the present study (HATTAS et al., 2011). There are tannin values which are considerably higher for mimosa, but resulting from other methodical approaches (LIAO and SHI, 2005; MAKINO et al., 2011). DREWES et al. (1966) indicated the mimosa monomer to be leucorobinetinidin (m/z 289), coupled either with catechin (dimer m/z 577) or gallo catechin (dimer m/z 593). This was confirmed by MAKINO et al. (2011). Apart from robinetinidol, fisetinidol units were also found to be attached to catechin (dimer m/z 561) (CRONJE et al., 1993; DUAN et al., 2005). The presence of these substances was confirmed by own measurements.

The dimeric components with a mass difference of 16 were somehow mirrored at m/z 833, 849, and 865 as well as at m/z 1137, 1153, and 1169, indicating the according trimers and tetramers. To the trimeric group, m/z 881 was added, containing one gallo catechin and two robinetinidol units. The tetrameric group was completed by m/z 1105, 1121, and 1185. Compounds like these were described before for mimosa heartwood (VIVIERS et al., 1982; YOUNG et al., 1985) and VENTER et al. (2012c) recently confirmed this grouping structure. The referred pentamers (m/z 1409, 1425, 1441, 1457,

1473, and 1495) occurred sporadically with low intensities in the tested mimosa extract, but as to be seen in Fig. 2, their amount adds up noticeably. Undecamer masses, found in the formerly mentioned study, did not rise significantly above background level. All fragments mentioned by VENTER et al. (2012c) could be seen in the present mimosa mass spectra.

A monomeric gallo catechin peak at 6 min (m/z 305) was found in low intensity, as well as the monomer unit m/z 289, which may be catechin or robinetinidol as suggested by VENTER et al. (2012c). A robinetinidol fragment at m/z 287 could also be observed. The fisetinidol monomer as well as the respective fisetinidol-containing oligomers were detected in low intensities, confirming the previous observations of their low proportion. While dimers and trimer were generally the most distinct peaks with high intensities, the signal intensity decreased with higher oligomers. Therefore, the general understanding of tannin compounds in mimosa rather being of lower molecular weight (PIZZI and STEPHANOU, 1993; MAKINO et al., 2011; VENTER et al., 2012c), can also be confirmed in the present study. Estimates for the average molecular weight are diverse, but with a range from 1284-1507 (ROUX and EVELYN, 1958) and 1343-1406 (THOMPSON and PIZZI, 1995) to 3200 (MAKINO et al., 2011) higher than for quebracho. Because robinetinidol has one additional OH group available for hydrogen bonding compared to fisetinidol, VENTER et al. (2012a) suspected it to be the reason for a better cold water solubility.

Extraction methods

DUAN et al. (2005) evaluated steaming methods for mimosa extraction and found that too high steaming temperatures or too much water can reduce isolation efficiency. Only total phenol content was observed for the optimization, so there is no information regarding influences on single tannins.

In the present study, no notable differences in mass intensity could be found for m/z 289, 305, 593, and 865 between the applied extraction methods. However, for m/z 561 and 849 the hot water method resulted in slightly higher intensity. Hot water and acetone extraction caused higher signal intensity of m/z 577 and 833 compared to the methanol extraction.

Tara

Tannin structure

To our knowledge, HPLC for estimation of tara tannin content was performed for the first time. Some data resulting from other methods state 59.7 and 25.5 % tannin in the extract, respectively (GALVEZ et al., 1997). PELLIKAN et al. (2011) relied on producer data reading 95.1 % tannin. Concluding, the present tara tannin content can be called plausible. Reported average molecular masses vary from 593 over 860 to 1736 (DEAVILLE et al., 2007; MANE et al., 2007), depending on the method.

Most hydrolyzable tannins contain sugar as a core unit, but tara tannins are based on quinic acid instead (HASLAM et al., 1962; GALVEZ et al., 1997). Quinic acid linked with up to eight galloyl moieties was identified as tara tannin (MANE et al., 2007), whereas depsidic and aliphatic linkage can appear together (CLIFFORD et al., 2007).

Tara tannins from one to eight galloyl moieties were detected in the present study, while 3-galloylquinic and 4-galloylquinic acids (m/z 647, 799) were the most abundant, which confirms previous studies (CLIFFORD et al., 2007). Single quinic acid (m/z 191), gallic acid (m/z 169), digallic acid (m/z 321), and glucogallin (m/z 331) were also observed in accordance with previous findings (HORLER and NURSTEN, 1961; HASLAM et al., 1962) in several peaks during the first half of the analysis run. The peaks partly corresponded with

galloylquinic and 2-galloylquinic acid (m/z 343, 647). With the ongoing analysis run, the smaller masses gradually disappeared, while 3-galloylquinic and 4-galloylquinic acid appeared in noticeably stronger intensity than the smaller tannins. With a retention time of about 15 min, 5-galloylquinic acid (m/z 951) appeared and reached its maximum at 16.5 min. With a delay of 0.5 min each, 6-, 7-, and 8-galloylquinic acid (m/z 1103, 1255, 1407) followed in exponentially decreasing intensity.

Alongside with these formerly described masses, corresponding well with 3-galloylquinic acid, unknown masses were detected at m/z 1295, 1447, and 1599 in minor intensity. Apparently, they continued the mass difference of 152, strongly suggesting the addition of galloyl moieties. Together with m/z 1599 the fragment m/z 1751 was observed, barely above the normal background, but consistent with all m/z 1599 peaks. Since these masses are not described for tara in the literature, it is assumed that these may be compounds containing two quinic acids in the molecule, because of the obvious mass difference of 192 between 6-galloylquinic acid and the smallest unknown compound.

Extraction methods

The extraction method had diverse effects on tara. While acetone treatment resulted in severe reduction of m/z 169, 191, 343, and 1295 compared to the hot water method; signals of m/z 1103, 1255, and 1407 increased noticeably. Methanol had a negative impact on all larger masses and m/z 169, except for m/z 1447, which increased compared to water.

Gambier

Tannin structure

Literature is inconsistent regarding the tannin content in gambier measured by HPLC. Catechin values from 25 % (SUHARSO et al., 2011) to 42–87 % are reported, depending on the solvent (KASSIM et al., 2011; TONG et al., 2011). No value for total tannin content could be found, but gambier is supposed to contain a high proportion of monomers anyway (PIZZI and STEPHANOU, 1993). All numbers are above of what was determined in the present study, which could be attributed to different sources.

NONAKA and NISHIOKA (1980) found gambiririin A1, A2, A3 (m/z 579), B1, B2, B3, and C (m/z 561) to be the major constituents of gambier tannins. Others claimed gambier tannin to consist mainly of catechin (m/z 289), with small amounts of galocatechin (m/z 305) and epigallocatechin gallate (m/z 457) (HUSSIN and KASSIM, 2011; KASSIM et al., 2011). TANIGUCHI et al. (2007b) as well as TANIGUCHI et al. (2008) corrected the assumptions on the isomeric structure of gambiririin tannins, added procyanidin B1 and B3 (both m/z 577) and gambiririin D3 (m/z 561), D4, D5 (m/z 579) to the list of constituents, and proved that these dimers and chalcane-flavan dimers formed out of catechin. EVELYN et al. (1960) suggested that 1530 may be the highest molecular weight occurring in gambier tannins, which corresponds with other findings of 502 as average molecular weight (THOMPSON and PIZZI, 1995). Therefore, gambier can be expected to mainly consist of tannins with smaller molecular weight.

All masses described above were found in the analyzed gambier extract, except for epigallocatechin gallate; the mass did not show intensities above normal background. This was also the case for uncariagambiririine (m/z 619) and gambircatechol (m/z 395) identified earlier in aqueous acetone extract of gambier (YOSHIKADO et al., 2009). SUHARSO et al. (2011) also determined 12 % of quercetin (m/z 447), which could only be observed in one peak at 19 min in the present study. Corresponding to chalcane-flavan dimers, additional masses appeared at m/z 833, 849, and 851, most likely representing the corresponding trimers. Because of the mass difference of 272,

the added unit has probably another chalcane structure. Tetramers were found as demonstrated by m/z 1105 (very low), 1121, 1123, with counterparts to the unknown trimeric masses at m/z 1137 and 1139. A possible pentamer was observed at m/z 1377.

Contrary to the other extracts, with gambier higher masses showed up quite early in the analysis run. There were four comparatively intensive peaks of almost solely m/z 849 with retention times between 3.5 and 5.8 min dominating the mass spectrum. From 9 min on, monomer and oligomeric masses appeared together, with dimeric and trimeric masses clearly the most abundant.

Extraction methods

Relative to hot water, the acetone treatment resulted in higher intensities for m/z 169, 561, 579, and 1123 and methanol treatment slightly increased m/z 305 and 561. Both methanol and acetone extraction lead to lower intensities for m/z 289, 833, 849, and 1377, while only methanol reduced m/z 577.

Influence of storage

There are experiments in the literature indicating an impact of light on tannin and phenolic content of aqueous extracts (NORDSTRÖM, 1956; DUCKSTEIN and STINTZING, 2011) as well as experiments indicating that light is not an important factor (PORTER et al., 1986). Results of the present study cannot confirm the imminent loss of phenols either, even during long term exposure (Tab. 6).

Tab. 6: Tannin content of the extracts in g kg^{-1} by HPLC-MS analysis as influenced by different storage conditions, quantified based on procyanidin C1 (C1) or epigallocatechin gallate (EG) as calibration standard (standard error = 1.3).

plant species	refrigerator	room ¹	oven ²
quebracho (C1)	164.3 ^a	157.6 ^b	163.3 ^a
mimosa (C1)	108.2 ^a	103.7 ^b	106.5 ^a
tara (EG)	647.5 ^a	648.8 ^a	651.1 ^a
gambier (C1)	169.3 ^a	160.1 ^b	163.1 ^b

¹ room temperature around 20 °C and normal daylight exposure

² storage at 60 °C for 5 days

^{a,b} different small letters indicate significant differences between storage conditions within one extract ($P < 0.05$)

In some studies, great effort is put into the prevention of light reaching the sample solution (FRANCISCO and RESURRECCION, 2009). The samples of the method evaluation were stored in the (dark) refrigerator, but otherwise no precautions were taken to protect them from light. After ten days, some sample solutions were remeasured and no degradation could be observed (data not presented).

For quebracho, storage conditions did not affect fragments m/z 289 and 409. Storage in the oven caused a decrease in intensity for m/z 561, 643, and 833, while for the other observed masses, both oven and room storage had negative effects. But even though the reduction was significant for the room storage, the loss only accounted for less than 1 % of measurable tannins in over a year.

With mimosa, the decrease was even smaller. Storage conditions had no visible effect on intensities of m/z 289, 305, 593, and 833. Higher storage temperature and daylight reduced intensities of the other observed masses in the order of refrigerator < room < oven.

Storage at room temperature and daylight had no effect on tannin masses of tara, except for a slight reduction of m/z 799, 951, and 1295. Five day storage in the oven reduced m/z 343, 647, 799, and

1295, while m/z 951, 1103, 1255, and 1447 actually increased. The other masses were not affected and overall, the tannin content did not change significantly.

For gambier, m/z 169, 289, 305, 833, 849, and 1377 were reduced by room storage, and m/z 577, 579, and 1123 by room storage as well as in the oven. The dimeric mass 561 was found increased in samples stored at room temperature compared to storage in the refrigerator, as were m/z 289, 561, and 849 for storage in the oven. As the only extract observed, gambier indicated a significant (negative) effect of oven storage, but losses remained in the same small margin.

Based on these findings, stored tannin plant extracts do not necessarily need to be kept in the dark, as long as they are kept dry. Also, extracts from different sources might show different sensitivities to storage conditions. Losses at room conditions are neglectable, but for analytical purposes storage in a refrigerator is recommended. Tannin content of individual extracts may shift between different origins within the same supplier as well as between different suppliers.

Conclusions

1. The photometrical methods did not give a satisfying picture of the plant materials tested. Folin-Ciocalteu method most likely overestimated the phenol content of the extracts. With butanol/HCl method, CT content could be roughly measured, but may get over- or underestimated, depending on the standard chosen. Apart from condensed tannins there are many other phenolic compounds probably contributing to the extracts properties, which would not be taken into account by sole CT determination. Therefore, an analytical solution aiming to include all contributing compounds is recommended when estimating effects of tannin extracts on protein metabolism.

Results differed remarkably between photometrical and HPLC-MS measurements. Since HPLC-MS provides much more information on the occurring tannin structure, it is more suitable to evaluate tannin content and to detect whether or not there are reasons for over- or underestimation.

2. The tested extracts were very diverse in their tannin structure and even among the condensed tannins there is no "common monomer" on which a standardized quantification could be based. From the results of HPLC-MS it is obvious that there cannot be one single standard to accurately fit "tannins" in general. This group of plant compounds is very heterogeneous and photometrical and spectroscopical reactions differ too much between different sources. Therefore, it is very difficult to employ an universally applicable standard. The optimal way would be to isolate the plant individual tannins as an individual standard for every tannin source, but this would require a high amount of sample material and maybe conflict with the comparability of results. Nevertheless, even one plant species would provide a large number of different tannins.

3. From the presented investigations it is evident, that there is no need for the use of organic solvents to prepare industrial tannin extracts for HPLC-MS analysis. In the literature, there are many different approaches to the extraction process, often involving hazardous chemicals and several methodical steps. Hot water treatment isolated more tannin from the tested extracts than preparation with methanol or acetone and also, matrix effects seemed to lessen. It is therefore suggested as a cheap and environmentally harmless solvent.

With hot water, more of the higher oligomers and polymers could be isolated in comparison to treatment with methanol or acetone. This was observed for quebracho and less obvious for mimosa

and gambier. Tara does not seem to react accordingly, so probably hydrolyzable tannins are not influenced in the same way as condensed tannins.

- Storage conditions had only minor impact on the tannin content of plant extracts. A slight reduction under the influence of light was observed, but this did not exceed 1 % in a year. Depending on the plant source, some tannins may decrease when exposed to high temperatures (tested for 60 °C). Therefore, oven drying of material should be avoided.
- There are indications of greater quality differences between extracts obtained from different suppliers as well as different extracts from the same supplier. With regards to animal feeding management, a particular chosen extract should be evaluated carefully, but will most likely maintain quality over time from the same source.

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Address of the corresponding author:

M. Kardel, University of Kiel, Institute of Crop Science and Plant Breeding – Grass and Forage Science / Organic Agriculture, Hermann-Rodewald-Straße 9, D-24118 Kiel, Germany.
E-mail: mkardel@gfo.uni-kiel.de