PERGAMON

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Phytochemistry 62 (2003) 1159–1170

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

MALDI-TOF mass spectrometry and PSD fragmentation as means for the analysis of condensed tannins in plant leaves and needles

Anke Behrens, Nagamitsu Maie[1], Heike Knicker*, Ingrid Kögel-Knabner

Lehrstuhl für Bodenkunde, Technische Universität München, 85350 Freising-Weihenstephan, Germany

Received 20 June 2002; received in revised form 11 November 2002

Abstract

MALDI-TOF mass spectrometry and ^{13}C NMR spectroscopy were applied to unveil typical characteristics of condensed tannins of leaves and needles from willow (*Salix alba*), spruce (*Picea abies*) and beech (*Fagus sylvatica*) of three tree species that are ubiquitous in German forests and landscapes. For further evaluation, lime (*Tilia cordata*) was included. The ^{13}C NMR spectroscopy confirmed the purity of the condensed tannin fractions and the efficiency of the procedure used for their extraction. While signals representative for procyanidin units are observable in all liquid-state ^{13}C NMR spectra, resonance lines of prodelphinidin were only detected in those obtained from the condensed tannins of spruce needles and beech leaves. Typical signals in the chemical shift region between 70 and 90 ppm demonstrated the presence of stereoisomers (catechin/epicatechin; gallocatechin/ epigallocatechin). The MALDI-TOF mass spectra of the condensed tannins show signals of polymers of up to undecamers. Supporting the observations from the NMR spectroscopy, the mass spectra of the willow and lime leaf condensed tannins were identified as polymers with mainly procyanidin units, while the polymers of the spruce needle and beech leaves exhibit varying procyanidin/prodelphinidin ratios. Post source decay (PSD) fragmentation lead to a sequential loss of monomers and allowed a detailed characterization and sequencing of individual chains. In the case of the condensed tannins of lime this technique clearly excludes a pelargonidin terminal unit followed by a prodelphinidin unit, which would result in the same molecular masses as a polymer solely built up of procyanidin units.

Keywords: *Salix alba*; *Picea abies*; *Fagus sylvatica*; *Tilia cordata*; Condensed tannins; MALDI-TOF; PSD fragmentation; NMR; Procyanidin; Prodelphinidin; Pelargonidin

1. Introduction

Condensed tannins are ubiquitous phenolic compounds, representing the second most abundant natural phenolics after lignin (Hemingway and Karchesy, 1989) and the fourth most widespread components in vascular plant tissues (Hernes and Hedges, 2000). They are secondary plant metabolites occurring in the bark and foliage of most plant species, but they are also found in stems, phloem, seeds, fruits, fruit pods and wood (Zucker, 1983; Hemingway and Karchesy, 1989). They are characterized by their astringent taste and by their ability to bind N-containing organic structures such as amino acids and proteins (Zucker, 1983) as well as carbohydrates and alkaloids. These same properties are thought to be responsible for their inhibitory effect on the growth of many microorganisms (Field and Lettinga, 1992). Due to the reduced digestibility of tannin-rich food for herbivores (Zucker, 1983) those biopolymers are often discussed as an efficient protection of plants against natural enemies (Bernays et al., 1989).

Aside from this, condensed tannins are considered to play an important ecological role in the carbon and nitrogen cycling in soils (Hemingway and Karchesy, 1989; van Breemen, 1993; Waterman and Mole, 1994; Chung et al., 1998, Kuiters, 1990). After litterfall, during decomposition of foliage and needles, condensed tannins are released into the soil where they were found to inhibit the degradation of organic matter and to influence the nitrogen availability (Baldwin et al., 1983;

---

* Corresponding author. Tel.: +49-8161-71-4423; fax: +49-8161-71-4466.

E-mail address: knicker@wzw-tum.de (H. Knicker).

[1] Present address: Department of Chemistry, Florida International University, University Park, Miami, FL 33199, USA.

0031-9422/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
PII: S0031-9422(02)00660-X

Kuiters, 1990; Northup et al., 1995, 1998). This is commonly assigned to their high protein binding affinity which allows the complexation of soil enzymes and proteins and, hence, prevents the mobilization and leaching of nitrogen (Northup et al., 1995, 1998) until these complexes are subjected to degradation by ectomycorrhizal fungi (Bending and Read, 1996; Northup et al., 1998). However, little is known about the mechanisms involved in the formation of tannin-protein complexes and the structural and chemical modifications implied in entailing a lower protein-binding affinity. Hydrophobic interactions were suggested to play an important role during tannin complexation. But also characteristics of the condensed tannins such as polymer chain length, number of hydroxyl groups, and conformational structures are expected to affect their protein binding (Zucker, 1983).

One problem is that the sophisticated structural elucidation of these compounds is difficult because of their heterogeneous character. Condensed tannins are formed of flavan-3-ol monomer units which are linked together inter-molecularly by C4–C8 or C4–C6 bonds to oligomers and high molecular weight polymers (Fig. 1; Hemingway and Karchesy, 1989; Waterman and Mole, 1994; Porter, 1988, 1993). The diversity of condensed tannins is given by the structural variability of the monomer units—different hydroxylation patterns of the aromatic rings A and B, different stereochemistry at the chiral centers C2 and C3—and the distinct location and stereochemistry of the interflavanoid bond (Fig. 1). Moreover, in recent years a vast diversity of derivatives of these above mentioned main chemical features has been identified as natural products (Porter, 1988, 1993). Especially the identification of higher polymers is therefore difficult regarding the higher probability of variation. Structural characterization has been performed mainly by means of ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy and by chemical degradation of the polymers and the subsequent analysis of the monomer units (Czochanska et al., 1980; Nonaka et al., 1981, 1983; Porter et al., 1982; Newman and Porter, 1992; Preston et al., 1997). However, these analytical techniques hardly allow the determination of the polymer chain length and the chemical constitution of individual chains. Moreover, the sequential succession of monomer units in individual chains cannot be elucidated. Those specific properties were found to be essential for the comprehension of the protein binding affinity of condensed tannins (Sarni-Manchado et al., 1999).

One alternative to common analytical approaches for the characterization of macropolymers is matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS), allowing the analysis of macropolymers and revealing information about their chain lengths. With this technique fragmentation of the analyte molecules upon laser irradiation can be substantially reduced by embedding them in a light adsorbing matrix. Since its introduction (Karas et al., 1987) the use of MALDI-TOF has revealed itself as powerful method for the characterization of synthetic and natural polymers and was also recently introduced for the analysis of condensed tannins, mainly, in food science (Ohnishi-Kameyama et al., 1997; Foo et al., 2000; Krüger et al., 2000; Pasch et al. 2001; Hedquist et al., 2000).

Thus, in the present study, MALDI-TOF MS and ^{13}C NMR spectroscopy were applied to unveil typical characteristics of condensed tannins that may be responsible for their protein binding ability during the first stages of

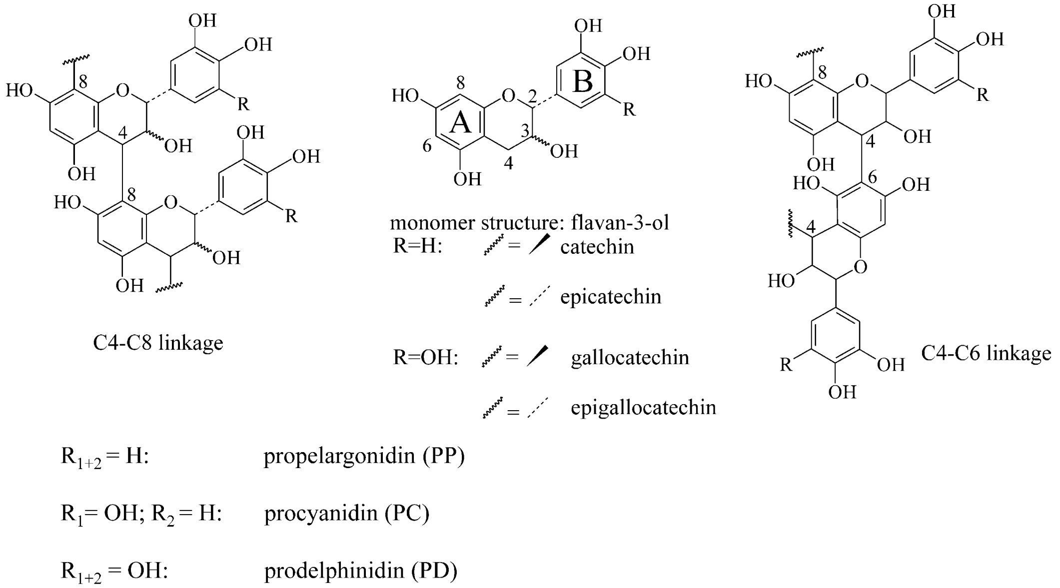

Fig. 1. Chemical structure of flavan-3-ol monomer units and condensed tannins.

soil organic matter formation. For this purpose, condensed tannins were extracted from leaves and needles of *Salix alba* (willow), *Picea abies* (spruce) and *Fagus sylvatica* (beech), representing tree species that are ubiquitous in German forests and landscapes. *Picea abies* is of particular interest, since it often grows on infertile and acidic soils where the production of condensed tannins was suggested to be vital in order to prevent nitrogen leaching and mineralization (Northup et al., 1995, 1998). Furthermore, *Tilia cordata* (lime) was included in this study to demonstrate the clear advantages of MALDI-TOF MS in revealing possible structural differences of condensed tannins from different species.

For a more specific sequential elucidation of individual chains, MALDI-TOF MS was extended to post source decay (PSD) fragmentation. This technique allows the selection of a precursor molecule in an distinct mass window and the subsequent analysis of its fragments. All ions with lower and higher masses are deflected by the electrostatic device. The precursor ions passing the mass window can spontaneously fragment on their way to the detector due to the application of higher laser energies. Up to now, MALDI-PSD has essentially been applied in peptide (Spengler, 1997; de Jong, 1998; Jespersen et al., 1999), and carbohydrate sequencing (Talbo and Mann, 1996; Harvey, 1999). As known to the authors, this technique is used for the first time with condensed tannins to help the elucidation of sequences of monomer units.

2. Results and discussion

Applying the extraction and purification scheme suggested by Preston (1999), a complete separation of the carbohydrates from the condensed tannin was achieved. Whereas the liquid-state ^{13}C NMR spectrum of the H_2O/acetone and the isolated carbohydrate fraction of the spruce needles show signals between 60 and 100 ppm that are typical for carbohydrates (Fig. 2A/B) the signals observed in this region in the liquid-state ^{13}C NMR spectrum of the purified extract are assignable to condensed tannin carbon (Fig. 2C). The amount of extracted condensed tannin was $7.6 \pm 3.7\%$ of the plant dry material. Subjecting the extraction residues to solid-state cross polarization magic angle spinning (CPMAS) ^{13}C NMR spectroscopy showed no characteristic signals of condensed tannins which indicated the efficient removal of those biopolymers by the used method (data not shown).

The liquid-state ^{13}C NMR spectrum of the purified condensed tannin fraction from *Picea abies* is depicted in Fig. 2. The signal assignment was performed according to Czochanska et al. (1980). The spectrum shows a distinct signal at 145 ppm which is assignable to C3′ and C4′ in procyanidin (PC) units (catechin/epicatechin). Their presence is supported by further signals at 115 ppm (C2′), 116 ppm (C5′) and 119 ppm (C6′). Comparable resonance lines are also observable in the liquid-state ^{13}C NMR spectra of the condensed tannins from willow and beech (Fig. 3). The resonance line at 146 ppm represents a typical indicator for the presence of prodelphinidin (PD) units (gallocatechin/epigallocatechin). It is clearly visible in the spectra of the condensed tannins from *Picea abies* (Fig. 2) and fairly distinguishable in that from *Fagus sylvatica* (Fig. 3). Using the ratio of the signal intensities at 145 ppm and at 146 ppm reveals that the spruce condensed tannins contain approximately 60% PC and 40% prodelphinidin (PD). In the case of condensed tannins extracted from beech, quantification was impossible due to signal overlapping. The absence of this signal in the spectra of the condensed tannins from willow further reveals that they are comprised of only PC monomers.

The region between 70 and 90 ppm is sensitive to the stereochemistry of the C ring. While C3 of both *cis* and *trans* isomers occurs at 73 ppm, C2 gives a resonance line at 76 ppm for the *cis* and at 84 ppm for the *trans* form. The latter is clearly visible in the spectrum of the condensed tannins from spruce (Fig. 2) indicating that here both stereoisomers (catechin/epicatechin; gallocatechin/epigallocatechin) are present.

The C3 in terminal units generally have their chemical shift around 67 ppm. Theoretically, its intensity relative to that of the signal of the C3 in extension monomer units at 73 ppm could be used for elucidating the polymer chain length. However, in the case of the spectra presented here, the signal-to-noise ratio is too low to allow for such quantification.

To obtain more detailed information on the chemical structure of the condensed tannins and to overcome the problems with determination of polymer chain lengths by NMR spectroscopy, further characterization was continued by means of MALDI-TOF MS. A crucial factor that influences the quality of MALDI-TOF mass spectra is the crystallization of the analyte during sample preparation and the behavior of the matrix during laser irradiation. Thus, α-cyano-4-hydroxycinnamic acid (HCCA), sinapinic acid (SA), dithranol, 3-β-indole acrylic acid (IAA) and dihydroxybenzoic acid (DHB) were tested as matrices. In agreement with the findings from Ohnishi-Kameyama et al. (1997) CCA, SA and dithranol did not result in reasonable spectra. These authors stated that best spectra are achieved by using DHB or IAA as matrices. Whereas DHB was reported to yield in two small signals for dimeric proanthocyanidins, IAA allowed the detection of peaks of monomeric units. Later, Foo et al. (2000) successfully applied DHB for the analysis of proanthocyanidins from cranberries and Krüger et al. (2000) used IAA for the investigation of condensed tannins from grape seed extracts. Due to

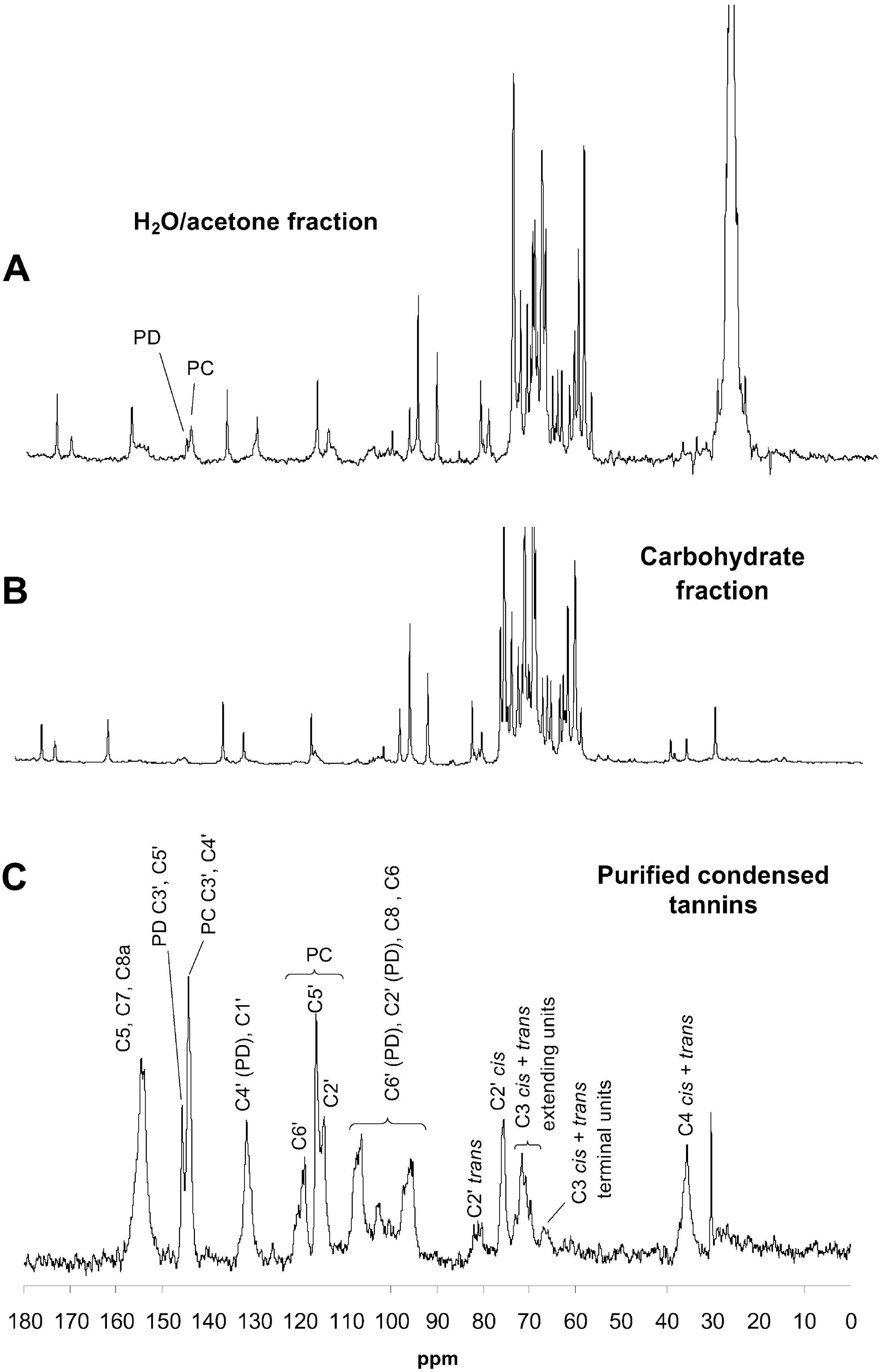

Fig. 2. Liquid-state ^{13}C NMR spectra of the H_2O/acetone fraction (A), the carbohydrate fraction (B) and the purified condensed tannin fraction (C) extracted from *Picea abies*.

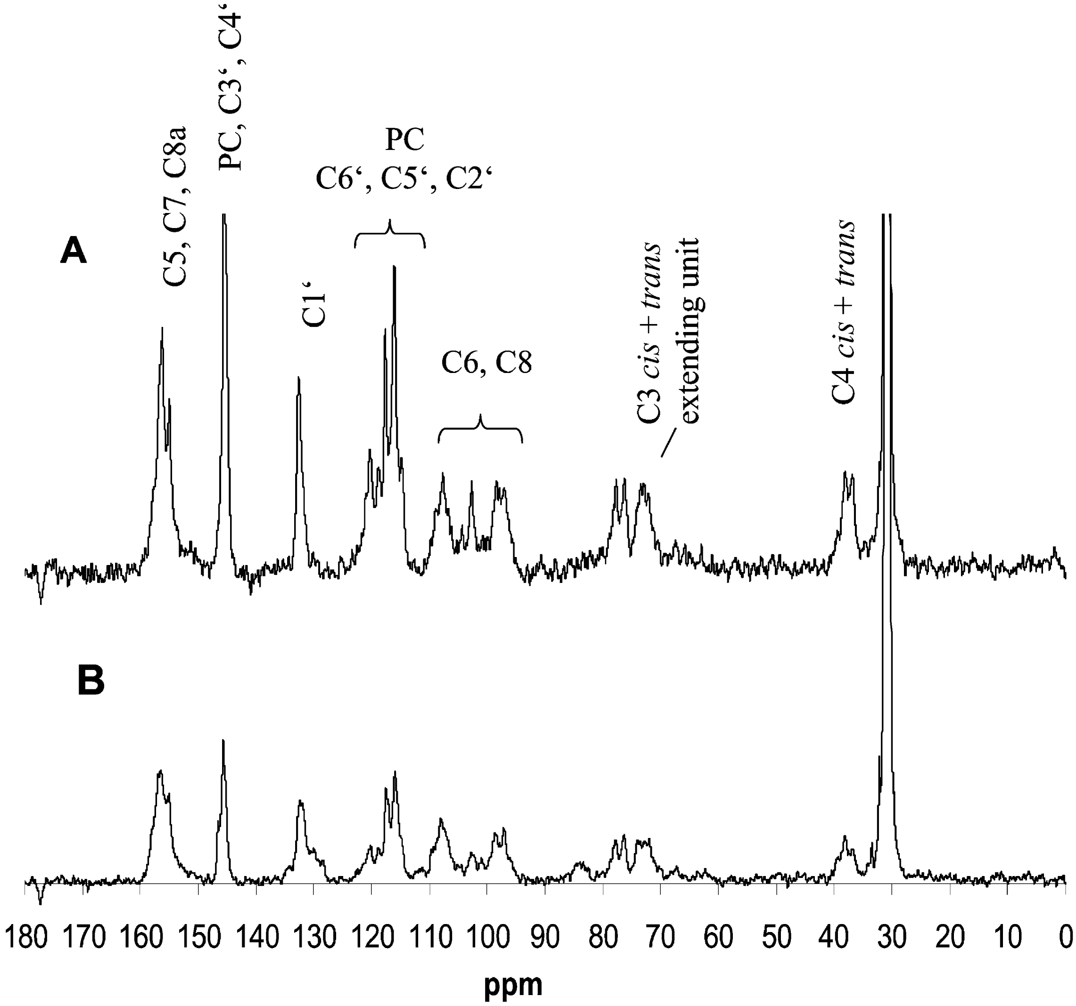

Fig. 3. Liquid-state ^{13}C NMR spectra of condensed tannins extracted from willow leaves (A, *Salix alba*) and beech leaves (B, *Fagus sylvatica*).

the fact that the best MALDI-TOF spectra of our tannin extracts were obtained with DHB, further optimization of the measurement conditions were performed with this matrix. Searching further improvement of the crystallization process and thus of the spectra quality, we tested the effect of solvent mixtures of acetone/water and methanol/water which are known to solubilize condensed tannins and which are often used for their extraction (Baldwin et al., 1983; Hagerman, 1987; Waterman and Mole, 1994; Mafongoya et al., 1998). Applying acetonitrile/water (1:1) for solubilizing the condensed tannins and the matrix allowed a homogenous sample preparation of our condensed tannins. This approach resulted in the best conditions for their MALDI-TOF analysis and resulted in mass spectra with low signal-to-noise ratios.

Figs. 4, 5 and 6 show the MALDI-TOF mass spectra of the polymeric tannin mixture of the four tree species. The included magnification demonstrate the good resolution of the spectra. Condensed tannins from lime and willow are characterized by mass spectra with a series of peaks with distances of 288 Da corresponding to a mass difference of one catechin/epicatechin between each polymer (Figs. 4 and 5). Therefore, prolongation of condensed tannins in lime and willow is due to the addition of catechin/epicatechin monomers. Given the absolute masses corresponding to each peak, it is further suggested that they contain only procyanidins, as was already indicated in the respective liquid-state ^{13}C NMR spectrum. The masses were calculated based on the Eq. 1 (Krüger et al., 2000):

$$m/z = 288\,n + 23 + a \qquad (1)$$

Here $m/z = 288$ corresponds to the molecular weight of one catechin unit, $m/z = 23$ to catechin and a to the number of 1H in the endgroups (corresponds to 2). The number of monomer units is given by n.

In contrast, the mass spectra of the spruce and beech tannins reflect higher structural complexity (Fig. 6). Here, too, the polymer character is reflected by the periodical occurrence of peak series representing different chain length (trimers, tetramers etc.). Comparing the

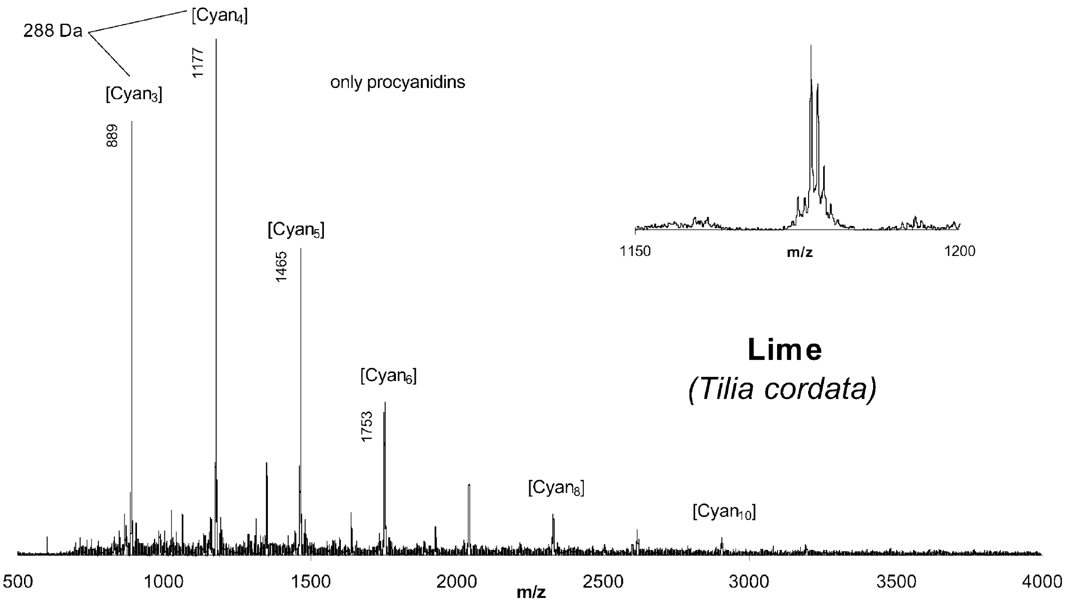

Fig. 4. MALDI-TOF mass spectra of condensed tannins extracted from lime leaves (*Tilia cordata*). Cyan$_x$ corresponds to the number of procyanidin units.

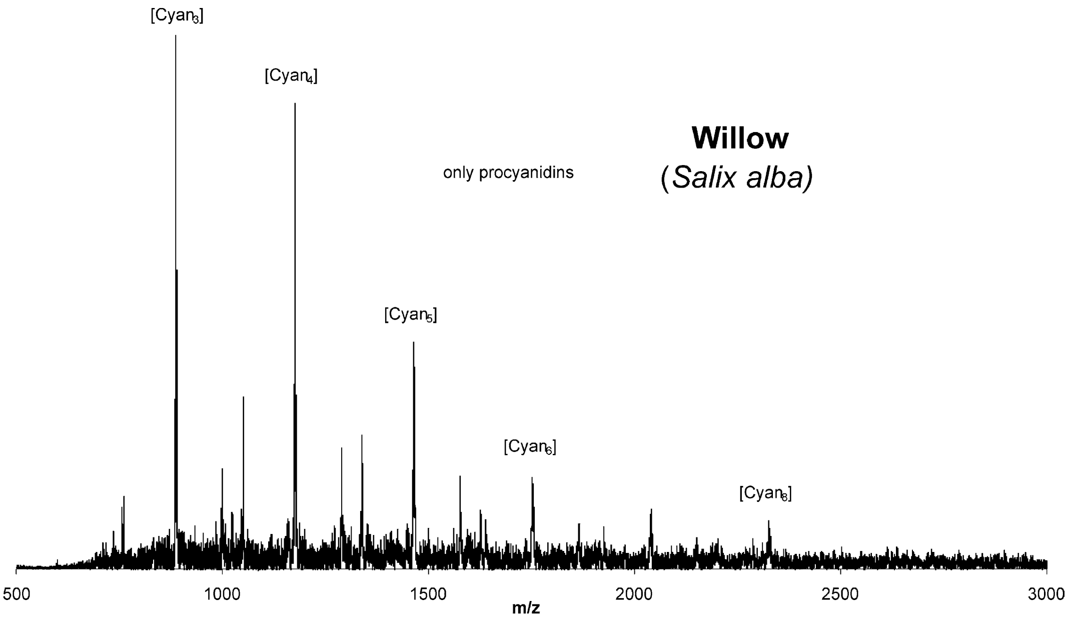

Fig. 5. MALDI-TOF mass spectra of condensed tannins extracted from willow leaves (*Salix alba*). Cyan$_x$ corresponds to the number of procyanidin units.

first and the highest peak, respectively, (Fig. 6) of each series with the one of the next series, the masses also increase by 288 Da, which corresponds to one catechin/epicatechin monomer unit. In the case of the condensed tannins from spruce, the first peak is always followed by mass signals in a distance of 16 Da corresponding to the addition of one hydroxy group at the aromatic ring B (see Fig. 1, gallocatechin/epigallocatechin). Therefore, MALDI-TOF mass spectrometry indicates the simultaneous occurrence of pure procyanidin polymers ($m/z=889$, 1177 etc.) and polymers with different ratios of procyandin and prodelphinidin ($m/z=905$, 921 etc.; Fig. 6A). A comparable observation was made for the condensed tannins of beech (Fig. 6B). However, this spectrum exhibits additional signals characterized by a mass decrease of 16 Da, which is indicative of polymer chains containing monomers with only one hydroxy group at the aromatic ring A or B, e.g. propelargonidin. Additionally, MALDI-TOF measurements clearly show different polymer chain length distributions of the

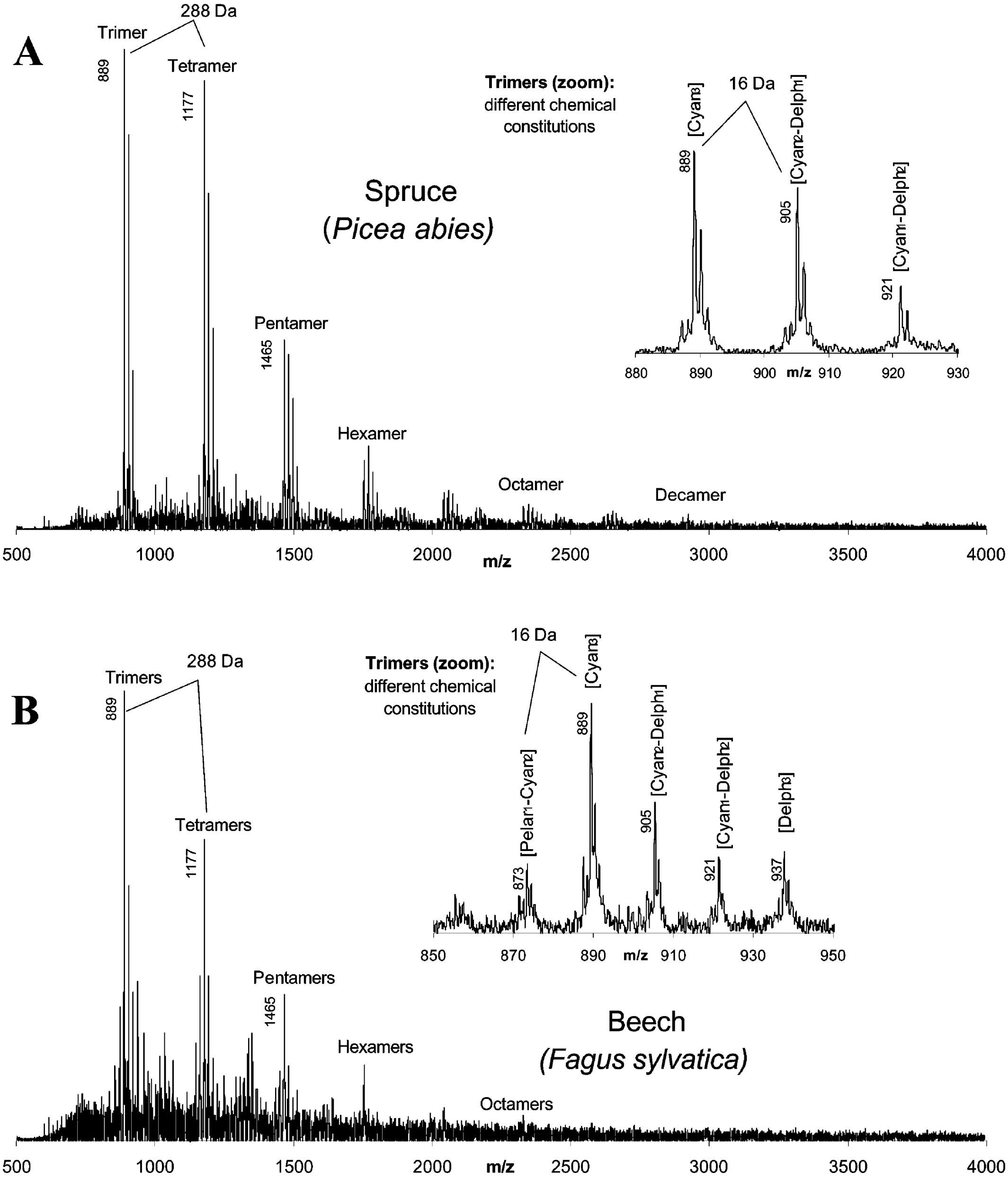

Fig. 6. MALDI-TOF mass spectra of condensed tannins extracted from (A) spruce needles (*Picea abies*) and (B) from beach leaves (*Fagus sylvatica*). Cyan_x corresponds to the number of procyanidin units, Delph_x to the number of prodelphinin units, and Pelar_x to the number of propelargonidin units.

condensed tannins from the four tree species varying from trimers to undecamers and decamers for lime and spruce and to octamers for willow and beech.

As mentioned above, fragmentation of specific molecular mass ions by post source decay (PSD) can help to elucidate the sequences of monomer units of individual chains. Since with the instrumentation used in our studies (Bruker Biflex III), it is only possible to select a mass window of ~60 Da, a sufficient separation of mass signals is necessary to allow the clear assignment of fragments with their precursor peaks. Therefore, we performed PSD fragmentation with condensed tannin samples from lime and spruce. The fragmentation of the tetramers ($m/z = 1177$) gives rise to signals at $m/z = 889$, 887 and $m/z = 601$, 599 (Fig. 7A). The peaks at $m/z = 889$ and 601 correspond to the loss of one and two catechin/epicatechin units respectively and, hence, represent trimeric and dimeric condensed tannins. The masses 887 and 599 may be explained by the fragmentation of A-type condensed tannins which are present in low concentrations. A-type condensed tannins are characterized by an ether bond between the hydroxy group at C7 and C2 in one monomer unit. However, regarding the low concentration of A-type tannins, these fragments

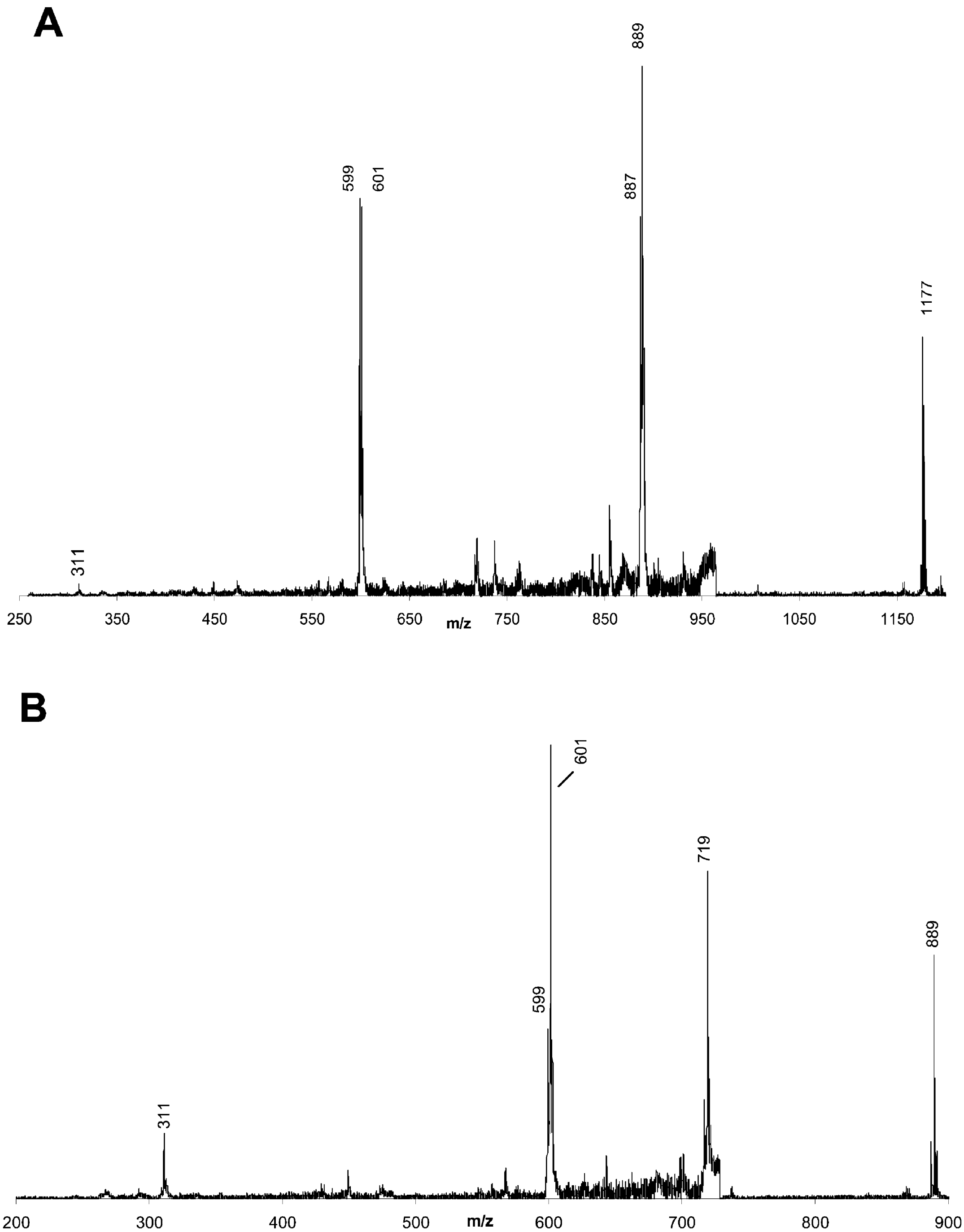

Fig. 7. PSD spectra of condensed tannin tetramer (A) and trimer (B) from lime (*Tilia cordata*).

may also be a consequence of the mode of fragmentation. Cleavage of an interflavanoid bond may lead to two molecular fragments (Fig. 8). The lower molecular part results in an unchanged trimeric, dimeric or monomeric condensed tannin respectively, whereas the upper part, a carbocation, may react to a condensed tannin with a flavanone terminal unit leading to a mass decrease of two Da. The trimer is characterized by a similar fragmentation (Fig. 7B). The loss of one monomer unit leads to dimeric condensed tannin and dimeric condensed tannin with a flavanone terminal unit ($m/z = 601$ and 599). The loss of two monomer units gives rise to $m/z = 311$ and to a small extent to $m/z = 313$. Unfortunately the peak $m/z = 719$ could not be explained. Fragmentation of co-polymers, e.g. tetramer from spruce condensed tannins with procyanidins and prodelphinidins (Fig. 9), also results in the formation of signals characteristic for trimers and dimers. The signals in the spectra of spruce and beech condensed tannins are not sufficiently separated (minimum ± 30

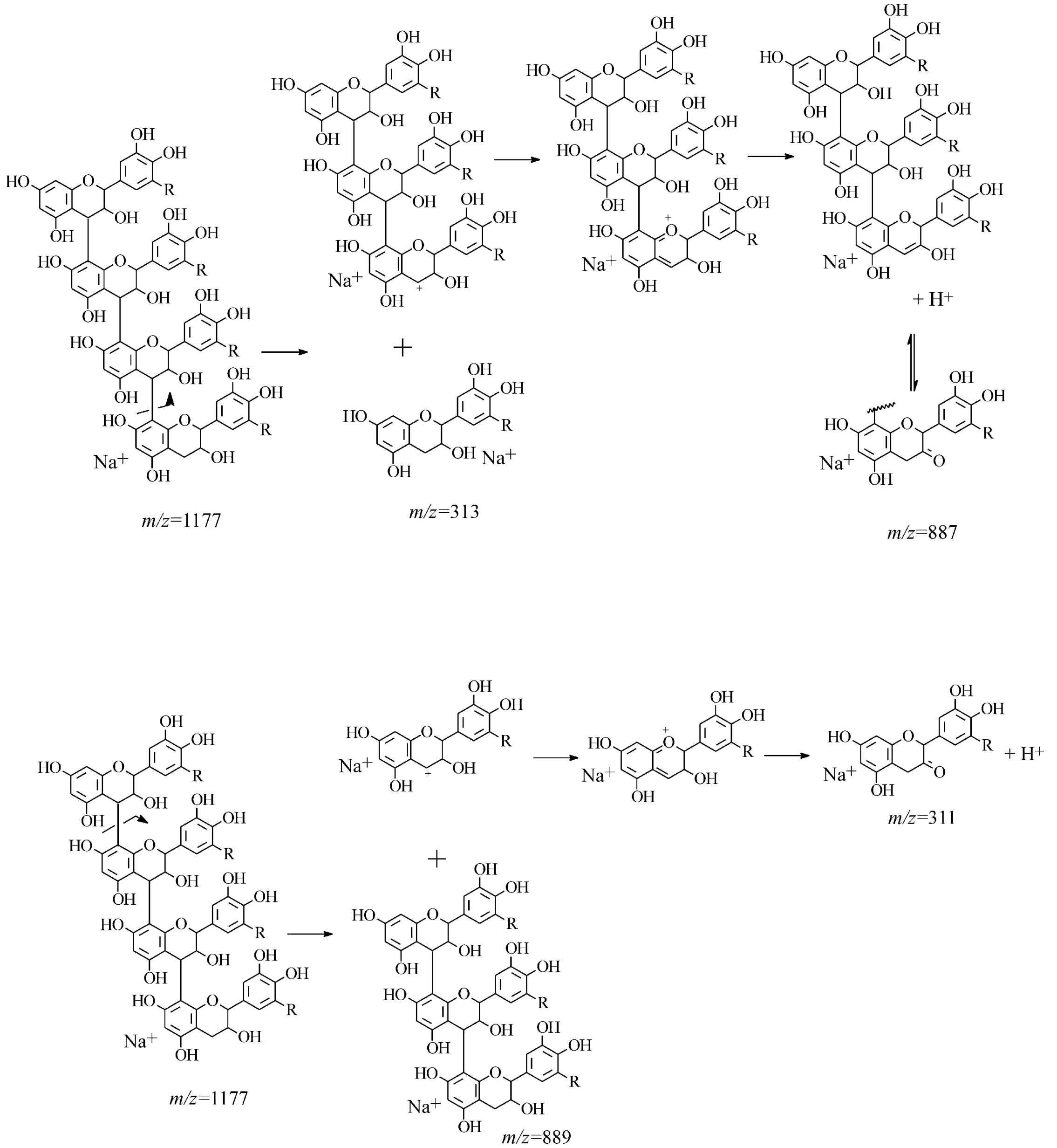

Fig. 8. Possible fragmentation pattern of condensed tannins in PSD MALDI-TOF mass spectrometry.

Da) to clearly assign their precursor mass. Here, the use of higher developed MALDI-TOF and MALDI-TOF/TOF instruments allowing smaller mass windows down to ±2.5 Da (i.e. ULTRAFLEX, Bruker, Saxonia) can overcome these problems. Alternatively, preparative separation of the condensed tannin mixture by, for example, high performance liquid chromatography may also approachable to circumvent such separation problems.

However, the obtained data demonstrate that PSD fragmentation leads to a sequential loss of one monomer unit after the other and therefore allows a detailed characterization and sequencing of individual chains when they are sufficiently separated. In the case of lime condensed tannins it further proves the procyanidin structure and excludes a pelargonidin terminal unit followed by a prodelphinidin unit, which would result in the same molecular masses.

In conclusion, different information of mutual importance are obtained from NMR and mass spectrometrical analysis of condensed tannins. NMR measurements give structural information about the molecules and their functional groups in this fraction and allow the determination of the purity of the sample. All information is averaged over all polymers present, average chemical structure and stereochemistry (amount of different monomer units) and average polymer chain length. On the other hand, the low intensity of the signal for terminal C3 in our NMR spectra prevented the determination of the polymerization degree of the studied tannins. In contrast, polymer chain length distribution and

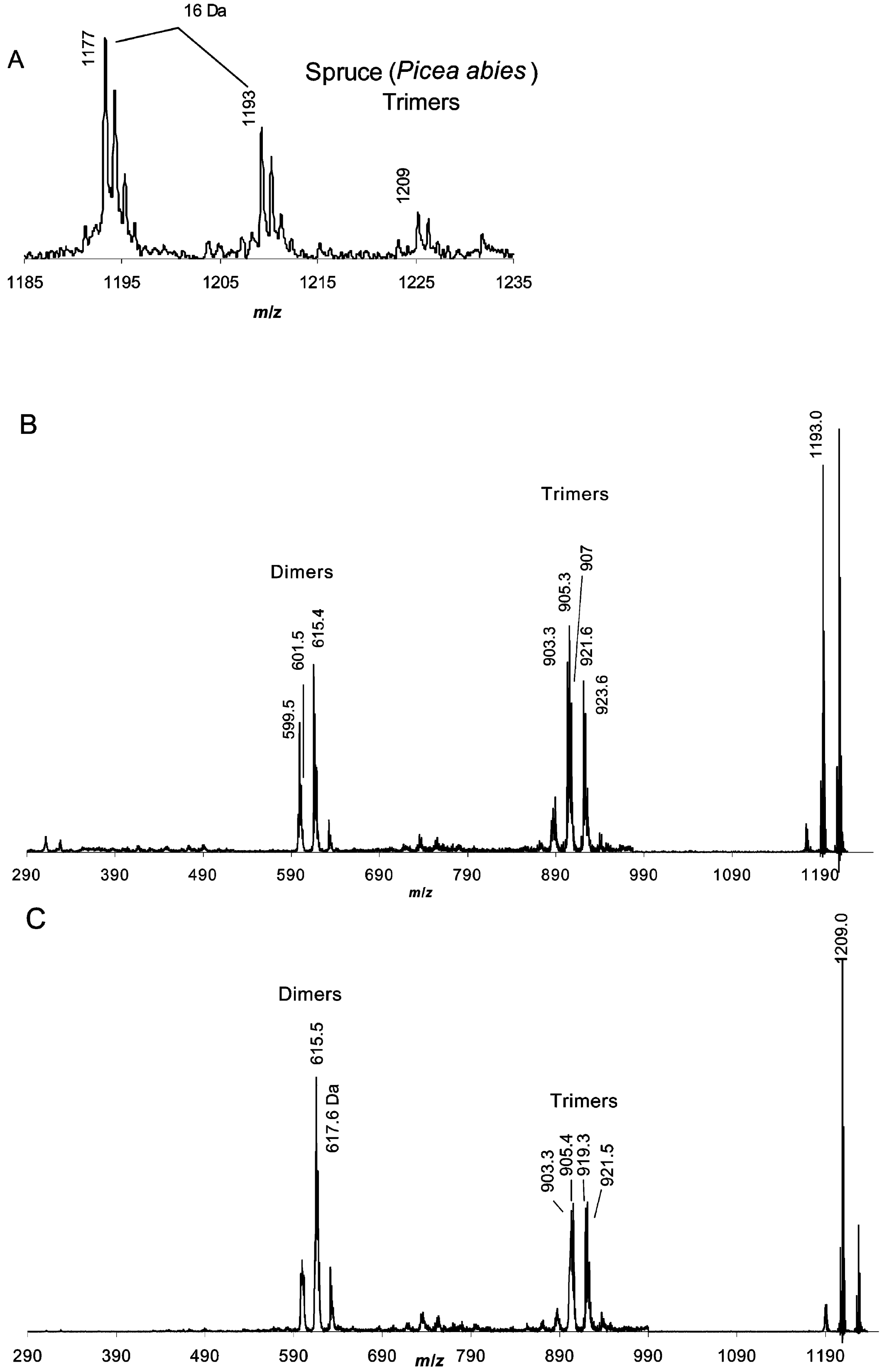

Fig. 9. A: Extention in the range $m/z = 1185$ to 1235 of the MALDI-TOF mass spectra of condensed tannins extracted from spruce. PSD spectra of condensed tannin tetramers from spruce. B: Fragmentation analysis and structural TOF (FAST) selection on mass 1193 corresponding to the mass of a copolymer of three procyanidins and one prodelphinidin, C: Fast selection on mass 1209 Da corresponding to the mass of a copolymer of two procyanidins and two prodelphinidins.

the chemical constitution of individual chains, as well as the sequences of monomer units, can easily be analyzed by MALDI-TOF mass spectrometry and PSD fragmentation.

The combined analyses indicated distinct differences between the examined condensed tannins. Those of willow and lime are composed solely of PC monomers, while those of spruce and beech contain additional polymers that are built up of a mixture of PC and PD units. The additional PD, on the other hand, is considered to have a higher reactivity than PC (Zucker, 1983), which is expected to influence the protein binding affinity and the degradability of the respective condensed tannins during the early stage of soil organic matter formation. The extent of this impact, however, needs further research and will be subject of ongoing examinations.

3. Experimental

3.1. MALDI-TOF MS

The MALDI-TOF mass spectra were recorded on a Bruker Biflex III (Bruker Daltonics, Bremen, Germany) equipped with a N_2-laser and employed in the linear mode. Peaks were calibrated using polyethylene glycol.

Matrices were purchased from Sigma-Aldrich (DHB: Aldrich; dithranol: Aldrich; IAA: Sigma; SA: Aldrich; CCA: Sigma) and were used without further purification except of IAA. IAA has been recrystalized with MeOH.

The liquid-state ^{13}C NMR spectra were obtained at 100.62 MHz on a Bruker DMX 400 Spectrometer (Bruker, Karlsruhe, Germany) using a 10 mm broad band probe. The sample material was dissolved in a D_2O/acetone mixture. A phase alternating ring down elimination pulse sequence combined with inverse-gated decoupling (RIDE-Gate) (Gerothanassis, 1986; Fründ, 1988) was applied to avoid distortion of the spectra by acoustic ringing of the probe and the Nuclear Overhauser effect. The free induction decay (FID) was acquired after a 90° ^{13}C-pulse during an acquisition time of 0.2 s. Relaxation time measurements indicated that the applied pulse delay of 5 s was sufficient to avoid saturation. Prior to Fourier transformation the FID was processed with a line broadening of 18 Hz. All ^{13}C NMR spectra are referenced to the tetramethyl silane scale (=0 ppm) and were adjusted with acetone (methyl-C = 30.7 ppm).

3.2. Extraction and isolation of condensed tannins

Leaves and needles of *Picea abies*, *Fagus sylvatica*, and *Tilia cordata* were collected from trees located in the Thalhauser forest close to Freising (Freising, Bavaria, Germany). The leaves of *Salix alba* were obtained from trees growing along a creek close to the aforementioned forest. After collection the foliage was immediately separated from the branches and freeze dried.

According to the procedure described by Preston (1999), the meshed and freeze dried foliage was soxhlet-extracted with hexane to remove lipids and lipophilic substances. Condensed tannins were subsequently extracted from the residue by a mixture of acetone/water (7:3). The acetone was evaporated and the water fraction was further purified by liquid/liquid extraction with EtOAc and chloroform in order to remove chlorophyll, carotenoids, and hydrolysable monomeric condensed tannins. Subsequently, the oligomeric and polymeric condensed tannins were separated from carbohydrates by liquid chromatography over Sephadex LH-20 column [carbohydrates: elution with MeOH/water (1:1); condensed tannins: elution with acetone/water (7:3)].

Acknowledgement

We wish to thank the Deutsche Forschungsgemeinscaft for financial support (Ko 1035/1d-1(A226)).

References

Baldwin, I.T., Olson, R.K., Reiners, W.A., 1983. Protein binding phenolics and the inhibition of nitrification in subalpine balsam fir soils. Soil Biology and Biochemistry 15, 419–423.

Bending, G.D., Read, D.J., 1996. Nitrogen mobilization from protein–polyphenol complex by eroid and ectomycorrhizal fungi. Soil Biology and Biochemistry 28, 1603–1612.

Bernays, E.A., Driver, G.C., Bilgener, M., 1989. Herbivores and plant tannins. Advances in Ecological Research 19, 263–302.

Chung, K.-T., Wei, C.-I., Johnson, M.G., 1998. Are tannins a double-edged sword in biology and health? Trends in Food Science & Technology 9, 168–175.

Czochanska, Z., Foo, L.Y., Newman, R.H., Porter, L.J., 1980. Polymeric proanthocyanidins. Stereochemistry, structural units and molecular weight. Journal of the Chemical Society Perkin Transaction 1, 2278–2286.

de Jong, A., 1998. Contribution of mass spectrometry to contemporary immunology. Mass Spectrometry Reviews 17, 311–335.

Field, J.A., Lettinga, G., 1992. Toxicity of tannic compounds to microorganisms. In: Hemmingway, R.E, Laks, P.E. (Eds.), Plant Polyphenols. Plenum Press, New York, pp. 673–692.

Foo, L.Y., Lu, Y., Howell, A.B., Vorsa, N., 2000. The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli* in vitro. Phytochemistry 54, 173–181.

Fründ, R., 1988. Quantitative Charakterisierung von organischen Bodeninhaltsstoffen mit Hilfe der ^{13}C-NMR-Spektroskopie. Dissertation, Universität Regensburg, Germany.

Gerothanassis, I.P., 1986. Simple reference baseline subtraction –90° pulse sequence for acoustic ringing elimination in pulsed fourier transform NMR spectroscopy. Magnetic Resonance in Chemistry 24, 428–433.

Hagerman, A.E., 1987. Radial diffusion method for determining tannin in plant extracts. Journal of Chemical Ecology 13, 437–449.

Harvey, D.J., 1999. Matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates. Mass Spectrometry Reviews 18, 349–451.

Hedquist, H., Mueller-Harvey, I., Reed, J.D., Krueger, C.G., Murphey, M., 2000. Characterization of tannins and *in vitro* protein digestibility of several *Lotus corniculatus* varieties. Animal Feed Science and Technology 87, 41–56.

Hemingway, R. W., Karchesy, J. J., 1989. Chemistry and significance of condensed tannins. In: Proceedings of the First North American Tannin Conference. Plenum Press, New York.

Hernes, P.J., Hedges, J.I., 2000. Determination of condensed tannin monomers in environmental samples by capillary gas chromatography of acid depolymerization extracts. Analytical Chemistry 72, 5115–5124.

Jespersen, S., Chaurand, P., Strien, F.J.C., 1999. Direct sequencing of neuropeptides in biological tissue by MALDI-PSD mass spectrometry. Analytical Chemistry 71, 660–666.

Karas, M., Bachmann, D., Bahr, U., Hillenkamp, F., 1987. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. International Journal of Mass Spectrometry and Ion Processes 78, 53–78.

Krüger, C.G., Dopke, N.C., Treichel, P.M., Folts, J., Reed, J.D., 2000. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. Journal of Agriculture and Food Chemistry 48, 1663–1667.

Kuiters, A.T., 1990. Role of phenolic substances from decomposing forest litter in plant-soil interactions. Acta Botanica Neerlandica 39, 329–348.

Mafongoya, P.L., Nair, P.K.R., Dzowela, B.H., 1998. Mineralization of nitrogen from decomposing leaves of multipurpose trees as affected by their chemical composition. Biology and Fertility of Soils 27, 143–148.

Newman, R.H., Porter, L.J., 1992. Solid state ^{13}C-NMR studies on condensed tannins. In: Hemingway, R.W., Laks, P.E. (Eds.), Plant Polyphenols. Plenum Press, New York, pp. 339–347.

Nonaka, G.-I., Hsu, F.-L., Nishioka, I., 1981. Structure of dimeric, trimeric, and tetrameric procyanindins from *Areca catechu* L. Journal of the Chemical Society Perkin Transaction 1, 781–783.

Northup, R.R., Yu, Z., Dahlgren, R.A., Vogt, K.A., 1995. Polyphenol control of nitrogen release from pine litter. Nature 377, 227–229.

Northup, R.R., Dahlgren, R.A., McColl, J.G., 1998. Polyphenols as regulators of plant-litter-soil interactions in northern California's pygmy forest: a positive feedback? Biogeochemistry 42, 189–220.

Ohnishi-Kameyama, M., Yanagida, A., Kanda, T., Nagata, T., 1997. Identification of catechin oligomers from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. Rapid Communications in Mass Spectrometry 11, 31–36.

Pasch, H., Pizzi, A., Rode, K., 2001. MALDI-TOF mass spectrometry of polyflavonoid tannins. Polymer 42, 7531–7539.

Porter, L.J., Newman, R.H., Foo, L.Y., Wong, H., Hemingway, R.W., 1982. Polymeric proanthocyanidins. Journal of the Chemical Society Perkin Transaction 1, 1217–1221.

Porter, L.J., 1988. Flavans and proanthocyanidins. In: Harborne, J.B. (Ed.), The Flavanoids. Chapman & Hall, London, pp. 21–62.

Porter, L.J., 1993. Flavans and proanthocyanidins. In: Harborne, J.B. (Ed.), The Flavanoids: Advances in Research Since 1986. Chapman & Hall, London, pp. 23–55.

Preston, C.M., Trofymow, J.A.T., Sayer, B.G., Niu, J., 1997. C13 nuclear magnetic resonance spectroscopy with cross-polarization and magic-angle spinning investigation of the proximate-analysis fractions used to assess litter quality in decomposition studies. Canadian Journal Botany 75, 1601–1613.

Preston, C.M., 1999. Condensed tannins of salal (*Gaultheria Shallon* Pursh): a contributing factor to seedling "growth check" on northern Vancouver Island? In: Gross, G.G., Hemingway, R.W., Yoshida, T. (Eds.), Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology. Kluwer Academic/Plenum Publishers, New York, pp. 825–841.

Sarni-Manchado, P., Cheynier, V., Moutounet, M., 1999. Interactions of grape seed tannins with salivary protein. Journal of Agricultural and Food Chemistry 47, 42–47.

Spengler, B., 1997. Post-source decay analysis in matrix-assisted laser desorption/ionization mass spectrometry of biomolecules. Journal of Mass Spectrometry 32, 1019–1036.

Talbo, G., Mann, M., 1996. Aspects of the sequencing of carbohydrates and oligonucleotides by matrix-assisted laser desorption/ionization post-source decay. Rapid Communications in Mass Spectrometry 10, 100–103.

van Breemen, N., 1993. Soils as biotic constructs favouring net primary productivity. Geoderma 57, 183–211.

Waterman, P.G., Mole, S., 1994. Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publications, Oxford.

Zucker, W.V., 1983. Tannins: Does structure determine function? An ecological perspective. The American Naturalist 121, 335–365.