Universidade Técnica de Lisboa
Instituto Superior de Agronomia

Caracterização do teor e composição estrutural da lenhina por espectroscopia de infravermelho próximo e pirólise analítica

Tese apresentada para a obtenção do grau de Doutor em Engenharia Florestal

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Resumo

Neste trabalho aplicou-se a pirólise analítica e a espectroscopia de infravermelho próximo (NIR) ao estudo do teor e da composição da lenhina, razão H/G (p-hidroxifenilo/guaiacilo), na madeira de três espécies de resinosas *Pinus pinaster*, *Picea abies* e *Larix* sp..

A pirólise analítica foi utilizada como método quantitativo, para determinar o teor de lenhina das madeiras (Py-lenhina), tendo-se obtido boas correlações (R^2=0,93) entre a Py-lenhina e o método Klason. Obteve-se um modelo comum para as três espécies (Py-lenhina=0,7188×lenhina Klason+4,3045). Este modelo permite-nos estimar o teor de lenhina Klason, a partir do valor de Py-lenhina usando apenas 75 µg de amostra.

A pirólise analítica em combinação com a análise de componentes principais (PCA) permitiu ainda a discriminação das três espécies e dentro da espécie *Pinus pinaster* a sua proveniência.

Neste trabalho desenvolveram-se ainda métodos expeditos de análise para a caracterização do teor (R^2=0,97) e composição (R^2=0,89) da lenhina da madeira de *Pinus pinaster*, por espectroscopia de infravermelho próximo (NIR) usando o método Klason e a pirólise analítica como métodos de referência respectivamente. Estes modelos foram usados para a caracterização do teor e composição da lenhina num programa de melhoramento de *Pinus pinaster*, tendo-se estimado uma elevada heritabilidade para o teor (0,75) e média a baixa (0,24) para a composição da lenhina.

Palavras-chave: Resinosas, *Pinus pinaster*, *Picea abies*, *Larix* sp., teor de lenhina, composição da lenhina, razão H/G, pirólise analítica, espectroscopia de infravermelho próximo, método Klason.

Abstract

Characterization of lignin amount and composition by near infrared spectroscopy and analytical pyrolysis

In this work analytical pyrolysis and near infrared spectroscopy (NIR) were used to assess lignin amount and composition, namely the H/G (p-hydroxyphenyl/guaiacyl) ratio, in the wood of three softwood species *Pinus pinaster*, *Picea abies* and *Larix* sp..

A method was developed for the quantification of lignin directly from analytical pyrolysis results (Py-lignin). A good correlation was found between Py-lignin and Klason lignin content (R^2=0,93). A commom model (Py-lignin=0,7188×Klason lignin+4,3045) was obtained for the three species studied, allowing the estimation of lignin in samples of 75 µg.

Analytical pyrolysis in combination with principal component analysis also allowed the identification of species and provenances within species.

Rapid, non-destructive methods were developed for the quantification of lignin (R^2=0,97) and determination of lignin composition (R^2=0,89) by near infrared spectroscopy, using Klason and analytical pyrolysis as reference methods. These NIR models were used for the characterization of samples from an improvement program for wood quality of *Pinus pinaster*, and revealed that lignin quantity is under stronger genetic control (0,75), whilst lignin composition is under moderate genetic control (0,24).

Keywords: Softwoods, *Pinus pinaster*, *Picea abies*, *Larix* sp., lignin quantification lignin composition, H/G ratio, analytical pyrolysis, near infrared spectroscopy, Klason method.

Resumo alargado

A lenhina é o segundo polímero natural mais abundante no nosso planeta logo a seguir à celulose, representando cerca de 30% do carbono da biosfera. A interligação entre a lenhina e os polissacáridos confere rigidez e resistência estrutural à parede celular das madeiras, permitindo o transporte da água e dos solutos através do sistema vascular. A deposição da lenhina nas paredes celulares tem ainda um papel importante na protecção da madeira contra os ataques de organismos xilófagos.

A lenhina é um polímero aromático complexo formado por três unidades fenilpropanóicas, nomeadamente *p*-hidroxifenilo (H), guaiacilo (G) e siringilo (S). A lenhina das resinosas é predominantemente composta por unidades G, ao passo que a lenhina das folhosas é composta por unidades S e G. As unidades H aparecem em pequena quantidade tanto nas folhosas como resinosas.

O interesse no conhecimento do teor e composição da lenhina resulta da sua influência no processo de deslenhificação no fabrico de pasta para papel a partir de madeira, afectando quer o rendimento em pasta, quer o consumo de reagentes. A lenhina composta maioritariamente por unidades siringílicas (S) apresenta uma maior reactividade no processo kraft do que as unidades (G), e estas que as unidades (H). A taxa de deslenhificação depende em primeiro lugar da estrutura química da lenhina e não da sua acessibilidade e é directamente proporcional à razão S/G nas folhosas e inversamente proporcional à razão H/G nas resinosas.

O desconhecimento em relação à variabilidade do teor e composição da lenhina entre árvores deve-se essencialmente às limitações dos métodos clássicos por via húmida: são demorados, caros, requerem mão-de-obra intensiva e qualificada e grandes quantidades de amostra que nem sempre estão disponíveis. A combinação de métodos expeditos e não destrutivos, permite a redução dos custos das análises, associados à mão-de-obra e ao consumo de reagentes, aumenta a produtividade e evita o abate das árvores.

Pretendeu-se com este trabalho desenvolver métodos expeditos de análise e sua utilização para a caracterização do teor e composição estrutural da lenhina nas madeiras, por espectroscopia de infravermelho próximo (NIR) e por pirólise analítica (Py-GC/MS e Py-GC/FID). A espectroscopia de infravermelho próximo (NIR) tem como vantagens: uma elevada produtividade; repetibilidade e versatilidade; é não destrutiva; a preparação das amostras é muito reduzida; apresenta um baixo custo de operação e manutenção; é não poluente. A pirólise analítica apresenta como vantagem a simplicidade de preparação das amostras, o reduzido período de tempo que a análise requer e o facto de necessitar de quantidades muito reduzidas de amostras (≈ 75 µg).

A parte prática da presente tese é apresentada na forma de seis artigos, publicados em revistas científicas internacionais com arbitragem científica.

No primeiro trabalho (artigo **I**) apresenta-se um método para a quantificação do teor de lenhina, em amostras extractadas de madeira de pinheiro bravo (*Pinus pinaster* Aiton) e espruce europeu (*Picea abies* (L.) Karst) a partir do cromatograma dos produtos de pirólise (pirograma). A razão entre a soma da área dos produtos da lenhina e a soma da área de todos os produtos identificados (lenhina e polissacáridos) foi usada como um estimador do teor de lenhina das amostras (Py-lenhina).
A precisão do método foi de 0,41% para o pinheiro bravo e de 0,34% para o espruce, valores que são próximos da precisão do método Klason, que segundo a norma TAPPI T222 OM-88 é de 0,34%. Estes resultados sugerem que o método permite descriminar amostras com pequenas diferenças no teor de lenhina.
Obteve-se uma elevada correlação entre os valores de Py-lenhina e os valores determinados pelo método de referência (Klason) para as mesmas amostras, quer para o pinheiro (R=0,95) quer para o espruce (R=0,95). Após a obtenção dos modelos individuais, obteve-se um modelo comum para as duas espécies (R^2=0,93).
A elevada correlação permite estimar o teor de lenhina Klason, com elevada fiabilidade, a partir do valor de Py-lenhina em amostras para as quais por falta de material não é possível usar o método de referência Klason.

No segundo trabalho (artigo **II**) estudou-se a aplicação do modelo comum (artigo **I**) para estimar o teor de lenhina da madeira de três espécies doutro género (*Larix*). A boa correlação obtida entre o teor de lenhina estimado pelo modelo e os resultados obtidos pelo método Klason (R^2=0,94) comprovam que o modelo comum pode ser usado para estimar o teor de lenhina também na madeira de larício.
Adicionalmente estudou-se a influência da presença de amostras com lenho de compressão nos resultados da pirólise, tendo-se verificado que apesar da diferente composição da lenhina, maior quantidade de unidades H no caso do lenho de compressão, também são bem preditas pelo modelo.
Obteve-se assim um modelo comum para as madeiras das espécies *Pinus pinaster*, *Picea abies* e *Larix* sp.. É esperado que este modelo possa predizer o teor de lenhina Klason na madeira de outras resinosas.

No terceiro trabalho (artigo **III**) procurou-se retirar mais informação dos resultados da pirólise analítica, partindo do princípio que, cada pirograma sendo único, como uma impressão digital da composição química, deve reflectir as diferenças de composição da lenhina dos diferentes géneros, espécies e tecidos, bem como a influência genética e ambiental. No entanto, esta informação está oculta num número elevado de variáveis (cerca de 60 produtos de pirólise) e portanto só com a utilização da análise multivariada é possível extraí-la. Utilizando a análise de componentes principais (PCA) foi possível separar as três espécies, *Pinus pinaster*, *Picea abies* e *Larix* sp., e dentro da mesma espécie (*Pinus pinaster*) foi possível separar as amostras de duas proveniências. Ficou assim demonstrado que a pirólise analítica combinada com a análise de componentes principais pode ser uma ferramenta útil na discriminação ao nível da espécie e da sua proveniência.

Apesar das vantagens da pirólise analítica como método para a caracterização da composição da lenhina, a sua principal limitação prende-se com o número limitado de amostras que é possível analisar por dia. Neste trabalho (artigo **IV**) desenvolveu-se um modelo por espectroscopia de infravermelho próximo, em combinação com a regressão pelo método dos mínimos quadrados parciais (NIR-PLSR), para a caracterização da composição estrutural da lenhina (razão H/G), usando a pirólise analítica

como método de referência em amostras de pinheiro bravo. O modelo obtido tem um elevado coeficiente de determinação (R^2=0,89) e um baixo erro médio quadrático da validação cruzada (RMSECV=0,0054), valor de erro que é semelhante ao da precisão do método de referência (0,005). Usando este modelo foi possível analisar cerca de 300 amostras por dia o que é uma considerável poupança de tempo e de recursos relativamente à pirólise analítica, em que só é possível analisar 6 amostras por dia.

No quinto trabalho (artigo **V**) desenvolveu-se um modelo para a determinação do teor de lenhina em amostras de pinheiro bravo (*Pinus pinaster*) por espectroscopia de infravermelho próximo (NIR) usando como referência o método Klason. O modelo foi obtido a partir de dados provenientes de um parceiro internacional da indústria de pasta para papel (AFOCEL). As estatísticas do modelo obtido com estes dados (R^2=0,50 e RMSECV=0,87) sugeriam uma fraca capacidade para estimar correctamente o teor de lenhina. Verificou-se, no entanto, que o modelo estimava correctamente o teor de lenhina de um conjunto de amostras independentes (R^2=0,92) tendo-se discutido as possíveis razões para este comportamento. Este trabalho, sendo uma aplicação clássica do NIR, mostra por um lado a importância da qualidade dos dados de referência na obtenção de bons modelos e por outro lado que os valores estimados podem ser mais precisos que os do método de referência.

No sexto trabalho (artigo **VI**) procedeu-se à aplicação de métodos expeditos (NIR) para a caracterização da composição química de 960 amostras de madeira de pinheiro bravo, de um programa de melhoramento francês, na 3ª geração de selecção. Pela primeira vez foi possível determinar a heritabilidade (sentido restrito) da composição da lenhina (razão H/G), que para esta população foi baixa (h^2=0,24), com ganhos genéticos correspondentes baixos (0,01). No entanto, para o teor de lenhina encontrou-se uma elevada heritabilidade (h^2=0,75), que corresponde uma diminuição do teor de lenhina de 3,8% para um índice de selecção de 1%.

Índice

1 - Introdução

1.1 – Enquadramento geral e justificação do interesse

A importância da floresta e do sector industrial associado é muito elevada em Portugal: i) pela extensão territorial ocupada, 3,4 milhões hectares, que correspondem a cerca de 38,4% da área total do país; ii) pela relevância das funções económicas, ambientais, sociais e culturais a ela associadas; iii) pela natureza da indústria transformadora que, baseada num recurso natural e renovável, assegura a existência de produtos recicláveis e reutilizáveis gerando emprego e riqueza; iv) pelo elevado número de agentes envolvidos na produção, transformação e comercialização de produtos florestais (Celpa, 2008).

O conhecimento da composição química da madeira é fundamental para a compreensão das suas propriedades, sobretudo nos casos em que ela se destina à transformação química para a produção de pasta para papel e produção de energia. Nesta intervêm não só os componentes estruturais das paredes celulares (a lenhina e os polissacáridos – celulose e hemiceluloses), mas também os materiais extractáveis, tanto do ponto de vista da sua composição estrutural, quanto dos respectivos teores. A introdução de parâmetros como a composição química da madeira em programas de melhoramento implicou o desenvolvimento de métodos expeditos de caracterização, reprodutíveis e de baixo custo, uma vez que é necessária a análise de muitas amostras e em que os métodos tradicionais são demorados, requerem muita mão-de-obra e são de elevado custo.

Se o conhecimento da influência das propriedades da madeira nas propriedades da pasta tem um papel importante na selecção de genótipos superiores para atingir as características desejadas, a produção de matéria-prima mais homogénea constitui, sempre, um objectivo desejável para a indústria, pois contribui para a eficiência do processo fabril. No entanto, e devido à sua natureza biológica, a madeira tem como característica marcante a sua variabilidade, qualquer que seja a propriedade considerada. Alterações, quer da estrutura macroscópica, quer da composição química, podem resultar de condições anormais de crescimento, mas ocorrem

também associadas à formação de lenho juvenil e adulto, de lenho de início e de fim de estação, de borne e cerne (Zobel e Buijtenen 1989).

O interesse na composição monomérica da lenhina resulta do facto de há muito se conhecer a sua influência no processo de deslenhificação da madeira (Fergus e Goring 1969, 1970; Chang e Sarkanen 1973). No entanto, pouco se sabe sobre a variabilidade da composição da lenhina entre árvores da mesma espécie. Este desconhecimento decorre em parte das limitações dos métodos clássicos de via húmida para a caracterização do teor e composição monomérica da lenhina. Estes métodos são demorados, requerem mão-de-obra intensiva e qualificada e são muito caros. Pretendeu-se neste trabalho desenvolver técnicas expeditas, de elevada fiabilidade e compatíveis com amostragens não destrutivas para estimar o teor e composição da lenhina. Estas técnicas incluíram a pirólise analítica e a espectroscopia de infravermelho próximo, em associação com a análise multivariada, nomeadamente a regressão pelo método dos mínimos quadrados parciais (PLS) e análise de componentes principais (PCA).

A pirólise analítica é um dos métodos que tem sido cada vez mais utilizado para a caracterização da composição da lenhina (Rodrigues *et al*. 1999; Rodrigues *et al*. 2001; Yokoi *et al*. 2001; Kuroda *et al*. 2002; del Rio *et al*. 2005; Meier *et al*. 2005;). Contudo, mesmo com a simplicidade e exigências de tempo muito inferiores à dos métodos de química húmida (Meier e Faix 1992; Rodrigues *et al*. 2001), é ainda assim exigente quando é necessário analisar um número elevado de amostras.

A simplicidade, rapidez e elevada reprodutibilidade da espectroscopia de infravermelho próximo tem levado à sua utilização para a caracterização química de materiais lenhocelulósicos, em substituição dos métodos químicos tradicionais (Bailleres *et al*. 2002; Gierlinger *et al*. 2002; Raymond e Schimleck 2002; Schimleck *et al*. 2003). A combinação da espectroscopia de infravermelho próximo (NIR) com a pirólise analítica abre a possibilidade de se analisar um grande número de amostras com elevada rapidez e precisão e a um custo que permite incluir este parâmetro em programas de melhoramento, permitindo aumentar os critérios de selecção pela redução

do tempo de análise e pela redução dos custos associados à medição destas propriedades. Dada a sua importância actual, as madeiras de pinheiro bravo, o espruce e o larício foram usadas como "modelos" neste estudo. Espera-se contudo que os resultados obtidos para estas três espécies possam ter aplicação noutras coníferas com interesse.

1.2 – Espécies estudadas

O pinheiro bravo (*Pinus pinaster* Aiton), ocupa em Portugal uma superfície aproximada de 711 mil hectares, isto é cerca de 20,9% da superfície arborizada do país (Celpa, 2008). É uma espécie de elevada importância no sector florestal português, pois o aproveitamento industrial da sua madeira para serração, fabrico de aglomerados e produção de pasta para papel representam um peso considerável na economia portuguesa. Em 2008, esta espécie contribuiu com 9,35% para o total de matéria–prima lenhosa usada no fabrico de 2,02 milhões de toneladas de pasta para papel, sendo a principal fonte de fibra longa usada na produção de Kraftliner (Celpa, 2008).

O espruce (*Picea abies* L.) ocorre naturalmente nos alpes europeus e nas montanhas dos Balcãs e dos Cárpatos, estendendo-se para o norte da Escandinávia e da Sibéria. Foi introduzida nas Ilhas Britânicas, nos anos 1500 d.C., e é muito plantada na América do Norte, particularmente no nordeste dos Estados Unidos, estados da Costa do Pacífico, das Montanhas Rochosas e no Sudeste do Canadá. A *Picea abies* é uma das espécies de coníferas economicamente mais importante da Europa. Por exemplo, na Suécia actualmente a indústria de pasta para papel pode ser dividida em três grupos, consoante o tipo de madeira utilizado: espruce, espruce misturado com pinheiro e folhosas (Duchesne *et al*. 1997).

O larício (*Larix* sp.) tem uma larga distribuição natural, ocorrendo desde as planícies na região circumpolar no Alasca, Canadá e Rússia, até altitudes moderadas a altas, nas montanhas da América do Norte, nos Alpes, Mongólia China, Coreia e Japão. Na Europa, o *Larix decidua* Mill. (Larício europeu) tem uma área de distribuição natural em quatro zonas geográficas Alpes, montanhas Sudetas, Cárpatos e centro da Polónia (Biswas e Mohri

1997). Esta espécie é apreciada para a produção de pasta devido ao seu crescimento rápido e à qualidade da madeira (Gierlinger 2003).

1.3 – Objectivos

Os objectivos deste trabalho foram:

- O desenvolvimento de métodos de análise por espectroscopia de infravermelho próximo (NIR) e por pirólise analítica (Py-GC/FID), para a caracterização do teor e composição da lenhina (razão H/G) em madeiras de resinosas com interesse para a produção de pasta para papel.

- A utilização destes métodos para caracterização fenotípica em programas de melhoramento.

1.4 - Estrutura do trabalho

A presente tese é uma compilação do trabalho apresentado em seis artigos científicos.

No capítulo 2 é feita a revisão de conhecimentos. Primeiro, sobre a composição química da madeira, com especial incidência sobre o teor e composição da lenhina; segundo sobre os métodos usados para a análise do teor e composição da lenhina, nomeadamente a pirólise analítica e a espectroscopia de infravermelho próximo (NIR).

O capítulo 3 constitui o trabalho de investigação desenvolvido, sendo apresentado na forma de seis artigos publicados em revistas científicas internacionais com arbitragem científica. Optou-se por incluir os artigos publicados com a formatação original com que foram publicados pelas revistas científicas e são referidos no texto por numeração romana.

I – Alves A, Schwanninger M, Pereira H, Rodrigues J (2006) Analytical pyrolysis as a direct method to determine the lignin content in wood
Part 1: Comparison of pyrolysis lignin with Klason lignin. **Journal of Analytical and Applied Pyrolysis** 76:209–213.

II – Alves A, Rodrigues J, Wimmer R, Schwanninger M (2008) Analytical pyrolysis as a direct method to determine the lignin content in wood
Part 2: Evaluation of the common model and the influence of compression wood. **Journal of Analytical and Applied Pyrolysis** 81:167–172.

III – Alves A, Gierlinger N, Schwanninger M, Rodrigues J (2009) Analytical pyrolysis as a direct method to determine the lignin content in wood
Part 3: Evaluation of species-specific and tissue-specific differences in softwood lignin composition using principal component analysis. **Journal of Analytical and Applied Pyrolysis** 85:30–37.

IV - Alves A, Schwanninger M, Pereira H, Rodrigues J (2006) Calibration of NIR to assess lignin composition (H/G ratio) in Maritime pine wood using analytical pyrolysis as the reference method. **Holzforschung** 60:29-31.

V – Rodrigues J, **Alves A**, Pereira H, Perez DDS, Chantre G, Schwanninger M (2006) NIR PLSR results obtained by calibration with noisy, low-precision reference values: Are the results acceptable? **Holzforschung** 60:402-408.

VI - Perez DD, Guillemain A, Alazard P, Plomion C, Rozenberg P, Rodrigues JC, **Alves A**, Chantre G (2007) Improvement of *Pinus pinaster* Ait. elite trees selection by combining near infrared spectroscopy and genetic tools. **Holzforschung** 61:611-622.

No capítulo 4 apresentam-se as conclusões.

2 – Revisão de conhecimentos

2.1 – Composição química da madeira

A madeira é constituída, maioritariamente, por duas classes de compostos estruturais de dimensões macromoleculares: polissacáridos (celulose e hemiceluloses), que representam entre 65 e 75% da massa seca da madeira; e lenhina, que representa entre 18 e 35%. Além destes compostos estruturais ocorrem também compostos de baixa massa molecular que podem ser removidos por extracção com solventes, designados extractivos, que representam entre 1 a 6% do peso seco e por uma fracção inorgânica (cinzas), inferior a 2% (Figura 2.1). No conjunto dos constituintes químicos genericamente designados por extractivos destacam-se os compostos aromáticos, terpenos, ácidos alifáticos e álcoois, entre outros (Fengel e Wegener 1984; Pereira *et al*. 2003).

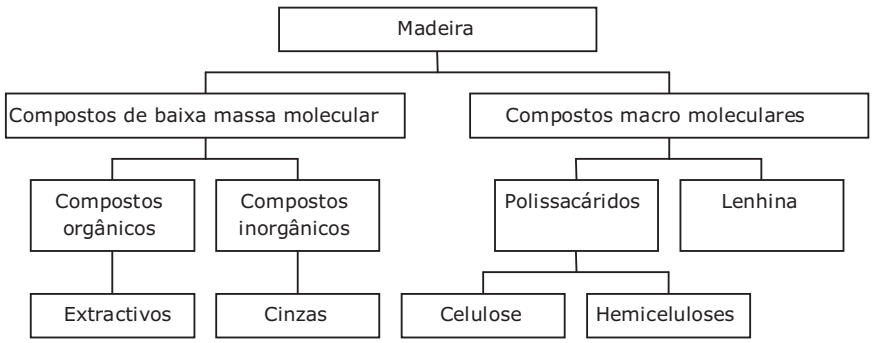

Figura 2.1 – Constituintes químicos da madeira (Fengel e Wegener 1984)

A celulose é o principal constituinte da madeira. Representa entre 40 a 45% do seu peso seco e está localizada, predominantemente, na parede secundária das células. No interior da parede secundária, a celulose e as hemiceluloses organizam-se em feixes orientados, as microfibrilas, nas quais a celulose se encontra maioritariamente na forma cristalina (Pereira *et al*. 2003).

A celulose é um polímero linear de elevada massa molecular, constituído exclusivamente, por unidades de β-D-glucopiranose. Duas moléculas de β-D-glucopiranose adjacentes ligam-se entre si pelos grupos hidroxílicos do

C1 e do C4, através da eliminação duma molécula de água, dando origem à molécula de celobiose que constitui a unidade estrutural de repetição da cadeia de celulose.

As hemiceluloses encontram-se associadas à celulose nas microfibrilas e estabelecem também ligações com a lenhina que as envolve. Os seus constituintes principais são cinco monossacáridos, três hexoses (glucose, manose e galactose) e duas pentoses (xilose e arabinose) (Fengel e Wegener 1984; Pereira *et al.* 2003).

2.1.1 – Lenhinas

A lenhina é um dos principais polímeros componentes da parede celular dos tecidos lenhosos, e é exclusiva do reino vegetal. Actua como agente de ligação entre as microfibrilas nas paredes celulares e entre células adjacentes, conferindo rigidez e impermeabilidade à parede celular, e tornando a madeira uma estrutura resistente ao impacto, à compressão e, até determinado grau, à flexão (Fengel e Wegener 1984; Pereira *et al.* 2003). A lenhina desempenha ainda um papel de protecção contra agentes patogénicos exteriores nas plantas superiores (Sjostrom 1981; Fengel e Wegener 1984; Sakakibara 1991; Pereira *et al.* 2003).

A lenhina não é um composto químico de fórmula e estrutura definido em termos de constituição mas, antes, é um conjunto de materiais amorfos com o mesmo tipo de constituição química, reactividade e estrutura molecular. É um polímero constituído por unidades de fenilpropano (também designadas por unidades C9) interligadas, formando uma estrutura entrecruzada. São três os percursores que vão originar a macromolécula por um processo de polimerização desidrogenativo: o álcool *p*-cumarílico, o álcool coniferílico e o álcool sinapílico (Figura 2.2) (Fengel e Wegener 1984; Pereira *et al.* 2003).

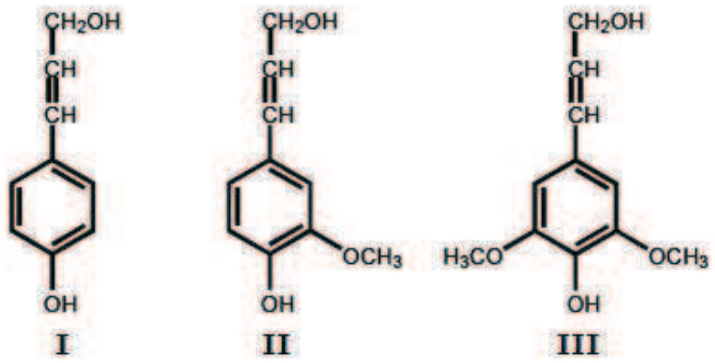

Figura 2.2 – Unidades precursoras da lenhina

(I) álcool *p*-cumarílico, (II) álcool coniferílico, (III) álcool sinapílico

O primeiro passo na construção da macromolécula de lenhina é a desidrogenação enzimática dum grupo fenólico OH dos precursores fenilpropano. Forma-se um radical por perda de um átomo de hidrogénio, o que constitui uma estrutura ressonante com formas mesoméricas. Dois radicais combinam-se, então, dando origem a um dímero.

Considerando os diferentes centros reactivos dos radicais, é possível formar diferentes tipos de ligação. As ligações β-O-4 são as mais abundantes constituindo 50 a 60% do total. Na Figura 2.3 são apresentados vários tipos de ligações entre os monómeros de lenhina (Fengel e Wegener 1984; Pereira *et al.* 2003).

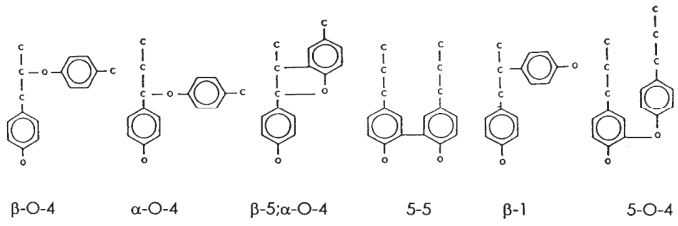

Figura 2.3 – Ligações entre monómeros da lenhina (Grace e Malcolm 1989)

Os dímeros formados podem, por sua vez, sofrer uma desidrogenação enzimática, dando origem a novos radicais que, por combinação entre si, dão origem a tetrâmeros ou, por combinação com um monómero, a trímeros. Um processo sucessivo de desidrogenação e acoplamento leva,

finalmente, ao desenvolvimento da macromolécula de lenhina. Na lenhina os monómeros encontram-se ligados entre si por dois tipos de ligações: ligações éter através do oxigénio do grupo hidroxilo do anel fenólico e ligações directas carbono-carbono (C–C), estas últimas aproximam mais os monómeros entre si, tornando a lenhina mais condensada (Pereira *et al.* 2003).

As lenhinas são constituídas por unidades do tipo guaiacílico (G, fenol com um grupo metoxilo), que resultam do percursor álcool *trans*-coniferílico, unidades do tipo *p*-hidroxifenilo (H, fenol sem grupos metoxilos), que resultam do percursor álcool *trans*-p-cumarílico e unidades do tipo siringílicas (S, fenol com dois grupos metoxilos) que resultam do percursor álcool sinapílico (Fengel e Wegener 1984; Biermann 1993). Sendo os precursores e o processo de polimerização da lenhina os mesmos, o que distingue as lenhinas entre espécies são basicamente duas componentes: a composição relativa dos três monómeros e a frequência dos vários tipos de interligações.

As lenhinas das madeiras das resinosas (gimnospérmicas), das folhosas (angiospérmicas, dicotiledóneas) e das gramíneas (angiospérmicas, monocotiledóneas) diferem na proporção relativa de unidades precursoras – guaiacilo (G), siringilo (S) e *p*-hidroxifenilo (H) (Fengel e Wegener 1984; Pereira *et al.* 2003). Apesar da variabilidade observada na composição das lenhinas, as madeiras das resinosas, folhosas e gramíneas apresentam, tendencialmente, lenhinas dos géneros G, GS e HGS, respectivamente (Fengel e Wegener 1984).

De acordo com um arranjo aleatório das subestruturas mais importantes, e segundo as frequências aproximadas das ligações entre unidades de fenilpropano, Adler (1977) apresentou uma proposta de modelo para a estrutura das lenhinas da madeira das resinosas (Figura 2.4).

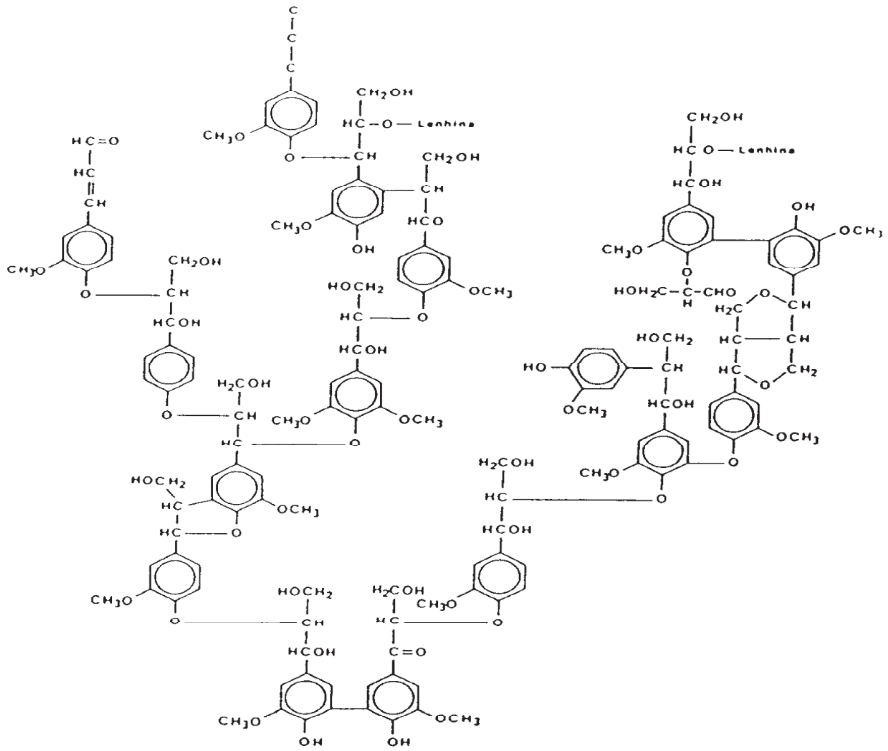

Figura 2.4 – Modelo estrutural da lenhina de uma resinosa de acordo com Adler (1977)

A lenhina é um polímero sem estrutura supramolecular organizada, não é elástica, não contribuindo, portanto, para a elasticidade da madeira, conferindo uma rigidez tridimensional ao conjunto da estrutura celular da madeira. Possui poucos grupos com carácter hidrofílico, pelo que a absorção da humidade é reduzida. Encontra-se no tecido lenhoso associada às hemiceluloses, com as quais estabelece ligações químicas pontuais (Fengel e Wegener 1984).

Os procedimentos para a determinação do teor de lenhina, foram objecto de estudo por diversos autores (Browning 1967; Lin e Dence 1992; Schwanninger e Hinterstoisser 2002) e objecto de normalização quer por parte de associações técnicas da indústria de pasta para papel (TAPPI e APPITA) quer por agências nacionais de normalização, como a AFNOR (Association française de Normalisation), ASTM (American Society for Testing and Materials), DIN (Deutsches Institut für Normung), NP (Normas

Portuguesas), etc. As instituições nacionais de normalização estão associadas numa organização internacional, a International Organization for Standardization (ISO).

O método mais utilizado para a determinação do teor de lenhina é o método Klason, que consiste na hidrólise dos polissacáridos em duas etapas, primeiro com ácido sulfúrico concentrado (72 %) durante duas horas num banho-maria a 20ºC, seguido de uma diluição a 3% e colocando a mistura num autoclave a 120ºC durante 1 hora (TAPPI T222 OM-88). O resíduo insolúvel da hidrólise corresponde à lenhina Klason. De acordo com a norma TAPPI T222 OM-88 a repetibilidade deste procedimento é de 0,34 e a reprodutibilidade é de 0,79 obtidos de acordo com a norma TAPPI T 1206.

2.2 - Pirólise analítica

2.2.1 – Introdução

A pirólise analítica é uma técnica que transforma compostos macromoleculares numa mistura de produtos voláteis por degradação térmica na ausência de oxigénio. A separação dos produtos da pirólise por cromatografia gasosa (GC) e a sua identificação por espectrometria de massa (MS) permitem obter informação sobre os compostos originais, nomeadamente o conhecimento das proporções relativas das suas unidades monoméricas constituintes (Meier e Faix 1992). A pirólise é executada normalmente através do aquecimento rápido numa atmosfera inerte, ou em vácuo, dos compostos a analisar (Galletti 1995). Geralmente, a pirólise realiza-se a temperaturas acima de 300ºC, mais frequentemente entre 500ºC e 800ºC (Moldoveanu 1998). O aquecimento rápido da amostra à temperatura de pirólise é um dos parâmetros mais importantes na obtenção da fragmentação térmica de forma a evitar reacções secundárias. A temperatura de pirólise deve ser suficientemente elevada para assegurar a degradação térmica das macromoléculas, para evitar reacções secundárias de recombinação, e as partículas fragmentadas devem ser removidas rapidamente da zona de pirólise. Isto é normalmente conseguido com a passagem de um gás inerte (hélio), que serve simultaneamente como gás de arraste na separação cromatográfica da análise por GC-MS (Meier e Faix 1992).

A informação obtida por pirólise analítica pode ser quantitativa, qualitativa ou estrutural. No entanto, a pirólise (Py nas iniciais inglesas) por si só não fornece nenhuma informação analítica acerca da estrutura inicial do material, a não ser que esteja associada a técnicas cromatográficas ou espectroscópicas (Moldoveanu, 1998). Os produtos da pirólise são, separados por cromatografia gasosa (GC) e identificados por espectrometria de massa (MS), originando um pirograma típico para cada amostra. A natureza dos fragmentos pode ser identificada pelos seus tempos de retenção e pelo seu espectro de massa (Rodrigues 1998).

A pirólise analítica apresenta as seguintes vantagens (Meier e Faix 1992):
1. a quantidade muito reduzida de amostra necessária ($\approx$ 75 μg);
2. a simplicidade de preparação das amostras, uma vez que só têm de ser submetidas a secagem e moagem;
3. o reduzido período de tempo que a análise requer (de escassos minutos até uma hora e trinta minutos);
4. a fácil identificação dos produtos da pirólise, a partir do momento em que existam bibliotecas dos espectros de massa dos compostos em causa.

A pirólise dos materiais lenhocelulósicos como a madeira origina produtos característicos dos polissacáridos e da lenhina. Relativamente a esta última é possível identificar os produtos característicos dos monómeros *p*-hidroxifenilo, guaiacilo e siringilo. A partir desta identificação é também possível quantificar a sua proporção relativa no material inicial, obtendo-se, deste modo, informação acerca da composição monomérica da lenhina.
Meier e Faix (1992) resumem a utilização da pirólise analítica para a caracterização de materiais lenhocelulósicos, referindo-se aos seguintes aspectos:
1. classificação do tipo de lenhina com base na presença e proporção relativa dos três percursores da lenhina, nomeadamente, *p*-hidroxifenilo, guaiacilo e siringilo;
2. microanálise de lenhina em células de madeira e em fragmentos de células de origem diferente;
3. análise de lenhina residual em pastas;

4. identificação e impressão digital de lenhinas de diversos processos de obtenção de pastas.

2.2.2 – Identificação dos produtos de pirólise

Os produtos da pirólise são identificados a partir dos respectivos espectros de massa de impacto electrónico. Os compostos pirolisados podem ser identificados pelos espectros de massa, ou mais frequentemente, por comparação com os espectros de padrões dos compostos em causa. A identificação dos produtos de pirólise está hoje facilitada pela existência de bibliotecas (NIST98 e Wiley) específicas de espectros de massa, e pela existência de diversas compilações desses produtos acompanhados dos respectivos espectros de massa, e dos tempos de retenção de acordo com o tipo de coluna e condições cromatográficas utilizadas.

A identificação dos produtos de pirólise derivados dos polissacáridos por espectrometria de massa é um processo complexo, devido aos seguintes aspectos: a fácil fragmentação dos seus produtos (usando o método de ionização por impacto de electrões) sendo dificultada a sua identificação devido à ausência dos iões moleculares, os espectros de massa dos produtos derivados dos polissacáridos são menos específicos, existem compostos diferentes, com espectros de massa semelhantes, e existem ainda isómeros com espectros de massa idênticos. Pelo contrário, os produtos da lenhina são normalmente identificados de forma inequívoca devido à abundância dos seus iões moleculares.

Faix *et al*. (1990 a, b) publicaram a abundância relativa dos principais fragmentos dos espectros de massa dos compostos derivados da lenhina e os respectivos tempos de retenção, bem como o espectro de massa dos 82 produtos da pirólise de materiais lenhocelulósicos sem isolamento prévio. O pirolisado foi separado numa coluna capilar DB-1701 (J&W Scientific) de 30 metros, com 0,25 mm de diâmetro interno e de 0,25 μm de espessura de filme.

Ralph e Hatfield (1991) publicaram os espectros de massa de 130 produtos de pirólise, derivados da lenhina e dos polissacáridos da *Medicago sativa* L. e do *Bromus inermus* L. O pirolisado foi separado numa coluna capilar DB-1 de 60 metros, com 0,25 mm de diâmetro interno.

Faix *et al.* (1991 a, b) publicaram os espectros de massa e os respectivos tempos de retenção de 104 produtos derivados dos polissacáridos. O pirolisado foi separado numa coluna capilar DB-1701 de 30 metros, com 0,25 mm de diâmetro interno e 0,25 μm de espessura de filme.

2.2.3 – Análise quantitativa

A pirólise analítica tem sido pouco utlizada para a análise quantitativa de materiais lenhocelulósicos embora alguns autores tenham mostrado o seu potencial como alternativa aos métodos de análise de química húmida. Destes trabalhos a maioria incidiu sobre a caracterização da composição da lenhina da madeira e de MWLs (YoKoi *et al*. 1999; Rodrigues *et al*. 1999; Rodrigues *et al*. 2001; del Rio *et al*. 2001; Kuroda *et al*. 2002; Barbosa *et al*. 2008; Lima *et al*. 2008; Nunes *et al*. 2010).

A utilização da pirólise analítica para a determinação do teor de lenhina (Kleen e Gellerstedt 1991; Kleen *et al*. 1993) e de monossacáridos (Kelly e Helleur 1992; Kleen *et al*. 1993; Syverud *et al*. 2003) foi utilizada sobretudo em pastas.

Poucos trabalhos referem a utilização da pirólise analítica para a determinação do teor de lenhina (Sonoda *et al*. 2001; Rodrigues *et al*. 2001; del Rio *et al*. 2001) e de polissacáridos (Rodrigues *et al*. 2001; del Rio *et al*. 2001) em madeiras.

Uma possível explicação para a falta de aplicação generalizada da pirólise analítica pode estar ligada ao facto de, apesar da simplicidade e da exigência de tempo muito inferiores à dos métodos de química húmida (Meier e Faix 1992; Rodrigues *et al*. 2001), o rendimento da pirólise em termos do número de análises por dia é muito limitado, sobretudo, quando é necessário analisar um número elevado de amostras. Contudo a pirólise analítica tem um grande potencial para ser utilizada como método de referência para a calibração da espectroscopia de infravermelho próximo.

2.3 - Espectroscopia de infravermelho próximo

A radiação no infravermelho próximo corresponde à gama de números de onda que vão desde 12 000 aos 4000 cm^{-1}, tendo sido a primeira radiação a ser detectada fora da região do visível. Esta descoberta remonta a 1800, quando Herschel procurava descobrir qual era a cor responsável pelo aquecimento. Usando um prisma de vidro equipado com termómetros, detectou a existência de radiação na gama de energias imediatamente abaixo da região visível, próximo do vermelho, do espectro solar (Herschel, 1800). O interesse na espectroscopia NIR como técnica analítica iniciou-se muito mais tarde, nos anos 70 (séc. XX), com os trabalhos de Norris e colaboradores para análise de produtos agrícolas incluindo o desenvolvimento de um aparelho para medir o teor de humidade em cereais (Osborne *et al*. 1993; Williams e Norris 2001).

A espectroscopia de infravermelho próximo (NIR), baseia-se na alteração do estado vibracional das moléculas por interacção com um feixe de fotões. O espectro de NIR é o resultado da absorção de combinações de banda (5200 - 4000 cm^{-1}) e sobretons (12000 - 5000 cm^{-1}) das vibrações fundamentais CH, NH OH e SH (Siesler *et al*. 2002). Estes grupos funcionais estão presentes em todas as moléculas biológicas, o que a torna uma técnica universal.

A informação contida nos espectros NIR é multivariada, composta por bandas largas e fortemente sobrepostas e é influenciada por um conjunto de variáveis físicas, químicas e estruturais das amostras. As interacções entre átomos em moléculas diferentes, como as ligações por pontes de hidrogénio, alteram os estados vibracionais, provocando desvios nas bandas de absorção e originando outras através de diferenças na estrutura cristalina. Isto permite distinguir formas cristalinas e determinar propriedades físicas (densidade, viscosidade e dimensões das partículas em sólidos granulares). Por outras palavras, o espectro NIR contém não só informação química como também contém informação física que se pode usar para a caracterização química e física das amostras.

A informação física contida nos espectros NIR, quer tenha origem nas amostras ou no processamento dos espectros (grau de compactação) e que permitem a utilização do NIR para a caracterização física de amostras, é um inconveniente, por interferirem com a informação da composição química. Esta interferência tem que ser removida ou minorada recorrendo ao pré-tratamento da informação espectral (Geladi *et al*. 1985; Osborne 1988; Krivácsy e Hlavay 1994; Martens *et al*. 2003). Os pré-tratamentos mais correntemente utilizados em espectroscopia incluem: correcção da linha de base, correcção multiplicativa de dispersão (MSC), derivadas (1ª e 2ª), normalização vectorial e combinações destes. O pré-tratamento óptimo deve ser encontrado empiricamente, depois de se terem testado todos os pré-tratamentos e comparados os resultados.

Para se extrair informação útil dos espectros NIR normalmente é necessário recorrer à análise multivariada (Massart. *et al*. 1988; Geladi 2003; Geladi *et al*. 2004). A informação analítica contida nos espectros NIR pode ser obtida por diferentes métodos de análise multivariada, tais como a regressão múltipla ou o método das componentes principais (PCA) ou, ainda, o método de regressão dos mínimos quadrados parciais (PLS) (Geladi *et al*. 2004). Estes métodos permitem, quer agrupar amostras semelhantes, e desse modo estabelecer métodos de classificação, quer relacionar a informação espectral com as propriedades dos analitos.

A espectroscopia NIR é uma técnica indirecta que requer calibração, isto é, estabelecer a relação (modelo) entre a informação espectral e a informação obtida com métodos de referência nas mesmas amostras (amostras de calibração). Os modelos assim obtidos têm depois que ser validados. A regressão pelo método dos mínimos quadrados parciais (PLS) é o principal método usado para modelar essa relação.

A análise de componentes principais (PCA) é uma técnica descritiva que permite encontrar estruturas subjacentes nos dados, estudar a correlação entre variáveis, detectar outliers ou observações extremas (Wold *et al*. 1987; Malinowski 1991).

Qualquer que seja o método a utilizar, a principal dificuldade é a escolha do número de componentes significativas: se insuficientes, os modelos não explicam toda a variância de interesse: se demasiadas, modelam sobretudo ruído que não tem informação relevante sobre a propriedade de interesse. A maioria dos softwares disponíveis sugere o número de componentes principais significativas, baseado na evolução dos erros quadráticos médios da validação cruzada. Este método, também conhecido por validação interna, é normalmente a única forma de validação quando o número de amostras é muito pequeno. No entanto, nestas situações deve usar-se os modelos com muita precaução. A única forma de validar realmente os modelos é usando um novo conjunto de amostras e comparar os valores preditos pelo modelo (NIR) com os dados de referência (validação externa).

2.3.1 – Aplicações da espectroscopia de infravermelho próximo NIR

A utilização da espectroscopia de infravermelho próximo (NIR) no sector florestal iniciou-se mais de 25 anos depois das primeiras aplicações do NIR no sector agro-alimentar, para a determinação do teor de humidade de cereais (Hart *et al*. 1962; Norris e Hart 1965).

As primeiras aplicações do NIR no sector florestal foram na caracterização química de pastas, nomeadamente a determinação do teor de lenhina (Birkett e Gambino 1989; Schultz e Burns 1990; Easty *et al*. 1990) de hemiceluloses e de celulose (Schultz e Burns 1990) e do rendimento em pasta (Wright *et al*. 1990). O interesse na utilização da técnica foi lento mas progressivo; passados 10 anos surgiu um artigo sobre a aplicação do NIR à investigação florestal e apenas são citados 8 artigos (Schimleck *et al*. 2000). Em 2004, uma revisão sobre a aplicação do NIR na indústria florestal cita 34 artigos (So *et al*. 2004). Apenas 3 anos depois, o número de citações da aplicação do NIR para a madeira e pasta chegou a 139 (Tsuchikawa 2007).

Neste trabalho, as aplicações do NIR foram separadas quanto ao tipo de aplicação que, para além da caracterização química de madeiras, incluíam a caracterização física: teor de humidade, densidade, ângulo de inclinação do fio e rugosidade superficial da madeira; caracterização mecânica: módulo

de elasticidade e resistência à tracção; parâmetros anatómicos: ângulo microfibrilar, comprimento dos traqueídos, espessura da parede celular e massa linear.

No que diz respeito apenas à determinação do teor e composição da lenhina da madeira, o número de trabalhos citados por Tsuchikawa (2007) não era muito elevado. No caso do teor de lenhina, embora não fossem apresentadas separadamente, estes incluíam quer aplicações na madeira moída (Schimleck *et al*. 1997; Bailleres *et al*. 2002; Hodge e Woodbridge 2004; Poke *et al*. 2004; Terdwongworakul *et al*. 2005) quer directamente na madeira sólida (Yeh *et al*. 2004; Kelley *et al*. 2004; Poke e Raymond 2006).

No que diz respeito à composição da lenhina, Tsuchikawa (2007) cita apenas dois trabalhos um para a determinação da razão S/G em eucalipto (Bailleres *et al*. 2002) e o outro para a determinação da razão H/G em pinheiro (Alves *et al*. 2006). Recentemente mais dois trabalhos foram publicados sobre a determinação da razão S/G em choupo (Maranan e Laborie 2008) e em eucalipto (Hein *et al*. 2010).

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3 – Trabalho prático

Artigo I

Analytical pyrolysis as a direct method to determine the lignin content in wood
Part 1: Comparison of pyrolysis lignin with Klason lignin

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Analytical pyrolysis as a direct method to determine the lignin content in wood
Part 1: Comparison of pyrolysis lignin with Klason lignin

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Abstract

In this work a method for the quantification of the lignin content (Py-lignin) of Maritime pine and spruce wood samples directly from the pyrograms is presented. The precision of the Py-lignin method was 0.41% for Maritime pine (*Pinus pinaster* Aiton) and 0.34% for spruce (*Picea abies* (L.) Karst), close to the stated repeatability of the reference Klason method (0.34%). Besides apparent species-specific deviations, an average difference of 3.9 ± 0.72% compared to the Klason lignin content was observed. Py-lignin retains between 70 (Maritime pine) and 89% (spruce) of the variation in the lignin amount found for the same samples by the Klason lignin method.

Good correlation was found between the Py-lignin and Klason lignin content, for both species but a good common model to both species was also found, which allows to calculate the Klason lignin content from Py-lignin content for both species using only one model.

Keywords: Analytical pyrolysis; Klason lignin; Lignin; *Pinus pinaster*; *Picea abies*; Py-lignin

1. Introduction

Analytical pyrolysis is being increasingly used as a quantitative method in the wood and pulp field to assess chemical composition of lignocellulosic materials. The main advantages of analytical pyrolysis over classical wet-chemical methods are an easy sample preparation (drying and milling), rapid analysis times and small sample sizes required (micrograms range) [1]. The fact that the structure of the major pyrolysis products of lignocellulosic materials are elucidated [2–7] also contributes to the spread of the technique.

Analytical pyrolysis was used to quantitatively assess content of carbohydrates [8–10], lignin [9,11] and extractives [17], as well as lignin composition [12–16] in wood and pulp.

Different approaches have been used to relate analytical pyrolysis information with classical wet-chemical data. In some cases a direct comparison of peak areas of characteristic pyrolysis products was used to determine carbohydrates and lignin in pulps [8,10,11,18]. This is the most often used method when the purpose is characterization rather than quantification. Calibration against wet-chemical data using multivariate techniques was used to assess carbohydrates and lignin in wood and pulps [2,9,19]. Absolute quantification of pyrolysis products using internal standards was also used both in on-line [20,21] and off-line systems [22].

The ratio of the sum of the lignin pyrolysis products relative to the sum of all pyrolysis products was thought-out to be correlated to the Klason lignin content. In this work we will refer to this ratio (sum of lignin products/sum of all pyrolysis products multiplied by 100%) as Py-lignin. Py-lignin was already used as an estimator of lignin amount in wood [14]. Two questions are raised: How does Py-lignin compare with the classical Klason lignin determination and How much of the original Klason lignin content variation is retained by using this procedure.

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In this work a method for the direct and precise quantification of the lignin content directly from the pyrogram is presented. The results of this method (Py-lignin) are further compared with the results obtained with the Klason lignin method for several *Pinus pinaster* and *Picea abies* wood samples.

2. Experimental

2.1. Sampling

A total of 81 (12–14 years old) Maritime pine (*P. pinaster* Aiton) wood discs (25-mm wide) from Blagon and Vacquey (France [23]) was collected between 1.3 and 2.0 m height of each tree. The discs were ground with a Thomas–Wiley mill model ED-5 to pass a 1 mm sieve, screened in a vibratory sieving apparatus and the 40–60 mesh wood meal fraction kept for analysis. The samples were successively extracted 16 h with water followed by a 12-h acetone extraction in a Soxhlet apparatus and dried at 60 °C over night. The Klason lignin content of these samples, determined using the sulphuric acid method [24], ranged from 25.8 to 35.3% based on oven-dry mass of extractive free material.

An aliquot of about 200 mg of each sample, used for pyrolysis, was milled in a vibratory ball mill (Mixer Mill MM, Retsch) for 30 min and kept in a desiccator until analysis.

A total of 57 spruce (*P. abies* (L.) Karst) wood samples (milled trough a sieve with a 80 μm hole width) was taken according to the Klason lignin content ranging from 24.9 to 29.5% based on oven-dry mass of extractive free material [24].

2.2. Analytical pyrolysis

Analytical pyrolysis (Py-GC/FID) was performed with a CDS Pyroprobe 1000 with a coil filament connected to a HP 5890 series II by a heated interface (270 °C). Each sample (75–80 μg) was pyrolyzed at 650 °C for 10 s with a temperature rise time of approximately 20.0 °C ms^{-1}. Capillary column: DB1701 (60 m × 0.25 mm, 0.25 μm film, J&W Scientific). GC conditions: injector 250 °C, detector 280 °C, temperature program 45 °C, 4 min isothermal, then heating rate 4–270 °C min^{-1}. The identification of pyrolysis products was performed previously using selected samples by Py-GC/MS (CDS Pyroprobe 1000 connected to a HP 6890 with a HP 5973 Mass Selective Detector). Products were identified by their mass spectra and retention time by comparison with NIST

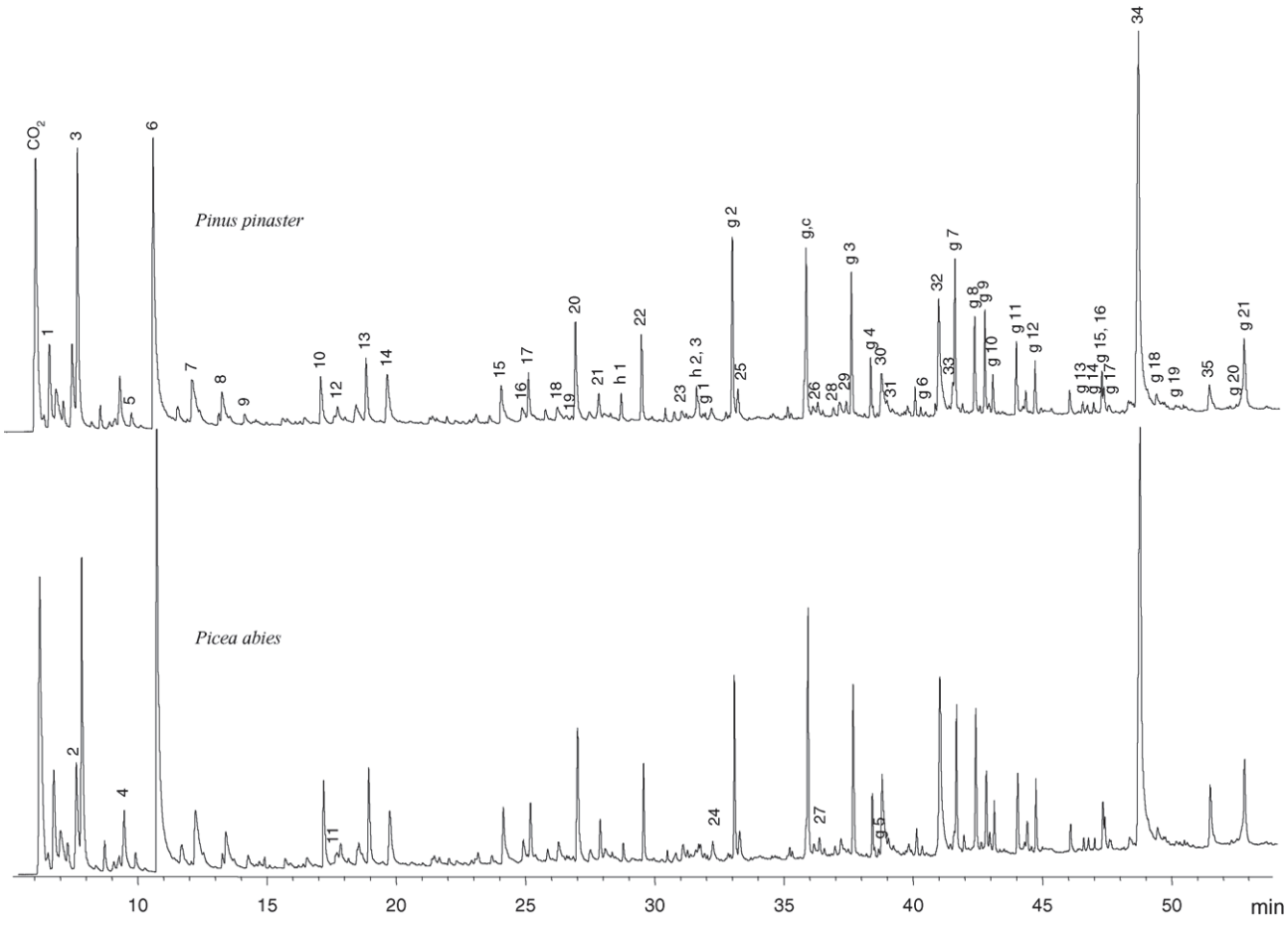

Fig. 1. Pyrograms of *Pinus pinaster* and *Picea abies*. The compounds belonging to the labeled peaks are listed in Table 1.

Table 1
The identification of the pyrolysis products used for the quantification

Label	RT (min)	Compound	Pine (%)	Spruce (%)
1	6.7	Acetaldehyde	2.9	2.7
2	7.6	2-Propenal	2.9	2.9
3	7.8	Propanal-2-one	7.4	7.3
4	9.5	2,3-Butanedione	1.9	1.8
5	9.9	Unknown	0.7	0.5
6	10.7	Hydroxyacetaldehyde	11.6	13.1
7	12.2	Acetic acid	3.0	2.9
8	13.4	Hydroxypropanone	1.9	1.5
9	14.3	Unknown	0.4	0.4
10	17.2	3-Hydroxypropanal	1.5	1.9
11	17.7	3-Butenal-2-one	0.3	0.7
12	17.9	(3H)-Furan-2-one	0.6	0.9
13	19.0	2-Hydroxy-3-oxobutanal	2.4	2.7
14	19.8	2-Furaldehyde	2.3	2.0
15	24.2	Dihydro-methyl-furanone	1.9	1.8
16	25.0	Dihydro-methyl-furanone	0.9	0.6
17	25.2	Isomer of compound #20	1.0	1.0
18	26.3	(5H)-Furan-2-one	1.1	0.8
19	26.9	Gamma-lactone and unknown	0.2	0.1
20	27.0	4-Hydroxy-5,6-dihydropyran-(2H)-2-one	2.6	2.6
21	27.9	2-Hydroxy-1-methyl-cyclopenten-(1)-3-one	1.1	0.9
h 1	28.8	Phenol	0.7	0.4
g 1	29.6	Guaiacol	2.1	1.6
23	31.1	Methyl-butyraldehyde derivative	0.5	0.5
h 2, 3	31.7	*m*-/*p*-Cresol	0.9	0.6
g 2	32.0	3-Methyl guaiacol	0.1	0.1
24	32.3	Gamma-lactone derivative	0.7	0.7
g 3	33.1	4-Methyl guaiacol	2.7	2.6
25	33.3	Anhydrosugar	0.9	0.8
g/c	35.9	Overlapping substances; 4-ethyl guaiacol and unknown	3.4[a]	4.2[a]
26	36.2	Unknown	0.3	0.4
27	36.4	Unknown	0.5	0.4
28	37.0	1,4:3,6-Dianhydro-glucopyranose	0.4	0.2
29	37.5	1,5-Anhydro-arabinofuranose	0.2	0.2
g 4	37.7	4-Vinyl guaiacol	2.9	2.5
g 5	38.4	Eugenol	0.9	0.8
g 6	38.5	4-Propyl guaiacol	0.2	0.1
30	38.8	5-Hydroxymethyl-2-furaldehyde	2.0	2.2
31	39.3	Gamma-lactone derivative	0.3	0.3
g 7	40.3	Isoeugenol (cis)	0.2	0.2
32	41.0	2-Hydroxymethyl-5-hydroxy-2,3-dihydro-(4H)-pyran-4-one	3.2	4.9
33	41.6	1,5-Anhydro-β-D-xylofuranose	1.0	0.3
g 8	41.7	Isoeugenol (*trans*)	2.0	2.2
g 9	42.4	Vanillin	1.9	2.3
g 10	42.8	G–C═C═C	1.6	1.4
g 11	43.1	G–C═C═C	0.6	0.8
g 12	44.0	Homovanillin	1.5	1.1
g 13	44.8	Acetoguaiacone	1.0	1.0
g 14	46.6	Guaiacyl acetone	0.5	0.2
g 15	47.0	Propioguaiacone	0.3	0.2
g 16	47.3	Isomer of coniferyl alcohol	0.6	0.6
g 17	47.4	G–CO–CH═CH_2	0.5	0.5
g 18	47.6	G–CO–CO–CH_3	0.2	0.2
34	48.7	1,6-Anydro-β-D-glucopyranose (levoglucosan)	12.5	11.1
g 19	49.4	Dihydroconiferyl alcohol	0.7	0.8
g 20	50.2	Coniferyl alcohol (*cis*)	0.3	0.2
35	51.5	Anhydrosugar: unknown	0.6	1.5
g 21	52.5	Coniferyl alcohol (*trans*)	0.4	0.1
g 22	52.8	Coniferylaldehyde	2.5	2.4
Total			100	100

RT: retention time.
[a] These peaks were not included in calculation.

library and with literature [3–7]. At least two runs were performed per sample.

2.3. Quantification

Py-lignin was calculated with Chemstation Software (Agilent Technologies, Palo Alto, USA) as the ratio of the sum of the areas of the peaks from lignin products divided by the sum of the area of all used peaks (lignin and polysaccharides, ca. 75% of the total area) multiplied by 100%. The 25% of the area not used for the calculation originate from CO_2 (40%), an overlapping peak from polysaccharides and lignin (25%), and minor products (35%). The retention time employed for quantification ranged from 6 to 53 min.

2.4. Precision of the analytical pyrolysis method

The precision of the method was assessed by the pooled standard deviation and also by repetition measurements of the same sample on the same day, and on different days, weeks and months.

3. Results and discussion

3.1. Pyrolysis

Two typical pyrograms of Maritime pine and spruce wood are presented in Fig. 1. Peaks labeled with numbers are of polysaccharide origin and the peaks labeled with letters are of lignin origin. The identification of the pyrolysis products used for the quantification is presented in Table 1. The two pyrograms are similar, the total number of peaks is the same and the difference between them is in the relative proportion of the peaks. The two main peaks that were not used for the quantification are the first peak (CO_2) and the peak labeled as g/c an overlapping peak from polysaccharides and lignin.

3.2. Precision of the method

Analytical pyrolysis proved to be a precise method for the determination of lignin (Py-lignin) with a low pooled standard deviation both for Maritime pine (0.41%) and spruce (0.34%). The standard deviation of repeated measurements of the same samples over different days, weeks and months gave the same result as the pooled standard deviation. These results are close to the stated repeatability (0.34%) of the Klason lignin method according to the standard test method (TAPPI T 222 om-88).

3.3. Py-lignin versus Klason lignin content

The Py-lignin and Klason lignin contents determined for Maritime pine and spruce wood listed in Table 2 show, besides apparent species-specific deviations, an average difference of $3.9 \pm 0.7\%$ compared to the Klason lignin content. One possible explanation lies in the fact that no individual response factors or correction factors [20] were used for quantitative evaluation. Therefore Py-lignin (the sum of lignin pyrolysis products divided by lignin and carbohydrate pyrolysis products) cannot truly reflect the ''real'' lignin content in the samples.

Table 2
Obtained ranges for Py-lignin and Klason lignin contents of Maritime pine (*Pinus pinaster*) and spruce (*Picea abies*) wood

Species (%)	*Pinus pinaster*	*Picea abies*	*Pinus* + *Picea*
Py-lignin	23.0–29.6	21.6–25.7	21.6–29.6
Py-lignin range	6.6	4.1	8
Klason lignin	25.8–35.3	24.9–29.5	24.9–35.3
Klason lignin range	9.5	4.6	10.4
Klason–Py average	4.2	3.5	3.9
Standard deviation	0.78	0.34	0.72
Retained variability	70	89	77

Retained variability (Py-lignin range/Klason lignin range × 100%).

The use of internal standards used by others [20–22] for quantitative analysis of pyrolysis products was avoided, not only to keep the method as simple as possible, but also because no improvement was expected since the ratio of the sum of lignin and lignin and carbohydrate type pyrolysis products was used here. Analytics is always a question of fitness for purpose and the use of internal standards is important if the absolute quantity of a single product is needed. However, the main objective of the presented study was to develop a precise direct method to assess the lignin content that only needs few micrograms of sample and can deliver the results in a shorter analysis time compared to the reference Klason lignin method.

The absolute amount of lignin is not crucial for comparative analysis of samples; often it is enough to get the samples correctly scaled according to the amount of the parameter of interest. Nevertheless, the question of absolute values is interesting and therefore a comparison between the Py-lignin content and Klason lignin content was performed for both species.

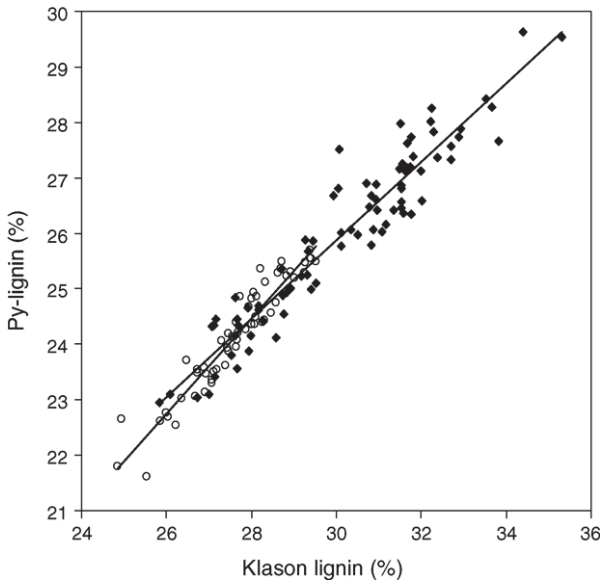

Fig. 2. Py-lignin vs. Klason lignin content of spruce (hollow circle) and Maritime pine (filled diamond) wood.

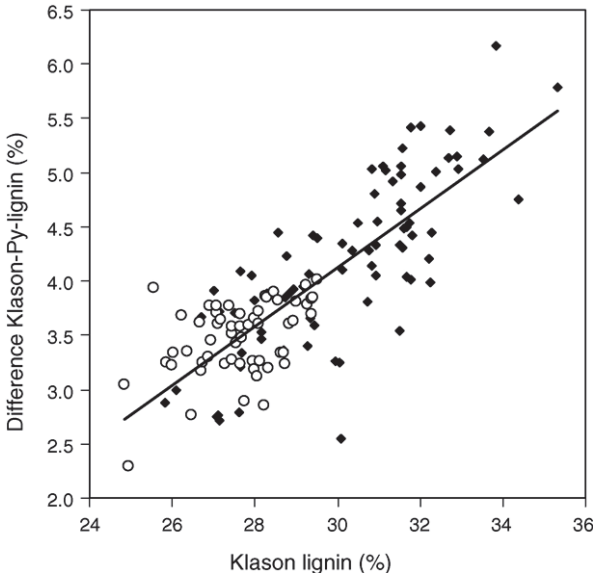

Fig. 3. Difference between Klason lignin and Py-lignin content vs. Klason lignin content. Maritime pine (filled diamond) and spruce (hollow circle).

Good correlations between Py-lignin results and those corresponding to acid insoluble lignin (Klason lignin) were obtained for both Maritime pine and spruce (Fig. 2). The coefficient of correlation obtained for the two species was high ($R = 0.95$), although slightly different slopes were obtained 0.86 (spruce) and 0.71 (Maritime pine). The second question raised in the introduction, how much of the original Klason lignin content variation is retained by using this procedure (Table 2), can now be answered: about 89% (spruce) and 70% (Maritime pine), which means that the true amount and the true variation are underestimated compared to the Klason lignin method. Nevertheless, the high correlation found allows calculating the true lignin amount with high accuracy using the regression equation obtained.

The scatter-plot of the difference between Klason lignin and Py-lignin content versus Klason lignin content shown in Fig. 3 reveals that the deviation from the reference value increases with increasing lignin content with a high correlation ($R = 0.81$), which could also be concluded from Fig. 2 and Table 2. Additionally different slopes of the regression lines were obtained for Maritime pine and spruce wood (Fig. 2).

The combination of the results of all samples (Maritime pine and spruce) led also to a good model ($R^2 = 0.93$ and Py-lignin = $0.7286 \times$ Klason lignin + 4.0132) (not shown). The average deviations between this model and the individual models presented in Fig. 2 are 0.08% for Maritime pine and −0.11% for spruce.

The relevance of this fact is that it is possible to estimate the Klason lignin content from the Py-lignin data for both species using the same model. This model could possibly be used for other softwoods too, which is speculative and has to be proved. Moreover, in principle the Py-lignin method should also be applicable to the quantification of lignin in hardwoods and grass. The remaining question is if the slope of the regression line is similar or different from the one reported in this work.

4. Conclusions

This study revealed that analytical pyrolysis can be used to assess the lignin amount in Maritime pine and spruce wood samples with a precision comparable to that of the reference Klason method. A good correlation was found between the Py-lignin and Klason lignin content, which allows calculating the Klason lignin content from Py-lignin content. Besides apparent species-specific deviations, an average difference of $3.9 \pm 0.72\%$ compared to the Klason lignin content was observed. About 89% (spruce) and 70% (Maritime pine) of the variability on the lignin content based on the Klason lignin method is retaining by using Py-lignin data.

Acknowledgements

The work was supported by funding from the EU (research projects GENIALITY-FAIR CT98 3953 and GEMINI-QLRT-1999-0942) and Fundação para a Ciência e Tecnologia (Portugal), under POCTI and FEDER programs (research projects POCTI/AGR/33967/99 and POCTI/ AGR/47353/ 2002) and was integrated in the activities of BIOPOL in Centro de Estudos Florestais (Portugal).

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Artigo II

Analytical pyrolysis as a direct method to determine the lignin content in wood
Part 2: Evaluation of the common model and the influence of compression wood

Alves A, Rodrigues J, Wimmer R, Schwanninger M

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Analytical pyrolysis as a direct method to determine the lignin content in wood
Part 2: Evaluation of the common model and the influence of compression wood

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Abstract

In Part 1, a method for the quantification of the lignin content (Py-lignin) of Maritime pine and spruce wood samples directly from the pyrograms was presented (A. Alves, M. Schwanninger, H. Pereira, J. Rodrigues, J. Anal. Appl. Pyrol. 76 (2006a) 209). The good correlation found between the Py-lignin and Klason lignin content gave a common model to both species.

In this work different larch species (*Larix* sp.) as well as varieties of European larch were used to evaluate this common model, revealing only small differences between the measured and the predicted Klason lignin contents. Compression wood was included due to the difference in lignin composition and content compared to normal wood. As the influence of compression wood was small a so-called ''softwood model'' including all samples was calculated (Py-lignin = 0.7325 × Klason lignin + 3.9195, $R^2 = 0.94$).

This can be used for pine, larch, and spruce wood with the limitation of the highest and lowest values where the species-specific models lead to better results, although more than 95% of the differences between the species-specific models and the ''softwood model'' lie within ±0.3%. It is expected that this model can predict the Klason lignin content of other softwoods.

Keywords: Analytical pyrolysis; Compression wood; Klason lignin; *Larix* sp.; *Pinus pinaster*; *Picea abies*; Py-lignin

1. Introduction

Analytical pyrolysis is being increasingly used as a quantitative method in the wood and pulp field to assess chemical composition of lignocellulosic materials [1–4]. The main advantages of analytical pyrolysis over classical wet-chemical methods are an easy sample preparation (drying and milling), rapid analysis times and small sample sizes required (μg range) [5]. Analytical pyrolysis data were related with classical wet-chemical data by direct comparison of peak areas of characteristic pyrolysis products [6–9], calibration using multivariate techniques [10,11], and absolute quantification of pyrolysis products using internal standards [12–14].

In Part 1, a method for the quantification of the lignin content (Py-lignin) of Maritime pine and spruce wood samples directly from the pyrograms was presented [1]. Good correlation was found between the Py-lignin and Klason lignin content for each species as well as a good common model to both species. In this work, the Py-lignin content of larch wood samples with known Klason lignin content was determined, which was further used to evaluate this common model. It was hypothesized that this

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common model that includes pine and spruce wood is also valid for other softwoods such as larch. Thereafter a ''softwood model'' including larch wood was calculated. Furthermore, the influence of so-called compression wood, a type of reaction wood formed under mechanical stresses [15], was assessed on the basis of existing differences in lignin composition and content, as compared to normal wood.

2. Experimental

The pine and spruce wood samples as well as the analytical pyrolysis analysis were previously described [1].

2.1. Sampling

2.1.1. Larch wood samples

A comprehensive sample set of mostly European larch (*Larix decidua*) varieties, of hybrid larch (*Larix eurolepis*) and of Japanese larch (*Larix kaempferi*) was used. Most trees were harvested at an age of 38, however, old-growth larch trees from high elevation were also included. Sampling sites are spread across Europe, further details of the 21 samples are given in Table 1; about sample preparation and processing in [16–19].

2.1.2. Spruce wood samples

One 30-year-old Norway spruce tree (*Picea abies* [L.] Karst) from Austria was harvested and a sample prepared as described by Gindl [20]. The compression wood of this tree with known Klason lignin content [20] was used in the study.

Additionally one wood sample containing compression wood from a 19-year-old spruce tree (*P. abies* (L.) Karst) from Sweden, with a Klason lignin content of 32.1%, based on oven-dry mass of extractive-free material was used [21].

The sample preparation of the extractives-free material prior analytical pyrolysis can be found in Part 1 [1].

2.2. Statistics

The root mean square error of prediction (RMSEP) was calculated as follows:

$$\mathrm{RMSEP} = \sqrt{\frac{\sum_{i=1}^{n} (K_{\mathrm{pred}_i} - K_{\mathrm{meas}_i})^2}{n}},$$

where n is the number of samples, K_{pred} is the Klason lignin content predicted with the common model, and K_{meas} is the measured Klason lignin content.

3. Results and discussion

3.1. Evaluation of a Py-lignin model based on pine and spruce

As shown in Part 1 [1] analytical pyrolysis can be used to predict the lignin content of pine and spruce wood. A good correlation was obtained between Py-lignin and Klason lignin content. It was anticipated that a model using both, pine and spruce wood, could also be valid for other softwood species, which was tested here with larch.

This common model obtained by combining the results of pine and spruce, as reported in Part 1 [1], ($R^2 = 0.93$ and Py-lignin = 0.7286 × Klason lignin + 4.0132; Fig. 1) was taken to predict Klason lignin content.

To evaluate this model 21 larch wood samples with known Klason lignin content (Table 1) were assessed by analytical pyrolysis according to Alves et al. [1]. The Klason lignin content

Table 1
Site, origin, species, age, Klason lignin and Py-lignin content (% extractive-free o.d.w.) of the larch wood samples

Sample	Origin	Growth site	Species-variety	Age	CW	Klason lignin (%)	Py-lignin (%)
342exf	Blizyn (PL)	Elm (DE)	*L. decidua polonica*	38	No	27.6	24.4
343exf	Blizyn (PL)	Elm (DE)	*L. decidua polonica*	38	No	30.7	26.1
326exf	Blizyn (PL)	Elm (DE)	*L. decidua polonica*	38	Low	30.1	25.5
035exf	Hybrid	Clanna (GB)	*L. x eurolepis*	39	Low	29.5	25.4
084exf	Hybrid	Coat-An-Noz (FR)	*L. x eurolepis*	38	Low	30.4	26
056exf	Hybrid	Coat-An-Noz (FR)	*L. x eurolepis*	38	Low	30.9	26.4
352exf	Ina (JP)	Coat-An-Noz (FR)	*L. kaempferi*	38	Low	30.1	25.4
160exf	Langau (AT)	Langau (AT)	*L. decidua alpica*	160	No	28.4	24.8
168exf	Langau (AT)	Langau (AT)	*L. decidua alpica*	160	No	29.1	25.2
235exf	Langau (AT)	Nassogne (AT)	*L. decidua alpica*	38	Low	26.6	23.6
151exf	Langau (AT)	Langau (AT)	*L. decidua alpica*	160	Low	28	24.3
221exf	Montgenevre (FR)	Coat-An-Noz (FR)	*L. decidua alpica*	38	No	28.1	24.7
222exf	Montgenevre (FR)	Coat-An-Noz (FR)	*L. decidua alpica*	38	High	26.8	24.2
217exf	Montgenevre (FR)	Coat-An-Noz (FR)	*L. decidua alpica*	38	High	27.2	24
255exf	Ruda (PL)	Nassogne (BE)	*L. decidua sudetica*	38	No	29.1	25.2
194exf	Ruda (PL)	Elm (DE)	*L. decidua sudetica*	38	No	32	27.2
192exf	Ruda (PL)	Elm (DE)	*L. decidua sudetica*	38	High	29.8	26
321exf	Zabreh (CZ)	Coat-An-Noz (FR)	*L. decidua sudetica*	38	No	27.2	24.2
288exf	Zabreh (CZ)	Nassogne (BE)	*L. decidua sudetica*	38	No	28.6	25.1
285exf	Zabreh (CZ)	Nassogne (BE)	*L. decidua sudetica*	38	Low	28.8	25.6
292exf	Zabreh (CZ)	Nassogne (BE)	*L. decidua sudetica*	38	High	31.6	26.7

CW, compression wood content; exf, extractives-free. Country codes: AT, Austria; BE, Belgium; CZ, Czech Republic; DE, Germany; FR, France; GB, United Kingdom; JP, Japan; PL, Poland.

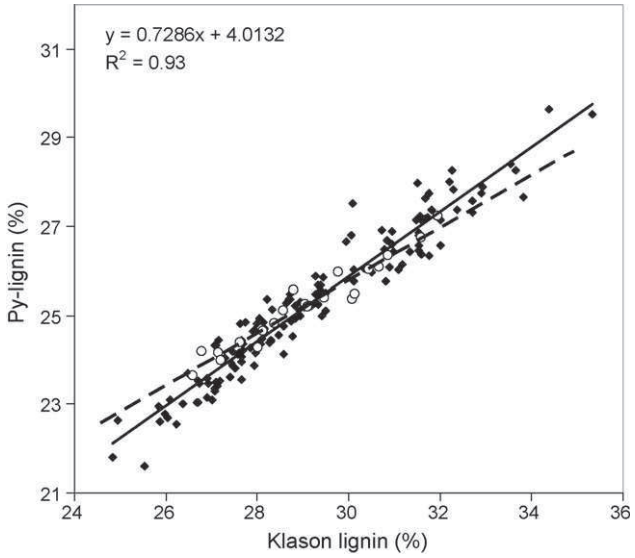

Fig. 1. Py-lignin versus Klason lignin content of pine and spruce samples (closed diamonds). The 21 larch samples are displayed with open circles. The broken line is the tendence line.

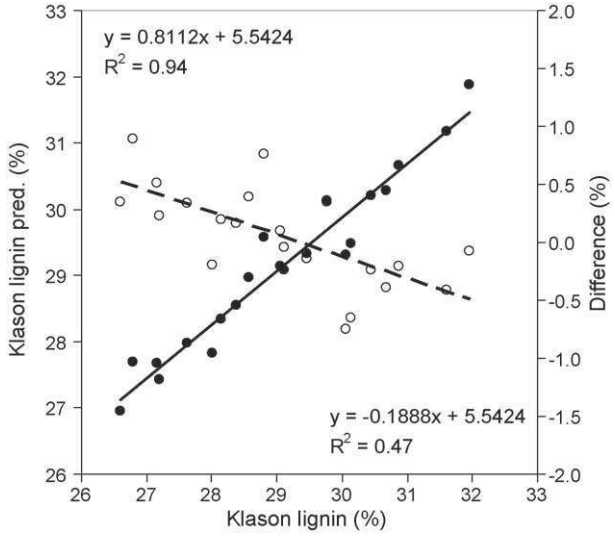

Fig. 2. Klason lignin content of Larch wood predicted with the common model (pine and spruce) versus Klason lignin (filled circles). Differences between predicted and the measured Klason lignin content versus Klason lignin content (open circles).

predicted with the obtained Py-lignin was compared to the measured Klason lignin content (Fig. 2). A good correlation with an R^2 of 0.94 and a slope of 0.8112 was obtained. It is known that Py-lignin increasingly underestimates the Klason lignin content

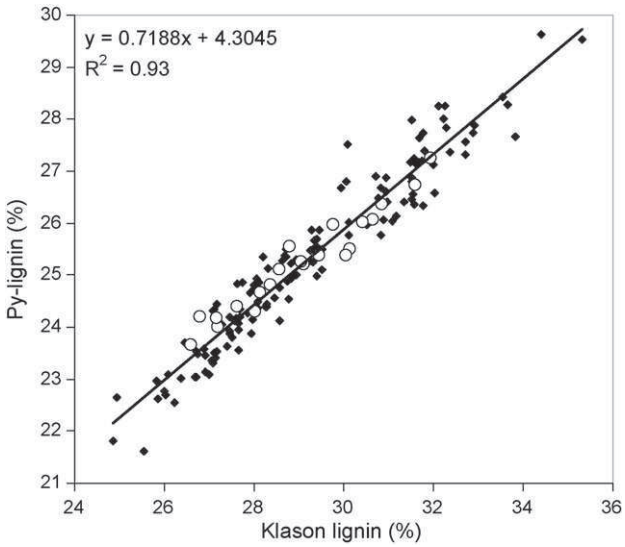

Fig. 3. Py-lignin versus Klason lignin content of pine, spruce, and larch samples. The larch samples are displayed with open circles.

[1] as the values get higher, a slope deviation from one was therefore expected. The slope (0.8112) possibly indicates a deviation of the regression between Py-lignin and Klason lignin of larch wood samples from the one used for prediction.

The differences between predicted and measured Klason lignin content, and the Klason lignin content are negatively correlated (Fig. 2). This was expected due to the lower slope of the tendence line for the larch wood samples compared to the regression line of the common model (Fig. 1).

The root mean square error of prediction of 0.42% is close to the stated repeatability of the Klason lignin TAPPI standard method [22], and 85% of the predicted values lie within ±RMSEP. The standard deviation expressed as a percentage of the mean is 1.45%.

A linear regression model with all samples (pine, spruce and larch), further on called model A, showed almost the same results ($R^2 = 0.93$ and Py-lignin = 0.7188 × Klason lignin + 4.3045; Fig. 3). The larch wood samples are closely positioned to the regression line (Fig. 3, open circles).

3.2. Influence of compression wood

Two spruce samples with compression wood with known Klason lignin content were accessed by analytical pyrolysis and the Klason lignin contents estimated using Py-lignin results and

Table 2
Klason lignin content of two spruce compression wood samples (CW) measured and predicted by the spruce model and model A

Sample	Py-lignin (%)	Klason lignin (%)				
		Measured	Spruce model Py = 0.8606 × Kla + 0.373		Model A Py = 0.7188 × Kla + 4.3045	
			Predicted	Difference	Predicted	Difference
CW1	31.7	37.2	36.4	−0.7	38.1	1.0
CW2	28.3	32.1	32.4	0.3	33.3	1.2

Py, Py-lignin; Kla, Klason lignin.

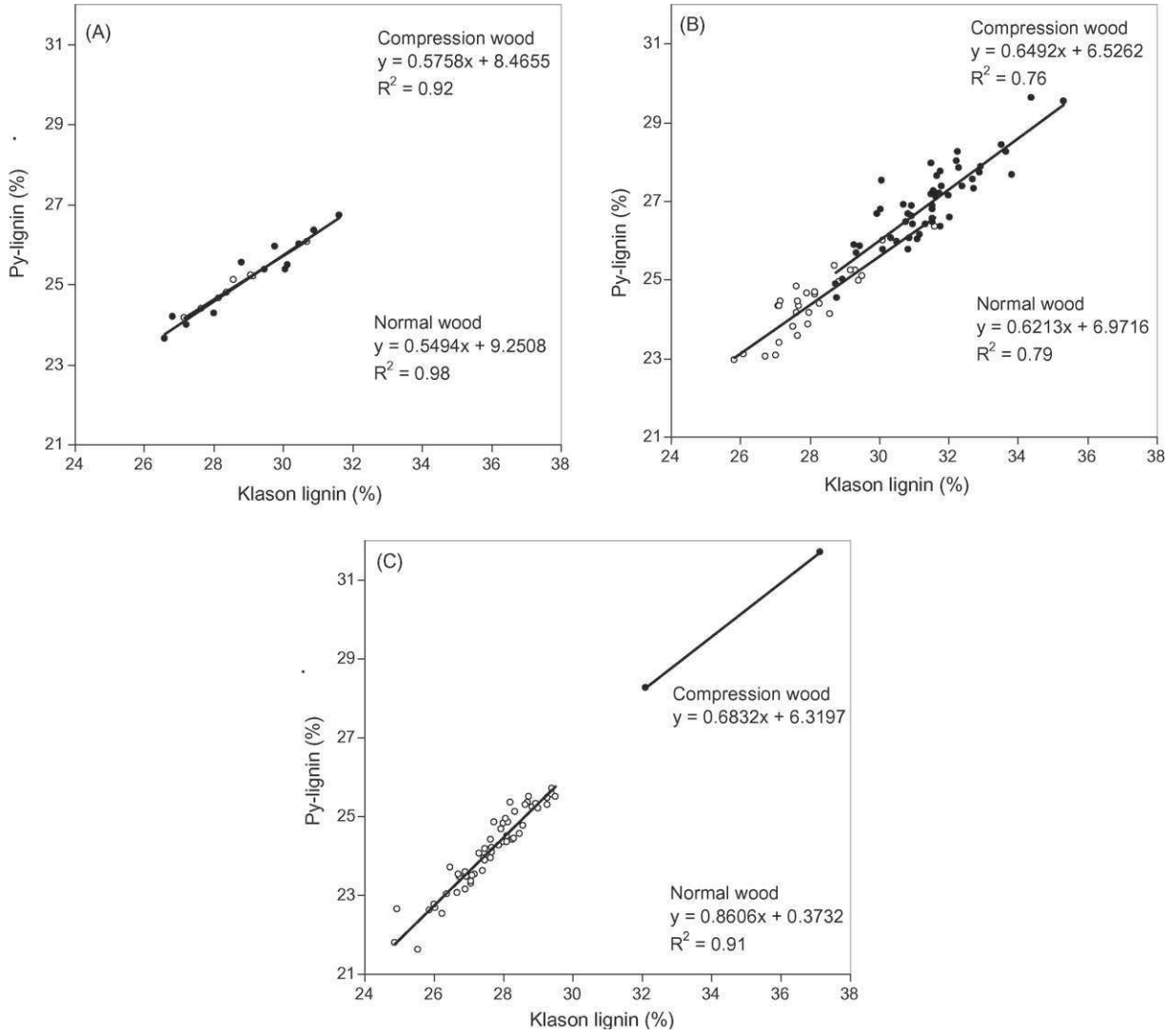

Fig. 4. Py-lignin versus Klason lignin content of larch (A), pine (B), and spruce (C) samples. Normal wood samples are displayed with open circles, compression wood samples with closed circles.

the spruce model [1] as well as the model A (Table 2). The differences between predicted and measured Klason lignin content are smaller for the spruce model than for the model A (Table 2). Since the spruce model does not contain compression wood (CW) samples and the model A contain samples with and without compression wood it was needed to investigate the influence of CW on the correlation between Py-lignin and Klason lignin. The pine and larch data sets were divided in normal wood (NW) and compression wood (Fig. 4).

The main differences were observed in the slope of the linear regression lines between spruce and the other two species. For larch almost identical regression lines were obtained for CW and NW (Fig. 4A). For pine almost identical slopes were obtained for CW and NW with a small shift between them (Fig. 4B). No explanation was found for this shift. Spruce NW slope line shows the highest slope (0.86). The sloping line of spruce CW (Fig. 4C) although only obtained with two samples with high compression wood was closer to the CW slopes of pine and larch.

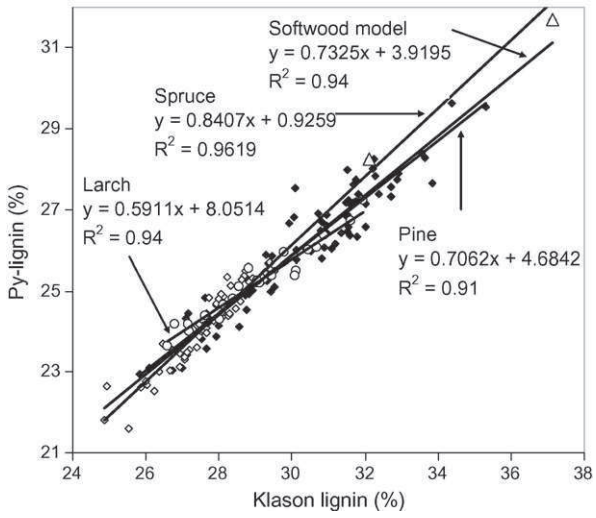

Fig. 5. Py-lignin versus Klason lignin content of larch (open circles), pine (closed diamonds), spruce (open circles) samples, and of all samples (“softwood model”). The two spruce CW samples are displayed with open triangles.

By adding the two CW samples (Fig. 5, triangles) to the spruce model only a small decrease in the slope from 0.86 to 0.84 was obtained (Fig. 5). This confirms the findings for pine and larch that CW and NW samples lies close to the species-specific regression lines.

The two CW samples were included and a ''softwood model'' calculated and compared with the species-specific models. The distribution of differences between the Klason lignin contents predicted with species-specific models and the ''softwood model'', as well as the distribution of differences between the Py-lignin contents predicted with species-specific models and the ''softwood model'' is shown in Fig. 6. Both show an approximately normal distribution. More than 95% of the differences lie within ±0.3%. This means that the softwood model can be used for the prediction of the Klason lignin content instead of the species-specific models. Only the spruce and larch samples with the lowest and the highest Klason lignin contents fell out of this range (±0.3%). These samples can be predicted more precisely by species-specific models.

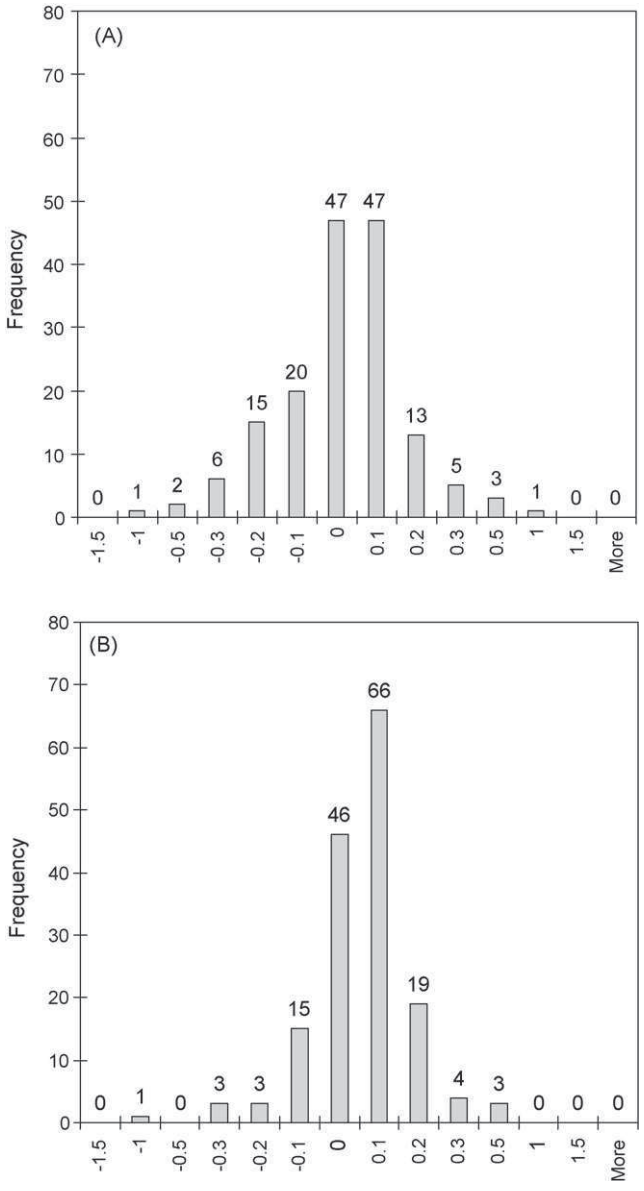

Fig. 6. (A) Distribution of differences between the Klason lignin contents predicted with species-specific models and the ''softwood model'', and (B) distribution of differences between the Py-lignin contents predicted with species-specific models and the ''softwood model''.

The slightly different slopes of the linear regression lines of pine and larch, and especially the one of spruce (Fig. 5) suggest possible differences in lignin and/or carbohydrate composition that should be investigated.

4. Conclusions

The evaluation of a Py-lignin model based on pine and spruce wood with larch wood samples revealed only small differences between the measured and the predicted Klason lignin contents of larch. Therefore, larch samples could be included in the model. The investigation of the influence of compression wood revealed small differences between normal wood and compression wood, with a so far unexplained shift between NW and CW within pine wood. Although, the slope of the spruce model was much higher compared to pine and larch, the spruce samples could be predicted well in general. Therefore, this so-called ''softwood model'' can be used for pine, larch, and spruce wood with the limitation of the highest and lowest values where the species-specific models have led to better results. Moreover, it is expected that this model can also be used to predict Klason lignin contents of other softwood species.

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Artigo III

Analytical pyrolysis as a direct method to determine the lignin content in wood
Part 3: Evaluation of species-specific and tissue-specific differences in softwood lignin composition using principal component analysis

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Analytical pyrolysis as a direct method to determine the lignin content in wood Part 3. Evaluation of species-specific and tissue-specific differences in softwood lignin composition using principal component analysis

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ABSTRACT

Both the genetics and the environment determine the chemical composition of wood. To assess the chemical composition analytical pyrolysis is being increasingly used. Each single pyrogram is a fingerprint of the chemical composition that should reflect tissue, species, and site related information although hidden in an amount of data.

Principal component analysis was applied to evaluate the pyrolysis results with respect to differences in lignin composition using G- and H-lignin-derived peaks from the pyrograms. The three species: pine, spruce and larch were separated in the first principal scores plot and the corresponding loadings plot revealed that it is vanillin (G 9) and G–C=C=C (G 11) on one hand and isoeugenol (G 8) and dihydroconiferyl alcohol (G 19) on the other hand that separate spruce and larch from pine. Beside others G 9 plus G 11 and G 8 plus G 19 separate spruce form larch as well as Vaquey pine from Blagon pine. In addition an investigation of the different tissues – normal wood and reaction wood – and the discussion of these results together with the loadings helped to reveal the differences in lignin composition between the species, tissues, and two sites. It was shown that analytical pyrolysis combined with principal component analysis could be useful for the identification of species and their origin.

1. Introduction

Analytical pyrolysis is being increasingly used as a quantitative method to assess chemical composition of lignocellulosic materials [1–5]. The main advantages of analytical pyrolysis over classical wet-chemical methods are an easy sample preparation (drying and milling), short analysis times and small sample sizes (μg range) [6]. Analytical pyrolysis data were related with classical wet chemical data by direct comparison of peak areas of characteristic pyrolysis products [7–10], using multivariate statistical techniques for qualitative analysis such as principal component analysis (PCA) [11] or for calibration [12,13], and absolute quantification of pyrolysis products using internal standards [14–16].

Unfortunately less is known about the lignin structures from which the analytical pyrolysis products derive [17]. Methods such as thioacidolysis [18–20], derivatization followed by reductive cleavage (DFRC) [21,22], hydrogenolysis [23], acidolysis [24], ozonation [25], selective tosylation (T) of primary hydroxyl group, iodination (I), and zinc-metal treatment (Z) (TIZ) to cleave β-*O*-4 linkage [26] were used to characterize lignin structure/composition. DFRC was combined with ^{1}H–^{13}C HMQC NMR spectroscopy [27] or ^{31}P NMR [28,29] and compared to thioacidolysis [27,28] to elucidate lignin structure. The two of the most effective methods for elucidating lignin structure have proven to be thioacidolysis and DFRC, whereas the latter method provides lower monomer yields from β-aryl ether bonds [28]. All these methods were developed to investigate the lignin composition of lignocellulosic materials in native as well as processed state, e.g. pulps.

In Part 1 of this series a method for the quantification of the lignin content (Py-lignin) of Maritime pine (*Pinus pinaster* Aiton)

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and spruce wood (*Picea abies* [L.] Karst.) samples directly from the pyrograms was presented [3]. The good correlation found between the Py-lignin and Klason lignin content gave a common model for both species. In Part 2 [5] five subspecies of larch wood (*Larix* sp.) were used to evaluate this common model, revealing only small differences between the measured and the predicted Klason lignin contents. Compression wood was included due to the difference in lignin composition and content compared to normal wood. As the influence of compression wood was small a so-called "softwood model" including all samples was calculated which can be used for pine, larch, and spruce wood with the limitation of the highest and lowest values were the species-specific models lead to better results. However, slightly different slopes of the linear regression lines of pine and larch, and especially the one of spruce suggested possible differences in lignin and/or carbohydrate composition that should be investigated.

In this Part 3 we investigated differences in lignin composition using analytical pyrolysis and PCA. The focus was on species-specific differences as well as tissue-specific differences including the influence of so-called compression wood, a type of reaction wood formed under mechanical stresses [30].

2. Experimental

2.1. Samples

The 74 (12–14-year-old) Maritime pine (*P. pinaster* Aiton) wood samples (48 from Blagon and 26 from Vacquey both France) [31]), 57 spruce (55 samples from about 19-year-old spruce trees (*P. abies* [L.] Karst.) from Sweden [32], two samples from a 30-year-old spruce tree (*P. abies* [L.] Karst.) from Austria [33]), and 18 larch wood samples (*Larix decidua*, *Larix eurolepis*, *Larix kaempferi*) harvested at an age of 38 years and three larch wood samples (*L. decidua*) harvested at an age of 160 years [34–37] were used. The range of Klason lignin and Py-lignin contents as well as the H/G ratios of the samples is compiled in Table 1. The determination of the Py-lignin content was described in Part 1 of this series [3]. Details about the samples, preparation, extraction, and Klason lignin determination have been published [3,5,32–37].

Except for the Austrian spruce compression wood sample where the compression wood was also investigated and identified by microscopy [33], the tissue type assignment was mainly done visually on large samples (mostly discs). The presence and the amount were confirmed by analytical pyrolysis using the H/G ratio [3,5], Klason lignin [3,5,32], and infrared spectroscopy [4,33,36,38].

The following labels for species/sites: spruce from Sweden (S), spruce from Austria (A), pine from France Blagon (B) and Vaquey (V), larch wood from Europe (E), and for types of wood tissues: normal wood (N), reaction (compression) wood (R), opposite wood (O), total wood (T) meaning the whole disc including compression wood, opposite wood, aside wood (wood aside compression wood and opposite wood), and normal wood and unknown amount of compression wood (X) were used. To simplify matters the latter will be called tissue types further on.

2.2. Analytical pyrolysis

Analytical pyrolysis (Py-GC/FID) was performed with a CDS Pyroprobe 1000 with a coil filament connected to a HP 5890 series II by a heated interface (270 °C). Each sample (75–80 μg) was pyrolysed at 650 °C for 10 s with a temperature rise time of approximately 20.0 °C ms^{-1} [3].

Details about the samples, their preparation and the analytical pyrolysis were previously described in Parts 1 and 2 [3,5]. Moreover, the identification table of all compounds (pyrolysis products) can be found in Part 1 [3]. The peaks labelled H 2,3 in the table in Part 2 were separated (recalculated) for data analysis in this paper and are now labelled H 2 and H 3, respectively.

2.3. Multivariate data analysis

PCA was performed using the Unscrambler™ Vsn. 9.7 (CAMO). Prior PCA the percentage of each peak from the pyrogram (the area of a peak divided by the sum of the area of all used peaks multiplied by 100% [3]) was standardized (weighted by the standard deviation of each variable – peak). To level off the influence of the carbohydrates and varying lignin contents (see Section 3.4) the percentage of the G- and H-lignin-derived peaks from the pyrogram (the area of a peak divided by the sum of the area of all used peaks multiplied by 100%) was standardized (weighted by the standard deviation of each variable – peak).

3. Results and discussion

3.1. G- and H-lignin of all samples

Subjecting all samples to PCA using G- and H-lignin-derived pyrolysis products (peaks from the pyrogram which will further be called variables) clustering according to species – as shown in the PC 1–PC 2 scores plot (Fig. 1A) – was obtained. The variances explained are 40% by PC 1 and 22% by PC 2, respectively. The three species, pine, spruce, and larch were separated into four clusters with a small overlapping region between pine and larch wood at about zero (the average) of PC 1 (Fig. 1A) as well as between pine wood sites. The Sweden spruce samples even in higher number showed a tighter cluster compared to the other species. This suggests a more homogeneous lignin composition for spruce normal wood, also confirmed by the smallest H/G ratio range (Table 1).

The loadings plot (Fig. 1C) reveals that vanillin (G 9) and G–C═C═C (G 11) on one hand and isoeugenol (G 8) and dihydro-coniferyl alcohol (G 19) on the other hand separate spruce and larch from pine. Beside others G 9 plus G 11 and G 8 plus G 19 separate spruce form larch as well as Vaquey pine from Blagon pine. Using only these four variables (G 8, G 9, G 11, and G 19) the

Table 1
Range of Klason lignin contents and H/G ratios of the samples.

Site	Species	Klason lignin (%)			Py-lignin (%)		H/G ratio	
		Min	Max	Average	Min	Max	Min	Max
Blagon, France	Pine	23.0	29.3	25.6	23.0	27.8	0.041	0.093
Vaquey, France	Pine	28.2	35.3	31.4	24.6	29.6	0.047	0.113
Europe	Larch	26.6	32.0	29.1	23.6	27.2	0.037	0.084
Sweden	Spruce	24.9	32.1	27.7	21.6	28.3	0.038	0.062
Austria[a]	Spruce	26.0	37.2	31.6	25.1	31.7	0.034	0.118

[a] Only two samples, one normal wood and one reaction (compression) wood.

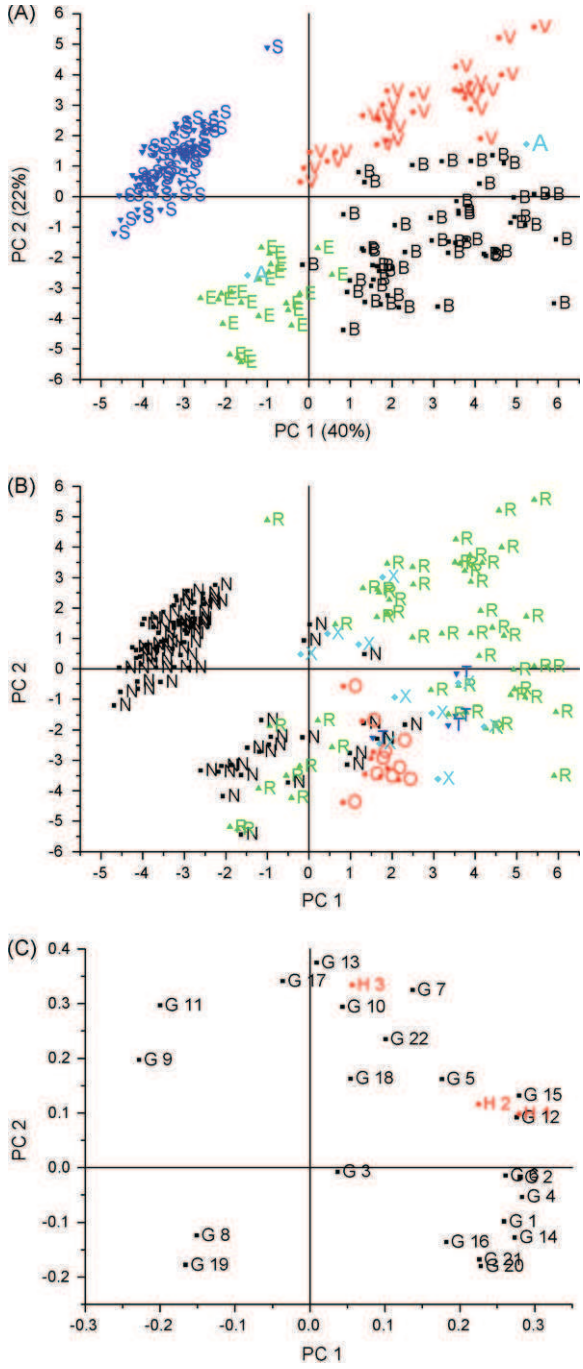

Fig. 1. Results of the PCA using all samples and G- and H-lignin variables (peaks from the pyrogram). (A and B) Show the PC 1 (40% explained variance) versus PC 2 (22% explained variance) scores plots, whereas in (A) the label indicates site and specie and in (B) the tissue type. (C) Shows the corresponding loadings plot of the G- and H-lignin variables. For abbreviations, see Section 2.1.

same pattern (not shown) was obtained. Of course also other variables contribute to the clustering but only the most important are mentioned in the text as the others are shown in the figures and the identification of the labels is given in Table 2.

Both Austrian spruce samples (A) are written in larger letters because they neither lie within the Sweden spruce cluster nor close to it. The main reason why the Austrian samples do not fit into the spruce cluster is the different lignin composition and the extreme high Klason lignin content (37.2%) of the compression wood (R) sample. Beside the high H-lignin content (H 1: phenol and H 2: *m*-cresol) and high homovanillin (G 12) and propioguaiacone (G 15) contents (Fig. 1C) this compression wood sample shows a high 5-hydroxymethyl-2-furaldehyde content and a lower hydroxyacetaldehyde, 3-hydroxypropanal, 3-butenal-2-one, (3*H*)-furan-2-one content. This is an extreme and unusual spruce sample that is hard to find, more samples like this would be interesting to analyse and necessary to draw better conclusions.

Table 2
The identification of the lignin-derived pyrolysis products used for the principal component analyses to reveal species-, tissue-, and/or site-specific differences. Separation of spruce and larch wood along PC 1 (52% explained variance) due to differences in lignin composition using only G- and H-lignin variables is indicated by S (more in spruce), 0 (average for both) or L (more in larch).

Label	Compound	S–L, PC 1
H 1	Phenol	L
H 2	*m*-Cresol	L
H 3	*p*-Cresol	S
G 1	Guaiacol	L
G 2	3-Methylguaiacol	L
G 3	4-Methylguaiacol	L
G 4	4-Vinylguaiacol	L
G 5	Eugenol	0
G 6	4-Propylguaiacol	L
G 7	Isoeugenol (*cis*)	S
G 8	Isoeugenol (*trans*)	L
G 9	Vanillin	S
G 10	G–C═C═C	S
G 11	G–C═C═C	S
G 12	Homovanillin	L
G 13	Acetoguaiacone	S
G 14	Guaiacyl acetone	L
G 15	Propioguaiacone	S
G 16	Structure isomer of coniferyl alcohol	0
G 17	G–CO–CH═CH_2	S
G 18	G–CO–CO–CH_3	S
G 19	Dihydroconiferyl alcohol	L
G 20	Coniferyl alcohol (*cis*)	L
G 21	Coniferyl alcohol (*trans*)	L
G 22	Coniferyl aldehyde	0

Besides the separation of the three species a kind of separation between pine from Vaquey and Blagon could be reached, although some of the Blagon samples look closer to Vaquey than to Blagon (Fig. 1A). This shows that besides species-specific differences analytical pyrolysis has the potential to reveal also site-specific differences. Labelling the samples according to tissue types in the PC 1–PC 2 scores plot shows some separation of the tissue types within the species clusters (Fig. 1B). Pine wood samples could be analysed in detail, because several different tissue types were available.

The PCA using only pine wood samples and only G- and H-lignin variables separates Blagon pine from Vaquey pine with the exception of one Vaquey and one Blagon sample in the PC 1–PC 2 scores plot (Fig. 2A). Labelling the samples according to tissue types shows a partial separation revealing a progression from normal wood, over opposite wood, total wood to reaction wood in the PC 1–PC 2 scores plot (Fig. 2B) within each site (Vaquey or Blagon) cluster. The loadings plot (Fig. 2C) presents which lignin-derived pyrolysis products are responsible for the separation, whereas on the rightmost of PC 1 the H-lignin products can be found.

To avoid the influence of pine wood on the separation of spruce from larch wood, a PCA without pine using only G- and H-lignin variables was calculated and the results are listed in Table 2.

3.2. G- and H-lignin of reaction wood

A PCA using all reaction wood samples and G- and H-lignin variables was calculated focusing on lignin composition

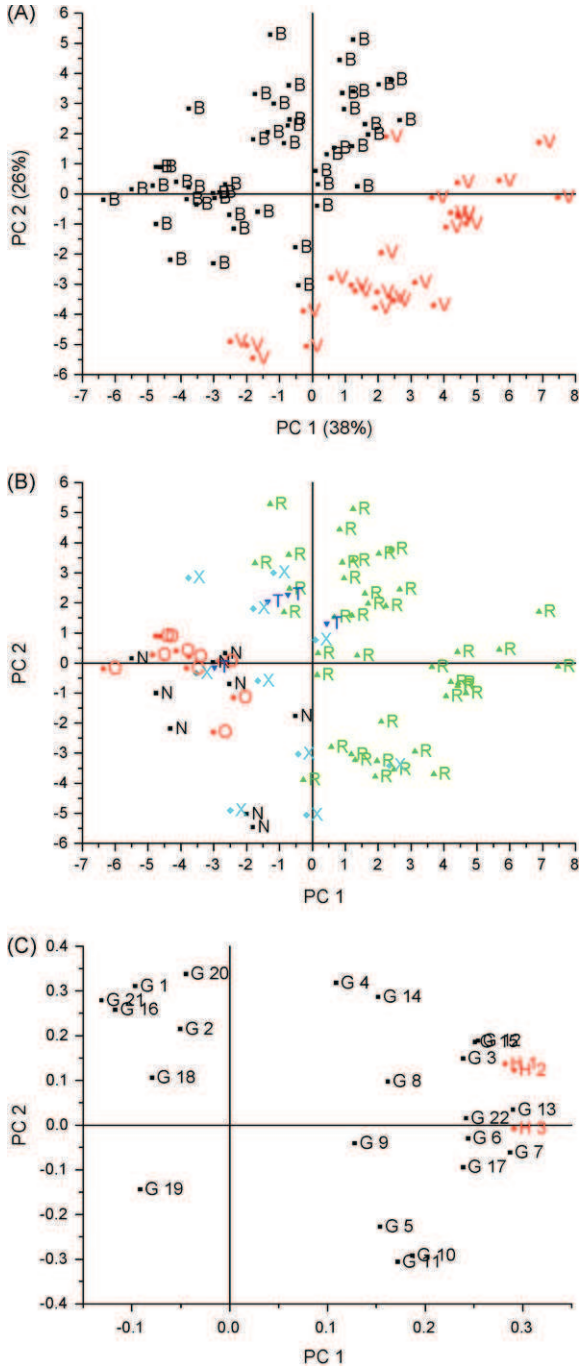

Fig. 2. Results of the PCA using only pine wood samples and G- and H-lignin variables (peaks from the pyrogram). (A and B) Show the PC 1 (38% explained variance) versus PC 2 (26% explained variance) scores plots, whereas in (A) the label indicates the site and in (B) the tissue type. (C) Shows the corresponding loadings plot of the G- and H-lignin variables. For abbreviations, see Section 2.1.

differences of the reaction wood samples. The PC 1 (33%)–PC 2 (22%) scores plot allows distinguishing between species and also between sites for pine (Fig. 3A). Additionally to G 9 and G 11 that allowed separating larch from pine (Fig. 1A) G 3 (4-methylguaiacol) and G 20 (*cis*-coniferyl alcohol) contribute to the reaction wood pattern being above average represented by larch. The H-lignin products are higher in pine with H 2 at the average, H 1 higher in Blagon pine and H 3 higher in Vaquey pine. The PC 1 (33%)–PC 3 (16%) scores plot shows positive values for the spruce samples (Fig. 3C) with the extreme one far from all others, which is due to the high H-lignin (mainly H 2) content (Fig. 3D).

3.3. G- and H-lignin of normal wood

Subjecting the normal wood samples to PCA using G- and H-lignin variables shows a clear clustering in the PC 1 (42%)–PC 2 (20%) scores plot (Fig. 4A). The Austrian spruce sample is close to H 2 and larch wood contains the most of H 2 besides G 3, G 8, and G 19, whereas much of H 1 can be found in pine and H 3 besides G 9, G 11, G 13, and G 17 contribute mainly to spruce (Fig. 4B). The PC 2 (20%)–PC 3 (13%) scores plot (Fig. 4C) that shows some separation within pine along PC 3 and between pine, spruce and larch along PC 2, whereas spruce is closer to pine, which is interesting from the genetic point of view (see also the loadings plot Fig. 4D), because phylogenetically spruce is closer to pine than to larch [39]. However, as the trees were grown on different sites (France and Sweden) a possible site effect cannot be excluded. Moreover it should be kept in mind that the average lignin contents of the species are different (Table 1) which may partly contribute to the separation.

3.4. G- and H-lignin of pine wood from one site

The pine wood samples from Blagon were used to investigate differences in the lignin composition between the tissue types. The carbohydrates-derived pyrolysis products self-evident contribute to the total sum of all peaks, which has an impact on the percentage of the lignin-derived peaks. To level off the influence of the carbohydrates and varying lignin contents, only G- and H-lignin-derived peaks were used (the area of each lignin peak divided by the sum of the area of all lignin peaks multiplied by 100%) and standardized (weighted by the standard deviation of each variable – peak) prior to PCA.

A PCA calculated using the Blagon pine wood samples and G- and H-lignin variables gave a PC 1 (31%)–PC 2 (19%) scores plot that allows distinguishing between tissues (Fig. 5A). Along PC 1 reaction wood could be separated from normal wood and opposite wood, and along PC 2 normal wood could be partly separated from opposite wood. It was expected that the separation of normal wood from opposite wood is difficult because, e.g. Lohrasebi et al. [40] found for black spruce that the differences in the chemical composition between them are small. The contribution of each variable is shown in the loadings plot (Fig. 5B). Around the average of PC 1 several tissue types can be found. The assignment of tissue type was done visually on large samples (mostly discs), which is not always unambiguous. Even using fluorescence microscopy [41,42] or digital imaging [43] only three kinds of wood-types in the images: normal wood, mild, and severe compression wood [42] can be discriminated normally from small samples. Nanayakkara [42] stated that the lignin content of mild compression wood was 18% higher while severe compression wood lignin content was 30–38% higher than that of opposite wood, and found a good agreement between the anatomical classification of the wood samples and their lignin content [42].

As it is known that the lignin composition is different in tissue-types [28] or cell wall types [19,44,45] analytical pyrolysis was shown to have the potential to discriminate them. The samples with unknown compression wood amount can now be tentatively assigned, and also the ones found around the average of PC 1 should maybe (re)assigned to the same tissue-type. Interestingly H 3 (*p*-cresol), which was found to be higher in the pine samples from site Vaquey (Fig. 2C) and the normal wood spruce samples (Fig. 4B), is about the average in all tissue types of Blagon pine. However, to reduce the number of variables focusing on the most relevant, the

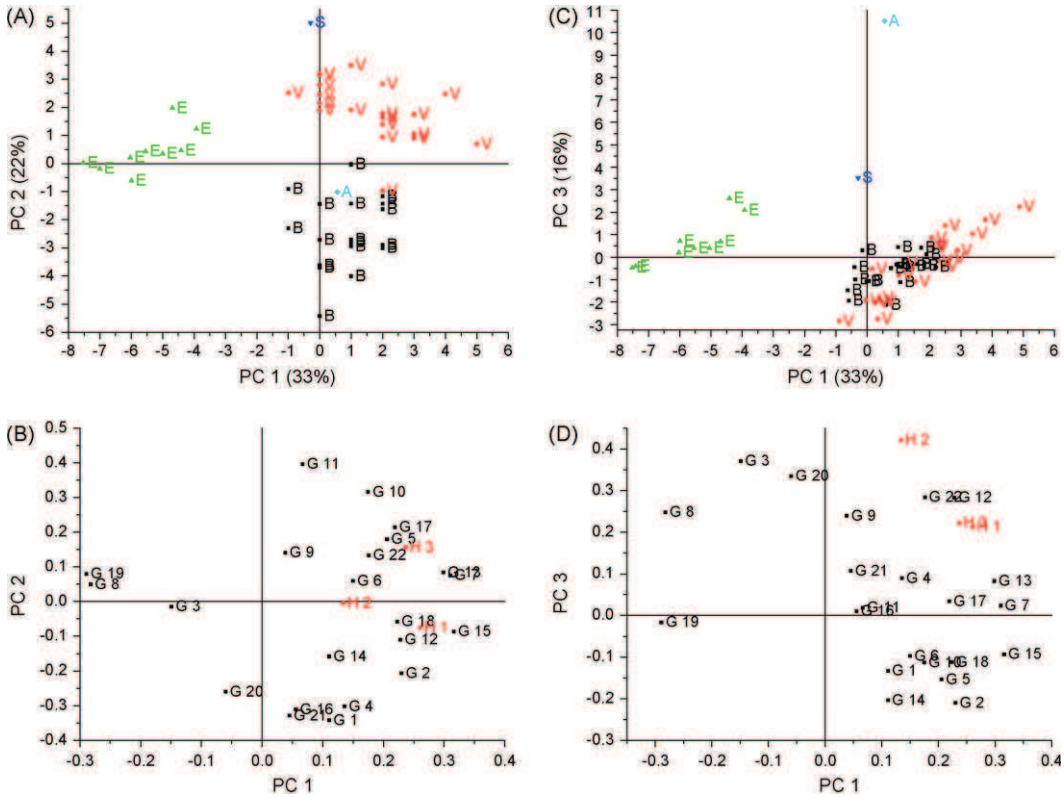

Fig. 3. Results of the PCA using all reaction wood samples and G- and H-lignin variables (peaks from the pyrogram). (A) Shows the PC 1 (33% explained variance) versus PC 2 (22% explained variance) scores plot and C the PC 1 (33% explained variance) versus PC 3 (16% explained variance) scores plot, whereas the label indicates the site. (B and D) Show the corresponding loadings plots of the G- and H-lignin variables. For abbreviations, see Section 2.1.

ones having loadings below ± 0.1 of the average of PC 1 (H 3 and G-lignin products written in light grey in Fig. 5B) were removed and the PCA recalculated without them. As expected a similar pattern was obtained for the PC 1 (45%) versus PC 2 (18%) scores plot (Fig. 5C). Reaction wood (R1, R2, and R3) and opposite wood (O1, O2, and O3) samples obtained from the same discs that are connected with arrows lie diametrically. Beside H-lignin (phenol (H 1) and *m*-cresol (H 2)) that is well known to be higher in compression wood, isoeugenol (G 7), homovanillin (G 12), propioguaiacone (G 15), and the coniferyl alcohols (*cis* G20 and *trans* G 21) were higher compared to normal and opposite wood.

Vanillin (G 9) and G–C═C═C (G 11) that separated spruce from the other species (Fig. 1A) using all samples were also important when only normal wood was investigated (Fig. 4A and B). Also within pine these products were enriched in normal and opposite wood (Fig. 5B and D). Isoeugenol (G 8) and dihydroconiferyl alcohol (G 19) that separated larch form the other species (Fig. 1A) were always important for the separation of larch meaning for reaction wood (Fig. 3A–D) as well as normal wood (Fig. 4A–D). Besides the high amounts of 4-methylguaiacol (G 3) formed from larch wood from both compression wood (Fig. 3) and normal wood (Fig. 4) compared to the others, *cis*-coniferyl alcohol (G 20) was also always found in reaction wood (Fig. 3B and D, and Fig. 5 B and D).

3.5. Lignin-derived pyrolysis products and lignin structure

Methods such as thioacidolysis, DFRC, and analytical pyrolysis were developed to investigate the lignin composition of lignocellulosic materials in native as well as processed state, e.g. pulps. As the lignin composition is known to influence the pulping and the bleaching efficiency [46–48] several attempts were undertaken to alter lignin content and composition by tree breeding [49] and genetic improvement [48,50,51], which by the way lead to new insights into the biosynthetic pathway [52].

Gellerstedt and Zhang [53] summarized some of the residual Kraft lignin features: a low remaining amount of β-*O*-4 structures [54], linkages between lignin and polysaccharides, the presence of reduced structures such as methylene and methyl groups [55], high degree in discoloration [56], successive increase of "condensed" structures with high degree of delignification [55], and an uneven distribution of lignin across the fibre wall. Although thioacidolysis and DFRC are the nowadays preferred methods to investigate lignin structure [27], analytical pyrolysis gives an overall picture of the sample [1,2,6,57–61] accounting for interactions between carbohydrates and lignin [62].

Pyrolytic degradation of lignin starts at about 200 and up 400 °C linkages between the lignin units are cleaved of which the α-*O*-4 ether bond is the weakest. Product yields increase with increasing temperatures but the decomposition chemistry becomes more complex as secondary decomposition also takes place resulting in, e.g. the conversion of guaiacols into catechols [16]. The various instrumental set-ups and different pyrolysis conditions, e.g. temperatures from 200 to 400 °C [63], 450 °C [64], 500 °C [65], 580 °C [57], 650 °C [3,5], and up to gasification temperature at 800 °C [62,66] used additionally complicate comparison of results obtained by several groups, which suggests a kind of standardisation. Moreover, care has to be taken by comparing the results with the literature, although most of them are from analytical pyrolysis works, some are from pyrolysis works for energy. Although in the past analytical pyrolysis was focused on the determination of G-, S-, and H-lignin contents and their alterations during processing, e.g. pulping [48], bleaching [67], and fungal treatment [68,69] based on

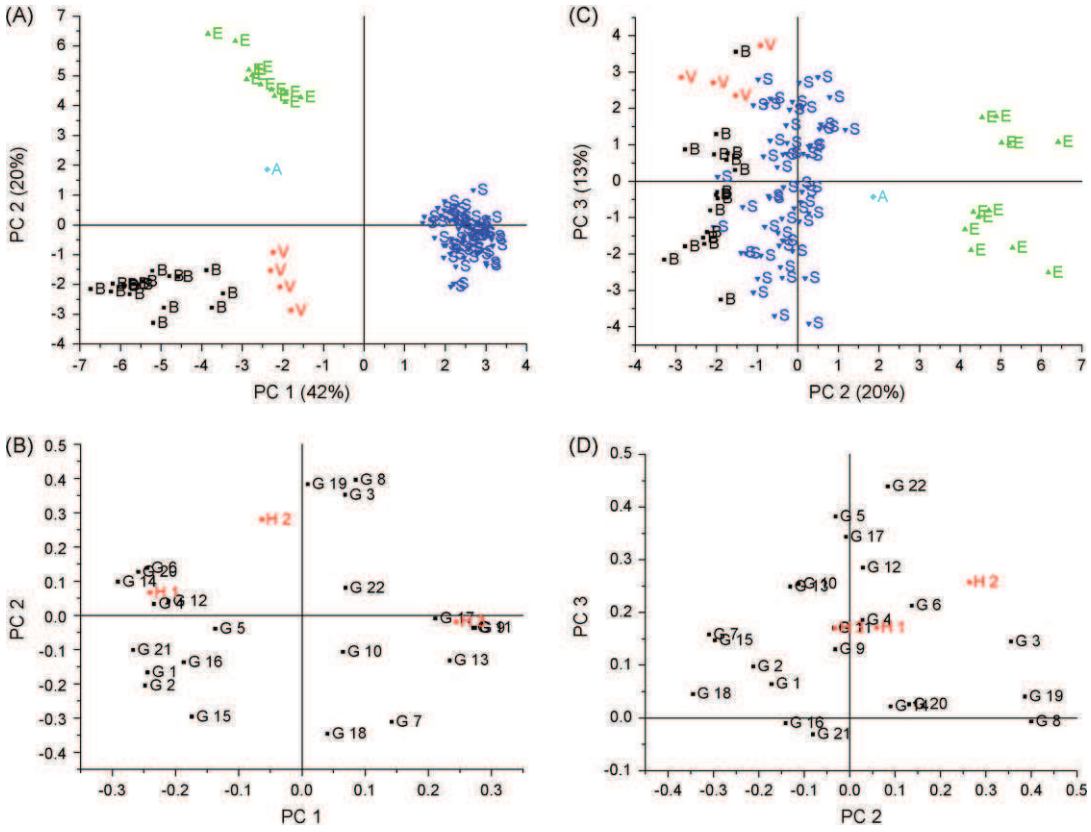

Fig. 4. Results of the PCA using normal wood samples and G- and H-lignin variables (peaks from the pyrogram). (A) Shows the PC 1 (42% explained variance) versus PC 2 (20% explained variance) scores plot and C the PC 2 (20% explained variance) versus PC 3 (13% explained variance) scores plot, whereas the label indicates the site. (B and D) Show the corresponding loadings plots of the G- and H-lignin variables. For abbreviations, see Section 2.1.

their pyrolysis products [6,59,67], the potential to provide more information is well known [11] even lacking an assignment of pyrolysis products to lignin structure [11]. Nevertheless several efforts were made to assign lignin-derived pyrolysis products using wood, lignin isolated from wood and/or lignin model compounds [17,57,58,63,70–74] especially in the last years [75–78]. Model compound studies have shown that the β-*O*-4 linkage is cleaved extensively in pyrolysis, but only partial cleavage of carbon–carbon bonds are reported to take place [57,73], and that the phenolic forms are in general much more reactive than the non-phenolic forms [75,76].

Unfortunately nothing could be found about the lignin structure of larch wood. The higher contents of 4-methylguaiacol (G 3) and dihydroconiferyl alcohol (G 19) may arise from higher amounts of β-5 structures as Kuroda and Nakagawa-izumi [72] found those products after pyrolysis of phenolic 2-arylcoumaran type lignin model compounds. Recently Kawamoto et al. [77] published that α-ether-type dimers and α,β-diether-type trimers give isoeugenol, *para*-substituted phenols, and guaiacol during pyrolysis which gives hint that possibly more of this structures are present in pine compression wood where higher contents of these pyrolysis products were found (Fig. 5B and D). According to Choi et al. [59] higher yields of isoeugenol found in spruce wood pulps can be interpreted as indication of the presence of β-*O*-4 linkages with OH groups in α-position from which water is split easily during pyrolysis. This reaction leads to degradation products with unsaturated side-chains. Therefore the β-*O*-4 linkages with OH groups in α-position can be a source of the high isoeugenol (G 8) contents found in larch wood (Figs. 3 and 4). The isoeugenol isomers were found to be of different importance for the differentiation between larch and pinewood. In larch reaction wood and pine normal as well as opposite wood the *trans* isomer dominates and in pine compression wood the *cis* isomer is higher. The *cis* isomer was also found to be higher in spruce wood than in larch wood (Table 2). The higher amount of coniferyl alcohol found in pine compression wood could arise from the cleavage of β-*O*-4 linkages as Nakamura et al. [63] found that coniferyl alcohol is one of the major pyrolysis products form phenolic β-ether dimers especially formed at higher temperatures. In contrast a decrease of uncondensed arylglycerol-β-aryl ether linkages in compression wood compared to normal wood of southern pine was reported [79,80] but they found higher phenolic and aliphatic OH, a higher number of etherified 5–5′ linkages, and the major difference in H-units were attributed to non-conjugated *p*-hydroxyphenyl moieties.

Using thioacidolysis and DFRC a much higher content of uncondensed β-*O*-4 linkages were found in southern pine normal wood compared to compression wood with both being higher than the ones found in spruce [28]. Yeh et al. [81] investigated loblolly pine compression wood of juvenile wood and mature wood with the main difference found was the higher content of aliphatic OH groups mature compression wood. Moreover, it is also reported that H-lignin pyrolysis products are formed not only from lignin but arise also from proteins [59] and from carbohydrates that can form aromatics during pulping [57]. Homovanillin – higher amounts obtained from compression wood – was found to increase during bleaching of softwood Kraft pulps [57]. As it is well known [40,80,82] that compression wood is more difficult to pulp and bleach, it is likely that lignin-structures giving more homovanillin during pyrolysis are enriched during compression wood formation.

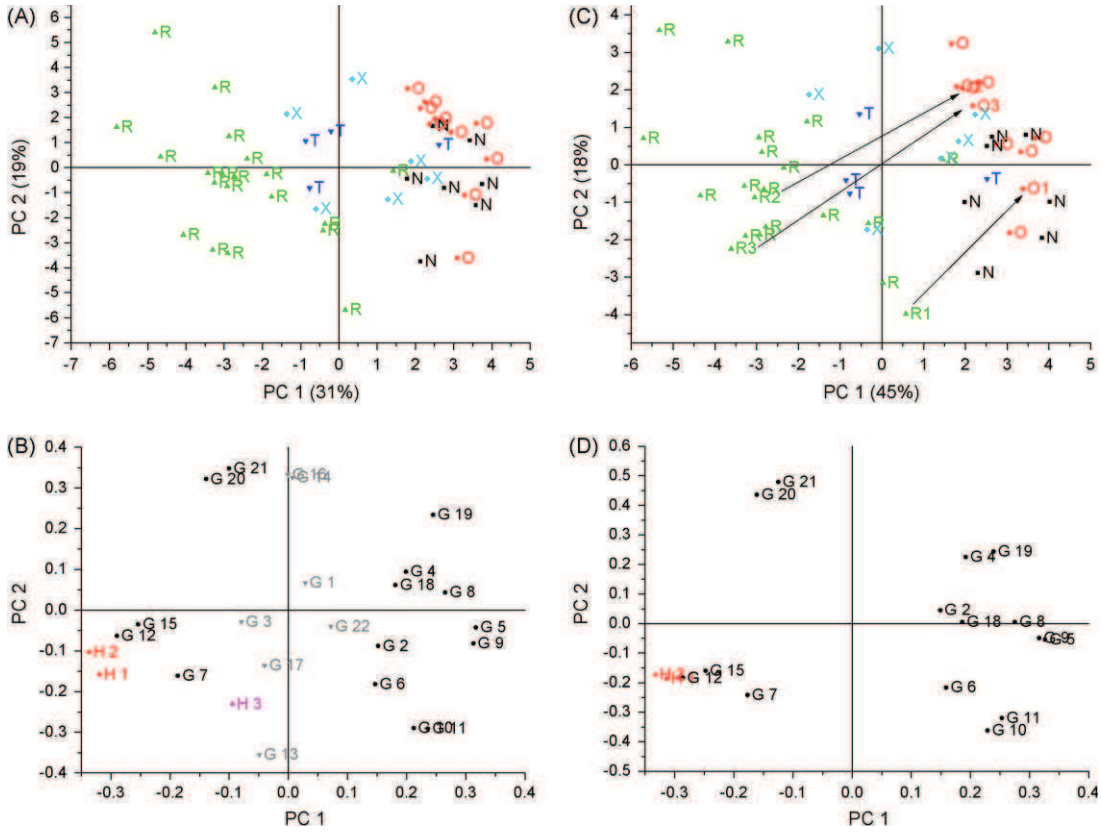

Fig. 5. Results of the PCAs using pine wood samples from Blagon and G- and H-lignin variables (peaks from the pyrogram). (A) Shows the PC 1 (31% explained variance) versus PC 2 (19% explained variance) scores plot with labels indicating the tissue type. (B) Shows the corresponding loadings plot of the G- and H-lignin variables. (C) Shows the PC 1 (45% explained variance) versus PC 2 (18% explained variance) scores plot with labels indicating the tissue type. The arrows connect the reaction wood with the opposite wood obtained from the same disc. (D) Shows the corresponding loadings plot of the G- and H-lignin variables. For abbreviations, see Section 2.1.

Although speculative and no direct or definitive conclusions can be drawn from the cited works (different species, conditions, model compounds) they give hints to probable structures and/or structural differences of lignin between species and tissues.

Moreover, differences in lignin composition were found, but no explanation can be provided at this time why the slopes of the correlations found between Py-lignin and Klason lignin in Part 2 [5] are slightly different for the species.

4. Conclusions

PCA performed using G- and H-lignin-derived pyrolysis products allowed to separate pine, spruce, and larch wood in the scores plot according to species. The corresponding loadings plot revealed the substances responsible for the clustering. Besides these species-specific differences, in the case of pine site-specific as well as tissue-specific differences could be shown. Tissue types could be separated partially showing a progression from normal wood, over opposite wood, total wood to reaction wood.

However, further investigations will reveal the differences in the carbohydrate composition, their contribution to the Py-lignin determination as well as their influence on the separation according to species, tissues and sites.

Analytical pyrolysis has proved to be a good technique to reveal species- and tissue-specific differences with the potential to disclose site-specific differences in lignin composition.

Although some hints on structural differences of lignin could be obtained from several studies, the assignment of pyrolysis products to lignin structures is still a challenge for the next decade.

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Artigo IV

Calibration of NIR to assess lignin composition (H/G ratio) in Maritime pine wood using analytical pyrolysis as the reference method

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Short Note

Calibration of NIR to assess lignin composition (H/G ratio) in maritime pine wood using analytical pyrolysis as the reference method

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Keywords: analytical pyrolysis; H/G ratio; lignin composition; maritime pine; NIR; partial least-squares regression (PLSR).

Introduction

Gymnosperm (softwood) lignins such as from maritime pine are complex aromatic heteropolymers composed of *p*-hydroxyphenyl (H), guaiacyl (G) and, if detectable (Saito et al. 2005), negligible (Obst and Landucci 1986) amounts of syringyl (S) phenylpropanoid units. The importance of lignin composition in terms of H/G ratio in softwoods for the pulping industry has led to attempts at genetic modification (MacKay et al. 1999; Wadenbäck et al. 2004). Nevertheless, very little is known about the natural variation of lignin composition. The lack of a suitable method to assess lignin composition in a large number of samples can in part explain this situation. Analytical pyrolysis is being increasingly used to assess lignin composition (Obst and Landucci 1986; Faix et al. 1993; Choi et al. 2001; Rodrigues et al. 2001; Yokoi et al. 2001; Kuroda et al. 2002; del Rio et al. 2005; Meier et al. 2005) but even if time requirements for the analytical procedure are well below those involved in wet-chemical analysis (Meier and Faix 1992) it is still demanding when large screening programs are necessary.

The simplicity, rapidity, and high reproducibility of near-infrared spectroscopy (NIRS) have widened its use to the determination of the chemical composition of lignocellulosic materials as a substitute for wet-chemistry techniques (Schwanninger and Hinterstoisser 2001; Gierlinger et al. 2002; Raymond and Schimleck 2002; Schimleck et al. 2003).

The aim of this work was to use analytical pyrolysis data to develop an NIR method for determining H/G ratios, with a focus on assessment of the natural variation of lignin composition in maritime pine wood.

Materials and methods

A total of 68 maritime pine (*Pinus pinaster* Aiton) wood discs (da Silva Perez et al. 2005) were ground with a Thomas-Wiley ED-5 mill to pass a 1-mm sieve, screened in a vibratory sieving apparatus and the 40–60-mesh wood meal fraction was retained for analysis. The samples were extracted for 16 h with water, followed by 12-h acetone extraction in a Soxhlet apparatus, and were then dried at 60°C overnight. Klason lignin was determined using the sulfuric acid method (Schwanninger and Hinterstoisser 2002).

Analytical pyrolysis was performed using a CDS Pyroprobe 1000 with a coil filament probe connected to a GC (HP 5890 FID) via a heated interface (280°C). The pyrolysis was carried out at 650°C for 10 s, using 85 μg of the extractive-free samples. At least duplicate analyses were performed. Details of the conditions and quantification procedures have been published elsewhere (Rodrigues et al. 1999, 2001).

NIR spectra of the extractive-free wood meal samples were recorded on a Bruker Vector 22/N using a spinning cup module with 50 scans per sample at spectral resolution of 8 cm^{-1} and two or three spectra per sample.

Samples were randomly divided into two groups (1 and 2). Each group was used for cross-validation (CV) and test set validation (TS), with group 1 as CV and group 2 as TS, and then the other way round, to evaluate if the model statistics were identical, or at least very similar, leading to the same rank. The sample spectra were pre-processed using first derivative-multiplicative scattering correction (MSC), and regressed against the calibration components using OPUS Quant 2 Software (Bruker). Then a partial least-squares regression (PLSR) model including all calibration spectra was calculated (Gierlinger et al. 2002).

Results and discussion

The H/G ratio of the 68 maritime pine wood samples determined by analytical pyrolysis ranged from 0.041 to 0.111, with an average of 0.064 and a standard deviation of 0.016. The precision of the method was 0.005 based on the pooled standard deviation of the replicates.

This data set was used for calibration of the NIR spectra. The wavenumber range (6100–5760 cm^{-1}) and the pre-processing method (first derivative-MSC) gave the best model for prediction of the H/G ratio (Table 1). The same parameters for wavenumber range and pre-processing method were applied for the determination of the

Table 1 PLSR results of the calibration, cross-validation (CV) and test set validation (TS) of the H/G ratio of the two data sets (1 and 2).

Data set (no. of samples)	Calibration		Cross-validation		Test set validation		Rank
	RMSEE	R^2 (%)	RMSECV	R^2 (%)	RMSEP	R^2 (%)	
CV_1 (34)	0.0046	92	0.0050	90	–	–	2
TS_2 (34)	–	–	–	–	0.0056	86	2
CV_2 (34)	0.0043	91	0.0048	88	–	–	2
TS_1 (34)	–	–	–	–	0.0060	85	2
CV_{all} H/G (68)	0.0051	90	0.0054	89	–	–	2

CV_{all}, cross-validation of all samples (both data sets together); RMSEE, root-mean-square error of estimation; R^2, coefficient of determination; RMSECV, root-mean-square error of cross-validation; RMSEP, root-mean-square error of prediction; rank, number of principal components.

lignin content of spruce wood (Schwanninger and Hinterstoisser 2001) and maritime pine (unpublished).

Comparison of the ranks determined by CV and TS gives a first indication of the predictive ability of the model, because models with large differences between the ranks are usually not satisfactory (Gierlinger et al. 2002). The rank for all models, as well as their statistics, is almost identical (Table 1). Therefore, a PLSR model including all the calibration spectra was calculated.

A good model with a CV root-mean-square error (RMSECV) of 0.0054 and a high coefficient of determination (89%) was obtained for the H/G ratio. This error is almost identical to the precision of the reference method (pooled standard deviation of 0.005). The small differences between replicate spectra of the same sample, shown as an example in the circle in Figure 1, indicate that the main proportion of the deviation between the true and predicted values can be attributed to the analytical pyrolysis (Figure 1).

This NIR model of the H/G ratio for maritime pine wood allows rapid and cheap determination of a large number of samples per day compared to the reference method. The natural variation in lignin composition can now be easily assessed, allowing the inclusion of this wood quality trait in tree breeding programs and the study of its impact on pulping.

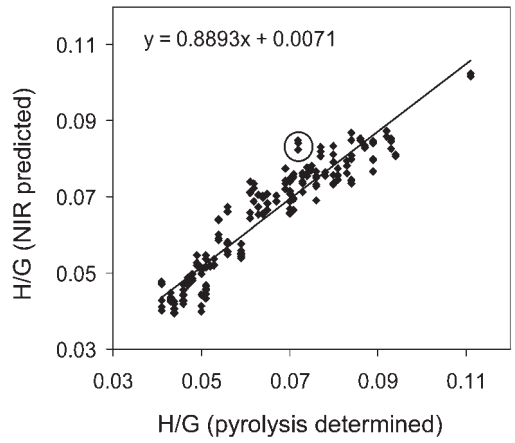

Figure 1 NIR predicted versus pyrolysis determined H/G ratio plot for maritime pine wood. The prediction model was obtained using all samples. The data points within the circle show the differences between repeated NIR measurements of the same sample.

Conclusions

NIR spectroscopy can be used to assess the lignin composition (H/G ratio) of maritime pine wood and its precision is comparable to that of the reference method.

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Artigo V

NIR PLSR results obtained by calibration with noisy, low-precision reference values: Are the results acceptable?

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NIR PLSR results obtained by calibration with noisy, low-precision reference values: Are the results acceptable?

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Abstract

Both spectral noise and reference method noise affect the accuracy and the precision NIR predicted values. The reference noise is often neglected, and the few reports dealing with it only consider random noise artificially added to the original sound reference data. A calibration for lignin content of maritime pine (*Pinus pinaster* Ait.) wood meal was developed, but due to low precision and accuracy in the reference data set, NIR partial least-squares regression (PLSR) yielded a slope of 0.51 and an intercept at 14% Klason lignin. We demonstrate with an independent data set for external validation, obtained with higher precision and accuracy, that the NIR PLSR model based on the noisy reference data led to better results. The slope of the correlation between predicted and reference values was 0.89 and the intercept was 3.9. Thus, the model performed much better than expected from the cross-validation results. The predictability can be explained by the facts that the loadings of the first principal component (PC) of the calibration and test samples are very similar and dominated by lignin-related bands, and that most of the variation in the test set can be explained by the first PC. This only explains why the Klason lignin content could be predicted with the model without giving many spectral outliers, but not the good result of the external validation. We show that the latter can be explained by the inverse calibration used for PLSR and that predicted values can be more accurate and precise than the reference values used for calibration.

Keywords: classical calibration; cross-validation; external validation; inverse calibration; Klason lignin; noisy reference data.

Introduction

Near-infrared spectroscopy (NIRS) has been increasingly applied to determine the chemical composition of lignocellulosic materials instead of time-consuming wet chemical techniques (Wright et al. 1990; Antti et al. 1996; Michell and Schimleck 1996; Raymond and Schimleck 2002; Gierlinger et al. 2004), which are impractical when a large number of samples has to be evaluated.

Being an indirect method, NIRS needs calibration: the relation between a spectral data set and known data for this set of samples (calibration data set) has to be established. It is generally recognised that the "true values" of NIR measurements are influenced by the precision of the reference method used for calibration (Sørensen 2002). The latter can be negligible if the precision of the reference method is higher than the accuracy of the NIR measurements, or if NIR estimations have better precision than the reference method (Næs et al. 2002). However, the question arises as to whether NIR estimations can also be more accurate, especially when the raw material has biotic or abiotic disturbances (noisy data) that may influence the accuracy of the reference method.

The accuracy of NIR spectroscopy is influenced by the spectral noise and the reference data noise (Geladi 2002). The latter is often neglected, and only a few studies deal with this problem. In studies of quantitative NIR spectroscopy, the effect of random noise artificially added to the reference data has mainly been the focus (DiFoggio 1995; Lu and McClure 1998). Statements such as "NIR predictions can never be better than the primary reference method" were refuted (DiFoggio 1995). Performance values of calibrations for natural products were found to be much more sensitive to added spectral noise than to the noise present in the reference data. Spectral noise levels greater than 30% produced unacceptable prediction errors (Lu and McClure 1998). The addition of Gaussian noise to the wavelength variables alone resulted in calibration models with improved accuracy and enhanced robustness. Improved model accuracy leads to smaller prediction errors for partial least-squares regression (PLSR) calibration models generated from noise-augmented data sets than those obtained from the original data set. Increased robustness means that the resultant calibration models are more tolerant of simulated process noise (Conlin et al. 1998).

The goal of this study was to demonstrate that predicted values can be better than expected from cross-validation results using lignin determination in maritime pine wood as a case study. We show the influence of

noise in the reference data on the slope and intercept of the cross-validation results, suggesting poor prediction ability. The real performance of the PLSR model in predicting an independent data set is shown to be better, and the reason for this behaviour is explained.

Materials and methods

Samples

For all samples, wood discs (25 mm wide) were collected from small logs taken within the first internodes of the trunk between a height of 1.3 and 2 m for each tree.

A total of 96 wood samples of 15-year-old maritime pine (*Pinus pinaster* Ait.) were collected in the Gironde region (France) from two different sites: half of the samples were from a half-diallel progeny test (known mother) from Cestas, and the other half from a full-sib progeny trial (known parents) from Malente The samples, with different colour shading owing to fungal activity and containing galleries of insect borers, which was selected as a noisy raw material set.

The discs were ground with a Thomas-Wiley mill model ED-5 to pass a 1-mm sieve and screened in a vibratory sieving apparatus; the 40–60-mesh wood meal fraction was retained for analysis. Samples were successively extracted for 16 h with water, followed by a 12-h acetone extraction in a Soxhlet apparatus and drying at 60°C overnight. Klason lignin was assessed in duplicate for each sample by five different operators using the Tappi T 222 om-88 standard reference method (Tappi 1994–1995). For principal component analysis (PCA) this sample set was split into two groups: group a, with differences between the replicate Klason lignin determinations of $\leq 0.63\%$, and group b, with differences of $> 0.63\%$.

An independent set of 38 wood samples without fungal or insect attack was prepared as described from wood discs of 12–14-year-old maritime pine trees from clonal trials from sites at Blagon and Vaquey in Gironde (France). In this set of samples, Klason lignin was assessed by only one operator using an improved method with higher precision and better reproducibility (Schwanninger and Hinterstoisser 2001, 2002) in a different laboratory. The sample set was split into three groups, c, d, and e, according to the Klason lignin content (high, medium, and low, respectively), covering the natural range of lignin content in maritime pine wood.

The experiments described here are part of an assessment of chemical genetic traits of *P. pinaster* Ait. (Pot et al. 2002; da Silva Perez et al. 2005) and identification of the genomic regions involved in the variability of chemical and physical wood properties.

FT-NIR spectroscopy

NIR spectra of the extractive-free wood meal samples were recorded on a Bruker Vector 22/N instrument using a spinning cup module with 50 scans per sample at spectral resolution of 8 cm^{-1} and two spectra per sample.

Principal component analysis and PLSR modelling

OPUS Quant (Bruker Optics, Germany) was used for data pre-processing [first and second derivatives applying the Savitzky and Golay (1964) algorithm using a 17-point filter and a second-order polynomial] for calculation of the PLSR models and prediction of the evaluation samples.

PCA was performed using the Unscrambler software 9 (CAMO, Norway). PCA was carried out for the calibration samples, as well as for all spectra, to see if the spectra comprised systematically different groups.

Calibration model (noisy data set)

In a first step, the pre-processed (first derivative) NIR data were regressed against the calibration component, and using full cross-validation with one sample omitted, a significant number of PLS components was obtained (Bruker 1996; Gierlinger et al. 2004).

In a second step (test set validation), the calibration data set was divided into two groups (a and b). Each group was used for both cross-validation (CV) and test set validation (TS). First, group a was used for CV and b for TS, and then vice versa, to evaluate whether the model statistics were identical or at least very similar, leading to the same rank. All models were calculated to a maximum rank of 10 and the results of the calibration (R^2, coefficient of determination, and RMSEE, root-mean-square error of estimation), the cross-validation (R^2 and RMSECV, root-mean-square error of cross-validation) and the test set validation (R^2 and RMSEP, root-mean-square error of prediction) were compared. Therefore, test set validation was performed not only using the calibration with optimal rank in the cross-validation (as usual in an external validation), but an optimal rank was also defined. Comparison of the ranks gives a first indication of the predictive ability of the model, because models with large differences between the ranks determined by CV and TS are never satisfactory (Gierlinger et al. 2004). A maximum of one outlier was detected in cross-validation and test set validation.

In a third step, a PLSR model including all calibration spectra was calculated for further investigations using an independent sample set.

External validation

Validation of the PLSR model obtained in the third step of the calibration was performed with a completely independent 38-sample set covering the natural range of lignin content of maritime pine wood.

Outlier detection

The Mahalanobis distance is a measure of the similarity between spectra of the analyte and the calibration spectra (Bruker 1996). The Mahalanobis distance is used as a diagnostic tool for outlier detection in multivariate calibration and to detect samples that would lead to extrapolation of the model. A more detailed description is given elsewhere (Bruker 1996; Gierlinger et al. 2004).

To calculate the limit of the Mahalanobis distance, a factor of two is often too restrictive for the prediction of unknown natural samples. As a consequence, too many samples are unjustifiably rejected and marked as outliers. Therefore, a factor of three was used instead.

Results and discussion

The 96 maritime pine wood samples with different levels of colour, which were analysed by five operators, were divided into two equal-sized groups with differences between replicate determinations of the Klason lignin content of $\leq 0.63\%$ (a) and $> 0.63\%$ (b). Figure 1 shows the wide range of differences between the replicate measurements and therefore a heteroscedastic variance, but no systematic trend in differences as a function of the lignin content can be observed, although a few more

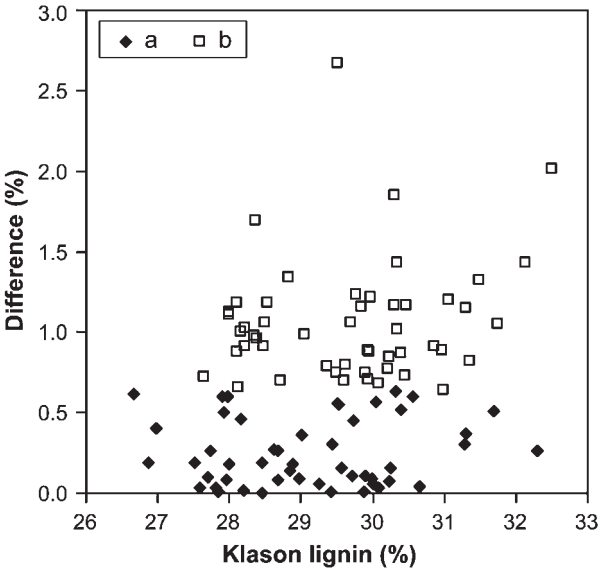

Figure 1 Differences between repeated determinations of the Klason lignin content: (a) $\leq 0.63\%$ and (b) $> 0.63\%$.

b samples than a samples have a lignin content above 31%.

Principal component analysis

First-derivative spectra in the wavenumber range 6102–5760 cm^{-1} that were useful for the prediction of the lignin content of softwoods (Schwanninger and Hinterstoisser 2001) were calculated. Other methods were also tested, but did not yield better results. However, using the second-derivative spectra, similar PLSR model statistics were obtained, which are useful in simplifying the explanation of the good prediction results.

The first-derivative spectra were subjected to PCA. The a and b samples are distributed over the whole scores plot of principal components 1 and 2, without showing any regular pattern (Figure 2a), although 62% of the variance is explained by PC 1 and 24% by PC 2. The scores plot for PC 2 versus PC 3 (7% variance explained) does not show a pattern either (data not shown).

The external validation set (38 samples) was divided into three groups according to lignin content (e, low; c, medium; d, high). The spectra of the external validation set were added to the calibration spectra set (hereafter called "all samples") and subjected together to PCA. The

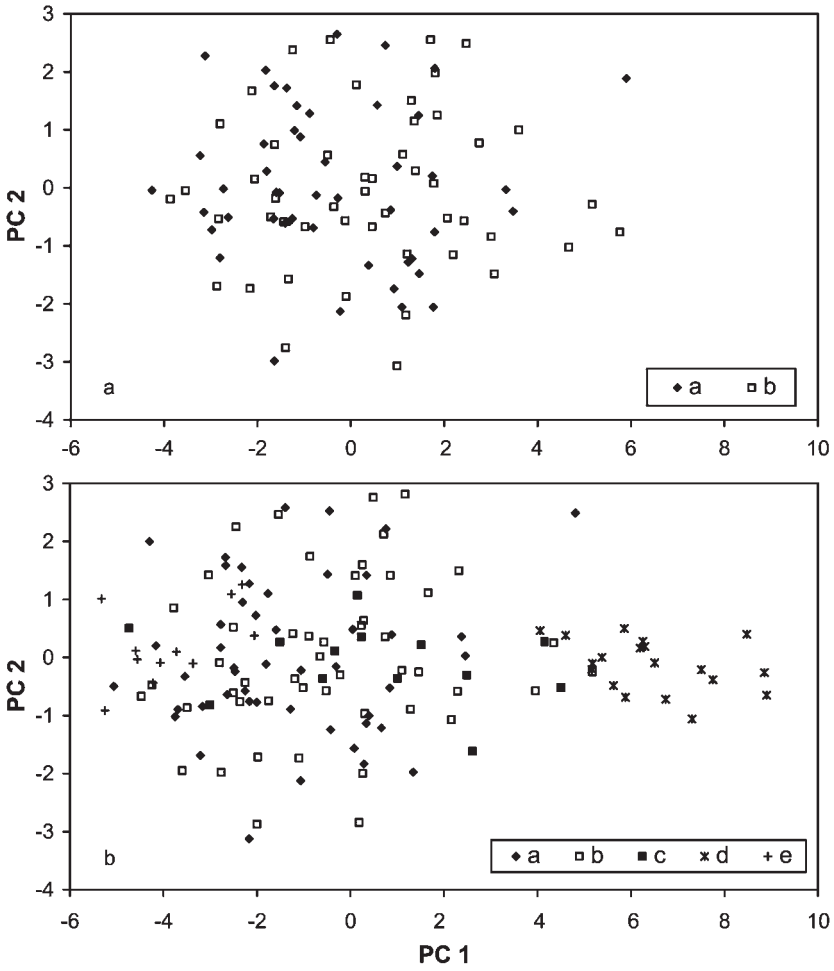

Figure 2 (a) Scores plot of principal components 1 and 2 of the pre-processed (first derivative) calibration spectra (reference data set, differences between the repeated determinations; a, $\leq 0.63\%$ and b, $> 0.63\%$) in the range from 6102 to 5760 cm^{-1}. (b) Scores plot of PC 1 and 2 of all pre-processed (first derivative) spectra (reference data set and independent validation data set) in the range from 6102 to 5760 cm^{-1}. The external validation set (38 samples) was divided into three groups according to lignin content (e, low; c, medium; d, high).

scores plot for all samples (Figure 2b) shows no systematic difference between samples from the calibration data set (a, b) and the independent data set (c, d, e). Along PC1 (80% variance explained) the samples are separated according to their lignin content. The separation of sample group d within the independent validation data set was expected, since these samples contained compression wood with a high lignin content. Furthermore, the samples of group e can be found to the left of PC 1 (low lignin content). The validation samples are within −1 and +1 of PC 2 (10% variance explained), whereas the calibration samples vary between −3 and +3. From the x-loading of PC 3, it is known (data not shown) that, apart from lignin, the range from 5890 to 5810 cm^{-1} contributes mostly to the variation. It is known for spruce wood that this range is influenced by fungal attack (Schwanninger et al. 2004). This could partly explain the higher spectral variation in the calibration samples. In addition, in the PC 2 versus PC 3 (4% variance explained) plot, the c, d, and e samples are much closer to the point of origin (data not shown).

Calibration, cross-validation and test set validation

The calibration model (PLSR) of a and b samples based on the pre-processed spectra in the range from 6102 to 5760 cm^{-1} and the noisy reference data show a poor coefficient of determination ($R^2 = 0.53$) with a RMSEE of 0.85% and poor cross-validation results ($R^2 = 0.50$; RMSECV = 0.87%) for the two PLS components (rank 2) used. Linear regression between the NIR cross-validation Klason lignin content and the reference values (Figure 3a) gave a slope of 0.51, an intercept of 14.25 (% Klason lignin) and R^2 of 0.5.

Calibrations (PLSR) using only a samples for calibration and CV and b samples for TS, and then vice versa (b samples for calibration and CV and a samples for TS) led to almost identical model statistics (not shown), which are very similar to those obtained as described above. This confirms the PCA result showing that no systematic difference between groups a and b exists. Interestingly, the larger differences between the replicate Klason lignin determinations found for group b do not influence the model statistics and therefore the average seems to be a good estimate.

Cross-validation with one sample omitted (leave-out-one CV) was performed for the internal validation. A large number of cancellation groups correspond to validation with a small perturbation of the statistical sample, whereas a small number of cancellation groups correspond to heavy perturbation. Perturbation of the model at each step is small when using a large number of samples combined with the leave-out-one method for cross-validation. This procedure tends to overfit the model. For this reason, the leave-more-out method for cross-validation may be preferable. The PLSR model obtained for all calibration samples was subjected to a further CV step by increasing the number of samples left out during CV (Table 1), as full CV may give over-optimistic results (Næs et al. 2002). When up to 30 samples were left out during CV, R^2 decreased from 0.5 to 0.44 and RMSECV increased from 0.87% to 0.91%. Leaving out more samples also led to a decrease in the rank from 2 to 1. Overall the results are very similar, and the robustness of the model seems to be proven. It can be concluded that the model is stable.

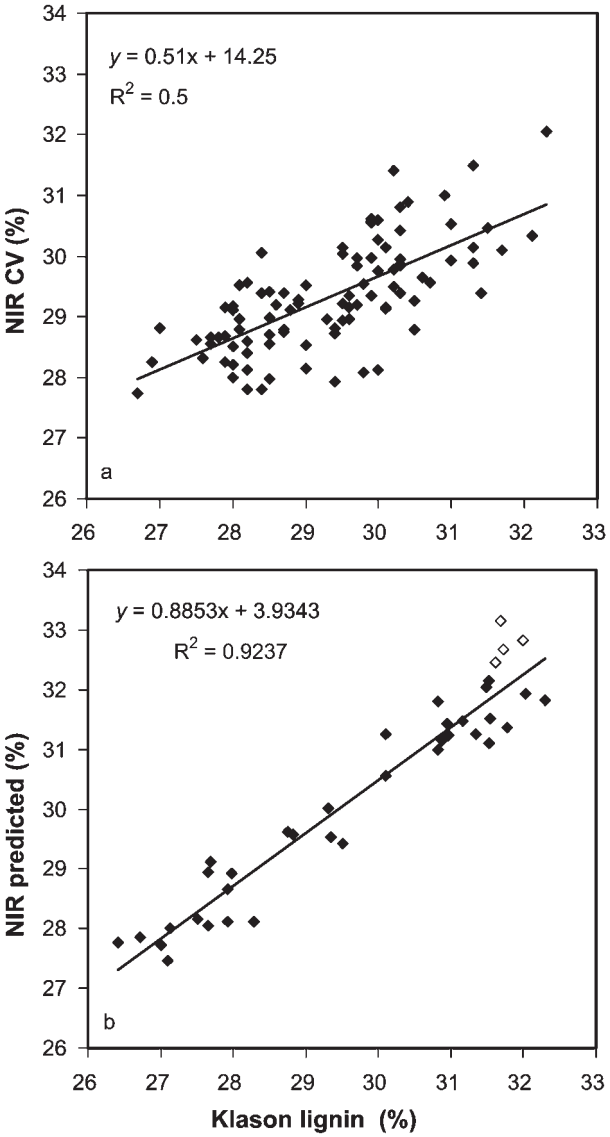

Figure 3 (a) Cross-validation (NIR CV) result for the calculated PLSR model for Klason lignin content and (b) external validation (NIR predicted) result for the calculated PLSR model for Klason lignin content. The unfilled symbols indicate outsiders.

Table 1 Results for cross-validation of the calibration samples: the number of samples left out during the cross-validation was increased from 1 to 40.

Leave out	Rank	R^2	RMSECV (%)
1	2	0.50	0.874
5	2	0.50	0.865
10	2	0.49	0.870
15	2	0.49	0.867
20	2	0.45	0.901
25	2	0.47	0.885
30	2	0.44	0.908
35	1	0.44	0.908
40	1	0.39	0.949

External validation

External validation was performed using the above model with 38 maritime pine wood samples and a better reference determination methodology (only one technician and an improved Klason lignin method; Schwanninger and Hinterstoisser 2002). Linear regression between the NIR predicted Klason lignin content and the reference values (Figure 3b) led to a linear equation with a slope of 0.89, an intercept of 3.9, R^2 of 0.92 and RMSEP of 0.74%. One sample identified as a spectral outlier was removed. The unfilled symbols in Figure 3b indicate samples that are not spectral outliers, but are outside the calibrated range. Nevertheless, the predicted values are very good and demonstrate that extrapolation is possible in a small range, which is in agreement with the literature (Estienne et al. 2001). The RMSEP is high, but compared to the RMSEE and the RMSECV an improvement is evident.

The most interesting observation is the change in slope found between true and predicted values from 0.51 (CV) to 0.89 (TS) and of the intercept from 14.25 (CV) to 3.9 (TS). Of course it is expected that the slope of the regression line between the reference data (Klason lignin) and the values predicted with NIR models would be close to one and the intercept close to zero, but it is not clear why this result was obtained from the calibration model. PCA of all samples (Figure 2b) showed that the external validation samples are distributed along the PC 1 axis in a small band according to their Klason lignin content, with increasing values from left (–) to right (+), whereas the calibration samples are well distributed along the PC 2 axis. Furthermore, it is known from the x-loading of PC 1 (Figure 4) that the most relevant variables are in the range of the first overtone of the aromatic C-H stretching vibration, which is dominated by lignin (Figure 4, peak 1), and overtones from C-H vibrations from lignin (Figure 4, peaks 2 and 3), whereas cellulose and hemicelluloses also contribute to peaks 2 and 3. Moreover, from the PCA of the data set (Figure 4, External) used for the external validation, it is known that the loadings of PC 1 (calibration samples and test samples; Figure 4, Cal + External) are very similar, meaning that they show a similar shape. The variance explained by PC 1 is 62% for the calibration samples and 96% for the test samples. Therefore, the contribution of PC 2 to explaining variance in the test set is low (2%). This explains why the Klason lignin content could be predicted with the model without giving many spectral outliers, but not the good result of the external validation.

Why can predicted values be more precise and accurate than the reference values used for calibration?

As mentioned above, using the second-derivative spectra, similar PLSR model statistics (RMSECV = 0.88, rank 2, $R^2 = 0.5$) were obtained for the calibration data set. However, this model produces more outliers in prediction. By using the external validation data set for calibration, the following PLSR model statistics were obtained: RMSECV = 0.52, rank 2, $R^2 = 0.92$. It was recently shown that good correlation exists between the lignin content

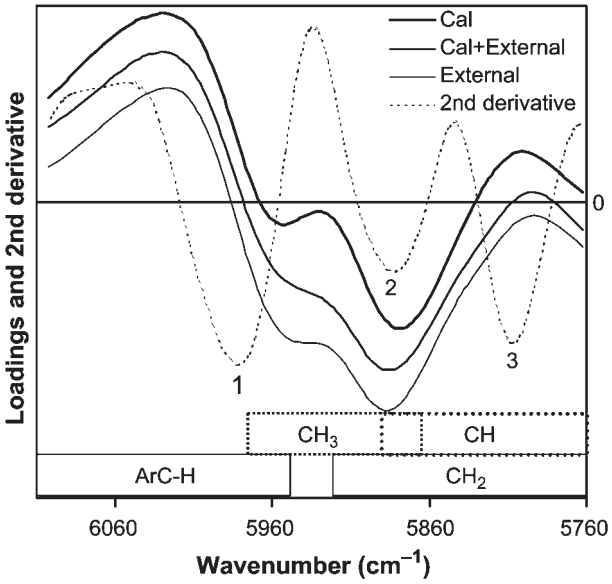

Figure 4 Loading plots for PC 1 of the calibration samples (thick continuous line), the external validation samples (thin continuous line), and both together (calibration plus external). The latter two are shifted parallel to the zero line. The ranges of the first overtones of C-H vibrations are presented. The second derivative of an NIR spectrum (dotted line) is shown for the band assignment of the first overtone of aromatic C-H stretching vibration, which is dominated by lignin (peak 1), and overtones from C-H vibrations from lignin (peak 2 and 3), whereas cellulose and hemicelluloses also contribute to peaks 2 and 3.

and the amplitude of the band at approximately 5978 cm^{-1} in second-derivative spectra (Schwanninger et al. 2004). To simplify explanation of the good prediction results presented above from a mathematical point of view, this amplitude (Figure 4, peak 1) was correlated with the Klason lignin content of the calibration and external validation data sets, respectively (Figure 5). The slope of the regression line for the calibration samples, in agreement with the results shown in Figure 3a, is less than that for the external validation samples. The coefficient of determination ($R^2 = 0.90$) of the latter is almost identical to that of the PLSR model ($R^2 = 0.92$). Correlating the Klason lignin content with the amplitude of the second-derivative spectra of the calibration samples and the external validation samples resulted in two parallel regression lines (Figure 6).

The first method (Figure 5: amplitude vs. Klason lignin), the classical calibration, assumes a linear model (Beer's law) for spectra as a function of the chemical concentration (Klason lignin content) and that the reference values are the accurate ones. The second method (Figure 6: Klason lignin vs. amplitude), the inverse calibration, assumes that the spectral data are more precise and that variation is due to the inaccuracy of the reference values, which in fact is true for the calibration samples used in the present study. The latter method is used in PLSR. Comparison of Figures 5 and 6 reveals a narrowing of the calibrated range. The classical method gives a calibrated range identical to the reference values (Figure 5), but application of the inverse calibration method narrows the calibrated range from 26.7–32.5 to 27.6–31.8. The range for the external validation samples is closer to the

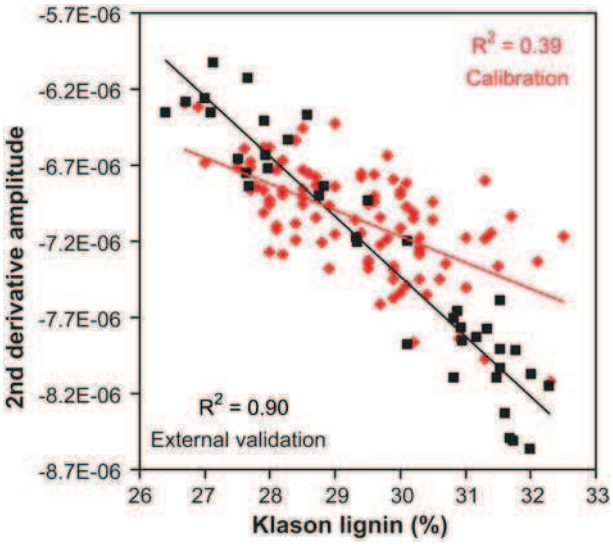

Figure 5 Correlation between the amplitude of the second-derivative spectra at approximately 5978 cm^{-1} and the Klason lignin content of the calibration samples (red) and the external validation samples (black).

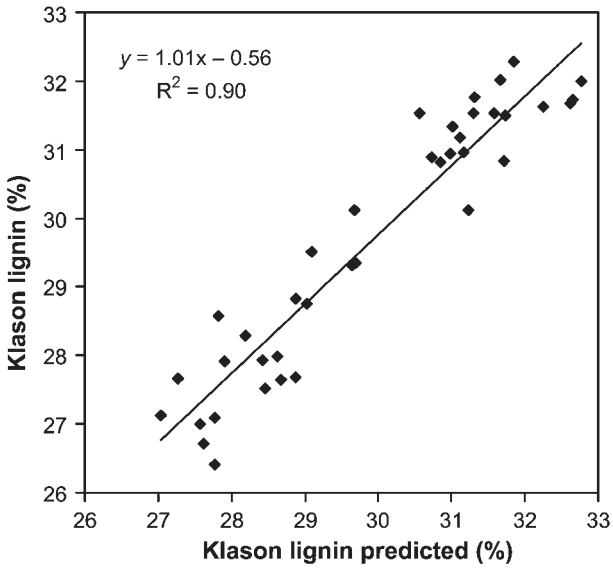

Figure 7 Klason lignin versus Klason lignin predicted with the regression equation obtained for calibration samples using the inverse calibration method.

reference values because of the better fit of the inverse model. The better the correlation between the reference values and the spectral data, the better is the fit, and the lesser is the difference between the classical method and the inverse method. Compared to the classical calibration, the inverse calibration "shrinks" predictions towards the mean, the so-called least-squares effect.

The regression equation obtained for inverse calibration of the calibration samples (Figure 6) was used for prediction of the external validation samples. A correlation of 0.95, slope of one, and an intercept of −0.5 (% Klason lignin) was found between Klason lignin and the predicted Klason lignin content (Figure 7). This was expected from Figure 6, because the regression lines are parallel, with a shift of approximately 0.5%.

As expected from PCA and shown by prediction of the external validation samples, only one sample was found to be a spectral outlier. Based on the fact that (a) the precision of the spectral data was much higher than for the calibration reference values, and (b) PLSR uses an inverse calibration, it is clear that predicted values can be better than expected from the calibration results using "noisy" reference data.

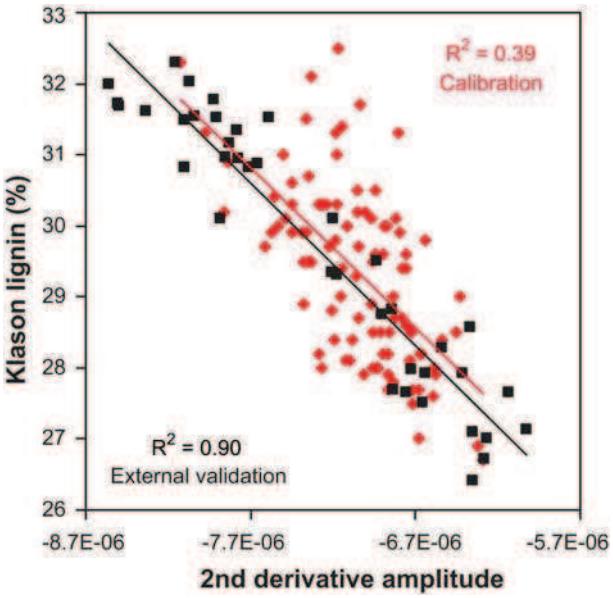

Figure 6 Correlation between the Klason lignin content of the calibration samples (red) and the external validation samples (black) and the amplitude of their second-derivative spectra at approximately 5978 cm^{-1}.

Conclusion

To the best of our knowledge, this is the first study dealing with real noise in the reference data obtained in one laboratory that compared these to a truly independent test data set prepared in another laboratory. Values predicted with a NIR PLSR model based on a noisy calibration data set can be more precise and accurate than expected from the cross-validation results, as has also been shown by other authors (DiFoggio 1995; Næs et al. 2002). PCA revealed that the loadings (calibration samples and test samples) of the first PC are very similar and dominated by lignin-related bands. Moreover, most of the variation in the test set can be explained by the first PC. This explains why the Klason lignin content could be predicted with the model without giving many spectral outliers, but not the good result of the external validation. The latter can be explained by the inverse calibration method used for PLSR.

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Artigo VI

Improvement of *Pinus pinaster* Ait elite trees selection by combining near infrared spectroscopy and genetic tools.

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Improvement of *Pinus pinaster* Ait elite trees selection by combining near infrared spectroscopy and genetic tools

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Abstract

The first and the second generation of improved maritime pine (*Pinus pinaster*) have produced gains of up to 30% in stem volume, a reduction of final harvesting age by 10 years and a considerable improvement of stem straightness. The third generation will include wood quality traits for different end-uses, including pulp and fibre properties. To facilitate this goal, near infrared spectroscopy (NIRS) was used to estimate chemical composition with regard to lignin, cellulose, hemicelluloses, and extractive content, and lignin quality with regard to 4-hydroxy-phenylpropane/guaiacylpropane ratio. A total of 960 samples were investigated which were collected from a large number of trees (belonging to 80 families obtained by crossing 18 mothers and 20 fathers; there are 12 trees per family). Good calibration data was obtained between NIRS and wet chemistry methods (R^2 values higher than 0.9 and good precision of prediction). To complete the NIRS work, kraft cookings in small scale, fibre morphology and microdensitometry investigations were also conducted. Genetic calculations indicated that for a 1% rate of selection on mothers and fathers, genetically induced changes are possible with lignin content (-3.8%), cellulose content (+1.3%), pulp yield (+1.8%), fibre length in pulps (+0.17 mm) and wood density (+50 kg m^{-3}).

Keywords: breeding; near infrared spectroscopy (NIRS); *Pinus pinaster*; quantitative genetics; wood quality.

[a] From June 1st 2007, AFOCEL and CTBA merged to become FCBA.

Introduction

Presently, considerable effort is invested in improving the wood and fibre quality of eucalyptus, pines, firs and poplar (Carnus et al. 2006), including maritime pine (*Pinus pinaster* Ait). The planted area of this species in France is approximately 1 million hectares and represents the largest plantation forest in Europe. The annual consumption of maritime pine for pulp production is approximately 3 million raw tons, of which 70% comes from thinnings (Chantre et al. 2004).

Intensive efforts have been undertaken by INRA and AFOCEL (now FCBA) in France for more than 40 years to improve wood quality of maritime pine (Alazard and Raffin 2002; Pot et al. 2002b). Although breeding has been the main focus for three decades on growth, soil adaptation and the creation of new varieties, wood and fibre quality have recently become important criteria in the selection of trees.

In view of pulp and paper production, elite trees can be defined as trees in which the wood has low lignin and extractives content and high polysaccharide (and especially high cellulose) content. Moreover, the wood of elite trees should have high density, long and coarse fibres with high proportion of earlywood fibres, and should give rise to pulps with good burst, tear and tensile strength.

The traditional analytical methods employed for wood, fibre and pulp evaluation are usually time-consuming and expensive. Rapid and non-destructive analysis methods, such as near infrared (NIRS) or Raman spectroscopy, provide an opportunity to shorten analysis time, elevate the throughput and lower the costs (Greaves et al. 1996; Schimleck et al. 2000; Chantre and Cahalan 2001; Baillères et al. 2002; Raymond 2002; da Silva Perez et al. 2004; Hannrup et al. 2004; Ona 2004; Schimleck and Workman 2004). Comprehensive literature reviews (Schimleck and Workman 2004; Tsuchikawa 2007) demonstrate the analytical power of NIRS for the rapid quality assessment of wood, fibres, pulp and paper.

A 4-year European research program, namely the GEMINI (Genetic determinism of maritime pulp and paper properties) project, was carried out in cooperation with different research institutes, universities and industrial partners. This research program was mainly aimed at the development of rapid assessment techniques for wood, pulp and paper quality, and the accelerated selection of elite maritime pine trees. In this article, the results concerning the genetic gains in wood and fibre quality with regard to chemical pulp production are described. The results were obtained by combining NIRS data and heritability calculations.

Material and methods

Trees and wood

A total of 960 samples from a progeny test in Tronquats (south-west of France) were analysed for quantitative genetic purposes (heritabilities and genotypic correlations) as part of the GEMINI project. The trees for this test were planted in 1988 and harvested in 2002. Accordingly, most of the vegetal material was composed of juvenile wood as expected for 14-year-old trees. The Tronquats test contained 155 G1 descendants (first generation of improved maritime pine) obtained from 18 selected mothers and 20 fathers by means of an incomplete factorial design. A total of 80 families were present, with at least two crossings per genitor. A total of 12 trees per family were studied, randomly distributed in five blocks. The planting density was 1250 trees/hectare (trees spaced of 4 m×2 m). One non-improved maritime pine family (280), also randomly distributed in the test, was used as a control. The organisation of the field test, in particular the connections of the families with their genitors are presented in Table 1.

After harvesting, a 1-m log was taken from each tree between the second and the third branch whorls. The logs were manually debarked and one disk (2.5 cm thick) was sampled in the middle of each log for microdensitometry. The disks were air-dried before cutting 2 mm wide×2.5 cm high wood strips with a twin-blade saw. After the disks sampling, each log was individually chipped and screened, i.e., large wood chips (bigger than 45 mm) and fines (smaller than 3 mm) were removed. Wood chips were then exhaustively mixed in a rotating drum for 15 min. A 500-g subsample was collected, air-dried and stored in a ventilated atmosphere. Wood chips were submitted to kraft pulping without any further treatment. For NIRS work and chemical analysis, 100 g of air-dried wood chips were ground in a 3-knife mill (Fritsch Pulverisette) and then sieved. Only the fraction passing the 40-mesh sieve and retained by the 60-mesh sieve was conserved and maintained in a controlled atmosphere (50% humidity, 23°C) for 1 week prior NIR spectra acquisition and analysis.

Measurements on trees

Height and circumference at breast height of the trees were measured before harvesting. The straightness of trees was evaluated as the projection of the deviation of the top of the trees to its base divided by tree height; the corresponding data ranged from 1 (seriously crooked) to 5 (straight).

Kraft cooking trials

Kraft pulping was conducted in an oil-heated multi-batch digester system in which wood was cooked in 150-ml mini-digesters containing 20 g (dry basis) of wood. For each cooking cycle, 12 mini-digesters can be used simultaneously. Kraft cooking conditions were alkali=18.5% on wood o.d.w. (based on Na_2O); sulphidity=30% of effective alkali; H-factor ~1600; liquor/wood ratio=4; temperature cooking profile=room temperature to 170°C in 90 min, plateau at 170°C for 90 min. Kappa number target was 80. The reason for the investigation on the high Kappa number level is that the kraft pulps of maritime pine are mainly used for packaging purposes. Pulp yield and Kappa number were measured for all the samples.

Chemical composition

Chemical characterisation of samples was performed by NIRS-partial least squares regression (PLSR) calibrations. Estimation of extractives content was performed by NIRS of non-extracted samples; otherwise the spectra of the extracted wood were recorded. To build NIRS calibrations, the yield of extractives were determined for each solvent (dichloromethane, ethanol and water). Extraction was carried out using 250-ml glass flasks equipped with condensers in two steps for each solvent. Firstly, the thimbles containing the samples were impregnated with the solvent for 30 min. Then they were suspended above the boiling solvent and below approximately 1 cm from the condensers basis for 60 min. Extractives content was calculated as % based on the dry whole wood powder. Klason lignin content determination was performed according to the procedure of Schwanninger and Hinterstoisser (2002). The content of cellulose and hemicelluloses were determined from HPLC analysis of monosaccharides after acidic hydrolysis according to Puls et al. (1995).

All NIRS-PLRS models yielded results with a precision which is comparable to that of the reference methods (Table 2). The externally validated models have been published elsewhere (Alves et al. 2006a; Rodrigues et al. 2006).

Microdensitometry

X-ray densitometry method was applied, as described first by Polge (1966) and subsequent improvements by Mothe et al. (1998) and Saranpää (2003). Microdensitometric profiles were obtained from 2-mm thick strips. The samples were X-rayed and the radiographic films digitalised with a high resolution scanner. Density levels were associated to grey levels in the image by means of a calibration curve. The earlywood and latewood densities were determined and average densities were calculated including the standard deviation.

Fibre morphology

Fibre morphology was evaluated using MorFi LB-01 apparatus (TechPap, Grenoble, France), as described by Eymin Petot Tourtollet et al. (2003) and Passas et al. (2004). Morphologic characteristics were obtained through the image analysis of the fibres in dilute suspension by means of a digital camera (resolution: 10 μm in image acquisition, 4 μm after software treatment). After the individual fibre image digitalisation, one virtual fibre skeleton was obtained and the length of this skeleton was the individual fibre length. Fibre width was calculated as the average of the width of all points along the skeleton fibre. Coarseness was estimated as the ratio between sample weight over the sum of all the individual length fibres. Curl was calculated as $(1\text{-}DE/L)\times 100$, where DE is the dimension of a straight line connecting the two extremities of the fibre and L the skeleton length. Kinks were zones of the fibres where the skeleton line undergoes abrupt changes of direction. Fine elements were the object images with lengths less than 200 μm or width less than 5 μm. As a minimum of 5000 fibres were analysed for each pulp, the distribution and the average values averages values can be calculated for all the properties. Arithmetic average values are used in this article, except for the fibre length (average weighted on length).

Analytical pyrolysis

CDS Pyroprobe 1000 with a coil filament probe connected to a GC (HP 5890 FID) via a heated interface 280°C was used for analytical pyrolysis. Pyrolysis temperature was 650°C for 10 s and sample amount was 75 μg of extractive-free samples. Up to three replicate analyses were performed per sample. The conditions and the quantification procedures have been published elsewhere (Rodrigues et al. 1999, 2001; Alves et al. 2006b).

Table 1 Incomplete factorial controlled crossing plan of selected fathers and mothers present at the Tronquats progeny test.

Mothers	Fathers																			
	22	29	41	70	123	152	156	1309	1317	1319	1328	3110	3111	3112	3113	3814	3823	3844	3847	4318
7				10				13			15					16		17	18	
24							25			26	27	28						30		
54												35			36		37			
56		40									42		43			44				
60			47		49	50		51	52											54
63			58				60							62						
113				69							71			73			74			75
128	78		79	80	81					82										
161		98						100		101						102				103
1901							123					124				125				
3116		140							141			142			143					144
3817						157	158	160		161		162								
3836	166			167						169			170						172	
3841		175			176	177			179										180	
4301												185		186		187		188	189	190
4309		193															194			
4324					203									206	207					
7101	212			213		214								216						

The numbers appearing at the intersection of one given father and one given mother are the family codes for descendants. Twelve trees per family were randomly distributed in five blocks. One non-improved family (280) was also present as control.

Table 2 Cross-validation performances of the NIRS calibrations for prediction of chemical composition of maritime pine wood.

Properties	Number of samples	Range		Rank	Calibration		Validation	
		Min.	Max.		R^2	RMSEC	R^2	RMSECV
Extractives content								
Dichloromethane	79	0.36%	2.96%	5	0.96	0.15	0.95	0.17
Ethanol	80	1.2%	2.4%	8	0.79	0.13	0.73	0.15
Water	80	1.4%	2.6%	5	0.88	0.11	0.84	0.12
Total	77	2.3%	7.1%	7	0.95	0.28	0.92	0.34
Lignin content	67	25.8%	32.7%	3	0.97	0.36	0.96	0.40
Cellulose content	67	39.9%	51.1%	2	0.93	0.77	0.92	0.80
Hemicelluloses content	68	23.3%	28.4%	4	0.77	0.54	0.71	0.59
Monosaccharide contents								
Mannose	62	14.5%	20.0%	1	0.89	0.53	0.87	0.55
Galactose	64	2.0%	10.9%	7	0.98	0.51	0.94	0.72
Xylose	67	9.1%	13.9%	5	0.77	0.41	0.63	0.50
Lignin quality								
Ratio of H/G units	62	0.041	0.111	2	0.90	0.005	0.89	0.005

Range data are reported for the non-extracted wood o.d.w. basis, except the calibration for the prediction of H/G lignin ratio.

Near infrared spectroscopy

A Bruker Vector 22N/I spectrometer equipped with a spinning cup module was used. NIR spectra were acquired using 50 scans per sample at 8 cm^{-1} spectral resolution and two spectra per sample. The spectra were not averaged. NIR spectra were acquired on wood powder prepared according to the protocol described in the Trees and wood section. PLSR was performed according to Workman et al. (1996) and Martens and Næs (2001). OPUS Quant (Bruker Optics, Ettlingen, Germany) was used for data pre-processing, calculation of the PLSR models and the prediction of the wood quality parameters.

The calibrations in this article were validated by the cross-validation method. The following parameters were considered: R^2 = coefficients of determination; rank = number of PLS components in the model retained for each calibration; RMSEE = root mean square error of estimation (calibration); RMSECV = root mean square error of cross-validation. NIR calibration for the prediction of 4-hydroxy-phenylpropane/guaiacylpropane (H/G) ratio has been published by Alves et al. (2006a).

Genetic calculations

The following model was applied for the estimation of genetic parameters, Eq. (1):

$$Y_{ijk} = \mu + M_i + P_j + b_k + (MP)_{ij} + e_{ijk}, \quad (1)$$

where Y_{ijk} is the property for the particular individual descendant from the i^{th} mother and the j^{th} father in the k^{th} block; μ is the general average of the test; M_i is the effect of i^{th} mother, P_j is the effect of j^{th} father, b_k is the effect of k^{th} block, $(MP)_{ij}$ is the effect of interactions of the i^{th} mother and the j^{th} father, and e_{ijk} is the residual.

The following relationships derived from the general theory of quantitative genetics were used, Eqs. (2) to (10):

Dominance variance: $\sigma^2_D = 4 \times \sigma^2_{MP}$ (2)

Additive variance: $\sigma^2_A = 4 \times \sigma^2_P$ or $4 \times \sigma^2_M$ (3)

Genetic variance: $\sigma^2_G = \sigma^2_A + \sigma^2_D$ (4)

Inter-family genetic variance: $\sigma^2_{Ginter} = \frac{1}{2} \times \sigma^2_A + \frac{1}{4} \times \sigma^2_D$ (5)

Intra-family genetic variance: $\sigma^2_{Gintra} = \sigma^2_G - \sigma^2_{Ginter}$ (6)

Environmental variance: $\sigma^2_E = \sigma^2_e - \sigma^2_{Gintra}$ (7)

Phenotypic variance: $\sigma^2_P = \sigma^2_G + \sigma^2_E$ (8)

Strict sense heritability: $h^2_{SS} = \sigma^2_A / \sigma^2_P$ (9)

Genetic gains: $\Delta G = i \times \theta \times h^2 \times \sigma_P$,
where $\theta = 2$ (2 genitors) (10)

Results and discussion

Near infrared spectroscopy

Maritime pine extractives are mainly composed of resin acids, fatty acids and their esters, and a variety of phenolic compounds (Hemingway et al. 1973). This particular chemical composition requires a combination of non-polar and polar solvents for a complete extraction. The sequence of solvents, dichloromethane-ethanol-water (DEW), was well suited for this purpose.

Individual and total extractives content obtained by DEW extraction were calibrated by NIR in combination with PLSR models. In addition, Klason lignin, cellulose, hemicelluloses, individual monosaccharides contents and lignin composition with regard to the ratio of the basic units (H/G ratio), were also predicted by NIRS calibrations.

Calibration data in Table 2 show that all components can be predicted by NIRS with high accuracy and precision. Some examples of cross-validation are given for the most abundant components of maritime pine wood in Figure 1. Good results were obtained for most wood components as indicated by statistical data presented in Table 2 (R^2 and RMSECV).

Calibrating NIRS with lignin content is quite common and a number of studies have addressed this topic (Easty et al. 1990; Greaves et al. 1996; Whiteman et al. 1996; Schimleck and Workman 2004; Rodrigues et al. 2006). Less common are the calibrations for polysaccharide analyses, with the exception of cellulose content (Schultz and Burns 1990; Wright et al. 1990; Raymond and

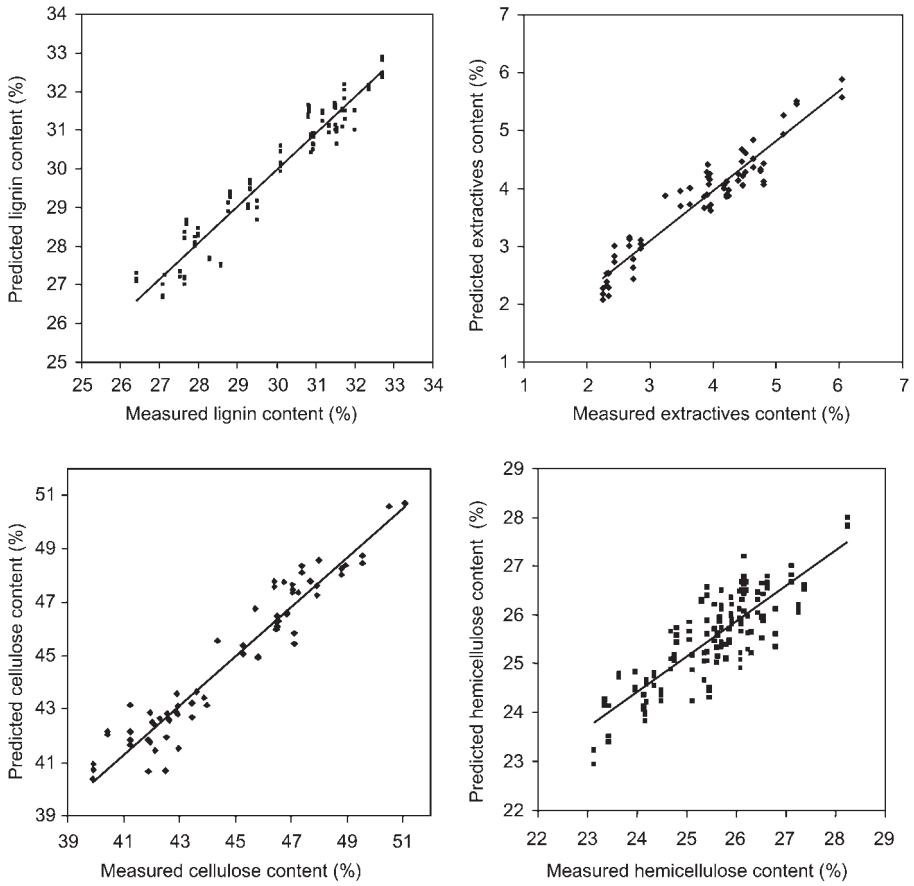

Figure 1 Cross-validation of NIRS calibration data for the prediction of the most important components of maritime pine wood.

Schimleck 2002; Kelly et al. 2004; Yeh et al. 2005) and extractive contents (Michell and Schimleck 1996; Gierlinger et al. 2002; Poke et al. 2004). To our knowledge, the NIRS calibration for the prediction of the basic lignin units estimated by analytical pyrolysis is unique (Alves et al. 2006a,b). Previously, Böttcher (1993) calibrated Fourier transform infrared data collected in the middle infrared range with pyrolytically determined basic lignin units. Good results were obtained for most wood components as indicated by statistical data (R^2 and RMSECV). H/G ratio for the samples in the present study, determined by analytical pyrolysis, ranged from 0.041 to 0.111, with an average of 0.064 and a standard deviation of 0.016. This data set was used for calibration of the NIR spectra. A good model with a RMSECV of 0.0054 and a high coefficient of determination of 89% was obtained (Alves et al. 2006a). This error is almost identical to the precision of the reference method (with a pooled standard deviation of 0.005).

Genetic determination of wood and pulp quality traits

Five main categories of wood quality traits were selected as input parameters for tree selection, with the aim of producing improved raw material: tree characteristics, wood density, chemical composition, kraft pulping and fibre morphology. Figure 2 presents averaged data calculated from 12 individual measurements of the descendants of each family. The corresponding error bars are constructed at the 95% confidence level (Students t-test). Table 3 presents the observed variation for all wood quality parameters.

Quantitative genetics was performed according to Eqs. (2) to (10) and the subsequent results are given in Table 4. The data include effects of block and parent (separate and combined) and analysis of variances and heritabilities. The possible gains if the best trees (1% or 5%) are selected for each property improvement are also presented. The block effect was observed to be significant for pulp yield, chemical composition (especially the contents of extractives and hemicelluloses) and density traits. Parent effects were significant for most of the properties, except for some fibre morphology traits, especially kinked fibres and fines.

Father×mother interactions were only significant for density traits and some isolated cases of chemical composition and tree characteristics. This information reveals certain symmetry of the heritable character in the crossings and also explains the relatively weak contribution of dominant variance to the total genetic variance for most traits. The model is significant for most of the parameters studied, except for some morphological traits. In terms of strict sense heritability (h^2_{SS}), the genetic effect is significant for a number of parameters, even if the heritabilities are only moderate for most parameters. A very slight effect of dominance on the genetic control of the parameters was observed. Most of the genetic variance is additive, except for hexose content and wood density (especially in earlywood).

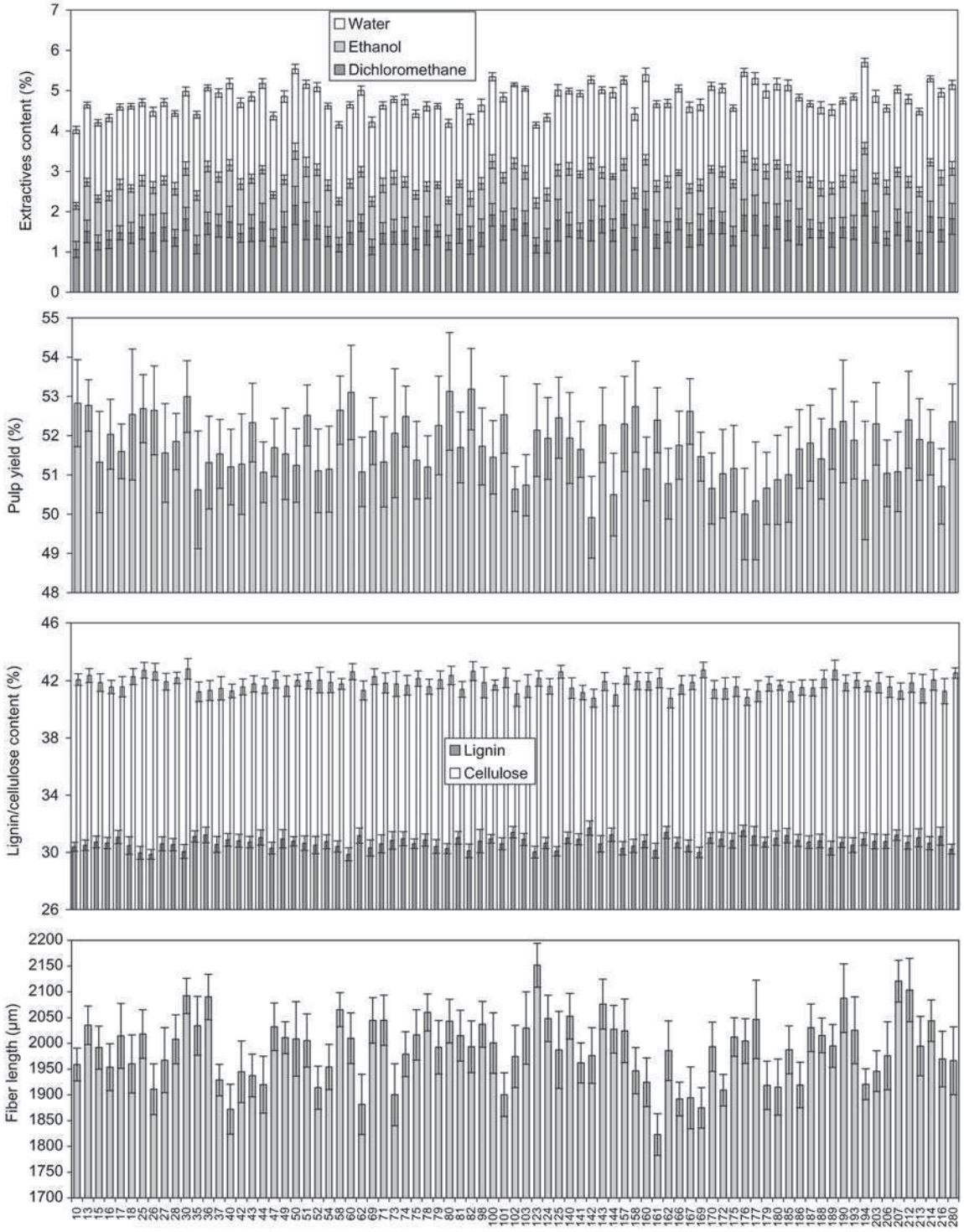

Figure 2 Average values and error bars (12 individuals per family, at the 95% confidence level) for selected quality traits of wood, pulp and fibres.

Tree characteristics

Height, circumference and straightness of the trees were all improved by the crossing of selected fathers and mothers for the Tronquats test. For 100% of the families present, height and circumference were greater than the non-improved family (280), with variation being from 48 to 59 cm for circumference and 9.4 and 11.7 m for height at the family level. On average, the best family gained 33% in volume compared to the non-improved family. The trees of the improved families were also considerably straighter. The average value of straightness ranged from 3.2 to 4.8 and only two families had average values lower than the non-improved family. Trees straightness is an important parameter for all forest-based industries as trees with high straightness values do not have compression wood and detrimental effects on pulping and fibre properties can be avoided (da Silva Perez and Fauchon 2003). Height, circumference and tree straightness had moderate heritability values of 0.3, 0.16 and 0.16, respectively. These values are in the same order of mag-

Table 3 Summary of the wood, pulp and fibre quality traits evaluated for the study of genetic determinism in *Pinus pinaster* based on the Tronquats progeny test data.

Wood, pulp and fibres quality parameters	Unit	Individual level				Family level		
		Min.	Max.	Aver.	SD	Min.	Max.	Aver. NI[b]
Diameter	cm	30.0	75.0	54.6	3.4	48.3	58.8	48.3
Height	m	7.2	12.6	10.5	0.9	9.4	11.7	9.4
Trees straightness	–	1.0	5.0	4.1	0.8	3.2	4.8	3.4
Pulp yield	%[a]	44.6	58.5	51.7	1.8	49.9	53.2	52.3
Kappa number	–	54.0	114.0	82.0	9.5	72.0	94.3	79.7
Extractives content								
Dichloromethane	%[a]	0.5	6.6	1.6	0.56	1.1	2.2	1.8
Ethanol	%[a]	0.4	2.3	1.2	0.23	1.0	1.5	1.3
Water	%[a]	1.6	3.4	2.0	0.18	1.9	2.1	2.1
Total	%[a]	2.7	12.3	4.8	0.85	4.0	5.7	5.2
Lignin content	%[a]	28.3	33.7	30.7	0.81	29.9	31.7	30.2
H/G lignin units ratio	–	0.055	0.096	0.073	0.007	0.061	0.084	0.070
Cellulose content	%[a]	37.8	44.8	41.8	1.07	40.8	42.8	42.5
Hemicelluloses content	%[a]	27.8	31.3	29.8	0.59	29.0	30.2	29.6
Pentoses content	%[a]	7.6	12.2	10.2	0.70	9.4	10.9	10.0
Hexoses content	%[a]	16.4	20.2	17.8	0.49	17.1	18.4	17.6
Mannose content	%[a]	9.7	15.9	13.8	0.75	12.8	14.4	14.2
Galactose content	%[a]	1.7	9.2	4.5	0.98	3.3	5.4	3.7
Xylose content	%[a]	7.1	9.6	8.5	0.43	8.0	9.1	8.6
Fibre length[c]	µm	1558	2344	1988	126	1823	2152	1966
Fibre width	µm	39.6	47.3	42.5	1.0	41.3	43.8	42.1
Coarseness	mg/m	0.193	0.449	0.289	0.038	0.256	0.328	0.298
Kink number/fibre	–	1.09	1.64	1.28	0.09	1.20	1.36	1.25
Curl index	%	4.62	7.81	5.71	0.55	5.05	6.07	5.54
Fines (surface)	% of total surface	1.07	8.72	2.18	1.48	1.76	4.96	2.34
Wood density	kg m^{-3}	330	664	449	53	397	501	472
Earlywood density	kg m^{-3}	269	587	377	46	331	423	401
Latewood density	kg m^{-3}	531	943	680	75	609	767	751
SD wood density	kg m^{-3}	14	159	55	19	37	64	69
SD earlywood density	kg m^{-3}	33	213	111	25	51	143	122
SD latewood density	kg m^{-3}	51	315	180	41	79	212	207
% of latewood	% of total surface	14.8	42.0	25.3	4.5	18.2	34.1	27.0

[a]% of o.d.w. wood; [b]non-improved family (280); [c]average weighted on length.

nitude as those reported in the literature for other pines (Balocchi et al. 1993; Fries and Ericsson 1998; Matziris 2000).

Microdensitometry

Wood density can be estimated by NIRS (Schimleck et al. 1999, 2001; Schimleck and Workman 2004). In the present study, the microdensitometry technique was preferred, because it allows the individual characterisation of growth rings, earlywood (EW) and latewood (LW) (da Silva Perez et al. 2005b). Traditional NIR spectroscopy does not deliver data with this high resolution. Interesting breeding potential was observed with this regard, both at the level of trees and families. The density variations with regard to the whole tree, EW and LW were considerable. At the family level, gains in whole tree density, compared to the non-improved family, were approximately 6%. However, the potential for density improvement was not the same for LW and EW. The number of families that had EW and LW densities higher than the control was 9 and 19, respectively. Density parameters had moderate heritabilities (0.16–0.31), and this finding concurs well with data in the literature (Whiteman et al. 1996; Pot et al. 2002b). LW heritability (0.31) was observed to be higher than EW heritability (0.16).

Chemical composition

The pulp yield of maritime pine is relatively low if compared to other softwoods. Its content of extractives and lignin is high and the cellulose content is low (Chantre and da Silva Perez 2002). Accordingly, breeding strategies must focus on increasing the polysaccharide content and decreasing the lignin and extractive contents. Native maritime pine can have extractive contents up to 13% (da Silva Perez et al. 2005a). In the present study, the extractive content was relatively small at the family level (4–5.7%). However, among trees the total extractives varied between 2.7% and 12.3%. It is important to note that the error of estimation of total extractive content by NIRS, expressed as CV and RMSECV, is 0.34% (Table 2). The within-family variation was higher for dichloromethane-soluble extractives than for the other fractions (see Figure 2).

Lignin content variation was more than 5% at the tree level and approximately 1.8% at the family level, while cellulose content variation was higher with a 7% difference between extremes among individual trees and 2% among families. The precision of estimation of the lignin content by NIRS (RMSECV) is 0.4% (Table 2).

Hemicelluloses variation was considerably smaller, with a difference of only 3.5% at the tree level and 1.2% at the family level. However, these values must be treated

Table 4 Genetic parameters calculated for wood, fibre and fibre quality traits (Tronquats progeny test).

Wood, pulp and fibres quality parameters	Block		Mother		Father		Mother × father		% Variance explained	Ratio variance σ^2A/σ^2G	Heritability h^2ss	Genetic gains	
	F	P	F	P	F	P	F	P				Gain 1%	Gain 5%
Diameter	0.82	ns	3.14	***	2.14	***	0.86	ns	15	1	0.16	5.22	4.04
Height	2	ns	4.16	***	6.39	***	1.4	*	25	0.77	0.31	13.12	10.16
Trees straightness	0.24	ns	2.4	***	2.2	***	1.3	ns	24	0.64	0.16	0.64	0.49
Pulp yield	3.28	*	3.19	***	3.34	***	1.38	ns	19	1	0.19	1.8	1.4
Kappa number	1.73	ns	2.33	***	2.26	***	1.33	ns	17	1	0.15	7.43	5.75
Extractives content									0				
Dichloromethane	4.33	***	2.4	***	4.37	***	0.88	ns	19	1	0.21	0.62	0.48
Ethanol	3.76	***	2.69	***	2.57	***	1.4	*	21	1	0.28	0.35	0.27
Water	28.48	***	2.49	***	2.73	***	1.01	ns	25	1	0.14	0.13	0.1
Total	8.14	***	2.46	***	3.71	***	0.88	ns	20	1	0.23	1.03	0.8
Lignin content	1.85	ns	3.49	***	5.07	***	1.27	ns	23	1	0.75	3.8	2.94
H/G lignin units ratio	1.51	ns	2.9	***	3.09	***	1.44	*	20	0.96	0.24	0.01	0.01
Cellulose content	2.2	ns	2.88	***	3.73	***	1.36	ns	20	1	0.23	1.3	1.01
Hemicelluloses content	6.27	***	2.56	***	3.31	***	1.06	ns	18	1	0.15	0.47	0.36
Pentoses content	1.34	ns	2.01	***	4.35	***	1.44	*	21	0.89	0.27	1.03	0.8
Hexoses content	1.12	ns	1.89	*	3.1	***	1.64	***	19	0.64	0.18	0.46	0.35
Mannose content	2.31	ns	4.6	***	3.84	***	1.28	ns	21	1	0.22	0.86	0.66
Galactose content	2.96	*	2.25	***	2.56	***	1.46	*	19	0.91	0.2	1.02	0.79
Xylose content	6.07	***	5.78	***	8.37	***	1.14	ns	33	1	0.64	1.52	1.17
Fibre length[a]	0.33	ns	3.97	***	6.03	***	1.25	ns	25	1	0.26	172.55	133.63
Fibre width	2.03	ns	4.53	***	4.86	***	1.06	ns	24	1	0.3	1.61	1.25
Coarseness	2.1	ns	1.57	ns	2.55	***	0.76	ns	14	1	0.07	0.01	0.01
Kink number/fibre	0.8	ns	1.19	ns	0.62	ns	1.29	ns	11				
Curl index	0.51	ns	2.17	***	1.44	ns	1.12	ns	14	1	0.06	0.17	0.13
Fines (surface)	0.36	ns	0.89	ns	0.82	ns	0.71	ns	8				
Wood density	16.56	***	2.37	***	3.83	***	2.05	***	25	0.42	0.17	0.05	0.04
Earlywood density	15.15	***	1.98	*	4.05	***	1.94	***	25	0.43	0.16	0.04	0.03
Latewood density	22.21	***	4.5	***	3.52	***	1.73	***	30	0.75	0.31	0.12	0.09
SD wood density	18.41	***	4.56	***	4.5	***	1.36	ns	28	1	0.28	0.03	0.02
SD earlywood density	24.63	***	3.91	***	4.29	***	1.7	***	29	0.46	0.2	0.01	0.01
SD latewood density	17.37	***	4.16	***	2.93	***	1.1	ns	25	1	0.2	0.01	0.01
% of latewood	1.74	ns	3.89	***	3.38	***	0.79	ns	20	1	0.28	6.84	5.3

***Highly significant effects ($P < 0.001$). [a]Average weighted on length.

with caution, because of statistical limitations. The data represent only twice the precision of estimation of hemicellulose content by NIRS (RMSECV), which is 0.59% (Table 2).

Strict sense heritability (h^2_{SS}) was quite high for lignin content (0.75) and xylose content (0.62). For lignin content, h^2_{SS} was considerably higher than that obtained in a previous study on maritime pine (0.47 measured on a half-diallel test; Pot et al. 2002b). The improved h^2_{SS} estimate can be attributed to the improved vegetal material and to a better precision of laboratory measurements according to the recommendations of Schwanninger and Hinterstoisser (2002). For other wood components, heritabilities were moderate, varying from 0.14 to 0.28 for extractives, depending on the fraction considered, and from 0.18 to 0.27 for the monosaccharide constituents of hemicellulose. Despite a good heritability estimate for hexose content, the heritability for cellulose was moderate (0.23), and suggests that an improvement in converting glucose content into cellulose and glucomannan hemicellulose fractions is still required. Lignin quality (H/G ratio) had a heritability of 0.24, also indicating potential for selecting trees for improved pulping performance.

Mini-kraft cooks

All samples were submitted to kraft cook in small digesters. Fibre morphology analyses, pulp yield and Kappa number determinations were performed. Improving pulp yield by crossbreeding appears possible at both the tree and family level. Differences between extreme yields were 3.3% at the family level and 14% at the tree level. According to Janin and Ory (1994), pulp yield gains higher than 0.13% are economically interesting provided that the pulp yield gains are heritable. Large variation in Kappa number was also observed between families (from 72 to 94) and at the tree level (from 54 to 114) under the same cooking conditions. Strict sense heritabilities for pulping characteristics were moderate and in agreement with those obtained for wood chemical composition (0.19 for pulp yield and 0.15 for Kappa number).

Fibre morphology

Maritime pine has relatively short fibre length compared to other softwoods despite high coarseness (Chantre and da Silva Perez 2002). Fibre length is directly related to physical properties of pulps, especially to tear index. As maritime pine is mainly a source of pulps for packaging grades, fibre morphology is an important quality parameter. Length weighted fibre length varied between 1.56 and 2.34 mm at the tree level and between 1.82 and 2.15 mm at the family level. The young age (14 years) explains the relatively short fibres. However, we suggest based on the variability of the data that a selection for fibre length is possible. Variation in average fibre width was small at the family level (41.3–43.8 μm). The measurement of fibre width was difficult as the high lignin content of the pulps led to difficulty in separating the fibres. The average number of kinks per fibre varied from 1.1 to 1.6 at the tree level and from 1.2 to 1.4 at the family level. For curl index, variation was from 4.6% to 7.8% at the tree level and 5% to 6.1% between families. Fibre length and fibre width revealed moderate values for h^2_{SS} (0.26 and 0.3, respectively). Variation in other morphology parameters were either not significant or had low heritability.

Phenotypic and genetic correlations

The most important phenotypic and genetic correlations are presented in Table 5. Only correlations significant at the 1% threshold ($P < 0.01$) between independent properties are shown. Among the few significant phenotypic correlations detected, cellulose and lignin contents (estimated by NIRS), and pulp yield (measured in laboratory) were the most interesting (Figure 3). As expected, cellulose and lignin contents were highly negatively correlated (-0.84). Pulp yield was positively correlated with cellulose content and negatively correlated with lignin content. However, no correlation existed between pulp yield and hemicellulose content.

Several genetic correlations were obtained. Surprisingly, cellulose content was positively correlated with extractive content. Total extractive content was negative-

Table 5 Phenotypic and genotypic correlations observed between the different wood, pulp and fibre quality traits for *Pinus pinaster*.

Property 1	Property 2	Type of correlation	
		Phenotypic	Genetic
Cellulose content	Extractives content		0.58
Cellulose content	Lignin content	-0.84	
Extractives content	EW density		-0.80
Extractives content	LW density		-0.54
Extractives content	Wood density		-0.70
Lignin content	Fibre length		0.98
Lignin content	Fibre width		-0.53
Lignin content	Hexoses content		-0.87
Lignin content	Pentoses content		-0.91
Pulp yield	Cellulose content	0.51	0.95
Pulp yield	Extractives content		-0.79
Pulp yield	Galactose content		-0.84
Pulp yield	Lignin content	-0.52	-0.87
Pulp yield	Mannose content		0.76
Pulp yield	Wood density SD		-0.78

EW, earlywood; LW, latewood.

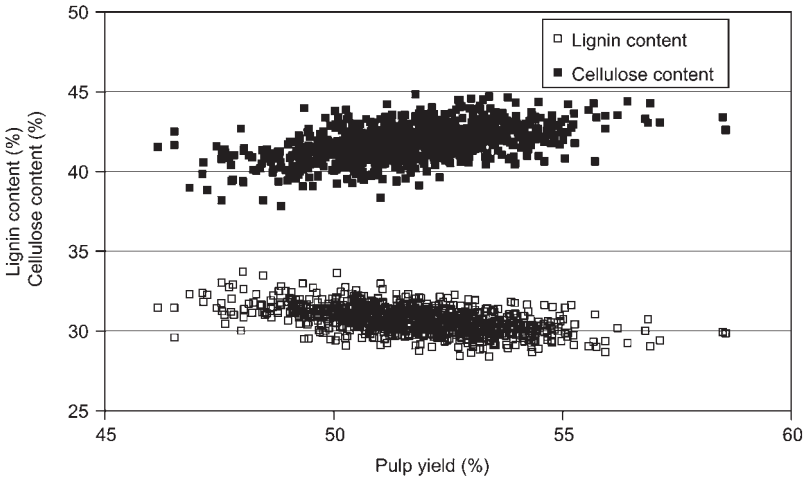

Figure 3 Phenotypic correlations between yield of kraft pulping and contents of lignin and cellulose as obtained by the Tronquats factorial test.

ly related to all density parameters, which can be explained by their relatively low specific weight and their low packing density in the cell wall compared to high molecular weight essential components. Although lignin content does not appear directly correlated with hemicelluloses content, highly negative correlations were observed with pentoses and hexoses. Lignin content was also related to fibre morphology parameter, showing a positive correlation with fibre length and a negative relationship with fibre width. Accordingly, if selection is based on lignin content, shorter and wider fibres will be produced. Finally, pulp yield was highly correlated with all wood components. Strong positive correlations were observed with cellulose (0.95) and partly with special hemicelluloses (0.76 for mannose content). Negative correlations were obtained with lignin (-0.87), extractives (-0.79) and some hemicelluloses constituents (-0.84 for galactose). No correlations were observed with wood density values. However, a negative correlation was observed between pulp yield and the standard deviation of wood density within growth rings. It is possible that the inhomogeneity of the tissue (large density differences between EW and LW) may lead to incomplete impregnation with a result of pulp yield decrement. However, this interpretation needs further proof.

The results reported here are in agreement with previous findings (Pot et al. 2002a,b). However, in an earlier study, a high positive correlation was found between lignin content and tree circumference. In the present study, however, with better characterised wood material and a better genetic material (more complete crossing between selected fathers and mothers), growth rate (the tree circumference) was independent of lignin content. It seems to be easier for tree breeders to increase growth rate, decrease lignin content, increase wood density and increase pulp yield at the same time.

Conclusions

NIRS calibrations based on analysis of 960 samples were successfully developed for the prediction of wood properties. The data facilitate the selection program of maritime pine (*Pinus pinaster* Ait) breeding. Good calibrations (high R^2 and low error or prediction) were observed for the content of different classes of extractives, lignin, cellulose and hemicelluloses. NIRS work was completed with trees measurements, kraft digestions, microdensitometry evaluation and fibre morphology characteristics. High variability was observed for most quality parameters of the wood, pulp and fibre quality, but only moderate heritabilities were calculated for most traits, except lignin and xylose content.

The selection rate was analysed at the 5% and 1% levels with regard to the best trees for each property. In the best case, for a 1% selection rate on mothers and fathers, the following genetic gains are possible (% of the average value for each wood property): 1.3 m (13%) for height, 5.2 cm (10%) for circumference, 3.8% (12.3%) for lignin content; for polysaccharide content: 1.3% for cellulose and 0.47 for hemicellulose content (3.2% and 1.6%), 1.03% (21.5%) for extractive content, 1.8 points (3.5%) for pulp yield; for fibre morphology: 0.17 mm (8.7%) for fibre length, 1.61 μm for fibre width (3%); for wood density: 50 kg m^{-3} for average density (11%), 40 kg m^{-3} for earlywood density (10.5%), and 120 kg m^{-3} for latewood density (17%).

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4 – Conclusões

A pirólise analítica demonstrou ser um método eficaz, reprodutível e preciso para a quantificação do teor de lenhina (Py-lenhina), em madeiras de três espécies de resinosas, *Pinus pinaster*, *Picea abies* e *Larix* sp..

Verificou-se que a pirólise analítica subestima o teor de lenhina quando comparado com o método Klason. No entanto, a boa correlação (R^2=0,93%) entre o teor de lenhina determinado pelos dois métodos (pirólise analítica e Klason) permite estimar o teor de lenhina Klason a partir da Py-lenhina.

A principal vantagem da pirólise analítica está na quantidade reduzida de amostra necessária na ordem dos 75 µg. Para além disso é uma técnica rápida e a preparação da amostra muito simples, uma vez que só têm de ser submetidas a moagem e extracção.

Cada pirograma é único, como uma impressão digital da composição química que reflecte o tipo de tecido, espécie, género, e proveniência. A pirólise analítica combinada com a análise de componentes principais (PCA) mostrou ser uma ferramenta útil para a descriminação de espécies e dentro da mesma espécie a descriminação da proveniência das amostras.
No entanto mesmo considerando as vantagens associadas à pirólise analítica, ainda assim é limitada em relação ao número de amostras que é possível analisar por dia.

Desenvolveu-se um modelo por espectroscopia de infravermelho próximo (NIR) em combinação com a análise multivariada e usando a pirólise analítica como método de referência. Este modelo foi desenvolvido em amostras de pinheiro bravo e permitiu obter um modelo para estimar a razão H/G com um erro médio da validação cruzada (0,0054). O erro do método é semelhante à precisão do método de referência (0,0050).

Desenvolveu-se um modelo por espectroscopia de infravermelho próximo (NIR) para a determinação do teor de lenhina em amostras de pinheiro bravo. O conjunto de amostras de calibração com baixa repetibilidade, originou um modelo PLSR NIR com estatísticas fracas (R^2=0,50 e

RMSECV=0,87). No entanto este modelo mostrou ser capaz de estimar correctamente o teor de lenhina num conjunto independente de amostras, como se verificou pelas estatísticas da validação externa (R^2=0,92 e RMSEP=0,74). Posteriormente obteve-se um novo modelo de calibração com o conjunto de dados da validação externa anterior, tendo-se obtido estatísticas muito boas (R^2=0,97 e RMSECV=0,36), sendo especialmente relevante o valor do erro quadrático médio muito próximo da repetibilidade do método Klason de acordo com a norma TAPPI T222 OM-88 (0,34%).

Usando os modelos desenvolvido por NIR para o teor e composição da lenhina é possível analisar mais de 300 amostras por dia o que é uma considerável poupança de tempo e de recursos relativamente à pirólise analítica, em que só era possível analisar 6 amostras por dia

Os modelos NIR foram usados para a caracterização do teor e composição da lenhina em madeira de pinheiro bravo, de um programa de melhoramento francês, na 3ª geração de selecção.
Esta técnica permitiu diminuir o tempo de análise e a redução dos custos associados à determinação do teor e composição da lenhina, usando os métodos tradicionais de química húmida. Verificou-se que, no caso desta população em particular, o teor de lenhina está sob um controle genético elevado de acordo com os valores de heritabilidade no sentido restrito (h^2=0,75), enquanto que a composição da lenhina está sob um controle genético moderado (h^2=0,24).