Extractives from Sitka spruce

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Abstract

The term *extractives* defines chemical compounds of different classes that can be extracted from wood or bark by means of polar or non-polar solvents. Extractives are derived mostly from the metabolic processes of the tree, particularly the sapwood to heartwood transformation.

The first objective of the research was to study the distribution of extractives within Sitka spruce trees at different heights in the trunk, as well as the distribution between bark, rootwood, knotwood, heartwood and sapwood. The second aim of the work was to learn about the influence of yield class, site elevation, North/East location and thinning on the extractives content and composition of Sitka spruce across Scotland.

The samples were sawdust obtained in different ways from either discs, knots or roots sawn from Sitka spruce trees freshly cut in the forest, or collected during the coring of trees from 64 sites all around Scotland and northern England.

The extraction was carried out on Soxhlet extractors using acetone as solvent. Two analytical techniques were used: gas chromatography and Fourier transform infrared spectroscopy.

The results of the research showed that the extractive content and composition of Sitka spruce differed according to the type of wood studied with the largest amount detected in bark and the lowest in heartwood and sapwood. The last two types of wood were studied in more detail, showing that the difference in extractive content between heartwood and sapwood was consistent at all heights in the trunk.

The chromatographic analysis of heartwood, sapwood, knotwood, rootwood and bark showed that their compositions differed slightly from published data on Norway spruce.

Across Scotland, the Sitka spruce extractive content was found to be low and stable, independent of forest management (yield class, thinning), site elevation and East or North location. However a slightly greater concentration of aromatic compounds relative to the aliphatic group was detected at sites in the West of Scotland.

Wood extractives are a major problem in regard to the pulp and paper industry. A low and stable extractive content can be a positive factor in the quality of the Scottish Sitka spruce resource, avoiding significant technical and economic problems.

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Author's declaration

This thesis and research described here are performed entirely by the author except where expressly stated. Furthermore, the work presented has not been submitted in whole or in part for any other degree.

Annabelle Caron-Decloquement

Definition

A.S.E : Accelerated solvent extraction
ANOVA : Analysis of variance
ATR : Attenuated total reflectance
BaF_2 : Barium fluoride
ECD : Electron capture detector
FID : Flame ionisation detector
FTIR : Fourier transform infrared spectroscopy
FTNIR : Fourier transform near infrared spectroscopy
GC : Gas chromatography
HPLC : High performance liquid chromatography
HPSEC : High performance size exclusion chromatography
IR : Infrared
LSD : Least significant difference
MC : Moisture content
MS : Mass spectrometer
MTBE : Methyl *tert.*-butyl ether
OD : Oven dried
RAPD : Random amplified polymorphic DNA
REML : Residual maximum likelihood
S.F.E : Supercritical fluid extraction
SPE : Solid phase extraction
TLC : Thin layer chromatography

Chapter 1: Introduction

1.1 Definition of wood extractives

1.1.1 What are extractives and how are they extracted?

The term *extractives* defines chemical compounds of different classes that can be extracted from wood or bark by means of polar and non-polar solvents. Extractives can also be water-soluble compounds such as carbohydrates and their derivatives. This definition covers a large number of components belonging to different classes (Fengel and Wegener, 1984; Hillis, 1987 and Baeza and Freer, 2001).

Following the above definition, extractives include a wide range of chemical compounds with different physical properties. As a result, the use of different solvents during extraction will extract different classes of extractives.

According to the solvent used in the extraction step, extractives can be divided into two different classes: lipophilic and hydrophilic compounds. The lipophilic extractives comprise mainly resin acids, diterpenyl alcohols, fatty acids, sterols, steryl esters and triglycerides which are extracted with non polar organic solvents such as hexane, pentane, petroleum ether or dichloromethane (Ekman, 1976; Ekman and Holmbom, 1989; Orsa and Holmbom, 1994; Willfor et al, 2003a; Willfor et al., 2003b; Willfor et al. 2004a and Willfor et al., 2006).

The hydrophilic extractives such as lignans, oligolignans and phenolic compounds are better extracted with polar solvents as acetone, methanol or ethanol. Sometimes water is mixed with a polar solvent to facilitate the penetration of the solvent through the wood and allow the extraction of even more polar compounds, such as lignan glycosides or polyphenols (Ekman, 1976; Ekman and Holmbom, 1989; Orsa and Holmbom, 1994; Awika et al., 2003; Willfor et al, 2003a; Willfor et al., 2003b; Willfor et al. 2004a and Willfor et al., 2006).

Kraft mill personnel, who are more interested in the impact of wood extractives on the pulping process, classify the extractives into saponifiable compounds that form soluble soaps under alkaline conditions, generally fatty acids, resin acids

and some steryl esters and glycerides; and unsaponifiable compounds which do not form soaps and have a tendency to precipitate and cause pitch problems, generally waxes, some steryl esters, diterpene alcohols and aldehydes, sterols, triterpene alcohols, fatty alcohols, etc… (Sithole, 1992). Pitch problems appear when wood resin precipitates during pulp and papermaking processes forming pitch deposits that affect both running properties and product quality (Blanco et al., 2005). Pitches are released from pulp into the process waters during the bleaching process. They can deposit in pulp and on different parts of the mill, or remain suspended in process waters and be discharged in the effluent (Gutierrez et al., 2001).

1.1.2 The role of extractants

1.1.2.1 How and why are they produced?

Extractives can come from at least two different sources. The first is compounds directly involved in the metabolic processes of the tree, such as the sapwood to heartwood transformation (Taylor et al., 2002). The second source would be as an artefact from further modifications of metabolites (Rowe and Corner, 1979). Due to the fact that the extractives have an important role during heartwood formation, they are therefore present in greater amount in the heartwood than in the sapwood (Hillis, 1972; Hillis, 1987 and Taylor et al., 2006).

1.1.2.2 What is their distribution within the tree?

The chemical composition of extractives is not uniform across the tree (Hillis, 1987). They are found in all morphological regions. But they can often be called “extraneous components” because they are mostly extraneous to the lignocellulose cell wall. They can be concentrated in resin canals and cell lumina, especially those of ray parenchyma cells (Kimland and Norin, 1972; Hillis, 1987; Gottlieb, 1990 and Obst, 1997). Extractives can be located in the capillaries in the cell walls, many compounds being small enough to diffuse into free space of this pore size. There is evidence from different studies that when the heartwood extractives are formed and the membranes containing them rupture, the smaller components may soak into the cell wall. So the cell wall extractives may be partly responsible for the differences in dimensional

stability, durability and strength that have been found between sapwood and heartwood. They may also have a direct influence on the ease of pulping by reacting with lignin (Kimland and Norin, 1972; Hillis, 1987; Gottlieb, 1990 and Obst, 1997).

Ekeberg et al. (2006) studied variation in the lipophilic extractives across stems of Scots pine and differences in the north–south and east-west directions in the stem to see if the wind or any external attacks have an influence on the lipophilic extractives. The results in the north–south and east–west directions showed no significant differences. There was a trend towards higher amount of extractives in the south quadrant compared to the other directions but the number of observations in this experiment was too small for clear determination of these differences.

Great variations occur also among species, from tree to tree and from season to season even in the same tissues. (Fengel and Wegener, 1984 and Baeza and Freer, 2001). Different species contain different total amounts of extractives. The total can vary from less than 1 per cent to more than one third of the dry weight (Fengel and Wegener, 1984, Obst, 1997 and Baeza and Freer, 2001). The proportion soluble in organic solvent is normally only a few percent in clear wood, but the concentration can be much higher in certain parts of the tree (Hillis 1972, Hillis 1987). It is generally higher in bark, heartwood, roots, branch bases and wound tissues (Obst, 1997). Heartwood contains extractives that can be used to define characteristics of the family, genus and even species (Hillis, 1987).

Generally, the extractive content varies from 1 to 10% for most European species and from 2 to 30% for tropical species (Baeza and Freer, 2001). The extractives content does not represent a large proportion of the mass of the wood in comparison with the cell wall polymers for example. However the identification of the extractives present allows the characterisation of each wood species chemically, just as the distribution of specific compounds provides the basis of chemotaxonomy of non-woody plants (Baeza and Freer, 2001).

1.1.3 What influences the content of extractants?

Silvicultural treatment may tend to improve vigour and heartwood formation, but these changes were not demonstrated to have any effect on heartwood quality, and so on extractive content in heartwood (Bergstrom et al., 1999; Gartner et al., 1999; Taylor et al., 2002 and Bergstrom 2003).

Possible factors influencing the amount of heartwood extractives have been summarised by Hillis (1968). He stated that the amount of heartwood extractives increased with the age of the tree and decreased with growth rate because of the repartition between heartwood and sapwood in the stem (Hillis et al. 1962 and Wilkes, 1984).

Philip et al. (1995), citing Scheffer and Cowling (1966) and Hafizoglu (1983) stated that the extractive content of wood is related to tree species, genetic differences between individual trees and different tree sizes. Taylor et al. (2006) who studied the extractives of Western red cedar did not find any relationship of growth rate and silvicultural treatments (thinning and soil fertilisation) with the wood extractives content (obtained after sequential extraction by toluene, ethanol and hot water).

1.1.3.1 Genetic

Due to the economic potential of extractives in some species (e.g. Maritime pine, Norway spruce and *Eucalyptus globules*) genetics and breeding have been investigated with a view to improve extractive production. For example, in a European project initiated in 2000 and entitled Genetic improvement of wood quality: increasing selection efficiency for different end uses (FAIR-CT97-3953). The genetic approach tended to improve extractive content more than silviculture, leading at the same time to more uniform durability of heartwood (Franklin et al., 1970 and Taylor et al., 2002).

In *Pinus sylvestris* Fries et al. (2000) and Elfving et al. (2001) established that the concentration of heartwood extractives varied between trees, although the extractive concentration was not related the amount of heartwood. Studying several progeny of *Pinus sylvestris*, they concluded that the variation of wood

extractives content was mostly under genetic control. Nevertheless for some specific extractive compounds the genetic expression can be influenced by environmental conditions such as soil fertility and thus nutrient balance and availability (Fries et al., 2000). With the objective of modifying wood extractive content further research was recommended to understand the molecular-genetic connection (Fries et al., 2000). Genetic selection at a young age may become an option to facilitate the production of more decay resistant raw material for construction and carpentry, as well as the production of various useful substances or alternatively, production of wood that is better suited for pulp and paper processing, these phenomena being closely related to the wood extractive content.

1.1.3.2 Wind

Strong wind induces the production of compression wood in the trunks of conifers and tension wood in hardwood species. Compression wood and tension wood contain less heartwood extractives than normal wood. In compression wood this is accompanied by a reduction in polysaccharides and an increase in lignin content (Hillis, 1987; Blanchette et al., 1994 and Taylor et al., 2002)

1.1.3.3 Pest and pathogen attacks

Several studies report that the extractives play a role against some forms of attack by pathogenic organisms (such as fungi or viruses). However Philip et al. (1995), citing Sheffer and Cowling (1966), stated that the durability of Sitka spruce heartwood is dependent on its high C:N ratio (which can be as high as 1250:1) and this rather than its extractive content may account for poor colonization in nature by micro-organisms.

1.1.3.4 Age of the tree

The extractive content seems to be related to the age of the tree, or more precisely related to the distance from the pith, the extractive content being smaller in the sapwood (Hillis, 1987; Krislow and Nault, 1988; Lasander, 1989; DeBell et al., 1999 and Taylor et al., 2002).

1.1.4 What are the human uses of extractives?

Wood colour, fragrance and durability depend on extractives composition. Extractives can also interfere with pulping for papermaking, with the drying and adhesion of the wood, and with its hygroscopic and acoustic properties (Umezawa, 2001). A number of extractives have been associated with specific biological properties, for example antitumor, antibacterial and antioxidant properties (Umezawa, 2001). Further details will be given for each class.

1.2 Extractives classes

1.2.1 Lignans, neolignans and related compounds

1.2.1.1 Introduction

Lignans and neolignans are phenylpropanoids, a group of compounds with two phenylpropane units linked by a covalent bond between the two propane side chains (Figure 1-1). If the linkage is C_8-C_8' (β-β) the molecule is classed as a lignan, while any other C-C bond leads to its classification as a neolignan.

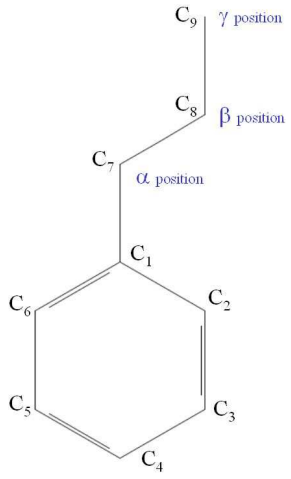


Figure 1-1: Phenylpropane unit

Lignans and neolignans are present in most plants including softwood and hardwood tree species and medicinal plants (MacRae and Towers, 1984; Umezawa, 2001 and Willfor et al., 2006). Norlignans are diphenyl pentane derivatives composed of a phenylpropane (C_6-C_3), and a phenylethane (C_2-C_6) unit connected via either C8-C7', C8-C8' or C9-C8' (Umezawa, 2001). This class

is of particular interest for the process of heartwood coloration (Umezawa, 2001)

Lignans often occur as glycosides. Some of these compounds represent dimeric structures that are also present in lignin molecules (Figure 1-2) (Fengel and Wegener, 1984; Umezawa, 2001). The main difference between lignin and lignans is the optical activity of lignans, which might suggest a difference in the mechanism of their biological synthesis (Umezawa, 2001).

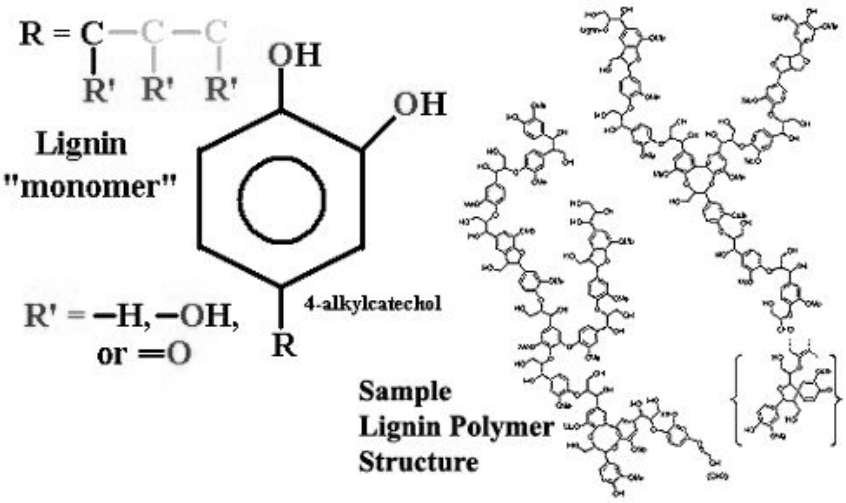


Figure 1-2: Example of Lignin structure (www.palaeos.com)

1.2.1.2 Presence in wood including Sitka spruce

Heartwood of softwood species (gymnosperms) contains mainly lignans (Figure 1-3) (Willfor et al., 2006). Heartwood contains a greater amount of lignans than sapwood (Obst, 1997). Many lignans that have been identified in the extract of *Picea*, *Pinus*, *Larix*, *Abies* and *Tsuga* species contain a tetrahydrofuran ring, such as pinoresinol, lariciresinol, matairesinol, conidendrin and liovil (Fengel and Wegener, 1984). Hydroxymatairesinol was the dominant lignan in Norway spruce and other major isomers were separated by several techniques (Mattinen et al., 1998 and Willfor et al., 2006).

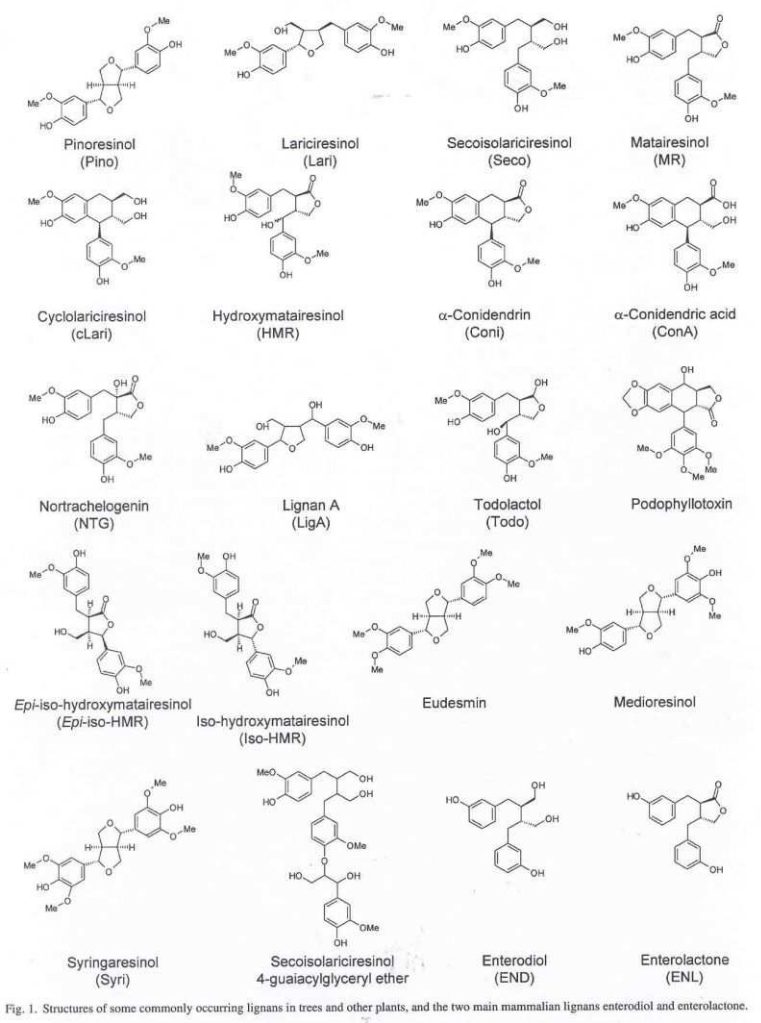


Fig. 1. Structures of some commonly occurring lignans in trees and other plants, and the two main mammalian lignans enterodiol and enterolactone.

Figure 1-3: Structure of some common lignans in trees and other plants, and the two main mammalian lignans enterodiol and enterolactone (Willfor et al., 2006)

Knots (i.e., branch bases inside tree stems) in Norway spruce trees contained much higher concentrations of lignans and oligolignans than the adjacent stemwood (6-24 % (w/w)) (Willfor et al., 2004a), with hydroxymatairesinol comprising 65-85 % of the lignans (Willfor et al., 2003a and Willfor et al., 2003b).

The lignans occur in free form (i.e. as aglycones) in knots and are easily extracted with polar solvents. Aside from the usual antioxidant and free radical scavenging properties, some lignans have specific activity against human diseases. Arctigenin for example is an effective antiretroviral agent, and inhibits the reproductive cycle of HIV. Consumption of the lignan 7-hydroxymatairesinol has been linked to lower incidences of breast cancer in women and prostate cancer in men. They provide a good opportunity to isolate sufficient amounts of softwood lignans for structural characterisation. 7-Hydroxymatairesinol was also

the predominant lignan in knots of *Picea abies, P. glauca, P. koraiensis, P. mariana,* and *P. omorika,* while liovil and secoisolariciresinol dominated in *P. sitchensis* (Sitka spruce) and *P. pungens* (Willfor et al., 2004a). 7-Hydroxymatairesinol, as well as other lignans, could be extracted in large amounts from spruce knots at pulp and paper mills. Other potentially important lignans could be produced from 7-hydroxymatairesinol by semi synthesis (Willfor et al., 2004a). The variation in the amount of lignans was large among knots, both within a single tree and between trees (Willfor et al., 2003a and Willfor et al., 2003b). The amount of lignans in the knots was approximately constant in the radial direction from the pith into the outer branch, but decreased outwards along the branch, until they almost disappeared after 10-20 cm. The ratio of the 2 epimers of hydroxymatairesinol differed between different knots and even within the knot. A novel spruce lignan, nortrachelogenin, or its enantiomer, wikstromol, was detected in knots from trees in northern Finland as opposed to samples from southern Finland (Willfor et al., 2003a and Willfor et al., 2003b). Some dilignans with four phenylpropanoid units were tentatively identified in the hydrophilic knotwood substances (Willfor et al., 2004b).

In addition to the true (dimeric) lignans, knots also contained particularly large amounts of lignan-related oligomeric aromatic substances (2-6 % (w/w)). These included a complex mixture of lignan-like compounds, called oligolignans and consisting of three or four, even up to six phenyl propane units (Willfor et al., 2004a).

Oligolignans have been found in substantial amounts in Norway spruce and Scots pine knots. Both heartwood and sapwood also contained oligolignans in small amounts (Willfor et al., 2004b).

The distribution of lignans in knots and a restricted area of the adjacent stemwood was studied in *Picea abies*. The distribution of lignans and some other extractives was determined in the radial direction within knots and into adjacent stemwood of a Norway spruce tree. The knots had an exceptionally high content of lignans (up to 15% w/w), compared to the stemwood (less than 0.05% w/w). The content decreased clearly in the radial direction from the pith of the knot towards the stemwood. In branches the lignan content also decreased in the radial direction from the branch pith outwards. The lignan

content decreased sharply outwards in emerging branches and came down to the same levels as in the stemwood by 20cm outside the stem (Willfor et al., 2005c).

1.2.1.3 Applications

The ready availability of large amounts of lignans and oligolignans now permits research to assess their bioactivity and provide the basis for applications in medicine and nutrition or as natural antioxidants and antimicrobial agents in a variety of technical products (Umezawa, 2001).

1.2.2Flavonoids

1.2.2.1 Introduction

Flavonoids are diphenyl propane (C_6-C_3-C_6) compounds, synthesised from C_6-C_3 (phenylpropane) precursors (Figure 1-1) (Umezawa, 2001).

The chalcones (Figure 1-4) are precursors for all the flavonoids (Umezawa, 2001).

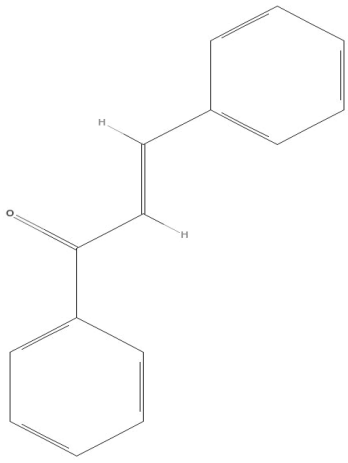


Figure 1-4: Chalcone structure (http://pubchem.ncbi.nlm.nih.gov)

The flavonoids are classified into flavanones, flavones, chalcones, dihydroflavonols (flavononols), flavonols, aurones, flavan-3-ols (catechins), flavan-3,4-diols (leucoanthocyanidins), anthocyanidins, isoflavonoids, and neoflavonoids (Figure 1-5). Flavonoids occur widely in the plant kingdom and are

present in foliage, bark, heartwood, sapwood, flowers, fruits, seeds and roots (Obst, 1997 and Umezawa, 2001).

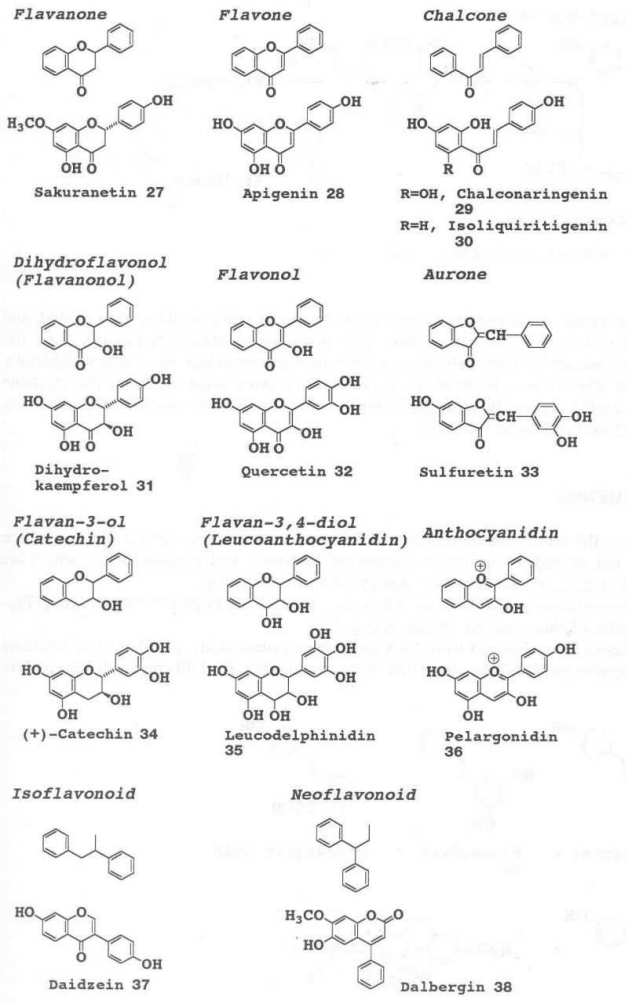


Figure 1-5: Structures of some commonly found flavonoids in trees and other plants (Umezawa, 2001)

1.2.2.2 Presence in wood including Sitka spruce

Flavonoids occur in foliage, bark, sapwood and heartwood of trees (Obst, 1997). Some flavonoids were identified in both wood and bark, e.g. Quercetin (Figure 1-6) is one of the most common flavonoids isolated from the bark of conifers. Willfor et al., (2004a) studied extractives from 7 different commercial spruce species, of which Sitka spruce was one, and managed to identify up to 5 different flavonoids in each species: Dihydrokaempferol, pentahydroxy-flavanone, cathechin, pinocembrin and pinobanksin.

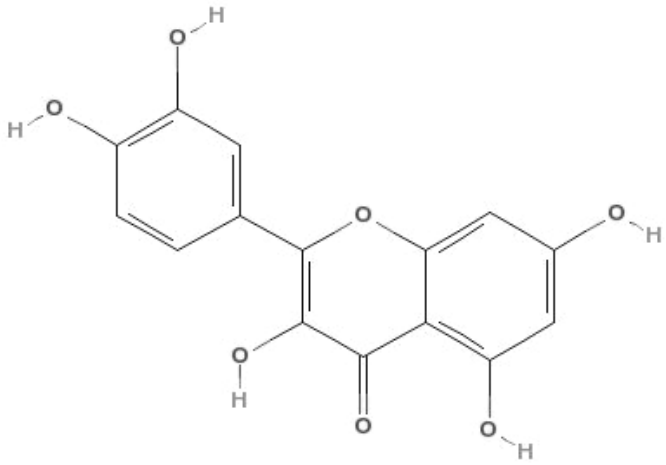


Figure 1-6: Quercetin structure (http://pubchem.ncbi.nlm.nih.gov)

1.2.2.3 Applications

Flavonoids have various biological activities (Pietta et al., 1998 and Umezawa, 2001). They are involved in the flower pigmentation process and in antioxidant activities. They are also biosynthesised in response to external stresses e.g., ultraviolet light in Scots Pine (Jungblut et al., 1995 and Umezawa, 2001), microbial attack and physical injury. Flavonoids are involved, amongst other extractives, during the process of heartwood formation (Umezawa, 2001). The flavonoids are the best-understood group of plant secondary metabolites, especially in term of biosynthesis (Umezawa, 2001).

1.2.3 Stilbenes

1.2.3.1 Introduction

Stilbenes are compounds based on the 1,2-diphenylethene structures. They have been grouped more recently with compounds having a C_6-C_2-C_6 skeleton such as bibenzyls and phenanthrenes (Umezawa, 2001). They occur in the Pinaceae, Moraceae, Betulaceae, Leguminosae and other families. Stilbenes (Figure 1-7are synthesised from the CoA esters of cinnamic acids (Umezawa, 2001).

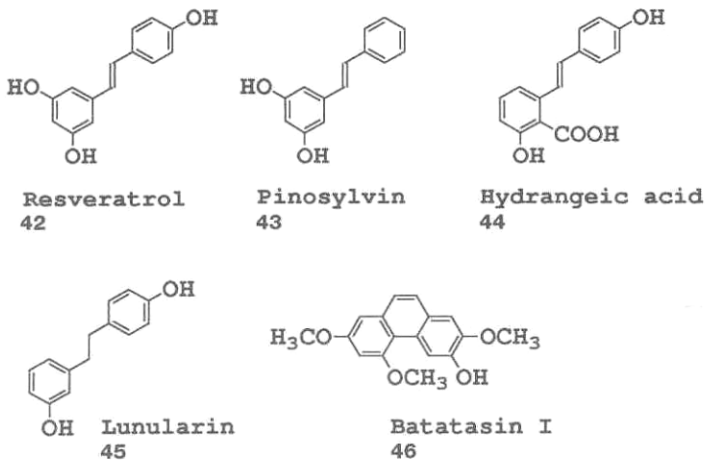


Figure 1-7: Examples of stilbenes (Umezawa, 2001).

1.2.3.2 Presence in wood including Sitka spruce

At least two stilbene glucosides have been identified in Sitka spruce: astringin (5,3',4'-trihydroxystilbene-3-β-D-glucoside) (Figure 1-8) and isorhapontin (5,4'-dihydroxy-3'-methoxystilbene-3-β-D-glucoside) (Figure 1-9).

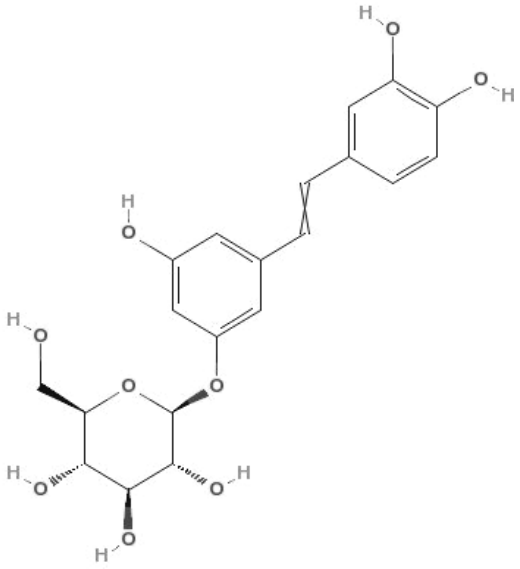


Figure 1-8: Astringin

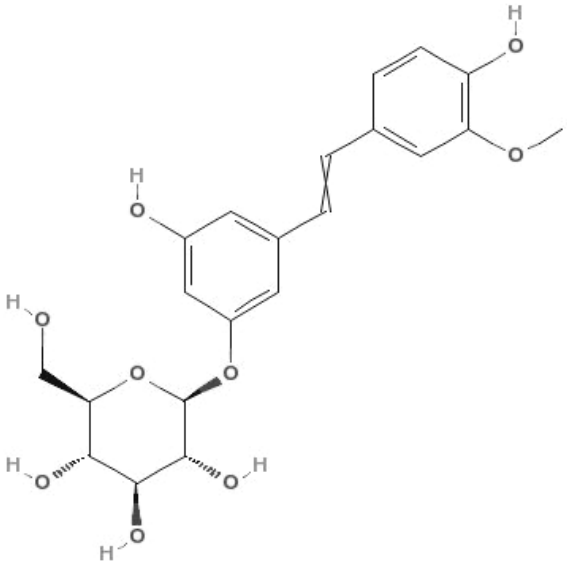


Figure 1-9: Isorhapontin

In the bark and young stem tissue of Sitka spruce, the stilbene glucosides astringin and isorhapontin were identified as the main constitutive antifungal compounds (Woodward and Pearce, 1988; Underwood and Pearce, 1992 and Pearce, 1996). They are present in high concentration and are hydrolysed to the more active stilbene aglycones astrengenin and isorharpontigenin on fungal

infection (Woodward and Pearce, 1988; Underwood and Pearce, 1992 and Pearce, 1996). These metabolites were identified by HPLC in methanol extracts of the bark (Underwood and Pearce, 1991a; Underwood and Pearce, 1991b and Pearce, 1996).

1.2.3.3 Applications

Some stilbenes are formed as a response to external stresses such as fungal infection or ultraviolet light (Umezawa, 2001). It is also known that some stilbenes such as pinosylvin and pinosylvin monomethyl ether are inhibitors of sulphite pulp cooking (Umezawa, 2001).

1.2.4 Isoprenoids

Isoprenoids are compounds synthesised from isoprene (C_5-H_8) units (Figure 1-10). The isoprenoid group includes terpenoids, steroids and tropolones (Umezawa, 2001). Isoprenoids represent the largest group of secondary metabolites in woody plants.

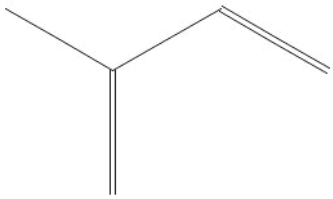


Figure 1-10: Isoprene

1.2.4.1 Terpenoids

The terpenoids are built from a number of five carbon isoprene units (Fengel and Wegener, 1984). The terpenes are pure hydrocarbons, usually linked intramolecularly to form one or more rings whereas the terpenoids bear functional groups such as hydroxyl, carboxylic acids, carbonyl, etc… Terpenoids are divided into several classes depending on the number of isoprene (C_5) units (Umezawa, 2001). They are mostly hydrophobic compounds and are usually

stored in plant resin ducts (Obst, 1997). Living cells within the phloem and the xylem in conifers can initiate the formation of chemical defences against insects or pathogens. The defence compounds synthesised are in most cases oleoresin terpenoids formed in the resin duct cells (Ralph et al., 2007).

The biosynthesis of terpenoids depends on the previous existence of resin ducts and the accumulation of phenolics in phloem polyphenolic parenchyma cells may result from a reaction to external attack (Ralph et al., 2007 and Franceschi et al., 1998).

The extractives of softwood contain all classes of terpenes from monoterpenes to tri- and tetraterpenes, except for seterterpenes, which are a very rare class.

In hardwoods it is mainly higher terpenes that are present. Monoterpenes are found only in some tropical hardwoods. Monoterpenes can be divided into acyclic, monocyclic and bicyclic components. Terpenes of all types can be found in the volatile fraction of softwood isolated by steam distillation. The volatile wood oil consists mainly of monoterpenes. The steam distillation of softwoods provides turpentine, a traditional solvent and paint ingredient. α–Pinene (Figure 1-11) and β–Pinene (Figure 1-12) are major components of turpentine (Umezawa, 2001).

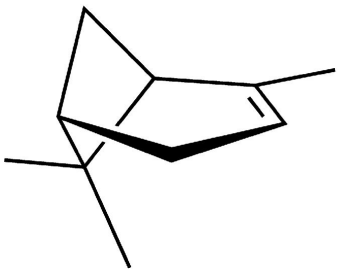


Figure 1-11: α-Pinene structure

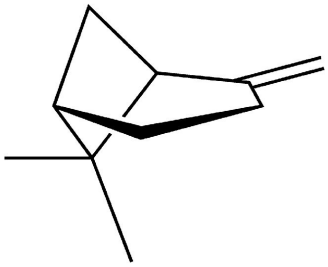


Figure 1-12: β-Pinene structure

The monoterpenes (C_{10}) and volatile sesquiterpenes (C_{15}) contribute to the specific fragrances of different wood (Umezawa, 2001). The sesquiterpenes are the most abundant and diverse members of the terpenoid group (Umezawa, 2001).

The acidic diterpenes from conifer species are known as resin acids, and are represented by abietic, pimaric acids and others (Figure 1-13).

Further components of volatile softwood oils are compounds belonging to the sesquiterpene group:

- Acyclic compounds: farnesene, nerolidol
- Monocyclic: germacrene
- Bicyclic: cadinene, cadinol and murolene
- Longifolene, longipinene and longicyclene

These components are present in pine and spruce species.

1.2.4.1.1 Presence in wood including Sitka spruce

In the pine family chemical defences involve constitutive and inducible terpenoid oleoresins (Ralph et al., 2007) that are formed and accumulated in special structures such as resin ducts, resin blisters and resin cells in stems, roots and needles. Terpenoids are inducible in response to insect or fungal attack (Ralph et al., 2007).

Resin acids are often implicated in defence against insect pests and pathogens (Tomlin et al., 1996). In Sitka spruce, the resin acid composition has been linked to resistance to the white pine weevil, a pest of Sitka spruce on the Pacific coast of North America (Tomlin et al., 1996). They identified pimaric, isopimaric, levopimaric, palustric dehydroabietic, abietic and neoabietic acids in Sitka spruce by extracting branches with acetone (Tomlin et al., 1996).

The diterpene acids, abietic, pimaric, communic and lambertianic acids are found in rosins (solid form of resin produced by heating fresh liquid resin to vaporize the volatile liquid terpene components) from gymnosperm wood (pine) (Bruce et al., 1998).

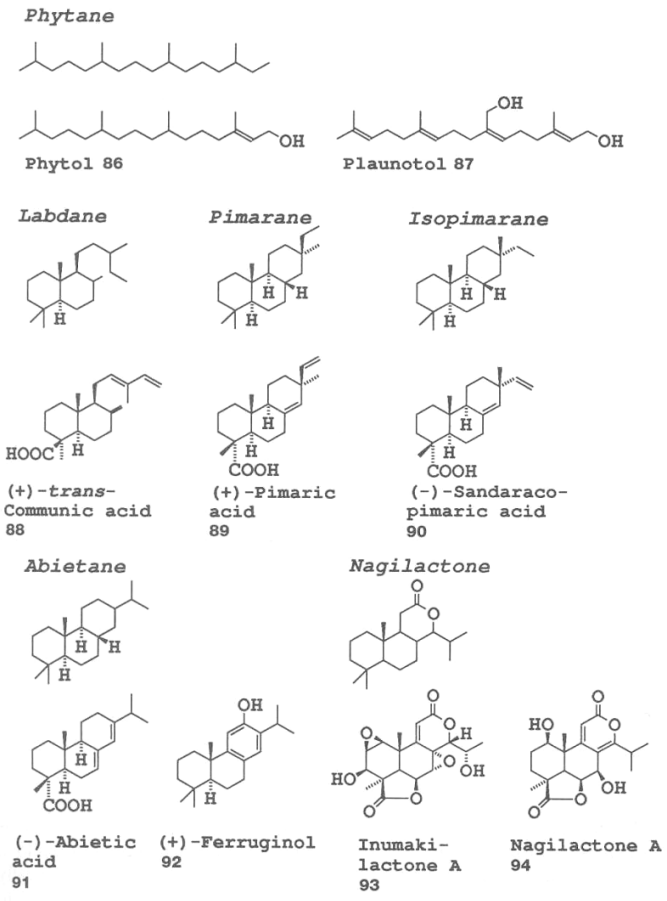


Figure 1-13: Example of diterpenes (Umezawa, 2001)

1.2.4.1.2 Applications

Many conifers accumulate large quantities of diterpene resin acids and monoterpenes in resin ducts as defences against fungal and pathogen attacks (Ralph et al., 2007). Some terpenoids such as the phytane diterpene plaunotol have anti-ulcer activities (Umezawa, 2001). Resin acids are also a by-product of the kraft pulping of wood, and when obtained from that source they are used as paper sizing agents, controlling absorption of water in paper (Obst, 1997).

1.2.4.2 Steroids

1.2.4.2.1 Introduction

Steroids are cyclic triterpenes derived from the precursor squalene (Figure 1-14), they are biosynthetized from squalene oxide (Obst, 1997); they are compounds with cyclo-pentano-per,hydro-phenanthrene skeleton (Figure 1-15). Sterols might occur as fatty acid esters or glycosides (Obst, 1997).

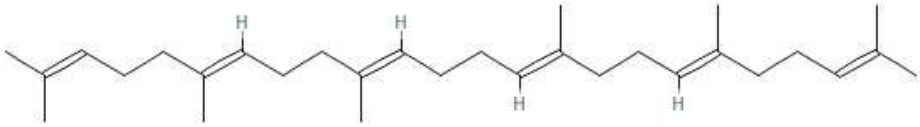


Figure 1-14: Squalene structure

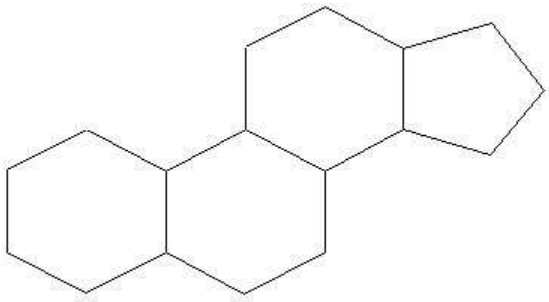


Figure 1-15: Cyclopentanoperhydrophenanthrene, the hydrocarbon skeleton of the steroids

β-sitosterol (or sitosterol) (Figure 1-16) along with campesterol represent a large part of the steroid group in conifers (Obst, 1997 and Umezawa, 2001). The concentration of sitosterol is low in heartwood, but large amounts may be isolated from tall oil, a by-product of the Kraft pulping process.

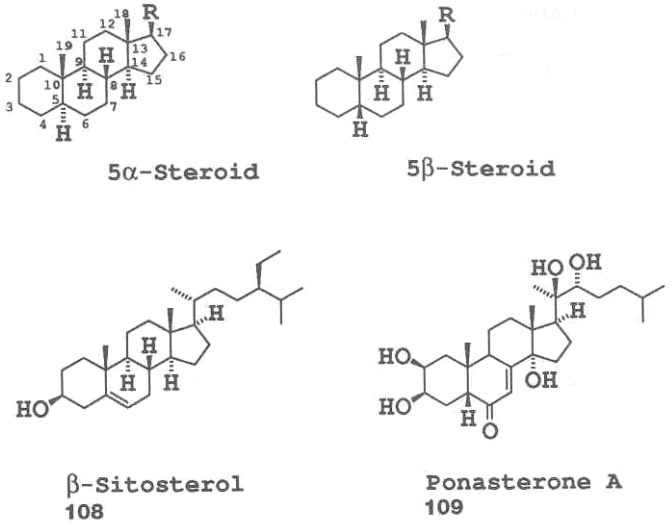


Figure 1-16: Examples of steroids (Umezawa, 2001).

1.2.4.2.2 Presence in wood including Sitka spruce

Sterols in wood occur mainly as esters. A study was done by Vikstrom et al. (2005) to compare the amount and composition of lipophilic extractives in six important wood species and in typical sulphate lye derived from these species in

Finland and the USA (Scots pine, Loblolly and Longleaf pine, Norway spruce, Siberian larch and Silver birch). Special emphasis was laid on the sterols. Sterols and triterpenyl alcohols were studied in common pulpwood and black liquor soaps. Isolation and analysis of the steryl esters confirmed the natural occurrence of sitostanol (3-β-, 5-α-stigmastan-3-ol) esters in wood (Vikstrom et al., 2005). In the sterol group, sitosterol and campesterol were the most abundant (Vikstrom et al., 2005). In general sterols have a low and consistent composition within the stem and knots of Norway spruce (Willfor et al., 2003b).

1.2.4.2.3 Applications

Some sterols have strong effects on the heart muscle and can be used as either therapeutic compounds or toxins (Obst, 1997). Sitosterol or sitostanol can be used as cholesterol-lowering components in food products, where a high ratio of sitosterol to campesterol is beneficial. This ratio was high in birch and pines but clearly lower in spruce and larch (Vikstrom et al., 2005).

1.2.4.3 Tropolones

Tropolones (Figure 1-17) are nonbenzenoid aromatic compounds having a seven-membered enolone structure (Umezawa, 2001). They are composed of 10 or 15 carbon atoms; they have been regarded as mevalonate origin, i.e., a subclass of isoprenoids (Umezawa, 2001). The tropolones are water soluble, colourless or pale yellow, and have a low molecular weight (Johansson et al., 2000).

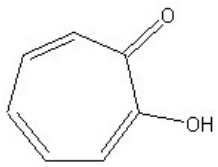


Figure 1-17: Tropolone

1.2.5 Quinones

Quinones are pigments and have several biological activities. For example juglone is a skin irritant, and is also known to be toxic towards other plants (Umezawa, 2001). Other quinones (e.g. tectoquinone and related compounds)

have anti-termite activities or mansonone and derivated compounds can cause allergies (Umezawa, 2001).

1.2.6 Tannins

1.2.6.1 Introduction

Tannins are oligomeric and polymeric, water-soluble polyphenolic compounds. (Haslam, 1996 and Obst, 1997). Their molecular weight is between 500 and 4000 g/Mol. They have the chemical capacity to precipitate some alkaloids, gelatine and other proteins out of solution, which has lead to the recognition of possible biological functions (Haslam, 1996).

Tannins are present in stemwood, bark, bud, foliage tissues, roots and seeds from many plants (Obst, 1997 and Umezawa, 2001). Bark and heartwood are the parts of the tree where tannins are found in largest concentration (Obst, 1997 and Umezawa, 2001).

There are two categories of tannins: hydrolysable and condensed tannins. Hydrolysable tannins are esters of an aliphatic polyol and a phenolic acid, such as gallic or ellagic acid. Condensed tannins (proanthocyanidins) are oligomers and polymers of polyhydroxyflvan-3-ol units derived from flavonoids (Obst, 1997 and Umezawa, 2001). Condensed tannins are the most common class and are involved in plant defence mechanisms. But like the hydrolysable tannins they are formed from phenolic units, and are therefore classified as polyphenols (Figure 1-18) (Haslam, 1996; Obst, 1997 and Umezawa, 2001).

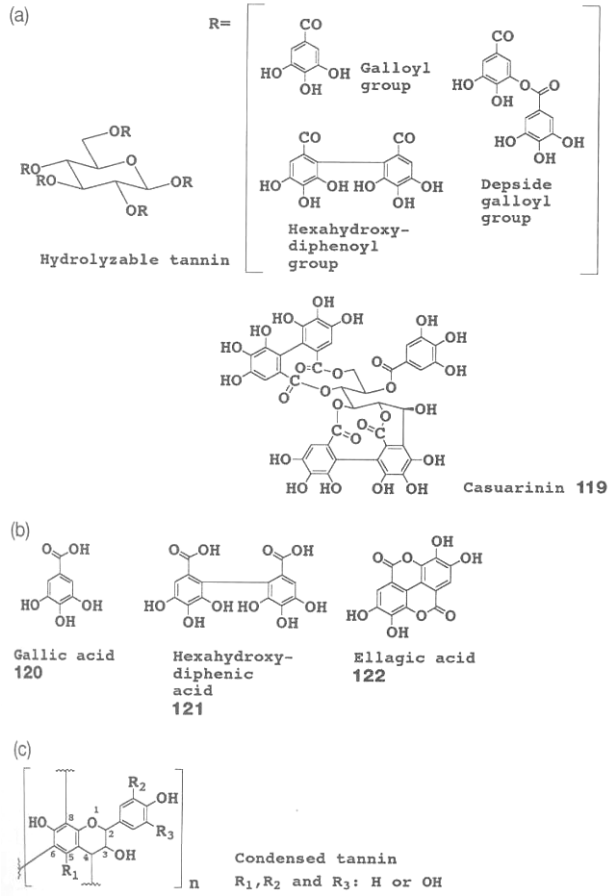


Figure 1-18: Hydrolysable and condensed tannins. (a): hydrolysable tannins (b): Phenolic acids which are components of (a) (c): condensed tannins.

1.2.6.2 Presence in wood including Sitka spruce

Tannins amongst other compounds were extracted from Norway spruce with ethanol after steam distillation (Ajuong and Birkinshaw, 2004).

1.2.6.3 Application

Tannins have antioxidant and radical-scavenging activities. They have also many biological and pharmacological activities (Haslam, 1996 and Umezawa, 2001). Tannins can be used as remedies for problems of the heart, circulatory system or digestive system. The consumption of beverages containing a lot of tannins, such as green tea or red wine, helps to reduce the risk of certain degenerative diseases (Haslam, 1996). The biological activity of tannins is dependent on the type of tannins and not all the tannin classes have the same properties (Haslam, 1996).

1.2.7Glycerides and waxes

1.2.7.1 Introduction

Glycerides are esters of glycerol with fatty acids. Triglycerides are the dominant group. Triglycerides are the cause of pitch problems in pulping of pine wood.

Waxes are a mixture of aliphatic compounds including fatty acids, fatty alcohols, hydrocarbons and their derivatives (Umezawa, 2001).

1.2.7.2 Presence in wood including Sitka spruce

The main esterified and free fatty acids identified in Norway spruce were linolenic, pinolenic, palmitic and oleic acid (Ekman and Holmbom, 1989 and Willfor et al., 2003b). The esterified fatty acids are composed of di- and triglycerides, steryl esters and diterpenyl alcohol esters. The stemwood contained more esterified fatty acids than knotwood (Ekman and Holmbom, 1989 and Willfor et al., 2003b). Fats and fatty acids are said to accumulate in woody tissues in order to provide food reserves (Obst, 1997).

1.2.8Monomeric aromatic compounds

1.2.8.1 Introduction

Wood metabolites also include phenylpropanoid monomers such as coniferin and syringin (Umezawa, 2001). Several phenylpropanoid monomers are compounds of essential oils (anethole, eugenol, safrole) and can be used as spices and in perfumes (Umezawa, 2001).

Coumarins, another class of phenylpropanoids are also widely distributed in plants and wood, and can confer several biological activities (Umezawa, 2001)

1.3 Introduction to Sitka spruce (*Picea sitchensis* (Bong.) Carr.)

Figure 1-19: Sitka spruce, Kershope Forest, Northumberland (UK)

Sitka spruce (*Picea sitchensis* (Bong.) Carr.) is a conifer native to the west coast of North America, and is still present from south Alaska to north California. It is named after Sitka Island (also known as Baranof Island) in southeast Alaska. The species was discovered and named by Europeans in 1832 (Harris, 2003).

Sitka spruce is widespread on the Northwest coast of North America. It needs humid conditions for growing, ideally a maritime climate with abundant moisture throughout the year, relatively mild winters and cool summers. The best development of Sitka spruce takes place on Queen Charlotte Island (Figure 1-20) where growth rate is one of the highest in North America.

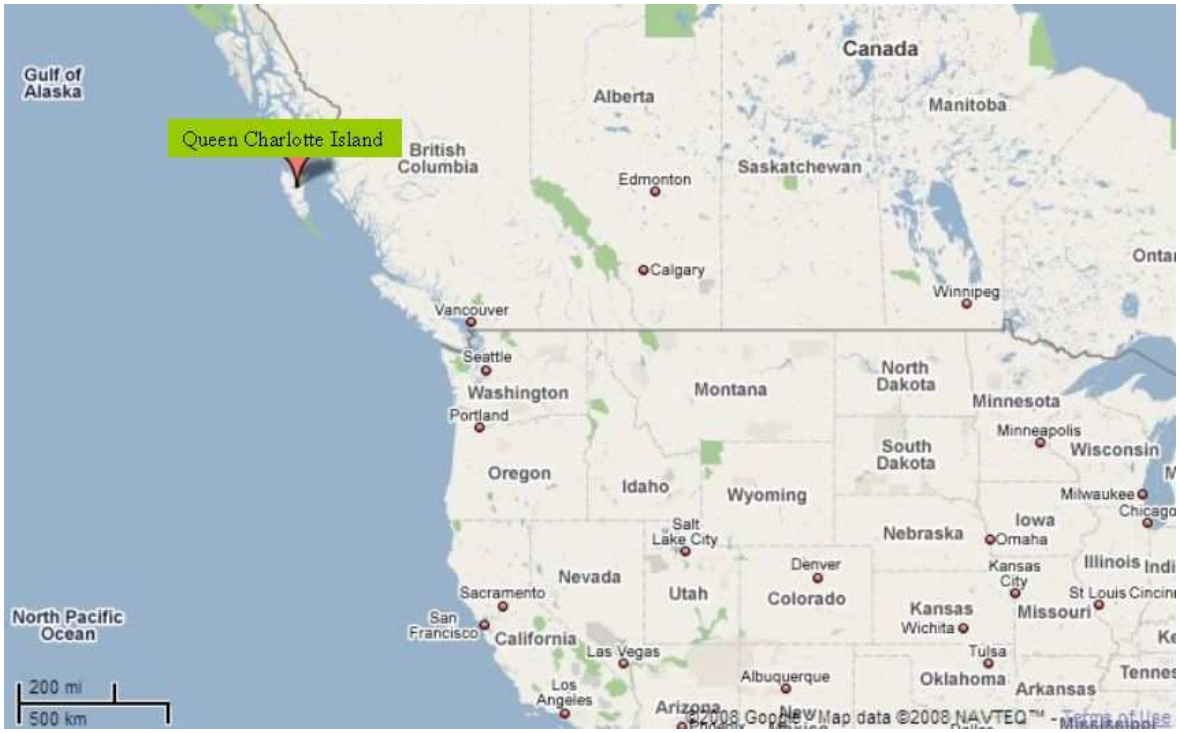


Figure 1-20: Map illustrating Queen Charlotte Island (http://maps.google.co.uk)

Sitka spruce is valuable species for timber and pulp (Harris, 2003). Sitka spruce wood has a uniform texture with little odour and relatively few resin ducts. The sapwood is a light yellow colour merging gradually into heartwood light brown in colour (Miller, 2002 and Harris, 2003).

In Great Britain, Sitka spruce has been planted extensively. It has been exploited in Great Britain because of its fast growth rate compared to other conifer species and its ability to develop at a wide variety of different sites (Brazier, 1970). Although it has a lifespan of several hundred years in its homeland, in Great Britain it is normally harvested on a 30-50 year rotation. These young trees are suitable for construction purposes, but not for joinery. With long, strong fibers, Sitka spruce makes excellent pulp; it bleaches easily making it suitable for newspaper print or high-grade printing. In the sulphate process, Sitka spruce makes high-grade kraft wrapping papers and fibreboard. In mechanical process, it is used for several purposes such as newsprint, low-grade papers and absorbent material (Harris, 2003).

About 89% of Sitka spruce in the United States grows in Alaska. The old growth natural forest trees, several hundred years old, are used for lumber, pulpwood, and more recently for sounding boards for high quality pianos, guitar faces,

ladders and components of experimental light aircraft (Anonymous, 2002 and Harris, 2003).

Sitka spruce was used for several purposes by native North American Indian tribes (MacKinnon et al., 1994 and Anonymous, 2002):

- The fleshy inner bark was eaten or dried into cakes.

- The powdered inner bark was used a soup thickener and added to cereals in bread.

- The roots were burnt over an open fire to remove the bark, then dried and used to make hats, ropes, and baskets to hold water.

- Pitch from the tree was used as glue or as a protective varnish-like coating on wood.

- Sitka spruce was used for several medicinal purposes as an antiseptic for lung complaints, sores and wounds; as a medicine for throat problems, coughs and colds and a palliative for gonorrhea, syphilis, internal swelling and toothache. The roots were used as a decoction in the treatment of diarrhoea.

1.4 Current methods used to analyse wood extractives

1.4.1Extraction apparatus

There are several extraction techniques available. They are based on the same principle: contact between the wood and a solvent in order to remove the extractives in solution.

1.4.1.1 Soxhlet extraction

This method is the classical method used for extraction and one of the oldest. The sample is placed inside a thimble made from filter paper, which is then loaded into the Soxhlet extractor. A wide-necked flask is fixed underneath the Soxhlet extractor. This flask contains the extraction solvent. On the top of the

extractor there is a condenser, which allows the hot solvent vapour to cool and trickle down onto the test material.

The chamber containing the test material is slowly filled with warm solvent until it is almost full; then it is emptied by siphon action back down into the flask. This cycle may be allowed to repeat many times. During each cycle, a portion of the extractives dissolves in the solvent. However, once the extractives reach the solvent heating flask, being involatile they remain there and do not participate in the extraction cycle any further. This is the key advantage of this type of extraction; only clean warm solvent is used to extract the solid in the thimble. This increases the efficiency of the extraction when compared with simply heating up the sample in a flask with the solvent (Figure 1-21).

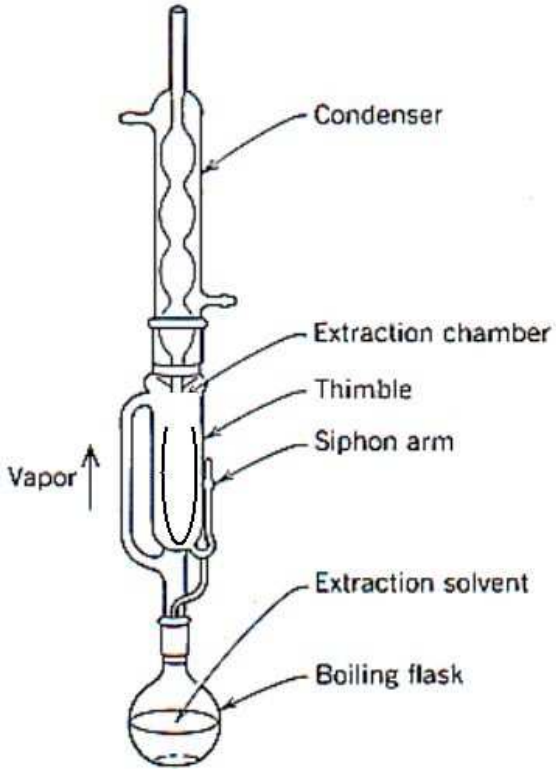


Figure 1-21: Soxhlet apparatus

1.4.1.2 Soxtec extraction

The Soxtec extraction units operate in three stages (Figure 1-22) (Sithole et al., 1991):

1. The sample in the thimble is immersed in the boiling solvent to begin extraction. This step solubilizes extractable matter from the sample.

2. The thimble is raised from the solvent, and the sample is rinsed with condensed solvent dropping through the sample (similar to conventional Soxhlet extraction).

3. The extract is concentrated by evaporation and the distilled solvent is collected for reuse or disposal. During evaporation, solvent is prevented from returning to the extraction cup by diversion into a collection tank.

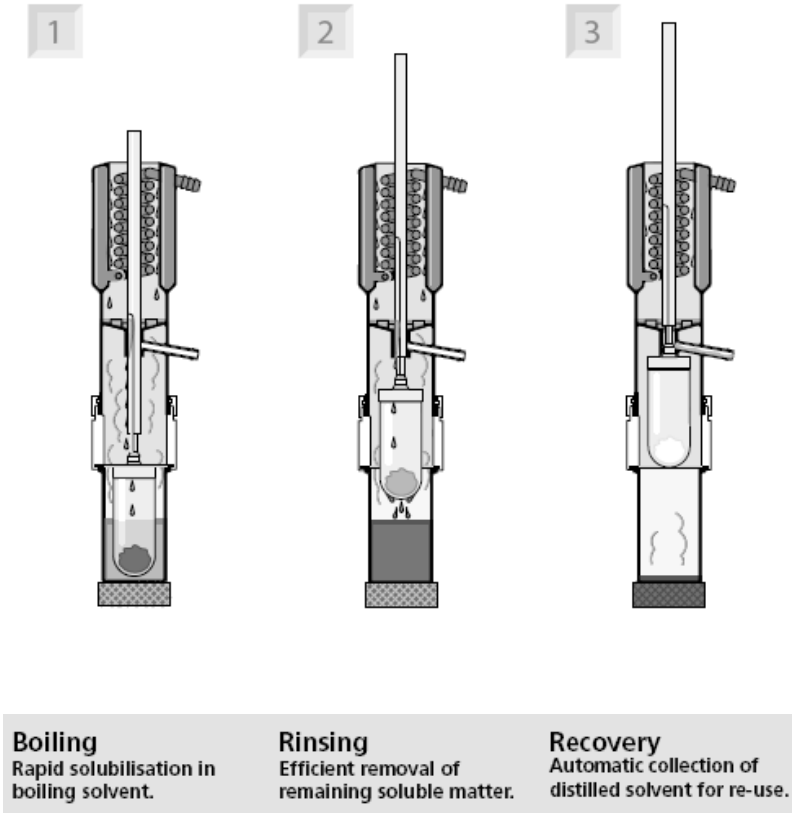


Figure 1-22: Soxtec extraction process (http://www.foss.dk)

This method provides quantities of extractives that are well correlated with those obtained by Soxhlet extraction. However the Soxtec values tend to be lower from those obtained by Soxhlet extraction. The reason is that washing during the rinsing stage of the procedure tends to not to be 100% efficient. To

overcome this problem double extraction of samples is performed and the combined extracts from the double extraction lead to results that are very close to the Soxhlet extraction. The main advantage of the Soxtec extraction is that is less time consuming even with the double rinsing and the consumption of solvent is also lower than with the conventional Soxhlet method (Sithole et al., 1991).

1.4.1.3 Accelerated solvent extraction

Accelerated solvent extraction (ASE) uses elevated temperatures and pressures in enclosed vessels, allowing extraction with a small amount of solvent to be completed in a very short time. It is believed that hot pressurized solvents are able to solubilize the extractives more effectively and penetrate the sample better (Gan et al., 1999 and Willfor et al., 2006). Applications of ASE have been reported for the extraction of conifer extractives at high temperatures and pressure under inert nitrogen atmosphere (Willfor et al., 2003a; Willfor et al. 2004b and Willfor et al., 2006).

The advantages of this method is that it is very fast, can be automated, and has particular advantages for successive extraction, as it only uses small quantities of solvent compared to the more traditional Soxhlet method (Willfor et al., 2006).

1.4.1.4 Extraction apparatus *fex*IKA

Another method, using the *fex*IKA apparatus, was compared to the Soxhlet extractor by Schwanninger et al. (2002).

Figure 1-23 shows the four phases of the *fex*IKA extraction method described below (Schwanninger et al. 2002).

- Phase 1: The material to be extracted is loaded into the extraction tube. The vessel is filled with solvent, a magnetic stirring rod is introduced and the extraction tube is mounted onto the basic vessel. The experimental conditions (temperature, number of cycles, filtration time) are controlled automatically.

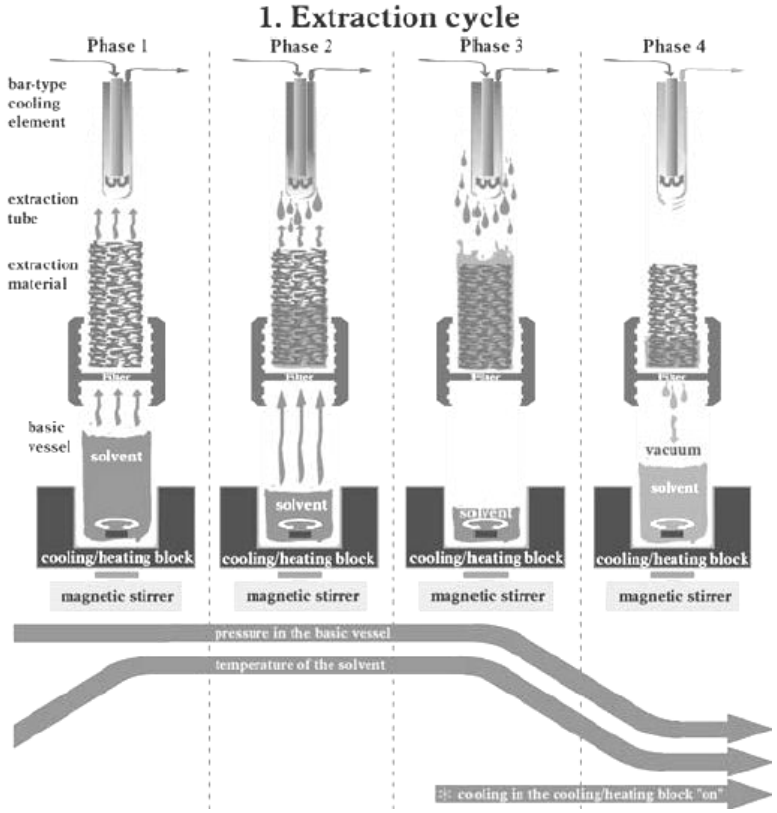


Figure 1-23: Illustration of the extraction cycle of the *fex*IKA extraction method

- Phase 2: When the solvent boils the vapor penetrates the membrane filter. The material is extracted with condensed solvent and excess vapor is condensed on the bar-type cooling element. The subsequent continuous stream of solvent vapor serves to heat up and vigorously fluidize the extraction material/mixed solvent at boiling temperature. This fluidized bed technique makes extraction particularly effective.

- Phase 3: Heating is switched off after several heating periods, the stirrer continues in operation and liquid coolant is directed through the cooling/heating block. This results in rapid cooling of the block, the lower vessel and its contents.

- Phase 4: This cooling and condensation create a vacuum in the lower vessel and the resulting pressure differential with respect to the atmospheric pressure conveys the extractive solution through the filter into the lower vessel.

This cycle may be repeated any number of times. With this extractor, the results for the total percentage of extractives obtained after extraction remain very

similar to the results obtained with the Soxhlet. The two major differences are in the volume of solvent consumed during the extraction, which is greater for the Soxhlet extractor, and also the need for more time to arrive at the same results with the Soxhlet extractor (Schwanninger et al., 2002).

1.4.1.5 Supercritical fluid extraction (SFE)

The principle of the SFE technique is based on the properties of supercritical fluids. A fluid in the supercritical state has penetration and transport properties approaching those of a gas but acts like a liquid when dissolving the analyte (Sithole, 1992) (Figure 1-24). Carbon dioxide is the solvent most commonly utilised for SFE extraction but organic solvents have also been used.

The advantages are: a decrease in the extraction time, 10 to 60 minutes instead of hours or days for liquid extraction, low use of hazardous solvent and large rate of extractives analysable. At constant temperature, extraction at low pressure will remove the less polar analytes and extraction at high pressure will favour more polar and higher molecular mass analytes. So by changing the pressure different classes of extractives can be analysed in the same procedure.

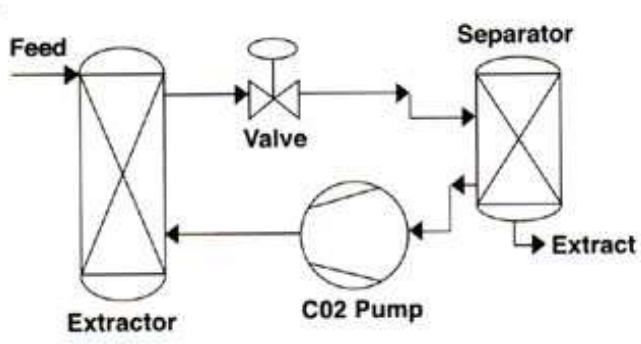


Figure 1-24: Supercritical fluid extraction process

Many supercritical fluids are gases under ambient conditions, which means that the extraction stages can be directly coupled to a chromatographic technique. Another advantage is that many supercritical fluids are relatively inert, pure, non toxic and inexpensive. So the waste generated and the exposure of the laboratory personnel to hazardous solvent can be reduced or eliminated.

Several research groups use SFE in the pulp and paper industry to extract resin and fatty acids (Lee, 1990). Demirbas (1991a and 1991b) found that SFE of

oriental spruce and oriental beech wood with acetone gave a gravimetric extractive content four times higher than soxhlet acetone extraction content for the same wood, with some differences depending on the extractive classes analysed, especially the resin acid class which is sensitive to high temperature.

1.4.2Analysis apparatus

As several analysis techniques are available for the extract, the choice of the technique to be used often depends on the extractives studied. Until now chromatography has been the most common approach for analysing wood extractives after solvent extraction. Chromatography is based on the separation of compounds in an appropriate mobile phase, which can be either gaseous in gas chromatography (GC) or liquid in high performance liquid chromatography (HPLC).

1.4.2.1 Gas chromatography (GC)

Gas chromatography (GC) is based on the volatility of the compounds to be analysed. They are separated by retention on the stationary liquid phase while travelling in the mobile gaseous phase along the column. Ideally the retention time is unique and reproducible for each compound, enabling their identification.

GC is widely used for the analysis of wood extracts. A number of protocols have been established and previous works can be used as a database allowing the identification of most of the extractive compounds (e.g. Sithole, 1992; Orsa and Holmbom, 1994; Charlet et al., 1997; Gutierrez et al., 1998; Delrio et al., 2004 and Willfor et al., 2006).

Several detectors are available with different detection principles. The most common is the flame ionisation detector (FID). The GC can also be coupled to a mass spectrometer (MS), which has the advantage of allowing the identification of the compounds within a mixture. When no authentic standard is available, a mass spectrometer coupled to the GC helps the identification of trace molecules. An electron capture detector (ECD) was used in Lee et al. (1990) to

study the chlorinated resin acids found in effluents from some pulp mills but is not appropriate for native extractives.

The GC method is based on the volatility of the materials being separated. However extractives are composed of polar and less-polar compounds that are not all volatile because of their chemical structure. In some cases the temperature used inside the column oven can be raised to separate less volatile molecules. But there are limits to the temperature increase, as at very high temperature the compounds can be degraded or the column may break down, especially with polar columns that do not have good resistance to high temperature.

Another drawback of GC is that several separation steps occur during the sample preparation, at the end of which not all components of the extract will necessarily remain for analysis.

One such separation step takes place when the sample has to be dissolved in a solvent compatible with the GC column. Chloroform can be used, but it has moderately low polarity and will only dissolve lipophilic compounds (Gutierrez et al., 1998). The polar fraction (including some phenolic compounds) will not be injected and therefore will not be detected. Orsa and Holmbom (1994) extracted wood extractives from the paper-making process water with methyl *tert*.-butyl ether (MTBE) and injected the sample after dilution with 50% toluene (MTBE/toluene is comparable in polarity with chloroform) (Ekman and Holmbom, 1989).

Further limiting factors are due to the GC instrument. Several components of the instrument can limit the analysis. The GC column and the injection chamber of the GC are two components sensitive to contamination. Molecules that are soluble in the mobile phase but not volatile enough to pass through the GC can contaminate the injection chamber or the column.

The column is chosen according to its polarity but more polar columns have less resistance to temperature. A compromise has to be found between polarity and temperature resistance.

In GC analysis the selectivity towards different classes of extractives analysed is due to their volatility and polarity. GC involves compromises with the fractionation during the sample preparation and therefore only a part of the extract present in wood is analysed. The sample preparation and the analysis are quite lengthy (up to 60min) (Rigol et al., 2003), which does not make GC analysis suitable for a large quantity of samples (Gierlinger et al., 2002).

1.4.2.2 High pressure liquid chromatography (HPLC)

HPLC uses a liquid as mobile phase and a monolayer coating on a porous solid as the stationary phase. Commonly a UV detector shows the retention times of the molecules. Retention times depend on the interactions between the stationary phase, the molecules being analyzed, and the solvents used.

Previous HPLC work led to the identification of lignans and phenolic compounds in particular (e.g. Suckling et al., 1990; Eklund et al., 2004; Eklund et al., 2005 and Willfor et al., 2006).

Willfor et al. (2006) reviewed the use of both normal-phase and reverse-phase C18 bonded silica columns. In normal-phase HPLC the separation of analytes is based on retention on a polar stationary phase from a non-polar mobile phase, and works effectively for relatively polar analytes such as lignans and oligolignans. Reversed phase HPLC utilises a non-polar stationary phase and a moderately polar mobile phase mixed from water and a polar organic solvent. Hence polar compounds are eluted first while non-polar compounds are retained (Braithwaite and Smith, 1985). For separating extractives the common mobile phases used for both normal-phase and reversed-phase separations utilise acidic solvent components (because of the acidity of the phenolic compounds) with gradient elution (Willfor et al., 2006).

UV detection (even at a single wavelength) may offer sufficient selectivity and sensitivity for the determination of lignans. High-pressure size exclusion chromatography, based on the hydrodynamic radii of the molecules, allows the identification of the molar mass distribution of the hydrophilic substances in the extract (Willfor et al., 2003b) But, like GC, HPLC can also be used in

combination with a mass spectrometer for a better identification of unknown individual compounds (Willfor et al., 2006).

HPLC columns have a lower resolution than GC columns, which leads to difficulties in separating extractive components (particularly isomers that have similar analyte-column interactions) (Rigol et al., 2003). This may lead to overlapping peaks or even co elution that precludes identification of the compounds concerned.

A mass spectrometer can be linked to the HPLC, which provides opportunities for identification of co-eluted material but a non-negligible portion of the extractives still remains non-analysed (McMartin et al., 2002 and Riggol et al., 2003).

Even if the HPLC method is less time consuming than the GC method, it still involves significant sample preparation time, and analysis time, which can vary from at least 15 to 60 minutes (Rigol et al., 2003).

Despite the fact that the chromatographic analysis of wood extractives allows the identification and quantification of the majority of wood extracts, it was intended to develop a technique which is capable of giving information on the whole extract and which will be fast and suitable for a large number of samples. FTIR spectroscopy was identified as the method of choice.

1.4.2.3 Solid phase extraction (SPE)

SPE uses the affinity of solutes dissolved in the solvent extract (the mobile phase) for a solid through which the sample is passed (the stationary phase) to separate the extract into classes or into desired and undesired components. The chemistry of the stationary and mobile phases is similar to HPLC but the separations are semi-preparative on small disposable columns of large enough particle size to make high pressures unnecessary.

SPE was used in the separation of lignans, as a purification step after hydrolysis of the solvent extract or to fractionate the oligomers. It was also used for separation of simple phenols and polyphenols. It was used for lignans and low to medium polarity compounds, usually in solvent systems containing methanol, to

separate lignans efficiently (Willfor et al., 2006). Gutierrez et al. (2004) used SPE to fractionate the extracts during wood lipid analysis.

1.4.2.4 Thin-layer chromatography (TLC)

TLC is mainly used for qualitative screening of a large number of extracts, and for monitoring isolation procedures as in Willfor et al. (2006). It involves a stationary phase consisting of a thin layer of adsorbent material, the most widely used phase being silica gel (Willfor et al., 2006) immobilized onto a flat, inert carrier sheet (Braithwaite and Smith, 1985). A liquid phase consisting of the solution to be separated is drawn up the plate by capillary action, separating the experimental mixture based on polarity (Braithwaite and Smith, 1985). TLC was used for the isolation and purification of small amounts of lignans and other polyphenols (Willfor et al., 2005b).

1.4.2.5 Fourier transform infrared spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is an analytical technique used to identify organic materials, often but not always qualitatively. This technique measures the absorption of infrared light at various wavelengths by the material of interest. The infrared absorption bands correspond to vibrational modes characteristic of specific structural features of the adsorbing molecules.

As a general rule, FTIR absorption bands in the frequency range 4000-1500cm^{-1} can be assigned to functional groups (e.g. -OH, C=O, N-H, CH_3, etc.). The region between 1500-400 cm^{-1} is referred to as the fingerprint region. Absorption bands in this region are generally due to more complex vibrational modes associated with larger intramolecular structural features, and are relatively specific for each molecule. For FTNIR (near infrared) analysis the absorption bands that are overtones and combinations of FTIR bands and lie in the range 11000-4000cm^{-1}. The FTIR technique will be further discussed in Chapter 3.

1.5 Aims and objectives

A large part of the UK forested area is devoted to exotic conifer species grown in short rotations of 30-40 years and clear felled. Much the most important of the imported conifer species is Sitka spruce. These Sitka spruce forests are regarded with mixed feelings because of their uniformity and artificial, straight lined appearance. This makes them highly unpopular as visual features of landscape, and they have been blamed for soil degradation and the obliteration of rural communities.

On the other hand they make a favourable contribution to the UK's CO_2 balance, being much more effective in this respect than either agricultural land or native woodland, and the timber that they produce meets all European Standards of forest stewardship. On balance the environmental value of UK Sitka forests is now considered to be negative and, as they are felled, they are being replaced with less uniform types of forest that include native species. This transformation will obviously take time. Over that next 20 years in the UK, very large amounts of Sitka spruce that are growing at the moment will be felled with production peaking around 2010. Low current prices for pulpwood and structural timber mean that any further income that can be derived from wood by-products will be very welcome. Extractives from European timbers have little commercial use today. The paper industry looks for low extractives levels in its raw materials because extractives represent unused biomass and may have to be removed from the waste stream to prevent pollution. In the past decade it has been realised that wood extractives of some kinds may represent an unexploited source of chemical or pharmaceutical raw materials.

The objectives of the research described in this thesis are as follows:

- Development and optimisation of a mass sampling method based on FTIR.

- To study the distribution of extractives within Sitka spruce trees, at different heights in the trunk, as well as the distribution between heartwood, sapwood and knotwood.

- To elucidate the influence of yield class, elevation, north/east location and thinning on the extractives content and composition of Sitka spruce across Scotland.

- To determine the extractive composition of Sitka spruce bark, rootwood, knotwood, heartwood and sapwood in detail by GC/MS.

- To compare the two analytical techniques used, GC/MS and FTIR spectroscopy.

Chapter 2: General methods

2.1 Moisture content

In order to determine the gravimetric extractive content in wood material, the moisture content of every sample needed to be measured before each extraction. This made it possible to calculate the same weight of dry wood for every sample, and the equivalent of 3.5g of dried wood was extracted on each occasion, as recommend by Philip et al. (1995).

According to the literature (Baeza and Freer, 2001), there are several ways to determine the moisture content of a wood sample (oven-dry method, electrical moisture detector, moisture determination by solvent distillation, by Karl Fisher method or by nuclear magnetic resonance). The most common one is the oven-drying method. About 2g of fresh sawdust is oven-dried at 105°C +/- 3°C until the weight does not change by more than 0.02g following a 1h heating period. The disadvantage is the loss of some of the volatile compounds, which then introduces an error in the determination of the quantity of water evaporated during drying.

The quantity of sample used for the determination of the moisture content was revised for each type of sample, because the material sampled was available in different quantity. As the Kershope experiment provided more than 100g of fresh sawdust, 5g of wet wood were used. But the average sample quantity available from the Benchmarking experiment represented only 15g of fresh wood, so only 1g of sawdust was used.

The amount of water in wood can be expressed in two different ways:

The moisture content MC is expressed as a percentage of the wet weight:

$$\% MC = (\text{Wet weight} - \text{OD weight}) / (\text{Wet weight}) * 100$$

The wet weight basis is generally used in the pulp and paper industry and when wood is used as fuel.

Alternatively the moisture content MC* is defined as the weight of water expressed as a percentage of the moisture-free or oven-dry (OD) weight of wood:

$$\% MC^* = (\text{Wet weight} - \text{OD weight}) / (\text{OD weight}) * 100$$

Because the denominator is dry weight, the moisture content calculated in this way can exceed 100%. This method of calculating moisture content is generally accepted as standard for wood-based materials such as lumber, plywood, particleboard, and fibreboard (Baeza and Freer, 2001).

Consequently the determination of the moisture content as the percentage of the oven dried weight has been preferred for the purpose of this study.

2.2 Soxhlet extraction

2.2.1 Solvent study

The extractives are mostly detected in rays and they can also form coatings on the cell wall and on the pits, or they can penetrate the cell wall itself (Hillis, 1972 and Taylor et al., 2002). These different locations inside the tree are one reason why one extraction solvent cannot extract all the extractives at once, and why the use of several solvents of different polarity can increase the total removal of extractives.

Standard methods exist for Soxhlet extraction to determine the extractives content of softwoods (for example: TAPPI Standard Method T-204 cm-97, SCAN (Scandinavian pulp, paper and board) SCAN-CM 49:03: Content of acetone-soluble matter methods, SCAN-CM 67:03: Content of extractable lipophilic matter or ASTM (American Society for Testing and Materials) method) (Baeza and Freer, 2001 and Nelson and Birkett, 2004). These authors emphasise the fact that one solvent does not remove all the extractives from the sample and that different solvent mixtures extract different combinations of extractives. They recommend using a mixture of ethanol and benzene, which provides the most complete removal of extractives. But due to the highly carcinogenic properties of benzene, the use of this solvent was avoided. A preliminary study comparing

different solvents was performed in order to choose the most suitable solvent for this study of Sitka spruce (Table 2-1).

All the solvents used were of HPLC grades. They were chosen according to their relative polarity, because this factor has an influence on the compounds extracted (chapter 1).

For this study the material was bulk sawdust from a frozen log of Sitka spruce grown in the Kershope forest. The equivalent of 3.5g of dried wood was extracted for 9 hours with the different solvents.

Solvent	Relative polarity	Percentage of dried wood of extractives obtained after extraction
Pentane	0.009	0.49
Hexane	0.009	0.62
Toluene	0.099	0.63
Ethyl acetate	0.228	1.27
Acetone	0.355	1.86
Isopropanol	0.546	1.52
Ethanol	0.654	2.17
Water	1.000	1.50

Table 2-1: Organic solvents tested for the extraction stage, during 9h, with their relative polarity (Reichardt, 1988) and the total percentage of extractives obtained.

This study showed that ethanol and acetone were the solvents that removed the largest proportion of extractives after 9 hours.

Nevertheless, we have some reluctance to use the ethanol solvent as it might extract higher molecular weight compounds, i.e. short polymers such as oligosaccharides/polysaccharides (Nelson and Birkett, 2004).

2.2.2Influence of the extraction time

The time of extraction has an influence on the amount of extractives collected. Another series of extractions with ethanol and acetone was performed to optimise the extraction time. For this study the material was also bulk sawdust from a frozen log of Sitka spruce grown in the Kershope forest.

Three extractions for 3 hours, 6 hours and 9 hours were carried out. Figure 2-1 shows the evolution of the percentage of extractives removed by acetone and by ethanol.

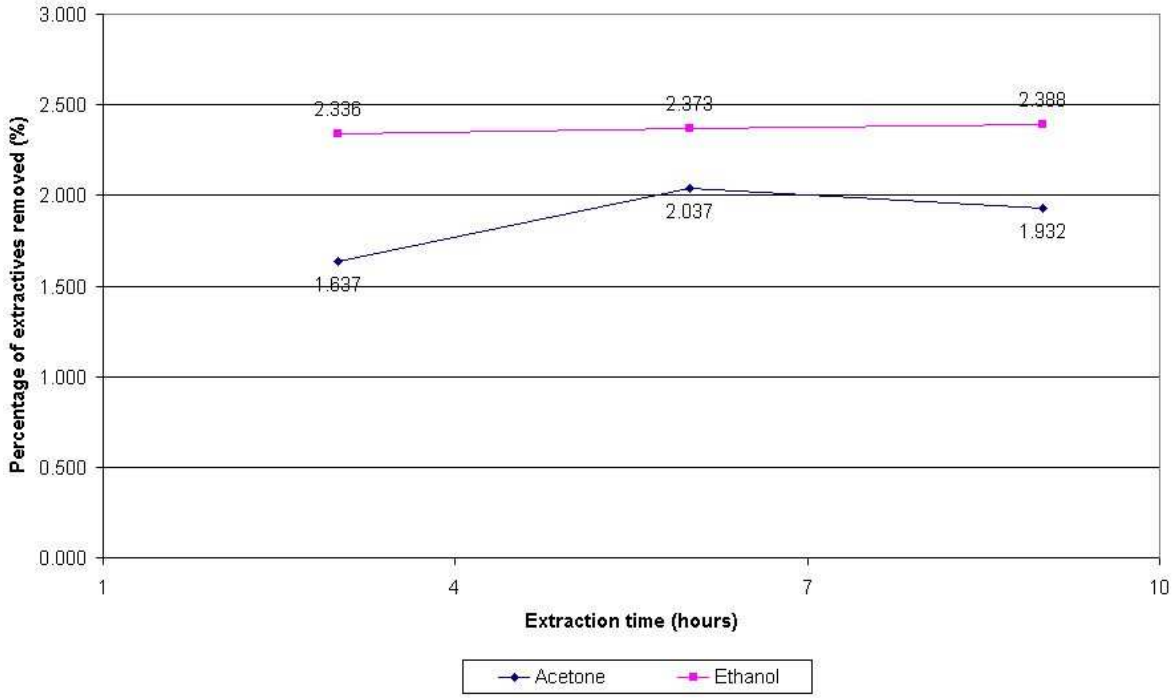


Figure 2-1: Comparison between acetone and ethanol for different extraction times

In this experiment we could see that ethanol removed more extractives than acetone in 3 hours of extraction and that the quantity extracted by ethanol remained stable. For acetone we estimated that full extraction is achieved after 9 hours.

2.2.3Finalised extraction protocol

The technique used for the extraction was the Soxhlet method. The protocol used was based on the equivalent of 3.5g of dried sawdust. During the experiment the moisture content of each sample was measured before extraction and the quantity of wet wood to analyse was deduced.

The calculated amount of wet wood was weighed out and added to the thimble, which was placed in a soxhlet extractor.

Before the experiment the glassware (soxhlet round-bottom flasks) was cleaned, oven-dried and weighed in order to determine the weight of extractives.

The sawdust was extracted with an excess of HPLC grade acetone (Fisher Scientific). After nine hours of extraction, the samples (extractives in acetone solution) were cooled to room temperature. The acetone volume was then adjusted to 200mL.

Once the sample was extracted, the solvent was evaporated with a rotary evaporator in order to remove the excess of the solvent. The temperature of the water bath was controlled at 34°C. The 200mL extract were divided into two 100mL aliquots. Half of the acetone extract (100ml) was evaporated to dryness to enable the determination of the weight of the extractive content in the sample (gravimetric analysis). The other half was concentrated to the desired volume for spectroscopic analysis.

2.3 Determination of total extractive content

The gravimetric determination of the extractives content in dried wood was based on the weight present in half of the volume of the sample extracted.

The calculation is based on expressing the moisture content as a percentage of the oven dried weight. The weight of the extractives obtained after evaporation of the solvent, was multiplied by two (because only half of the volume of the sample was used for this step). This weight was then divided by the weight of the dried wood. The dried weight of the wood was obtained by correcting the weight of the wet sample for the moisture content. The percentage of extractives in the dried wood is the ratio of the weight of the extractives to the dried weight of the wood (Table 2-2).

Weight of milled wood (g)	Moisture content (%)	Weight of dried wood (g)	Weight of the round flask (g)	Weight of the round flask + extract (g)	Weight of extractives (g)	**% Extractives for dry wood**
F	E Calculated	G=F*(1/(1+E/100))	H	I	J=I-H	K=(2*J)/G*100
Example 5.3095	50.62	3.5251	54.8242	54.8607	0.0365	**2.0708**

Table 2-2: Sample calculations

2.4 Statistical analyses

Three kinds of statistical analysis were used to analyse the experimental data:

1. Two-way analysis of variance (ANOVA), which makes it possible to study two main effects and their interaction:

    - For example for the Kershope experiment: Type of wood (heartwood/sapwood), and the height of the sample (between 0 to 12 meters) and also the interaction between these.

2. One way analysis of variance for the sapwood between 0 to 18 meters in order to study the effect of the height on sapwood composition.

The ANOVA analysis yielded a p-value which, when compared to the threshold of 5% adopted here, allows the identification of significant differences between the values studied. The p-value is the probability of obtaining a result at least as extreme as the one that was actually observed, given that the null hypothesis is true, here “there is no significant differences between the samples”. If the p-value is below the threshold of 5%, differences within the wood data are considered significant (Sterne and Smith, 2001).

3. Residual maximum likelihood with Wald test (REML) analysis provides a least significant difference (L.S.D) value, which compared to the difference between the mean values calculated makes it possible to determine if there is a significant difference between the means calculated and to find where the differences come from.

    - For example, the benchmarking experiment is based on the study of 5 factors (Yield class, thinning, elevation, north and east location) with two levels each. The experimental means for each level of each factor were calculated, and compared to the least significant difference in order to detect any influence of the factors on the results.

The REML and ANOVA analyses are generally interchangeable here, as the data are usually balanced. The REML analysis has been preferred because it makes it

possible to calculate a variance component that would be observed after removing all the other sources of noise. The REML analysis was implemented within the statistical software package GenStat.

2.5 Heartwood/sapwood distribution method

During the research for a previous thesis by McLean (2008) scanned colour images of Sitka spruce discs were collected with the aim of measuring compression wood content. These images were re-analysed to measure the proportion of heartwood to sapwood. The discs were from Sitka spruce trees of Queen Charlotte Island provenance grown in Kershope forest, Northumberland (NY473 477, Latitude 55° 05'N, Longitude 2° 50'W, 190m elevation). The images were obtained after scanning the discs collected at different heights in the trunk (McLean, 2008). In total 7 discs from 4 trees of the same family were analysed.

For each disc the mean radii of the heartwood and of the disc itself were measured, based on the colour difference between heartwood and sapwood as illustrated (Figure 2-2). Then the areas of heartwood and sapwood were calculated (the sapwood area being the disc area minus the heartwood area). From the radii and areas, ratios between heartwood and sapwood were established as functions of the sample height in the trunk in order to follow the distribution of heartwood and sapwood with height.

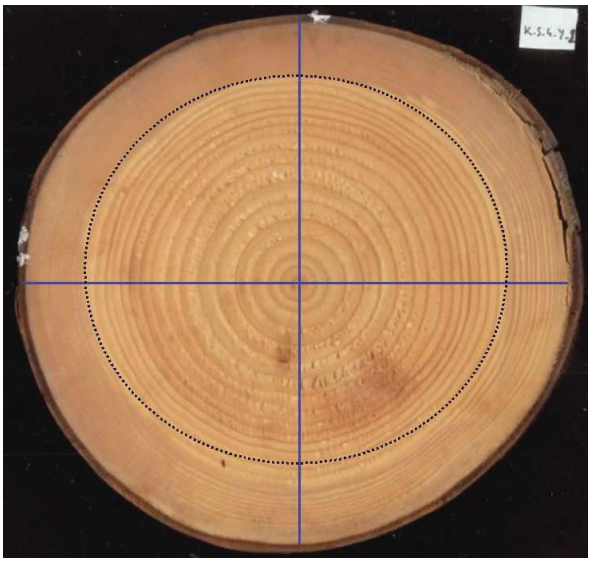


Figure 2-2: Image of disc from Sitka spruce

Chapter 3: Development of a mass sampling technique based on FTIR

3.1 Introduction

FTIR spectroscopy has been used for wood extractives analysis by Ajuong and Breese (1998), Ajuong and Birkinshaw (2004) and Ajuong and Redington (2004), who have studied respectively Pai wood, bog and modern oak, spruce and larch wood extractives.

We tried to optimise the sampling technique in terms of preparation time and sample amount. We wished to achieve a cheap, fast and reliable system to analyse the wood extractives without any separation step before bringing them to the FTIR, so several approaches involving different types of sample holders were tested.

One approach is to obtain spectra from the solid wood itself and to use the "difference" spectra between the non-extracted wood and extracted wood. The difference spectra represent the extractives (Nault and Manville, 1992). Alternatively, the liquid extractives have been analysed by applying the organic extract to a sodium chloride disc or incorporating hydrophilic extracts into potassium bromide (KBr) discs, by Ajuong and Breese (1998), Ajuong and Birkinshaw (2004) and Ajuong and Redington (2004).

Fourier Transform Infrared Spectroscopy (FTIR) is sensitive to the nature of bonding within a molecule. FTIR detects functional groups and characterizes covalent bonding information.

FTIR spectroscopy includes two modes of analyses: transmission and reflectance mode. In transmission mode, the infrared beam passes through the sample. In reflection mode, the signal passes through the sample, then is reflected and redirected to the detector. In diffuse reflectance and ATR (Attenuated Total Reflectance) the reflection occurs within the sample, while in specular reflectance a thin layer of sample overlies a polished reflective surface.

In transmission mode, 3 systems were tested with 2 different supports. The first support was the KBr disc. This method is considered as the reference FTIR method for several reasons (Hauser and Oelichmann, 1988). First, the infrared beam is much wider (3 mm) than the one used in the microscopy technique (50 μm), and covers a larger analysed area. Secondly, the KBr disc method allows the extract to be distributed evenly throughout the disc. The amount of sample incorporated in the disc can be kept constant from one KBr disc to another and the amount of sample in the infrared beam is then also constant, allowing a quantitative approach.

The second support was the barium fluoride (BaF_2) window. BaF_2 is an infrared transparent material. The BaF_2 windows were tested unmodified and as part of a multilayer system. These trials were carried out in order to make the link with work already done in this domain (Ajuong and Breese, 1998).

In reflectance mode, two major techniques were tested: ATR (Attenuated Total Reflectance) spectroscopy and specular reflectance microscopy techniques using different supports. The support for the extract has to be flat and to have good reflectance properties. Special physical and chemical properties are also needed in order to maintain the sample droplet confined reproducibly on the reflective surface to improve the data collection.

We studied FTIR spectroscopy in order to find a fast and cheap reliable method to identify wood extractives.

3.2 Materials and methods

3.2.1 Wood samples

The wood sample used to develop the FTIR technique was sawdust originating from a Sitka spruce trunk from Kershope forest (Northumberland - NY473 477, Latitude 55° 05'N, Longitude 2° 50'W, 190m elevation). The sawdust (the equivalent of 3.5g of dried wood) was extracted for 9 hours with either acetone or ethanol solvent (HPLC grade from Fisher Scientific) and then the volume was concentrated to one tenth of the volume (so from 100ml to 10ml) with the

rotary evaporator. In most of the tests both solvents were analysed, as they both appeared suitable for the subsequent research.

3.2.2Fourier Transform Infrared spectroscopy (FTIR) analysis

The samples were analysed on a Nicolet Nexus Fourier Transform infrared Spectrometer (FTIR) spectrometer equipped with a Nicolet Continuum microscope attachment, with a liquid-nitrogen-cooled MCT detector. The spectroscopy was in specular reflectance mode with the sample dried onto a polished aluminium plate. Five spectra were measured for each acetone extract. An appropriate background spectrum was collected and automatically subtracted by the Omnic software. Each spectrum represents an average of 128 scans at the resolution of 4cm^{-1}.

A few droplets of concentrated acetone extract were placed (usually 3 times 1µL on the same spot) on the polished aluminium plate and allowed to dry at room temperature. The sample formed a stain on the plate representing the extractives to be analysed (Figure 3-1).

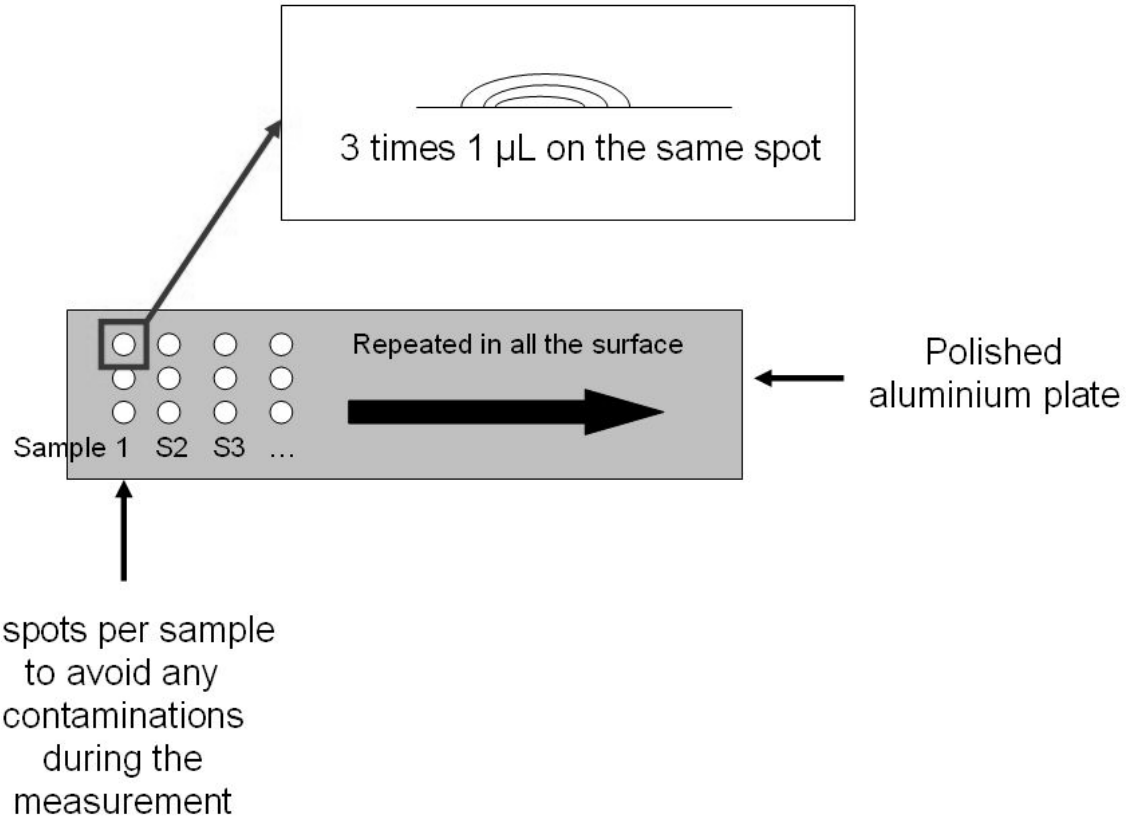


Figure 3-1: FTIR sample preparation

All the spectra were then processed in the same way in Microsoft Excel software as described in the following paragraphs.

3.2.3 Data collection from the FTIR experiments

The spectra were collected with the FTIR microscope operating in specular reflectance mode. They were collected in absorbance (or more accurately, reflectance) using the Nicolet Omnic software package.

The data were then transferred as .CSV files into Microsoft Excel, where the data were baseline-corrected using a linear algorithm connecting 7 different wavelengths (3690, 3040, 2740, 1810, 1545, 1480 and 840 cm^{-1}) in the spectra.

The total area of the baseline corrected spectra was normalised to one to enable the comparison between the spectra when required.

Wavelengths that are characteristic of certain functional groups were chosen (Table 3-1), and the area of the peaks was calculated.

Wavelengths		Functional group identified
3400cm^{-1}	OH stretching mode	Hydroxyl function
2930cm^{-1} and 2850cm^{-1}	CH and CH_2 stretching modes	Methylene and methyl groups
1698cm^{-1}	COOR carbonyl stretching mode	Carboxylic function in ester group
1510cm^{-1}	Ring deformation mode	Aromatic compounds

Table 3-1: Wavelengths of functional groups of extractives

3.3 Results

3.3.1 Transmission mode

There are several ways to prepare the sample for the analysis in transmission mode. The first consists of preparing a disc with KBr; the properties of this material allow the signal to pass through the sample.

The second way to prepare the sample is to apply it to a barium fluoride window. In this case, the liquid extract is dropped on the window, then is allowed to dry and the residue is detected with the microscope.

3.3.1.1 Potassium bromide discs

3.3.1.1.1 Introduction

The chemical properties of potassium bromide (KBr) allow the formation of KBr disc using finely powdered dried KBr under very high pressure. The disc formed is transparent to visible and infrared radiation. These characteristics meet the constraint to do transmission measurement of the samples, when the sample was previously added to KBr powder (Smith, 1996) and pressed into a disc.

Ajuong and Birkinshaw (2004) and Ajuong and Redington (2004) analysed respectively the water-soluble extractives content of bog and modern Oak wood and the acetylated extractives of Sitka spruce and Larch, using the same method as Ajuong and Breese (1998) who analysed the water-soluble extractives of Pai wood in KBr discs. They mixed 5mg of dry, powdered extract with 0.5g of dry, powdered KBr after sequential toluene and toluene/ethanol extraction. The KBr disc method can also be used for solid wood provided that it has first been very finely powdered. Sun and Sun (2003) also analysed directly dried and powdered methyl tert-butyl ether extract of straw mixed with KBr. Owen and Thomas (1989) used the KBr disc method to study the holocellulose/lignin ratio and the lignin type present in 24 different woods.

3.3.1.1.2 Experimental

To form a perfectly transparent disc, the extract was mixed with KBr powder and pressed to form a 12 mm disc of controlled dimensions. Therefore the quantity of extract in the beam is known. The KBr powder mixed with the sample must be completely dry before going into the press. This was a necessary precaution to eliminate the absorption band due to the water in the spectrum and to avoid any recombination of the water with extractive compounds (esters for example). As the samples were in solution, a drying step was added. The volume needed was up to 50μL, which took a few minutes to evaporate at room temperature. Acetone and ethanol liquid extracts were tested using KBr discs.

3.3.1.1.3 Results

The spectra obtained were baseline corrected and normalised by the total area (Figure 3-2).

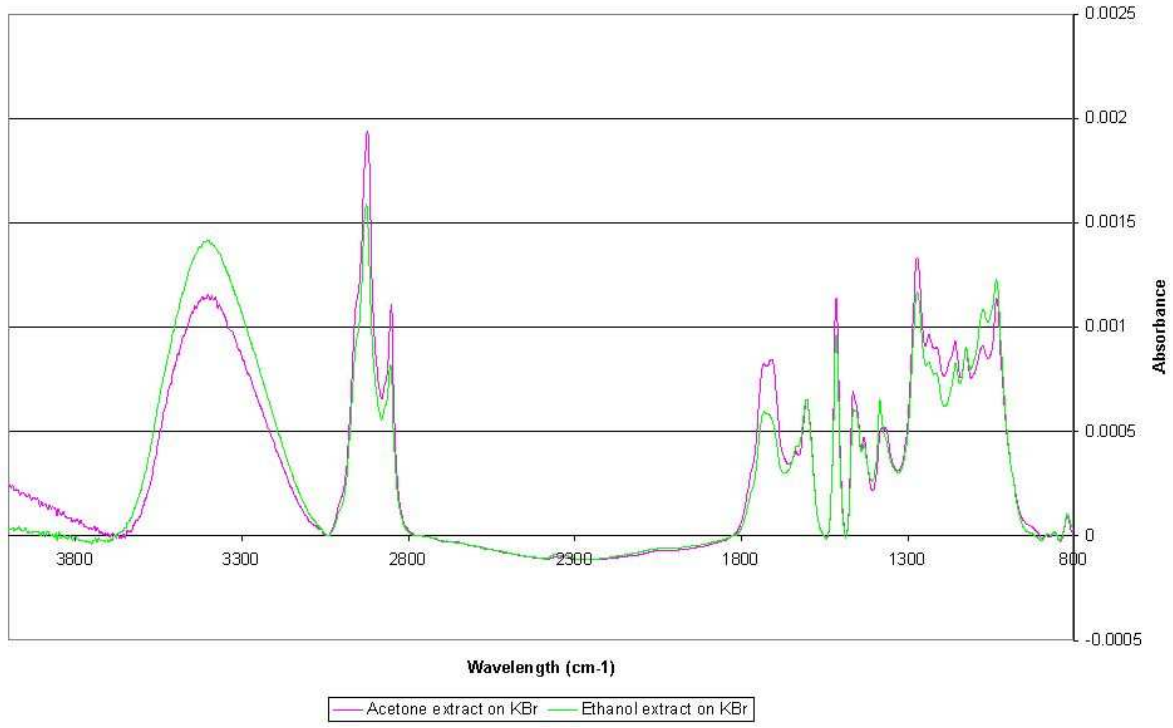


Figure 3-2: Spectra, normalised and baseline corrected, of acetone and ethanol extracts from Sitka spruce analysed by FTIR spectroscopy using KBr discs

The assignment of the FTIR spectra is based on: general references about FTIR band assignment (Pecsok and Shields 1968; Williams and Fleming, 1989 and Lin-Vien et al., 1991), previous work studying either wood, cellulose or lignin (Holgrem et al., 1999; Silva et al., 1999; Schwanniger et al., 2004 and Pan, 2007) and comparing wood extractives from several species (Ajuong and Breese, 1998; Nuopponen et al., 2003; Sun and Sun, 2003; Ajuong and Birkinshaw, 2004 and Ajuong and Redington, 2004).

The first peak around 3400cm^{-1}, represents the stretching vibration of hydrogen-bonded OH groups (Williams and Fleming, 1989), characteristic of intermolecularly bonded hydroxylated extractives (Ajuong and Breese, 1998; Ajuong and Birkinshaw, 2004 and Ajuong and Redington, 2004) as can be found in sterols, glycerides, in some polysaccharides or in water in moist samples (Sun and Sun, 2003).

At 2930 and 2850cm^{-1} there are two very strong peaks characteristic of the symmetric and antisymmetric C-H stretching vibrations of CH_2 (methylene) groups (Lin-Vien et al., 1991). The C-H stretching vibrations of other saturated and unsaturated hydrocarbons and methyl groups are found between the CH_2 stretching peaks.

The bands at 1730 cm^{-1} are the C=O stretching vibrations produced by the ester carbonyl (Williams and Fleming, 1989 and Holmgren et al., 1999). These peaks appear when the lipophilic fraction of extractive is studied; they may come from fat, wax compounds or in esterified resin acids (Holmgren et al., 1999; Nuopponen et al., 2003 and Sun and Sun, 2003). This band may shift to 1698cm^{-1} in presence of free carboxylic acid carbonyl groups in wood extractives (Holmgren et al., 1999 and Nuopponen et al., 2003).

The peak around 1600cm^{-1} can be assigned to either C=C stretching at lower than usual frequency, but with increased intensity because of conjugation with a carbonyl group; or an aromatic ring deformation mode (Ajuong and Breese, 1998). In our case, the hypothesis of the aromatic rings is less probable because of the strong absorption at 1510cm^{-1} from lignin and associated compounds (Schwanniger et al., 2004).

The strong peak at 1510cm^{-1} is assigned to the deformation vibration within benzene rings (Williams and Fleming, 1989 and Lin-Vien et al., 1991). This peak is characteristic of aromatic compounds in wood and wood extractives (Silva et al., 1999 and Schwanniger et al., 2004).

At lower frequencies than this the vibrational modes become progressively more complex, with the participation of more than two atoms so that any band in this part of the spectrum cannot necessarily be assigned to a single functional group.

The C-H bending mode appears at 1460cm^{-1} and a symmetrical deformation of C-H_3 at 1380cm^{-1}. These bands are related to the two previous bands at 2930 and 2850cm^{-1} (Ajuong and Redington, 2004).

The strong band at 1270cm^{-1} originates from the C-O stretching vibrations of softwood resin acids (Williams and Fleming, 1989 and Nuopponen et al., 2003).

The 1155cm^{-1} peak is produced by C-O-C asymmetric valence vibrations as in carbohydrates (Schwanniger et al., 2004).

3.3.1.1.4 Discussion

The spectra of the acetone and ethanol extracts both contained the same peaks at the same frequencies. The main distinction that could be identified was at around 1730 and 1710 cm^{-1}, where the acetone extract showed two distinguishable peaks but the ethanol extract showed a flat broad peak. It was also noticeable that the broad O-H stretching band was of lower intensity in the acetone than the ethanol spectrum. This difference could be due to greater extraction or subsequent absorption of water by ethanol.

Good spectra were obtained for both samples. These two spectra will be taken as reference to compare with other sampling systems, as the KBr disc method has already been widely used in FTIR (Hauser and Oelichmann, 1988).

3.3.1.2 Barium fluoride window

3.3.1.2.1 Introduction

Barium fluoride (BaF_2) windows are used in infrared spectroscopy in transmission mode. This material is transparent to infrared light. BaF_2 has non-hygroscopic properties, unlike KBr and NaCl (Wetzel, 2002).

McCann et al. (1992) studied plant cell walls by FTIR. They deposited a suspension of finely ground cell walls or a solution of polymers to form a thin layer on top of a BaF_2 window. Areas with little depth of material were selected in the microscope and spectra recorded from these.

3.3.1.2.2 Experimental

The barium fluoride windows used (13 mm diameter x 2 mm thick) were purchased from Spectroscopy Central Ltd. Acetone and ethanol extracts were analysed using BaF_2 windows.

A solvent extract droplet of several microlitres volume was deposited on the BaF_2 window and the solvent evaporated at room temperature. A light stain remained on the window and represented the extractives (Figure 3-3). The

microscope was then focused on the stain and the spectrum collected. This part of the procedure is not easy to carry out because the detection of the stain remained difficult (Figure 3-4). The utilisation of pigments was not possible, as these would produce infrared spectra too.

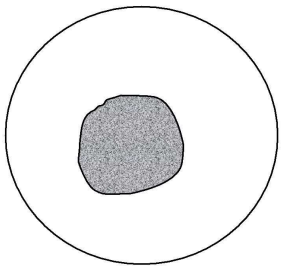


Figure 3-3: Sketch of acetone extract of Sitka spruce on Barium fluoride window

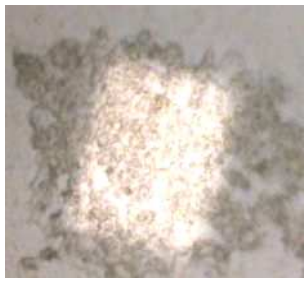


Figure 3-4: Light micrograph of acetone extract of Sitka spruce on Barium fluoride window

3.3.1.2.3 Results

Once the stain had been located, spectra were collected from several regions of the stain. The spectra from the middle of the stain had less intensity than that obtained on the border. That lead us to think that the sample does not dry uniformly and the extractives are more concentrated at the border than in the middle of the stain.

A comparison was made between the KBr disc procedure and the barium fluoride window (Figure 3-5).

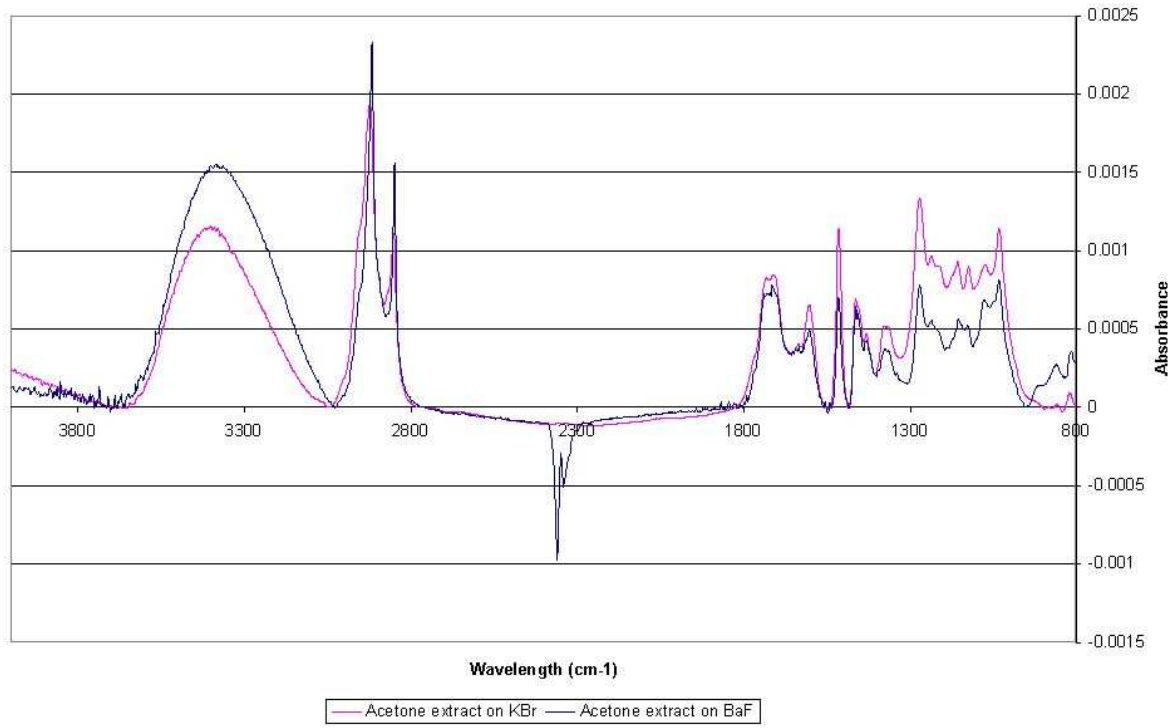


Figure 3-5: Spectra, normalised and baseline corrected, of acetone extracts from Sitka spruce analysed by FTIR spectroscopy using KBr discs and Barium fluoride window

Comparing the spectra obtained by both methods, KBr disc and BaF_2 window, (after the baseline correction and the normalisation of the area to 1), all the peaks previously detected with the KBr disc were also present with the BaF_2 window procedure. The difference is a more pronounced distinction between the pairs of peaks at 1730 and 1710cm^{-1} and at 1155 and 1125cm^{-1}. The signal to noise ratio was greater for the BaF_2 window than for the KBr disc technique.

The ethanol extract was also tested on BaF_2 windows. It was evident that the drying of the sample on the window was different. While the acetone droplet remained approximately circular, the ethanol extract spread over a bigger area with irregular borders, and the formation of micro-droplets containing very high concentrations of extractives (Figure 3-6).

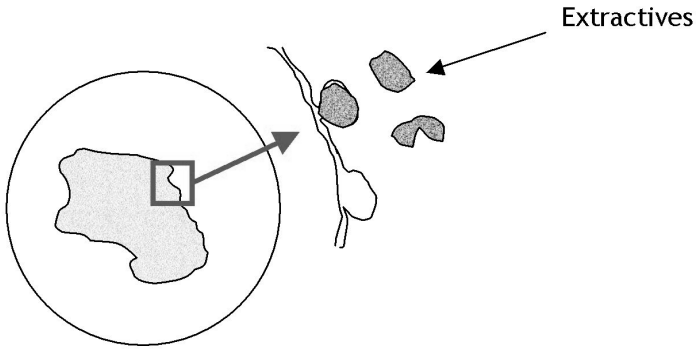


Figure 3-6: Sketch of ethanol extract of Sitka spruce on Barium fluoride window

As the ethanol extract did not dry uniformly, the collection of representative spectra was not easy and we could not be sure that all the classes of extractives would be equally represented in these micro-droplets (the size of the micro-droplets was too small to give a good repeatability of the spectrum with all the samples).

3.3.1.2.4 Discussion

The BaF_2 window gave good spectra from the acetone extract. The major drawback was the difficulty locating the sample on the window. The sample had to be as concentrated as possible to be detected, which involved a longer duration for the evaporation step and a risk of overheating the sample and losing volatile compounds.

3.3.1.3 Barium fluoride window system 1

3.3.1.3.1 Introduction

It was difficult to keep the extract droplet confined on the Barium fluoride window and to prevent possible fractionation of the sample according to 'chromatographic' phenomena. In order to solve this problem, we tried to build a sample holder, which would retain the droplet in a restricted area.

3.3.1.3.2 Experimental

Two systems based on the same principle were constructed. The first was built using a polyethylene sealing layer (Figure 3-7). The polyethylene was contained between two layers of aluminium, the bottom layer being the original sample holder with the barium fluoride window; the second layer was screwed to the

first layer to seal the system. The problem with system 1 was that it was not watertight enough to keep the droplet on the barium fluoride window and the extract diffused into the gap between the polyethylene and the aluminium sample holder.

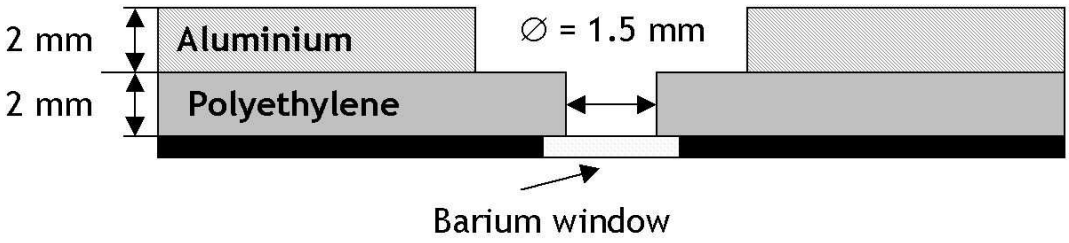


Figure 3-7: Sketch of system 1 where a polyethylene layer is intended to maintain the droplet confined on the barium fluoride window.

A second system was established using a polished silicon wafer instead of polyethylene as sealing but the result was the same; the system was not watertight enough to contain the extract droplet.

During all the trial, particular care was taken to only use sealing material that could not be affected by the solvent or interact with the extractives.

3.3.1.3.3 Discussion

With the BaF_2 window method, all the peaks previously analysed with the KBr disc method were detected. The major disadvantage was the difficulty in placing the sample onto the BaF_2 window and locating the residue under the microscope. The shape of the residue depended on the solvent used for the extraction. As the results obtained using barium fluoride windows were not very promising, we decided to investigate the other modes available in FTIR.

3.3.2Reflectance mode

In specular reflectance mode the infrared beam travels through the sample and is reflected by the surface of the sample holder.

There are three types of reflectance mode used in infrared spectroscopy and microscopy: ATR, diffuse reflectance and specular reflectance. These differ in the angle of reflectance formed after reflection (Figure 3-8). If the angle of

incidence equals the angle of reflectance, this constitutes specular reflectance. For specular reflectance the sample holder must be optically mirror-smooth at the wavelength of midrange infrared radiation (1-10 μm). If the incidence angle is fixed but the reflectance angle varies from 0 to 360 degrees, we have diffuse reflectance, which occurs on rough surfaces (Smith, 1996). The objective of the FTIR microscope that we used works in specular reflectance.

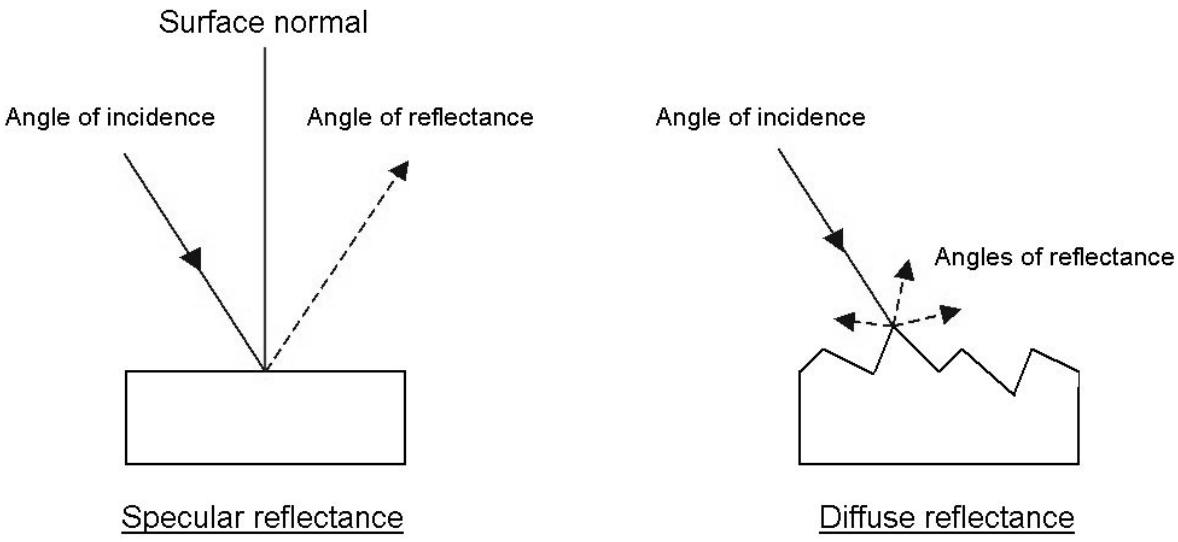


Figure 3-8: Examples of specular and diffuse reflectance

The key issue in this mode is the reflectiveness of the sample holder. The physical properties of the surface are also important because the aim of the study is to use a small volume of sample during the analysis, so the concentration of the sample has to be suitable to collect representative spectra.

The ATR technique is used in infrared spectroscopy to measure IR spectra at surfaces. This technique makes it possible to directly analyse samples in the solid or liquid state. The technique uses the internal reflection properties of a crystal to generate a standing wave of radiation, called an evanescent wave (Figure 3-9). The sample when pressed against the crystal will modify the optic properties of the interface and will absorb infrared radiation. The evanescent wave intensity will be attenuated by the sample, and the result will be given by the difference between the original and modified signals. The key is to have good contact between the crystal and the sample (Smith, 1996).

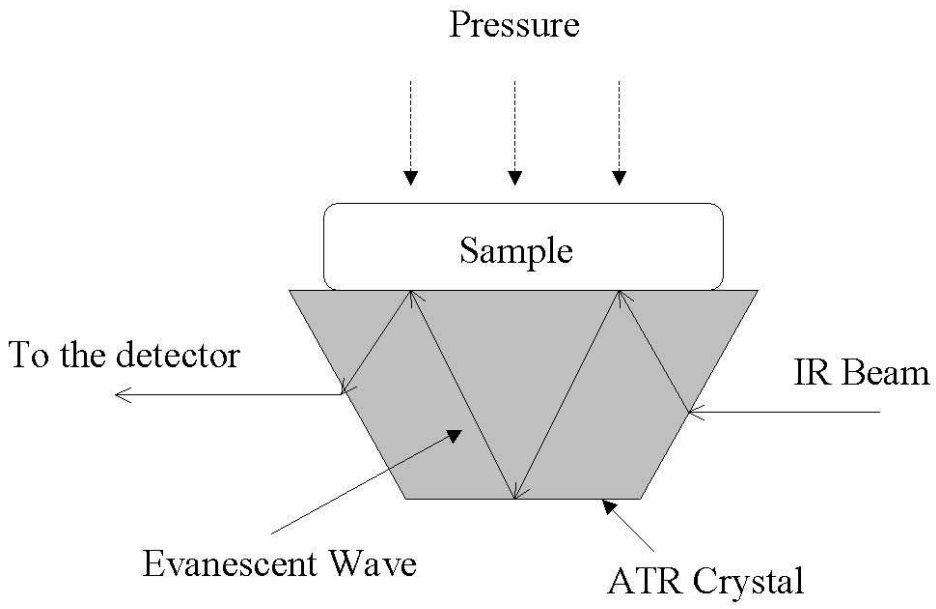


Figure 3-9: Sketch of ATR principle

3.3.2.1 Attenuated Total Reflectance (ATR) with aluminium foil

3.3.2.1.1 Introduction

Spectra were obtained on ATR and a Bio-Rad FTS40 FTIR spectrometer. All spectra were obtained at a resolution of 8 cm^{-1}, with 256 co-added interferograms.

3.3.2.1.2 Experimental

To analyse samples by ATR spectroscopy, the liquid extract was dropped onto aluminium foil and allowed to dry. The dried sample on the aluminium foil was pressed onto the ATR crystal. The quantity needed was around 5µL.

3.3.2.1.3 Results

Acetone and ethanol extracts at the same concentration were tested by the ATR method on aluminium foil and analysed again in KBr discs (Figure 3-10 and Figure 3-11).

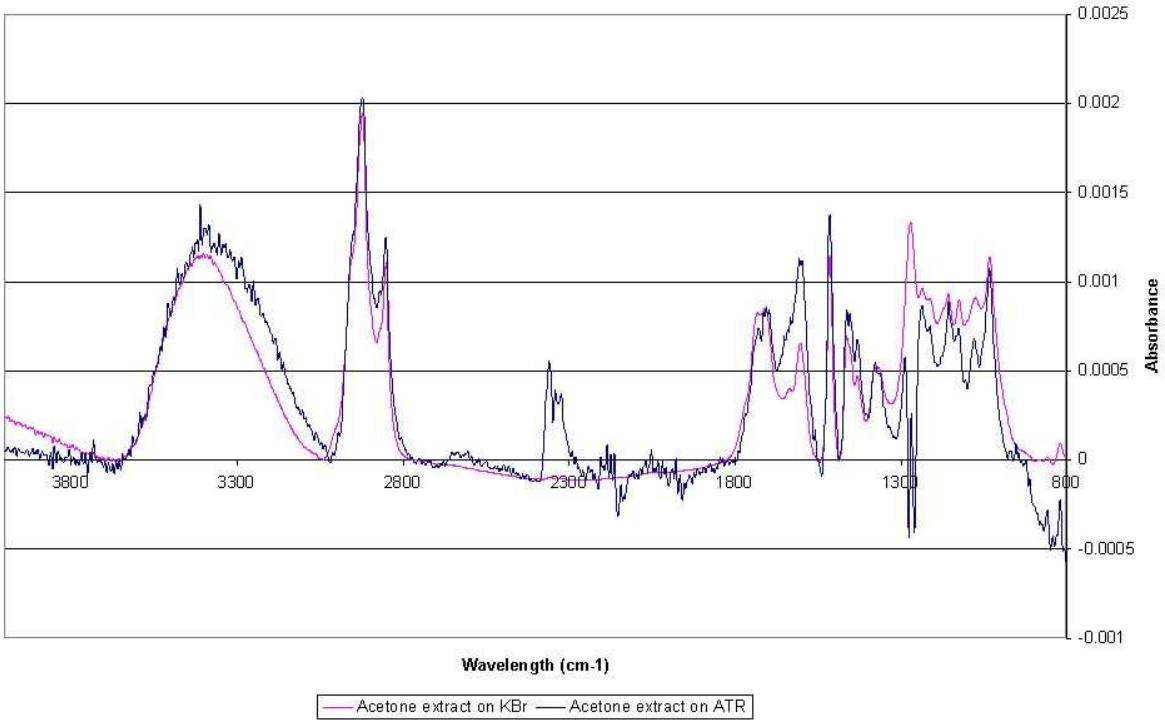


Figure 3-10: Spectra, normalised and baseline corrected, of acetone extracts from Sitka spruce analysed by FTIR spectroscopy using KBr discs and ATR on aluminium foil

The acetone sample analysed with ATR gave spectra showing a lot of noise. There was an unexpected negative signal around 1300cm^{-1}. Around 1700cm^{-1}, the shape of the peak was different probably due also to the signal/noise ratio, and in the fingerprint region the signals appeared to have slightly different intensities compared to KBr.

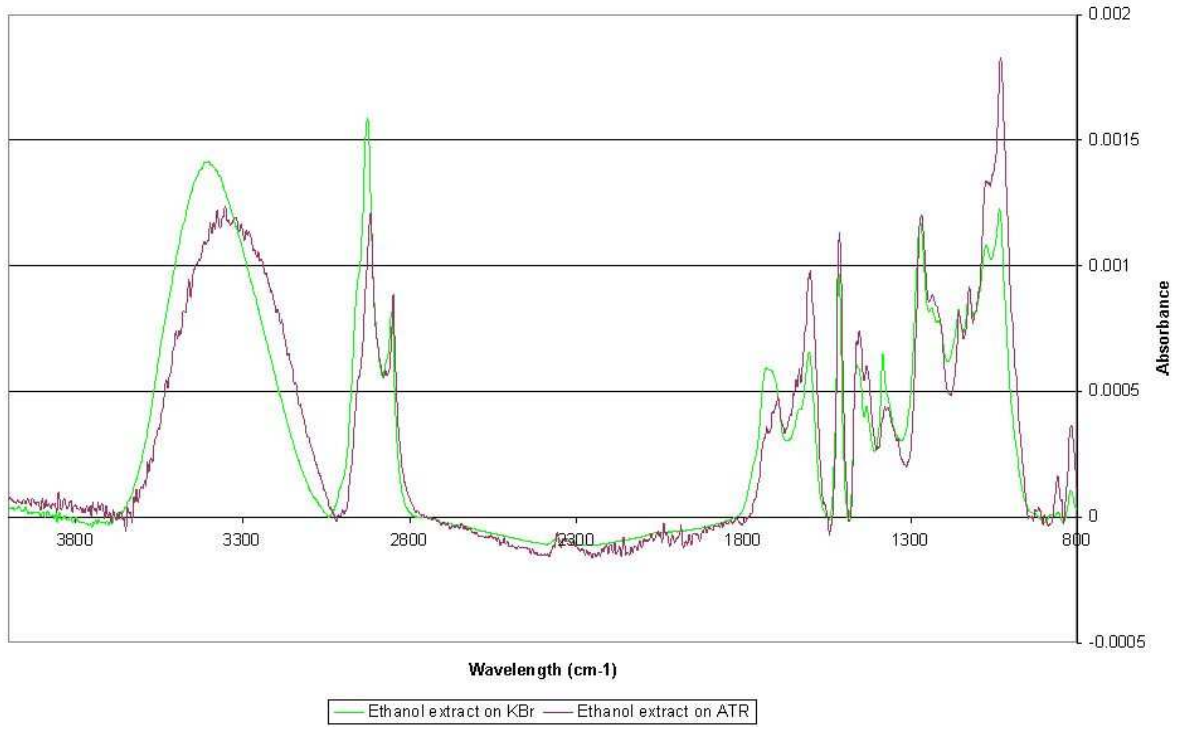


Figure 3-11: Spectra, normalised and baseline corrected, of ethanol extracts from Sitka spruce analysed by FTIR spectroscopy using KBr discs and ATR with aluminium foil

For the ethanol sample, the ATR spectrum again had more noise than the KBr disc spectrum. The differences between the two methods (KBr discs and ATR) for the ethanol extract are:

- At around 1700cm^{-1}, the peaks were more distinct for the KBr method.

- Also around 1230cm^{-1}, the two peaks were more distinct for the KBr method.

But these differences are probably due to the signal to noise ratio and that the method was more difficult to carry out compared to the KBr disc method, because good contact between crystal and sample was needed and this was difficult to achieve.

3.3.2.1.4 Discussion

The preparation of the sample for the ATR method was faster because only a few microlitres are needed while the volume of sample to prepare a KBr disc is close to one millilitre. But the drawback of the ATR method was the difficulty of putting a second droplet at the same place as the first one. It was necessary to

be careful that the extract stayed concentrated at the same place and did not expand too much as the surface which will be analysed cannot be not expanded. Also the aluminium foil is smooth and it was essential not to crease it, to avoid too much noise in the background. With the ATR method, the signal obtained was noisier than with the KBr disc method and the method is also not 'quantifiable' because it is not know how much sample the spectrum is derived from.

3.3.2.2 Aluminium foil with FTIR microscopy

The results obtained after the ATR method were not as conclusive as expected. The investigation was continued further in the reflectance mode, using microscopy techniques.

3.3.2.2.1 Experimental

Aluminium foil was used as the sample holder and was taped onto a flat surface with double-sided tape. The droplets of the acetone or ethanol extract were dropped directly onto the aluminium foil and allowed to dry.

This technique provided a cheap and fast system to analyse the sample. However the background was quite dark on looking through the microscope, so we were not very confident that good spectra would be obtained (Figure 3-12 and Figure 3-13).

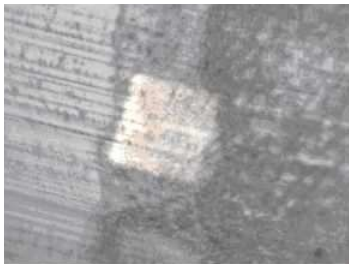


Figure 3-12: Aluminium foil holder with ethanol extract for FTIR microscopy on the border of the sample droplet.

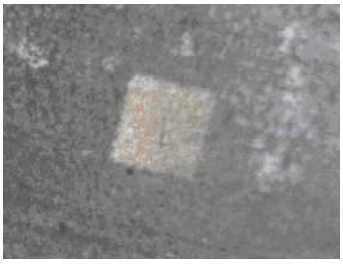


Figure 3-13: Aluminium foil holder with ethanol sample for FTIR microscopy in the middle of the sample droplet.

Ethanol and acetone extracts were analysed on aluminium foil. As the background was dark under the microscope, more than 3µL was necessary to obtain sufficient amount of sample for a spectrum.

3.3.2.2.2 Results

Despite the fact that the background was dark and showed some black spots, it was possible to obtain good quality spectra (Figure 3-14).

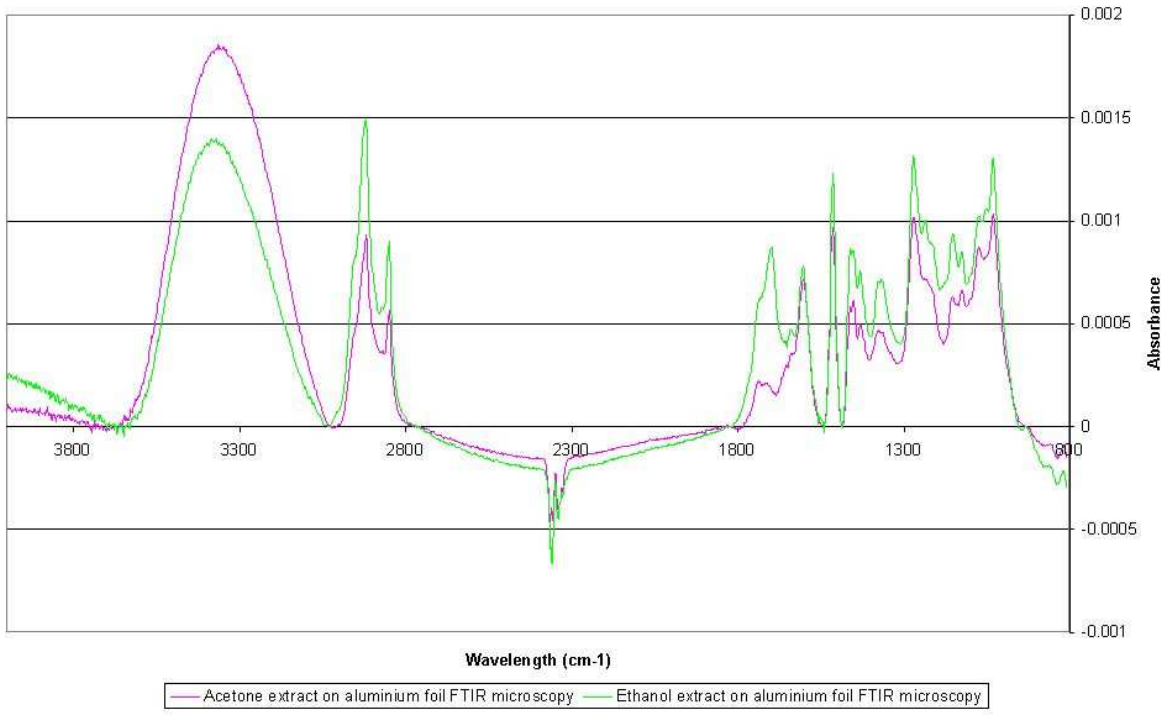


Figure 3-14: Spectra of acetone and ethanol extracts on aluminium foil analysed by FTIR microscopy

Some differences were evident between the ethanol and acetone extracts. The peak at 1700cm^{-1} in the ethanol extract was not detected in the acetone extract. It may have been present but at a very low intensity. The peak at 1230cm^{-1} was also detected in the ethanol extract but was not as intense in the acetone extract. These peaks were present in the spectra from the acetone and ethanol extracts measured as KBr discs (Figure 3-2).

3.3.2.2.3 Discussion

The way in which the droplets dried differed between the acetone and ethanol samples. Acetone droplets dried more uniformly than ethanol droplets leaving a circular residue on the aluminium foil. The extractives from the ethanol extract were located at the borders and good spectra were obtained from that region.

The drawback of the method is the difficulty of placing a second droplet of sample at the same place as the first, so as to avoid the sample spreading over a

larger area. It was preferred to repeat the deposition of smaller quantities so that the sampling area stayed relatively small and the concentration of extractives was sufficient for detection of minor components, especially for the ethanol extract.

The fact that the acetone and ethanol extract did not give the same result, in terms of spectra quality, might be due to difficulties in sample preparation.

3.3.2.3 Gold mirror system

3.3.2.3.1 Experimental

A gold mirror supplied by the Thermo Nicolet Company was tested using acetone extract.

The surface was reflective but had a golden colour. Despite the reflective surface the yellowish colour made the background dark, which was not good for our purpose because the detection of the sample on the sample holder was even more difficult (Figure 3-15). A few droplets of acetone extract were dropped onto the gold surface.

Figure 3-15: Acetone extract on gold mirror under the microscope

3.3.2.3.2 Results

We choose to compare the spectra obtained with the gold mirror sample holder against the reference technique, the KBr disc method (Figure 3-16).

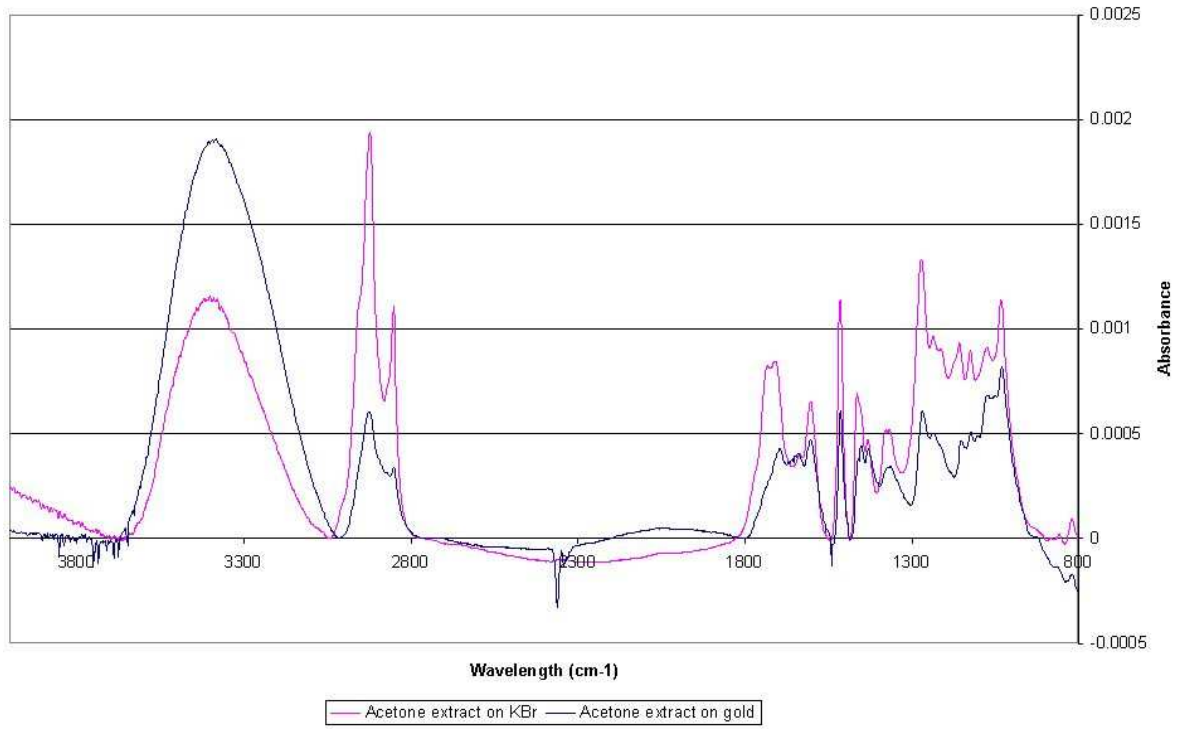


Figure 3-16: Spectra, normalised and baseline corrected, of acetone extract on gold sample holder analysed by FTIR microscopy and analysed by Kbr disc

It can be seen that around 1700cm^{-1} the spectra from the gold mirror and the KBr disc differ with a lot of overlapping for the golden mirror. A difference is also visible around 1120cm^{-1}, where despite the baseline correction; detection of the peak is not easy.

3.3.2.3.3 Discussion

The quality of the spectra obtained with the gold mirror sample holder was not as good as was obtained with the KBr disc method. The signals did not show the same resolution. There was a lot of overlap around an important area of the spectrum, which would be a problem later when quantification is being established.

The sample preparation was not very easy due to the fragility of the sample holder, which was easily scratched during the cleaning step, so we have chosen to continue the investigation on the reflectance mode with sample holders of other materials to find a more suitable technique.

3.3.2.4 Silicon plate system

3.3.2.4.1 Introduction

On a low surface energy material, a droplet has a smaller area in contact with the material than with a high surface energy. In that case after the droplet evaporates, the concentration of the sample should be higher within the area of the deposit.

3.3.2.4.2 Experimental

A Silicon wafer (100 plan cristallin, p-doped (boron), resistivity 1-50), purchased from University Wafer Inc., was tested as sample holder in order to provide a lower surface energy and thus confine the liquid sample in a restricted area.

3.3.2.4.3 Results

For the acetone sample, the dried droplet was not as uniform as with other techniques (Figure 3-17 and Figure 3-18), and the deposit was less circular. The solution left a white stain when it dried. The results obtained on the silicon plate did not agree with the original expectation of a droplet as spherical as possible with a minimum of contact with the surface.

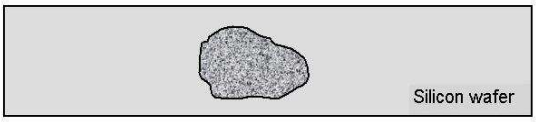


Figure 3-17: Sketch of dried acetone extract on silicon sample holder

Figure 3-18: Picture of dried acetone extract on silicon sample holder

The silicon method allowed the detection of all the compounds already detected by the KBr disc method (Figure 3-19). The only difference was that at around 1700cm^{-1}, there was a clearer distinction between the closely spaced peaks than with the KBr disc method.

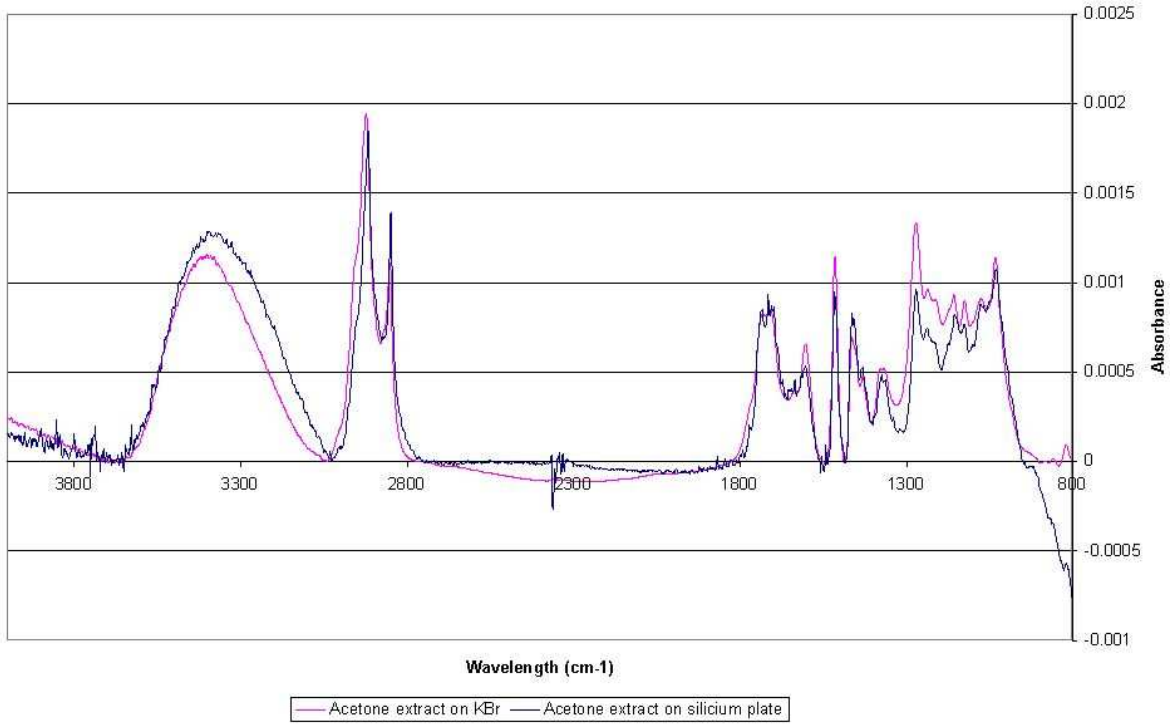


Figure 3-19: Spectra, normalised and baseline corrected, of acetone extract on silicon sample holder and on KBr disc

No results were acquired with the ethanol sample due to the adsorption and spreading of the ethanol solvent on the silicon surface (Figure 3-20 and Figure 3-21) (Mizukami et al., 2002).

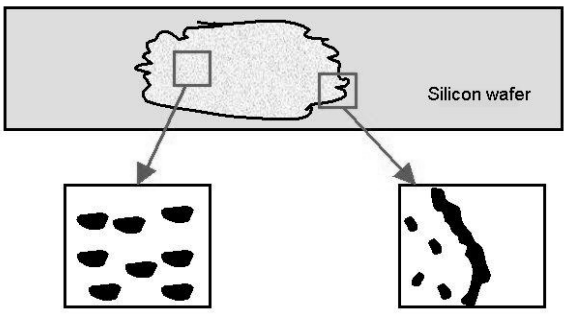


Figure 3-20: Sketch of dried ethanol extract on silicon sample holder

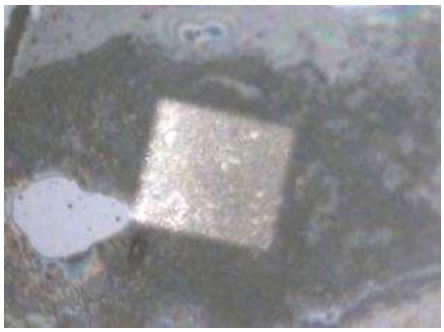


Figure 3-21: Picture of dried ethanol extract on silicon sample holder

3.3.2.4.4 Discussion

This sample holder seemed to be the most reflective system tested until that point. However the spectra obtained showed some noise and was not appreciably better than the spectra obtained with the gold mirror plate.

Unfortunately the silicon surface reacted with the ethanol so we were unable to obtain any spectrum from the ethanol extract.

This material was also the most expensive of the materials tested and was too expensive to use a new sample holder for each experiment.

3.3.2.5 Non-polished aluminium plate

3.3.2.5.1 Introduction

A holder made from raw aluminium strip was considered, as this material is quite reflective and very cheap.

Goodacre et al. (1998) used milled wells in sandblasted aluminium plates to analyse microbial systems. They chose this technique because it made it possible to analyse a large number of sample (> 100) in one dataset collection. This was why we decided to try to use strip aluminium, as the aim of the study was to speed the analysis process due to the large number of samples to by analysed.

3.3.2.5.2 Experiment

The sample holders were cut to 75 mm length from 25mm x 2mm raw aluminium strip (B&Q Ltd).

3.3.2.5.3 Results

When the unpolished aluminium holder was examined under the microscope, the background was very dark and the surface was very uneven (Figure 3-22). It was not easy to find the sample on the holder. It seemed likely that the spectra were influenced by the roughness of the surface.

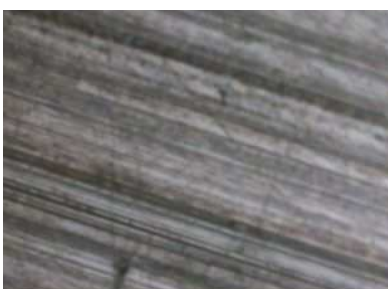


Figure 3-22: Background of aluminium sample holder for FTIR microscope

3.3.2.5.4 Discussion

It was decided that the surface needed to be altered in order to make it more uniform and more reflective.

3.3.2.6 Polished aluminium plate

3.3.2.6.1 Experimental

To obtain better reflectance, the aluminium plate was polished to a bright finish with few flaws. Steel wool and Brasso metal polish (by Reckitt Benckisser Ltd) were used. This treatment gave the desired result, an optically smooth mirror surface (Figure 3-23). The same concept was used by Thomas et al. (2000) who used a sand-blasted aluminium plate with FTIR to study follicular fluids from antral follicles.

Figure 3-23: Background of polished aluminium sample holder for FTIR microscope

We were concerned that the metal polish liquid would interfere with the sample. Background spectra of the polished and non-polished aluminium holder were therefore compared, and no difference was evident between the two surfaces. When samples were applied there was no noticeable interaction between the sample and the sample holder.

The next concern was the way in which the sample droplet would dry on this surface. The acetone sample dried uniformly, remaining circular with no spreading into the microscopic scratches still present, while the ethanol sample spread somewhat and dried heterogeneously (Figure 3-24).

Figure 3-24: Sketch of the acetone and ethanol samples dried on a polished aluminium plate

3.3.2.6.2 Results

The acetone extract was analysed on a polished aluminium plate.

The polished aluminium plate sample holder gave a good result for the analysis of the Sitka spruce extractives, comparable with the KBr disc method (Figure 3-25).

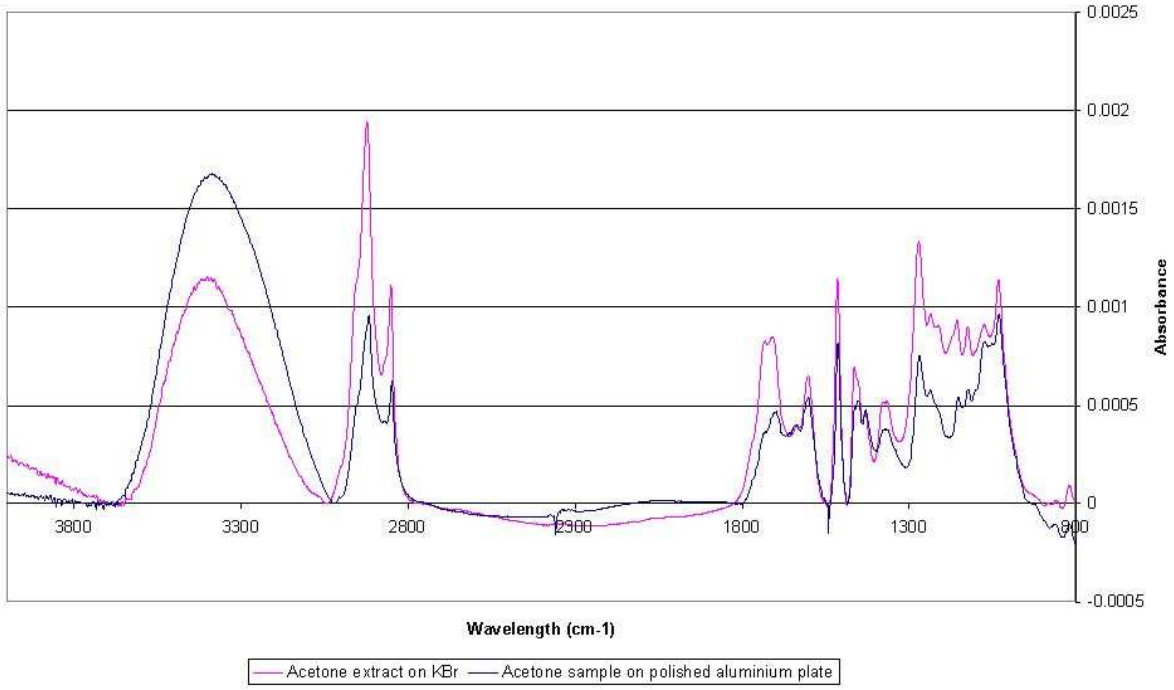


Figure 3-25: Spectra of acetone extract analysed by FTIR microscopy on polished aluminium plate and by the KBr disc method

All the peaks detected with the KBr disc method were present in the spectra from the polished aluminium plate method. There was also better definition of the peak at 1700cm^{-1}.

With the ethanol extract, the liquid did not dry in the same way as the acetone extract but spread rapidly, making it more difficult to find an area in which the extractives were concentrated.

3.3.2.6.3 Discussion

The background was now more reflective than with the aluminium foil and the non-polished plate. Acetone droplets dried very well and remained circular. Most of the extractives were present at the border. However ethanol droplets spread out very quickly: the area to be analysed was wide and a greater volume of sample was needed to obtain a good spectrum, which made the analysis difficult and less repeatable.

The system was best suited to the acetone extracts, because it was very reflective and gave well-resolved spectra of good intensity. In term of sample preparation, several microlitres of sample were enough (around 3 times 1 μL droplet), which reduced the sample preparation prior to analysis. The time saving was significant in comparison with the preparation of KBr disc as the volume of sample needed for KBr discs was much greater (around 50μL) and the solvent had to be completely evaporated without absorption of moisture.

On the polished aluminium plate, if the sample was deposited carefully it stayed confined in a restricted area and so a small volume of sample is enough to obtain good and reproducible spectra. 30 sample droplets (3 times 1μL sample) can be analysed on a single aluminium plate.

The spectra obtained with the polished aluminium plate were equivalent to those obtained with KBr disc (the reference method), and some of the peaks were better resolved on the polished aluminium plate.

3.3.2.7 SU-8 and polished aluminium plate system

3.3.2.7.1 Introduction

In order to improve and facilitate the concentration of the sample on a restricted area, we attempted to add a second layer onto the polished aluminium plate; this second layer had different physico-chemical properties intended to retain the sample on the open aluminium space.

3.3.2.7.2 Experimental

The product SU-8 2002 (Microchem) is a chemically and thermally stable epoxy based photoresist. A 2 microns layer of SU-8 was spin coated and patterned by

photolithography on to a polished aluminium plate by Dr Francois Caron. The pattern was a 2mm diameter circular hole in the SU-8.

The SU-8 has a lower surface tension than the aluminium plate leading to the confinement of the droplet on the 2mm aluminium spot (Figure 3-26).

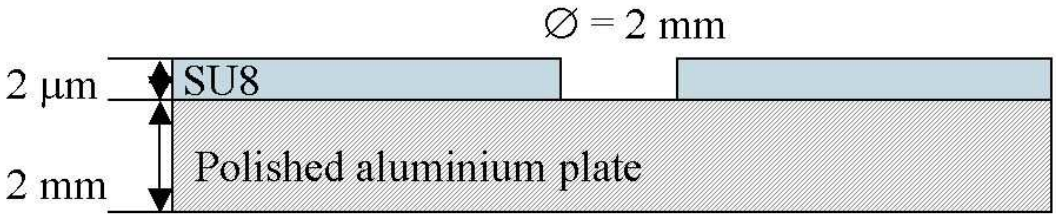


Figure 3-26: schema of SU8 and aluminium sample holder

3.3.2.7.3 Results

Acetone and ethanol extracts were tested.

The droplet of acetone extract remained confined on the open area of aluminium. Two microlitres of sample were needed to obtain a good spectrum (Figure 3-27).

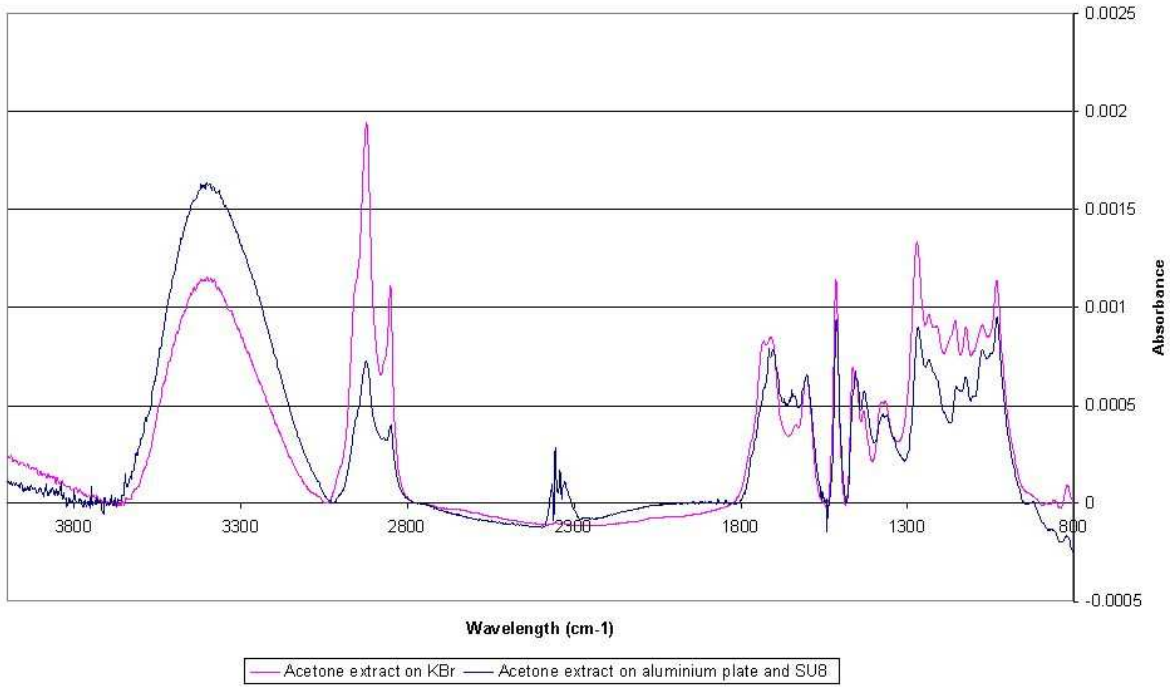


Figure 3-27: Spectrum of acetone extract on SU8 and aluminium sample holder and KBr disc

As the sample holder background is the polished aluminium plate, we obtained similar spectra to the 3.3.2.6 polished aluminium plate paragraph. All the peaks previously detected with the KBr disc method were also detected with the polished aluminium plate, and there was also better definition of the peaks around 1700cm^{-1}.

The ethanol sample was also analysed, but the solvent being more polar expanded outside the analysis area, so it was not possible to use this system to confine the sample within a designated area.

3.3.2.7.4 Discussion

This system was effective because it allowed us to confine a very small droplet of sample in the area intended.

But the system was very expensive because of the need to pattern the layer of SU-8 in a clean room environment. Despite the advantage of the well-defined sample area, equivalent results could be obtained when the liquid sample was deposited carefully on the polished, uncoated aluminium plate. The SU-8 coating method would be worth considering if the method were to be developed into a microarray format with spectra from large numbers of samples being recorded in parallel.

3.3.2.8 Systems with wells

3.3.2.8.1 Introduction

Another potential way to concentrate the extractives into the analysis area is to drill a hole into the polished aluminium surface. This way the droplet would stay inside and it might be possible to collect the extractives on the bottom of the hole.

However as the analysis was undertaken in specular reflectance mode, the bottom of the well would have to be flat and have a good reflectance. Several shapes were considered (Figure 3-28, Figure 3-29 and Figure 3-30). These systems were very difficult to build because of the need for a reflecting flat bottom.

The first well shape was the simplest (Figure 3-28), a deep narrow hole with a flat bottom. This shape was suitable but the drilling operation led to a lack of reflection from the bottom surface. Also the convergent light beam from the microscope did not illuminate the whole bottom surface.

Figure 3-28: Well shape 1

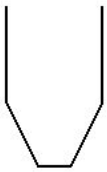


Figure 3-29: Well shape 2

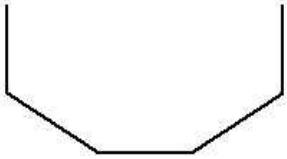


Figure 3-30: Well shape 3

To reduce the lighting problem we tried a V shape close to the bottom (Figure 3-29), but again it was difficult to get the bottom aluminium surface reflective enough to obtain quality spectra.

Then we tried to enlarge the hole in order to get more light inside (Figure 3-30), but the problem was that the deposition of extractives stopped randomly on the sloping border or at the bottom.

As the first structure had a square elevation, some other concepts with a round bottom were considered. Unfortunatly they were not capable of being constructed, at least with the materials available. Further work would have been needed to find a appropriate watertight, reflective material but we estimated that it was not necessary to pursue this line of study.

In order to find a better way to retain the droplet of sample confined in a restricted area, systems constructed with multiple layers were tested. The polished aluminium plate sample holder was good in reflectance so we attempted to add a second layer of aluminium to restrict the area where the droplet might expand. The possibility to dismantle multilayer system would allow the reflective surface to be cleaned and re-polished readily. But the limited space available below the objective lens of the FTIR microscope restricted the maximum thickness of the whole assembly to about 7 mm.

3.3.2.8.2 Two-aluminium plates system

3.3.2.8.2.1 Experimental

The first system is composed of two layers of aluminium held together with two sets of screws (Figure 3-31).

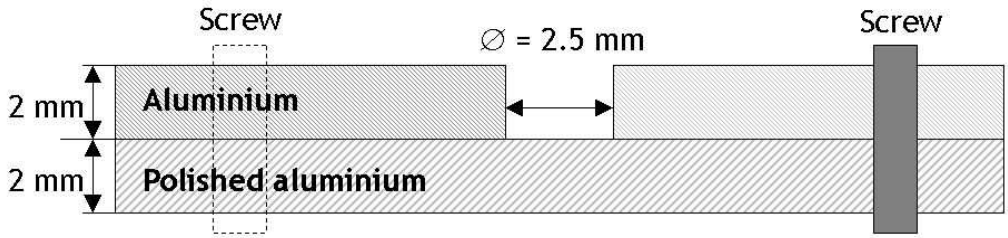


Figure 3-31: Sketch of the double-aluminium plate sample holder

3.3.2.8.2.2 Results

A small volume of acetone extract was dropped inside the well. Despite the fact that the top aluminium plate was screwed down as tightly as possible, the system was not sealed and it was not possible to get the sample to stay inside the required area.

3.3.2.8.2.3 Discussion

As the system is not solvent-tight, a sealing layer is necessary between the two layers of aluminium.

3.3.2.8.3 Two aluminium plates and one polyethylene plate

3.3.2.8.3.1 Introduction

The second system was also based on polished aluminium plates, but this time a third layer will be added to improve the sealing of the system.

3.3.2.8.3.2 Experimental

Special care was taken to use a sealing material that would not have any interaction with the solvent or the sample (Figure 3-32).

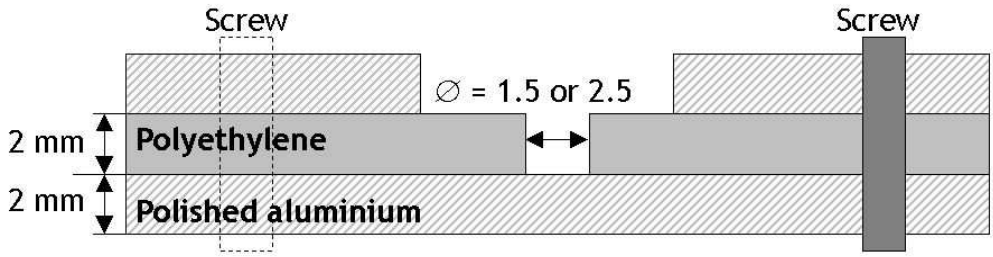


Figure 3-32: Sketch of sample holder constructed from two aluminium plates and one polyethylene plate

This system was tested with two different well diameters. The first one was 1.5mm diameter; this size was too small and did not allow the divergent reflected light to pass through the hole (Figure 3-33).

Figure 3-33: Image of acetone extract

As the depth of the well was increased (by adding a second layer of sealing material), the diameter of the hole had to be larger to allow the light to penetrate through the hole.

The second diameter tested was 2.5mm. This permitted the light to pass through the hole but the system was still not watertight enough to contain a small quantity of solvent on the area required.

3.3.2.8.3.3 Results

We did not succeed in constructing a multilayer system, solvent-tight enough to contain 2 or 3µL of acetone in the restricted area.

3.4 Conclusion on FTIR spectroscopy methods

The KBr disc method in transmission mode remains the reference method; it is the only method tested that allowed a possible quantification of the extractive classes by having a uniform volume of extract in each KBr disc.

However transmission mode does not seem the most appropriate method for the analysis of large numbers, as the KBr discs are very fragile and need a longer sample preparation time compared to the other methods tested.

Acetone is a better solvent to work with than ethanol, since the latter interacts more strongly with several of the sample supports tested. For example, the ethanol extract spreads irregularly when it dries on a BaF_2 window (transmission mode) and does not permit a representative spectrum to be obtained.

The construction of a system (in reflectance and in transmission modes) to retain the sample droplet confined in a restricted area was not successful. However this line of research was not pursued, as a single-layer system performed well enough.

The ATR method on aluminium foil allowed the identification of all the peaks already detected with the KBr disc method, but the disadvantage was the difficulty of depositing the sample on the aluminium foil and maintaining the aluminium foil free of creasing to minimise the background noise.

In reflectance mode coupled with microscopy, the results obtained with several sample holders were close to the results obtained with the KBr disc, the main differences being due to the sample deposition step. With aluminium foil and the non-polished aluminium plate, the reflected intensity was low and the background showed a lot of noise due to the irregularity of the surfaces. The gold mirror system was too sensitive to sample preparation and gave a lot of overlap in the spectra. The silicon plate interacted with the ethanol solvent, and was too expensive.

After running these comparisons, it appeared that the polished-aluminium plate system was the most suitable for the purpose of the study. It allows easy and fast deposition of the sample on the plate. The spectra obtained match every peak detected by the KBr disc method. The system is cheap and easy to construct. The way to deposit the sample using several small applications of solvent was simple and efficient and allowed some concentration of the sample. With the addition of the SU-8 epoxy coating it performed even better with the acetone extract but the added cost was not worth it, as the system is meant to be disposable and it did not work with ethanol as the solvent.

3.5 Validation of the technique chosen

Further tests were run in order to confirm the reliability of the polished aluminium plate system.

Spectra were taken in different places within the dried droplet (Figure 3-34). It appeared that the spectra obtained in the middle of the droplet were comparable but had a lower intensity than the spectra taken at the border of the dried droplet. The measurements were therefore always taken at the border of the dried extract droplet.

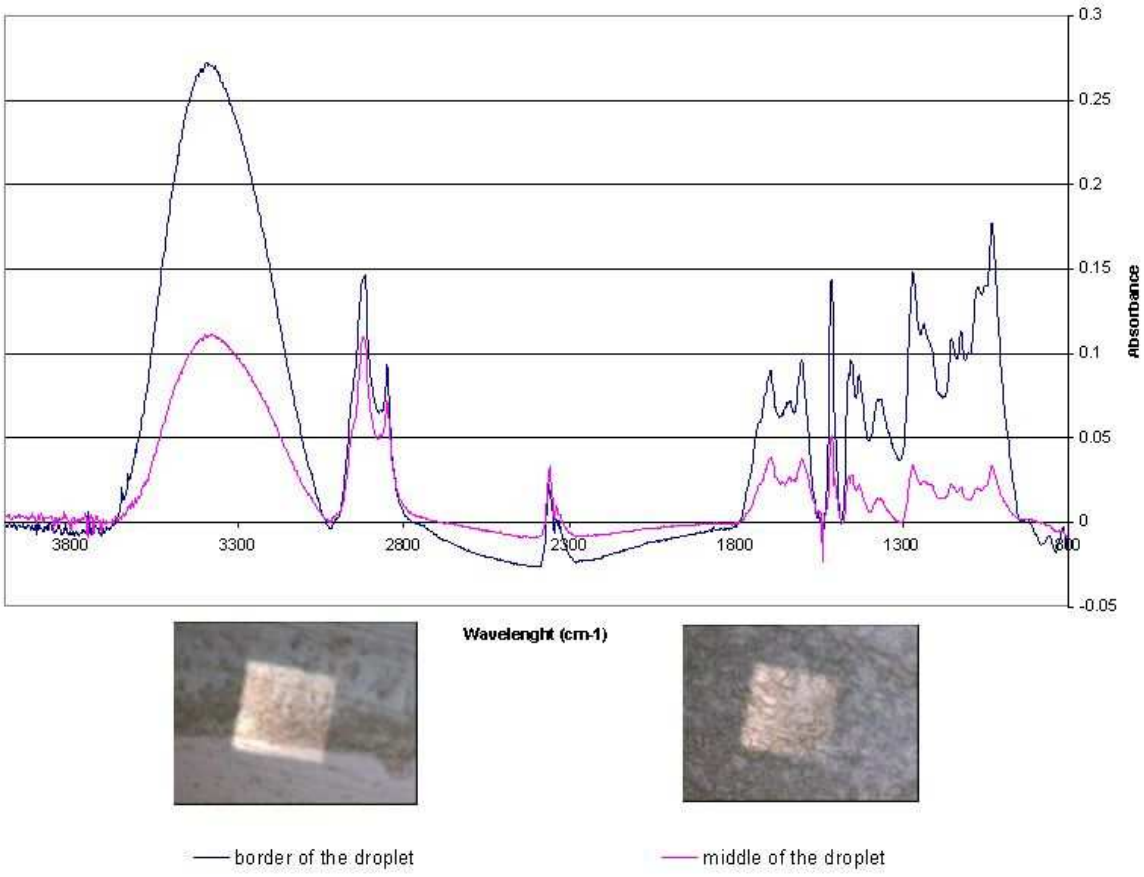


Figure 3-34: FTIR spectra from different place in acetone extract droplet

In order to confirm that there is no separation of extractive compounds during the drying of the droplet, peak areas centred on 3 selected wavelengths (2930-2840cm^{-1}, 1698cm^{-1} and 1510cm^{-1}) were measured. The same wavelengths will be used later in the study for the quantification. Two ratios were calculated from the area values for the measurement taken at the border and in the middle of the droplet and also for the KBr disk analysis. The results are presented in Table 3-2.

	KBr disc method	Border of the droplet	Middle of the droplet
Ratio 1	0.47	0.50	0.24
Ratio 2	0.14	0.22	0.10
Ratio 1: Area of 1698cm^{-1} peak/ Area 2930-2840cm^{-1} peak			
Ratio 2: Area of 1510cm^{-1} peak/ Area 2930-2840cm^{-1} peak			

Table 3-2: Ratio from FTIR analysis of acetone extract by three different analysis methods

There was a large difference in ratio 1 for the spectra obtained from the middle of the droplet. Higher values of both ratios were found from the spectra obtained from the border of the droplet, where most of the mass of the extract was concentrated. It would appear that the hydrocarbon extractives represented by the 2930-2840cm^{-1} peak have less tendency to move out to the border during the drying of the droplet. It was considered better to measure the sample at the border, as the results were more comparable to what was found in the KBr disc and the extract was so sparse in the middle of the droplet that it was difficult to obtain spectra there.

The samples were analysed several times, in order to establish the repeatability of the measurements. The results obtained are quite repeatable. The Figure 3-35 shows six different measurements of three different samples of acetone extract on polished aluminium plate.

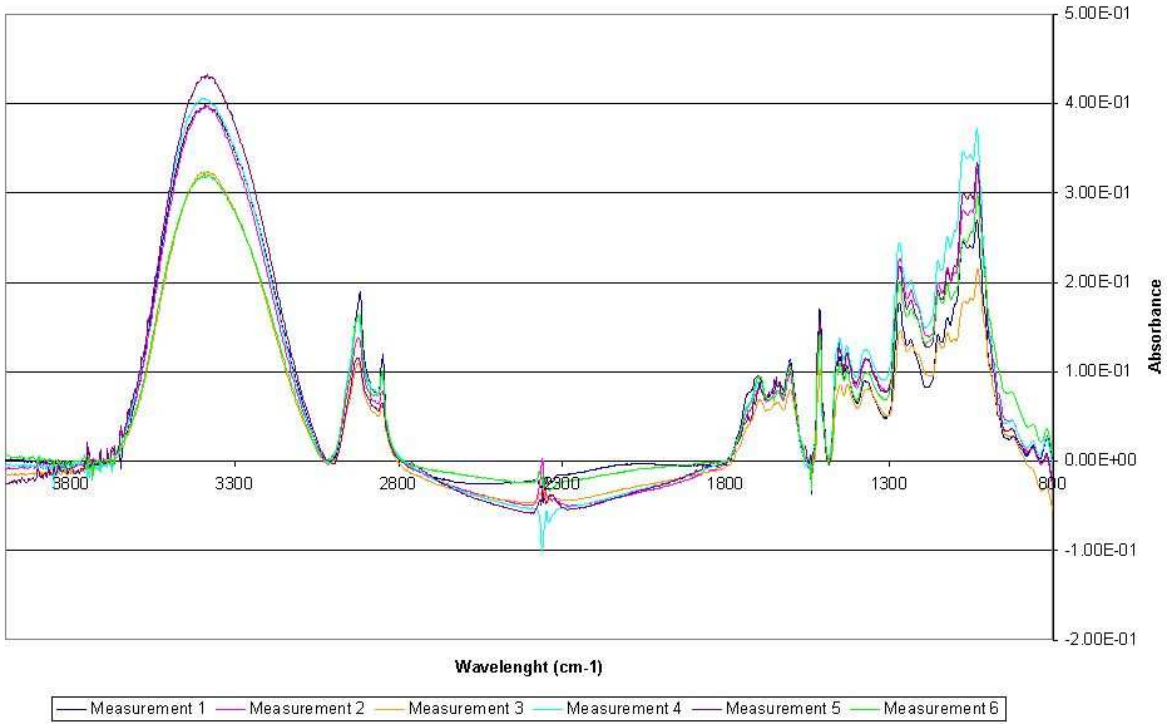


Figure 3-35: 6 different measurements of the same acetone extract on polished aluminium plate analysed by FTIR microscopy

If we look at the finger print region in more detail we obtain the following spectra (Figure 3-36).

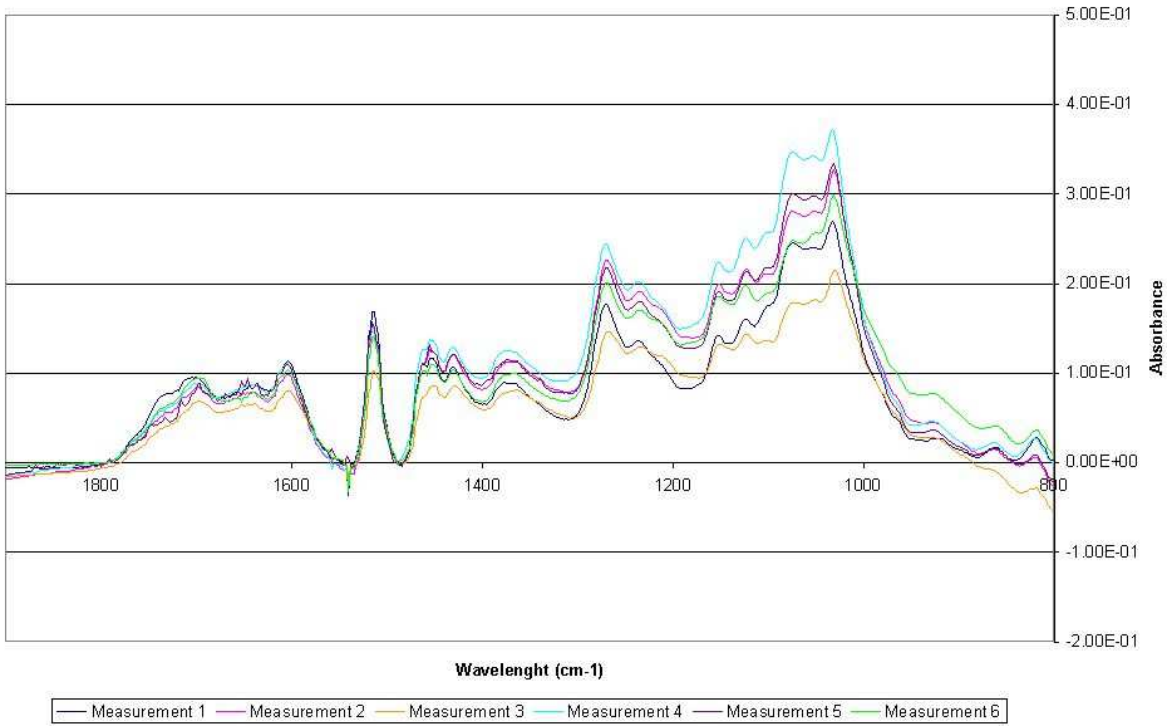


Figure 3-36: Finger print region of the FTIR spectra of the Figure 3-35

In the fingerprint, we remark a small difference in intensity between the samples, but all the peaks are detected. The variation between the spectra in the fingerprint region is mostly due to the difficulty of obtaining accurate baseline correction right across the spectrum. But there is least difficulty in getting good baseline correction close to the frequencies of the peaks that were used for quantification presented later.

3.6 Finalised method for FTIR analyses

The sample holder is an aluminium plate (size: 750 x 250 mm) purchased in B&Q. The plate is polished using fine steel wool wads followed by Brasso metal polish (by Reckitt Benckisser Ltd), the excess is removed with paper and the plate is rinsed with acetone before the analysis. Then the extract is applied in three droplets of one microlitre using a 10 microlitres gas chromatography syringe, letting the extract evaporate at room temperature between droplets. The FTIR spectra are then collected in reflectance mode at 5 points at the border of the dried droplet and normalised and baseline corrected as required.

3.7 Data processing from the FTIR experiments

FTIR spectroscopy in reflectance mode does not allow an absolute quantification of the functional groups detected. With the technique making use of acetone droplets deposited on a polished aluminium plate, quantification was not possible because of the irregular nature of the dried deposits. It was not practicable to measure the absolute quantity of extractives in each droplet. However, relative quantities of different species within each sample could be assessed.

The area of each peak (Table 3-1) of interest was calculated. The peaks representing methylene and methyl groups were chosen as common denominator as they were present in all samples and appeared to be the most stable in terms of intensity. The peak at 3400cm^{-1}, which represents the hydroxyl function was set aside because it intensity was too dependent on the relative humidity of the room atmosphere.

The ratio named (COOR/CH) between the carboxylic peak at 1698cm^{-1} and the combined C-H stretching peaks at 2930cm^{-1} and 2850cm^{-1} represents the proportion of esters to the aliphatic fraction.

The ratio named (Ring/CH) between the aromatic ring vibration at 1510cm^{-1} and the C-H stretching peak at 2930cm^{-1} and 2850cm^{-1} represents the proportion of aromatic compounds relative to the aliphatic fraction.

Chapter 4: Height study

4.1 Introduction and aims

Originally the aim of the Kershope Experiment was a "sawmilling" study, which assessed the benefit of selective breeding for timber quality and wood mechanical properties. We had access to this knowledge from the tree progeny trial and a good quality of timber to study the variation in extractive content within a tree, in heartwood, sapwood and knot wood at different heights on the stem. The data lead to conclusions on the distribution of extractives within the tree.

The aim of the work was to study the distribution of extractives within the tree, which means at different heights in the trunk, as well as the distribution between heartwood, sapwood and knotwood.

A gravimetric analysis was performed to determine the total extractive content, which was further analysed by FTIR spectroscopy in order to identify differences in the composition of the extracts between the samples.

For the heartwood and the sapwood it was possible to select samples from several heights of the trunk, making it possible to determine the distribution of the extractives within the trunk. In addition, FTIR spectroscopy provided the distribution of the extractive classes identified.

Then a comparison between heartwood, sapwood and knotwood was made to study the composition and distribution of extractives between these different types of wood.

4.2 Methods and procedures

Ekeberg et al. (2006) examined the influence of the particle size of the wood on the extraction results. They recommended using sawdust, because it is the best compromise to obtain the largest yield of extractives (pinosylvin, pinosylvin monomethyl ether, resin acids and free fatty acids) compared to solid wood or small particles.

Sawdust also allows a better penetration of the solvent, which will lead to a better extraction of the desired material, therefore it was decided to analyse extractives from sawdust (i.e. particles that pass through a 4mm sieve) in this and the following experiments.

4.2.1Wood samples from the Kershope forest

The Kershope experiment was based on sawdust obtained from discs sawn from Sitka spruce trunks freshly cut in the forest. The discs were drilled parallel to the grain using a wood bit to produce sawdust. This experiment made it possible to study the distribution of extractives within a tree, i.e. the differences between heartwood, sapwood and knotwood extractive composition as well as the variation in heartwood and sapwood extractives at different heights within the trunk.

Figure 4-1: Tree sampled from Kershope forest (UK)

Figure 4-2: Felled tree without branches

Three trees were chosen from one experimental area in Kershope forest, Northumberland (NY473 477, Latitude 55° 05'N, Longitude 2° 50'W, 190m elevation) (Figure 4-1 and Figure 4-2). This area was part of a field experiment that has been monitored and the genetic background of the trees is well known

(Mochan et al., 2008). The experiment was a progeny by plot size trial planted in 1968 and reaching harvesting maturity in 2004. The trees were Sitka spruce (*Picea sitchensis* (Bong.) Carr.) of a range of provenances from Oregon to Queen Charlotte Island (details about Sitka spruce can be found in chapter 1).

The trees were chosen to be as straight as possible, avoiding any fungus infection or visible defects as these factors could have an impact on the extractive content. Shaun Mochan and Dr. Paul McLean cut the trees specifically for this study.

Three trees were sampled and discs of 20cm thickness were collected every three meters from the base of the trunk to about 18m (Figure 4-3). Above 18 meters the trunk was too thin (i.e. diameter less than 7cm) for sampling. After the collection of the discs in the forest, the samples were brought back to the lab to be processed.

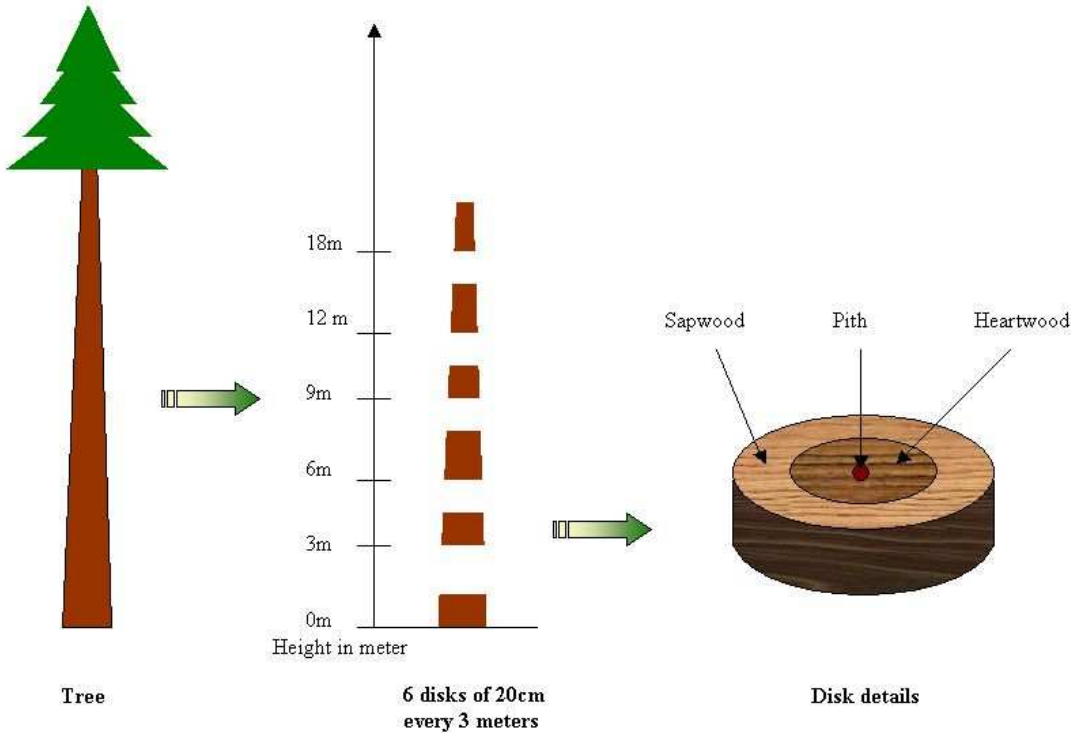


Figure 4-3: Tree sampling description

This allowed us to work on freshly cut (green) wood, that had never been dried, which is the best way to avoid the loss of volatile compounds and to avoid any kind of chemical reaction between the extractive compounds. In order to

maintain this fresh condition, the samples were kept in a cold room at about 4°C, in plastic bags to avoid drying out, until further processing.

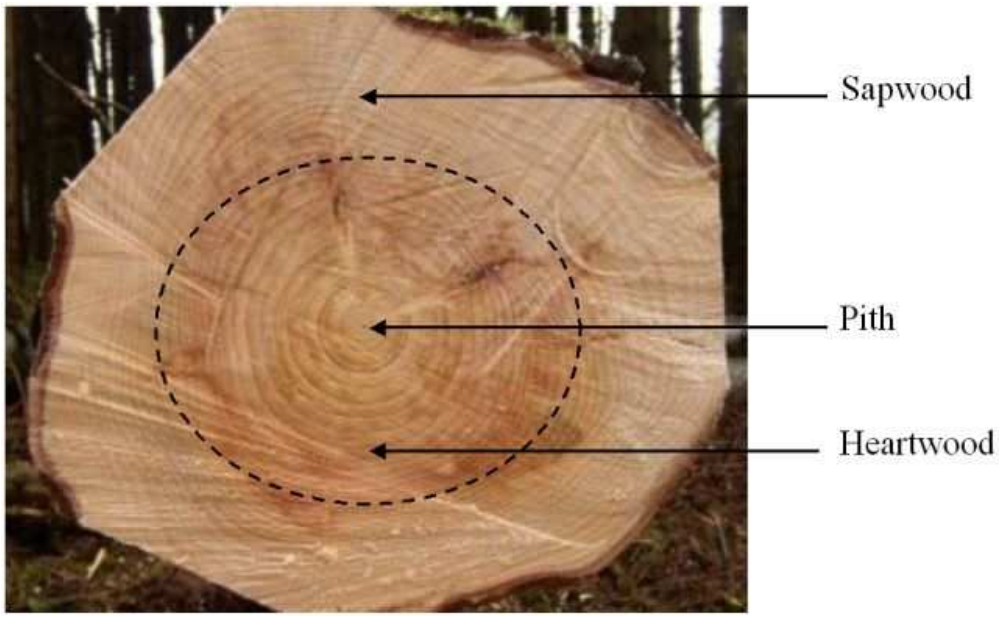


Figure 4-4: Illustration of the location of heartwood and sapwood in Sitka spruce

Within each disc, heartwood and sapwood were distinguished by eye (Figure 4-4). Sawdust of the two wood types was produced separately by drilling into the flat face of the disc using an electric drill fitted with a 10 mm flat woodworking bit. The sawdust comprised a representative sample of each type of wood at the corresponding height in the tree.

To resume: The samples come from 3 trees, 5 discs coming from different heights, from either heartwood or sapwood, with 3 replicates per sample. We had in total 90 samples to analyse.

Sampling of knotwood was different. Originally it was planned to collect individual whorls, (a whorl is an arrangement of several knots radiating at a certain height of the trunk), at different heights up the trees. Unfortunately the material collected from one whorl was not sufficient to generate enough sample material. Therefore the knotwood of each tree was pooled resulting in one knotwood sample for each tree. The “drilling method” was inappropriate for the knotwood, as the material was harder and too small. However the knots could be isolated by splitting them out of the discs. They were subsequently ground in ten second runs using an IKA Labortechnik A10 water-cooled grinder until the

particles passed through a 4mm sieve. In order to avoid any impact on wood composition by the increase of temperature during milling, particular care was taken to not overheat the sample during the milling. Furthermore the same protocol was always used in order to reduce errors inducing during the sample preparation (Schwanninger et al., 2004).

Once the samples were in the form of sawdust they were kept in airtight glass jars in a cold room at 4°C until they could be extracted.

4.2.2 Extractives analysis

The samples were extracted with acetone for 9 hours. They were concentrated to be analysed by FTIR in reflectance mode on polished aluminium plates. The general method is described in chapter 2 and chapter 3.

The samples were taken from five discs cut at different heights from each of three trees. Within each disc three replicate samples were cut from each wood type, heartwood and sapwood. A total of 90 samples were analysed (3 trees x 5 discs x 2 wood types x 3 replicates).

4.2.3 Statistical analysis

The extractive results were analysed using REML analysis (Patterson and Thompson, 1971) in the statistical software Genstat (Payne et al., 2008). The analysis model considered height, wood type and the height x wood type interaction as the fixed effects to be tested by sequentially adding these terms to the model. The variation between each level of the nested strata tree, disc, wood type and replicate were included as random effects, for which variance components were estimated.

In the analysis model heights are compared across all wood types and wood types are compared across all heights. Furthermore, the height x wood type interaction allows comparison between heights for each wood type and vice versa.

It is recognised that the use of simple, unweighted averages in these cases ignores the different quantities of heartwood and sapwood in each disc and the

variation in their ratio with height. This approach was considered to be more appropriate when the aim was to examine the extractives in heartwood and sapwood as the end result of two different physiological processes. Weighted averages would have been more appropriate had the aim been, for example, to determine whether height affected extractives content of logs for pulping where heartwood and sapwood are mixed. Preliminary examination of the data showed that any effect of height was smaller when weighted averages were used.

4.3 Results

4.3.1 Comparison between heartwood and sapwood at different heights

4.3.1.1 Gravimetric analysis

The Figure 4-5 represents the distribution of extractives as a function of height for heartwood and sapwood. Each of the three trees is shown separately.

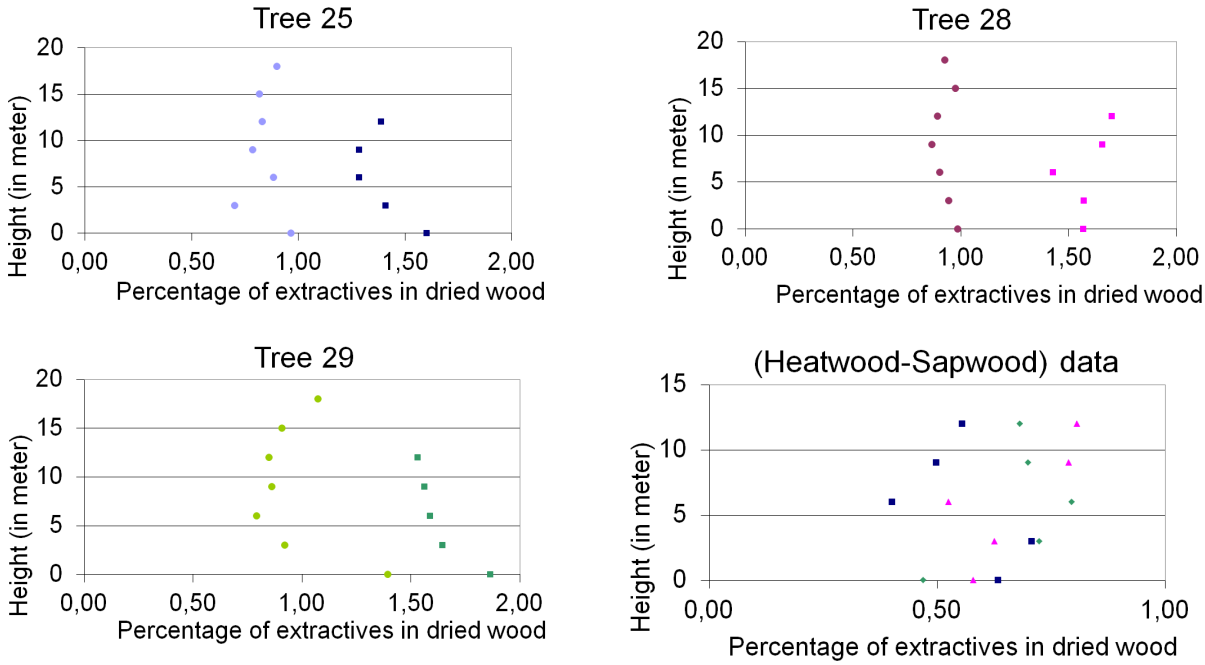


Figure 4-5: Percentage of extractives in heartwood and sapwood depending of the height of the sample in the trunk for each tree.

Figure 1-5 shows the extractives content of the heartwood was consistently greater than that of sapwood, with some variation from tree to tree in the effect of height. In the REML variance components analysis, the fixed effects of

height and wood type were tested by sequentially adding terms to the fixed model (Table 4-1).

Sequentially adding terms to fixed model		
Fixed term	F	P
Height	3.44	0.064
Wood type	340.5	<0.001
Height x wood type	0.62	0.656

Table 4-1: Tests for fixed effects in REML analysis for gravimetric extractives content.

The REML analysis confirms that the difference in gravimetric extractives content between heartwood and sapwood is significant and the absence of a significant height x wood type interaction term shows that the relationship between the two wood types does not change significantly with height. This is visually evident in the plot of differences (heartwood – sapwood) in Figure 4-5 which shows no evident pattern with height. The REML analysis gave a LSD (SL = 0.05) of 0.1759 for the heartwood/sapwood averages at different heights. The average value at the base of the trees exceeds that at some of the intermediate heights by more than this LSD (Table 4-2) but, in the absence of a significant effect of height in Table 4-1, this cannot be regarded as significant. The mean percentages of extractives in dry heartwood and sapwood depending on height are summarised in Table 4-2.

	Mean percentages of extractives in dry wood	
Height of the sample in the trunk (in meter)	Heartwood	Sapwood
0	1.6775	1.1163
3	1.5420	0.8558
6	1.4336	0.8595
9	1.5012	0.8386
12	1.5401	0.8583
15		0.9019
18		0.9673

Table 4-2: Means of extractives (% of dry mass) in Sitka spruce for heartwood and sapwood depending on the height of the sample

4.3.1.1.1 Discussion on gravimetric analysis

From the gravimetric analysis, it was concluded that Sitka spruce heartwood from the Kershope experiment contained more extractives than sapwood and that the difference was consistent for all heights in the trunk. Elevated extractives levels in heartwood have been noted and discussed in many other species (Fengel and Wegener, 1984; Hillis, 1987 and Baeza and Freer, 2001).

There was considerable variation between the three trees in the pattern with height, especially in the extent to which the extractives content rose at the base of the trunk where other data such as microfibril angle (McLean, 2008) suggest an influence of rootwood. The variance in the data is dominated by tree to tree variation and with hindsight, more replicate trees would have been desirable.

From the point of view of the paper industry, where extractives lead to processing problems and heartwood and sapwood are not separated, this experiment does not suggest that problems would arise from the use of the upper part of the trunk when the lower part is converted to sawn timber. The extractives content throughout was much lower than in alternative species such as Scots pine.

4.3.1.2 FTIR analysis

According to the method described in Chapter 3.7, spectral areas at chosen wavelengths were calculated. Figure 4-6 illustrates the FTIR spectra of heartwood and sapwood samples from the same tree at the same height. The wavelengths chosen are characteristic of vibrational modes dominated by stretching vibrations of specific structural features of the extractive molecules. The first area calculated is between 2930 and 2850 cm^{-1}, corresponding to the CH and CH_2 stretching modes of methylene and methyl groups. At 1698cm^{-1} is the carbonyl stretching mode from the carboxylic function COOR in ester groups, and at 1510cm^{-1} the benzene ring deformation mode of aromatic compounds.

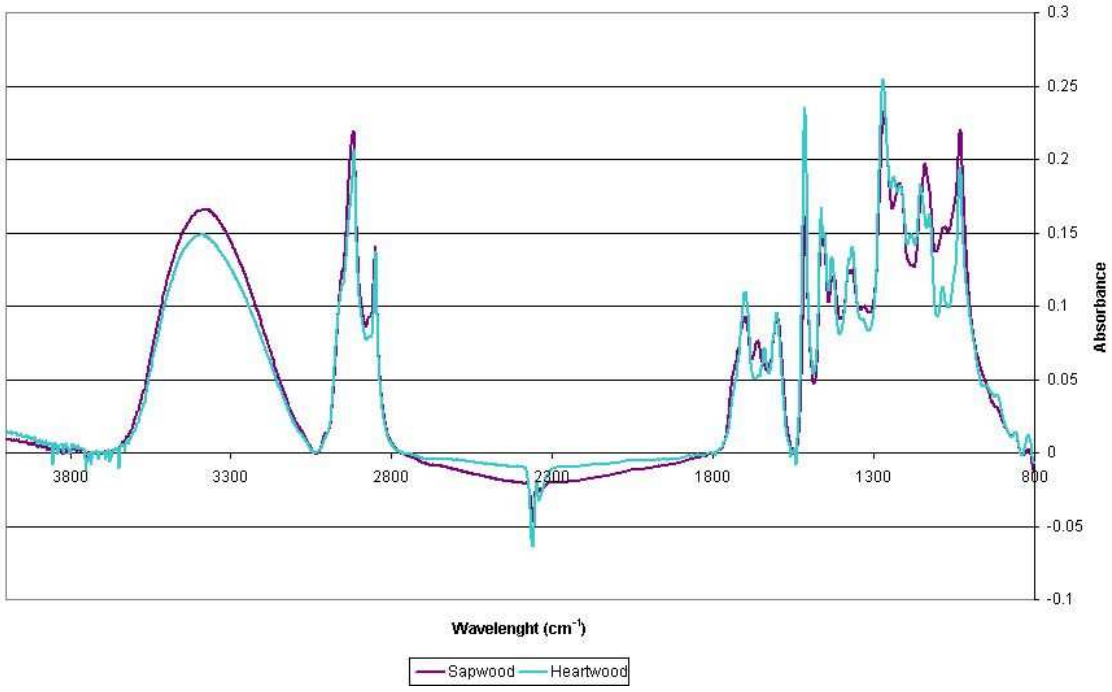


Figure 4-6: FTIR spectra baseline corrected of sapwood and heartwood acetone extracts from the same Sitka spruce tree at the equal height

A fully quantitative study leading to absolute mass of extractives classes was not possible using FTIR in reflectance mode because the mass of sample in the beam could not be controlled, and because any structural feature may be present in a range of molecules differing in molecular mass. Instead the ratio between the peak areas corresponding to methylene and methyl groups and the peak areas corresponding to the two other functional groups identified, namely the carboxylic function and benzene rings, were calculated. These ratios were used to study the variation in extractive composition in heartwood and sapwood depending on the height of the sample within the trunk.

For each type of wood at every height, 5 spectra were taken, then the peak area ratios were calculated and the results were statistically tested by ANOVA and REML analyses. The statistical analyses were performed for each ratio separately.

4.3.1.2.1 Ratio (COOR/CH)

For each height and each type of wood, 3 replicates per sample were analysed, and from each replicate 5 spectra were taken, so 15 values were obtained for each of the 9 samples. Figure 4-7 shows the variation in the ratio (COOR/CH) depending on the height of the sample for heartwood and sapwood.

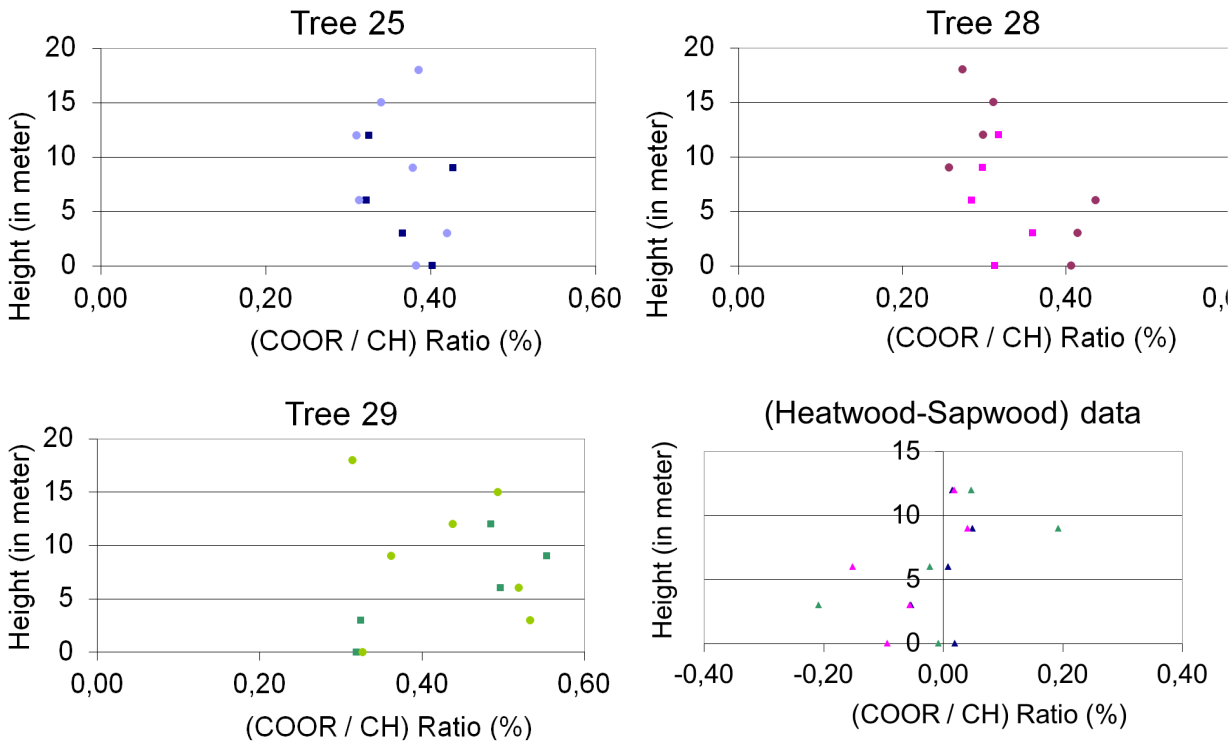


Figure 4-7: Ratio (COOR/CH) from FTIR in heartwood and sapwood acetone extracts depending of the height of the sample for each tree

Figure 4-7 shows that the ratio (COOH/CH) does not differ markedly between heartwood and sapwood nor with height.

The ANOVA analysis and the differences between the mean ratios for each height for the heartwood and sapwood were calculated and compared to the corresponding least significant difference (as explained previously for the gravimetric analysis). The results are summarised in Table 4-3 and Table 4-4.

Sequentially adding terms to fixed model		
Fixed term	F	P
Height	0.31	0.862
Wood type	0.53	0.483
Height x wood type	3.40	0.053

Table 4-3: Tests for fixed effects in REML analysis for Ratio (COOR/CH).

	Mean of the (COOR/CH) ratio	
Height of the sample in the trunk (in meter)	Heartwood	Sapwood
0	0.3445	0.3719
3	0.3499	0.4561
6	0.3678	0.4230
9	0.4265	0.3326
12	0.3759	0.3490
15		0.3817
18		0.3247

Table 4-4: Means of the ratio (COOR/CH) in Sitka spruce in heartwood and sapwood depending on the height of the sample

According the p-values obtained after the ANOVA analysis (Table 4-3), neither the type of wood or the height of the samples lead to a significant difference between the (COOR/CH) ratio values.

Even though the p-value describing the interaction between the wood type and height is close to the 5% threshold, the interaction is not statistically significant and we cannot conclude that the interaction between the type of wood and the sample heights has an impact on the (COOR/CH) ratio.

4.3.1.2.2 Ratio (Ring/CH)

The same statistical analyses were performed for the ratio (Ring/CH).

Figure 4-8 shows a trend where the heartwood and sapwood sample compositions differ.

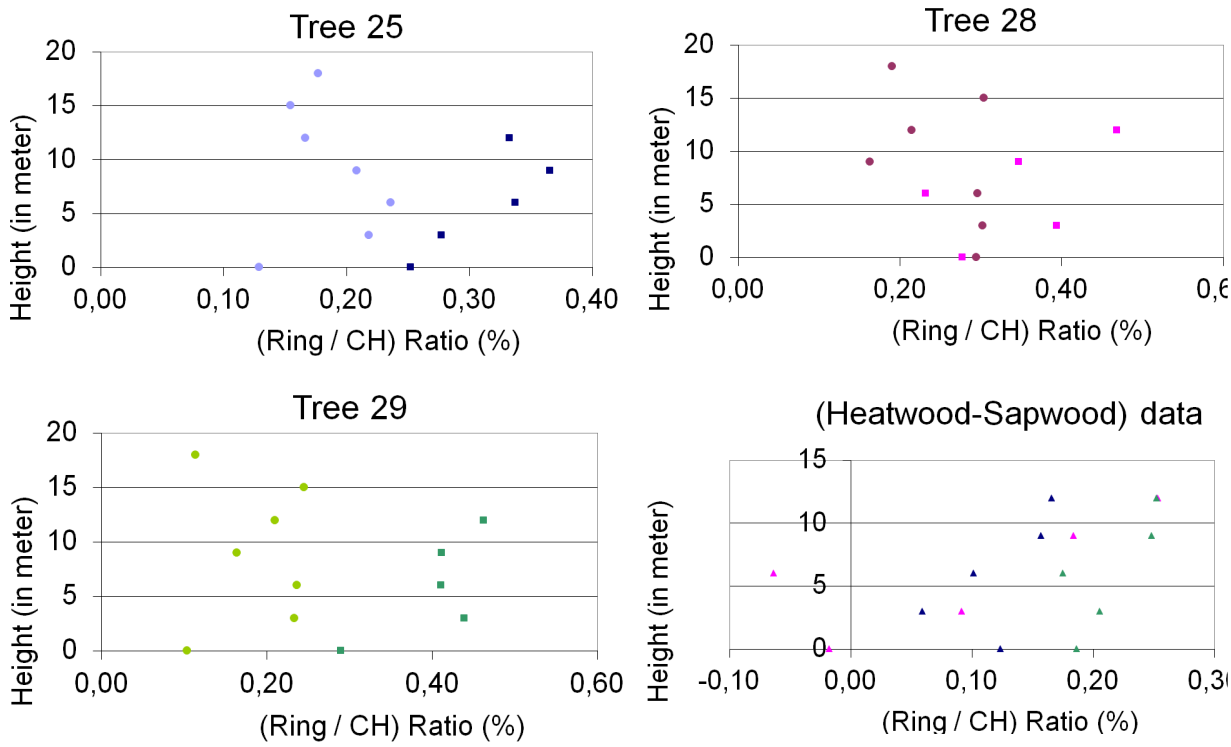


Figure 4-8: Ratio (Ring/CH) from FTIR in heartwood and sapwood acetone extracts depending of the height of the sample for each tree

The analysis of variance was performed on ratios at different heights, separately for the heartwood and the sapwood. The calculated differences between ratios were compared to the corresponding least significant different values. The results are summarised Table 4-6 and Table 4-6.

Sequentially adding terms to fixed model		
Fixed term	F	P
Height	2.19	0.111
Wood type	43.75	<0.001
Height x wood type	1.89	0.157

Table 4-5: Tests for fixed effects in REML analysis for Ratio (Ring/CH).

	Mean of the (Ring/CH) ratio	
Height of the sample in the trunk (in meter)	Heartwood	Sapwood
0	0.2728	0.1753
3	0.3699	0.2511
6	0.3268	0.2559
9	0.3746	0.1781
12	0.4210	0.1970
15		0.2350
18		0.1578

Table 4-6: Means of the ratio (Ring/CH) in Sitka spruce in heartwood and sapwood acetone extracts depending on the height of the sample

If we look at the overall comparison between heartwood and sapwood excluding the influence of the height, Table 4-5 shows a p-value smaller than the 5% threshold, so clear differences in the (Ring/CH) ratio values between heartwood and sapwood are inferred. The (Ring/CH) ratio is greater in heartwood than in sapwood.

This difference of composition is confirmed by the REML analysis. Comparing heartwood with sapwood at each height with respect to the ratio (Ring/CH), it may be noted that four values out of five are greater than the Least significant difference (LSD= 0.0710 for SL=0.05). Heartwood and sapwood thus differ significantly in composition at 0, 3, 9 and 12 meters.

4.3.1.2.3 Conclusions from the FTIR analysis

For the (COOR/CH) ratio, the statistical analysis does not reveal any significant differences between the ratio values, which means that the proportion of esters within the aliphatic fraction stays constant with height and no difference is evident between heartwood and sapwood.

The (Ring/CH) ratio represents the proportion of aromatic compounds relative to the aliphatic fraction. There are differences between heartwood and sapwood. The (Ring/CH) ratio is bigger in the heartwood than in the sapwood, which means that the heartwood contains a greater amount of aromatic compounds relative to the aliphatic fraction. Thus the tendency for heartwood to contain more extractives is particularly evident for the aromatic extractives fraction.

The composition of aromatic compounds should be further investigated because the phenolics are often related to the compounds provided antioxidant properties as in other species (in Norway spruce for example) (Umezawa, 2001).

4.3.2 Comparison between heartwood, sapwood and knotwood

As previously mentioned it was not possible to analyse the knotwood extractives as a function of height. However one sample per tree was collected and the total extractive content and the FTIR ratios were calculated. The results were compared to the averages for the heartwood and sapwood samples from the Kershope experiment. The results are summarised in Table 4-7.

The total extractive content for the knotwood is about ten times larger than the extractive content of heartwood and sapwood.

	Mean extractive content in dry wood (in %)	Mean (COOR/CH) ratio	Mean (Ring/CH) ratio
Heartwood	1.54	0.37	0.35
Sapwood	0.91	0.39	0.21
Knotwood	10.68	0.03	0.40

Table 4-7: Results from the heartwood, sapwood and knotwood comparison

The (COOR/CH) ratio illustrates the relative composition of esters within the aliphatic group. The knotwood ratio is about ten times smaller than the heartwood and sapwood ratios.

The (Ring/CH) ratio represents the proportion of aromatic to aliphatic groups. A statistical analysis was performed on the (Ring/CH) ratios, a REML with variation between heights as a random effect and the fixed effect being the comparison of the wood source (heartwood versus sapwood versus knotwood). The least significant difference calculated is 0.08. It transpires that the knotwood (Ring/CH) ratio is greater than in the heartwood and sapwood samples, but the difference is significant only for the sapwood sample. The heartwood ratio is also significantly higher than the sapwood ratio.

The knotwood has a much higher extractive content than the heartwood and sapwood samples, but contains very little ester based on the (COOR/CH) ratios.

Relative to the aliphatic groups, knotwood has a significantly higher proportion of aromatic extractives than sapwood.

4.3.3 Heartwood/sapwood distribution results

From the disc images measured (method described in chapter 2.5), the heartwood/sapwood ratios were calculated in terms of radius and of cross-sectional area. The results are presented in Figure 4-9 and Figure 4-10.

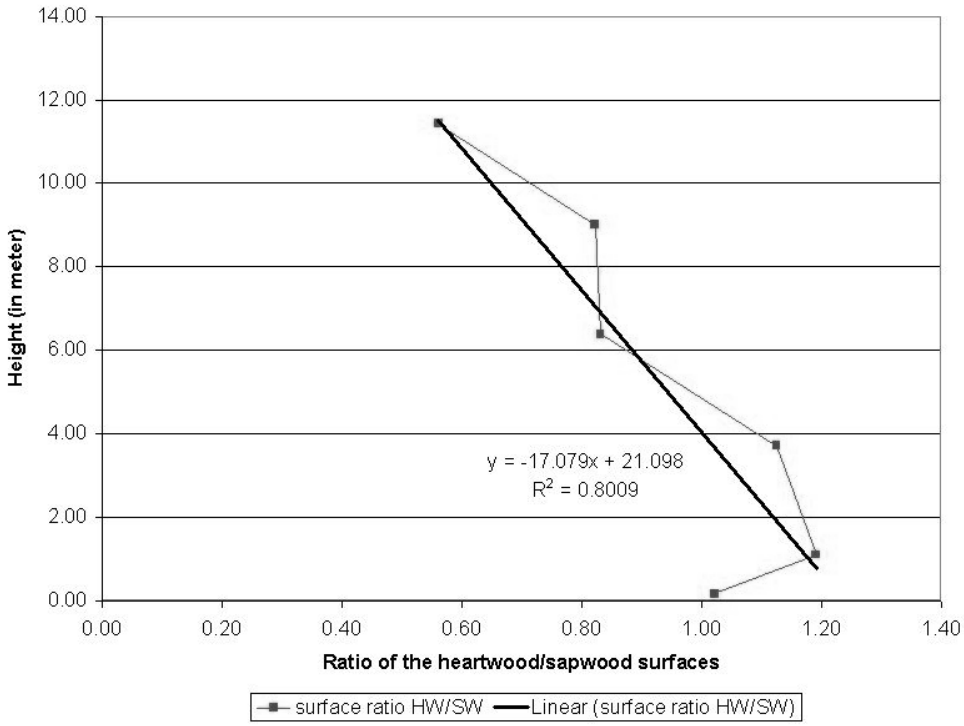


Figure 4-9: Ratio of the heartwood/sapwood areas from Sitka spruce from Kershope forest

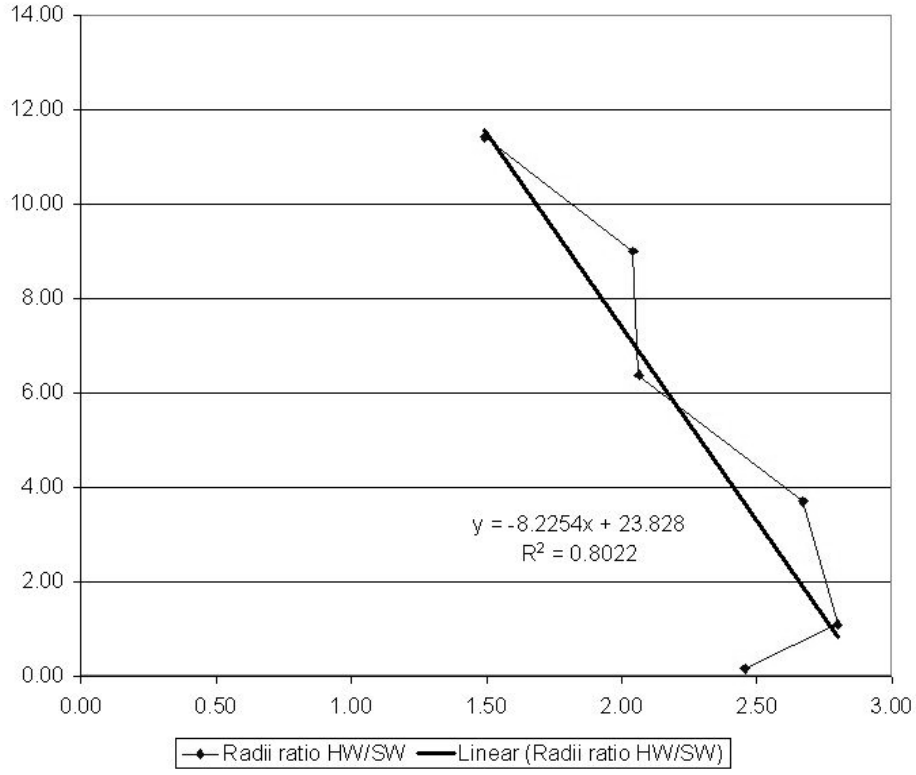


Figure 4-10: Ratio of the heartwood/sapwood radii from Sitka spruce from Kershope forest

From Figure 4-9 and Figure 4-10, it is evident that the heartwood/sapwood ratio, expressed as either radius or area, generally decreases upwards within the tree. This trend is reversed where the trunk expands into the roots.

4.4 Discussion

It was established that the total extractive content differed between heartwood and sapwood. In the height study, it was confirmed that this difference was consistent throughout the range of heights examined in the trunk.

Philip, (1995) found in Scots pine and Sitka spruce that the distribution of extractives varied with height and across the diameter of the tree with a larger concentration of extractives at the boundary between heartwood and sapwood. The variation may be caused by natural oxidation, biological detoxification of heartwood extractives or continued polymerisation of extractive material (Philip, 1995).

From the FTIR analysis in reflectance mode, two ratios were calculated, representing the relative ester content within the aliphatic compounds

((COOR/CH) ratio) and the aromatic compounds relative to the aliphatic compounds ((Ring/CH) ratio).

In the overall comparison between heartwood and sapwood samples, relative to the aliphatic fraction no difference was observed in ester content but the aromatic content was significantly higher in heartwood. Previously the gravimetric study demonstrated greater total extractives content in heartwood than in sapwood. The experiment as a whole shows that the elevated total extractive content of heartwood is due principally to aromatic compounds.

The 'aliphatic' class of organic compounds includes all compounds composed of carbon and hydrogen that are not aromatic compounds (Anonymous, 2000). The functional definition for our experiments is slightly different in that all C-H bonds contributed to the 'aliphatic' content of the extractives whatever the nature of the rest of the molecule of which the CH, CH_2 or CH_3 groups formed a part. With this definition the aliphatic compounds comprise the largest chemical class present in the extractives.

If we made the assumption that the majority of extractives come from the aliphatic compounds, we can calculate a relative amount of aromatic and esters at any location within the tree by multiplying the total content of extractives by respectively the (Ring/CH) ratio and the (COOR/CH) ratio established through the FTIR analyses. The results are presented in Figure 4-11 and Figure 4-12.

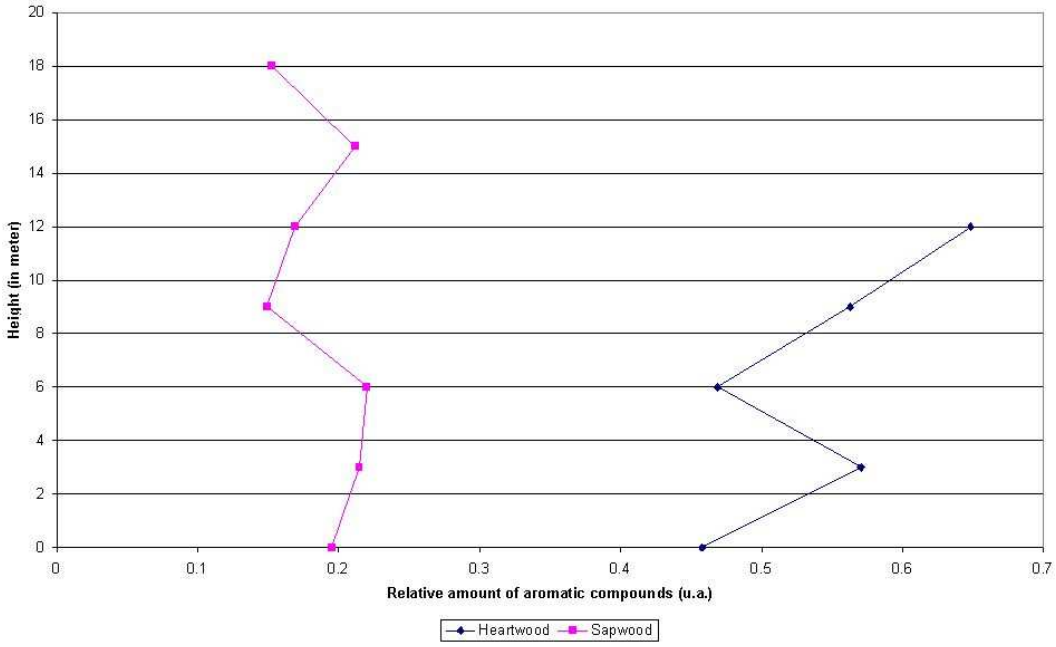


Figure 4-11: The relative amount of aromatic compounds for the heartwood and sapwood sample for the different heights

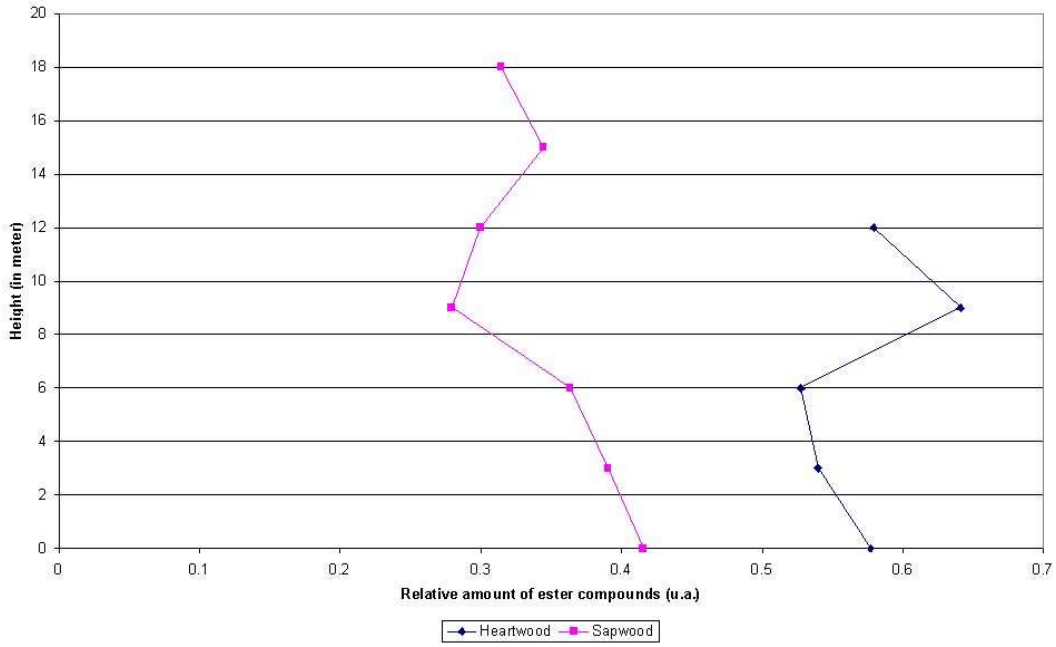


Figure 4-12: The relative amount of ester compounds for the heartwood and sapwood sample for the different heights

Heartwood formation involves the formation of extractives (Philip, 1995; Scheffer and Cowling, 1996), but the significance of this process for the distribution of extractives between heartwood and sapwood remains unclear (Andrews and Siccana, 1995). The distribution of extractives between heartwood and sapwood does not change when the tree is felled (Philip, 1995).

The data in Figure 4-11 and Figure 4-12, are consistent with the idea that the aromatic compounds are generated during heartwood formation and contribute to the difference in extractive content between the heartwood and the sapwood in Sitka spruce.

Chapter 5: Benchmarking

5.1 Introduction and aims

This section deals with the 'Benchmarking' experiment undertaken by Edinburgh Napier University. The aim of this benchmarking study was to learn about the influence of yield class, elevation, North/East location and thinning on the extractives content and composition of Sitka spruce across Scotland. This experiment was part of a wider study on the effect of environment and forest management on selected mechanical wood properties of Sitka spruce. The study provided the benefit of a wide geographical sample area and detailed knowledge of the trees studied.

The aim of the benchmarking study was to learn about the influence of yield class, elevation, North/East location and thinning on the extractives content and composition of Sitka spruce across Scotland.

5.2 Methods and procedures

5.2.1 Wood samples from the Benchmarking experiment

The benchmarking experiment is a national study of the influence of forest management and geographical criteria on physical properties of Sitka spruce timber. For this study, sawdust was collected during the coring of trees from 64 sites all around Scotland and northern England (Figure 5-1).

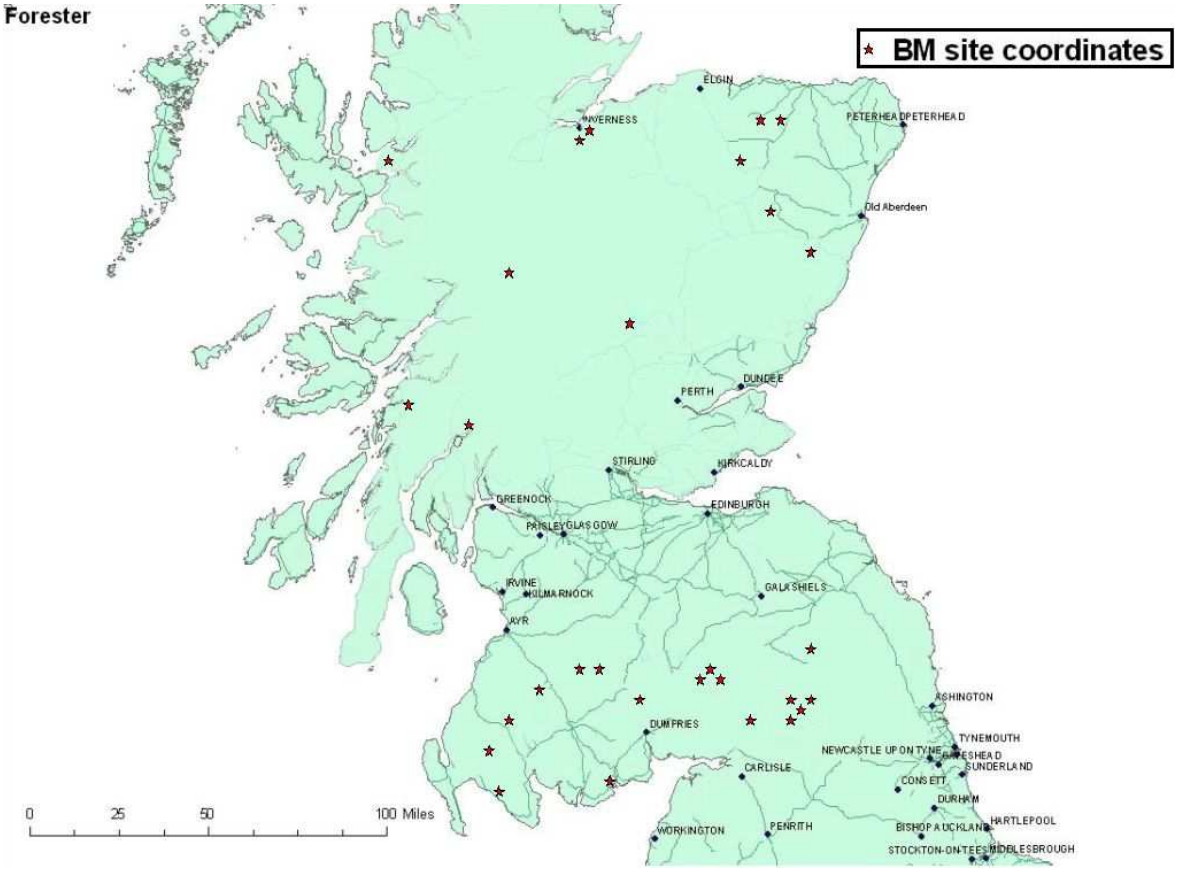


Figure 5-1: Location of the sites of the Benchmarking experiment

The benchmarking experiment is part of a wider study on the effect of environment and management on selected wood properties of Sitka spruce (collaboration between Edinburgh Napier University, Forest Research and the University of Glasgow, Moore et al., in review).

The trial was designed according to the following criteria:

- 2 Yield classes (<14, >14)
- 2 silvicultural regimes (with or without thinning)
- 2 elevation classes (<280m, >280m)
- 2 initial spacing classes (<2500 tree/ha, >2500 tree/ha – this is 2*2m spacing)
- 2 latitude classes (<600km, >600km north from OS grid datum)
- 2 longitude classes (<300km, >300km east from OS grid datum)

The OS grid datum reference being NS 900 000 (equivalent to Longitude 3°43.9'W and Latitude 55°16.9'N).

The experiment is based on a fractional factorial design 2^{6-1} with 2 replicates from a study of 64 sites with 10 trees per site (640 samples). The number of samples was reduced to 96 by taking a sub sample of 6 trees per site and excluding the spacing factor (which we presumed was a factor having a minor effect on our study) (Shupe et al., 1997 and Taylor et al., 2006) as well as one replicate. These 96 samples are representative of the Sitka spruce trees currently growing at sites across Scotland and northern England.

Each factor in the design had 2 levels. It was decided to look at 16 combinations (recovering up to the two-way interactions). For each combination 6 trees from 1, 2 or 3 sites were sampled.

The sampling included trees between 35 to 45 years old, with a stem straightness score (Macdonald et al., 2002) between 2 and 4 and breast height diameter more than 20cm in order to provide enough material for extraction.

Wood material for isolation of extractives is not normally obtained from a coring operation. However coring with a hollow drill bit yields a substantial amount of sawdust from the wood surrounding each core.

We were aware of the fact that the temperature of the wood would increase during the sampling, which might influence the extractives. But each sample was taken according to the same protocol and any changes in the extractives would be similar in each sample. Therefore a comparison between the site factors is still valid. And it is acceptable because the benchmarking study offered the possibility to analyse the extractives coming from 32 sites located across Scotland and northern England. Without this opportunity it would not have been feasible to collect as many samples covering most of the Scottish geographical conditions.

Within this experiment, it was not possible to distinguish heartwood and sapwood because each core was taken from bark to bark in a North-South direction across the trunk. Therefore the samples were a mixture of heartwood and sapwood.

The experimental protocol of the benchmarking experiment made it necessary to collect the cores at breast height, normally defined as 1.3m (4.3 feet) above the forest floor on the uphill side of the tree. Breast height is traditionally the height on a tree where measurements are taken to determine parameters like growth and volume yield providing uniformity across the range of samples collected.

The orientation of the coring axis was predefined as North-South. It is common to use one direction (e.g. North facing) when making measurements for comparing treatments between trees (McLean, 2008). In general the East quadrant should be avoided; the trend in Scotland to have wind coming from Southwest leads to the formation of compression wood on the East side of the trunk which modifies most of the timber characteristics (McLean, 2008).

The cores were obtained by Leena Vihermaa and Gregory Searles using a Trecor™ wood corer attached to a hand-held, motor-driven drill. The sawdust was carefully collected from the coring operation on 6 trees with a diameter larger than 20cm to ensure that each coring operation provided enough material to constitute a sample.

The sawdust from the coring operation contained small amounts of foreign material (bark, needles, insects etc…) (Figure 5-2). Contaminants were removed using a pair of tweezers. Due to the irregular size of the sawdust particles, a grinding step was necessary to homogenise the sample. An IKA Labortechnik A10 water-cooled grinder was used, with several runs of ten seconds to avoid high temperatures until the particles passed through a 4mm sieve (Figure 5-3).

Figure 5-2: Sawdust as collected by coring

Figure 5-3: Sawdust ready to be extracted

As the sampling extended over several months, and in order to standardize the conditions for sample preservation, as soon as the sawdust was brought back from the forest to the lab it was kept frozen (-18°C) until processing and extraction.

An analysis of variance, completed with a residual maximum likelihood (REML) analysis was performed on the data in order to determine if there were significant differences between the two levels chosen for each criterion tested.

The data set is comprised of 16 combinations with 6 trees per combination. The 16 combinations are studying the variation of the 5 factors.

5.2.2Sample analysis

The sawdust samples were extracted with acetone during 9 hours (as described in chapter 2). The samples were then analysed by FTIR on reflectance mode on polished aluminium plate and the data were collected and analysed as explained in chapter 3.

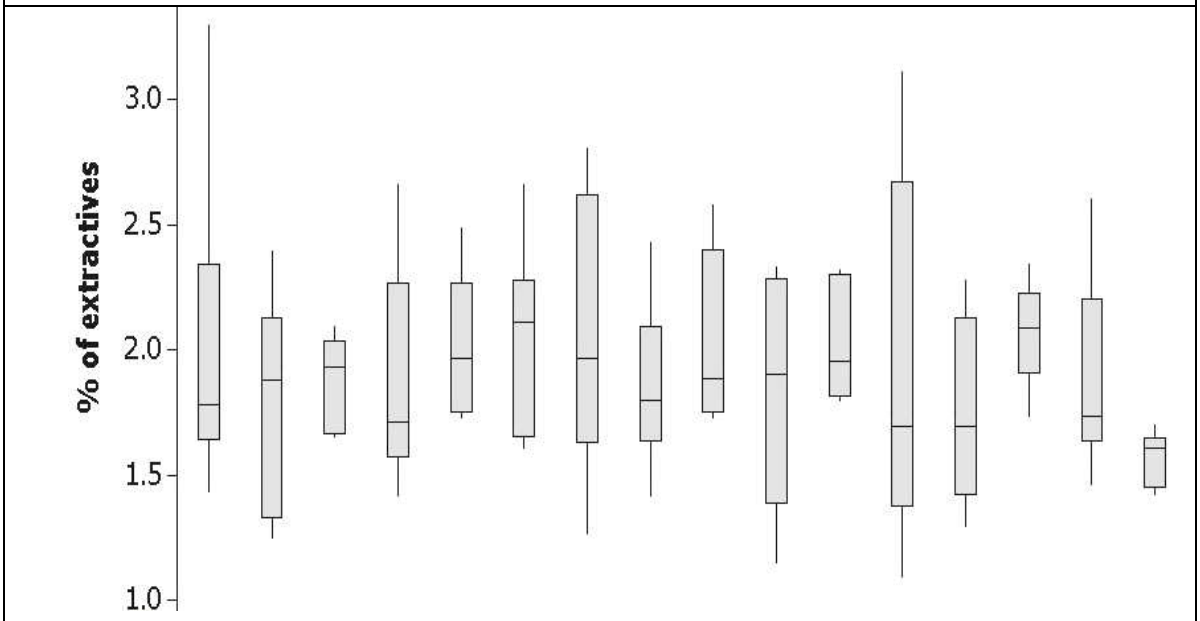
5.3 Results

5.3.1 Gravimetric results

Figure 5-4 represents the boxplot of the extractive content in acetone extract for the 16 combinations. The boxplot, as explained in

Figure 5-5 allows the representation of the range of the data collected.

Representation of the extractive content for the 16 site combinations



Combination	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
% of extractives	*2.0*	*1.8*	*1.9*	*1.9*	*2.0*	*2.0*	*2.0*	*1.8*	*2.0*	*1.8*	*2.0*	*1.9*	*1.7*	*2.0*	*1.8*	*1.6*
Yield class	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1
Elevation	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1
East	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	1	1	1
North	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	1
Thinning	-1	1	1	-1	1	-1	-1	1	1	-1	-1	1	-1	1	1	-1

	-1	***1***
Yield class	***<14***	***>14***
Elevation	***<280m***	***>280m***
East	***<300km***	***>300km east from OS Grid datum***
North	***<600km***	***>600km north from OS Grid datum***
Thinning	***Without***	***With***

Figure 5-4: Extractives content in Sitka spruce originating from 16 different site conditions

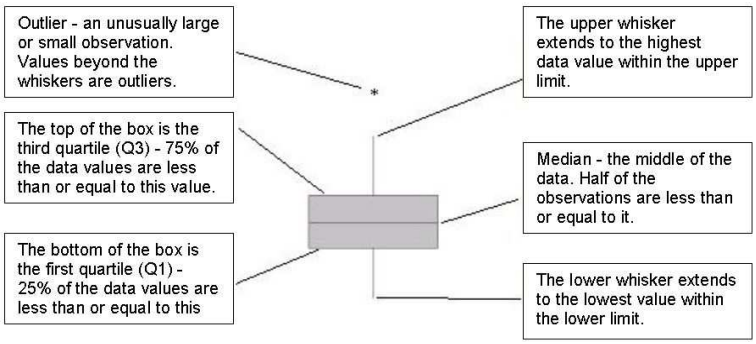


Figure 5-5: Box plot explanation (Anonymous, 1993)

The mean percentages of extractives in dry wood were calculated for both levels of every factor. The differences between these values were compared to the least significant difference (L.S.D) obtained after the REML analysis. The results are summarised in Table 5-1.

Criterion	Means of extractive content for each level for each factor tested		Least significant difference (L.S.D)
Yield class	**< 14**	**> 14**	
	1.957	1.895	0.199
Elevation	**< 280 m**	**> 280 m**	
	1.947	1.905	0.199
East	**< 300 km**	**> 300 km**	
	1.926	1.927	0.199
North	**< 600 km**	**> 600 km**	
	1.945	1.906	0.199
Thinning	**With**	**Without**	
	1.916	1.937	0.199

Table 5-1: Mean percentage of extractives in dry wood for each level of every factor studied

The differences between the means for each factor were calculated and compared to the L.S.D value to detect any significant differences between the samples.

All the values calculated are smaller than the least significant differences, which means that there is no significant difference in total extractives content between the 2 levels of each factor.

5.3.2 FTIR analysis

For each tree extract, 5 FTIR spectra were taken and baseline corrected as mentioned in Chapter 3.7 to represent the average composition of the sample (example Figure 5-6).

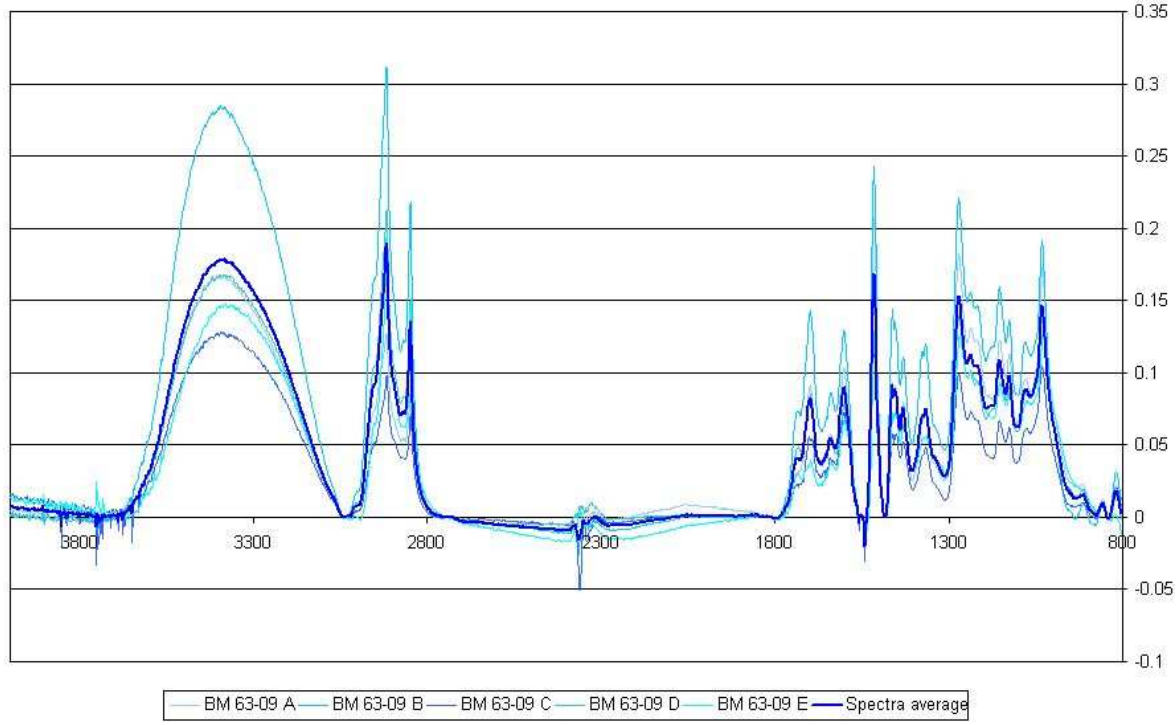


Figure 5-6: Example of a FTIR spectra represented acetone extract of tree 9 from the site 63 of the benchmarking experiment

Six trees, and thus 30 spectra, represent each of the 16 combinations. Figure 5-7 is an example of the FTIR spectra from the first combination with the peaks used to calculate the ratios highlighted.

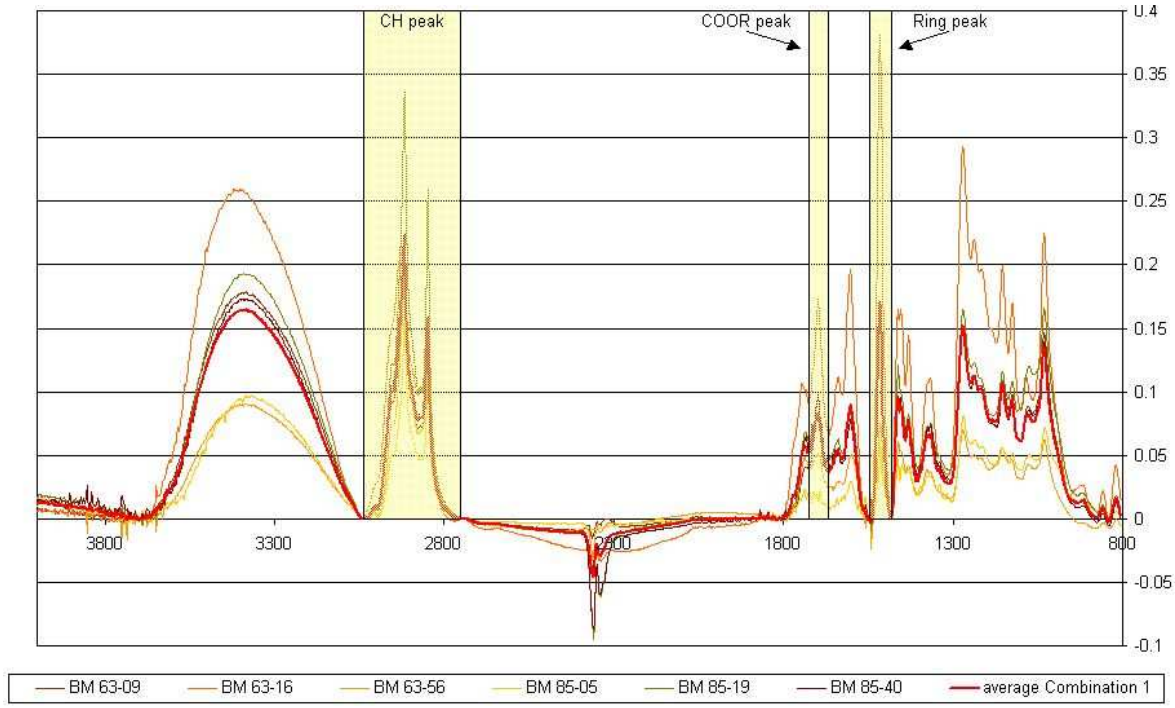


Figure 5-7: FTIR spectra represented combination 1 (with 3 trees coming from the site 63 and 3 trees coming from the site 85) with highlighted peak used for the ratio calculation

Ratios between the representative wavelengths chosen were calculated and analysed in order to determine if there was any variation in composition depending on the criteria tested.

Two ratios (ratio (COOR/CH) and ratio (Ring/CH)) were calculated and analysed by analysis of variance and REML.

5.3.2.1 Ratio COOR/CH

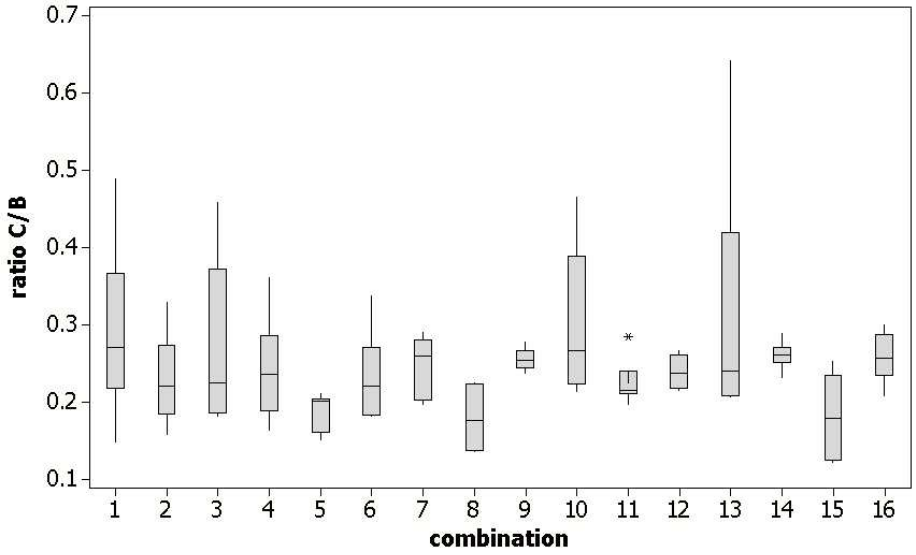


Figure 5-8:Ratio (COOR/CH) boxplot for the 16 combinations

Figure 5-8 is a boxplot of the distribution of ratio (COOR/CH) for the 16 combinations tested. It allows us to look at the distribution of the (COOR/CH) ratio for the 16 combinations. We can see that the distribution of the value is not uniform and that the spread of the data depends on the combination studied. The variance of data being large, the square roots of the data were calculated and this transformation was used to complete the analysis.

Criterion	Square root of means of the (COOR/CH) ratio for each level for each factor tested		Least significant difference (L.S.D)
Yield class	**< 14**	**> 14**	
	0.4905	0.4901	0.041
Elevation	**< 280 m**	**> 280 m**	
	0.5035	0.4762	0.041
East	**< 300 km**	**> 300 km**	
	0.5032	0.4765	0.041
North	**< 600 km**	**> 600 km**	
	0.4810	0.5002	0.041
Thinning	**With**	**Without**	
	0.5098	0.4720	0.041

Table 5-2: Means of (COOR/CH) ratio values in dry wood extracts for each level of every factor studied

Analysis of variance and REML analysis were done on the square root ratio (COOR/CH) values. A mean representative of each level of the factors studied was calculated. The data are summarised in Table 5-2.

If we compare the differences between the square roots of the ratios and the least significant difference (Table 5-2), they are all less than the L.S.D. No differences were found between the 2 levels of each factor for the ratio (COOR/CH), which means that the geographical factors (North and East location, elevation) and the silvicultural factors tested (thinning of the forest, yield class) did not significantly influence the relative amounts of ester and hydrocarbon structures in extractives in Sitka spruce in Scotland.

5.3.2.2 Ratio Ring/CH

The same analyses were performed for the second ratio (Ring/CH). Figure 5-9 illustrates the distribution of the (Ring/CH) ratio for the 16 combinations.

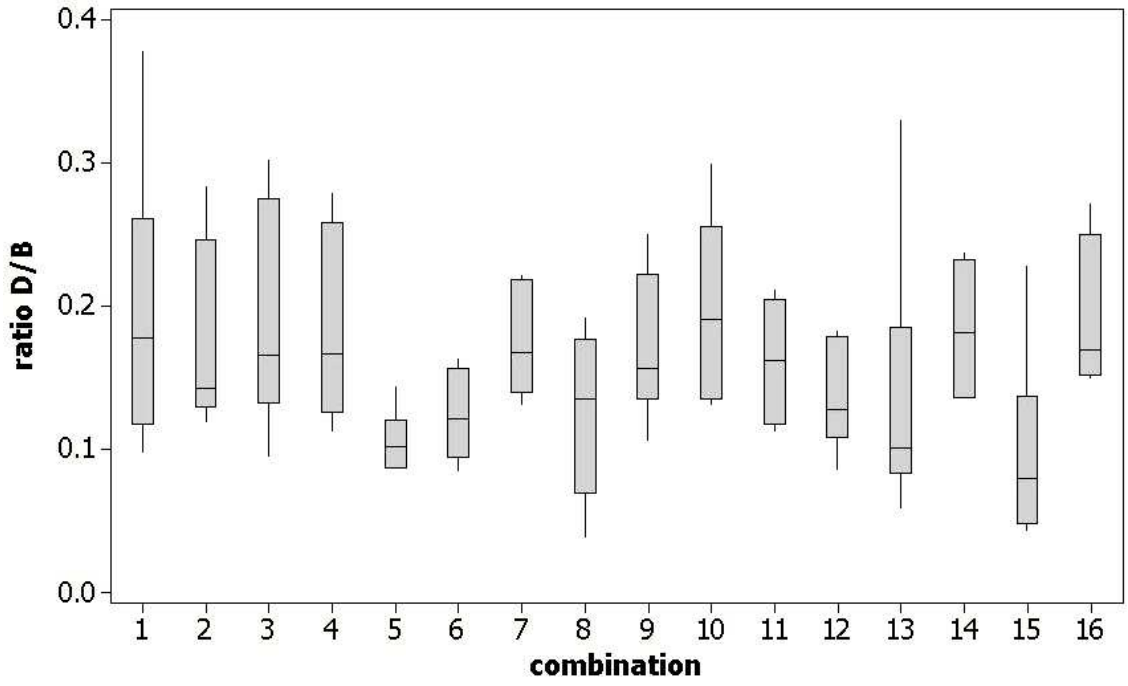


Figure 5-9: Box plot of the (Ring/CH) ratio of the 16 combinations studied

As for the (COOR/CH) ratio, the square roots of the value were calculated in order to reduce the variance in the data to complete the statistical analysis.

Criterion	Square root of means of (Ring/CH) ratio for each level for each factor tested		Least significant difference (L.S.D)
Yield class	**< 14**	**> 14**	
	0.3829	0.3998	0.044
Elevation	**< 280 m**	**> 280 m**	
	0.3936	0.3884	0.044
East	**< 300 km**	**> 300 km**	
	0.4140	0.3667	0.044
North	**< 600 km**	**> 600 km**	
	0.3920	0.3901	0.044
Thinning	**With**	**Without**	
	0.4059	0.3772	0.044

Table 5-3: Means (Ring/CH) ratios in spectra from dry wood for each level of every factor studied

Table 5-3 includes the results obtained after REML analysis. The differences between the mean values for the 2 levels for each factor were compared to the least significant difference value. No factors seem to have an influence on the ratio (Ring/CH). The factors tested do not influence the relative proportions of phenolic and aliphatic compounds in Sitka spruce in Scotland.

5.3.3 Conclusion

The total extractive content in Sitka spruce in dried wood across Scotland was shown to be low and stable. Neither the management of the forest (yield class, thinning) nor the East or North location, nor the elevation had a significant impact on the extractives content across the wide range of Scottish sites examined. Looking at the observed data on Figure 5-4, it may be remarked that the lack of significant effects within the experiment was not due to excessive variability within replicates. The stability of extractives content across Scotland (represented by the samples of 16 combinations) appeared to be genuine.

The study of the (COOR/CH) ratio reflects the ester composition within the aliphatic fraction and the (Ring/CH) ratios provides information about the composition of aromatic compounds relative to the aliphatic fraction that account for most of the extractive mass. It appears that none of the criteria studied has an influence on the relative extractive composition.

5.4 Discussion

It appeared that the total extractives content of extractives in Sitka spruce stayed stable across Scotland and did not seem to depend on the geographical and silvicultural factors set up for this experiment, as no significant differences were found in total extractive content between the factors tested. It should be noted that the trees at any one site are genetically heterogeneous and that random or genetic differences in growth rate may be amplified through dominance relationships with adjacent trees.

Reports of similar studies are sparse, and none were located on spruces. Phelps et al. (1983) explored the influence of soil composition in heartwood colour (related to the presence of some extractives) in black walnut. They came to the conclusion that there were significant differences in heartwood colour within-site, but within-tree variations were also observed. The variation between sites was considered less important than the within-site variation.

Ellagitannin extractives from young clones of European Oak grown at two sites with contrasting soil composition were studied by Mosedale et al. (1996). They emphasize the high degree of variation in heartwood ellagitannins found between individual trees, even when grown under very constant conditions (Mosedale et al., 1996). Their results show that these properties are under strong genetic control.

Spacing, thinning, regeneration method and site quality were also tested by Bjorklund (1999) on *Pinus* heartwood extractives. Much variation was found between individual trees and between stands, poorly correlated to site, stand and tree variables

Kim et al. (1989) worked on chemical variation in lodgepole pine timber with latitude (between 40^{o} to 60^{o} north latitude divided into nine sampling zones at 2.5^{o} intervals), elevation (3 altitude grades not given) and diameter class. Lodgepole pine is one of the most abundant forest resources in the Northwest United States and Canada. Kim et al. (1989) found only small, non-significant chemical variations in extractive content (after ethanol-toluene extraction) despite a large number of samples (279 trees). The variation observed had no impact in the utilisation of lodgepole pine in the timber industry.

We can conclude that as no significant variation in Sitka spruce extractive content was found despite the consistent sampling protocol (same height in the tree, same orientation in the trunk), the geographical or silvicultural criteria tested could not have affected Sitka spruce extractives.

However as previously mentioned heartwood contains more extractives than sapwood. As the ratio between the heartwood and sapwood area (presented in chapter 3) varies more sharply with height than the variation with height in either heartwood or sapwood extractive content, it can be deduced that the total extractive content will decrease with the increasing height of the trunk. Therefore when the extractive content was measured during the Benchmarking experiment from only one height (breast height), the results constitute an overestimate of the real extractive content present in the tree.

Further work would be needed to estimate the variation in the volume of heartwood and sapwood along the trunk as well as the variation in total extractive content in the same tree. This would show whether variation in extractive content develops in the same way as the distribution of heartwood and sapwood volume.

Chapter 6: Study of the five components

6.1 Introduction and aims

In this part, several other samples coming from different parts of the tree were analysed by a wider range of methods. In particular, gas chromatography added details of the individual identification of extractives and also direct quantification of some compounds. These analyses allow the comparison between the results obtained by FTIR spectroscopy and gas chromatography.

The chromatographic study was carried out in collaboration with the author in the laboratory of Dr José C. del Río at the Instituto de Recursos Naturales Agrobiologia de Sevilla (IRNAS), during a short term scientific mission financed by the European Cooperation in the field of Scientific and Technical Research (COST) action E41: "Analytical tools with applications for wood and pulping chemistry".

There were two aims in this study. The first was the measurement of the extractive composition of five samples (bark, rootwood, knotwood, heartwood and sapwood) of Sitka spruce by GC/MS.

The second aim of the study was to establish a comparison between the two analytical techniques used, GC/MS and FTIR spectroscopy. It was suspected that due to the chemical nature of the extractives, the multiple separation steps involved in the GC/MS protocol and the nature of the GC technique itself some extractives might be non-analysed or lost, whereas the direct analysis of the acetone extract by FTIR spectroscopy should measure the totality of extractives collected after acetone extraction. The GC/MS analysis allows the identification of each compound individually, whereas the FTIR only detects vibrational modes of specific functional groups or larger structural features, which are then assigned to extractive classes. This made it possible, in approximate terms, to quantify the extractive fraction actually analysed by GC/MS, making the assumption that the FTIR spectra were derived from all components of the extract. The GC/MS and FTIR analyses were both carried out on one extract of each type of tissue. So the comparison of the two analytical techniques is direct but the comparison of the five kinds of wood is only valid for the samples

analysed and constitutes a preliminary study. A larger number of samples of each material would be needed to confirm the FTIR semi-quantitative analysis and extend the results to Sitka spruce in general.

6.2 Methods and procedures

6.2.1 Wood samples

The comparative study was done on several types of Sitka spruce samples grown at a range of British locations: heartwood and sapwood coming from the grounds of the Forestry Commission Northern Research Station, Penicuik, Lothian; knotwood from Kershope forest, Northumbria; roots and bark from the forest of Ae, Galloway. Collection of these samples from different locations was unfortunately necessary due to time constraints on fieldwork when arrangements were being made for the COST collaboration (for the gas chromatography study) that made the analysis possible.

Rectangular segments (4 by 10 cm) of bark were peeled from the tree using a knife. Several pieces of roots from an uprooted tree were collected. The material was processed in the lab by cutting it into smaller pieces and subsequent milling using an IKA Labortechnik A10 water-cooled grinder, with several runs of ten seconds until the particles passed through a 4mm sieve.

The heartwood, sapwood and knotwood samples were processed as in Chapter 4.

The samples were kept in a freezer at –20°C until further processing.

The samples were first extracted with acetone for 9h, then the extract was concentrated to around 1/10 of the initial extract volume to be analysed by FTIR spectroscopy. One part of each acetone sample was evaporated to dryness and re-diluted into chloroform to be analysed by gas chromatography and gas chromatography coupled with mass spectroscopy.

6.2.2 Gas Chromatography (GC) method

The acetone extracts were dried and redissolved in chloroform. This separated the compounds into two fractions according to their polarity.

The acetone extracts contained both polar and non-polar compounds. The non-polar lipophilic fraction in the chloroform fraction was analysed by GC. The polar fraction, insoluble in chloroform, was not analysed by GC.

Before injection into the GC the sample was filtered through glass wool in order to remove insoluble material and washed with excess of chloroform. The purified sample was dried and redissolved in chloroform to standardise the amount injected in the GC.

The GC analyses of the extracts were performed using a Hewlett-Packard HP-5890 GC (Hewlett-Packard, Hoofddorp, Netherlands) with a short fused-silica capillary column (DB-5HT; 5m x 0.25mm i.d., 0.1μm film thickness) from J&W Scientific (Folsom, CA, USA). The temperature program started at 100ºC with a 1 min hold and then was raised to the final temperature of 350ºC at 150ºC/min were it was held for 3 min. The injector (split-splitless) and detector (flame ionization detector, FID) temperatures were set at 300ºC and 350ºC, respectively. The carrier gas was helium at a rate of 2 mL/min and the injection was performed in splitless mode. Peaks were quantified by GC/FID peak area.

The GC/MS analysis was performed using a Varian Saturn 2000 gas chromatograph (Varian, Walnut Creek, CA, USA) equipped with a fused-silica capillary column (DB-5HT, J&W; 12mx0.25mm i.d., 0.1 mm film thickness) and an ion trap detector. The oven was heated from 120ºC (1 min) to 380ºC at 10ºC/min and held for 5 min. The transfer line was kept at 300ºC. The injector was temperature programmed from 120ºC (0.1 min) to 380ºC at a rate of 200ºC/min and held until the end of the analysis. Helium was used as carrier gas at a rate of 2 mL/min. Samples were silylated using with Bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) with pyridine and heated for 1 hour at 70ºC to produce the appropriate derivatives (Rencoret et al., 2007).

6.2.3FTIR Data collection method

FTIR spectroscopy records spectral contributions from all extractives. Not all of the signals of the individual extractive compounds are sufficiently well resolved within the spectrum to be measurable. Furthermore FTIR spectroscopy does not

allow direct quantification of individual compounds in the same way as chromatographic analysis. A correlation between the classes of extractives identified with GC/MS and the extractive groups identified by FTIR spectroscopy needed to be established. No precedent for this approach was found in the literature on extractives.

To allow the comparison between GC and FTIR spectroscopy, relative FTIR response factors for each functional group in a small number of standard compounds were measured. The following two approximations were assumed:

- Each FTIR band used is derived from a vibrational mode associated exclusively with one functional group.

- The intensity of each vibrational band is unaffected by the environment of the molecule.

The first approximation is reasonable for vibrational modes used to quantify the aliphatic and the aromatic groups. Because hydrocarbon chains comprise a large part of the molecules compared to the other functional groups, the C-H stretching vibrations of CH_2 and CH_3 groups represent a considerable proportion of the mass of the entire molecule. Because of the large imbalance in mass between carbons and hydrogen atoms, the C-H stretching modes are not strongly coupled to vibrations elsewhere in the molecule (although there is a strong coupling between the stretching vibrations of the two C-H bonds in a CH_2 group). For the aromatic ring vibration, although the 1510cm^{-1} peak chosen belongs to the fingerprint region of the FTIR spectrum, this peak is very specific for the aromatic molecules and has been used previously for the quantification of lignin (Owen and Pawlak, 1989; Machado et al., 1996 and Silva et al., 1999).

The second assumption is more doubtful as the intensity of ester carbonyl stretching modes is known to be affected by hydrogen bonding. More precisely, hydrogen bonding leads to simultaneous changes in frequency and intensity. Smith and Hartley (1983) state that the C=O peak (at 1695cm^{-1}) is at lower frequency than usual (1717-1730cm^{-1}) for the ester C=O stretching mode of ferulates due to hydrogen bonding. This matches the band position already known for the esters of resin acids. Holgrem et al. (1999) and Nuopponen et al. (2003) state that the band at 1697cm^{-1} is characteristic of the resin acids of the

resin canals in Norway spruce. In this study the carboxylic C=O stretching band was always located around 1697cm^{-1} and no peaks were detected in the usual 1717-1730cm^{-1} region. The lack of significant variation in frequency suggests that the intensity of this band is also relatively constant.

Standard compounds were analysed by transmission FTIR spectroscopy using KBr discs. The technique was more time consuming but allowed quantification (absorbance units per mg of standard in each 12 mm KBr disc) whereas only relative data can be obtained by reflectance method. Each of the standards contains a functional group characteristic of an extractive class (Table 6-1).

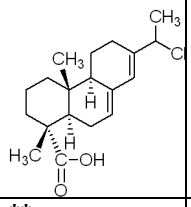
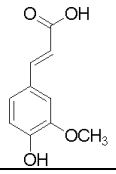
Wavelength	**Extractive class**	**Standard molecule**	**Response factor** (FTIR area of functional group (a.u.) /mg of functional group/ KBr disc)
3037-2780 cm^{-1}	Aliphatic group	Abietic acid * * 	48.1
1800-1622 cm^{-1}	Carboxyl group	Ferulic acid ** ** 	160.3
1544-1485 cm^{-1}	Phenolic group	Ferulic acid **	33.0

Table 6-1: Standard molecules used for the determination of the response factor for the FTIR analysis

6.2.4FTIR data processing for the comparative study

A response factor was calculated from the absorbance integrated across each wavelength band for each standard spectrum. The response factors allowed the correlation between the absorbance for each functional group, deduced from the FTIR spectrum, and the relative mass of the functional group within the molecule. The response factor was used later to quantify functional groups in the acetone extracts.

The acetone extracts were also analysed by FTIR spectroscopy using KBr pellets. The spectra were baseline corrected and areas assigned to vibrational modes associated with specific functional groups (aliphatic, carboxylic and phenolic

groups) were calculated as explained above. Each area was then corrected by the relevant response factor, the volume incorporated in the KBr pellet, the volume of the acetone extract and the weight of dry wood. These corrections allowed the calculation of the content of each functional group in the extractives from the corresponding mass of dry wood.

6.2.5 GC and GC/MS data processing

Retention times and mass spectra were used to identify extractive compounds in gas chromatography coupled with mass spectrometry. Reference mass spectra were derived from the NIST library as well as a previous thesis (Ekman, 1980b) and publications on the subject (Ekman and Sjoholm, 1979; Ekman, 1980a; Bannwart et al., 1989; Meagher et al., 1999; Willfor et al., 2003b; Willfor et al., 2003c; DelRio et al., 2004; Willfor et al., 2004a; Willfor et al., 2004b; and Willfor et al., 2005c and Rencoret et al., 2007). Additionally standard compounds were used when it was possible. The peak area of each component in the total ion current chromatograms was measured using MS Spectrometry Workstation Version 6.9. The molecules identified were grouped by classes of extractives (Ekman, 1980b):

- Fatty acids
- Resin acids
- Lignans and sterols
- Steroid ketones
- Esters
- Triglycerides

The peak areas (A) for all compounds within each class of extracts were added (S), and the sum represents the total abundance of the extractive group (Class 1) (example Table 6-2).

Standard compounds were used in order to calculate a response factor for each group of extractives.

The concentration of each extractive class was determined by the product of the combined peak area (S) of the extractives in the class corrected by the response factor and the dilution factor.

The weight of the extractives belonging to the class in question was then divided by the dried weight of wood in order to obtain the percentage of the extractive class in dried wood.

Extractive classes	**Compounds**	**GC/MS Peak area**	**Sum of area**
Class 1	X	A	S= A + B + C
	Y	B	
	Z	C	
Example: For the resin acid group (Sapwood sample)	Pimaric acid	38459427	368573645
	Isopimaric acid	67046000	
	Dehydroabietic acid	172836218	
	Abietic acid	90232000	

Table 6-2: Example of presentation of GC/MS data

6.3 Results

6.3.1 Gravimetric results

The percentage of extractives in the dry mass determined for each sample is illustrated in Figure 6-1.

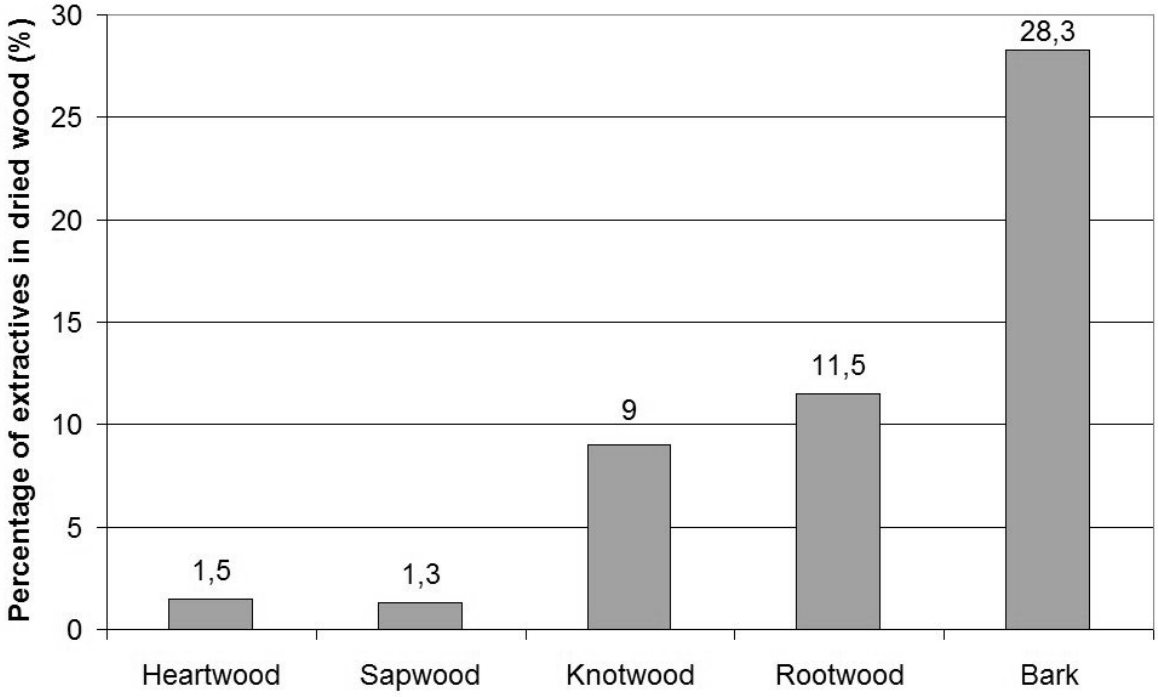


Figure 6-1: Extractive content in different tissues of Sitka spruce (in % of dried mass)

The bark contained the largest total amount of extractives. Knotwood and the rootwood samples contained about one-third of the amount of extractives detected in the bark. Heartwood and sapwood were the parts of the tree where the total extractive content was lowest.

The heartwood and sapwood extractives contents were similar to those found in the Kershope experiment, while the root and bark samples proved to differ so radically in extractives content that the influence of the different sample site locations is unlikely to be important, given the low level of variation from site to site found in the benchmarking experiment (Chapter 5).

6.3.2 Fourier transformed infrared spectroscopy (FTIR)

The 5 samples were analysed by FTIR spectroscopy with KBr discs. As explained in chapter 3, ratios between selected peak areas were calculated in order to obtain information on the quantity of esters and aromatic compounds relative to the aliphatic compounds, which account for the largest part of the extractives. The results are summarised in Figure 6-2.

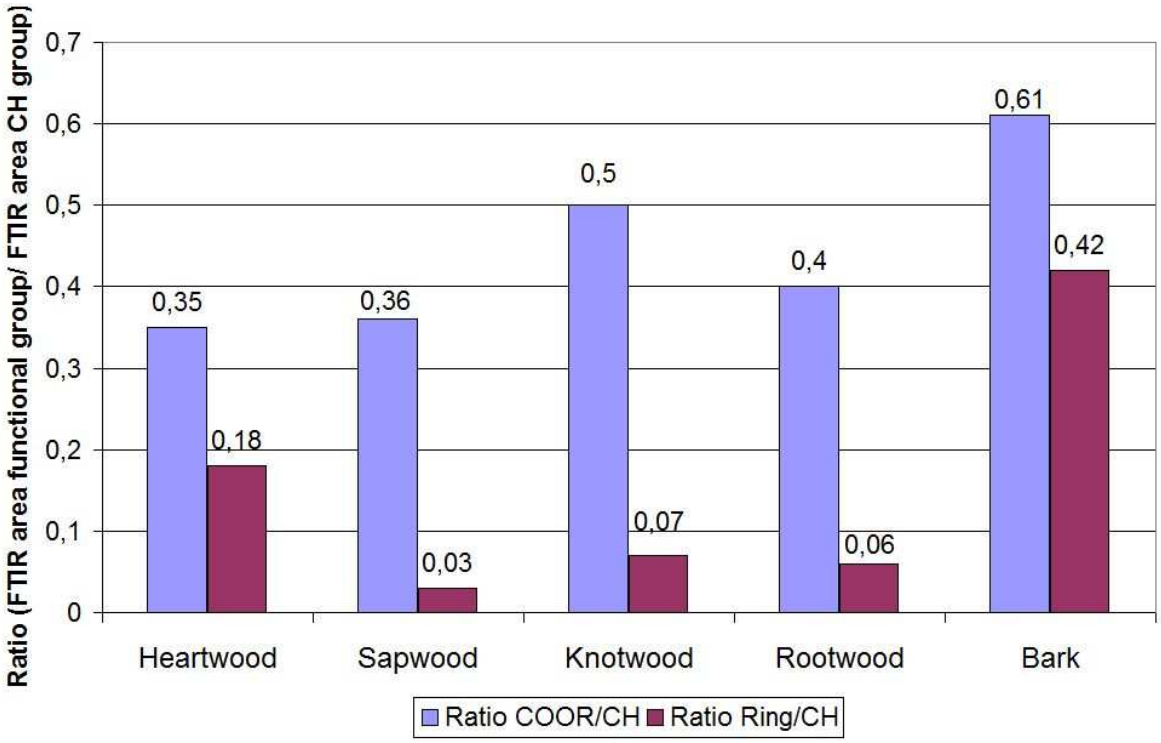


Figure 6-2: FTIR ratios of the five samples

Figure 6-2 illustrates the ratios between the intensities of the peaks, obtained for each of the samples after FTIR analyses. The (COOR/CH) and the (Ring/CH) ratios illustrate respectively the content of esters and aromatic compounds relative to the aliphatic fraction.

For the five samples, the global variation of the (COOR/CH) ratio was small (less than a factor of two) compared to what was observed for the (Ring/CH) ratio. The (Ring/CH) ratio was considerably higher in heartwood than in sapwood, knotwood or root wood, and higher still by a factor of two in bark. The total extractives content was much higher in the bark than in the other four samples (Figure 6-1). The higher (Ring/CH) ratio implies that the bark had a much higher content of phenolics.

6.3.3 Gas chromatography analysis

6.3.3.1 Qualitative analysis

Four different types of wood (knotwood, heartwood, sapwood, roots) and one bark sample were analysed by GC and GC/MS.

	Peak number	Knotwood	Roots wood	Heartwood	Sapwood	Bark
α-linolenic acid	**1**		S			
palmitic acid	**2**			X	X	
levopimaric acid	**3**	X		X		X
pimaric acid	**4**	S	S	S	S	S
isopimaric acid	**5**	X/S	S	S	S	S
dehydroabietic acid	**6**	X/S	X/S	X/S	X/S	X/S
abietic acid	**7**	X/S	S	X/S	X/S	X/S
eicosanoic acid	**8**		S			
palustric acid	**9**			X	X	
stigmast-5-en-3 oleate	**10**					X
α-tocopherol (Vit E)	**11**					X
campesterol	**12**		X	X	X	X/S
isolariciresinol	**13**	S		S		
seicosalariciresinol	**14**	S	S	S	S	
divanillytetrahydrofuran	**15**		S			
matairesinol	**16**	S	X/S	X	X	
lariciresinol	**17**	S		S	S	
nortrachelogenin	**18**	X				
β-sitosterol	**19**	X	X/S	X/S	X/S	X/S
pinoresinol	**20**	S		S	S	
7-hydroxymatairesinol	**21**	X		X	X	
stigmast-3,5-dien-7-one	**22**			X	X	
stigmastone-3,6-dione	**23**		X	X	X	
7-oxositosterol	**24**			X	X	
cis eicosanyl ferulate	**25**			X	X	
trans eicosanyl ferulate	**26**			X	X	X
cis docosanyl ferulate	**27**			X	X	X
trans docosanyl ferulate	**28**			X	X	X
cis tetracosanyl ferulate	**29**			X	X	X
trans tetracosanyl ferulate	**30**			X	X	X
β-sitosterol acetate	**31**			X	X	
campesterol ester	**32**		X	X	X	X
sitosterol ester	**33**		X	X	X	X
triglycerides	**34**			X	X	X
X = compounds identified by GC MS without silylation						
S = compounds identified by GC MS with silylation						
X/S = compounds identified by GC MS without silylation AND with silylation						

Table 6-3: Compounds identified in Sitka spruce extracts

At least 34 compounds from different classes were identified in the samples (Table 6-3), though some of the peaks could not be identified.

α-linolenic acid was only detected in the rootwood silylated extract (Figure 6-8). No fatty acids were identified in the other samples (from Figure 6-3 to Figure 6-12). However, according to Ekman (1980b) (who used sequential extraction with petroleum ether and then acetone/water mixture 95:5 v/v and GC analyses with glass capillary columns (SE-30 or BDS) and packed glass column (1% XE-60)) several fatty acids were found in the heartwood and sapwood extracts of Norway spruce. It is possible that Soxhlet extraction with pure acetone is not the most suitable method to extract this class of compounds or that the quantity present in Sitka spruce was below the detection limit.

The composition of the resin acids family was quite similar for all five tissues. Although not all of the resin acid compounds were identified by the GC/MS analysis on non-silylated samples, silylation confirmed the results (from Figure 6-3 to Figure 6-12).

Only 2 out of 4 sterols present in Norway spruce (Ekman, 1980b) were identified in Sitka spruce extracts: sitosterol and campesterol. Campesterol was not found in knotwood (Figure 6-3 and Figure 6-4).

No diterpene alcohols and diterpene aldehydes were identified by the methods used (described in chapter 3) for this experiment. The presence of several diterpene alcohols and diterpene aldehydes has been reported in Norway spruce wood (Ekman, 1980b).

The composition of the lignan fraction varied among the 5 samples (Table 6-3). The presence of the lignans was confirmed after silylation (Figure 6-4, Figure 6-6, Figure 6-8, Figure 6-10 and Figure 6-12). All the lignans identified in Sitka spruce knotwood, heartwood and sapwood are in accordance with literature on Norway spruce extractives (Willfor et al., 2003b). Root wood contained different types of lignans (Figure 6-8). It was not possible to identify any of the lignans in the bark. The chromatogram appeared to show some form of grease contamination with several distinct repeated peaks of the sample (Figure 6-6) and the amount of sample was not sufficient to perform another analysis.

A group of esters of ferulic acid with fatty alcohols were identified in bark, sapwood and heartwood (Figure 6-5, Figure 6-9 and Figure 6-11). In the samples as analysed the feruloyl moiety was present in both cis and trans conformations. But these interconvert photochemically in free ferulate and ester ferulate solution when exposed to daylight. UV irradiation causes –trans to -cis isomerisation of hydroxycinnamic acids (Smith and Hartley, 1983). Only the even-numbered fatty alcohols (from C_{20} to C_{24}) were identified in the samples (DelRio et al., 2004). These molecules were described by Ekman and Sjoholm (1979) in Norway spruce.

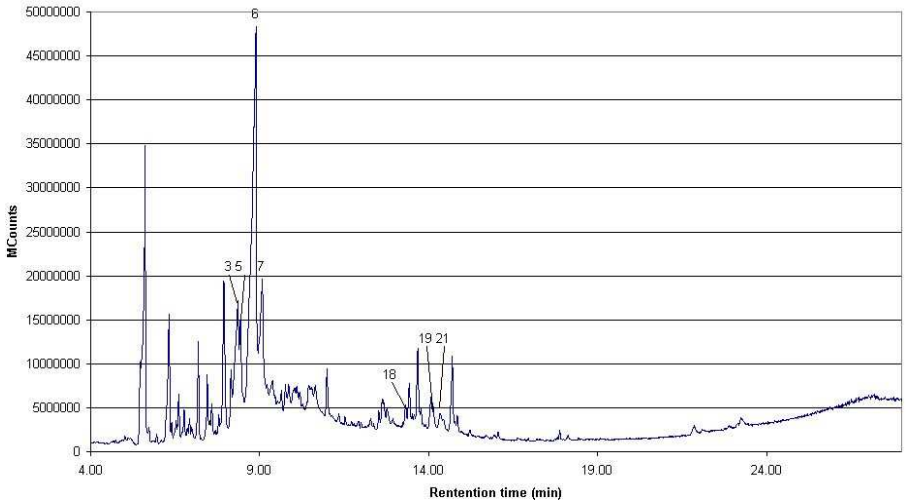


Figure 6-3: GC/MS chromatogram from knotwood sample

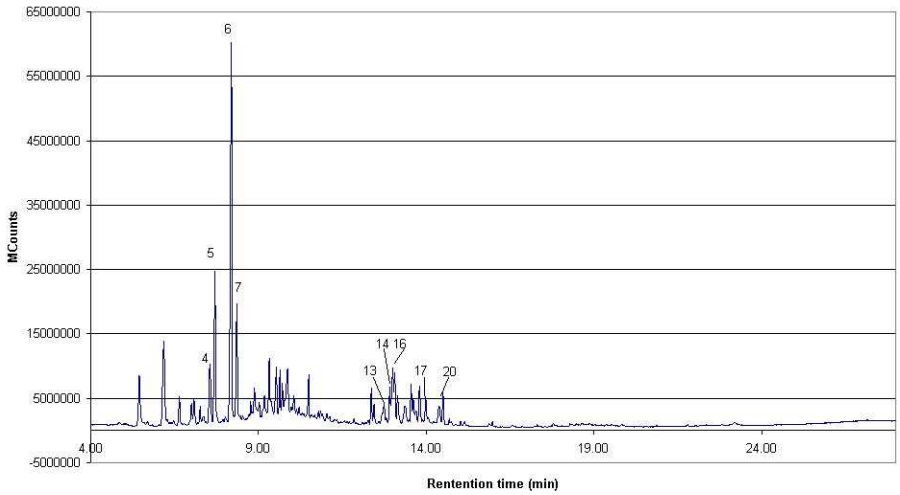


Figure 6-4: GC/MS chromatogram of silylated knotwood

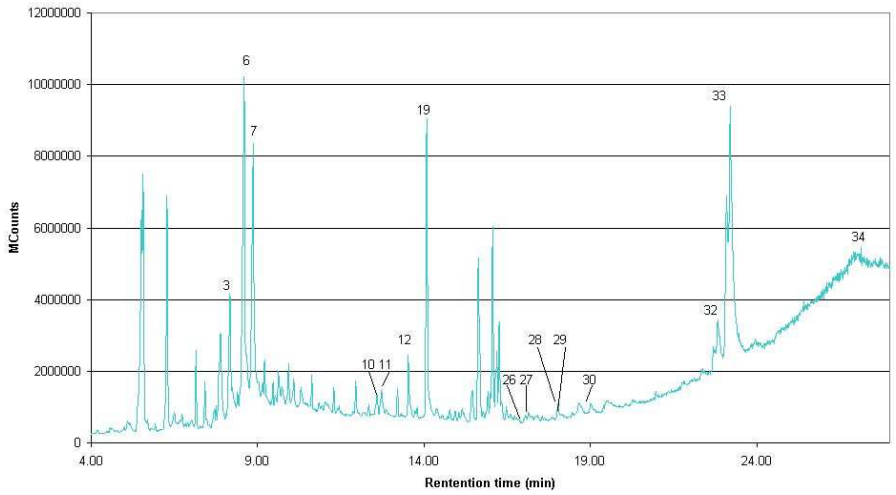


Figure 6-5: GC/MS chromatogram from bark sample

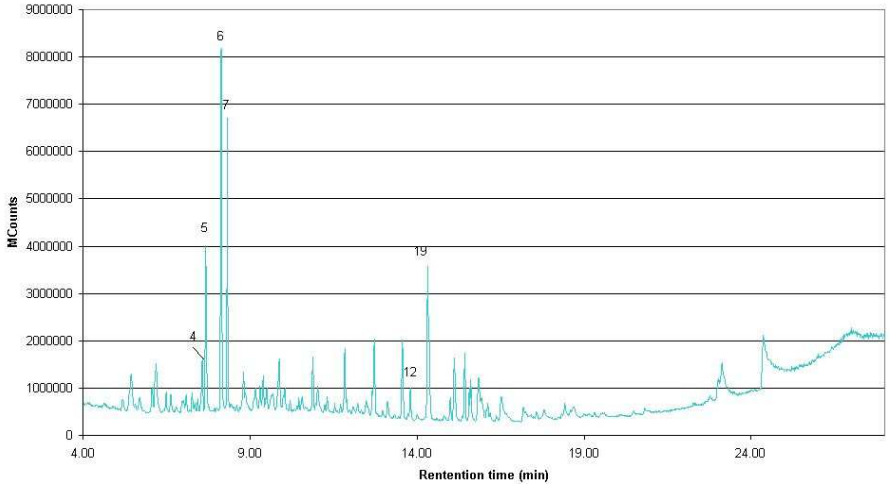


Figure 6-6: GC/MS chromatogram of silylated bark

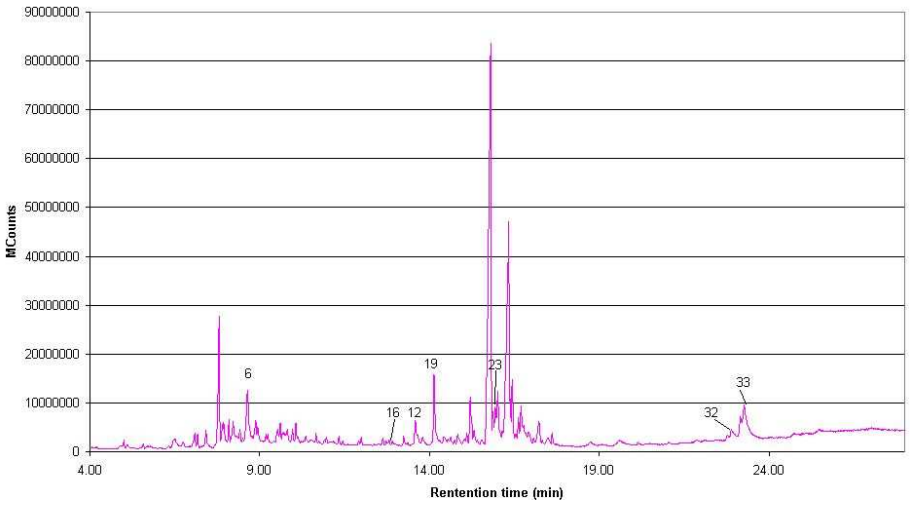


Figure 6-7: GC/MS chromatogram from rootwood sample

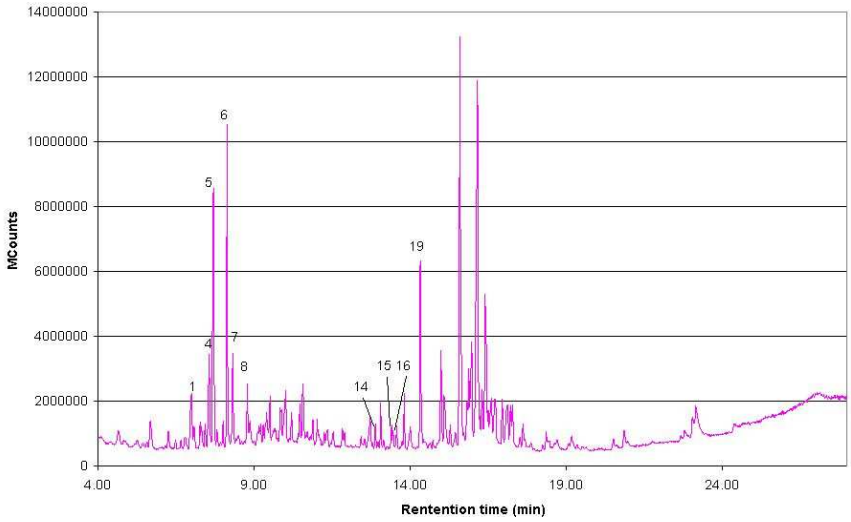


Figure 6-8: GC/MS chromatogram of silylated rootwood

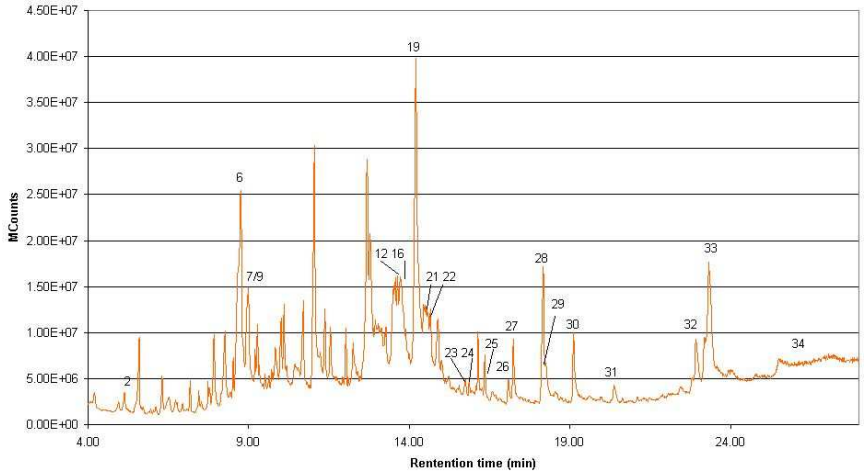


Figure 6-9: GC/MS chromatogram from sapwood sample

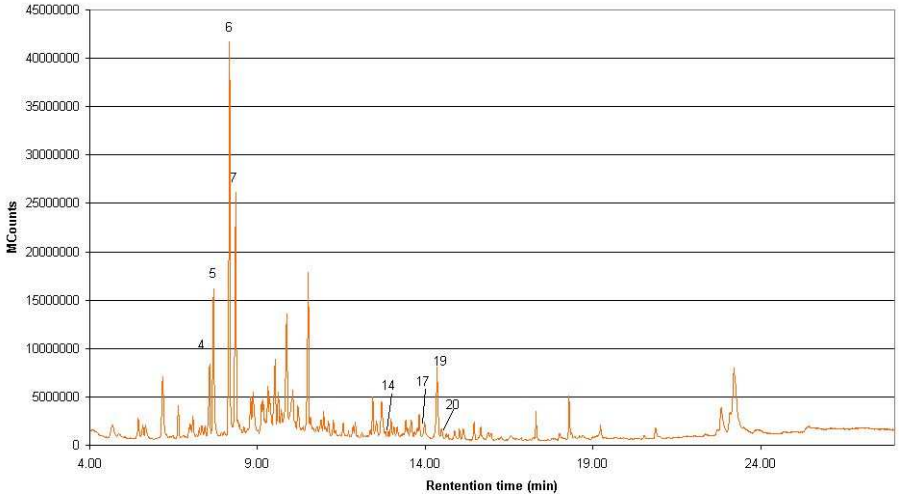


Figure 6-10: GC/MS chromatogram of silylated sapwood

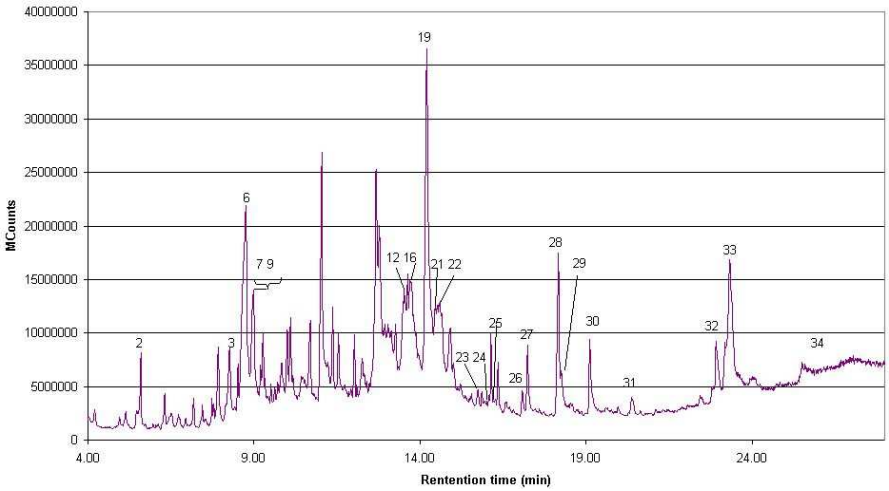


Figure 6-11: GC/MS chromatogram from heartwood sample

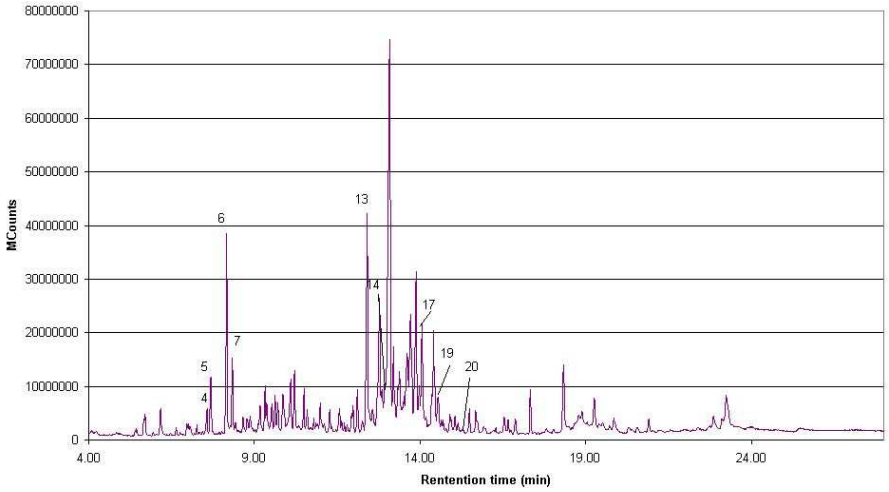


Figure 6-12: GC/MS chromatogram of silylated heartwood

6.3.3.2 Quantitative study

Quantification by classes was established, adding together the weight of individual compounds identified by GC-MS within each class for the 5 samples.

Content each class in dried mass (%)	Heartwood	Sapwood	Knotwood	Roots	Bark
Sterols	0.02%	0.01%	NI*	0.01%	0.01%
Resin acids	0.22%	0.31%	0.43%	0.11%	0.07%
Lignans and phenolic compounds	0.09%	0.02%	0.04%	0.01%	NI*
Esters	0.25%	0.23%	NI*	0.07%	0.07%
TOTAL	0.58%	0.57%	0.47%	0.18%	0.14%
** NI: non identified*					
Content of extractives in dried mass (%) from the gravimetric analysis	1.48%	1.33%	9.04%	11.53%	28.31%

Table 6-4: Summary by extractive classes identified by gas chromatography

From the 34 compounds identified by the GC/MS analysis in Table 6-3, the percentage of each class of extractives in dried material was calculated (Table 6-4). The heartwood sample contained the largest relative amount of sterols. The largest relative amounts of the resin acid compounds were identified in knotwood, sapwood and heartwood. Lignans and phenolic compounds were found in greatest relative concentration in heartwood. Sapwood and heartwood contain more esters, relative to the total extractives identified, than root and bark samples.

6.3.3.3 Comparison between gas chromatography and FTIR spectroscopy

Following the calculation method presented in chapter 3, the absolute content of identifiable functional groups in extracts of dry wood was established by FTIR in transmission mode with the extracts incorporated into KBr discs. The results are shown in Table 6-5.

The advantage of the transmission mode using KBr discs was that the volume to the concentrated extract and thus the mass of extractives mixed in the disc is known. This lead to semi-quantification, as a full calibration is not possible with

FTIR, which detects vibrational modes that vary in intensity from one individual compound to another.

Content of functional group in dried mass (%)	Heartwood	Sapwood	Knotwood	Rootwood	Bark
Aliphatic group	1.65%	1.27%	9.84%	8.68%	8.36%
Carboxylic group	0.18%	0.13%	1.50%	1.05%	1.54%
Aromatic group	1.02%	0.33%	1.02%	0.72%	5.14%
TOTAL	2.85%	1.73%	12.36%	10.45%	15.04%
Content of total extractives in dried mass (%) from the gravimetric analysis	1.48%	1.33%	9.04%	11.53%	28.31%

Table 6-5: Content of each functional group in dry wood calculated from the KBr FTIR analysis

Bearing in mind that we had to make several assumptions to complete the calculation (Chapter 6.2.3), the accuracy of the results is not expected to be 100%.

We can see that for the heartwood, sapwood, knot and the root samples the FTIR analysis allows the identification of most of the acetone extract, with a majority of the extractives coming from the aliphatic group. A higher aliphatic content is found in root, knot and bark samples.

The quantification established with FTIR seems to over-estimate the total extractive content slightly except for bark. This might come from the fact that the drying procedures differed between the gravimetric analysis and the FTIR KBr disc analysis (with rotary evaporator with a water bath at 34^{o}C for the gravimetric analysis and under room temperature for the KBr disc analysis).

The other possibility is that a part of the aliphatic compounds could represent a fraction of the ester class. Based on the chromatographic analysis, the esters identified represent 15% of the extract in heartwood and about 0.2% of the bark sample, which could contribute somewhat to the over-estimation especially for heartwood and sapwood samples.

Alternatively if the standards chosen were not fully representative of the extractives measured, the response factor of the aliphatic compounds present in

the heartwood and sapwood samples could differ from the response factor used for FTIR calibration, leading to an overestimation of the aliphatic fraction.

For the bark sample, we recovered half of the gravimetric content of extractives. The aliphatic fraction recovered represents the same percentage of the dry mass as in knotwood and rootwood. The bark sample contained the greatest relative amount of phenolic group. The largest relative amount of carboxylic groups was found in knotwood and bark samples.

These results obtained by the KBr FTIR technique (Table 6-5) may be compared to the distribution of extractives classes analysed by GC/MS (Table 6-4). We will compare the aromatic to the lignans and phenolic compounds and the carboxylate group to the resin acid and ester groups.

First, for the GC/MS, the fraction of the extract identified from the bark and rootwood samples are quite low. To explain that, from the high content of aliphatic chains and carboxylic functions, it may be suggested that these samples contain some resin or triglyceride molecules that cannot be detected by GC/MS.

We then look at phenolics and lignans identified with GC/MS and compare them with the aromatic groups detected by KBr FTIR. In GC/MS, most of the lignans and phenolic compounds are detected in the heartwood sample. With the FTIR the largest amount is detected in the bark sample and in this sample the lignans and phenolics identified by GC/MS do not account for all the aromatic compounds detected by FTIR. This might be due to the molecular mass of some compounds being too great to permit sufficient volatility for these molecules to pass through the GC/MS column.

Finally we will look at the carboxylic group (FTIR) and compare it to the resin acid and ester classes (GC/MS). The calculation for the KBr FTIR analysis is based on the formula mass of the functional group, and not on the molecular mass of the entire molecule as in GC/MS. This explained the lower percentage found in the FTIR analysis. But we can remark that most of the esters and resin acids compounds are found in heartwood and sapwood by GC/MS. A high content of carboxylic ester functions detected by FTIR is associated with low recoveries of total mass and aliphatics by GC/MS in the knot and bark samples.

Two FTIR spectra of standard molecules (abietic acid and methyl palmitate) are presented in Figure 6-13 and Figure 6-14.

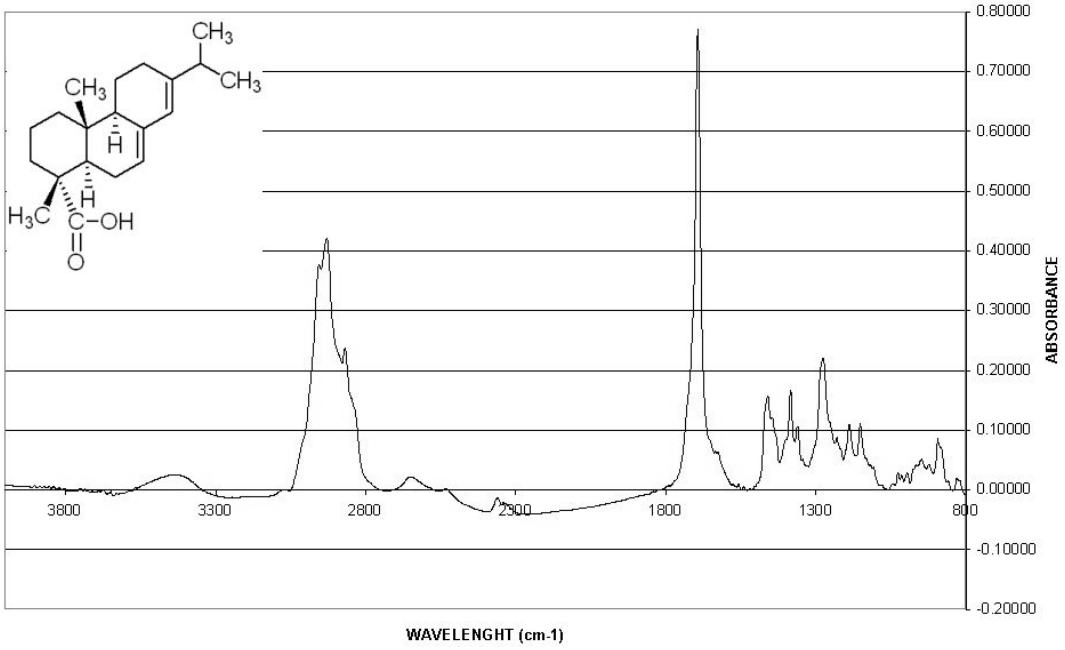


Figure 6-13: FTIR spectra of abietic acid with KBr disc

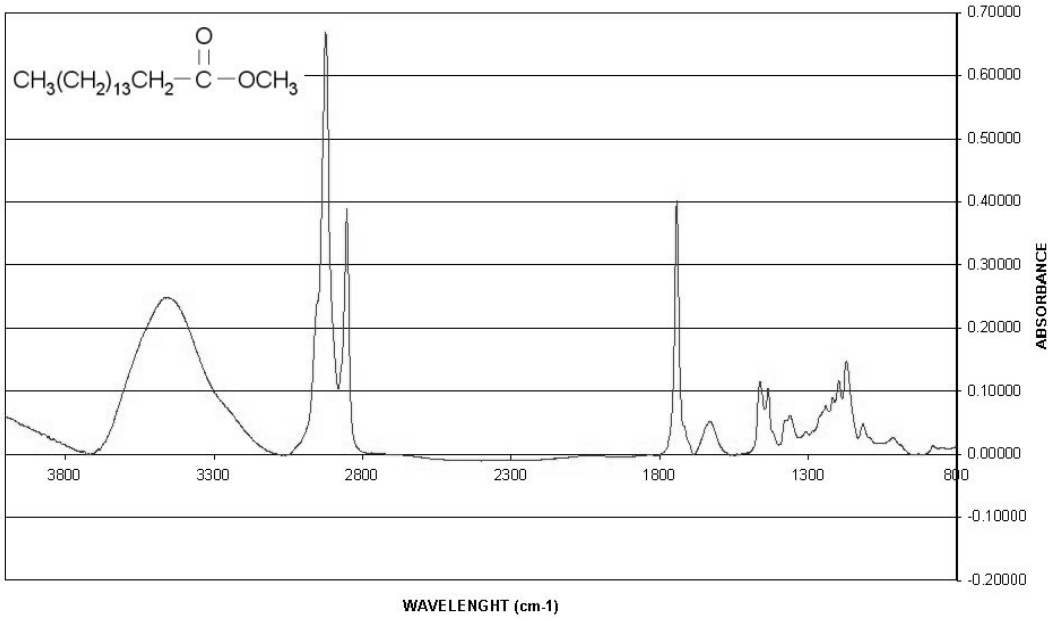


Figure 6-14: FTIR spectra of methyl palmitate with KBr disc

Abietic acid ($C_{20}H_{30}O_2$) and methyl palmitate ($C_{17}H_{34}O_2$) are close in composition, but their structures differ with a ring formation for the abietic acid and a long aliphatic chain in methyl palmitate. These differences are represented in the FTIR spectra in the 3050-2800cm^{-1} area characteristic of the C-H stretching region, with one broad peak with a shoulder for the ring formation and nearly

two separate peaks for the aliphatic structure. The shape of the FTIR spectra in that area will provide information about the nature of the aliphatic compounds that will be detected in the Sitka spruce extractives. If the aliphatic peak is present as one broad peak the presence of resin acids and their polymers will be expected. If the aliphatic fraction comes as two separated peaks triglycerides or fatty acids will be probable.

The FTIR spectra of the five samples analysed by KBr discs are shown in Figure 6-15, and the detailed spectra are Figure 6-16 to Figure 6-17.

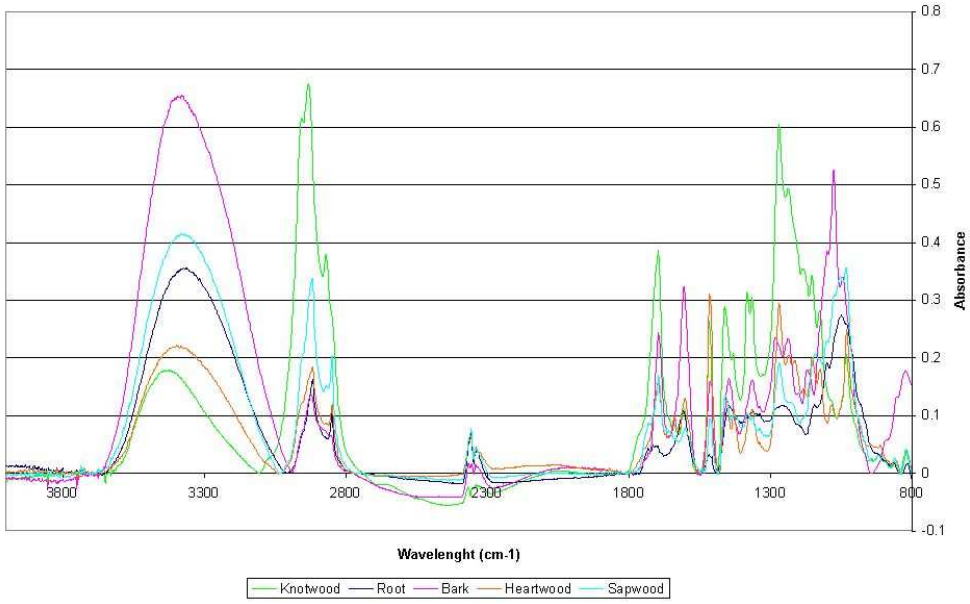


Figure 6-15: FTIR spectra in transmission mode for the 5 samples

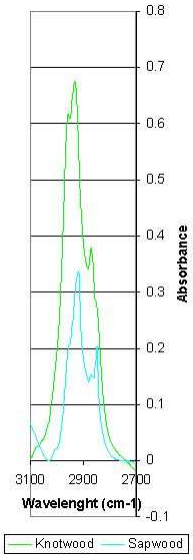


Figure 6-16: FTIR spectra of knotwood and sapwood between 3100 and 2800cm^{-1}

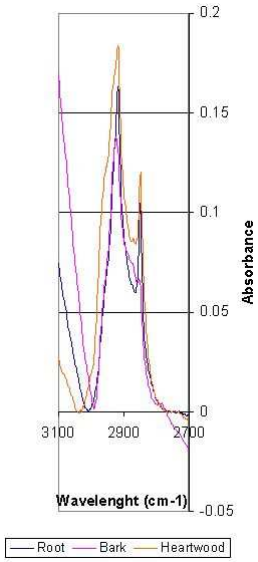


Figure 6-17: FTIR spectra of heartwood, rootwood and bark between 3100 and 2800cm^{-1}

It appears that in the rootwood FTIR spectrum, the peaks between 3100-2800cm^{-1} are clearly separated, so it seems to contain more triglycerides or fatty acids type of aliphatic group. From the knotwood and bark sample, they should contain resin acids and their polymers. For the heartwood and sapwood, the peaks obtained are intermediate, so a mixture of triglycerides and resin acids is expected.

Also the 1000-1200 cm^{-1} region of the bark and rootwood spectra suggests the presence of some carbohydrate (Figure 6-18).

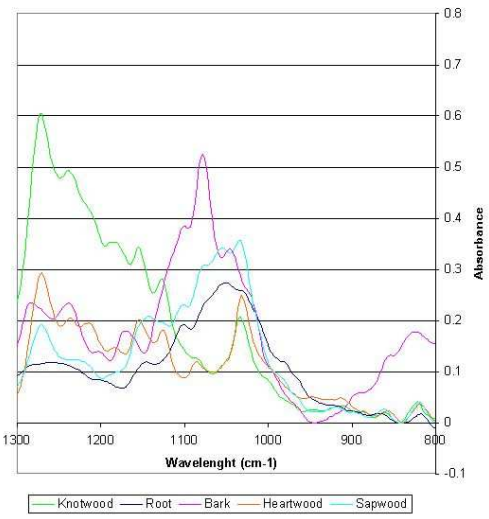


Figure 6-18: FTIR spectra of the five samples between 1300 and 800cm^{-1}

6.3.4 Conclusion

The aim of this chapter was to study the differences in extractives content between bark, rootwood, knotwood, heartwood and sapwood samples, and then compare the GC/MS and the FTIR methods to study the extractive content.

In the five samples analysed, major differences in extractive composition were found. Bark was the sample that contained the greatest amount of extractives, with about ten times the extractive content found in heartwood or sapwood.

From the FTIR, it was apparent that the (COOR/CH) ratio, related to the ester compound composition within the aliphatic fraction, remained rather constant. For the (Ring/CH) ratio, associated with the content of aromatic compounds relative to the aliphatics, the variation between the samples was greater. However the variation in total extractive content between the five samples was very much more than the variation between heartwood and sapwood samples observed in the Kershope experiment.

Based on the GC/MS analysis, the composition of the resin acid fraction was comparable for the five samples analysed. Differences were observed in lignan composition, with a large number of lignans detected in the knotwood and none detected in the bark sample. The data obtained here confimed that, as observed in the Chapter 4, heartwood had a relatively greater aromatic extractive content than sapwood and this difference was attributable largely to the lignans and other phenolic compounds. A relatively large content of ferulic esters in heartwood and sapwood samples and some in the bark were found.

The last part of the study was focused on the comparison of the GC/MS and FTIR using KBr disc methods. Although a comparison of individual compounds was not possible with FTIR, semi-quantification was established in order to compare the classes of extractives detected with both methods. Despite some over-estimation obtained by the FTIR analysis, it was still possible to obtain a recovery closer to the total extractive content than was achieved by GC/MS analysis.

6.4 Discussion

6.4.1Distribution of extractive content within the tree

6.4.1.1 Heartwood and sapwood

The heartwood and sapwood of the trunk were the parts of the tree where the total extractive content in dry wood was the lowest, with even less extractives in sapwood (1.3%) than in heartwood (1.5%) on a dried wood basis.

Ekman (1980b) extracted Norway spruce with petroleum ether and found 1% of extractives in sapwood and 0.8% in heartwood in dried wood. The petroleum ether solvent only allows the extraction of the lipophilic part of the extractives. This explains the lower extractive content found by Ekman (1980b) in Norway spruce compared to what was found here in Sitka spruce heartwood and sapwood. Acetone solvent removes both hydrophilic and lipophilic compounds.

Heartwood represents the inner layers of wood in the growing tree, which no longer contain living cells and where the reserve materials (e.g. starch) have been removed or transformed into heartwood extractives (Taylor et al., 2002).

Sapwood is defined as the part of the tree containing living cells. The role of the sapwood is to transport water from the root to the crown, and act as a water and energy reserve material for the tree. Sapwood is also the location of living cells that can respond to exterior attack or physical injuries (Hillis, 1987 and Taylor et al., 2002).

The process of heartwood formation in conifers includes two major biological phenomena in the tissue: death of the parenchyma and extractive formation (Taylor et al., 2002). Some physical changes inside the wood also take place. The moisture content decreases at the heartwood/sapwood boundary, the stored starch in sapwood disappears in heartwood and the cell wall becomes lignified (Hillis et al., 1962 and Bergstrom et al., 1999). The heartwood becomes impregnated with extractives. The extractives are formed close to the heartwood/sapwood boundary by using local available compounds (such as carbohydrates) and material translocated from the phloem and sapwood (Hillis et al., 1962; Hillis, 1987; Bergstrom et al., 1999 and Taylor et al., 2002).

The main physical differences between heartwood and sapwood are reviewed by Taylor et al. (2002). Heartwood has a darker colour, a lower permeability and greater decay resistance than the sapwood. These differences are related to the higher extractive content of the heartwood.

Although extractives contribute to the natural durability of wood, the importance of physical barriers versus the role of extractives is poorly understood (Taylor et al., 2002).

Obst (1997) summarised the evidence that heartwood contains more extractives than sapwood in conifers, which is in accordance with the results obtained for Sitka spruce.

6.4.1.2 Knotwood

Knotwood comprises the traces left by the formation of both live and dead branches on the main stem. When a branch moves, it generates tension and formation of free radicals at the branch base (Pietarinen et al., 2006). Also an open wound is formed when a branch is broken close to the stem, leaving the knot susceptible to attack by fungi. Therefore the biosynthesis of compounds with fungicidal and bactericidal properties is needed (Pietarinen et al., 2006).

Sitka spruce knotwood was found to contain about 9% of total extractives in dry wood, which is a very large amount compared to the Sitka spruce heartwood and sapwood samples.

6.4.1.3 Rootwood

The spruce root has been little studied, only in relation to fungal attack (e.g. sensitivity of roots to blue stain fungi (Evensen et al., 2000) or root rot fungi (Lindberg et al., 1992; Pan and Lundgren, 1995 and Evensen et al., 2000).

From the gravimetric analysis, it was shown that the rootwood sample had the second largest extractives content, with about one third of the bark extractive content, but ten times more than the heartwood and sapwood extractive content.

6.4.1.4 Bark

The bark constitutes a barrier between the tree and the exterior environment. As extractives are active in defence against exterior attack, a higher concentration of extractives within the bark was expected (Ralph et al., 2007).

According to the gravimetric analysis, the bark sample contained the largest total amount of extractive content with about 28% of extractives in dry wood.

6.4.2 Comparison of extractive classes according to position in the tree

A quantification of extractive classes was attempted by summing the individual components identified in the GC/MS analysis of the samples. The results are summarized in Table 6-4.

If we compare the extractive content identified by GC/MS and the gravimetric extractive content in the dry mass, there is a large difference between them. Particularly in knotwood, roots and bark the fraction identified by GC/MS is much too small to be representative of the entire extractive composition.

Ekman et al. (1979a) analysed heartwood and sapwood from a 26 year old Norway spruce by methods described in Ekman (1979b). The method consisted of a sequential extraction with petroleum ether followed by acetone/water mixture (9:1 v/v). The analyses were carried out by GC packed glass column (1% XE-60) and glass capillary columns coated with 1,4-butanediol succinate (BDS) with FID detector. The comparison of the results is presented in Table 6-6.

In order to make the comparison easier the Sitka spruce results are repeated in this and subsequent tables, along with the Norway spruce data obtained by other teams.

	Sitka spruce (From Table 6-4)		Norway spruce (Ekman et al., 1979a)	
	Heartwood	Sapwood	Heartwood	Sapwood
Content of extractives in dried mass (%) from the gravimetric analysis	1.48%	1.33%	2.33%	2.42%
Fatty acids			0.25%	0.52%
Resin acids	0.23%	0.31%	0.12%	0.16%
Diterpene alcohols			0.02%	0.03%
Esters	0.25%	0.23%		
Sterols and triterpenes alcohols	0.02%	0.01%	0.11%	0.11%
Total extractive analysed by GC/MS	0.50%	0.55%	0.50%	0.82%
Percentage of extractives analysed by GC/MS from the gravimetric content	33.78%	41.35%	21.65%	33.87%

Table 6-6: Comparison of the extractives analysed by GC/MS for Sitka spruce and Norway spruce (Ekman et al., 1979a)

The percentage of extractives analysed by GC/MS was somewhat higher for the Sitka spruce samples. A large part of the extractive analysed by GC/MS in Norway spruce comes from the quantity of fatty acids, none of which were detected in Sitka spruce.

The differences between the two measurements of extractives content may come from the differences in method. Petroleum ether is a less polar solvent than acetone and will allow a better extraction of the non-polar compounds such as triglycerides. Ekman (1979b) then used methylation by diazomethane to detect the free fatty acids and saponification by KOH solution in 90% ethanol to identify the esters of fatty acids. Fatty acids from triglycerides account for a large part of the difference in total extractives between Sitka and Norway spruce (for both heartwood and sapwood). The differences between the heartwood and sapwood composition will be explained in the next paragraph 6.4.2.1.

The ferulic acid esters were identified in Sitka spruce but not in Norway spruce and their quantity was not negligible within the total amount of compounds analysed by GC/MS.

Nevertheless we remark that even with the method used by Ekman et al. (1979a) in Norway spruce, between 60 to 70% of the compounds extracted are not analysed by GC/MS.

Willfor et al. (2003b) analysed Norway spruce at different ages. The lignans and phenolic compounds were extracted by acetone/water (95:5 v/v) and analysed by high performance size exclusion chromatography (HPSEC), and the lipophilic compounds were extracted by hexane and analysed by GC/MS (with an HP1 column). The extractive content in dried wood analysed by GC/MS for the heartwood, sapwood and knotwood is summarised in Table 6-7 and represents between 0.50 to 1.65% of the dried wood for the heartwood and sapwood in Norway spruce samples. Extractive content analysed by GC/MS is probably lower than the gravimetric content of extract that they might have obtained just after the solvent extraction, if we refer to the amount of gravimetric extractives in Norway spruce found by Ekman et al. (1979a) in Table 6-6.

	Total extractive content in dried wood analysed by GC/MS		
Age of the tree	Heartwood	Sapwood	Knotwood
66	0.39%	0.98%	14.92%
71	0.50%	0.87%	15.66%
64	0.62%	0.78%	12.39%
17	X	1.11%	X
17	X	1.12%	X
150	0.66%	1.52%	17.42%
134	0.80%	1.65%	22.16%
X states for no sample			

Table 6-7: Total extractive content in dried wood analysed by GC/MS in Norway spruce by Willfor et al. (2003b)

However the quantity of extractives identified by GC/MS by Willfor et al. (2003b) appears to be greater than what was recovered in the present Sitka spruce experiment or by Ekman et al. (1979a) in Norway spruce. It is due principally to the use of HPSEC which recovers a large fraction of phenolic compounds, as will be mentioned in paragraph 6.4.1.2.

A comparison between the gravimetric results and the extractive content analysed by GC/MS and HPSEC would be useful but unfortunately Willfor et al. (2003b) do not state their gravimetric results.

6.4.2.1 Heartwood and sapwood

If we look at the distribution of Sitka spruce extractive classes within the fraction analysed by GC/MS (Table 6-4), the heartwood extractives contained more lignans and phenolic compounds than the sapwood extractives. The heartwood and sapwood extracts contained a greater percentage of sterols and esters, relative to the mass of extractives recovered, than the knotwood, rootwood and bark samples. Heartwood and sapwood had the second largest percentage of resin acids.

A large percentage of lignans and aromatic compounds was expected in the heartwood sample in Sitka spruce. Heartwood formation involves the formation of several classes of extractives: terpenoids, tropolones, flavonoids, stilbenes and other aromatic compounds (Philip, 1995; Scheffer and Cowling, 1966). Lignans and phenolic compounds have several biological activities such as antifungal and antioxidant properties (Hon 2001; MacRae and Towers, 1984; Clark et al., 1981; Goettlieb 1990). Previous work (Obst, 1997) showed that heartwood contains more extractives than the sapwood and has higher resistance to decay or attack by other organisms.

Sapwood was found to contain more lipophilic extractives than heartwood by Bertaud and Holmbom (2004) who analysed acetone micro-extraction products from milled Norway spruce sawdust by GC, after silylation, on a DB1-Column (capillary column coated with cross-linked dimethyl polysiloxane) between 80^{o}C to 340^{o}C (Orsa and Holmbom, 1994). The higher lipophilic extractive content in sapwood is due to the fact that triglycerides, as well as steryl esters, occur in the living parenchyma cells. When heartwood is formed the parenchyma cells die and the triglycerides are hydrolysed (Bertaud and Holmbom, 2004).

The distribution of sterols and resin acids found in Sitka spruce was consistent with the Norway spruce lipophilic extractives. But looking at the detailed composition, the major difference is a higher content of triglycerides in sapwood (Table 6-8) but the GC/MS analysis technique chosen for our study only allowed the quantification of the most volatile triglycerides in very small quantities from Sitka spruce. The difference between Bertaud and Holmbom (2004) work and the present Sitka spruce study was the analysis method. They used a DB1 non-polar

column, which is more resistant to higher temperature. The higher temperature used in their method will allow the detection of larger molecules as triglycerides, but with a less good separation of the more volatile compounds.

	Sitka spruce (From Table 6-4)		Norway spruce (Bertaud and Holmbom, 2004)	
Content each class in dried mass (%)	Heartwood	Sapwood	Heartwood	Sapwood
Free fatty acids			0.11%	0.06%
Sterols	0.02%	0.01%	0.02%	0.01%
Steryl esters			0.11%	0.14%
Esters	0.25%	0.23%		
Triglycerides			0.07%	0.45%
Resin acids	0.23%	0.31%	0.19%	0.22%
TOTAL	0.50%	0.55%	0.51%	0.88%

Table 6-8: Lipophilic extractives in the different samples determined by extraction and GC/MS for Sitka spruce (this study) and for Norway spruce by Bertaud and Holmbom (2004)

Willfor et al. (2003b) also analysed heartwood and sapwood of Norway spruce. The lignans and phenolic compounds were extracted by acetone/water (95:5 v/v) and analysed by HPSEC, and the lipophilic compounds were extracted by hexane, alkaline hydrolysed, silylated and analysed by GC/MS (on an HP1 column). The results are shown in Table 6-9 and Table 6-10, alongside the Sitka spruce results repeated from Table 6-4.

The samples are not fully comparable as the age of the tree influences the extractives composition (Fengel, 1970). Even within the same age range the variation between trees was substantial as shown for the lignan composition of Norway spruce (lignans for samples HW1, HW2 and HW3 in Table 6-9). Nevertheless the lignan composition detected in Sitka spruce at 35 years old is within the range of lignan contents detected in Norway spruce at around 70 years old. The 'lignan' class for Sitka spruce includes all the lignans and phenolic compounds identified. The amount of resin acids is comparable between Sitka spruce and Norway spruce. The quantity of sterol compounds is smaller, as in Sitka only two sterols were identified against four in Norway spruce (Ekman, 1980b). In Sitka spruce, a large range of ferulic esters was also quantified that Willfor et al. (2003b) do not mention.

	Age of the tree	Lignans	Oligolignans	Resin acids	Diterpenyl alcohols	Sterols	Fatty acids	Esters	Total
Sitka spruce (From Table 6-	35	0.09%		0.23%		0.02%		0.25%	0.59%
Norway spruce (Willfor et al., 2003b)	HW1 66	0.00%	0.04%	0.09%	0.01%	0.08%	0.17%		0.39%
	HW2 71	0.03%	0.01%	0.25%	0.01%	0.08%	0.12%		0.50%
	HW3 64	0.19%	0.01%	0.20%	0.01%	0.07%	0.14%		0.62%
	HW6 150	0.25%	0.02%	0.17%	0.01%	0.08%	0.13%		0.66%
	HW7 134	0.12%	0.04%	0.22%	0.02%	0.12%	0.28%		0.80%

Table 6-9: Heartwood extractives analysed by GC/MS in Sitka spruce and in Norway spruce by Willfor et al. (2003b)

	Age of the tree	Lignans	Oligolignans	Resin acids	Diterpenyl alcohols	Sterols	Fatty acids	Esters	Total
Sitka spruce (From Table 6--4)	35	0.02%		0.31%		0.01%		0.23%	0.57%
Norway spruce (Willfor et al., 2003b)	SW1 66	0.06%	0.03%	0.09%	0.03%	0.10%	0.67%		0.98%
	SW2 71	0.01%	0.02%	0.22%	0.03%	0.08%	0.51%		0.87%
	SW3 64	0.01%	0.04%	0.16%	0.03%	0.08%	0.46%		0.78%
	SW4 17	0.03%	0.04%	0.20%	0.01%	0.08%	0.75%		1.11%
	SW5 17	0.06%	0.04%	0.23%	0.01%	0.15%	0.63%		1.12%
	SW6 150	0.02%	0.09%	0.17%	0.02%	0.13%	1.09%		1.52%
	SW7 134	0.06%	0.09%	0.22%	0.09%	0.14%	1.05%		1.65%

Table 6-10: Sapwood extractives analysed by GC/MS in Sitka spruce and in Norway spruce by Willfor et al. (2003b)

For the sapwood, the quantity of lignans detected in Sitka spruce was small compared to the lignan content detected in Norway spruce, with again a large variability in the lignan class between trees of the same age. A greater amount of resin acids was detected in the Sitka spruce extractives sample from sapwood.

The resin acid class is where the difference with the heartwood sample is the largest with only 0.23% detected in heartwood against 0.31% in sapwood. Fatty acids detected after hydrolysis and derivatisation by Willfor et al (2003b) are likely to be derived from the triglycerides measured directly by Ekman (1980a) These Sitka spruce results are in accordance with the previous work of Bertaud and Holmbom (2004) in Norway spruce discussed earlier in this paragraph.

6.4.2.2 Knotwood

The knotwood extractives analysed in Sitka spruce by GC/MS are compared to the Norway spruce extractives identified by GC/MS or HPSEC. Knotwood samples were analysed by Willfor et al. (2003b) at different heights. The average extractive content has been calculated to enable the comparison, and the results are summarised in Table 6-11.

	Age of the tree	Lignans	Oligolignans	Resin acids	Diterpenyl alcohols	Sterols	Fatty acids	Total
Sitka spruce (From Table 6--4)	35	0.04%		0.43%				0.47%
Norway spruce (Willfor et al., 2003b)	KW1 66	11.75%	2.85%	0.02%	0.01%	0.10%	0.19%	14.92%
	KW2 71	11.88%	2.75%	0.69%	0.03%	0.12%	0.19%	15.66%
	KW3 64	8.72%	2.38%	1.02%	0.03%	0.08%	0.15%	12.39%
	KW6 150	13.85%	3.25%	0.05%	0.01%	0.09%	0.18%	17.42%
	KW7 134	17.15%	4.55%	0.18%	0.02%	0.10%	0.16%	22.16%

Table 6-11: Knotwood extractives analysed by GC/MS and HPSEC in Sitka spruce and in Norway spruce by Willfor et al. (2003b)

Only two extractive classes were detected in Sitka spruce knotwood by GC/MS, the lignans and the resin acids. In Norway spruce, much more variation was found in resin acids in knotwood than in heartwood and sapwood samples. The resin acid content found in Sitka spruce is within the range of values found in Norway spruce. We can remark that the knotwood sample from Sitka spruce yielded a greater amount of resin acid than the Sitka spruce heartwood or sapwood samples.

In Sitka spruce samples, the knotwood lignan content measured was higher than in sapwood, but smaller than heartwood.

As previously mentioned, in Norway spruce the lignans and oligolignans were detected by HPSEC by Willfor et al. (2003b). The amount of lignans detected in Norway spruce knotwood (between 8 to 17%) was much larger than the quantities detected in heartwood and sapwood of Norway spruce (Table 6-11), and much larger than was detected in this study of Sitka spruce by GC/MS. Indeed Willfor et al. (2003b) detected more lignans in the HPSEC study of most of their Norway spruce knotwood samples than the total extractives content of Sitka spruce knotwood. Some of the individual lignans detected by Willfor et al. (2003b) were not on the list that was detected in Sitka spruce samples because either their molecular weight was too high to be detected with our method or their quantities were below the detection threshold.

However no gravimetric content for Norway spruce knotwood extractives is given by Willfor et al. (2003b) so it is not possible to ascertain whether their recovery was complete.

Sitka and Norway spruce are quite similar physiologically, but it is their taxonomic relatedness that is more likely to determine how much similarity can be expected in their extractives Nkongolo (1999) studied the genomic relationships among 10 taxa of *Picea* spp. including Sitka spruce and Norway spruce by random amplified polymorphic DNA (RAPD) and cytological analyses. Figure 6-19 illustrates the dendrogram of genetic relationships linking the ten spruce species.

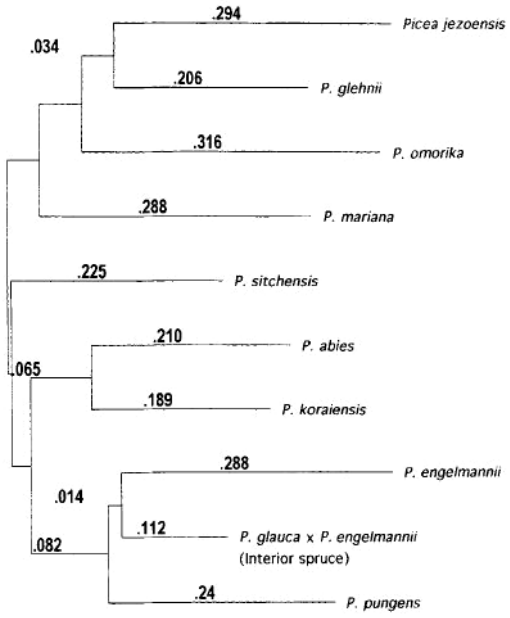


Figure 6-19: Dendrogram of genetic relationships between the ten spruce species based on the Jaccard similarity matrix from Nkongolo (1999)

The dendrogram shows the genetic relationships between spruce species based on RAPD distance. Sitka spruce (*P. sitchensis*) was found to be in the middle of the two principal clusters, and is not particularly closely related to Norway spruce (*P. abies*).

6.4.2.3 Rootwood

The root sample was the only sample where α-linolenic acid, from the fatty acid class identified by GC/MS in Sitka spruce study. The resin acid composition was the same in rootwood as in the other samples, but fewer lignans were identified compared to the heartwood, sapwood or knotwood samples (Table 6-4).

There is little published information on the extractives of spruce rootwood. Most of it relates to stilbenes and their role during the defence mechanism against exterior attacks. Stilbenes were not detected as major components in the present study.

Woodward and Pearce (1988) studied the role of stilbenes in resistance of Sitka spruce saplings to entry of fungal pathogens. They found that the rootwood was particularly rich in glucoside stilbenes with the two major constituents being the glucosides astringin and rhaponticin. The stilbenes were suggested to be

involved during the defence response against fungi attack through formation of structural barriers formed from stilbene aglycones, among other polyphenols, following the synthesis of β-glucosidase enzyme (Woodward and Pearce, 1988; Lindberg et al., 1992). Other resin extractive compounds such as terpenes, resin acids and fatty acids were suggested as being involved during the rootwood response to fungal attacks of a 9 year old trees (Woodward and Pearce, 1988 and Pearce, 1996).

No evidence of such high levels of stilbenes in mature Sitka spruce roots were found, therefore this defence mechanism must be restricted to very young trees

In general, however, such an active mechanism against external attack might justify the high extractive content in rootwood of Sitka spruce that was found in this study.

6.4.2.4 Bark

The Sitka spruce bark sample shows the greatest amount of total extractive content (28.3% in Table 6-4) in dry bark. Within this total only 0.5% of the extractives were analysed by GC/MS. The quantity of compounds detected by GC/MS in the bark was similar to the quantity detected in rootwood. Some sterols and esters, very few resin acids and none of the lignans were identified.

Pietarinen et al. (2006) sequentially extracted bark of Norway spruce first with hexane to remove the lipophilic compounds and then with acetone/water (95:5 v/v) mixture to extract the hydrophilic fraction. They found that the gravimetric yield of the hydrophilic extract from Norway spruce was 12% of the dried bark, which corresponds to about the half of the total extractive content detected in Sitka spruce (lipophilic and hydrophilic extractives together). Unfortunately Pietarinen et al. (2006) do not mention the gravimetric yield of the lipophilic extractives nor did they quantify individual compounds in the hydrophilic fraction of bark extractives.

The gravimetric content of resin material and polyphenols in Norway spruce bark seedlings was established by Wainhouse et al. (2004). They state that the spruce bark was rich in polyphenols (about 13.6% in the dry mass after extraction with

50% aqueous ethanol (v/v) mixture) close to the 12% of hydrophilic fraction found by Pietarinen et al. (2006).

Wainhouse et al. (2004) detected 1.9% of resin material in dry mass after pentane extraction. They referred to the method used by Wainhouse et al. (1998) who considered as resin material all the material weight extracted from needle resin ducts by pentane, which should be close to the weight of what we previously called the lipophilic fraction.

If we combine the results obtained by Wainhouse et al. (2004) we should get an estimation of the total extractive content of the Norway spruce bark, i.e. 15.5%. That extractive content represents half of the gravimetric extractive content measured in the mature bark from Sitka spruce (28.3% in dry mass in Table 6-4)

The bark composition of Norway spruce was analysed by Lindberg et al. (1992) in relation to the resistance of bark to fungal attack. They used ethanol extraction followed by solvent partition (with petroleum ether and water mixture and with methanol) and GC analysis (SE-30 column, 220°C). Their results are presented in Table 6-12 beside the relevant Sitka spruce results.

	Resin acid	Analysis
Sitka spruce Bark of 35 years old (From Table 6-4)	0.07% of dry bark	- Extracted in acetone - Evaporated to dryness - Chloroform fraction analysed by GC/MS
Norway spruce Bark of 4 years old seedling (Lindberg et al., 1992)	0.19% of fresh bark	- Extracted 5 min in ethanol, - Evaporated to dryness - Partition in petroleum ether and water - Petroleum ether fraction analysed by GC/MS with fused silica capillary column at 220°C
	Stilbene glucosides	Analysis
Norway spruce Bark of 4 years old (Lindberg et al., 1992)	25.1% of fresh bark	- Evaporation and redilution in methanol of the aqueous fraction, - Analysed by HPLC, C_{18} column

Table 6-12: Resin acids content of bark of Sitka spruce and Norway spruce analysed by Lindberg et al. (1992)

The results presented by Lindberg et al. (1992) were presented as the weight of extract in fresh material. Therefore Lindberg et al.'s (1992) results should be

corrected upwards by an indefinite factor. But the comparison will still be difficult as the Norway spruce samples are young seedlings (4 years old) compare to the mature Sitka spruce (35 years old).

6.4.3 Comparison between GC/MS and FTIR spectroscopy

Using the results in Table 6-4 showing the amount of extractives analysed by GC/MS compared to the total gravimetric amount of Sitka spruce extractives, we can conclude that the entire extracted fraction was not analysed by GC/MS. Comparison with the results of Ekman et al (1979a) showed that triglycerides were a group of extractives present, particularly in sapwood, but not identified in this study, while Willfor et al's (2003b) HPSEC method gave much larger recoveries of lignans, especially from knotwood, than were possible by GC/MS. It was not clear whether these were the only extractive fractions under-represented in the GC/MS data. The data from the FTIR spectroscopy of the same samples illuminate this question.

Table 6-13 summarises the FTIR results obtained in transmission mode from Sitka spruce extract. The semi-quantification of each functional group was possible through previous establishment of response factors using standard molecules. The results in Table 6-13 represent the equivalent of standard functional group in dry mass.

On average the total extractive content predicted using a single calibration standard for each functional group was in the same range as the gravimetric measurements, although the heartwood and sapwood totals were overestimated and the bark total underestimated by FTIR spectroscopy.

The FTIR results (Table 6-13) indicated that in Sitka spruce samples, aliphatics predominated, followed by aromatics in the bark sample. The GC/MS data (Table 6-4) showed that a wide variety of linear and cyclic, saturated and unsaturated hydrocarbons were present in the aliphatic fraction. These would undoubtedly give different spectra in the C-H stretching region from that used for semi-quantitative estimation. Using a single standard was therefore a considerable approximation but sufficient agreement was obtained to allow the

different types of wood and positions in the tree to be compared with one another.

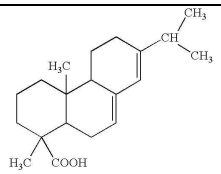
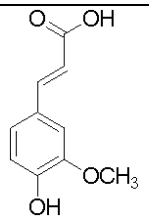
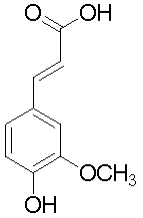
Content each functional group in dried mass (%)	Standard molecule	Heartwood	Sapwood	Knotwood	Rootwood	Bark
Aliphatic group	 Abietic acid	1.65%	1.27%	9.84%	8.68%	8.36%
Carboxylic group	 Ferulic acid	0.18%	0.13%	1.50%	1.05%	1.54%
Aromatic group	 Ferulic acid	1.02%	0.33%	1.02%	0.72%	5.14%
TOTAL		2.85%	1.73%	12.36%	10.45%	15.04%
Content of extractives in dried mass (%) from the gravimetric analysis		1.48%	1.33%	9.04%	11.53%	28.31%

Table 6-13: Content of each functional group in dry wood calculated from the KBr FTIR analysis in Sitka spruce

6.4.3.1 Heartwood and sapwood

The results from heartwood and sapwood FTIR analyses are similar in terms of amount of aliphatic and carboxylic compound detected (Table 6-13), but differ for the aromatic group with 0.33% of aromatic in sapwood against 1.02% in heartwood. The results for the aromatic groups are in accordance with GC/MS analysis (as cited in paragraph 6.4.2.1) as more lignans are expected in heartwood compared to sapwood.

Compare to knotwood, rootwood and bark samples the amount of aliphatic and carboxylic groups are about ten times smaller in heartwood and sapwood

samples (Table 6-13). The combination of aliphatic and carboxylic groups is found in resin acids, or triglyceride molecules, consequently these compounds are expected to be in lower quantity in heartwood and sapwood. Referring to the FTIR peaks between 3000-2800cm^{-1} (chapter 4), heartwood and sapwood seem to contain both classes.

The detailed GC/MS data from this study are a better guide to the composition of the heartwood and sapwood lipophilic extractives because they account for a much larger proportion of the total mass than in knotwood, rootwood and bark.

	Resin acids content in dried wood analysed by GC/MS			
	Age of the tree	Heartwood	Sapwood	Knotwood
Sitka spruce (From Table 6-4)	35	0.23%	0.31%	0.43%
Norway spruce [a] (Ekman et al. (1979a) [b] (Willfor et al., 2003b)	26[a]	0.12%	0.16%	
	66[b]	0.09%	0.09%	0.02%
	71[b]	0.25%	0.22%	0.69%
	64[b]	0.20%	0.16%	1.02%
	150[b]	0.17%	0.17%	0.05%
	134[b]	0.22%	0.22%	0.18%

Table 6-14: Resin acid content in dried wood of Sitka spruce and Norway spruce (Willfor et al., 2003b and Ekman et al., 1979a)

Ekman et al. (1979a) and Willfor et al. (2003b) referring to Ekman (1979b), state that in Norway spruce sapwood should contain more resin acids than heartwood and that knotwood contains less resin acids than stemwood (Table 6-14). The Sitka spruce heartwood, sapwood and knotwood samples do not seem to follow this trend. It appears that knotwood contained the most resin acids and sapwood least.

6.4.3.2 Knotwood

For the knotwood sample, a large part of the mass of extract is accounted for in the FTIR spectra by aliphatic (9.8%) and carboxylic compounds (1.5%). A high representation of both groups (aliphatic and carboxylic) and the shape of the FTIR peaks between 3000-2800cm^{-1} (chapter 4.3.3.3) might be related to a high resin acid content in knotwood sample. Polymerisation of these resin acids would account for the much smaller recovery by GC/MS.

The amount of aromatic groups analysed in knotwood by FTIR is similar to that in the heartwood sample (1.02% in Table 6-13). Consequently we cannot expect a large difference in the content of lignan and aromatic compounds between knotwood and heartwood, as was found in Norway spruce knotwood by Willfor et al. (2003b).

The qualitative GC/MS analyses of knotwood and heartwood did not show a lot of differences in the compounds detected. But the amount of lignans recovered by GC/MS from heartwood (0.09% in Table 6-4) was double the amount detected in knotwood (0.04% in Table 6-4). Some lignans present in Sitka spruce knotwood were probably not detectable by GC/MS because of their molecular weight being too great to permit sufficient volatility for these molecules to pass through the GC/MS column.

6.4.3.3 Bark

From the FTIR analysis, the bark sample was where the largest amount of aromatic substances was detected, about 5%. In the GC/MS chromatogram from bark no lignans or phenolic compounds were identified. Because the FTIR spectrum allowed the detection of a large content of aromatic groups, we suspect the presence of aromatic substances of higher molecular mass than would be detected by GC/MS. These could be oligolignans. A large percentage of oligolignans in the Sitka spruce bark sample would be consistent with what has been found by HPSEC in bark from Norway spruce by Pietarinen et al. (2006) and Wainhouse et al. (2004).

The presence of more resin acids or triglycerides than could be analysed by GC/MS is suspected in Sitka spruce bark, because of the large representation of aliphatic and carboxylic compounds. A closer look at the FTIR spectra presented in chapter 4.3.3.3, and particularly at the 3000-2800cm^{-1} area help to confirm the presence of resin acids or polymers of resin acids.

Chapter 7: Conclusions

7.1 General conclusions

The extractives content and composition differed between Sitka spruce heartwood, sapwood, knotwood, rootwood and bark samples. The bark contained the largest amount of extractives while the heartwood and sapwood were the parts of the tree where the extractive content was the lowest.

Heartwood and sapwood samples from the Kershope experiment were studied in more detail. It was found that more extractives were contained in heartwood than in sapwood and that the difference was consistent at all heights in the trunk. The FTIR analysis showed that the relative quantity of aromatic compounds in heartwood tended to increase with height, while there was a more uniform distribution in sapwood. The relative ester content did not vary with height for either type of wood.

The total extractive content in Sitka spruce in dried wood across Scotland was shown to be low and stable with rather constant proportions of carboxylic and aromatic compounds. Neither the management of the forest (yield class, thinning) nor the East or North location, nor the elevation had a significant impact on the extractives content across the wide range of Scottish sites examined.

The range of individual compounds identified in Sitka spruce by GC/MS was similar to, but slightly narrower than, what has previously been reported for Norway spruce. Only half as many sterols were recovered from Sitka spruce. No diterpene alcohols and diterpene aldehydes were found in the Sitka samples. However the lignans detected and a group of ferulic esters were similar in Sitka spruce and Norway spruce.

The gravimetric total and ester content of extractives in Sitka spruce increment cores was stable across Scotland and did not seem to depend on yield class, site elevation, North/East location or thinning. Geography did not seem to have an influence on the aromatic composition. Because heartwood is over-represented in increment cores and has higher extractive levels, these data slightly

overestimate the mean extractives content of the clear wood in the whole trunk, which is predicted to decrease with height in parallel with the decreasing proportion of heartwood.

A low and stable extractive content can be a positive factor in the quality of the Scottish Sitka spruce resource for the pulp and paper industry, where wood extractives interfere with the process and cause significant technical and economic problems.

7.2 Application of extractives

7.2.1In biolomedical industry

Lignans have a large range of biological properties such as: antitumor, antimitotic, antioxidant, antiviral activities, fungal growth inhibition, fish toxicity, insect antifeedant and muscle relaxant activities and juvenile hormone functions (Clark et al., 1981; MacRae and Towers, 1984; Goettlieb 1990 and Umezawa, 2001).

Flavonoids have antioxidant, anti-inflammatory, antihistamic and antiviral properties (Obst, 1997).

Some terpenoids such as the phytane diterpene plaunotol have anti-ulcer activities (Umezawa, 2001). Resin acids are also a by-product of the kraft pulping of wood, and when obtained from that source they are used as paper sizing agents, controlling absorption of water in paper (Obst, 1997).

Some sterols have strong effects on the heart muscle and can be used as either therapeutic compounds or toxins (Obst, 1997). Sitosterol or sitostanol can be used as cholesterol-lowering components in food products, where a high ratio of sitosterol to campesterol is beneficial. This ratio was high in birch and pines but clearly lower in spruce and larch (Vikstrom et al., 2005).

7.2.2In pulp and paper industry

Wood extractives can cause problems especially in the pulp and paper industry. The lipophilic fraction of the wood extractives, coming from the resin canals and ray parenchyma cells, is composed of low molecular mass resins and fatty acids and higher molecular mass waxes, sterol esters and triglycerides (Gutierrez et al., 1998). These can be can be released and form colloidal 'pitch' during the pulp and paper making process. Pitch particles may initially be small in size and then coalesce into larger droplets, which deposit on the surface of the fibers or the equipment, or remain suspended in the washing water to be discharged in the effluent (Gutierrez et al., 2001). Pitch may deposit alone or with other compounds such as inorganic salts, defoamers and coating binders derived from the pulp and papermaking process (Gutierrez and Del Rio, 2005)

Conventional approaches used to reduce wood extractives interaction during the process are debarking or seasoning logs, but these measures are often not sufficient to eliminate pitch problems (Gutierrez et al., 1998). Other precautions such as conventional filtration, membrane filtration with or without reverse osmosis, flotation, biological treatments, precipitation or evaporation have been introduced into the process. Their sequence will depend on the identification of the streams to be treated and the characterisation of contaminants in each stream of the water system (Gutierrez et al., 2001). The ability of different wood-inhabiting fungi to degrade lipophilic compounds using enzymes are already being exploited in commercial products for wood and pulp depitching in order to degrade the lipophilic extractives before the pulp and pulping process (Fischer and Messner, 1992; Farrell et al., 1993 and Gutierrez et al., 1998). However these methods are specific to certain wood species under specific pulping conditions.

Pitch problems cause significant technical and economic problems for pulp and paper manufacturers regardless of the pulping and bleaching procedures used, and have a detrimental impact on the environment (Chen et al., 1994). The pitch problems result in a low quality final product and problems in mill operations with significant economic losses.

7.2.3 Other outlets

While the lipophilic extractive fraction is not wanted in the pulp and paper industry, wood extractives can be still be valuable compounds for other purposes.

The bark constitutes a large quantity of available material, as approximately 10 to 15% of each log consists of bark. It imperative that bark is no longer considered as waste material. Conifer bark is a valuable product in its own right, with a profitable market in the horticultural industry. Roffael et al (2000) studied the possibility of using bark extract as an adhesive or binder in particleboards and medium density fibreboards. The preparation of extract-based adhesives is based on the reaction between the phenolic extractives and formaldehyde. Despite the fact that the content of extractives, particularly water-soluble extractives in spruce is low, their amount can be increased by higher temperature extraction. Roffael et al. (2000) found that water soluble extractives from spruce bark can substituted at up to 20% for Quebracho extracts, widely used as particleboard binders and at up to 60% in binders for medium density fibreboard without impairing the physical-mechanical properties of the board.

Bark has been studied for its bioactive chemicals to add value to its use as an energy resource (Kahkonen et al., 1999 and Pietarinen et al., 2006). Pietarinen et al. (2006) deduced that bark extract of Norway spruce had the strongest antioxidant potential of several species (such as *Abies*, *lasiocarpa*, *Pinus banksiana*, *Populus tremula*), although it contained a large amount of stilbenes and stilbene glycosides with the potential to inhibit the antioxidant properties.

Knotwood is of little value in the manufacturing of pulp and paper industry. The knots have a negative effect on pulping processes and pulp quality. It is better that they are separated out before the pulping process and in that case they are normally burnt. But the knotwood of Norway spruce constitutes also a very valuable raw material for the extraction of lignans, stilbenes, flavonoids, and other phenolic substances that could be utilised as natural antioxidants in various technical applications and as an active ingredient in health promoting food and pharmaceuticals (Willfor et al. 2004a). Two patents related to the use

of knotwood are known: The “use of knotwood extract” by Willfor et al., 2005a and the “method for isolating phenolic substances or juvabiones from wood comprising knotwood” by Holmbom et al., 2002.

The high content and distinctive composition of extractives in Sitka spruce roots was a novel finding in this research. New harvesting methods make root biomass readily available, currently as fuel. Root extractives are worth investigating for technical applications. There is evidence that the influence of roots on wood composition extends further up into the stem in Sitka spruce than in other conifer species. The possibility of higher and more variable extractive content in the lowest metre of the stem is worth investigating, since it might influence how stems are divided for pulpwood production.

7.3 Recommendations for future work

Further work would be needed to estimate the variation in the volume of heartwood and sapwood along the trunk as well as the variation in total extractive content in the same tree. This would show whether variation in extractive content develops in the same way as the distribution of heartwood and sapwood volume.

Further tests would be needed also to continue the comparison between the chromatographic and spectroscopic methods.

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