

SPECTROSCOPIC ANALYSIS OF THE LIVERS IN RAINBOW TROUT
(*ONCORHYNCHUS MYKISS*) EXPOSED TO NONYLPHENOL

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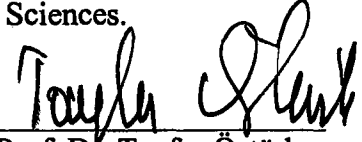
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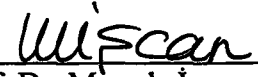
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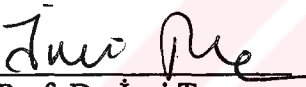
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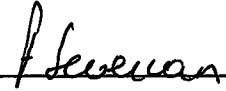
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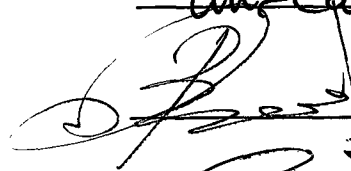
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ABSTRACT

SPECTROSCOPIC ANALYSIS OF THE LIVERS IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) EXPOSED TO NONYLPHENOL

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Nonylphenol (NP) is a biodegradation product of nonylphenol ethoxylates (NPEs) belonging to the alkylphenol ethoxylates (APEs) group, which are widely used non-ionic surfactants in detergents, herbicides, pesticides, paints and cosmetics. APEs are one of the important groups of environmental endocrine disrupters. It has been shown that, besides its estrogenic effect, as an endocrine disrupter NP, has also toxic and carcinogenic effects.

Rainbow trout (*Oncorhynchus mykiss*) is one of the widely consumed fish and NP is the most abundant APEs derivatives in fresh water. Since, liver has an essential role in detoxification of toxic substances and has a role in the synthesis of vitellogenin, which is egg-yolk precursor protein in female fish, the present

study was conducted to determine the effects of NP on rainbow trout liver by using FT-IR spectroscopy. In order to compare the effects of NP with the estrogen, livers of the fish treated with 17 β -Estradiol (natural estrogen in vertebrates) were also analyzed.

The FT-IR spectra revealed dramatic differences between the NP-treated and control tissues at the molecular level. Glycogen level was significantly decreased and the relative content of nucleic acids increased, especially, DNA became dominant component in the NP-treated liver. Moreover, the nucleic acids exhibited an increase in the number of the hydrogen-bonded phosphodiester groups in treated fish. The level of the fatty acids, particularly, unsaturated fatty acids, were largely increased and the membrane structure and permeability were altered by NP, possibly, due to the toxic effect of this chemical in this tissue. Furthermore, the presence of an unfolding of the molecules in the NP-treated liver may also be the result of the toxic effect of NP. The protein synthesis in the treated tissue was increased. The observation could be attributed to the estrogenic effect of this compound. The estrogenic effect of NP was also supported by the spectra of the E2-treated fish liver which exhibited very similar spectral pattern with that of NP-treated fish.

Thus, FT-IR spectroscopy appears to be useful tool to rapidly investigate the metabolic, structural and conformational alterations induced by NP treatment in liver and to correlate them with biochemical and physiological data.

Keywords: Nonylphenol, Rainbow Trout, FT-IR, Liver

ÖZ

NONYLPHENOLE MARUZ BIRAKILMIŞ GÖKKUŞAĞI ALABALIĞI KARACİĞERİNİN SPEKTROSKOPİK ANALİZİ

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Nonilfenol (NP), deterjanlar, herbisit, pestisit, boyalar ve kozmetiklerin üretiminde yaygın olarak kullanılan alkilfenol etoksilatlarına ait nonilfenol etoksilatların bir biyolojik parçalanma ürünüdür. Alkilfenol etoksilatlar çevredeki hormonal dengeyi bozan maddelerden önemli bir gruptur. Önceki çalışmalarda, hormonal dengeyi bozucu bir madde olarak NP'nin estrojenik etkisinin yanında kanserojenik ve toksik etkilerinin de olduğu gösterilmiştir.

Gökkuşığı alabalığı (*Oncorhynchus mykiss*) yaygın olarak tüketilen bir balıktır ve NP tatlı sularda en fazla bulunan bir alkilfenol etoksilat türevidir. Karaciğer toksik maddelerin detoksifikasyonunda önemli bir role sahip olduğundan ve dişi balıklara özgü bir protein olan vitellojenin sentezinde rol

oynadığından, bu çalışmada, NP'ün Gökkuşığı alabalığı karaciğeri üzerindeki etkileri Kızıl Ötesi Spektroskopisi kullanılarak araştırılmıştır. Ayrıca NP ile estrogenin etkilerini karşılaştırmak için 17- β Estradiol'e (omurgalılarıdaki doğal estrojene) tabii tutulan balık karaciğerleri de incelenmiştir.

Kızıl Ötesi Spektroskopisi sonuçları kontrol ve NP' e maruz bırakılmış balık dokuları arasında moleküler düzeyde önemli farklılıklar olduğunu göstermiştir. NP'e maruz bırakılmış karaciğerdeki glikojen miktarında önemli oranda bir azalma ve nükleik asitlerin miktarında bir artma gözlemlendi; özellikle DNA baskın hale geçti. Ayrıca, nükleik asitlerin fosfodiester gruplarına büyük oranda hidrojen bağlanması oldu. Yağ asitlerinin, özellikle doymamış yağ asitlerinin miktarının önemli oranda artması ve hücre zarının yapısının ve geçirgenliğinin NP tarafından değiştirilmesi NP'ün toksik etkisinden kaynaklanmış olabilir. Bunun yanında NP'e maruz bırakılmış dokulardaki moleküllerde gözlenen açılma da NP'ün toksik etkisinin bir sonucu olabilir. NP'e maruz bırakılmış dokularda protein sentezinin artması NP'ün estrogenik etkisinin bir sonucu olarak değerlendirilebilir. Bu bulgu ayrıca estrojene maruz bırakılmış balık karaciğerinin NP'e maruz bırakılmışla çok benzer spektral özellikler göstermesi ile de desteklenmektedir.

Sonuç olarak, Kızıl Ötesi Spektroskopisi NP'ün karaciğerde meydana getirdiği metabolik, yapısal, ve konformosyonel değişiklikleri hızlı bir şekilde incelemek ve bu sonuçları biyokimyasal ve fizyolojik bulgularla birleştirmek için kullanışlı bir araç gibi görünmektedir.

Anahtar Kelimeler: Nonilfenol, Gökkuşığı Alabalığı, Karaciğer, Kızıl Ötesi Spektroskopisi

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ABBREVIATIONS

AP: Alkylphenol
APE: Alkylphenol ethoxylates
CD: Circular Dichroism
CYP: Cytochrom P450
DDT: Dichlorodiphenyltrichloroethane
E2: 17 β -Estradiol
ER: Estrogen Receptor
ERE: Estrogen Responsive Element
ESR: Electron Spin Resonance
FT-IR: Fourier Transform Infrared Spectroscopy
IR: Infrared Spectroscopy
KBr: Potassium Bromide
LDL: Low Density Lipoprotein
MCF-7: Human Breast Cancer Cell
NMR: Nuclear Magnetic Resonance
NP: Nonylphenol
NPE: Nonylphenol Ethoxylate
NPEC: Nonylphenoxy Carboxylate
Vtg: Vitellogenin

CHAPTER 1

INTRODUCTION

1.1 Endocrine Disrupters

Nonylphenol ethoxylates (NPEs) are non-ionic, polymeric surfactants (synthetic surface active substances) belonging to the alylphenol ethoxylates (APEs) group, which are the second largest group of non-ionic surfactants in commercial production. Nonylphenol (NP) is the predominant and persistent biodegradation product of NPEs in the environment and digested sewage sludges. Due to their ability to mimic the effects of female sex hormones (estrogens), NPEs and their degradation products in particular NP are commonly associated with a class of toxicants referred to as endocrine disrupters. NP is toxic to aquatic organisms and has been found to affect endocrine systems in fishes, birds and mammals through weak estrogen-like properties (Thibout *et al.*, 1999).

Endocrine disrupters are defined as exogenous compounds, which interfere with the production, release, transport, metabolism, binding action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (Ferguson *et al.*, 2000). Other terms used to describe these chemicals include xenoestrogens, environmental estrogens, estrogenic, hormone mimicking and endocrine disrupting chemicals. Examples of potential endocrine disrupters include the APEs, phytoestrogens (natural hormones present in many plants), and certain pesticide and insecticides.

Many of these endocrine disrupters have estrogenic activity and can disrupt normal functions of sex steroids by causing feminization phenomena in all classes of vertebrates (Kloas *et al.*, 1999). These compounds are suspected to cause adverse effects in wildlife such as abnormal reproductive systems development, decreased reproductive success in males and females and feminization of males. There are many reports of declines in human sperm quality and quantity and increased breast cancer and testicular cancer rates. The estrogenic effect was discovered long after some of the endocrine disrupters were released into the environment. For example the insecticide dichlorodiphenyltrichloroethane (DDT) was the first heavily released chemical and much later it was found to be estrogenic (Sonnenschein and Soto, 1998).

The toxicity of NPEs are mainly attributed to NP and their other biodegradation products which are generally ten times more toxic than the parent compound. These biodegradation products are persistent and have been shown to bioaccumulate in both fresh water and marine organisms. Because many of the endocrine disrupters bioaccumulate due to their lipophilicity and they are very persistent in the environment, the potential for exposure of humans and animals to environmental estrogen like chemicals is very high (Roy *et al.*, 1997 and Kloas *et al.*, 1999). Therefore the effects of these endocrine disrupting chemicals on animals need to be considered urgently. Xenoestrogens represent the most characterized class of endocrine disrupters. NP, one of the xenoestrogens was identified to possess some capacity to influence the estrogen receptor pathway (Gagne *et al.*, 1999).

1.2 Classification of Estrogenic Chemicals

Ovarian estrogens (e.g., estradiol, estrone) occur naturally and are steroidal in nature. Nonovarian estrogens are either synthetic (diethylstilbestrol) or produced by plants (equaol, coumestrol) or microbes (enterolactone). Synthetic estrogens are either steroidal or nonsteroidal in nature and can be categorized into two groups:

1. Chemicals synthesized as estrogens and used for pharmacological and other purposes (eg., diethylstilbestrol, 17 α -ethinylestradiol, mestranol).

2. Chemicals synthesized not as estrogens, but they have estrogenic properties that have been identified with time (NP, bisphenol A, chlorinated hydrocarbons).

Structures of some of the natural and environmental estrogens are shown in figure1 (Roy *et al.*, 1997).



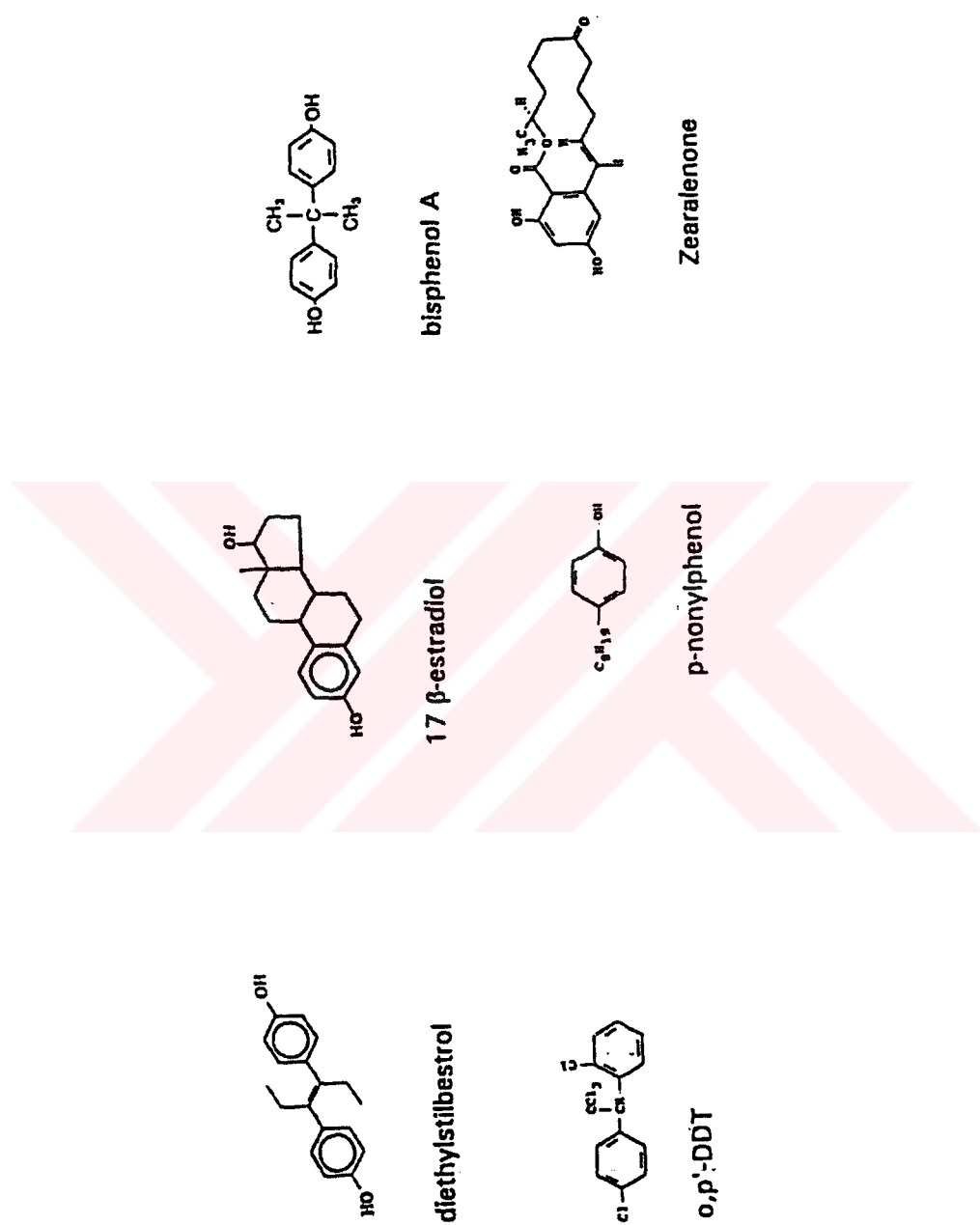


Figure1. Structures of Some of the Natural and Environmental Estrogens (Roy *et al.*, 1997).

It has been reported that the number of estrogen-like chemicals of the latter group has been growing rapidly. A large number of pesticides and industrial chemicals possessing estrogen-like activity are persistent in the environment and make their way into the food chain. Some of these chemicals, such as APEs, need to be metabolized before they can bind to estrogen receptors. The pesticide DDT, even though banned and not in use, is still present in the environment due to its previous widespread use and relatively long half-life.

Principal sources of some of the environmental estrogen-like chemicals are listed in table 1 (Roy *et al.*, 1997).

Table 1. Partial List of Environmental Estrogen-like Chemicals (Roy *et al.*, 1997).

Agricultural and Industrial Estrogen-like Chemicals:	Pesticides: Kepone (chlordecone), methoxychlor, DDE, a metabolite of o,p'-DDT, endosulfan, toxaphene, dieldrin, dicofol, 1-hydroxychlordecone
	Polychlorinated Industrial by-products: Some polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins, and chlorofluorocarbon
	Alkylphenols: Nonylphenol, 6-bromo-naphtol-2, 4-octylphenol, 4-nonylphenoxycarboxylic acid, bisphenol A
	Polycyclic Aromatic Hydrocarbons: 3,9-dihydroxybenz(a)anthracene
Drugs:	Cimetidine, digitalis, diethylstilbestrol
Chemical Constituents of Oral Contraceptives:	17 α -ethynilestradiol, equilenin,menstranol
Natural and Synthetic Mycotoxins:	Zeranol, zearalenone
Microbial Intestinal Fauna:	Enterolactone
Plant Estrogens:	Equol, diadzein, formononetin, biochanin A, genistein, coumesterol, giberillic acid.

1.3 Alkylphenol Ethoxylates (APEs)

Surfactants are very common water pollutants, because they are primarily used in aqueous solutions which are later introduced into the environment via waste water discharge. APEs are an important class of non-ionic surfactants which have found many industrial, commercial, institutional and household uses in the world (Nimrod and Benson, 1996). Worldwide more than 500.000 tons/year of APEs are produced of which approximately 60% can be found in the aquatic environment as various breakdown products (eg. NP, octylphenol). Among APEs, NPEs which account for approximately 80 percent of the world's production, are the most abundant products (Mann and Boddy, 2000). NPEs are commonly used in a large variety of industries and manufacturing processes (Pedersen *et al.*, 1999 and Richerdson *et al.*, 1999).

1.3.1 Nonylphenol Ethoxylates (NPEs)

1.3.1.1 Industrial, Household and Commercial Usage of NPEs

NPEs have been in use for over 40 years. The NPEs are used both as detergents and in many formulated products such as herbicides, pesticides and paints. They are widely used as detergents, emulsifiers, wetting agents in household products and in agricultural and in industrial applications such as in the manufacturing of plastics, cosmetics, lubricant oils and fuels, metals, petroleum textile and leather processing, construction materials, pulp and paper. For instance in the plastic industry NPEs are used as softeners and stabilizer additives, and as UV filters to prevent yellowing (Herrero-Martinez *et al.*, 2001). NPEs are also used as spermicides in contraceptive applications (Nimrod and Benson, 1996) and found in latex coatings and adhesives (Ferguson *et al.*, 2000). NPEs also have been approved by the U.S. Food and Drug Administration to be used in the manufacture of food packaging (Nimrod and Benson, 1996 and Roy *et al.*, 1997).

1.3.1.2 Chemical Structure of NPEs

The structure of NPE is shown in figure 2 (Hao *et al.*, 2000). A NPE molecule consists of two parts; a hydrophobic nonylphenol and a hydrophilic polyethylene oxide chain which has one to 100 repeating (Pederson *et al.*, 1999). The nonylphenol portion of a NPE molecule consists of a hydrocarbon chain, which has 9 carbon atoms, attached to phenol. This part gives NPEs the ability to dissolve grease and other substances that are not soluble in water. The ethoxylate portion of NPEs is a long chain of two-carbon units connected by oxygen atoms. This structure makes NPEs soluble in water and helps remove dirt and grease from soiled surfaces into water (Ahel *et al.*, 1984).

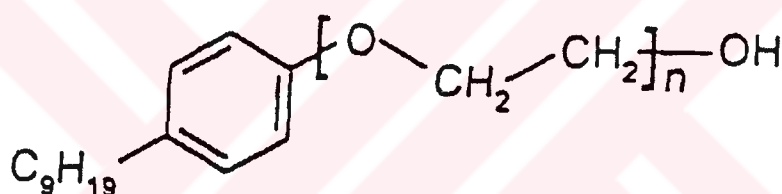


Figure 2. The structure of NPE (Hao *et al.*, 2000).

1.3.1.3 Biodegradation Pattern of NPEs

NPEs can be degraded aerobically and anaerobically by microorganisms in natural environments and sewage treatment plants. The biodegradation mechanism involves stepwise loss of ethoxy groups to lower NPE intermediates, followed by production of NPEC and NP. The degradation pathway is shown in figure 3. This pathway is an oversimplification because it does not include NPnEC where $n > 2$ or NPEs with carboxyl groups attached to the nonyl chain (Nimrod and Benson, 1996). The final degradation product, NP is more persistent, more toxic, more lipophilic, less water soluble and more estrogenic than the parent compounds (Bennie, 1999).

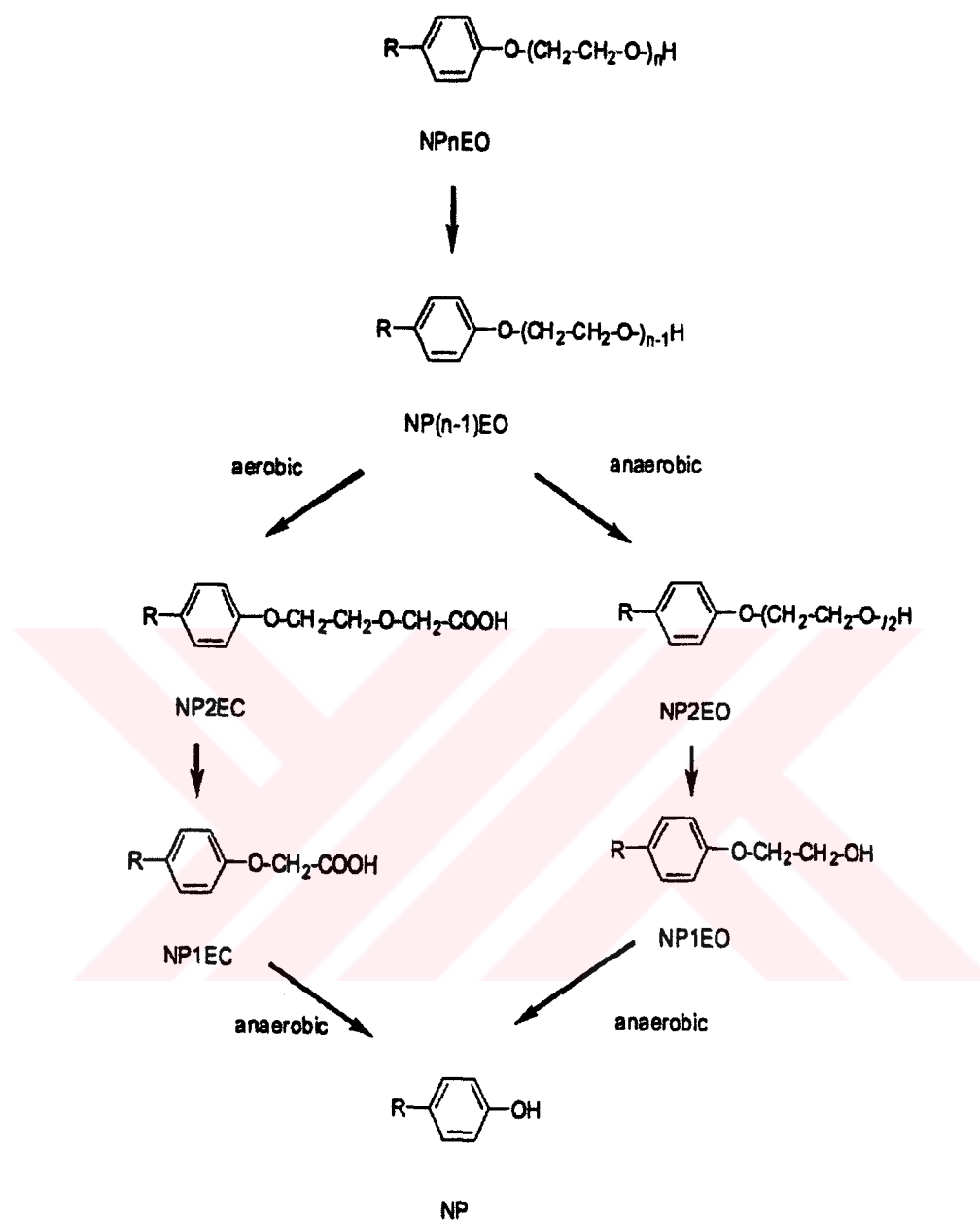


Figure 3. Biological Degradation Pathway for NPE (R = branched nonyl group) (Nimrod and Benson, 1996).

1.3.1.4 Presence of NP in the Environment:

NPEs enter the environment primarily through discharges of wastewater from sewage treatment plants or through secondary release from landfills, farming, or grassland properties. NP, which is the most abundant degradation products occur in the anaerobically digested sludge of sewage treatment plants. Sludge from these sewage treatment plants may be used as amendment to agricultural soils (Bennie, 1999). Due to its high lipophilicity the NP accumulate in sewage sludge, and sediments where they are sufficiently persistent to be transferred to agricultural soils manured with sludge from sewage treatment plants (Mann and Boddy, 2000). Although NP has been found in high concentrations (0.45-2.53 g/kg) in anaerobically stabilized sewage sludge, concentration of NP in river water, sediments and sludge-amended soils are typically several orders of magnitude lower. The environmental distribution of NP is estimated to be 25% in water, >60% in sediment and >10% in soil (Pederson *et al.*, 1999). Clark *et al.*, (1992) have reported the presence of both NPEs and their carboxylic acid derivatives in drinking water. The concentrations that have been reported are extremely variable and range from nanograms to milligrams/litre. NP has been measured in rivers and lakes in many countries. For example the concentration of NP were around 2-336 µg/L in British rivers (Blackburn and Waldock 1995); 0.3-45 µg/L in Swiss river (Ahel *et al.*, 1994); 3-300 µg/L in Canadian rivers (O'Halloran *et al.*, 1999). NP accumulates in a wide range of marine and aquatic life including algae, molluscs, and fish. A recent study demonstrated NP uptake from water into rainbow trout tissues have a high bioaccumulation factor and significant deposition in many tissues, including muscle and liver (Coldham *et al.*, 1998).

Humans have the potential to be exposed to NPEs and their degradation products through the water supply by drinking of contaminated drinking water, sewage sludge used for fertilizer, aquatic flora and fauna serving as food. Also human exposure may occur by absorption through skin/mucosae, from shampoos,

detergents, cosmetics and spermicidal lubricants; inhalation or ingestion from pesticide sprays; and ingestion of soil or contaminated food from fields spread with sludge containing NPEs (Nimrod and Benson, 1996).

1.3.1.5 Physicochemical Properties of NP

Description: NP is a clear to pale yellow viscous liquid with a slight phenolic odour.

Purity: 90% w/w.

Melting Point: Substances of this type (oily) do not have a clear melting point. Various values have been reported, probably owing to differences in the alkyl chain structure. The values are: -10 °C, -8 °C, 10 °C, and <20 °C.

Boiling Point: 290-310 °C. However, some thermal decomposition probably occurs before this temperature is reached.

Density: 0.95 g/ml (at 20 °C).

Vapour Pressure: 0.0023 mmHg (0.3 Pa) at 20 °C.

Flash Point: 141-155 °C.

Autoignition Temperature: 370 °C.

Solubility: Water: Practically insoluble, 6 mg/100 ml (at 20 °C).

LogP octanol/water: 4.48.

Henry's Constant: 1.02 Pa m³/mole.

pKa-Value: Estimated value 10.25 (Groves *et al.*, 1972; Talmage, 1994 and Miyamoto and Klein, 1998).

1.3.1.6 Toxicity of NPEs

The acute toxicity of APEs to mammalian species is quite low; oral LD₅₀ values for rats and mice range from 2 to 4 g/kg (Nimrod and Benson, 1996). In contrast to their effects in mammals, non-ionic surfactants are highly toxic to aquatic organisms. The toxicity of APEs to aquatic organisms increases with a decreases number of ethylene oxide units and increasing hydrophobic chain length. Therefore the toxicity of the parent surfactants is less than the degradation

products NP, NP1EO, and NP2EO. Naylor (1992), has summarised the toxicity of NP to aquatic organisms, including crustaceans, fish, invertebrates, and algae. No observable effect concentrations (NOECs) ranged from 6.7 µg/L for *Mysidopsis bahia* reproduction to 24 µg/L for *Daphnia magna* reproduction. The 96-h LC50 value for the alga *Skeletonema costatum* was 27 µg/L, while for *Oncorhynchus mykiss* (rainbow trout) it was 480 µg/L. Limited toxicity data on the carboxylic acid degradation products NP1EC and NP2EC suggest the toxicity is low, comparable to NP1EO, probably due to their higher water solubility (Nimrod and Benson, 1996).

1.3.1.7 Metabolisation of NP in the Organisms

In order to understand the mechanism of APs' effects, it is crucial to understand the metabolic fate of APs in the organisms. APs were degraded or metabolised to H₂O and CO₂ or uptaken by living organisms.

Since liver has an essential role in detoxification of toxic substances, it contains enzyme systems capable of chemically altering toxic compounds, usually to a less toxic form. When a toxic compound enter the liver cells, the specific enzymes which metabolize this compound such as cytochrom P450 enzymes are started to synthesize.

Arukwe *et al.*, (1997) has shown that NP cause variations in isoforms of hepatic cytochrom P450-dependent xenobiotic metabolizing enzymes. Recently Coldham *et al.*, identified some NP metabolites in rainbow trout bile. This finding suggests that NP is detoxified in liver (Coldham *et al.*, 1998). They have found the highest concentrations of NP residues in trout bile and intestinal contents/faeces, indicating a biliary/faecal route of excretion. Moreover, in a previous study, a significant increase was observed in the activity of the plasma enzyme glutamate pyruvate transaminase (GPT), which is frequently been used to detect an eventual damage to the liver cells, in the plasma of the 4-NP-treated males (Christensen *et al.*, 1999).

NP is hydroxylated by cytochrome P450 monooxygenase enzymes (CYP) at C-8 and C-9 positions of the nonyl chain to give catechols or conjugated catechol structure namely (8- or 9-hydroxy nonylphenol) (Pederson *et al.*, 2000; Arukwe *et al.*, 2000). Hydroxylation of the NP by CYP enzymes is the phase I of the metabolic pathway from NP metabolism. Then, glucouronidation of the NP occurs by β -glucouronidase enzyme. For example, Thibaut *et al.*, (1998) reported that 4-NP is modified by β -glucouronidase hydrolysis. Therefore, most of the 4-NP metabolites are to be found glucuronic conjugates in an organism (figure 4). NP is hydroxylated at both omega-1 and omega-2 positions of the alkyl chain, giving 9-hydroxynonylphenol and 8-hydroxynonylphenol. 9-hydroxynonylphenol is oxidized to and subsequently beta-oxidized to yield 7-(4-hydroxyphenyl) heptanoic acid, 5-(4- hydroxyphenyl) pentonoic acid, 3-(4-hydroxyphenyl) propionic acid and 3-(4-hydroxyphenyl)-2-propenoic acid.

Predominant metabolites of 4-NP were found in bile as a glucuronid conjugate (Coldham *et al.*, 1998; Ferreria-Leach and Hill, 2001). Other metabolites included glucuronid conjugates of ring or side chain of hydroxylated 4-NP. Despite relatively rapid metabolism and excretion, a substantial remain of the parent compound found in muscle which will have implications for the maintenance of 4-NP residues and associated biological activity in the organisms (Coldham *et al.*, 1998).

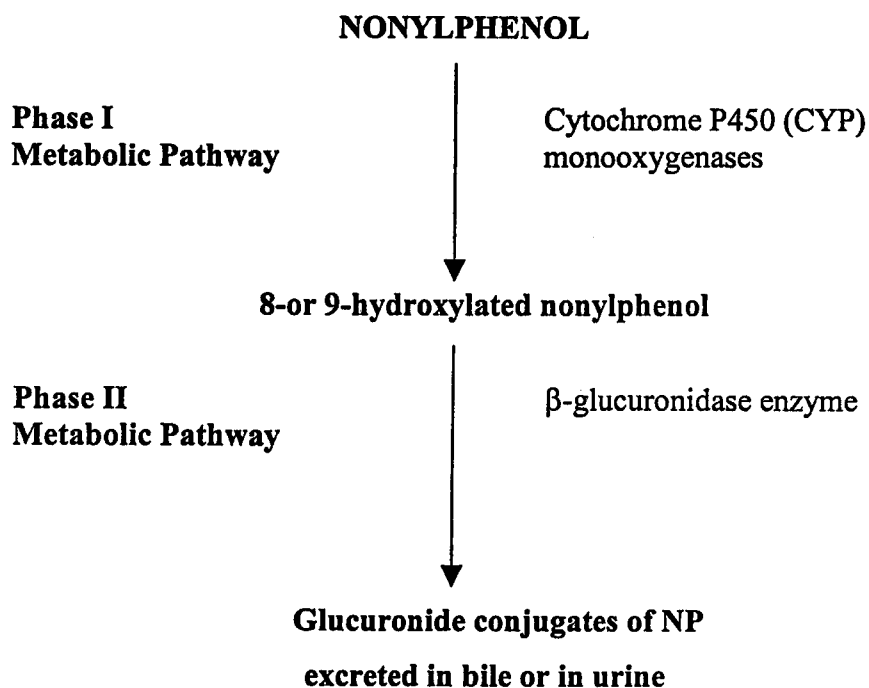


Figure 4. Metabolism and excretion of Nonylphenol (adapted from Arukwe et al., 2000 and Pederson et al., 2000)

1.4. Major Effects of NP on Animals

As well as NP has a well documented estrogenic effect, it has toxic and carcinogenic effects on animals. It has been reported that NP is acutely toxic to fish (17-3000 µg/L), invertebrates (20-300 µg/L) and algae (27-2500 µg/L) (Bennie, 1999). NP and NPEs bind to the estrogen receptor resulting in the expression of several responses both in vitro and in vivo, including induction of vitellogenin. The threshold for vitellogenin induction in fish is 10 µg/L (Bennie, 1999).

When estrogenic activities of interest are examined, trout are a very sensitive model for tumor studies. Estrogens promote liver tumors in trout. This is particularly relevant considering that human and trout estrogen receptors have similar specificities for binding estrogens (Oganesia *et al.*, 1999).

There is a general agreement that human populations are continually exposed to a wide variety of environmental alkylphenols and therefore it is important to understand the effects of environmental estrogen-like chemicals at the cellular level. According to Soto *et al.*, (1991) NP displays similar estrogenic effects in inducing the proliferation of MCF-7 cells (human breast cancer cells) causing feminisation of male fish and affect adversely testicular size and spermatogenesis in rats. Recently, toxicity results have shown that the nonylphenolic compounds are endocrine disrupters that can pose a health risk to humans and to wildlife.

1.4.1 Estrogenic Effects of NP on Animals

In 1991, Soto *et al.*, showed the estrogenicity of NP in mammals. Recent studies using a wide range of in vitro assay systems, including induction of vitellogenin secretion by trout hepatocytes, confirmed that NP is weak estrogen (Coldham *et al.*, 1998). Several in vitro studies demonstrated that NP stimulates the synthesis of vitellogenin in rainbow trout hepatocytes. For example, Lech *et al.*, (1996) reported the in vivo induction of liver vitellogenin mRNA in both male and immature female rainbow trout exposed to NP under flow-through conditions. Jobling *et al.*, (1996) confirmed that exposure of male rainbow trout to NP caused synthesis of vitellogenin and a concomitant inhibition of testicular growth. Estrogenic effects have also been shown on chicken embryo fibroblasts and a mouse estrogen receptor (Jobling and Sumpter, 1999 and White *et al.*, 1994).

1.4.1.1 Comparison of Estrogenic Compounds and Natural Estrogens

Natural estrogens play a major role in controlling reproduction in females and, to a lesser extent, males. Additionally, physiological concentrations of estrogen are essential for the maintenance of cell growth and several other biological activities. For example, normal physiological levels of estrogen are known to be involved in the control of cell proliferation, transcription and DNA synthesis. Besides estrogen-responsive organs (such as uterus, breast, and pituitary), estrogens exert their effect at multiple sites (kidney, liver, skeletal

tissues, etc.) depending upon the species. Imbalance of the steady-state concentrations or pharmacological dose or concentration higher than the physiological level of estrogens is known to produce adverse effects (Roy *et al.*, 1997).

Many of the biodegradation products of APEs are estrogenic to fish cells when tested at micromolar concentrations. Although these compounds are weak estrogens, this does not mean that they are not biologically important. Soto *et al.*, (1992) demonstrated that the APs are three-times more potent than the weakly estrogenic pesticides DDT and kepone. Because of the bioaccumulative properties of the APs, it is likely that they will be potent at much lower concentrations in vivo than in vitro (Jobling and Sumpter, 1993).

A recent review summarized that APs' relative potencies were approximately 1000-100,000 times less than that of the natural estrogen 17 β -estradiol (E2). It was tested by representative APE degradation products in mammalian, avian, and fish in vitro estrogen assays and was demonstrated that, at the molecular level, their action is mediated by the estrogen receptor and that this interaction is via a similar region of the hormone-binding domain as estradiol itself (Jobling *et al.*, 1996 ; Christensen *et al.*, 1999 and Coldham *et al.*, 1998).

Yadiete *et al.*, (1999) indicated that NP could be more potent than reported for APs in the literature from in vivo studies in fish.

1.4.1.2 Vitellogenin

Vitellogenin is a large lipoglycophosphoprotein, which is specifically synthesized, lipidated, phosphorylated and glycosylated in the liver of oviparous female vertebrates under the control of 17 β -estradiol (E2), secreted into the blood stream and finally taken up by oocytes via receptor-mediated endocytosis. Normally, vitellogenin is undetectable in male and immature female fish; however, its synthesis can be induced by exogenous stimulation of estrogenic

compounds (Islinger *et al.*, 1999; Jobling and Sumpter, 1993). In many previous studies, it has been shown that exposure of NP resulted in producing vitellogenin in both males and females. So the presence of vitellogenin in liver and serum of male fish may be used as a biomarker of exposure the (xeno)estrogenic chemicals (Jobling *et al.*, 1996)).

In amphibians, like in all other oviparous vertebrates, vitellogenin gene expression is mediated by binding of E2 to cytosolic estrogen receptors in target cells leading to dimerization of the ligand-receptor complex. In target cells E2 binds to estradiol receptor (ER) and the E2-ER complex binds to estrogen responsive elements (ERE) in E2 controlled genes modifying their transcriptional rates. Consequently the expression of ER is essential for cellular estrogenic responses (Ahel *et al.*, 1994). Figure 5 shows vitellogenesis at the cellular level. In one of the previous study, it was shown that ER was induced by NP, in a dose-dependent manner. NP and other APs were shown to compete with E2 in binding to ER and also to activate reporter genes with ERE in cell cultures. ER induction was followed by induction of vitellogenin mRNA and protein synthesis. (Yadeite *et al.*, 1999). The rainbow trout ER gene promoter contains estrogen-responsive elements (EREs) that regulate its own transcription. It is likely that NP activated ER binds to the same EREs (in the promoters of E2 regulated genes) as E2 in modulating the transcription of estrogen responsive genes (Yadeite *et al.*, 1999).

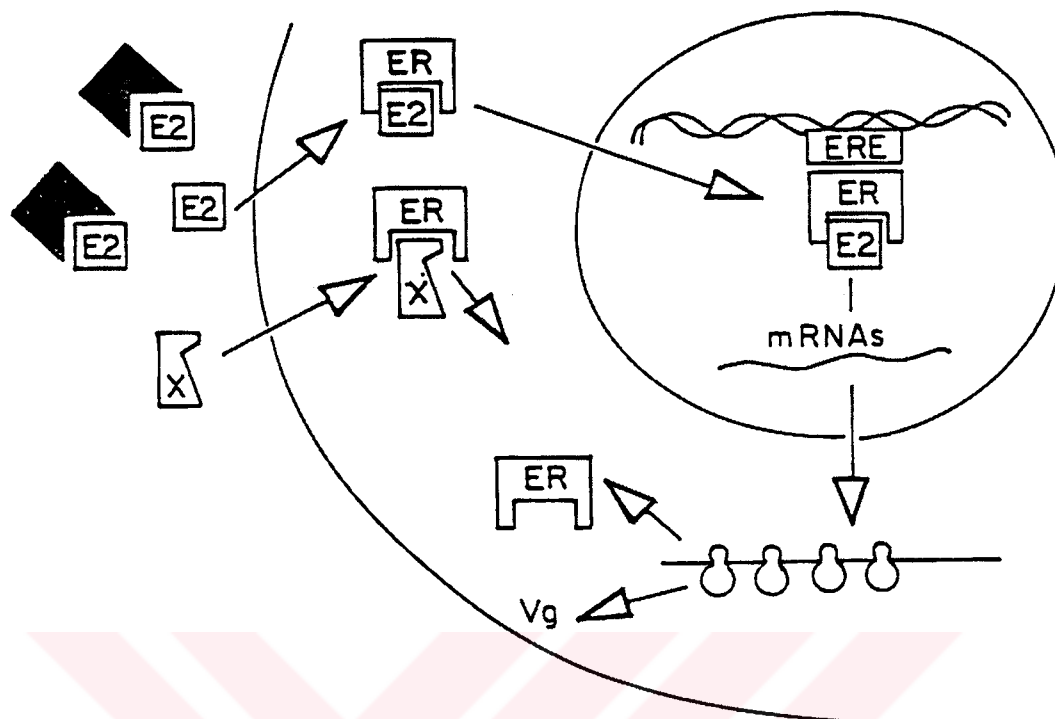


Figure 5. Vitellogenesis at the cellular level. Estradiol (E2) enters the cell and binds to the estrogen receptor (ER). In the nucleus, it induces mRNAs by interaction with the estrogen response element (ERE) on DNA. Xenoestrogens (X) may interfere with this process by interaction with the ER (Nimrod and Benson, 1996).

1.4.2 Toxic Effects of NP on Animals

Since NP is a bulky hydrophobic molecule it increases membrane permeability and causes cell leakage and loss of ions. Increased ion permeability and sodium efflux of gill epithelial cells due to NP-10 ethoxylate were reported for rainbow trout. Calcium especially seems to play an important role in direct, as well as indirect, effects of NP. In the sarcoplasmic reticulum of the rat, CaMg-ATPase is inhibited by NP due to a decrease of the enzyme affinity to calcium. Nonylphenol was reported to inhibit L-type calcium channels in vascular smooth muscle cells of the rat. Also Bragadin *et al.*, (1999), reported that NP enhances

the potassium permeability of mitochondrial membrane and inhibits or reduces the ATP synthesis. (Michelangeli *et al.*, 1990; Bragadin *et al.*, 1999 and Burkhardt-Holm *et al.*, 2000).

Schwaiger *et al.*, (2000) showed that chronic exposure to environmentally relevant concentrations of NP severely affects haematological parameters in juvenile carp. They found that NP induces anaemia as a consequence of the interaction between NP and erythrocyte membrane.

APs have been shown to induce apoptosis in a wide variety of cells including rat primary germ and Sertoli cell cultures (Christensen *et al.*, 1998 and Hughes *et al.*, 2000). Hughes *et al.*, (2000), has also shown that NP caused cell death by the inhibition of calcium pump in endoplasmic reticulum.

Since, similar adverse effects on testicular structure and function have been observed following prenatal exposure of both humans and rodents to artificial estrogens such as diethylstilbestrol or ethinylestradiol, it has been suggested that the toxic effects of alkylphenols are mediated via estrogen receptors (Hughes *et al.*, 2000)

1.4.3 Carcinogenic Effects of NP on Animals

In animals, APs act as both initiators and promoters of various types of cancers. Recently, NP has been shown to cause breast cancer (Bloom *et al.*, 1998 and Legler *et al.*, 1999), testicular and ovarian cancer (Skakkeback *et al.*, 1998).

It has been shown that, estrogen-like chemicals increase cell proliferation leading to disturbances in cell cycle. This concept may provide a necessary environment for the development of some cancers particularly breast cancer in human (Roy *et al.*, 1997). Almost certainly, the xenobiotic-induced DNA damage created a considerable degree of genetic instability. This may well be pivotal in the activation of oncogenes and the deregulation of tumor-suppressor genes, such

as p53 and it may at least partially explain the timing of oncogenesis (Malins and Gunselman, 1994). Increased cell proliferation and alteration in cell cycle kinetics are considered important factors for the development of genetic instability. For example, cell proliferation may allow mitotic recombination to occur, which may result in more profound changes than those of single mutation. Damage to DNA, such as incorporation of a wrong nucleotide, can occur during DNA synthesis. Damaged DNA is considered to be repaired during the G1 phase of the cell cycle. It has been reported that, some alkylphenols can directly produce DNA damage. The massive burst in the synthesis phase of the cell cycle in response to exposure to an alkylphenol such as NP coupled with a decrease in DNA repair may allow DNA damage, acquired during synthesis, to accumulate and fix the genetic instability. Most cancer cells exhibit telomeric associations. The telomeric associations have been implicated to contribute instability in the genome. The major function of the telomer is to provide stability to chromosomes and protect them from illegitimate recombination. There are some findings suggests that exposure of cells to NP may potentially be involved in the induction of instability in the genome through telomeric associations and/or reduction in telomeric length (Roy *et al.*, 1997).

1.5 Basic Principles of Optical Spectroscopy

Spectroscopy is defined as the study of interaction of electromagnetic radiation with matter, excluding chemical effects. Light is called electromagnetic radiation because it has electric and magnetic parts. Electromagnetic radiation can be characterized by either a frequency or a wavelength. Different types of light are denoted by their wavelength (λ). A wavelength is the distance between adjacent crests of a wave. The frequency (ν) of a light wave equals the number of cycles the wave undergoes in a second (Smith, 1999). The frequency, wavelength, and the speed of the light (c) are related to each other via the following equation:

$$c = \nu\lambda$$

where c is the speed of light (3×10^{10} cm/second), ν is frequency in Hertz (sec^{-1}) and λ is wavelength in cm.

Light is an electromagnetic wave and the energy of the wave is shown by Bohr equation:

$$\Delta E = E_2 - E_1 = hc/\lambda = h\nu \text{ (Bohr Equation)}$$

where h is Planck's constant ($6.62 \cdot 10^{-27}$ erg.s), c is the speed of the light ($3 \cdot 10^{10}$ cm/s), λ is the wavelength, and ν is the vibrational frequency. Bohr equation shows the change in molecular vibrational energy after infrared absorption as E_1 is the energy level of the molecule before absorption and E_2 is the energy reached by absorption (Freifelder, 1982).

In infrared spectroscopy, to denote different types of light the units called wavenumbers ($\bar{\nu}$) are normally used (Smith, 1999). Wavenumber is a measure of the number of cycles in a light wave per centimetre and defined as the reciprocal of the wavelength as follow:

$$\bar{\nu} = 1/\lambda$$

where $\bar{\nu}$ is wavenumber in cm^{-1} , λ is wavelength in cm.

The wavenumbers of several different types of light are shown in figure 6.

> 14,000 cm⁻¹ Visible UV & X-rays	14,000 to 4000 cm⁻¹ Near Infrared	4000 to 400 cm⁻¹ Mid Infrared	400 to 4 cm⁻¹ Far Infrared	< 4 cm⁻¹ Microwaves Radio Waves
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Higher Wavenumber	Lower Wavenumber
Higher Frequency	Lower Frequency
Higher Energy	Lower Energy
Shorter Wavelength	Longer Wavelength

Figure 6. The electromagnetic spectrum, showing the wavenumber ranges for different types of light (Smith, 1999).

As can be seen from this figure, the infrared regions include radiation at wavenumbers between 14 000 and 4 cm^{-1} . The infrared spectrum can be divided into three regions: the far infrared ($<400 \text{ cm}^{-1}$), the mid infrared (4000-400 cm^{-1}) and the near infrared (14000-4000 cm^{-1}). The spectral range of greatest use is the mid-infrared region which covers the frequency range from 400 to 4000 cm^{-1} because the vast majority of molecules in the universe absorb mid-infrared light but the near- and far-infrared regions can also provide valuable information about certain materials (Stuart, 1996).

For most purposes, it is convenient to treat a molecule as if it possesses several distinct reservoirs of energy. The total energy is given by the following equation (Campbell and Dwek, 1984).

$$E_{\text{total}} = E_{\text{transition}} + E_{\text{electronic}} + E_{\text{rotation}} + E_{\text{vibration}} + E_{\text{electron spin orientation}} + E_{\text{nuclear spin orientation}}$$

Energy can reside in molecules in a number of forms, of which the most important, are translational, electronic, vibrational, and rotational energy. Infrared spectroscopy is based on molecular vibrations and monitors the transition between vibrational energy level (Wilson *et al.*, 1995).

When an electromagnetic wave encounters a molecule, it can be either scattered (i.e., its direction of propagation changes) or absorbed (i.e., its energy is transferred to the molecule). If the electromagnetic radiation of the light is absorbed, the molecule is said to be excited or in an excited state. The major energy levels are determined by the possible spatial distributions of the electrons and are called electronic energy levels; on these are superimposed vibrational levels, which indicate the various modes of the vibration of the molecule (i.e., the stretching and bending of various covalent bonds). All these energy levels are usually described by an energy level diagram like the one in figure 7. The lowest electronic level is called the ground state and all others are excited states. Horizontal lines indicate vibrational levels. A change in the energy levels is called transition. Electronic transition between the ground state and the third vibrational level of the first excited state is indicated by the long arrow. A vibrational transition within the ground state is indicated by the short arrow (Freifelder *et al.*, 1982).

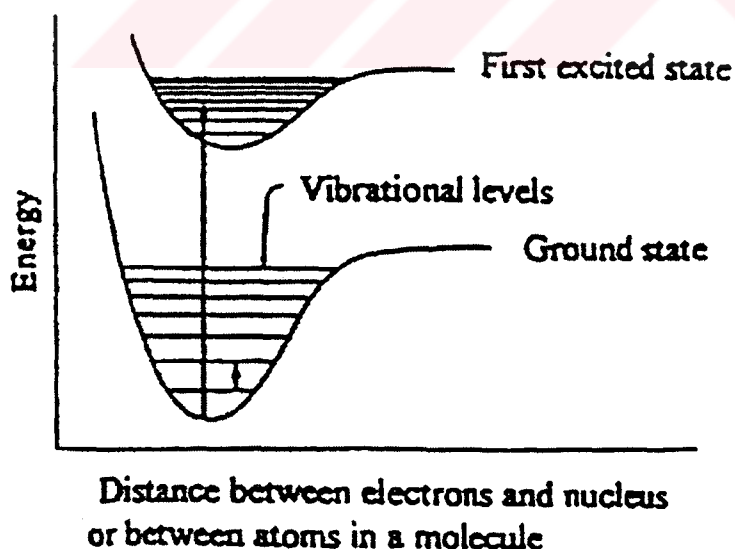


Figure 7. Typical energy level diagram showing the ground state and the first excited state (Freifelder *et al.*, 1982).

1.5.1 Infrared Spectroscopy

1.5.1.1 Basis of Infrared Spectroscopy

Matter is composed of atoms that are in a continuous motion with respect to each other. These atoms are held together by valence forces and they can vibrate because of their thermal energy. When infrared radiation interacts with matter, it can be absorbed, causing the chemical bonds in the material to vibrate. The presence of chemical bonds in a material is a necessary condition for infrared absorbance to occur. Functional groups tend to absorb infrared radiation in the same wavenumber range. This means that there is correlation between the wavenumbers at which molecule absorbs infrared radiation and its structure. This correlation allows the structure of an unknown molecule to be determined from its infrared spectrum and allows spectra of different samples to be compared to each other to see if they are the same (Stuart, 1997). The correlation between infrared band positions and chemical structure makes infrared spectrum a useful chemical analysis tool. An infrared spectrum usually consists of a plot of absorption of radiation as a function of wavenumber and is characterised only in terms of the positions of the maxima of each of the absorption bands $\bar{\nu}_{\text{max}}$, expressed in cm^{-1} (Smith, 1998).

The infrared spectra are generated by the characteristic motions of various functional groups like methyl, amide and carbonyl groups. Vibrations can involve either a change in bond length (stretching) or bond angle (bending). Some bonds can stretch in-phase (symmetric stretching) or out of phase (asymmetric stretching) (Stuart, 1997). Figure 8 shows the main types of variations schematically (Arrondo *et al.*, 1993). The value of infrared spectral analysis comes from the fact that the modes of vibration of each group are very sensitive to changes in chemical structure, conformation, and environment (Freifelder *et al.*, 1993).

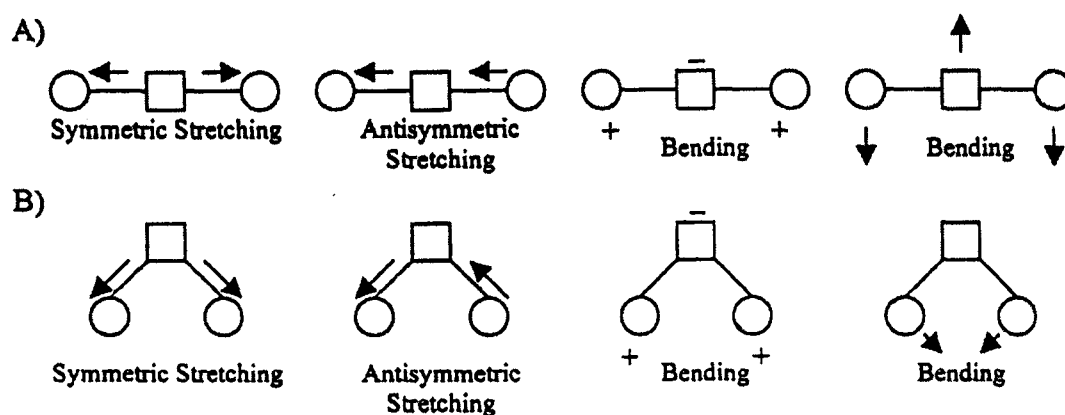


Figure 8. Types of normal vibration in a linear (A) and non-linear (B) triatomic molecule. Atomic displacements are represented by arrows (in plane of page) and by + and – symbols (out of page plane) (Arrondo et al., 1993).

1.5.1.2 Fourier Transform Infrared Spectroscopy

In recent years FT-IR spectroscopy has found increasing favour in laboratories. This method is based on the interference of radiation between two beams to yield an interferogram. An interferogram is a signal produced as a function of the change of pathlength between two beams. The two domains of distance and frequency are interconvertible by the mathematical method of Fourier transformation. The basic components of an FT-IR spectrometer are shown schematically in figure 9. The radiation emerging from the source is passed to the sample through an interferometer before reaching a detector. The scanning interferometer modulates each IR wavelength at a characteristic frequency which allows all wavelengths to reach the detector at the same time (Stuart,1997).

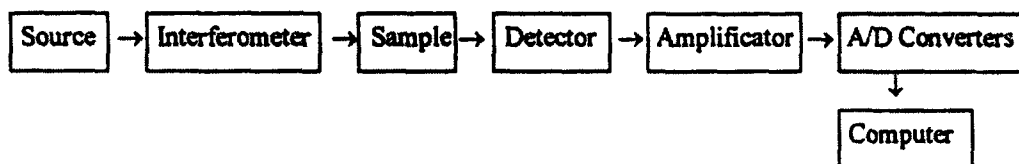


Figure 9. The components of FT-IR spectrometer (Stuart, 1997).

Upon amplification of the signal in which high frequency contributions have been eliminated by a filter the data is converted to a digital form by an analog to digital converter and then transferred to the computer for Fourier transformation to be carried out (Stuart,1997).

FT-IR spectroscopy is based upon the absorption of infrared light by covalent bonds as they vibrate. The frequency of light that is absorbed depends upon the nature of the bond (e.g., C-C versus C=C), the atoms involved in the bond (e.g., C=C versus C=O), the type of vibration (e.g., bending versus stretching), and factors such as strength of any hydrogen bonding interactions. Furthermore, as with other forms of optical spectroscopy, the amount of light absorbed by a vibrating bond is linearly related to concentration. The infrared spectrum of a sample is therefore a direct indicator of its chemical composition (Jackson *et al.*, 1999).

1.5.1.3 Advantages of FT-IR Spectroscopy

FT-IR spectroscopy produces conventional spectrum and it has several important advantages.

- i) The most important advantage of FT-IR spectroscopy for biological studies is that spectra of almost any biological material can be obtained in a wide variety of environments.

- ii) FT-IR method is a rapid and sensitive technique with sampling techniques that are easy to use.
- iii) The amount of sample required is relatively small.
- iv) The instrumentation is inexpensive compared to the cost of X-Ray diffraction, NMR, ESR and CD spectroscopic equipment and the operation of the equipment is simple. Interpretation of the spectra is not particularly difficult and can be learned easily.
- v) Since a computer is already used to obtain the Fourier transform, it is easy to perform many scans to improve the signal-to-noise ratio (noise adds up as the square root of the number of scans, whereas signal adds linearly).
- vi) Digital subtraction (that is, point-by-point subtraction of the separate spectra by a computer) can also be used to produce good difference spectra. This method has great advantages in obtaining infrared spectra in aqueous solutions.
- vii) A non-perturbing technique, since it can be applied to biological membrane preparations in a straightforward manner, which does not involve introduction of exogenous probes or potentially harmful preparative procedures. This eliminates problems that occur with other techniques involving extrapolation of results from the probe containing to the native system.
- viii) Lipids are easily studied in the gel or liquid-crystalline phases.
- ix) The FT-IR has advantage in terms of spectral regions which originate from molecular vibrations and different molecular moieties. For instance “head group” and “hydrocarbon tails” have spectral regions for membranes.

- x) There is no light scattering or fluorescent effects.
- xi) Kinetic and time-resolved studies are possible (Mantsch *et al.*, 1986; Mendelson and Mantsch, 1986 and Haris and Severcan, 1999).

1.5.1.4 FT-IR Spectroscopy in Tissue Studies

Infrared spectroscopy is becoming an increasingly powerful tool for the study of the composition and structure of cellular components within intact tissues (Takahashi *et al.*, 1991). In biological systems, functional groups that strongly absorb infrared light include C=O, N-H, C-H, and P=O groups. FT-IR spectroscopy is a valuable technique due to its high sensitivity in detecting changes in the functional groups belonging to tissue components, such as lipids and proteins. The shift in the peak positions, bandwidths, and the intensity of the bands all give valuable structural and functional information which may have diagnostic value (Yano *et al.*, 1996, Liu *et al.*, 1996, Ci *et al.*, 1999 and Severcan *et al.*, 2000). The intensity of the absorption bands in the infrared spectrum of a tissue sample provides information concerning lipid, protein, and nucleic acid content of the sample, whereas the frequency of the absorption provides information relating to structure/conformation and intermolecular interactions. In other words, the infrared spectrum of tissues provides information that reflects the biochemistry of the tissue (Jackson *et al.*, 1999).

In the last three decades, IR spectroscopy has become an accepted tool in the biological sciences, finding an important place in studies of lipid structure and phase properties, protein structure and nucleic acid structure. More recently, the information obtained from these studies has been transferred to studies of normal and diseased human tissues and fluids, which have demonstrated that IR spectroscopy, may be applied to the characterisation of pathological conditions (Liu *et al.*, 1996).

It has been established that FT-IR spectroscopy has an increasingly important role to play in the field of pathology. Disease-induced changes can easily be detected with this technique. FT-IR spectroscopy has been used in characterisation of tissues from cancer, Alzheimer's disease, arthritis and multiple sclerosis. These characterisations have been largely based on band assignments gleaned from empirical studies of the biological building blocks of these tissues, namely proteins, lipids and polynucleotides (Gentner *et al*, 1998). If the infrared spectrum of a tissue sample can provide information with regard to tissue biochemistry, then the changes in tissue biochemistry accompanying a disease process should be reflected in changes in the infrared spectrum of diseased tissue. It has been recently demonstrated that indeed so, identifying spectroscopic features characteristic of Alzheimer's disease, chronic lymphocytic leukaemia, multiple sclerosis, and scar formation in ventricular tissue following infarction. By extension, it is reasonable to speculate that tumors of differing grades will also show different spectroscopic features, in principle allowing characterisation of tumor spectra by grade (Jackson *et al.*, 1999). It has been documented that FT-IR spectroscopy can be used to detect malignant, dysplastic, or inflammatory cells in cervical smears (Fung *et al.*, 1997).

Using FT-IR spectroscopy in tissue studies has a number of advantages. For instance, fixation and staining of tissues are necessary before a histological assessment of tissue sections can be performed. No fixation and staining is required for infrared studies. In other words it examines tissues in the natural states and it is not limited by the quality of the slide preparation. Indeed, staining is undesirable, as the stain will itself contribute significantly to the IR spectrum of the sample. Fixation may also result in artefactual results if care is not exercised in the choice of fixative. Furthermore, an IR spectroscopic approach to pathology has the major advantage that it provides information concerning the molecular structure of the tissue. It relies on objective criteria such as absorption frequency and intensity changes in various IR absorption bands due to vibrations of the functional groups in cellular molecules (Jackson *et al.*, 1997 and Fung *et al.*,

1997). IR spectroscopy enabled us to investigate subtle changes in the structure of the tissues by analyzing the frequency, intensity and bandwidth changes of the vibrational modes. (Lester *et al.*, 1998).

1.6 Aim of the Study

Review of the literature revealed that APEs are heavily produced on all over the world and they are eventually mixed in wastewaters. One of their breakdown products is NP and it is the most abundantly found breakdown product in nature. Because of its hydrophobic structure it accumulates in the sediment and in aquatic biota.

Numerous studies showed that NP has toxic, carcinogenic and estrogenic effects on aquatic organisms. In vivo studies on the effects of NP on freshwater fish have a special importance. Such studies will help us to estimate the degree of adverse effects experienced upon the exposure to various levels of NP. Furthermore, better understanding about the mechanisms of the NP effects can be understood by these studies. Additionally, since the estrogen receptors of the fish are very similar to those of the humans it is highly likely understanding of the possible estrogenic effect mechanisms of NP on humans will also benefit from these studies.

Because rainbow trout is one of the most commonly consumed fresh-water fish and since liver has an essential role in detoxification of toxic substances and has a role in synthesis of vitellogenin, which is expected to increase upon exposure to NP as a part of its estrogenic effect, the present study is conducted to determine the effects of NP on rainbow trout liver. For this purpose FT-IR spectroscopy will be employed.

FT-IR spectroscopy is a valuable technique due to its high sensitivity in detecting changes in the functional groups belonging to tissue components, such as lipids and proteins. The shift in the peak positions, bandwidth, and the intensity

of the bands all give valuable structural and functional information. This technique, which requires neither reagent nor sample preparation, is non-destructive, and highly selective because of its ability to be a spectral fingerprint for molecular components.

The purpose of the this work is to use FT-IR spectroscopy as a rapid and suitable method to evaluate the effect of NP on trout liver at the molecular level and to correlate these findings with biochemical and physiological data.



CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

Nonylphenol (4-NP) was purchased from Aldrich Company. Potassium Bromide (KBr) was purchased from Merck.

2.2 Fish

Eight-month-old juvenile rainbow trout were employed. Fish were transferred to the laboratory in plastic sacs with aeration. Fish were then acclimated for several hours and put into 35 L tanks. Prior to experiment, fish were rested for 2 days. During the whole experiment fish were fed with trout chow.

2.3 Experimentation

Experiments were conducted in a static aquaria system. Charcoal filter was used to generate fish water. Prior to use, fish water was rested for 24 hours with aerator. In the present study the range for the treatment of NP was 66 µg/L and 660 µg/L and it was chosen in accordance with environmentally observed values. Upper limit of the range proved to be lethal in our system. In vivo effects of various concentrations of NP exposed rainbow trout for two weeks period was

determined in laboratory conditions and it was found that 220 µg/L NP is the most effective dose while 660 µg/L NP induce 100% mortality in four days. For this reason, fish treated with 0 (control), 66 µg/L and 220 µg/L NP for 2 weeks in 35 L aquaria. To able to compare the effects of NP with those of estrogen a group of fish was exposed for two weeks 22 µg/L estrogen (17-β-estradiol). NP and 17-β-estradiol were dissolved in absolute ethanol and then introduced into the aquaria. The same amount of ethanol without NP was also added in to the tank of control group. 6 fish were put in each aquarium. 2 fish from each aquarium were sampled at the end of the first week and 4 fish from each of the treatment tanks were sampled at the end of the second week of the experiment. Five liter of water siphoned and replaced with the same volume of water and NP concentration was adjusted in accordance with the replacement everyday. Temperature was 9 ± 1 °C in each aquarium. Livers of the sampled fish were removed for FT-IR study and they were stored at -22 °C.

2.4 Sample Preparation for FT-IR Studies

5 mg liver samples taken from fish were dried in a HETO-MAXI dry lyo freeze drier for 12 hours. The samples then were ground in agate mortar containing liquid nitrogen to obtain liver powder. 1 mg liver powder was mixed 100 mg potassium bromide (at the ratio of 1/100). KBr is most commonly used alkali halide disk which is used as a beam condensing system. It is completely transparent in the mid-infrared region (Stuart, 1997). The mixture was then dried again in HETO- MAXI dry lyo freeze drier for 6 hours. In this procedure the water solution of the sample and halide is frozen and a strong vacuum is applied to the frozen solid. The mixture then was subjected to a pressure of $\sim 1100 \text{ kg/cm}^2$ in a evacuated die. This sinters the mixture and produces a clear transparent disc (Stuart, 1997).

2.5 FT-IR Studies

IR spectra were obtained using Bomem 157 FT-IR Spectrophotometer (MB Series, Canada). Dry air has been continuously purged into the spectrometer to get rid of water vapor, which disturbs the signals. Water and carbon dioxide molecules in the air affect the IR spectrum. To overcome this problem the spectrum of air was recorded as background and subtracted automatically by using appropriate software. Figure 10 shows the infrared spectrum of air used as a background in this study. Before doing any operation with FT-IR spectroscopy, background spectrum should be renewed in order to obtain more precise data. FT-IR spectra of liver samples were recorded in the $4000\text{--}400\text{ cm}^{-1}$ region at room temperature. 200 scans were taken for each interferogram at 4 cm^{-1} resolution. These spectra were rationed against a background air spectrum which was recorded by 200 scans at 4 cm^{-1} resolution in the region of $4000\text{--}400\text{ cm}^{-1}$. To make comparison between the control and treated livers, the spectra were normalized. The purpose of the normalization is to remove differences in peak heights between the spectra acquired under different conditions. It allows an point-to-point comparison to be made (Smith,1999). The ratios of the intensities and shiftings of the frequencies were examined before the normalization process.

2.6 Statistical Test:

The difference in the means of the treated and control fish were compared by means of t-test (Daniel, 1999). The p value of less than 0.05 was considered significant.

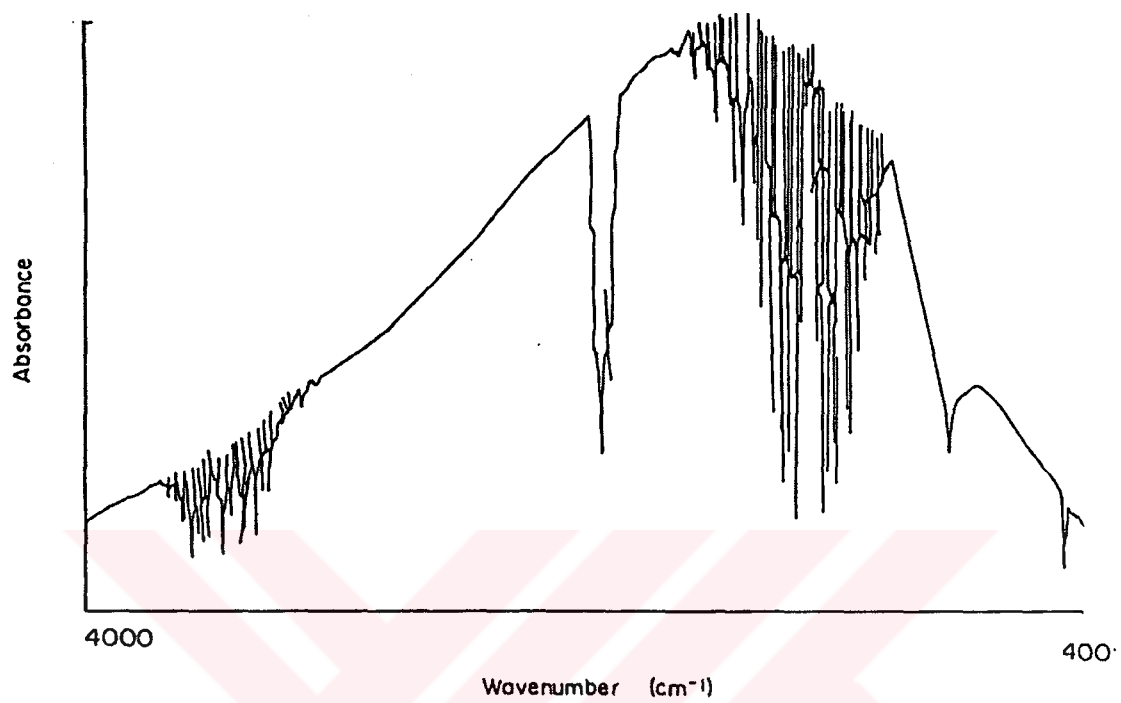


Figure 10. The Infrared Spectrum of Air.

CHAPTER 3

RESULTS

3.1 Band Assignment of the Rainbow Trout Liver Spectrum

This work was carried out to investigate the effects of Nonylphenol (NP) on the structure of rainbow trout liver by using FT-IR spectroscopic technique.

Traditionally, interpretation of infrared spectra of complex samples such as tissues follows the classical “group frequency” approach, in which absorption bands are assigned to specific vibrational modes of particular functional groups based on comparison with model systems. Using the group frequency approach to spectral analysis, the absorptions can be assigned to vibrational modes of chromophores present within proteins, lipids, carbohydrates and nucleic acids (Fabian *et al.*, 1995). In the present study, the same approach was employed.

Figure 11 shows the representative infrared spectrum of untreated (control) trout liver in the 3600-800 cm^{-1} region. The main bands were labeled in this figure and detailed spectral band assignments based upon work from this and other laboratories for liver were given in table 2.

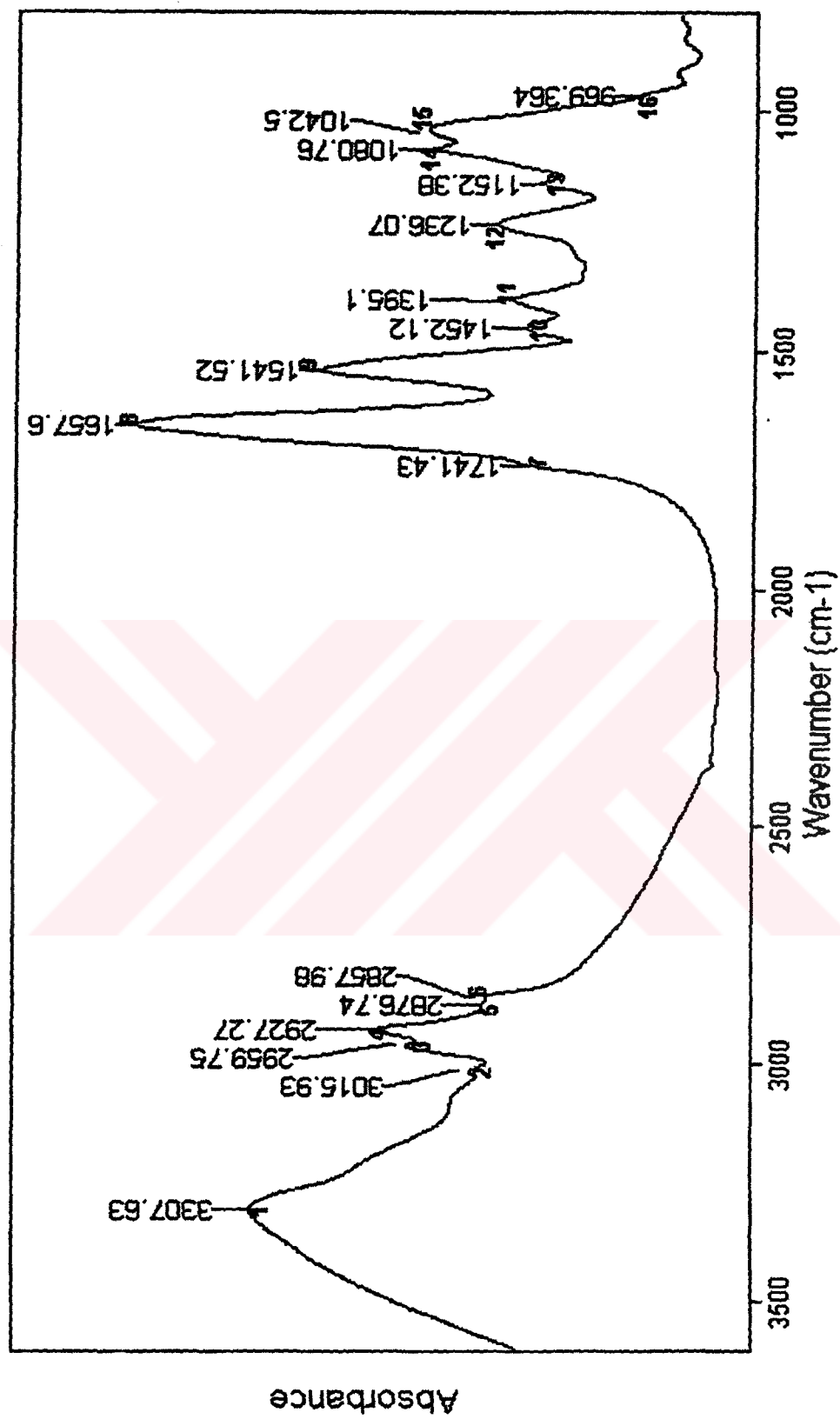


Figure11. FT-IR spectrum of the control rainbow trout liver.

Table 2. General band assignment of liver cited in literature (Rigas *et al.*, 1990; Takahashi *et al.*, 1991; Wong *et al.*, 1991; Wang *et al.*, 1997; Jamin *et al.*, 1998; Menashi *et al.*, 1998; Jackson *et al.*, 1998; Ci *et al.*, 1998; Melin *et al.*, 1999; Lyman *et al.*, 1999; Chiriboga *et al.*, 2000).

Peak numbers	Wavenumbers (cm ⁻¹)	Definition of the spectral assignment
1	3307	N-H and O-H stretching vibration: polysaccharides, proteins,
2	3015	Olefinic=CH stretching vibration: lipids, cholesterol esters
3	2959	CH ₃ asymmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
4	2927	CH ₂ asymmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
5	2876	CH ₃ symmetric stretch: mainly proteins, with the little contribution from lipids, carbohydrates, nucleic acids
6	2857	CH ₂ symmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
7	1739-1744	Ester C=O stretch: lipid, cholesterol esters
8	1657	Amide I (protein C=O stretching): α -helices
9	1541	Amide II (protein N-H bend, C-N stretch): α -helices
10	1452	CH ₂ Bending: proteins, lipids,
11	1391	COO ⁻ symmetric stretch: aminoacid side chains, fatty acids
12	1236	PO ₂ ⁻ asymmetric stretch: mainly nucleic acids with the little contribution from phospholipids
13	1152	CO-O-C asymmetric stretching: glycogen and nucleic acids
14	1080	PO ₂ ⁻ symmetric stretching: nucleic acids and phospholipids C-O stretch: glycogen
15	1042	C-O stretching: Polysaccharides
16	969	C-N ⁺ -C stretch: nucleic acids

As an example to show that 2876 cm^{-1} band is mainly from protein, pure lipid spectrum was presented in Appendix A figure 1 which contains very weak band at 2876 cm^{-1} . Whilst, the spectrum in Appendix A figure 2 belongs to a membrane spectrum with protein which has a strong band at 2876 cm^{-1} . These figures show that the band at 2876 cm^{-1} is mainly from protein.

3.2 Comparison of Liver Spectra of Control, Nonylphenol and Estrogen Treated Fish

3.2.1 Control and Nonylphenol Spectra

FT-IR spectral data were collected over the frequency range of $3600\text{--}800\text{ cm}^{-1}$. In order to display the details of spectral changes analysis were performed in two distinct frequency ranges. The first range was between $3600\text{--}2800\text{ cm}^{-1}$ and the second was between $1800\text{--}800\text{ cm}^{-1}$. The spectra of all control and two-week- $220\text{ }\mu\text{g/L}$ -NP-treated liver tissues were presented in Appendix B. As seen from these figures all control spectra are overlapped. All NP treated spectra are different then control spectra which are also overlapped. For this reason, for the following discussions only one control and NP-treated spectra will be chosen as a representative spectrum.

Figure 12 and Figure 13 present in the infrared spectra of rainbow trout livers for control, $66\text{ }\mu\text{g/L}$ NP and $220\text{ }\mu\text{g/L}$ NP treatments in the $3600\text{--}2800\text{ cm}^{-1}$ region. In these figures, treatment times were one and two weeks respectively. As it could be seen from these figures fish exposed to different NP concentrations for various exposure times yielded spectra considerably differing in peak positions, peak heights and the widths in the first region ($3600\text{--}2800\text{ cm}^{-1}$) of the analysis. Dose dependent progressive different from the control could be seen in the first week (Figure 12). The difference was even more pronounced for $220\text{ }\mu\text{g/L}$ NP treatment in the second week (Figure 13).

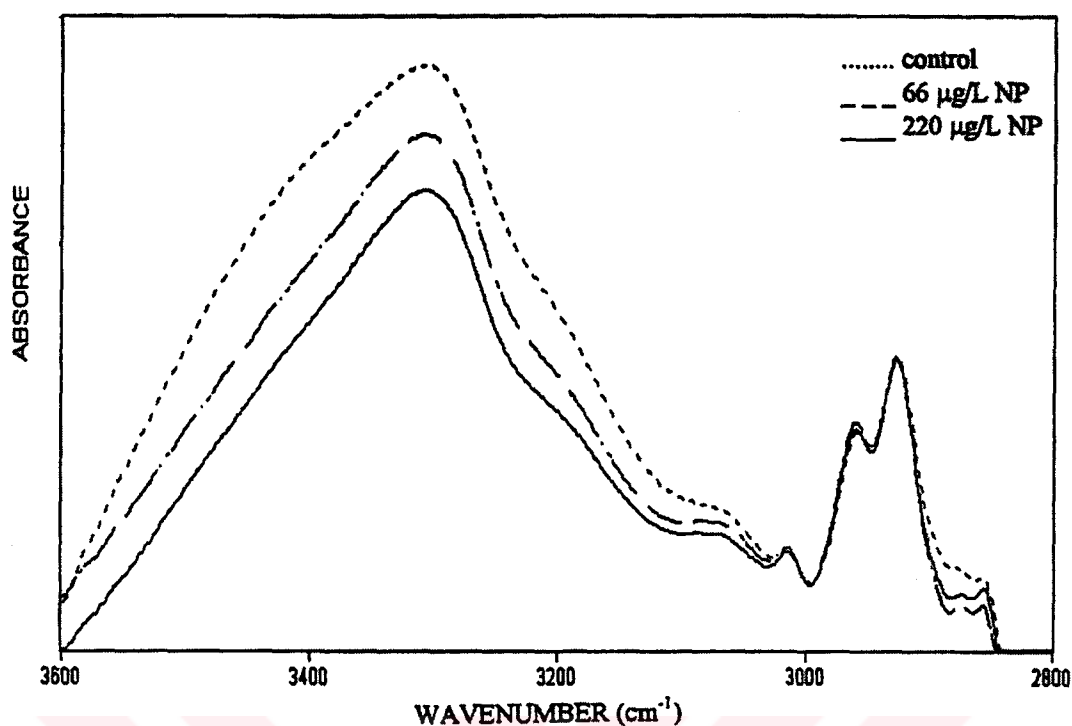


Figure 12. 3600-2800 cm^{-1} region of the infrared spectra of rainbow trout livers treated with 0 (control), 66 and 220 $\mu\text{g/L}$ NP for one week (the spectra were normalized with respect to the CH_2 asymmetric mode which is observed at 2927 cm^{-1}).

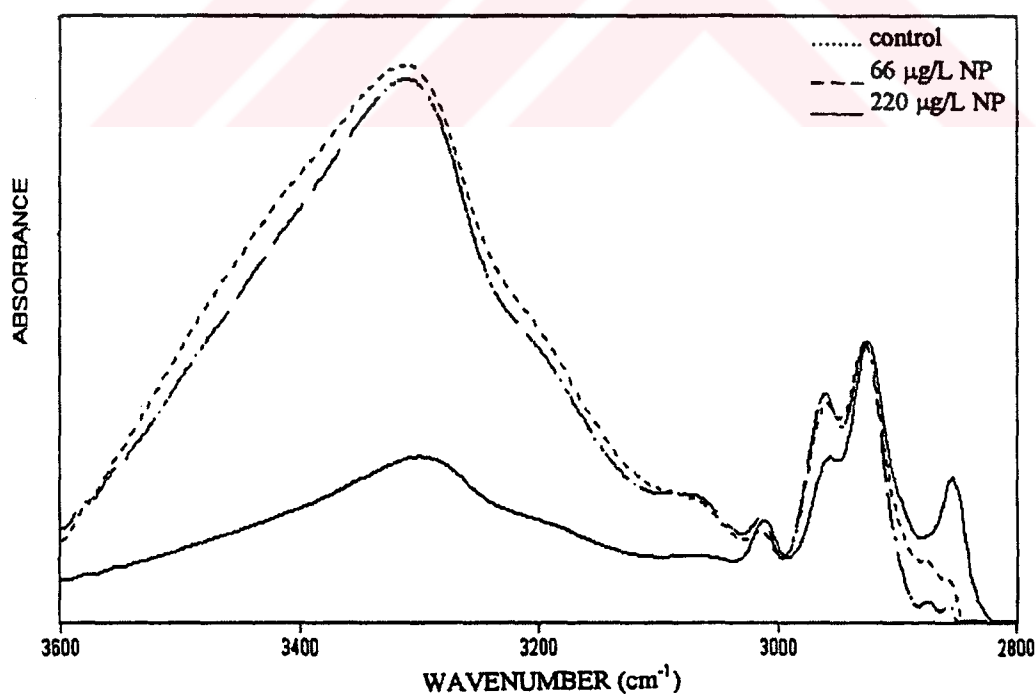


Figure 13. 3600-2800 cm^{-1} region of the infrared spectra of rainbow trout livers treated with 0 (control), 66 and 220 $\mu\text{g/L}$ NP for two weeks (the spectra were normalized with respect to the CH_2 asymmetric mode which is observed at 2927 cm^{-1}).

Figure 14 and 15 show the infrared spectra of the liver tissues at the treatments of 0 (control), 66 and 220 $\mu\text{g/L}$ NP in the $1800\text{-}800\text{ cm}^{-1}$ region after one and two week treatments, respectively. As it could be seen from these figures, different NP concentrations induced remarkable changes, in the bandwidth, intensity and frequency values of the FT-IR bands after one and two week exposure times, which will be later discussed more specifically. The differences are, in parallel to the ones observed in the first region, more significant in the 220 $\mu\text{g/L}$ NP-treated liver spectrum.



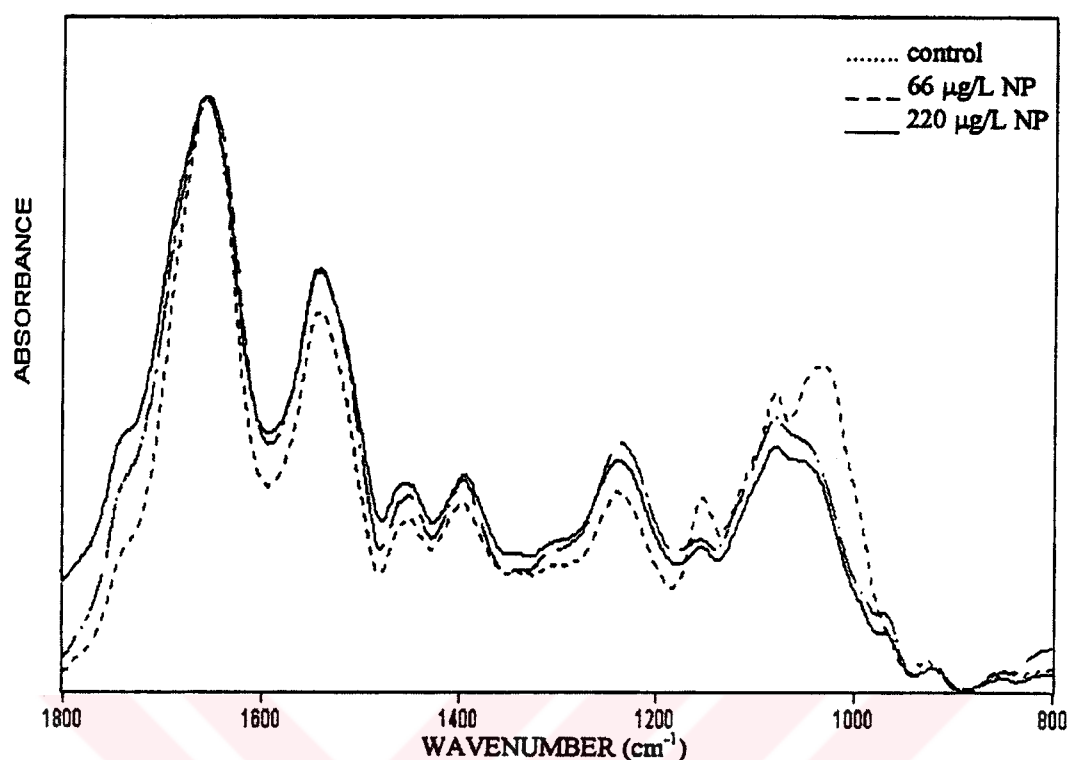


Figure 14. 1800-800 cm^{-1} region of the infrared spectra of rainbow trout livers treated with 0 (control), 66 and 220 $\mu\text{g/L}$ NP for one week (the spectra were normalized with respect to the amide I band which is observed at 1657 cm^{-1}).

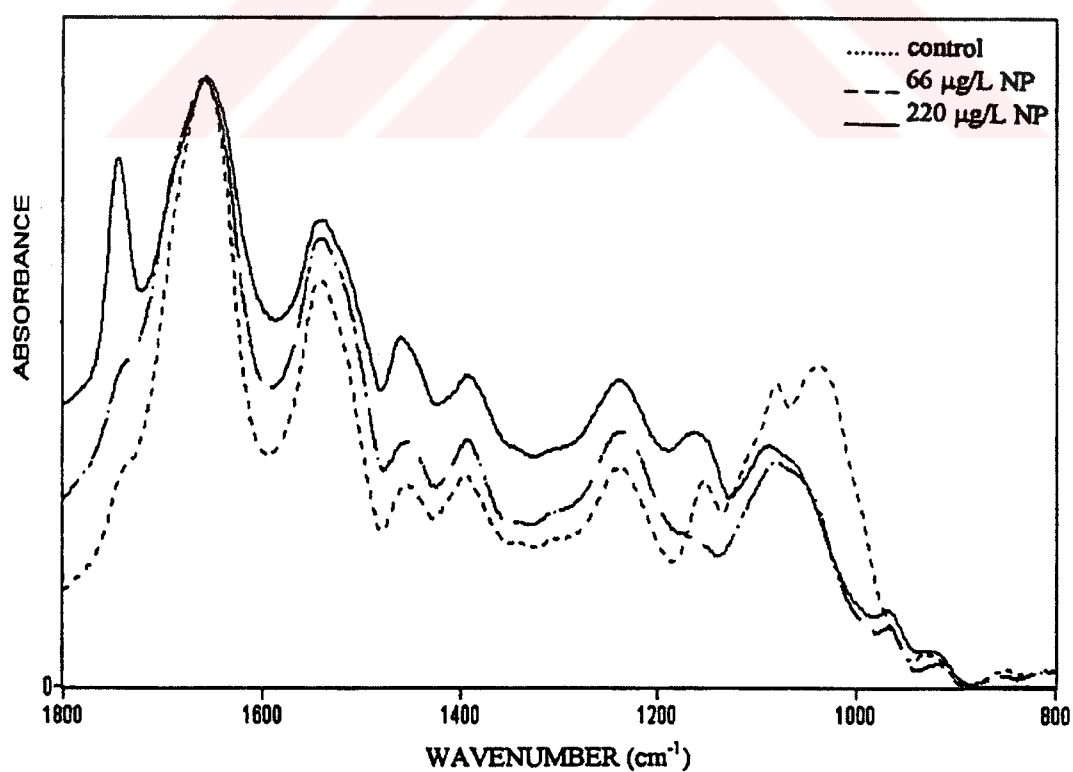


Figure 15. 1800-800 cm^{-1} region of the infrared spectra of rainbow trout livers treated with 0 (control), 66 and 220 $\mu\text{g/L}$ NP for two weeks (the spectra were normalized with respect to the amide I band which is observed at 1657 cm^{-1}).

3.2.2. Control, Nonylphenol and Estrogen Spectra

In order to compare the effects of NP which are detected by FT-IR with those of livers of the fish treated with 22 µg/L 17-β Estradiol (E2) for two weeks were also analyzed. Because the estrogenic compounds' relative potencies were approximately 100.000 fold less than that of the natural estrogen E2 (Jobling *et al.*, 1996), we chose the 22 µg/L concentration of E2 to expose the juvenile fish. Infrared spectra from both E2 and various concentrations of NP treatments were shown in two regions of the spectra in figures 16 and 17 As it can be seen from these figures, the 22 µg/L E2 treated liver spectrum showed similar changes to those generated by NP-treated spectra, which will be discussed later in more detail, and the spectrum from E2 treatment is between 66 and 220 µg/L NP treated spectra.

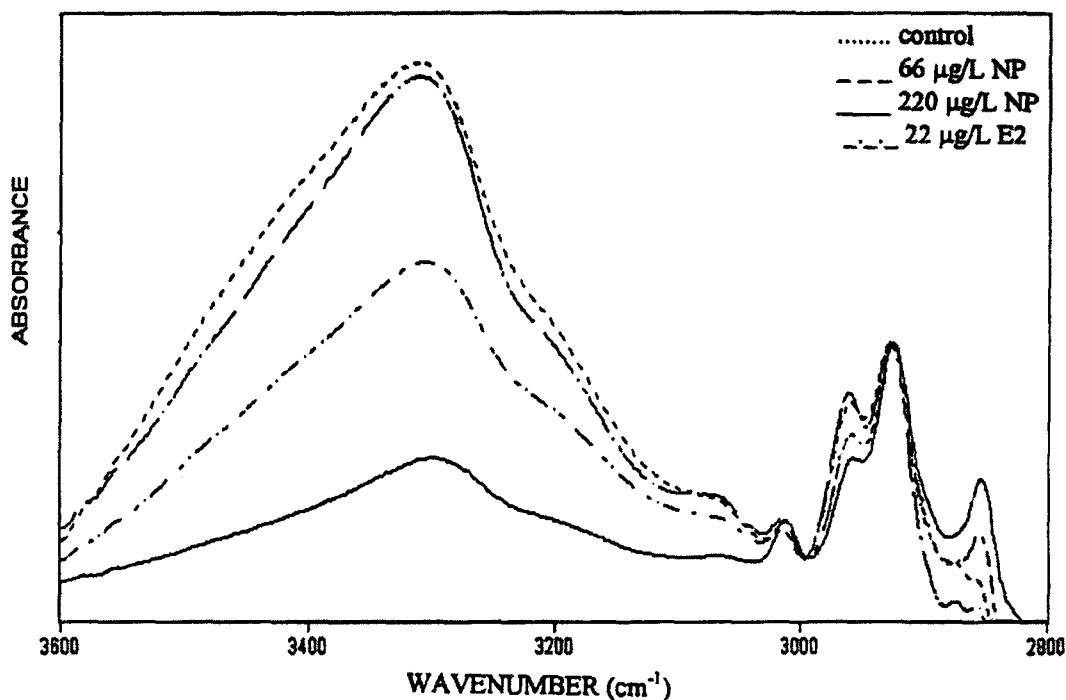


Figure 16. 3600-2800 cm^{-1} region of the infrared spectra of rainbow trout livers treated with 0 (control), 66 and 220 $\mu\text{g/L}$ NP and 22 $\mu\text{g/L}$ E2 for two weeks (the spectra were normalized with respect to the CH_2 asymmetric mode which is observed at 2927 cm^{-1}).

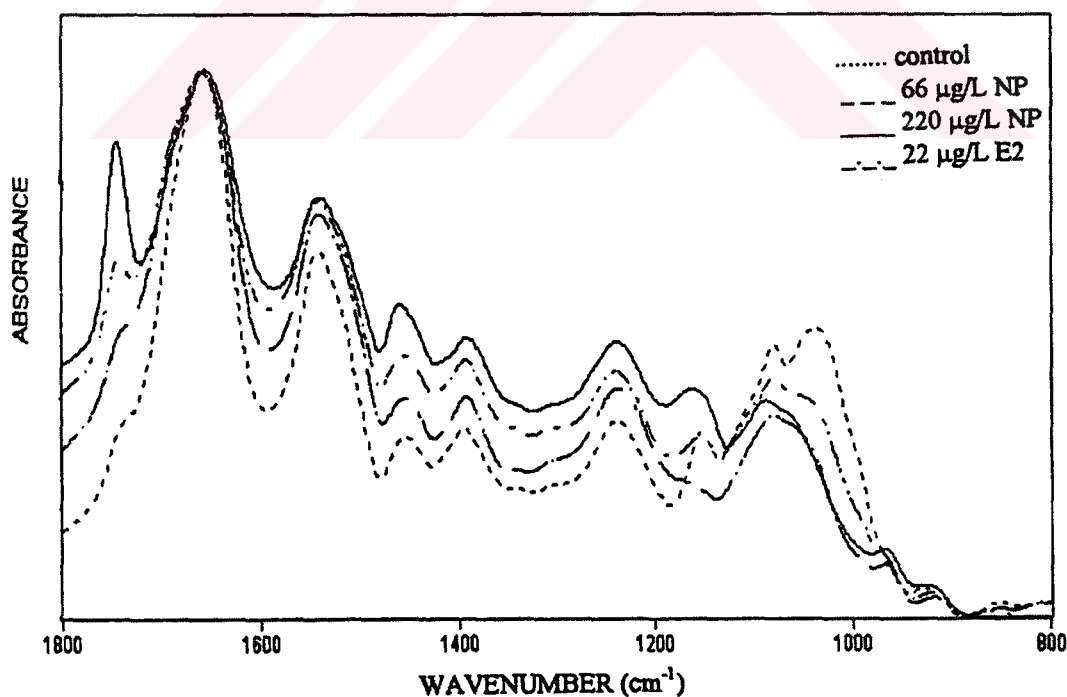


Figure 17. 1800-800 cm^{-1} region of the infrared spectra of rainbow trout livers treated with 0 (control), 66 and 220 $\mu\text{g/L}$ NP and 22 $\mu\text{g/L}$ E2 for two weeks (the spectra were normalized with respect to the amide I band which is observed at 1657 cm^{-1}).

3.2.3 Numerical Comparisons of the Bands of Control, Nonylphenol and Estrogen Spectra

In order to take into account of possible spectral differences that could be observed between the individuals of even same treatment group, the means and the standard deviations of the band intensities, bandwidths and direction of the shifts with respect to the control were obtained for control, 220 µg/L NP and 22 µg/L E2 treatments. NP, E2 treatment means were compared with those of control means by the help of t-test. Results were given in table 3.

The table illustrated the presence of significant ($p < 0.05$ – $p < 0.001$) changes in the spectra of both 220 µg/L NP and 22 µg/L E2 treated fish for especially 220 µg/L NP. In the few instances (band no 4 and 8) comparisons could not be carried out because of the normalization process. Again for band numbers 5, 15 and 16 because there were no band intensities obtained for some treatment or control groups, comparisons were not possible. In 7 cases where both of the treatments (NP and E2) were significantly different from those of the control's changes in band intensities and bandwidths were parallel to each other. When the directions of the shifts with respect to control were examined out of 13 comparisons 7 of them were in parallel to each other for NP and E2 treatments and only 2 of them were opposite to each other. In two of the comparisons there were no differences in E2 spectrum compared to control spectrum.

As a summary it can be said that both NP and E2 treatments generated significant changes in the spectra and most of the time they were parallel to each other.

Because the most considerable differences were observed after two week exposure time in the 220 µg/L NP-treated liver spectrum, the spectrum of control and two-week-220 µg/L-NP-treated liver spectrum will be compared in the following section.

Table 3. Numerical summary of the detailed differences for 220 µg/L NP and 22µg/L E2 treated spectra from that of the control spectra. The values are the mean ± Standard Deviation for each sample. Upper p values were for NP vs control and lower p values were for E2 vs control comparisons. Arrows to the left indicated a decrease and to the right indicated an increase with respect to the control.

Band No	BAND FREQUENCY			BAND INTENSITY			BANDWIDTH		
	CONTROL (n=6)	220 µg/L NP (n=4)	22µg/L E2 (n=3)	P value	CONTROL	220µg/L NP	22µg/L E2	P value	P value
1	3311.13 ± 2.076	3302.7 ± 1.46 ←	3306.36 ± 1.25 ←	<0.001 <0.01	2.123 ± 0.212	0.461 ± 0.042	1.67 ± 0.115	<0.001 <0.01	<0.01 <0.01
2	3015.86 ± 0.854	3012.4 ± 0.287 ←	3014.9 ± 0.643 ←	<0.001	0.137 ± 0.01	0.152 ± 0.047	0.16 ± 0.025		
3	2959.23 ± 0.492	2956.8 ± 0.341 ←	2959.2 ± 0.378 ←	<0.001	0.694 ± 0.025	0.455 ± 0.013	0.665 ± 0.04	<0.001	
4	2927.51 ± 0.519	2925.7 ± 0.408 ←	2927.4 ± 0.462 ←	<0.001	1	1	1		
5	2877.45 ± 1.347	Not observed	Not observed		0.036 ± 0.027	Not observed	Not observed		
6	2857.48 ± 0.746	2854.5 ± 0.238 ←	2856.53 ± 0.55 ←	<0.001	0.027 ± 0.032	0.353 ± 0.014	0.249 ± 0.07	<0.001 <0.01	
7	1741.32 ± 0.694	1745.05 ± 0.31 →	1741.5 ± 1.703 →	<0.001	0.294 ± 0.073	0.861 ± 0.014	0.464 ± 0.118	<0.001	<0.05
8	1657.43 ± 0.413	1656.5 ± 0.708 ←	1658.03 ± 0.45 →	<0.05	1	1	1		
9	1540.31 ± 0.371	1541.6 ± 1.302 →	1539.96 ± 0.85 ←		0.666 ± 0.023	0.721 ± 0.044	0.713 ± 0.029	<0.05 <0.05	
10	1452.45 ± 0.595	1457.9 ± 0.294 →	1453.9 ± 0.832 →	<0.001 <0.05	0.311 ± 0.02	0.514 ± 0.045	0.357 ± 0.06	<0.001	
11	1396.35 ± 0.755	1391.55 ± 0.45 ←	1393.23 ± 0.49 ←	<0.001 <0.01	0.334 ± 0.017	0.455 ± 0.046	0.381 ± 0.06	<0.001	
12	1236.96 ± 1.83	1237.05 ± 1.51 →	1238.7 ± 1.115 →		0.367 ± 0.025	0.502 ± 0.023	0.416 ± 0.031	<0.001	
13	1152.5 ± 0.563	1161.07 ± 1.37 →	1152.96 ± 0.55 →	<0.01	0.313 ± 0.031	0.451 ± 0.051	0.236 ± 0.058	<0.01	
14	1080.33 ± 0.512	1090.05 ± 1.55 →	1081.8 ± 1.942 →	<0.001	0.486 ± 0.029	0.415 ± 0.046	0.391 ± 0.039	<0.05 <0.001	
15	1039.96 ± 1.011	Not observed	Not observed		0.504 ± 0.049	Not Observed	Not observed		
16	Not observed	969.69 ± 0.224	968.073 ± 0.17		Not observed	0.138 ± 0.027	0.096 ± 0.014		

3.3 Detailed Spectral Analysis

3.3.1 Comparison of Control and NP-Treated Liver Spectra in the 3600-2800 cm^{-1} Region

The 3600-2800 cm^{-1} region contains different bands centered at 3307, 3015, 2959, 2927, 2876 and 2857 cm^{-1} , corresponding to the stretching mode of OH and/or NH, H-C=C-H, both asymmetrical CH_3 and CH_2 , and both symmetrical CH_3 and CH_2 vibrations, respectively (Melin *et al.*, 1999).

Figure 18 shows the infrared spectra of control rainbow trout liver and 220 $\mu\text{g/L}$ NP-treated samples in the 3600-3050 cm^{-1} region after two week treatment. As can be seen from this figure in the treated liver spectrum there was a dramatic reduction ($p < 0.001$) in the intensity of the band at 3307 cm^{-1} , which contains strong absorptions arising from N-H and O-H stretching modes of proteins and polysaccharides. We also observed a reduction in the bandwidth of the treated spectrum. The bandwidth values were listed in table 3.

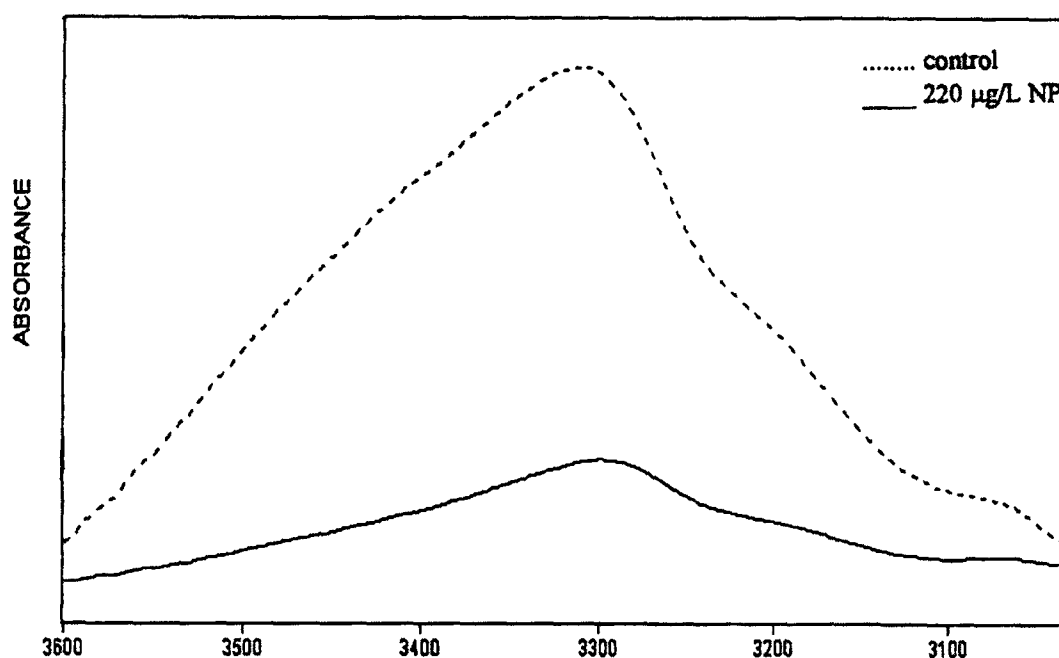


Figure 18. 3600-3030 cm^{-1} region of the infrared spectra of rainbow trout livers treated with 0 (control) and 220 $\mu\text{g/L}$ NP for two weeks (the spectra were normalized with respect to the CH_2 asymmetric mode which is observed at 2927 cm^{-1}).

Figure 19 shows the normalized infrared spectra of control rainbow trout liver and 220 $\mu\text{g/L}$ NP-treated samples in the 3030-2800 cm^{-1} region after two week treatment. The intensity of the band at 3015 cm^{-1} , which is due to the CH stretching mode of $\text{HC}=\text{CH}$ groups, can be used as a measure of unsaturation in the acyl chains (Takahashi *et al.*, 1991). As it can be seen from this figure, the intensity of this band increased slightly in the 220 $\mu\text{g/L}$ NP-exposed liver spectrum during two week. Also the maximum of this band was shifted to lower frequency values in the 220 $\mu\text{g/L}$ NP-treated liver from $3015.8 \pm 0.854 \text{ cm}^{-1}$ to $3012.4 \pm 0.287 \text{ cm}^{-1}$ ($p < 0.001$).

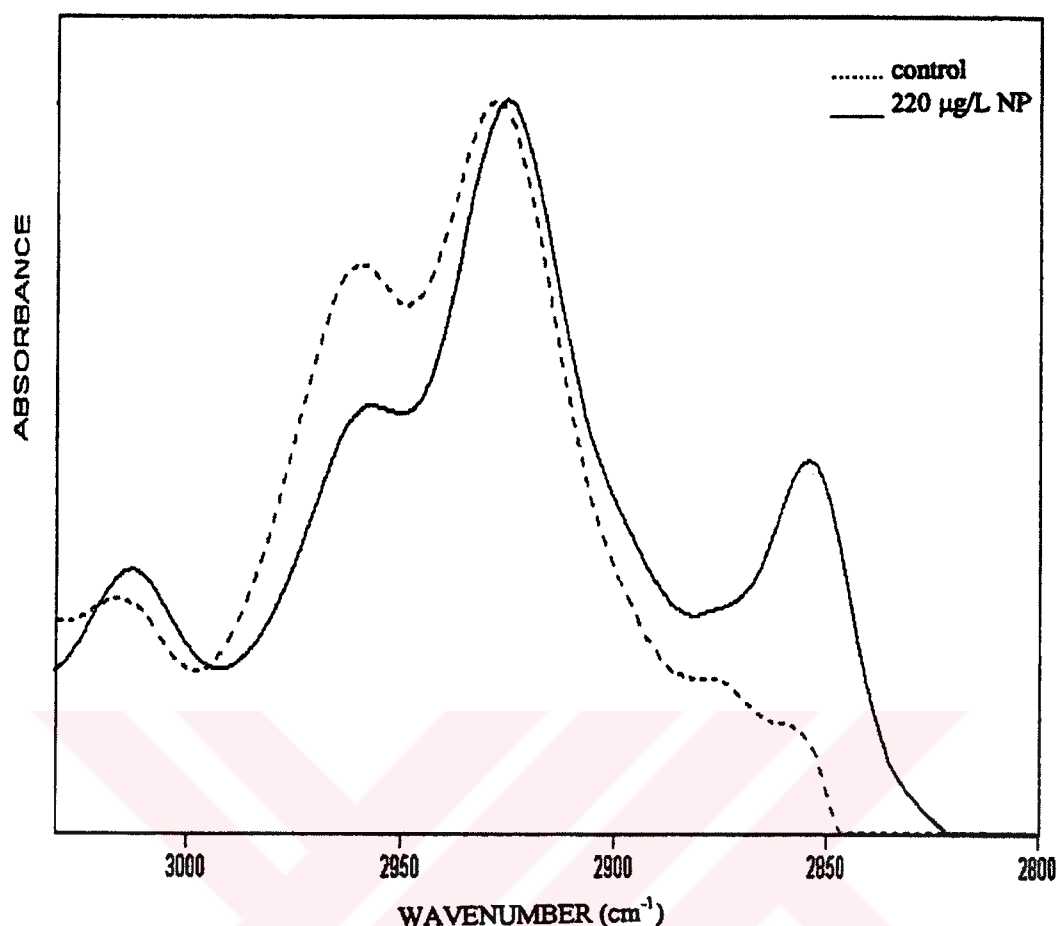


Figure 19. The C-H stretching region of the infrared spectra of rainbow trout livers treated with 0 (control) and 220 $\mu\text{g/L}$ NP for two weeks (the spectra were normalized with respect to the CH_2 asymmetric mode which is observed at 2927 cm^{-1}).

As a consequence of NP treatment for two weeks, the absorption intensity of the CH_3 asymmetric stretching vibration at 2959 cm^{-1} was decreased dramatically ($p < 0.001$) and the wavenumber of this and the CH_2 symmetric (at 2857 cm^{-1}) band was dramatically ($p < 0.001$) shifted to lower frequency values for NP-exposed liver spectrum. Moreover, the wavenumber of the CH_2 asymmetric stretching band shifted significantly ($p < 0.001$) to lower values from 2927.5 ± 0.519 to $2925.7 \pm 0.408 \text{ cm}^{-1}$. Also the bandwidth of this band decreased dramatically ($p < 0.001$) in the 220 $\mu\text{g/L}$ NP treated group as it can be seen from table 3.

Figure 19 displayed that the intensity of the CH₂ symmetric stretching vibration band at 2857 cm⁻¹ is dramatically increased in 220 µg/L NP-treated liver (p<0.001) and the shapes of this band is more symmetric compared to that of control liver. The frequency of this band was shifted dramatically (p<0.001) to lower value after 220 µg/L NP treatment. The CH₂ stretching frequency reflects mainly conformational disorder and they increase with the introduction of gauche conformers in the fatty acyl chains (Rana *et al.*, 1990; Schultz *et al.*, 1991 and Melin *et al.*, 1999).

It is also observed that the band at 2876 cm⁻¹, which is assigned as CH₃ symmetric stretching is almost diminished in 220 µg/L NP- treated liver spectrum after two-week-treatment time.

3.3.2 Comparison of Control and NP-Treated Liver Spectra in the 1800-800 cm⁻¹ Region

The second frequency range, which is under consideration, was that of 1800-800 cm⁻¹ region. Figure 20 shows the 1800-800 cm⁻¹ region of the normalized FT-IR spectra of control and 220 µg/L NP-treated livers after two weeks of exposure. The band centered at 1739 cm⁻¹ was mainly assigned to the >C=O ester stretching vibration in phospholipids (Melin *et al.*, 1999). The bands at 1657 and 1541 cm⁻¹ are attributable to the amide I and II vibrations of structural proteins. In addition, the intense band at 1452 cm⁻¹ can be assigned to the CH₂ bending mode of proteins and lipids (Manoharan *et al.*, 1993). The band at 1391 cm⁻¹ is due to COO⁻ symmetric stretching vibration of amino acid side chains and fatty acids (Jackson *et al.*, 1998). The relatively strong bands at 1236 and 1080 cm⁻¹ are mainly due to the asymmetric and symmetric stretching modes of phosphodiester groups in nucleic acids rather than in phospholipids, respectively (Wong *et al.*, 1991; Wang *et al.*, 1997 and Menashi *et al.*, 1998). However, the symmetric phosphate stretching band might be masked by spectral contributions of glycogen, which exhibits three strong peaks at 1042, 1080, and 1152 cm⁻¹ (Chiriboga *et al.*, 2000). The relatively weak band at 1152 cm⁻¹ is due to

stretching mode of CO-O-C groups present in glycogen and nucleic acids. (Rigas *et al.*, 1990). The band at 1042 cm^{-1} is assigned as mainly to C-O stretching vibrations in polysaccharides (Malin *et al.*, 1999). The band centered at 969 cm^{-1} is generally assigned to symmetric stretching mode of dianionic phosphate monoester of cellular nucleic acids, especially for DNA (Ci *et al.*, 1999 and Chiriboga *et al.*, 2000).

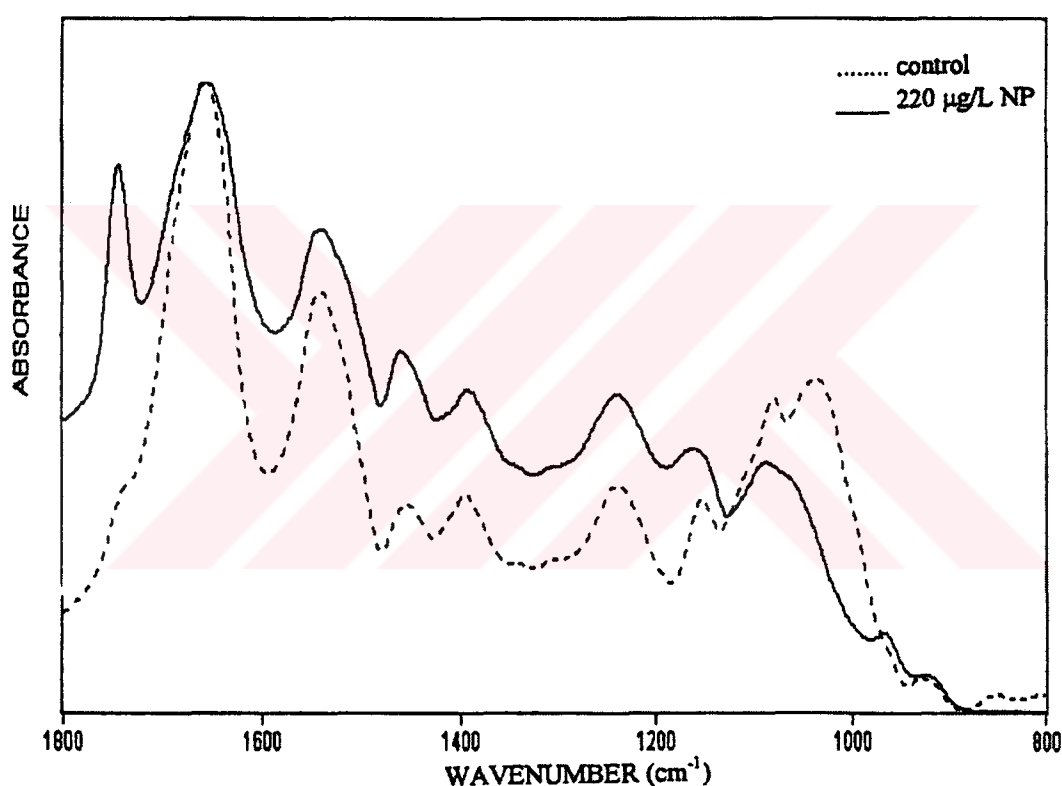


Figure 20. $1800\text{--}800\text{ cm}^{-1}$ region of the infrared spectra of rainbow trout livers treated with 0 (control) and $220\text{ }\mu\text{g/L}$ NP for two weeks (the spectra were normalized with respect to amide I band which is observed at 1657 cm^{-1}).

As it was illustrated in figure 20, the intensity of the lipid C=O stretching vibration at 1739 cm^{-1} was increased dramatically in the spectrum of $220\text{ }\mu\text{g/L}$ NP-treated liver ($p < 0.001$). In addition to an increase in the intensity, the

frequency of the absorption maximum of the ester C=O stretching vibration was shifted from 1741.32 ± 0.694 to $1745.05 \pm 0.31 \text{ cm}^{-1}$ ($p < 0.001$), indicating a difference in packing of ester groups within the tissue (Jackson *et al.*, 1998).

The intensities of the bands in protein amide groups centered at 1657 cm^{-1} corresponding to the stretching C=O and bending C-N (amide I) vibrational modes of the polypeptide and protein backbone and at 1541 cm^{-1} assigned to the bending N-H and stretching C-N (amide II) ones were also altered after $220 \mu\text{g/L}$ NP exposing during two week. The amide I region is useful for determination of protein secondary structure because the frequency of vibration is very sensitive to changes in the nature of hydrogen bonds in the different types of protein secondary structures (Lyman *et al.*, 1998). As could be easily seen from figure 20 and table 3, there was a broadening in the bandwidth of the amide I band in the $220 \mu\text{g/L}$ NP-treated liver ($p < 0.05$). The absorbance for amide II (at 1541 cm^{-1}) band increased and the bandwidth of this band was broaden significantly ($p < 0.01$) in $220 \mu\text{g/L}$ NP-treated liver compared to that of the control group.

The ratios of the absorbance of some bands in this region were calculated and listed in table 4. As it can be seen from this table the intensity ratio between amide I and amide II bands ($1657/1541$) decreased in the NP-treated group. This reduction in intensity reflected a change in the intensity of the C=O stretching vibration located at 1657 cm^{-1} after two-week NP treatment (Melin *et al.*, 1999).

Table 4. Ratios of the Intensities. The means and the standard deviations and the sample sizes of the groups (n) are given.

Ratio of Band Intensities	Control Groups (n=6)	220 $\mu\text{g/L}$ NP-treated livers (n=4)	p Values
I_{1657}/I_{1541}	1.496 ± 0.051	1.362 ± 0.051	< 0.01
I_{1042}/I_{1080}	1.028 ± 0.047	0.931 ± 0.082	< 0.1

Moreover, an increase in the intensity was observed at 1452 cm^{-1} that was due to the bending vibration of CH_2 in the lipids and proteins. As seen from table 3 there was a dramatic ($p < 0.001$) shifting from $1452.45 \pm 0.595\text{ cm}^{-1}$ to $1457.9 \pm 0.294\text{ cm}^{-1}$ in the frequency of this band.

Also, we observed a dramatic increase in the intensity of COO^- symmetric stretching vibration band at 1395 cm^{-1} that was due to amino acid side chains and fatty acids. In addition, there was a significant ($p < 0.001$) shifting to lower value in the frequency of $220\text{ }\mu\text{g/L}$ NP-treated liver (table 3).

In the $1280\text{-}900\text{ cm}^{-1}$ frequency range, mainly several macromolecules (e.g. polysaccharides and phosphate carrying compounds such as phospholipids and nucleic acids) show absorption bands (Melin *et al.*, 1999). Study of the frequency of this region revealed significant differences in the infrared spectra between control and treated tissues. In the present work, figure 20, also indicated remarkable differences in the bands located $1280\text{-}900\text{ cm}^{-1}$. In these spectra the strongest band were at 1236 cm^{-1} and 1080 cm^{-1} . They were due to the asymmetric and symmetric phosphate stretching modes, respectively. These bands originated mainly due to in the phosphodiester backbone of cellular nucleic acids (Rigas *et al.*, 1990). In all cases, when compared with control tissues, the treated tissues, displayed dramatically increased intensity of the phosphate asymmetric stretching band ($p < 0.001$), decreased intensity of the symmetric phosphate stretching band ($p < 0.01$), and changes in the shape of these bands, as shown in figure 20. The ratio of the absorption at 1042 cm^{-1} (C-O stretching vibration) to that at 1080 cm^{-1} (phosphate symmetric stretching vibration) was calculated and listed in table 4. The value of this ratio for $220\text{ }\mu\text{g/L}$ NP-treated samples was very small while the control group has the higher ratio. In addition, the frequencies of these bands were shifted in the treated tissues. The phosphate asymmetric band was shifted from 1238 in the normal tissue to 1239 , while the phosphate symmetric band was shifted from 1080 to 1088 cm^{-1} . Of particular interest is the effect of NP in the spectral region between 1152 and 967 cm^{-1} where we observed a large decrease in the intensity, ~ 1080 and 1042 cm^{-1} that are assigned mainly to

the C-O stretching vibrations in polysaccharides. On the other hand, in the 900-1238 cm^{-1} region there were significant nucleic acid vibrations. The 900-1100 cm^{-1} regions of nucleic acids may be masked by spectral contributions of glycogen, which exhibited three strong peaks at 1042, 1080 and 1152 cm^{-1} . In addition, the infrared spectrum of control sample in the CO-O-C asymmetric stretching region consisted of a relatively narrow and symmetric band near 1152 cm^{-1} where treated samples gave relatively broader and asymmetric band near 1163 cm^{-1} .

It was found that the intensity of the band at 1080 cm^{-1} in control liver spectrum was much higher than that of treated one as could be seen from figure 20. The maximum frequency of this band shifted dramatically to higher value in the 220 $\mu\text{g/L}$ NP-treated liver spectrum (table 3).

It is also seen that, the band at 1042 cm^{-1} is assigned mainly to C-O stretching vibrations in polysaccharides. The peak of this band was almost lost in treated liver spectrum indicating a change in the types of glycoproteins in the treated tissues (Lyman *et al.*, 1998).

The band at 969 cm^{-1} which is due to nucleic acids was sharper and stronger in the NP-treated liver spectrum than that in the control group spectrum.

CHAPTER 4

DISCUSSION

In this study, FT-IR technique has been used to investigate the effect of NP on rainbow trout liver tissues. Experiments carried out on the rainbow trout liver demonstrated that the degree of differences observed between control and treated group in trout exposed to NP is both dose-dependent and affected by the duration of exposure. When it was compared the 66 µg/L NP treated liver spectrum with the 220 µg/L NP treated liver spectrum, it was seen that 220 µg/L NP induced more significant changes than that of 66 µg/L NP. So different concentrations of NP have different effect on the system. As seen from figure 12 and 14, the treatment of NP at 66 µg/L and 220 µg/L during one week induced some changes in the liver tissues. However, figure 13 and 15 show that exposing to especially high NP concentrations for two weeks induce more pronounced changes in the tissues. Also we treated juvenile fish E2 during two weeks to see whether NP shows estrogenic effects. As it can be seen from figure 16 and 17, the E2-treated liver spectrum and 220 µg/L NP treated liver spectrum exhibited many similar changes in these tissues. More detailed comparisons will be made in a future study.

In a parallel study which is not published yet, significant ($p < 0.05$) bioaccumulation of NP in the body of the rainbow trout in a dose and time dependent manner and histopathologic changes in the liver of the fish were observed. Severity of the pathologic changes in livers also increased in a time and dose dependent manner (Uğuz *et al.*, unpublished).

With the FT-IR analysis the effects of NP on the macromolecules in tissues will be assessed. Since the effects of NP were more clearly seen in the spectra of 220 µg/L NP treated fish, the detailed comparisons with the treated and control fish were carried out only for this NP concentration.

As seen from table 3, we observed statistically significant differences in band intensities, bandwidths, band frequency values between the control and 220 µg/L-NP-exposed group. Also, figures 18, 19 and 20 showed that a number of differences were immediately apparent between the spectra of these two groups.

The 3307 cm⁻¹ band arises from both the NH and OH stretching modes in H₂O, proteins and polysaccharides (Jamin *et al.*, 1998). Here in our case since water was removed by freeze drying its contribution to this band can be neglected and the contribution was considered due only to proteins and polysaccharides. As it can be seen from figure 18, there was a dramatic reduction in the intensity of this band in the NP treated liver spectrum. One explanation for the dramatic reduction in the intensity of this band might be due to a reduced contribution from glycogen OH absorption (Melin *et al.*, 1999). As a strong support to the suggested explanation for the reduced intensity of 3307 cm⁻¹ band, the peak of the band at 1042 cm⁻¹ that is assigned mainly to C-O stretching vibrations in glycogen, was almost lost in treated liver spectrum (figure 20) (Lyman *et al.*, 1998). So, NP treatment could result in the decrease of glycogen level.

As it can be seen from figure 19, the pronounced differences are seen between 3050-2800 cm⁻¹, a region of the spectrum populated by absorptions arising from C-H stretching vibrations of olefinic=CH, -CH₂ and -CH₃ groups. The increase in intensity of each of these absorptions suggests an increased concentration of these chromophores within NP-treated tissue (Jackson *et al.*, 1998). The band at 3015 cm⁻¹ is due to the CH stretching mode of HC=CH groups. Therefore the intensity of this band can be used as a measure of unsaturation in the acyl chains (Takahashi *et al.*, 1991). As it can be seen from figure 19, the intensity of this band increased for 220 µg/L NP exposed liver in

two weeks. Here, in accordance to Takahashi *et al.*, (1991), this result indicated that the population of unsaturation was increased in the 220 µg/L NP-exposed liver. Following the NP toxication the total content of fatty acids was enhanced, particularly unsaturated fatty acids in NP treated fish. This might be due to alterations in the rates of synthesis or it might be attributed to the increased storage of unsaturated fatty acids or to the reduced metabolism and transport of lipids. The maximum of this band was also shifted to lower frequency values in the 220 µg/L NP-exposed liver. Thus, the increasing in the intensity and shifting the frequency value may be resulted from a change in lipid metabolism induced by NP. The conclusion is in agreement with the results obtained from 1800-800 cm^{-1} which was illustrated in figure 20. As seen from this figure, the intensity of the lipid C=O stretching vibration at 1739 cm^{-1} is increased dramatically ($p < 0.001$) in the spectrum of the 220 µg/L NP-treated liver. This dramatic increase in the intensity of this band suggests an increased concentration of ester groups within 220 µg/L NP exposed liver during two weeks. This was also confirmed by an increase in the intensity of the asymmetric CO-O-C stretching vibration at 1152 cm^{-1} . In addition to an increase in intensity, the frequency of the absorption maximum of the ester C=O stretching vibration was shifted from 1739 to 1745 cm^{-1} , indicating a difference in packing of ester groups within the tissue (Jackson *et al.*, 1998). This difference in packing is presumably the result of the increased proportion of unsaturated acyl chains in the 220 µg/L NP-treated liver as also suggested by the increased intensity of the olefinic=CH stretching absorption at 3015 cm^{-1} . The increased intensity of the ester and CH_2 absorption may suggest an increase in phospholipid and acylglyceride concentration. An increased phospholipid content might be responsible for the spectral changes discussed above, as it is expected that the asymmetric and symmetric PO_2 stretching vibrations at 1240 and 1080 were increased in intensity as the ester C=O absorption increased in intensity. These findings suggested that the changes described are result from phospholipid accumulation as reported by Jackson *et al.*, (1998). The enhanced intensity of the peak near 1739 cm^{-1} concomitant with that at 1452 cm^{-1} could also suggest an increased concentration of lipid ester groups within the liver (Melin *et al.*, 1999).

Scwaiger *et al.*, (2000) demonstrated that alkylphenolic compounds exert highly toxic effects on the organisms. Liver is a site where significant xenobiotic metabolism occurs. Since the liver is the site of synthesis, storage and release of lipids, it stands to reason that interference with these processes by a toxic compound could lead to an accumulation of fats in the liver itself. The exposure of xenobiotics can block the secretion of a type of lipid called triglycerides, leading to the development of what is commonly called a “fatty liver” where anywhere from 5 to 50% of the liver’s weight is fat (Stine and Brown, 1996). Consequently, in our studies the changes at 3015, 1739, 1452, and 1152 cm^{-1} (figure 19 and 20) might be partly assigned to high concentration of cholesterol esters present in the lipoprotein complexes synthesized in the liver or to triglyceride accumulation as a consequence of the inhibitory transfer of triglyceride-rich low density lipoproteins (LDL) from liver to plasma (Melin *et al.*, 2000). The increased intensity of C=O, CO-O-C and fatty acyl CH_2 groups might be attributed to an elevated level of cholesterol esters/LDL in the liver (Jackson *et al.*, 1998).

Another interesting observation derived from Figure 19 that, as a consequence of NP treatment for two weeks, the absorption intensity of the CH_3 asymmetric stretching vibration at 2959 cm^{-1} was decreased dramatically. So we can say that the composition of acyl chains was also changed in the NP-treated livers. The number of methyl groups in each acyl chain was decreased as indicated by the decrease in the intensity of the methyl CH stretching band at $\sim 2959 \text{ cm}^{-1}$ in the spectrum of the two-week-NP-treated livers (Takahashi *et al.*, 1991).

As seen from table 2, the frequency of the symmetric CH_2 stretching band at 2857 cm^{-1} was shifted to lower value (from 2857.48 ± 0.746 to 2854.55 ± 0.238) after NP treatment. Decreases in the frequency of the CH_2 symmetric stretching mode for this system in the presence of NP indicated a reorganization of the membrane to an average bilayer structure (Lewis *et al.*, 1989). In addition to this shifting, the intensity of this band was increased significantly ($p < 0.001$) in treated tissues. This finding suggested an increased proportion of CH_2 groups in these tissues. Such changes are typical of an increase in the concentration of fatty acyl chains in tissues. This was confirmed by an increased intensity of the CH_2

bending vibration at 1452 cm^{-1} (figure 20) in the treated liver (Jackson *et al.*, 1998). They increase with the introduction of gauche conformers in the fatty acyl chains. Argese and coworkers (1994) have been showed that alkylphenols are toxic to animals, plants, and microorganisms, probably due to their hydrophobic alkyl residue that induces alteration of the cell membranes. Bulky hydrophobic molecules, like NP, increase membrane permeability and cause cell leakage and loss of ions. Increased ion permeability and sodium efflux of gill epithelial cells due to NP-10 ethoxylate were reported for rainbow trout as well (Burkhardt-Holm *et al.*, 2000). The activities of many membrane proteins have been found to be affected by a wide range of hydrophobic molecules. Although some of these effects may arise from specific interactions between the hydrophobic additive and distinct side on the protein, the majority of the effects are likely to be nonspecific. NP behaves as an uncoupler by increasing the membrane permeability to the protons. This is not unexpected since NP is a phenol and some uncouplers are phenols (Bragadin *et al.*, 1999). So the spectral changes observed in figure 19 implies the fact that NP alters the membrane structure (the relations between membrane dynamics and permeability were presented in appendix C).

As it can be seen from figure 20, the intensities of amide II (1541), CH_2 bending (1452) and COO^- symmetric stretching (1391) band increased dramatically in $220\text{ }\mu\text{g/L}$ NP-treated liver. These bands are mainly due to proteins in tissues. In addition, treated sample spectrum gave relatively broader and asymmetric band near 1163 cm^{-1} which was not appeared in control spectrum. The relatively weak band at 1163 cm^{-1} is attributed C- O stretching modes of the C-OH groups in cell proteins. The C-O band of the acyl chains of membrane lipids appears in this frequency region as well. However the contribution to this band by membrane lipids is negligible (Rigas *et al.*, 1990). NP and other alkylphenols are shown to have estrogenic effects in vivo and in vitro assays (Jobling and Sumpter, 1993; White *et al.*, 1994). In fish, estrogenic effects, such as synthesis of egg yolk precursor protein, vitellogenin (Vtg) and inhibition of testicular growth in male rainbow trout have been reported (Christensen *et al.*, 1999 and Jobling *et al.*, 1996). 4-NP treatment induces a dose-dependent synthesis

of Vtg as indicated by increased levels of circulating Vtg and hepatic RNA concentrations (Christensen *et al.*, 1999). As the Vtg is the only circulating protein which increases by exogenous estrogen induction, this increase in the protein synthesizing capacity of the liver is most likely due to an increase in the synthesis of Vtg. Therefore the dramatic increase observed in the intensities of the amide II, CH₂ bending, COO⁻ symmetric stretching and C-O stretching bands in our studies can be interpreted as the conclusion of the increasing protein synthesis capacity of the NP-treated tissues. If the synthesized protein was Vtg the increased protein synthesis observed in the present study could be interpreted on the detection of the estrogenic effect of NP treatment. As a support of this argument it was seen from figures 16 and 17 that the spectra of E2 and NP exhibit very similar changes in most of the peaks.

In figure 20, the strongest bands at 1238 cm⁻¹ and 1080 cm⁻¹ are due to the asymmetric and symmetric phosphate stretching modes, respectively. These bands originate mainly in the phosphodiester backbone of cellular nucleic acids. The contribution of these bands by phosphate residues present in membrane lipids is negligible (Rigas *et al.*, 1990 and Wang *et al.*, 1997). The frequencies of these bands were shifted in the treated tissues. The phosphate asymmetric band was shifted slightly to higher frequency values and became broader in treated tissue. This slight shift and broadening observed in the phosphate asymmetric stretching band may be due to the increase in the number of hydrogen-bonded phosphodiester groups of the DNA in molecules of the treated cells (Fung *et al.*, 1997; Wong *et al.*, 1991). These results suggest that in NP treated tissues PO₂⁻ groups become hydrogen bonded (Wong *et al.*, 1991). In addition, as seen from table 4, the ratio of the absorption at 1042 cm⁻¹ (C-O stretching vibration) to that at 1080 cm⁻¹ (phosphate symmetric stretching vibration) for 220 µg/L NP-treated samples had very small ratio when compared to the control group. This result implied an increase in the relative content of nucleic acids in NP-treated tissues (Ci *et al.*, 1999). The band at 969 cm⁻¹ in the NP-treated liver spectrum is sharper and stronger than that in the control group spectra. This result also implied an increase in the relative content of the nucleic acids, especially the DNA, in the treated tissues (Ci *et al.*, 1999). Thus it is clear that DNA became the dominant

component in NP-treated tissues. The increased DNA contribution in NP-treated tissues must originate from the cell nucleus. Therefore the increased DNA signal is due to differences in nuclear morphology, organization, and architecture (increased nuclear/cytoplasm ratio, hyperchromicity, chromatin aggregation, cell division, perhaps decreased DNA condensation) between control and NP-treated cells (Chiriboga *et al.*, 2000).

As it can be seen from Fig 20, all the IR bands (amide I and II) are much broader than those observed on control cells. This broadening indicates unfolding of the molecules containing NP and an increased disorder in the molecular environment (Jamin *et al.*, 1998). In the liver, alkylphenols causes cell death by inhibiting sarcoplasmic reticulum Ca^{2+} pump. Roy *et al.*, (1997) reported that alkylphenol can induce cell death in variety of cells. According to some histological findings after two weeks of exposure, necrosis of hepatocytes started to occur (Uğuz *et al.*, unpublished). So the broadening of the amide I and amide II bands could be attributed to already started necrosis in the liver tissues.

CONCLUSION

In the present study, the results of the FT-IR spectral analysis indicated that when the rainbow trout were treated by a sublethal dose (220 µg/L) of NP for two weeks, it induced significant alterations in the livers.

The detailed examination of the FT-IR spectra revealed the following observations:

Glycogen level was much lower in the treated liver. The relative content of nucleic acids were increased, especially, DNA became dominant component in the liver. Nucleic acids exhibited increased number of hydrogen-bonded phosphodiester groups in treated fish.

It has been also observed that the total content of fatty acids, particularly unsaturated fatty acids, was enhanced in NP-treated liver indicating a change in lipid metabolism of the treated liver. It was also shown that NP altered the membrane structure and permeability. Furthermore, there was an unfolding of the molecules in the NP-treated liver.

NP caused an increase in the synthesis of the protein, possibly the synthesis of vitellogenin was enhanced. This in turn could be considered as then estrogenic effect of NP. Possible estrogenic effect of NP was also supported by the many parallel spectral alterations observed in NP and E2 treated fish livers.

In conclusion; this study shows that FT-IR spectroscopy could be a convenient tool for monitoring structural, conformational and metabolic changes in tissues at molecular level after in vivo exposure to toxic chemicals, and that it might be used in conjunction with biochemical and physiological data.



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APPENDIX A

FT-IR SPECTRA FOR THE C-H STRETCHING REGION OF A PURE LIPOSOME WITHOUT PROTEIN AND A MEMBRANE WITH PROTEIN

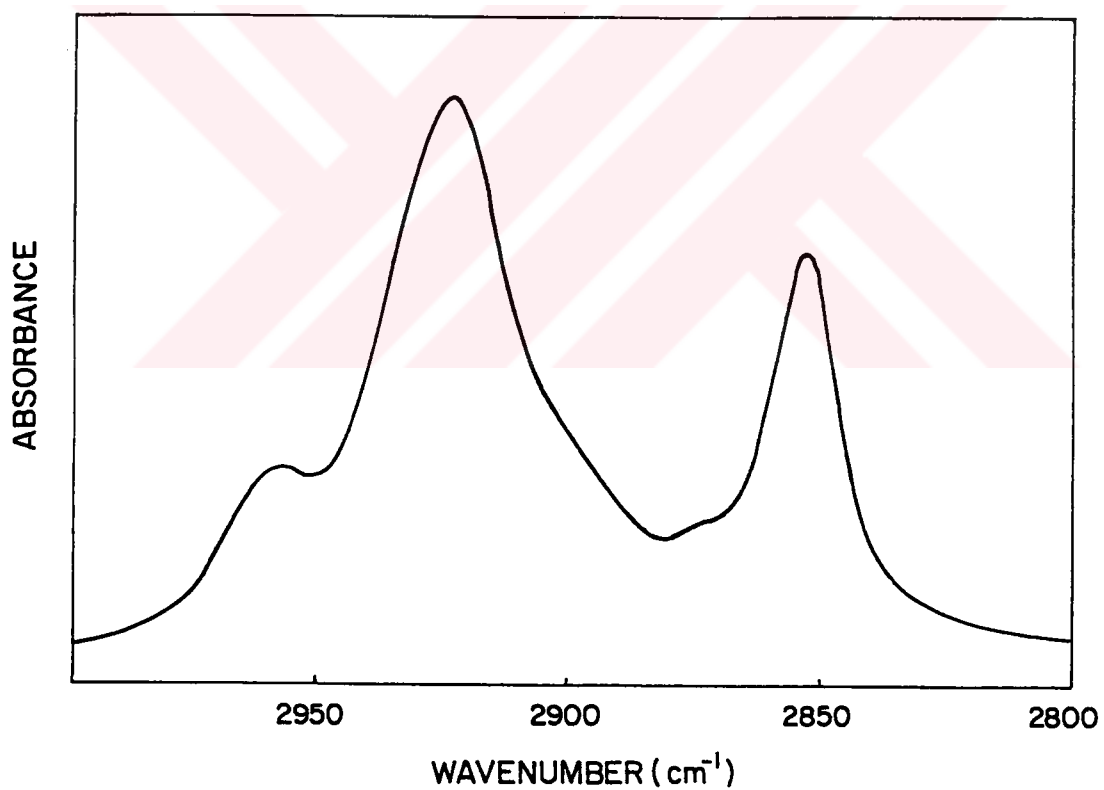


Figure 1. The infrared spectrum for the C-H stretching region of a pure lipid liposom.

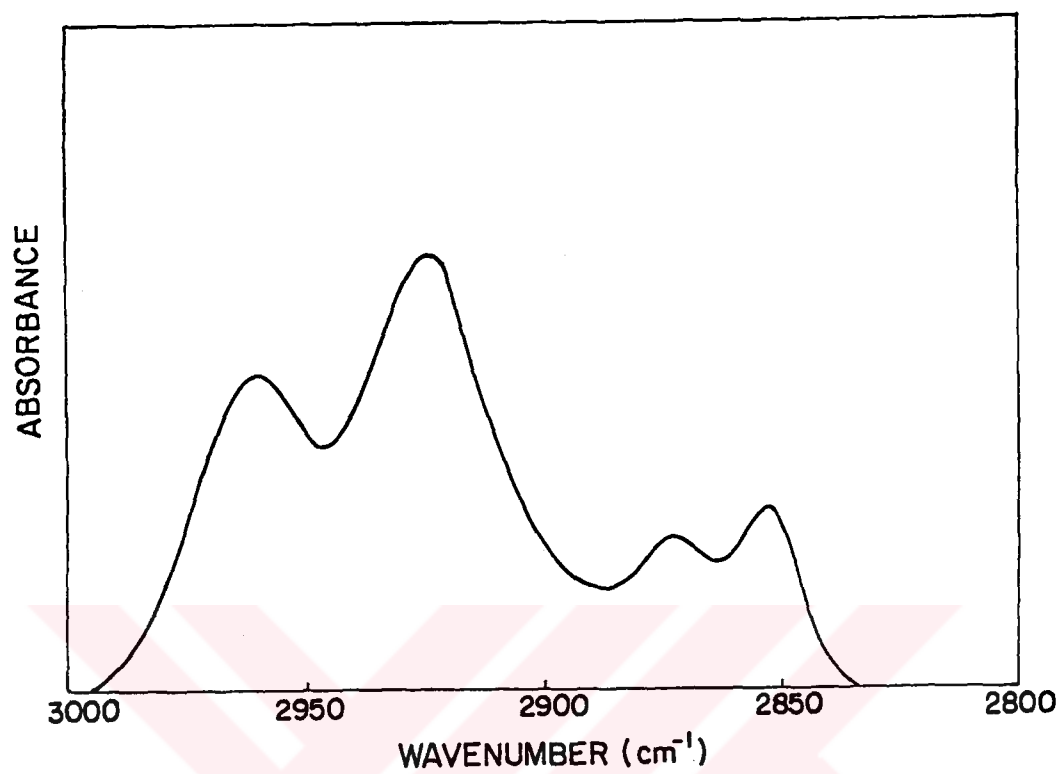


Figure 2. The infrared spectrum of a liver membrane which includes protein.

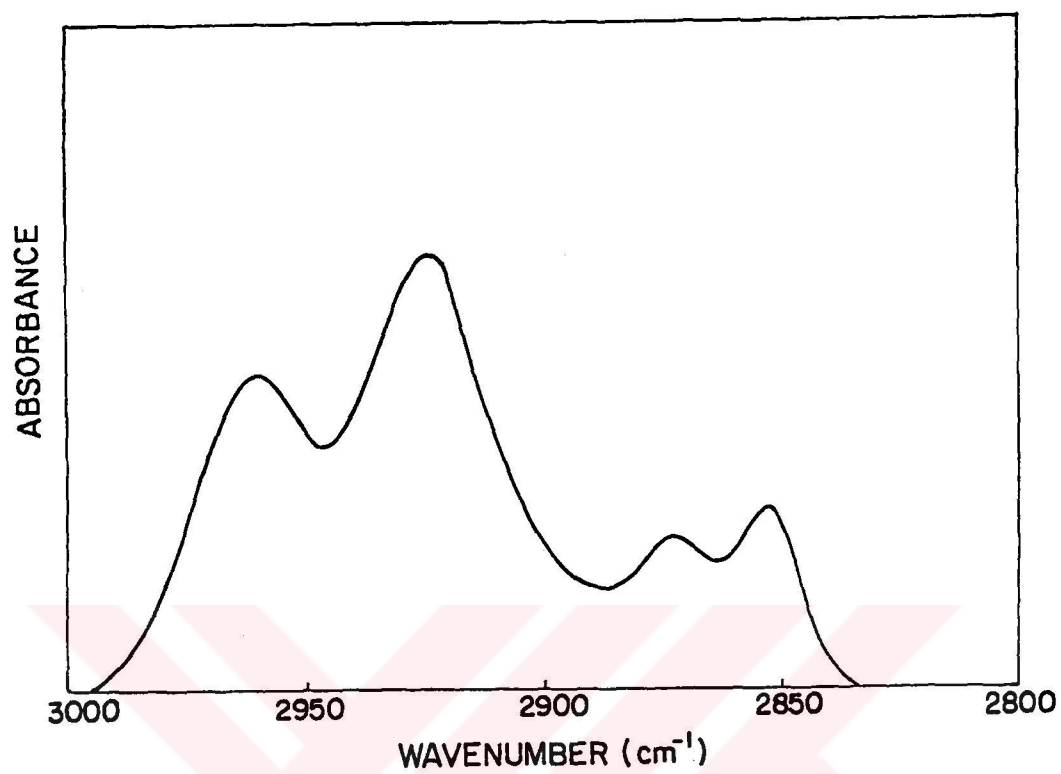


Figure 2. The infrared spectrum of a liver membrane which includes protein.

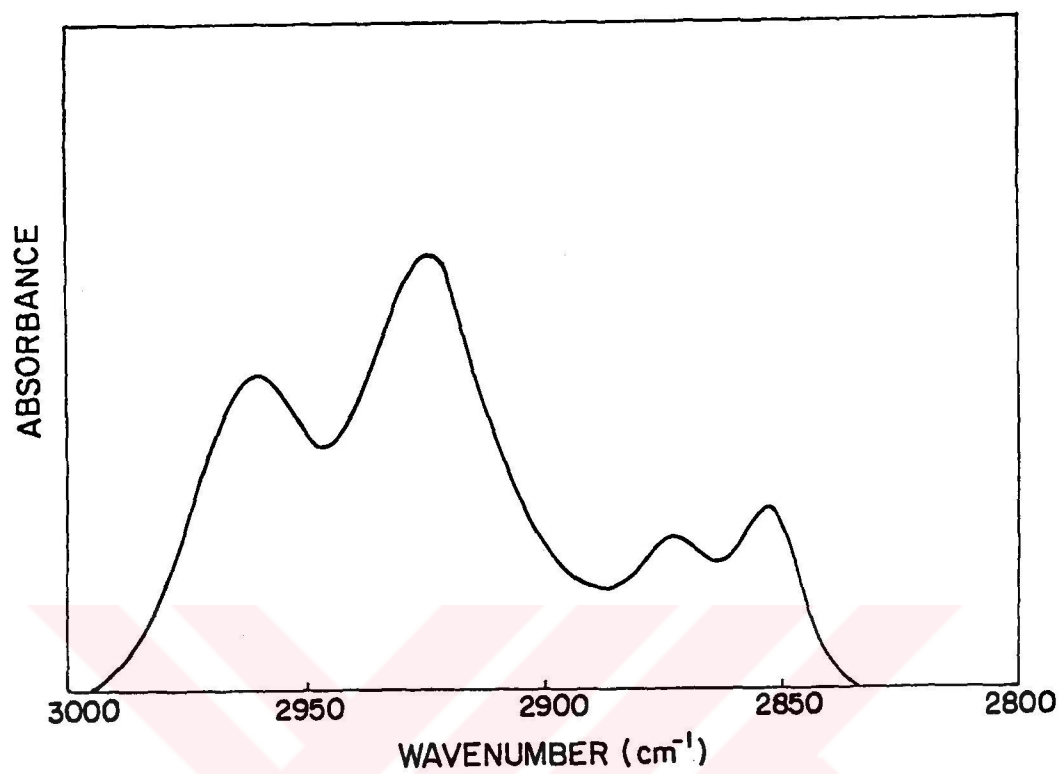


Figure 2. The infrared spectrum of a liver membrane which includes protein.

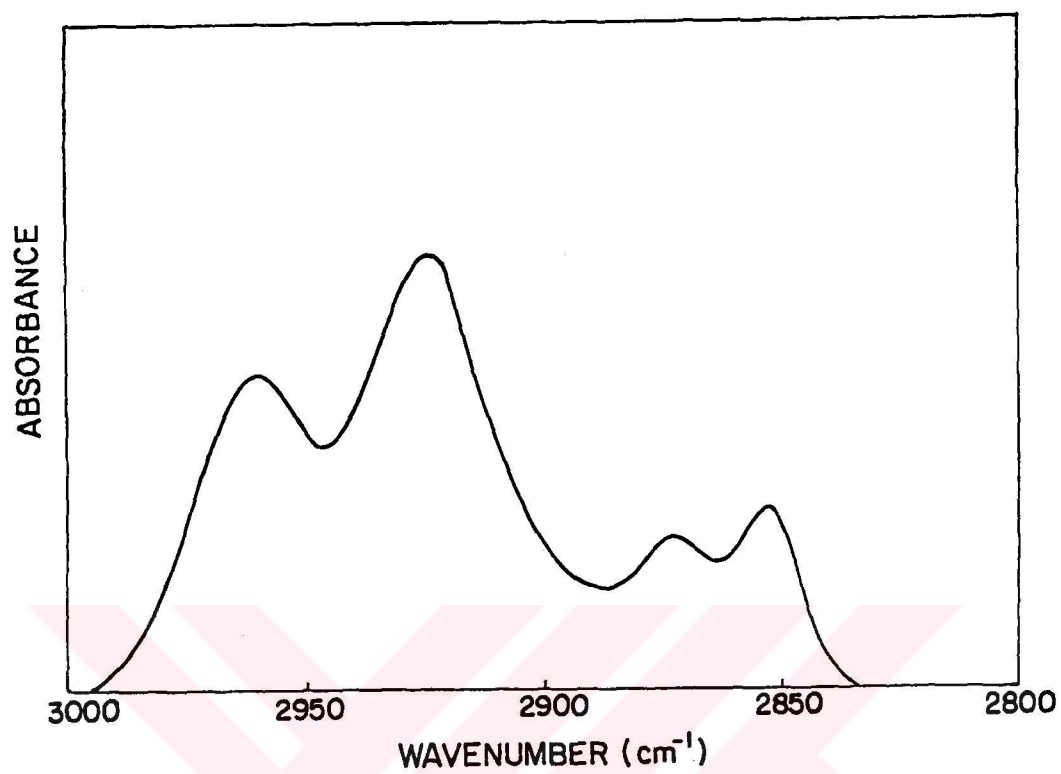


Figure 2. The infrared spectrum of a liver membrane which includes protein.

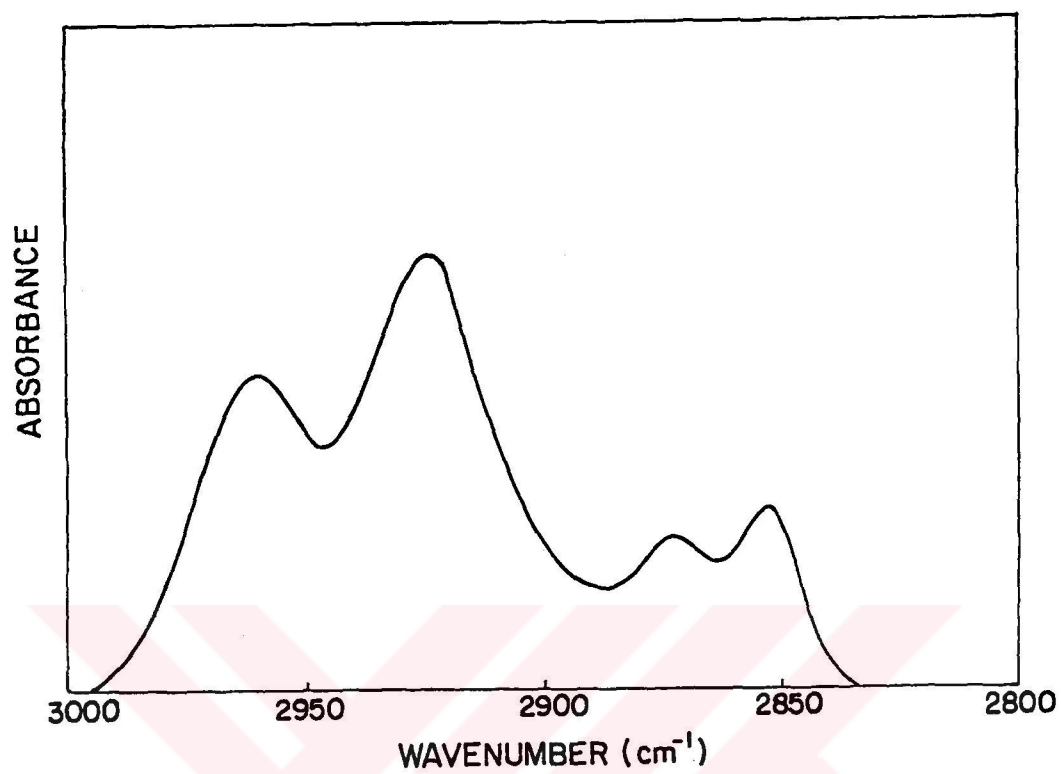


Figure 2. The infrared spectrum of a liver membrane which includes protein.