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STABILITY AND DEGRADATION STUDIES OF CHOLESTEROL-LOWERING STATIN DRUGS

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DISSERTATION

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These last pages of a PhD thesis are usually the most widely read pages of the entire publication. It is here where you think that you will find out whether you have meant something in the life of the PhD candidate. While this may be true to some level, you have to weigh my verdict with the disturbingly low level of sanity left in this PhD candidate after several years of studying toxic molecules…

Pursuing a PhD project is a both painful and enjoyable experience. It is just like climbing a high peak, step by step, accompanied with bitterness, hardships, frustration, encouragement and trust. When I found myself at the top enjoying beautiful scenery, I realized that it was, in fact, teamwork that got me there.

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Thank you all.

You make a living by what you get. You make a life by what you give (Winston Churchill).

Tym, których kocham....

SUMMARY

Cholesterol-lowering statin drugs are potent inhibitors of HMGCoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase, which are highly effective in reducing total cholesterol and the low-density lipoprotein cholesterol levels in the body. They are usually used to remedy several forms of hypercholesterolemic patients and could reduce morbidity and mortality associated with coronary heart disease (CHD) significantly. They have been introduced into the market for the past thirty years, but despite of common use the fate and effects of statin in the environment are largely unknown. Statin residues might enter wastewater treatment facilities and the incomplete removal in sewage treatment plants (STP's) might be a major source of discharge of these compounds to the environment, affecting various aqueous organisms. Although these compounds can be degraded in the environment, it is assumed that they could act as persistent compounds simply because of their continual infusion into aquatic media via STP effluents.

Three representatives of statin drugs: simvastatin, lovastatin and pravastatin were chosen as a model compounds for our study. Three major goals of the research were:

- development of sensitive and selective analytical methods for the determination of statin compounds in aqueous solutions. Determination studies were combined with stability experiments, since statin are significantly affected by various conditions such as pH, temperature, solvent systems, sun light, etc. Wide range of applied concentrations allowed determination of statins at the environmental concentrations level (ng L^{-1}) and concentrations applied in further degradation experiments (mg L^{-1}) in order to monitor initial compounds and their degradation products without time-requiring sample preparation
- application of different degradation techniques (AOP'S - advanced oxidation processes as photocatalytic degradation and ozonation) in order to investigate potential methods of statin removal in the case of their presence in STP's.
- toxicity measurements were performed for standard statin compounds, as well as for by-products, formed during mentioned above degradation

experiments. The acute bioluminescence inhibition assay using the marine bacterium *Vibrio fischeri* as the test organism was used to evaluate short term toxicity. Biodegradation (manometric respirometry test) was involved in evaluation of statin characterisation in terms of their accessibility to microbial degradation and unwanted effects in the environment.

Experimental results revealed that lactone forms of statins can be partially or completely converted to their corresponding hydroxy acid forms under different conditions such as high pH, as well as under presence of different solvents, which might lead to quantification errors. At any stage of our research no pravastatin in lactone form was found. This compound was determined only in its free hydroxy acid form. Various solvent systems were tested and sample acidification was investigated in order to eliminate or at least minimize interconversions for simvastatin and lovastatin.

Despite of non-toxicity towards *Vibrio fischeri*, aerobic biodegradation at the rate significantly lower than 60 % of ThOD (theoretical oxygen demand) pointed out statin as potentially persistent. Obtained toxicity results of 12 months sun-exposed samples demonstrate the importance of toxicity monitoring, raising concerns about statin transformation in surface waters.

First irradiation studies were performed with 125 W Cermax Xenon parabolic lamp, emitting light with broad spectral range of 260 nm – 1000 nm. Further experiments involved low pressure mercury lamp emitting at 254 nm, 310 nm, and 365 nm. For the determination of the quantum yields, a parallel 254 nm beam was used. For analytical studies, a cylindrical setup emitting at 254 nm was employed in order to monitor the formation of by-products and the disappearance kinetics of the parent compound. LC-MS instrument was used to determine photolysis products. Under all our experimental conditions, the obtained data show that the most susceptible compound for UV degradation is pravastatin most likely owing to its high absorbance at the excitation wavelength.

Subsequent experiments were mainly focused on potential methods of statin degradation. Aqueous solution of drugs were irradiated in the presence of 6 glass slides with immobilized sol-gel derived TiO_2. LC-MS analysis confirms that formation of irradiation products (for photocatalysed irradiation as well as ozonation) proceeds through the hydroxyl radical attack on the double bond of the ring, which leads to different isomers. Consequently the molecule is oxidized and $HO_2^{\bullet}$ radical is eliminated, resulting in conjugated double bond reconstruction. Under experimental conditions we observed that the ozonation process could be a powerful tool for statin removal, which could be implemented for example in the sewage treatment plants (STPs) after further, supplementary studies covering this topic.

POVZETEK

Statini so zdravila, ki se uporabljajo za zniževanje ravni holesterola v krvi. Kot izjemno učinkoviti inhibitorji encima HMGCoA (3-hidroksi-3-metil-glutaril-CoA) reduktaze prispevajo k znižanju telesne ravni skupnega holesterola ter lipoproteinov nizke gostote. V medicini se najpogosteje uporabljajo za zdravljenje hiperholesterolemije ter tako prispevajo k manjši umrljivosti zaradi bolezni srca in ožilja. Kljub tridesetletni prisotnosti na trgu ostajajo razgradnja, pretvorbe in učinki statinov ter njihov vpliv na okolje še vedno nepoznani. S prisotnostjo v odpadnih vodah iz čistilnih naprav, ki nastajo zaradi neučinkovitega čiščenja slednjih, namreč lahko predstavljajo pomemben izvor kontaminacije vodnih teles, ki ogroža številne vodne organizme.

V našo raziskavo smo kot modelne spojine vključili tri reprezentativne predstavnike statinov poznane pod imeni simvastatin, lovastatin in pravastatin. Z raziskavo smo želeli:

- razviti novo, selektivno in občutljivo analizno metodo za določanje statinov v vodnih raztopinah. Raziskavo smo dopolnili s testi stabilnosti omenjenili spojin, saj so slednji izjemno občutljivi na različne okoljske pogoje, kot so: sprememba pH vrednosti, temperature, različna topila, sončna svetloba, itd. Uporabljeno široko koncentracijsko območje metode je omogočilo detekcijo statinov tako na nivoju okoljskih koncentracij (ng L^{-1}) kot višjih koncentracij v dodatno izvedenih poskusih razgradnje statinov (mg L^{-1}), saj smo želeli spremljati začetne spojine in njihove razgradne produkte brez predhodne, časovno zahtevne priprave vzorcev za analizo.
- preveriti učinkovitost različnih postopkov razgradnje statinov (NOP - naprednih oksidacijskih procesov kot sta fotoliza in ozonacija) z namenom izbora potencialno najučinkovitejše metode za odstranjevanje statinov v primeru njihove prisotnosti v odplakah čistilnih naprav.
- določiti strupenost statinov (standardnih spojin) kot tudi njihovih razgradnih produktov, nastalih med zgoraj omenjenimi razgradnimi poskusi. Pri tem smo uporabili metodo akutne bioluminiscenčne inhibicije z uporabo morske bakterije *Vibrio fischeri* z namenom ovrednotenja kratkoročne toksičnosti na zgoraj omenjeni testni organizem. Preverili smo tudi, kako se statini razgrajujejo

naravno, in sicer z uporabo manometričnega respiratornega testa in na tak način ocenili sposobnost biorazgradnje statinov kot tudi možnost neželjenih učinkov na okolje.

Rezultati raziskave so pokazali da se laktonska oblika statinov lahko delno ali popolnoma pretvori v ustrezno hidroksi kislinsko obliko pod različnimi eksperimentalnimi pogoji, kot so visok pH ter prisotnost različnih topil, kar vodi v napake kvantitativne analize naših spojin. Ker rezultati analize pravastatina niso potrdili prisotnost laktonske oblike, smo ga kvantificirali le v prosti hidroksi kislinski obliki. Da bi zmanjšali verjetnost interkonverzij preostalih dveh, tj. simvastatina in lovastatina, smo testirali, kako različni sistemi topil ter sprememba pH vrednosti vplivata na pretvarjanje statinov.

Kljub netoksičnosti statinov na bakterijo *Vibrio fischeri*, so rezultati hitrosti aerobne biorazgradnje pokazali vrednosti nižje od 60 % TPK (teoretične potrebe po kisiku), kar jasno kaže na možnost obstojnosti statinov v okolju. Prav tako so rezultati strupenosti enoletnega poskusa v prisotnosti sončne svetlobe potrdili pomembnost merjenja strupenosti, saj upravičeno vzbujajo skrb potencialne transformacije statinov v površinskih vodah.

Prvi eksperimenti obsevanja vzorcev so bili opravljeni z uporabo 125 W parabolične Xenonove žarnice, ki seva v širokem spektralnem območju (260 nm – 1000 nm). Vsi nadaljni eksperimenti so vključevali uporabo nizkotlačnih Hg žarnic, ki emitirajo svetlobo pri 254 nm, 310 nm in 365 nm. Za določevanje kvantnih izkoristkov smo uporabili sijalke s paralelni žarki valovne dolžine 254 nm. V eksperimentih, kjer smo spremljali reakcijsko kinetiko in ugotavljali prisotnost razgradnih produktov, smo uporabili cilindričen fotoreaktor s sevajočo svetlobo pri 254 nm. Detekcija produktov fotolize je bila izvedena z LC-MS analizami. Rezultati eksperimentov, izvedenih pri zgoraj omenjenih pogojih, so pokazali največjo fotolabilnost pravastatina, verjetno zaradi njegove absorbcije pri uporabljeni vzbujevalni valovni dolžini.

Vsi nadaljni eksperimenti so bili usmerjeni v raziskave potencialnih metod za razgradnjo statinov. Vodne raztopine statinov smo obsevali v prisotnosti 6-ih steklenih ploščic z nanešenim TiO_2 po sol gel postopku.. LC-MS analize so potrdile nastanek

razgradnih produktov obsevanja tako pri fotokatalizi kot ozonaciji, preko napada hidroksilnega radikala na dvojne vezi obroča, kar vodi v nastanek različnih izomer, pri čemer poteče oksidacija molekule statina in eliminacija $HO_2^{\bullet}$ radikalov, kar se odraža v rekonstrukciji konjugiranih dvojnih vezi. Rezultati eksperimentov so pokazali, da ozonacija lahko predstavlja učinkovito metodo za razgradnjo statinov, ki bi jo lahko uporabili v komunalnih čistilnih napravah, za kar pa bi bilo potrebno opraviti dodatne študije.

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ABBREVIATIONS

HMGCoA = 3-hydroxy-3-methyl-glutaryl-CoA

CHD - coronary heart disease

AOP = advanced oxidation process

VUV radiation = vacuum ultraviolet radiation

VOC = volatile organic compound

O_3 = ozonation

O_3/UV = ozonation, coupled with UV radiation

O_2/TiO_2/UV = photocatalysis

LD_{50} = median lethal dose

EC_{50} = half maximal effective concentration

DT_{50}= time for 50% decomposition (i.e. half-life)

MCIG = minimal concentration to inhibit growth

ADME = adsorption, distribution, metabolism, excretion

TI = teratogenic index (LC_{50}/EC_{50})

NOEC = No Observed Effect Concentration

LOEC = Lowest Observed Effect Concentration

EPA = Environmental Protection Agency

ChV = chronic toxicity value

QSAR – Quantitative Structure-Activity Relationship

PBT = Persistent, Bioaccumulative, Toxic

BCF = bioconcentration factor

vPvB = very persistent and very bioaccumulative

CMR = carcinogenic, mutagenic or toxic to reproduction

OECD = Organization for Economic Co-operation and Development

BOD – biochemical oxygen demand

ThOD – theoretical oxygen demand

STP’s – sewage treatment plants

RNA – ribonucleic acid

Sim-L – simvastatin in its lactone form

Sim-H – simvastatin in its hydroxy acid form

Lov-L- lovastatin in its lactone form

Lov-H – lovastatin in its hydroxy acid form

Pra-L – pravastatin in its lactone form

Pra-H – pravastatin in its hydroxy acid form

LOD- limit of detection

LOQ- limit of quantification

I. INTRODUCTION

Physiologically active substances (pharmaceuticals) are a class of so called "emerging" contaminants that have raised great concern in the last years. Even the amount of pharmaceuticals and their bioactive metabolites being introduced into environment is likely low, their continuous environmental input may lead to high long-term concentration and promote continual, but unnoticed adverse effects on aquatic and terrestrial organisms (Petrovic et al., 2005). Sources of pharmaceuticals to surface waters also include direct runoff of on-ground faecal material from pets and livestock (which is not the source of contamination with statins, since they are not used in veterinary medicine), release from industrial production of pharmaceuticals and municipal sewage treatment plants (Cooper et al., 2008; Boxall et al., 2003; Chen et al., 2005; Kemper, 2008; Pedersen et al., 2003; Pedersen et al., 2005; Rogers, 1996; Velagaleti, 1997).

Lipid-lowering drugs can be divided into two main groups: "fibrate" class of the lipid regulators and "statin" class. Cholesterol lowering statins are a group of pharmaceuticals, which are the most frequently prescribed agents for reducing morbidity and mortality related to coronary heart disease. Lovastatin (Mevacor®), pravastatin (Pravachol®) and simvastatin (Zocor®) have been introduced to the market since 1987. Lovastatin is a natural product; simvastatin and pravastatin are semi-synthetic products (Metcalfe and Miao, 2003). Statins exist in two forms, lactone and hydroxy acid in general. In vivo, the hydroxy acid forms are the active drugs to lower plasma cholesterol while the lactone forms are inactive (prodrug). Lactone forms can be absorbed from the gastrointestinal tract and transformed to the active drugs in liver and non-hepatic tissues (Hwang and Yang, 2006). The instability of the lactone compounds (lovastatin and simvastatin), reflected in their partial or complete transformation to hydroxy acid forms, can be due to hydrolysis in aqueous media; while for acidic compound (pravastatin) – due to ester formation by reaction with alcohols (Metcalfe and Miao, 2003).

Due to the widespread use of statins and their large scale production municipal sewage treatment plants, as well as sewage treatment plants of the pharmaceutical industry might therefore be important point sources of contamination. A multiresidue

analytical method is prerequisite to provide reliable information on the fate of pharmaceuticals (and statins among them) in sewage treatment plants (STPs) and surface water and to assess drug removal, partition and fate in the environment (Zuccato et al., 2005b). Between 30 and 90 % of an administrated dose of many pharmaceuticals ingested by humans is excreted in the urine as the active substance and many of them do not undergo biodegradation in STPs in active form (Herberer, 2002; Velagaleti, 1997; Joss et al., 2005; Joss et al., 2006), and up to 90 % of drug residues may remain in effluent after treatment (Ternes, 1998). Studies of the biodegradability of several clinically important pharmaceuticals as antibiotics found that none of the antibiotics tested in Closed Bottle Tests could be classified as ready biodegradable (Al-Ahmad et al., 1999, Alexy et al., 2004). Available data for one of the cholesterol-lowering drugs - clorofibric acid, refer to its estimated persistence in the environment of 21 years. The compound is still detected in lakes and rivers after it was withdrawn from the market (Molinari et al., 2006). Following treatment, the sewage effluent containing pharmaceutical parent compounds and transformation products can be discharged from the STP directly into aquatic environments, where pharmaceuticals that may have been transformed during treatment could be converted back into parent forms (Ternes, 1998; Lester et al., 2005). Reconnaissance of organic wastewater contaminants (United States), including pharmaceuticals, in fluvial ecosystems detected contaminants in 80% of 139 streams sampled (Kolpin et al., 2002). To our knowledge, the data, regarding pollution with pharmaceutical residues in Slovenia are limited to non-steroidal anti-inflamatory drug (NSAIDs) residues (Kosjek et al., 2005).

Pharmaceuticals, and statin drugs among them, are potent bioactive chemicals that are designed to target specific metabolic and molecular pathways in humans and animals, but they can also have noteworthy side effects (Fent et al., 2006). Residues of pharmaceuticals may have substantial effects on the environment comparable to or greater than those agricultural chemicals (Cleuvers, 2003; Daughton and Ternes, 1999). Unknown modes of action of pharmaceutical contaminants on lower organisms make toxicity prediction difficult. Pharmaceutical concentrations measured in surface waters are generally well below concentrations that are known to cause acute toxicity to aquatic organisms. However, chronic exposure to pharmaceuticals has the potential for numerous effects, such as metabolic or

reproductive changes on non-target organisms (Daughton and Ternes, 1999). Some recent studies have seen subtle effects at concentrations similar to those measured in the environment. Low, sublethal concentrations of simvastatin (0.16 µg L^{-1} – 5.0 µg L^{-1}) were found to affect the development time, body length, RNA content, and growth rate of harpacticoid copepods (Dahl et al., 2006).

For any pollutant, including pharmaceuticals, abiotic transformations in surface water may occur via hydrolysis and photolysis. Pharmaceuticals, usually designed for oral intake, are as a rule resistant to hydrolysis suggesting the mechanism of direct and indirect photolysis as a primary pathway for their abiotic transformation in surface waters (Petrovic and Barcelo, 2007). Despite of common use the fate and effects of statins in the environment are largely unknown.

II. Theoretical background

2.1. Pharmaceuticals

2.1.1. General overview

In recent years, the occurrence and fate of pharmaceutical residues in the environment has become a subject of public interest. The reason why these medical substances are of environmental concern can be deduced from their specific biological effects not only on patients, but also on aquatic biota. Due to the variety and amount of their applications pharmaceuticals belong to the environmentally relevant compounds (Halling-Sorensen et al., 1998). When applying pharmaceuticals to humans, many of their constituents are excreted unchanged through urine and faeces or as metabolites via municipal sewage system, many pharmaceutical compounds and their residues can frequently be found in effluents of wastewater treatment plants, in rivers and lakes.

Balances of the input and the output of drugs and diagnostic agents in sewage treatment plants reveal that many pharmaceuticals are not removed quantitatively (Halling-Sorensen et al., 1998). Therefore sewage treatment plants act as point sources for surface water contamination.

2.1.2. Pharmaceuticals in the environment

The drugs used by humans will be discharged to the sewer systems together with the urine and faeces (Fig. 1, F1) and enter the sewage treatment plant (STP) (Fig. 1, F4). The incomplete removal in STP's is pointed out by many authors, as the major source of discharge of these compounds to the environment (Ternes, 1998; Daughton and Ternes, 1999; Joss et al., 2005; Miao et al., 2002). The possible fate of drugs in the sewage treatment plant as for all other xenobiotics may be divided as follows (Jorgensen and Halling-Sorensen, 2000):

(i) The drug or metabolites of the parent drug is mineralised by micro-organisms to carbon dioxide and water, e.g., aspirin (Richardson and Bowron, 1985)

(ii) The drug or metabolites of the parent drug are more or less persistent in the STP, which implies that depending on the lipophilicity or other binding possibilities e.g. ionic bindings, a part of the substance will be retained in the sludge. If the sludge is used as soil conditioner (Fig. 1, F5) drugs may be dispersed on agricultural fields (which is according to our knowledge against the law in Slovenia). Again the fate of the drugs depends on the lipophilicity or other ability of binding to sludge or soil. Drug molecules often have many functional groups e.g., carboxylic, carbonyl and amino groups, which makes the binding capacities of the molecules to solids dependent on pH or other constituents (e.g., complexation) in the solids matrix. Drugs that are mobile in the soil may be a threat to the ground water (Fig. 1, F6) or leach to a nearby stream (Fig. 1, F7). Depending on the ability of the drug to bind solids, either organisms in the terrestrial ecosystem (Fig. 1, F8) or aquatic ecosystem (Fig. 1, F9) may be exposed.

(iii) The drug or metabolites of the parent drug is persistent and at the same time very polar and nonbinding to solids. The substance will thus not be retained neither degraded in the STP and therefore easily reach the aquatic environment (Fig. 1, F5), and may affect the aquatic organisms.

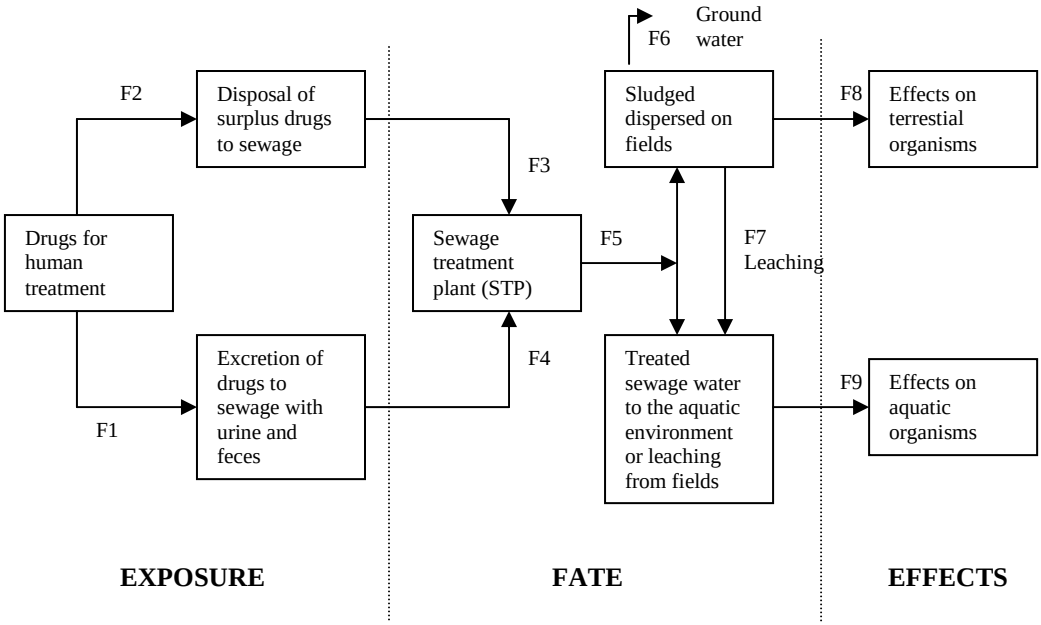


Fig 1: Anticipated exposure route of drugs for human treatment in the environment (Jorgensen and Halling-Sorensen, 2000)

An unknown portion of drugs marketed for human treatment ends in the sewer system as surplus medical substances (Fig. 1, F2). After entering the STP (Fig. 1, F3) the fate of these drugs will be almost identical with the excreted drugs. The only difference is that the waste water will not include the drug metabolites often produced by humans before excretion (Jorgensen and Halling-Sorensen, 2000).

Langford and Thomas (2009) reported on the determination of pharmaceutical compounds, and statins among them, in hospital effluents and their contribution to wastewater treatment works. This assessment shows that point sources discharges from hospitals typically make a small contribution to the overall pharmaceutical load when compared to municipal areas; however this varies from substance to substance and is not the case when a drug's use is primarily hospital based. Atorvastatin and simvastatin were found to contribute approximately 2% to the daily inputs to the waste water treatment works.

2.1.3. Analytical methods for the determination of pharmaceutical residues in the environment

The actual pharmaceutical residues concentrations in the environment are in the ng L^{-1} range and often associated with complex matrices (sediment, soil, industrial wastewaters etc.), that makes heavy demands on the analytical work and the preconcentration procedures. Consequently, it is recommended to apply the most demanding quality control of the analytical results, for instance by use of two or more different analytical methods to determine the same substances (Jorgensen and Halling-Sorensen, 2000).

Pharmaceuticals are mainly polar compounds containing acidic or basic functional groups (e.g., carboxylic, carbonyl, and amino groups) that may be subject to direct and indirect photolysis. The result can be a complex mixture of intermediates and transformation products. Elucidation of photolysis-reaction pathways and identification of transformation products are therefore of crucial importance in understanding their fate in the aquatic environment (Petrovic and Barcelo, 2007).

Another analytical complication is the determination of drug metabolites, since many drugs are partially metabolized in the human body before excretion. The metabolites may also be harmful to the environment and it is therefore necessary to include them in the investigations. All by all, analytical chemistry has in the coming years two challenging tasks: to develop and to validate analytical methods that may determine low concentrations of drugs and their metabolites in complex environmental matrices (Jorgensen and Halling-Sorensen, 2000).

A lot of research has been dedicated already to develop and validate analytical methods that may determine low concentrations of pharmaceuticals in biological samples or in complex environmental matrices (Jorgensen and Halling-Sorensen, 2000; Halling-Sorensen et al., 1998; Ling et al., 2005; Pan et al., 2006; Metcalfe and Miao, 2003). However, environmental detection of human metabolites or their environmental transformation products is still very limited (Hernando et al., 2007; Petrovic et al., 2005). Their identification is complicated and cumbersome, requiring the application of advanced instrumental methods (e.g., liquid chromatography with

mass spectrometry (LC-MS), LC with ultraviolet or fluorescence (UV-FL) detection, nuclear magnetic resonance (NMR), matrix-assisted laser desorption-ionization (MALDI-MS), gas chromatography with mass spectrometry (GC-MS), ion chromatography (IC) or infrared (IR) spectroscopy) (Grahek et al., 2001; Alonso et al., 2005; Bauer et al., 2005; Erturk et al., 2003; Huclova et al., 2006; Jemal et al., 2000a and 2000b; Montenegro et al., 2006; Vuletic et al., 2005; Wang and Asgharnejad, 2000). Of these, LC-MS has gained popularity and has become one of the preferred techniques also for statin analyzing because of its permission for rapid identification and quantification as well as the fact that the samples do not need to be derivatized (Petrovic and Barcelo, 2007; Hwang and Yang, 2006; Zhu and Neirinck, 2003). The major sample preparation methods applied in these studies are liquid–liquid extraction (Carlucci et al., 1992; Zhang et al., 2004) and solid-phase extraction (Metcalfe and Miao, 2003; Ochiai et al., 1997; Petrovic et al., 2005; Zuccato et al., 2005a; Bauer et al., 2005).

The main advantages of LC-MS are its sensitivity and its suitability for compounds with a wide range of masses and polarities. Two common strategies are applied, depending on the LC-MS instrumentation used. One relies on MS measurements of accurate molecular mass, and, subsequently the determination of the empirical formula using orthogonal acceleration time-of-flight (oaTOF) instruments, whereas the other involves elucidation on the basis of structural information gained in tandem MS (MS^2) experiments that can be accomplished either by coupling mass analyzers in series (e.g. triplequadrupole (OgQ)) or by using a single ion-trap (IT) analyzer (Petrovic and Barcelo, 2007).

2.2. Methods for pharmaceuticals removal. Fundamentals of Advanced Oxidation Methods

Once released into the environment via the discharge of treated or untreated wastewater, pharmaceuticals are subjected to processes (e.g., dilution, photolysis, biodegradation, and sorption to bed sediments) that contribute to their elimination from the environmental waters (Petrovic and Barcelo, 2007). Abiotic processes are of great importance in determining fate of pharmaceutical compounds, which might take place in the aquatic environment. Because pharmaceutical compounds or their degradation products are expected to be resistant to hydrolytic processes, direct and indirect photolysis may be the only relevant abiotic loss process in sunlit aquatic systems (Petrovic and Barcelo, 2007).

Photolytic reactions are often complex involving various competing or parallel pathways and leading to multiple reaction products that may (Petrovic and Barcelo, 2007):

- be more toxic than the parent compound;
- retain the properties of the parent compound or,
- lose antimicrobial activity of parent compound

The need for ‘‘clean’’ treatment technologies, e.g., techniques which do not result in the creation of either more hazardous or environment damaging by-products or more concentrated waste streams, has led to increased interest in advanced oxidation processes. Advanced oxidation processes (AOPs) have typically been defined as those water cleaning technologies that involve the formation of powerful oxidizing agents (commonly hydroxyl radicals) capable of unselectively reacting with the organic (and in some cases inorganic) content of the aqueous effluents (Guwy et al., 2007; Gunten et al., 2006). AOPs are usually applied to eliminate micropollutants present in relatively “clean” waters, although under some circumstances they have also been used to improve the biodegradability properties of wastewaters. Application of AOPs is governed by several factors (organic load, water transparency to radiation, presence of radical inhibitors, etc.). AOPs are established as “destructive” water treatment technologies. Thus, contrarily to other tertiary

processes (i.e. adsorption processes, membrane processes, etc.) they transform harmful substances into water and carbon dioxide, or, alternatively into other harmless byproducts. They constitute an alternative to biological processes when biotoxic substances are present in the effluents. Additionally, AOPs are capable of mineralizing a wide range of micropollutants, avoiding the accumulation of oxidation end products. AOPs consist of "clean" methods and more friendly to the environment reagents and technologies, e.g. ultraviolet irradiation, ultrasound treatment, ozonation, hydrogen peroxide oxidation, which can efficiently be applied also in combination. Advanced Oxidation Process might be combined together with UV light, increasing the efficiency of mineralization. Photochemical Advanced Oxidation process has gained a lot of interest lately.

Although studies have examined and compared degradation products resulting from direct and indirect photochemical reactions (Gomez et al., 2008; Mabury and Lam, 2005; Andreozzi et al., 2003; Frimmel and Doll, 2003; Burrows et al., 2002) fewer studies have used pharmaceuticals as test compounds. Since drugs are considered environmental contaminants, it is important to investigate what these compounds degrade into, the persistence of products relative to the starting material, and whether the degradation products retain the activity of the parent molecule to elicit a toxicological effect on non-target organisms in aqueous systems.

2.2.1. H_2O_2/UV process

Among the well known advanced oxidation processes (AOP) and beside TiO_2/UV, the activation of hydrogen peroxide by UV has been extensively studied from analytical and kinetic points of view.

The mechanism of organic molecule destruction in AOPs is based on the formation of hydroxyl radicals ($^{\bullet}OH$), with an oxidation potential of 2.80 V that can oxidize a broad range of organic compounds. In comparison with other AOPs, i.e. Fenton, O_3, UV/O_3, TiO_2/UV, etc., the photolysis of hydrogen peroxide shows some advantages such as the complete miscibility of H_2O_2 with water, the stability and commercial availability of hydrogen peroxide, the absence of phase transfer problems and lower investment costs. Theoretical quantum yield of $^{\bullet}OH$ generation is 2 for

H_2O_2/UVprocess, which is due to the generation of 2 mol of hydroxyl radicals/mol of hydrogen peroxide photolyzed (Aleboyeh at al., 2008):

$$H_2O_2 + h\nu \rightarrow 2\ ^{\bullet}OH \qquad (1)$$

However, generated hydroxyl radicals also react with hydrogen peroxide that limits the efficiency of the process in terms of pollutants degradation (Eq. 2) and are prone to react or to recombine according to the following scheme (Aleboyeh at al., 2008):

$$^{\bullet}OH + H_2O_2 \rightarrow H_2O + HO^{\bullet}_2 \qquad (2)$$

$$HO^{\bullet}_2 + H_2O_2 \rightarrow {}^{\bullet}OH + H_2O + O_2 \qquad (3)$$

$$2\ HO^{\bullet}_2 \rightarrow H_2O_2 + O_2 \qquad (4)$$

$$HO^{\bullet}_2 + {}^{\bullet}OH \rightarrow H_2O + O_2 \qquad (5)$$

Therefore, an important step in the optimization of the method is the determination of the adequate amount of H_2O_2, to avoid an excess amount of reagent mineralization of organic carbon into CO_2. Unfortunately, the molar absorptivity of H_2O_2 is low in the readily accessible UV region. Hydrogen peroxide does not absorb significantly beyond 300 nm and absorbs only weakly in the range of 200-300 nm.

2.2.2. Photolysis of water

For any pollutant, including pharmaceuticals, abiotic transformations in surfaces waters may occur via hydrolysis and photolysis. Pharmaceuticals, usually designed for oral intake, are as a rule resistant to hydrolysis suggesting the mechanism of direct and indirect photolysis as a primary pathway for their abiotic transformation in surface waters. Direct photolysis of chemical species is caused by direct absorption of solar light (Zepp and Cline, 1977).

One of the most powerful AOP for the degradation of organic compounds in aqueous solutions is the vacuum-UV (VUV) photolysis. Due to the high absorption cross-section of water, VUV-radiation, emitted by a Xe_2-excimer radiation source

(λ_{em}: 172±14 nm), is almost exclusively absorbed by H_2O, leading primarily to the homolysis of the O–H bond (Eq. 6)

$$H_2O + h\nu \rightarrow {}^{\bullet}OH + H^{\bullet} \qquad (6)$$

The quantum yield of reaction (2.2.2.1) in water depends on the wavelength of irradiation (0.42 at 172 nm) (Quici et al., 2008). Hydroxyl radicals react primarily by hydrogen abstraction (generally from aliphatic carbon atoms) or by electrophilic addition to π-systems. With standard redox potentials of 2.7 V in acidic solution and of 1.8V in neutral solution, ${}^{\bullet}OH$ may also oxidize organic compounds by electron transfer reactions. Beside homolysis, VUV-irradiation (172 nm) of H_2O also produces solvated electrons with a quantum yield of 0.05 (Eq. 7), the e_{aq}^- being converted to $H^{\bullet}$ by H_3O^+ (reaction 8) (Quici et al., 2008):

$$H_2O + h\nu \rightarrow {}^{\bullet}OH + H^+ + e_{aq}^- \qquad (7)$$

$$e_{aq}^- + H_3O^+ \rightarrow H^{\bullet} + H_2O \qquad (8)$$

Hydrogen atoms may be involved in the reduction of organic substrates, but are quantitatively trapped by molecular oxygen (O_2) in aerated solutions (reaction 9). O_2 may also be reduced by e_{aq}^- (reaction leading to the formation of the superoxide anion) ($O_2^{\bullet -}$, reaction (10)). The disproportionation of $O_2^{\bullet -}$ and its conjugated acid $HO_2^{\bullet}$ (reaction (11)) yields hydrogen peroxide (reaction (12)). Production of H_2O_2 during VUV-photolysis of water has been demonstrated (Quici et al., 2008):

$$O_2 + H^{\bullet} \rightarrow H^{\bullet} + HO_2^{\bullet} \qquad (9)$$

$$O_2 + e_{aq}^- \rightarrow O_2^{\bullet -} \qquad (10)$$

$$O_2^{\bullet -} + H_2O \leftrightarrow HO_2^{\bullet} + HO^- \qquad (11)$$

$$O_2^{\bullet -} + HO_2^{\bullet} + H_3O^+ \rightarrow O_2 + H_2O_2 + H_2O \qquad (12)$$

The VUV-photolysis of water or aqueous systems may be used for ultrapure water production and for the degradation of pollutants dissolved in water and known to be mineralized only in reaction systems combining reductive and oxidative steps. The high absorption cross-section of water and the present state of technology for generating high potentials at high frequencies prevent the development of the VUV-photolysis to the level of large-scale units. However, the high quantum efficiency of $HO^{\bullet}$ generation without addition of any oxidant other than O_2 favors this process for production and treatment units of limited photonic fluxes.

2.2.3. Homogenous photocatalysis

Fenton's oxidation is one of the best known metal catalyzed oxidation reactions of water-miscible organic compounds. The mixture of $FeSO_4$ or any other ferrous complex and H_2O_2 (Fenton's reagent) at low enough pH, results in Fe^{2+} catalytic decomposition of H_2O_2 and proceeds via a free radical chain process that produces hydroxyl radicals, which have extremely high oxidizing ability and can oxidize very stable organic compounds in a short time. The Fenton's reagent does not have only oxidation function but also coagulation by the formation of ferric-hydroxo complexes.

The suggested overall reaction for Fenton's oxidation is given below (Yetis et al., 2006):

$$2\ Fe^{2+} + H_2O_2 + 2\ H^{+} \rightarrow 2\ Fe^{3+} + 2\ H_2O \qquad (13)$$

According to Eq. 13, the pH value has to be in the acidic range to generate the maximum amount of hydroxyl radicals to oxidize organic compounds. However, pH value should not be too low since at very low pH values (<2.0) the reaction is slowed down due to the formation of complex iron species and formation of oxonium ion $[H_3O]^{+}$.

On the other hand, at high pH (pH > 4), the generation of hydroxyl radicals becomes slower because of the formation of the ferric-hydroxo complexes. Therefore, the initial pH value has to be between 2 and 4 to generate the maximum amount of

hydroxyl radicals to oxidize organic compounds (Tang and Tassos, 1997; Kang.and Chang, 1997; Solozhenko et al., 1995; Tang and Huang, 1996).

A combination of hydrogen peroxide and UV radiation with Fe^{2+}, the so-called photo-Fenton process, produces more hydroxyl radicals in comparison to the conventional Fenton method or the photolysis, thus promoting the degradation of organic pollutants. The higher production of $^{\bullet}OH$ due to the combination of oxidant compounds and metallic catalysts in presence of UV radiation and the potential applicability of sun light as UV light source are some attractive advantages of this system (Pignatello et al., 2006)

The important role of UV in such system is reduction of Fe^{3+} to Fe^{2+}, thus finishing the photocatalytic cycle (Eq. 14), Fe^{2+} adsorbs UVB and UVA radiation with the broad maximum at 300 nm.

$$[Fe(OH)]^{2+} + h\nu \rightarrow Fe^{2+} + {}^{\bullet}OH \qquad (14)$$

The use of ferrioxalate (FeOx) in the photo-Fenton process for the degradation of organic pollutants was first reported by Safarzadeh-Amiri et al. (1997). Ferrioxalate complex is highly suitable for solar applications since it absorbs strongly between 250 and 500 nm and has a high quantum efficiency of Fe^{2+} generation ($^{\circ}Fe^{2+}$ = 1.24 at 300 nm). The Fe^{3+}– polycarboxylate complexes undergo rapid photochemical reaction under irradiation generating Fe^{3+} in the following equation: (Safarzadeh-Amiri et al., 1997):

$$[Fe(C_2O_4)_3]^{3-} + h\nu \rightarrow Fe^{2+} + 2C_2O_4^{2-} + C_2O_4^{\bullet-} \qquad (15)$$

$$[Fe(C_2O_4)_3]^{3-} + C_2O_4^{\bullet-} \rightarrow Fe^{2+} + 3C_2O_4^{2-} + 2\ CO_2 \qquad (16)$$

$$C_2O_4^{\bullet-} + O_2 \rightarrow O_2^{\bullet-} + 2\ CO_2 \qquad (17)$$

2.2.4. Heterogenous photocatalysis

Heterogeneous photocatalysis represents an example of AOP capable of achieving a complete oxidation of organic and inorganic species, including also pharmaceutical substances. It takes advantage of some semiconductor solids, which can be used as photocatalysts suspended in the water effluent to be treated, or immobilised on various types of supports. Among the various solids, polycrystalline anatase TiO_2 is largely used because of its low cost and its photostability. Unfortunately, from the economic point of view the treatment of polluted waters with the photocatalytic process can be competitive, with respect to conventional processes, only under particularly favorable circumstances. The use of membrane technology processes, instead, has already been demonstrated to be competitive with respect to the other separation processes, owing to low energy cost and environment impact (Molinari et al., 2006).

Photocatalysis of organic compounds using TiO_2 particles presents many advantages: the large number of organic compounds dissolved, or dispersed in water undergo complete mineralization (Molinari et al., 2006). TiO_2 is used as a photocatalyst because of its nontoxicity, and photochemical stability.

When aqueous TiO_2 suspension is irradiated with light energy greater than the band gap energy of the semiconductor, conduction band electrons (e_{cb}^-) and valence band holes (h_{vb}^+) are formed. The photogenerated electrons react with adsorbed molecular O_2, which is reduced to superoxide radical anion $O_2^{\bullet-}$, and the photogenerated holes either can oxidize the organic molecules directly, or can oxidize OH^- ions and water molecules adsorbed on the TiO_2 surface to $^{\bullet}OH$ radicals. These will act as strong oxidizing agents that can easily attack any organic molecules adsorbed on, or located close to, the surface of the catalyst, thus leading to their complete degradation into small inorganic species (Abdelmalek et al., 2007):

$$TiO_2 + h\nu \rightarrow TiO_2\,(e_{cb}^- + h_{vb}^+) \qquad (18)$$

$$O_2 + e_{cb}^- \rightarrow O_2^{\bullet-} \qquad (19)$$

$$H_2O + h_{vb}^{+} \rightarrow {}^{\bullet}OH + H^{+} \qquad (20)$$

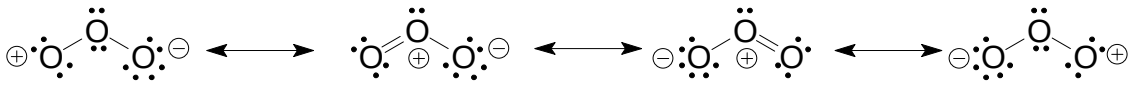
$$^{\bullet}OH + RH \rightarrow R^{\bullet} + H_2O \qquad (21)$$

$$R^{\bullet} + O_2 \rightarrow R'COO^{\bullet} \rightarrow R'^{\bullet} + CO_2 \qquad (22)$$

Organic pollutants are attacked and oxidized by the radicals formed through the above mechanisms. In addition to hydroxyl radicals, superoxide radical anions and positive holes are also suggested as possible oxidizing species that can attack organic contaminants present at or near the surface of TiO_2 (Lu et al., 2008).

2.2.5. Ozonation

Ozone, a triatomic molecule is an extremely unstable gas. It is the strongest oxidant of the common oxidizing agents, manufactured by passing air or oxygen through two electrodes with high, alternating potential difference.



Ozone may be produced by electrolysis, photochemical reaction, radiochemical reaction, or by “electric discharge” in a gas that contains oxygen. The electric discharge principle has been used in most commercial applications and in all known water- and wastewater-treatment applications.

At ordinary temperatures ozone is a blue gas, but at typical concentrations its colour is not noticeable. The stability of ozone is greater in air than in water but is not excessively long in either case. The half-life of residual ozone in water has been reported to range from 8 min to 14 h depending on the phosphate and carbonate concentration of the water. In the absence of phosphates and carbonates, water having a pH of 7 was found to have an ozone half-life of 8 min (Shammas and Wang, 1999). Ozone solubility in water is important because ozone disinfection is dependent on the amount of ozone transferred to the water. Henry’s law relative to ozone systems states that the mass of ozone that will dissolve in a given volume of

water, at constant temperature, is directly proportional to the partial pressure of the ozone gas above the water.

Ozone is a very strong oxidizing agent, having an oxidation potential of 2.07 V. It will react with many organic and inorganic compounds in water or wastewater. These reactions are typically called “ozone demand” reactions. They are important in ozone disinfection system design because the reacted ozone is no longer available for disinfection. Waters or wastewaters that have high concentrations of organics or inorganics may require high ozone dosages to achieve disinfection. It is very important to conduct pilot plant studies on these wastewaters during ozone disinfection system design in order to determine the ozone reaction kinetics for the level of treatment prior to ozone disinfection. The goal of the oxidation reactions is to produce an end product that is less toxic than the original compound; therefore toxicity of oxidation products should be monitored. Numerous studies have been completed describing the reactions with ozone and various inorganic and organic compounds (Sotelo et al., 1987; Goel et al., 1995; Ternes et al., 2003; Hoigne, 1998).

Because it is such a strong oxidant, ozone is also a powerful disinfectant. When exposed to a neutral or alkaline environment (pH above 6), UV light, or hydrogen peroxide, it decomposes in water to more reactive hydroxyl free radicals as shown in the Eq. 23 below (Shammas and Wang, 1999):

$$O_3 + H_2O \rightarrow O_2 + 2\ ^{\bullet}OH \qquad (23)$$

This reaction is accelerated at pH values above 8. It was found that ozone will react directly with solutes in the water, and that hydroxide ions (OH^-) and hydroxyl radicals ($OH^{\bullet}$) will provide a catalyst for the decomposition of ozone into intermediate compounds that are also reactive, such as peroxide ions (O_2^-) and the radical $HO_2^{\bullet}$ (Shammas and Wang, 1999). These results suggest that ozone disinfection is influenced by raw water chemistry characteristics, in addition to the better-known influences of wastewater pollutants. These influences are important to keep in mind when a comparison is made of ozone disinfection performance at different treatment plants.

Because ozone is unstable at ambient temperatures and pressures, it must be generated onsite and used quickly. Ozone is generated by applying energy to oxygen (pure oxygen or dried air). A high-energy electrical field causes oxygen to dissociate according to the Eq. 24 below:

$$O_2 + e^- \rightarrow 2\,[O] + e^- \qquad (24)$$

These oxygen "fragments" are highly reactive and combine rapidly with molecular oxygen to form the triatomic molecule, ozone:

$$2\,[O] + 2\,O_2 \rightarrow 2\,O_3 \qquad (25)$$

The overall reaction that produces ozone is the sum of the above reactions:

$$3\,O_2 + e^- \rightleftharpoons 2\,O_3 + e^- \qquad (26)$$

This reaction is reversible; once formed, ozone decomposes to oxygen. This reverse reaction increases with temperature and occurs quite rapidly above 35ºC. Because of this, ozone generators have cooling components to minimize ozone losses by thermal decomposition. Ozone is only slightly soluble in water depending on the temperature and its concentration as it enters the ozone contactor. The higher the concentration of ozone generated, the more ozone dissolves in water. Increasing pressure in the ozone contactor system increases its solubility.

Ozonation has been demonstrated to be successfully used to treat water containing antibiotics (Andreozzi et al., 2005) and also as a pretreatment step to improve the biodegradability of wastewater containing them (Balcioglu and Otker, 2003). The efficiency of ozone degradation is well known, however the stability and the toxicity of the intermediate degradation products have to be adequately evaluated.

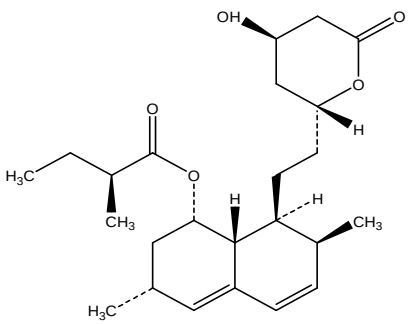
2.3. Statin compounds

2.3.1. General overview

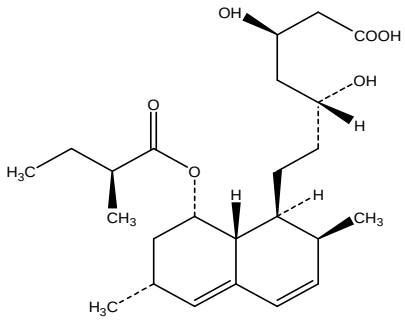
Statins are commonly used to treat several forms of hypercholesterolemia. Simvastatin, lovastatin and pravastatin (Fig. 2) are the most commonly available pharmaceutical formations used for the clinical treatment of hypercholesterolemia. Simvastatin and pravastatin are nowadays produced semi-synthetically from lovastatin and mevastatin (Metcalfe and Miao, 2003). Depending upon chemical structure, statin have different affinities for HMG-CoA reductase, which determines their pharmacological effects and different pharmacokinetic properties (eg., tissue distribution and metabolic stability).

Statins exist in two forms, lactone (Sim-L, Lov-L, Pra-L) and hydroxy acid (Sim-H, Lov-H, Pra-H) in general (Hwang and Yang, 2006). However our research revealed pravastatin only as Pra-H. In vivo, the hydroxy acid forms are the active drugs to lower plasma cholesterol while the lactone forms are inactive (prodrug). Lactone form of statin can be absorbed from the gastrointestinal tract and hydrolysed to active β-hydroxy acid in the liver and non-hepatic tissues (Hwang and Yang, 2006; Erturk, et al., 2003).

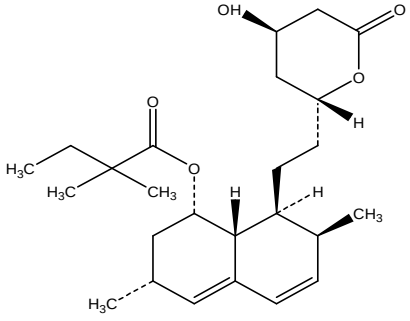
The instability of the lactone compounds (for lovastatin and simvastatin), is reflected in their hydrolysis in aqueous media, while for acidic compound (pravastatin) in ester formation by reaction with alcohols. If Sim-L and Lov-L are hydrolyzed to Sim-H and Lov-H they might form esters, for example with methanol, during extraction procedure, since there is one active OH group, positioned on β location in the statin molecule. Interconversions between closed lactone and open hydroxy acid forms may take place in the biological matrix before collecting aliquots of samples for the analysis; during extraction procedure, during evaporation to dryness or reconstitution; in the solution in the injection vial and in the case of MS, in the ion source as well.



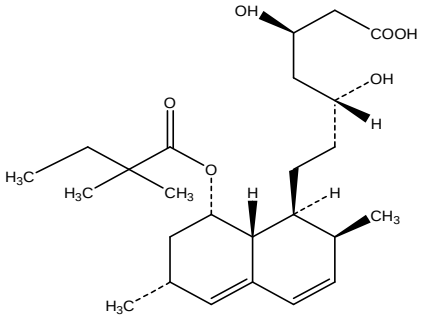
Formula: $C_{24}H_{36}O_5$ (MW=404)
Lovastatin (lactone form)
Lov-L



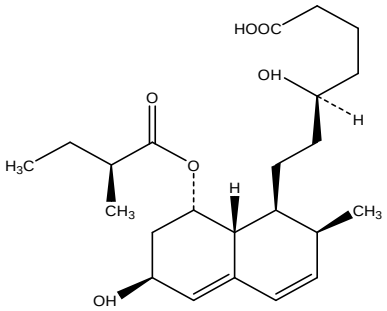
Formula: $C_{24}H_{36}O_6$ (MW=422)
Lovastatin (hydroxy acid form)
Lov-H



Formula: $C_{25}H_{35}O_5$ (MW=418)
Simvastatin (lactone form)
Sim-L



Formula: $C_{25}H_{40}O_6$ (MW=436)
Simvastatin (hydroxy acid form)
Sim-H



Formula: $C_{23}H_{36}O_7$ (MW=424)
Pravastatin (hydroxy acid form)
Pra-H

Fig 2: Chemical structure of statin in lactone and hydroxy acid form, their formulae, molecular mass and name abbreviation

2.3.2. Statin occurrence in biological and environmental samples

The presence of pharmaceutical compounds in general in surface water is an emerging environmental issue. Sewage treatment plants (STPs) are recognized as being main point discharge sources of these substances to environment. Due to high persistence and widespread occurrence of lipid-regulating agents in aquatic environments, their presence in drinking water has also been reported. (Hernando et al., 2007). In contrast to the extensive information related to the fibrate class drugs in the environment, very few papers have been published reporting the presence of pharmaceuticals belonging to the "statin" class. Available data for the statin class refers to the detection of atorvastatin (Lipitor) in wastewater from municipal STP (1-117 ng L^{-1}) and in rivers at low levels of ng L^{-1} (Metcalfe and Miao, 2003; Hernando et al., 2007). Table 1 summarizes the data of occurrence of statin compounds detected in surface waters, STP's effluents and influents (Metcalfe and Miao, 2003).

Table 1: Available data of presence of statin drugs in the environment – method detection limits, mean concentrations and standard deviations

Analyte	**STP influent (ng L^{-1}, n=3)**		**STP effluent (ng L^{-1}, n=3)**		**Surface water (ng L^{-1}, n=3)**		**Reference**
	Mean conc.	**MDL**	**Mean conc.**	**MDL**	**Mean conc.**	**MDL**	
Atorvastatin	76±3	1.2	37±2	0.5	1±0	0.1	Metcalfe and Miao, 2003; Metcalfe and Miao, 2002
Lovastatin	49±2	1.2	14±1	0.9	nd	0.1	Metcalfe and Miao, 2003
Pravastatin	117±6	15.4	59±2	9.8	nd	1.0	Metcalfe and Miao, 2003
Simvastatin	4±0	1.0	1±0	0.2	nd	0.1	Metcalfe and Miao, 2003

nd=not detected

Pravastatin is active in the administrated sodium salt, and is absorbed from the gastrointestinal tract, undergoing extensive first-pass metabolism in the liver. Approximately 50 % of it is bound to plasma proteins, while the rest is mainly excreted in the feces via the bile, with a smaller proportion excreted in the urine. In

plasma, urine, feces, it is found as Pra-H (free form of sodium salt) or one of its isomeric metabolites (R-416) (Erturk et al., 2003; Whigan et al., 1989; Kawabata et al., 2005). Available data for environmental detection of pravastatin refers to its presence in STP influents and effluents in Pra-H form (Metcalfe and Miao, 2003).

Lovastatin and simvastatin (Lov-L and Sim-L) are prodrugs that are administrated as inactive lactone forms. Their lactone forms are absorbed from the gastrointestinal tract and hydrolysed to active β-hydroxy acid form in the liver. Both drugs and β-hydroxy acid metabolites are extensively (95 %) bound to plasma proteins. The substances undergo extensive first-pass metabolism in the liver and are mainly excreted in the bile; about 85 % of administrated dose has been recovered from the feces as metabolite and about 10 to 15 % from the urine, mainly as inactive lactone forms (Erturk et al., 2003).

Statin compounds may enter the environment through different pathways, resulting in the contamination of waste water or fresh water, where bacteria are most likely the primarily affected organisms. Regarding toxicity of these compounds to other non-target organisms, there is almost no data. The current use of statins is not likely to cause acute environmental toxic effects, however they are considered for long-term use and their human metabolites might reach natural waters through sewage treatments plants. Again, chronic ecotoxicity data, which would be more relevant for risk assessment of compounds that are continuously released into the environment, are lacking (Carlsson et al., 2005).

While transformation of pharmaceuticals in the human body and in other mammals has been studied extensively, the microbial degradation of such compounds, its degradation pathways and products, have rarely been investigated and are seldom known. OECD biodegradation tests are commonly used to obtain a first characterisation of organic compounds in terms of their accessibility to microbial degradation and play an important role in the EU environmental classification of chemicals (Stasinakis et al., 2007).

2.3.3. Persistence, toxicity and bioaccumulation of lipid-regulating agents

At present, little data is available on the persistence of pharmaceuticals in the aquatic environment. This information could be obtained using a combination of experimental investigations (OECD, Organization for Economic Co-operation and Development, guidance 301 and 308). For instance, studies based on the laboratory degradation tests in water/sediment systems have shown which pharmaceuticals can persist a long time in water and sediments (>100 days) (Oppel et al., 2004), but these experiments can be costly and time consuming. An alternative approach is to use predictive models developed using factors related to chemical structure or environmental conditions (Estimation programs interface (EPI) version 3.11 for Windows (2003)). The vast majority of publications deal with quantitative structure–biodegradation relationships (QSBRs), which rely on octanol/water partition coefficients (K_{ow}), van der Waal's radii, alkaline (abiotic) hydrolysis rate constants and various molecular connectivity indices. The application of these models could be limited to a very specific class, and could be inappropriate for predicting the biodegradation rates for chemicals outside a determined class (Hernando et al., 2007).

The PBT Profiler (Persistent, Bioaccumulative, Toxic) is using a chronic (long-term) toxicity value so called chronic value (ChV) to estimate a chemical's relative toxicity for fish following the quantitative structure-activity relationship (QSAR). The ChV value corresponds to the geometric mean of the lowest observed effect concentration (LOEC) and non-observed effect concentration (NOEC). PBT Profiler estimates the half-life for the degradation of a chemical in water, soil and sediments by using the biodegradation survey module of the BIOWIN estimation program (Hernando et al., 2007).

By applying EU vPvB (very persistent and very bioaccumulative) criteria for sediments and freshwaters (half time should not exceed 180 days (in marine or freshwater sediments) and 60 days (for marine or freshwater) in order to classify the compound as non-persistent (see Table 2) pravastatin and mevastatin comply with requirements. The half-life $DT_{50\ sed}$ for both compounds were calculated as 140 days

and $DT_{50\ water}$ as 15 days (Table 3). All the other statins exceed the cut-off values (Table 3).

Table 2: EU and EPA guidelines for substance toxicity classification

PBT criteria	Persistence	Bioaccumulation	Toxicity
EU PBT criteria	Half-life > 60 days in marine water > 40 days in freshwater > 180 days in marine sediments	BCF > 2000	Chronic NOEC<0.01 mg L^{-1} or CMR or endocrine-disrupting effect
EU vPvB criteria	Half-life > 60 days in marine or freshwater > 180 days in marine or freshwater sediments	BCF > 5000	Not applicable
EPA criteria	≥ 60 days (persistent) ≥ 180 days (very persistent)	BCF ≥ 1000 (bioaccumulative) BCF > 5000 (very bioaccumulative)	> 10 mg L^{-1}, low concern 0.1 – 10 mg L^{-1}, moderate concern < 0.1 mg L^{-1}, high concern

BCF bioconcentration factor
NOEC no observed effect concentration
vPvB very persistent and very bioaccumulative
CMR carcinogenic, mutagenic or toxic to reproduction
PBT - persistent, bioaccumulative, toxic)

The rate of adsorption (K_{oc}) of simvastatin, estimated as 8400 (Table 3), should also indicate that simvastatin is expected to be immobile in soil (Hansch et al., 1995). The half-life (DT_{50}) values of lipid-regulating agents in water, soil and sediments are shown in the Table 3.

In the case of the statin class, the major metabolites are hydroxylated and hydroxy acid derivatives, but to our knowledge, the data reporting detection of statin residues in the environment is limited to one study in Canada (Metcalfe and Miao, 2003). For the pharmaceuticals which are highly metabolized (e.g. atorvastatin) and therefore poorly excreted, it should be noted that a negative correlation may occur, i.e. having a low degradability in the environment, such as in the case of clofibrate and clofibric acid (Hernando et al., 2007).

Wastewater and sewage sludge are the major vectors for the monitoring of environmental entrance of these compounds, which is dependable on their physico-chemical properties. The most widely accepted measure of bioaccumulation is the bioconcentration factor (BCF), but in the absence of BCF values, the bioaccumulation potential may be indicated from $\log K_{ow}$ values. Another alternative to the measured values of BCF is the use of predictive models such as quantitative structure–property relationship (QSPR)-based models or by the use of molecular connectivity indices and polarity correction factors (Liu et al., 2006). The PBT Profiler estimates a BCF based on a chemical's physical and chemical properties.

In the statin class, BCF values were calculated (EPI software, version 3.11 for Windows US EPA) (Hernando et al., 2007) and their values were determined as: 800 (simvastatin); 2000 (fluvastatin); 56 (atorvastatin); 380 (lovastatin), 220 (mevastatin) and 3.2 (pravastatin). All of those compounds (besides fluvastatin) meet both EU and EPA criteria, giving BCF value lower than 1000. Fluvastatin is the only pharmaceutical of this group which exceeds EU and EPA criteria (BCF$\geq$1000) and would be considered as bioaccumulative (Table 2 and Table 3).

Most of the studies dedicated to assess the hazard posed by pharmaceuticals in the environment have been based on acute toxicity data. Pharmaceuticals occur in the environment at very low concentrations and for short exposure times which might not show acute toxicity effects. However, these compounds could induce disturbances which should achieve higher relevance and biological responses being detectable as adverse effects after a long time period of exposure. In this sense, further data on chronic toxicity analysis should contribute to the assessment of their potential impact in the environment. Extensive knowledge concerning adverse effects at the genetic level, related to biological signaling cascade effects, or effects on the ecosystem is still needed. Regarding acute toxicity, most studies have been carried out with aquatic organisms, determining the effective concentration (EC_{50}) through standardized methods. Different *in vivo* (algae, crustaceans and bacteria) and *in vitro* tests (cytotoxicity, enzyme-based assays) have been applied to evaluate acute toxicity, in particular for compounds of the fibrate class (Hernando et al., 2004; Halling-Sorensen et al., 1998; Emblidge and DeLorenzo, 2006). Till now, the ecotoxicity studies developed for lipid-regulating agents have been limited.

To our knowledge, chronic toxicity studies on aquatic organisms have mainly been performed for fibrate class lipid regulators. The development of experiments with chronic toxicity tests can be unachievable in most cases due to the characteristics of this kind of test limiting the knowledge of their potential effects. Supplementary information can be achieved through predictive models, although we should be aware that they are giving only preliminary, predictive data. Using PBT Profiler approach, the ChV values calculated for lipid regulators exceed the EU and EPA criteria with NOEC (no observed effect concentration) and would show for several test species toxicity, at the ng L^{-1} level (Hernando et al., 2007).

The estimated NOEC values for statin class of lipid regulators: for fluvastatin (1.4 μg L^{-1}), simvastatin (56 μg L^{-1}) and atorvastatin (86 μg L^{-1}) (Table 3), according to EU PBT guidelines (Table 2) classify them as chronically toxic (with NOEC<0.01 mg L^{-1}). The same criteria, but applied for lovastatin (130 μg L^{-1}), pravastatin (180 μg L^{-1}) and mevastatin (270 μg L^{-1}) (Table 3) do not point them out as chronically toxic or endocrine-disrupting compounds.

The type of activity and mode of action of these pharmaceuticals, which are designed for the treatment of diseases in humans, could serve as important basis and source of information for deriving risks in the environment, since the pharmacodynamic activity (on receptors, tissues and organs) and pharmacokinetic properties of pharmaceuticals are well known. In this sense, this information can help in the selection of the appropriate test organisms to assess chronic toxicity. Thus, from the mammalian toxicology database of the pharmaceuticals, the ratio of acute to chronic toxicity, target organs, genotoxicity, reproduction or immunotoxicity could be useful for deriving environmental risk. For example, vertebrates (fish) have many physiological functions in common with mammals and it is possible to observe similar endpoints in both organisms (Bjerselius et al., 2001). Other relevant data are obtained from pharmacokinetics studies (i.e. the process of absorption, distribution, metabolism and excretion (ADME)) which provides information about the kinetics of pharmaceutical in human body (formation of metabolites, which can help to predict expected amount and form of these compounds released to the environment). This could be a reference of the exposure which would be necessary for evaluating in eco-chronic toxicity tests (Hernando et al., 2007).

It should be noted that pharmacological and pharmacokinetic information from mammalian investigations could support ecotoxicity evaluations in vertebrates (fish); however, for invertebrates, approaches tailored in the pharmacological function and activity may be assumed by taking into consideration several aspects such as those related to the presence of enzymes, receptors, etc. for detecting the pharmacological activity or toxicological effects (secondary effects) in the test organisms. Thus, for the generation of effects in organisms, these must possess the structure or function which is targeted in the therapeutic indication. In particular, some of compounds of the statin class have a mechanism of action by inhibiting the target enzyme hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) in a relatively non-specific way related to its localization in different tissues which could be responsible for no target effects. This is the case with cerivastatin which was withdrawn from the market because of its relation with rhabdomyolysis. On the other hand, other compounds of the statin class which act through a specific mechanism reducing the possibilities of adverse effects do not show a direct correlation with damage in ecological species, but the hazard of these pharmaceuticals could be assessed by taking into consideration the implications of the adverse effects. Secondary effects of statins in humans have been described related to cholesterol biosynthesis, effects on gene expression and effects on immunologic parameters, which may not be relevant for the characterization of environmental hazards (Hernando et al., 2007).

The teratogenic potential of a compound can be estimated qualitatively by examining embryonic growth of the exposed organisms, including type and severity of the induced malformations, and quantitatively by creating the Teratogenic Index (TI = LC_{50}/EC_{50}). Generally, TI values greater than 1.5 indicate a teratogenic potential (Fabro et al., 1982). Richards and Cole (2006) investigated lovastatin toxicity, teratogenity, minimum concentration to inhibit growth, and types and severity of associated malformation to Hgnopus blastulae, Stage 9. Experimental data proved that lovastatin displays teratogenic effects - teratogenicity index based on the LC_{50}/EC_{50} is equal to 2.5 (LC_{50} = 52.2 mg L^{-1}, EC_{50}= 20.5 mg L^{-1}). Minimal concentration to inhibit growth (MCIG) has been determined at 20.0 mg L^{-1}. The malformation observed for lovastatin compound was abnormal gut coiling. Some recent studies have seen subtle effects at concentrations similar to those measured in the environment.

Low, sublethal concentrations of simvastatin (0.16 µg L^{-1} – 5.0 µg L^{-1}) were found to affect the development time, body length, RNA content, and growth rate of harpacticoid copepods (Dahl et al., 2006)

Predictive models, such as PBT profiler, using factors related to chemical structure or environmental conditions (Estimation programs interface EPI version 3.11 for Windows) give only initial information about potential persistence, toxicity and bioaccumulation of the compound. In order to confirm if values, obtained with PBT profiler are reflected in real data, laboratory investigations should be performed.

Table 3: Profile of lipid-regulating agents. Reported bioaccumulation, persistence and toxicity data

Lipid-regulating agents	**Acute toxicity**			**Chronic toxicity**			**Persist. DT_{50} (days)**	**Bioaccum. BCF and/or $logK_{ow}$**	**Ref.**
	Test organism/endpoint toxicity	**EC_{50}**	**Ref.**	**Test organisms/endpoint toxicity**	**NOEC (mg L^{-1})**	**Ref.**			
"Fibrate" class									
Clorofibrate	Gambusia holbrooki, effects on AChE, LDH and CAT	7.7 mg L^{-1} (96 h)	Nunes et al., 2004	Gambusia holbrooki, effects on AChE, LDH and CAT	–	Nunes et al., 2004	DT_{50} water=38 DT_{50} soil=75 DT_{50} sed=340	BCF=120 $LogK_{ow}$=3.6	PBT Profiler, Lee et al., 2005
	Rainbow trout, fatty acyl-CoA oxidase (FOA)	46–152 mg/kg/day	Daughton and Ternes, 1999	Daphnia magna./reproduction	0.01	Halling-Sorensen et al., 1998			
	Cytotoxicity in fish cell	0.46 mg L^{-1} (24 h)	Andreozzi et al., 2003	Fish ChV (estimated)	0.64				
	Algae/growth inhibition	12 mg L^{-1}	Halling-Sorensen et al., 1998						
	Algae (estimated	0.50 mg L^{-1}	Gagne et al., 2006						
	Fish (estimated)	5 mg L^{-1}	Gagne et al., 2006						

Lipid-regulating agents	Acute toxicity			Chronic toxicity			Persist. DT_{50} (days)	Bioaccum. BCF and/or $logK_{ow}$	Ref.
	Test organism/endpoint toxicity	**EC_{50}**	**Ref.**	**Test organisms/endpoint toxicity**	**NOEC (mg L^{-1})**	**Ref.**			
	Daphnia magna	28.2 mg L^{-1}	Halling-Sorensen et al., 1998						
	Daphnid (estimated)	6.50 mg L^{-1}	Gagne et al., 2006						
	EROD	96 mg L^{-1}	Andreozzi et al., 2003						
Fenofibrate	Cytotoxicity in fish cell	3.25 mg L^{-1} (24 h)	Andreozzi et al., 2003	Fish ChV (estimated)	0.048	Halling-Sorensen et al., 1998	DT_{50} water=60 DT_{50} soil=120 DT_{50} sed=540	BCF=290 $LogK_{ow}$=5.19	PBT Profiler, Lee et al., 2005
	Daphnia magna	50 mg L^{-1}	Hernando et at., 2004						
	Daphnid (estimated)	0.35 mg L^{-1}	Gagne et al., 2006						
	Fish (estimated)	0.8 mg L^{-1}	Gagne et al., 2006						
	Algae (estimated)	0.10 mg L^{-1}	Gagne et al., 2006						
	EROD	25 mg L^{-1}	Andreozzi et al., 2003						
Bezafibrate	Daphnia magna	30 mg L^{-1}	Han et at., 2006	Fish ChV (estimated)	3.8	Han et at., 2006	DT_{50} water=60 DT_{50} soil=120 DT_{50} sed=540	BCF=3.2 $LogK_{ow}$=4.25	PBT Profiler, Khan and Ongerth, 2002;
	Immobilization	>200 mg L^{-1}	Hernando et at., 2004						
	Daphnid (estimated)	25 mg L^{-1}	Gagne et al., 2006						
	Fish (estimated)	5.30 mg L^{-1}	Gagne et al., 2006						

Lipid-regulating agents	**Acute toxicity**			**Chronic toxicity**			**Persist. DT_{50} (days)**	**Bioaccum. BCF and/or $logK_{ow}$**	**Ref.**
	Test organism/endpoint toxicity	**EC_{50}**	**Ref.**	**Test organisms/endpoint toxicity**	**NOEC (mg L^{-1})**	**Ref.**			
	Algae (estimated)	18 mg L^{-1}	Gagne et al., 2006	Daphnia magna Reproduction 21 days	ND	Han et al., 2006			
Gemfibrozil	Daphnia magna	10.4 mg L^{-1}	Han et at., 2006	Fish ChV (estimated)	0.93	Han et al., 2006	DT_{50} water=38	113 times in blood (goldfish)	Zuccato et al., 2000
	Immobilization	100 mg L^{-1}	Hernando et at., 2004				DT_{50} soil=75 DT_{50} sed=340	BCF=3.2	PBT Profiler
	Daphnid (estimated)	6 mg L^{-1}	Gagne et al., 2006					$LogK_{ow}$=4.77	Khan and Ongerth, 2002
	Algae (estimated)	4 mg L^{-1}	Gagne et al., 2006						
	Fish (estimated)	0.9 mg L^{-1}	Gagne et al., 2006						
"Statin" class									
Atorvastatin				Fish ChV (estimated)	0.086	PBT Profiler	DT_{50} water=60 DT_{50} soil=120 DT_{50} sed=540	BCF=56 $LogK_{ow}$=4.46	PBT Profiler, Jjemba, 2006

Lipid-regulating agents	**Acute toxicity**			**Chronic toxicity**			**Persist. DT_{50} (days)**	**Bioaccum. BCF and/or $logK_{ow}$**	**Ref.**
	Test organism/endpoint toxicity	**EC_{50}**	**Ref.**	**Test organisms/endpoint toxicity**	**NOEC (mg L^{-1})**	**Ref.**			
Simvastatin				Fish ChV (estimated)	0.056	PBT Profiler	DT_{50} water=38 DT_{50} soil=75 DT_{50} sed=340	BCF=800 $LogK_{ow}$=4.68 Koc=8400	PBT Profiler. Laville et al., 2004
Fluvastatin				Fish ChV (estimated)	0.0014	PBT Profiler	DT_{50} water=38 DT_{50} soil=75 DT_{50} sed=340	BCF=2000 $LogK_{ow}$=4.85	Lee et al., 2005, Laville et al., 2004
Lovastatin				Fish ChV (estimated)	0.13	PBT Profiler	DT_{50} water=38 DT_{50} soil=75 DT_{50} sed=340	BCF=380 $LogK_{ow}$=4.26	PBT Profiler, Laville et al., 2004
Pravastatin				Fish ChV (estimated)	0.18	PBT Profiler	DT_{50} water=15 DT_{50} soil=30 DT_{50} sed=140	BCF=3.2 $LogK_{ow}$=n.a.	PBT Profiler
Mevastatin				Fish ChV (estimated)	0.27	PBT Profiler	DT_{50} water=15 DT_{50} soil=30 DT_{50} sed=140	BCF=220 $LogK_{ow}$=3.95	PBT Profiler, Lee et al., 2005

Lipid-regulating agents	**Acute toxicity**			**Chronic toxicity**			**Persist. DT_{50} (days)**	**Bioaccum. BCF and/or $logK_{ow}$**	**Ref.**
	Test organism/endpoint toxicity	**EC_{50}**	**Ref.**	**Test organisms/endpoint toxicity**	**NOEC (mg L^{-1})**	**Ref.**			
Metabolites									
Clorofibric acid	Gambusia holbrooki, effects on AChE, LDH and CAT	–	Nunes et al., 2004	C. dubia	0.64 (7 days)	Ferrari et al., 2003	DT_{50}water=100 DT_{50} water=38 DT_{50}soil=75 DT_{50} sed=340	BCF=3.2 $LogK_{ow}$=2.84–2.50	PBT Profiler
	Scenedesmus subspicatus/ growth inhibition	89 mg L^{-1} (96 h)	Herberer, 1997	B. calyciflorus	0.246	Ferrari et al., 2003			
	Photobacterium phosphoreum	NOEC 5-40 μg L^{-1}	Emblidge and DeLorenzo, 2006	D. rerio	0.70 (10 days)	Ferrari et al., 2003	DT_{50}water=100 DT_{50} water=38 DT_{50}soil=75 DT_{50} sed=340	BCF=3.2 $LogK_{ow}$=2.84–2.50	PBT Profiler
	P. Pugio	No effect <1000 μg L^{-1}	Emblidge and DeLorenzo, 2006	Fish ChV (estimated)	38	PBT Profiler			
	F. Heteroclitus	No effect <1000 μg L^{-1}	Emblidge and DeLorenzo, 2006	Daphnia magna Reproduction 21 days	40	Han et al., 2006			
	D. tertiolecta	No effect	Emblidge and DeLorenzo, 2006	P. subcapitata 96 h	0.075				
	Cell density or growth rate	<1000 μg L^{-1}							
	Microtox 30 min	91 mg L^{-1}	Ferrari et al., 2003						
	Daphnia magna	106 μg L^{-1}	Ferrari et al., 2003	Gambusia holbrooki, effects on AChE,	0.075	Nunes et al., 2004			
	Immobilization	141 mg L^{-1}	Han et al., 2006						

Lipid-regulating agents	**Acute toxicity**			**Chronic toxicity**			**Persist. DT_{50} (days)**	**Bioaccum. BCF and/or $logK_{ow}$**	**Ref.**
	Test organism/endpoint toxicity	**EC_{50}**	**Ref.**	**Test organisms/endpoint toxicity**	**NOEC (mg L^{-1})**	**Ref.**			
	Daphnid (estimated)	200 mg L^{-1} 150 mg L^{-1} 293 mg L^{-1}	Hernando et at., 2004	LDH and CAT					
	Fish (estimated)	53 mg L^{-1}	Gagne et al., 2006						
	Algae (estimated)	192 mg L^{-1}	Gagne et al., 2006						
Fenofibric acid	Daphnid (estimated)	38 mg L^{-1}	Gagne et al., 2006	Fish ChV (estimated)	5.5	PBT Profiler	DT_{50} water=100 DT_{50} water=38 DT_{50} soil=75 DT_{50} sed=340	BCF=3.2 $LogK_{ow}$=4.0	PBT Profiler, Gagne et al., 2006
	Fish (estimated)	7.7 mg L^{-1}	Gagne et al., 2006						
	Algae (estimated)	26 mg L^{-1}	Gagne et al., 2006						

n.d. – not determined

2.3.3.1. Expected environmental occurrence of statin compounds

Once a chemical is released to the environment, it may move from one environmental compartment to another. The PBT Profiler, broadly described already in the previous chapter, can also be applied for the estimation of distribution of a chemical in air, water, soil, and sediment (estimated percentage in each medium relative to the total amount in the environment). The PBT Profiler determines the amount in each medium at steady state using a multimedia mass balance model (also called a fugacity model) that is based on a chemical's physical/chemical properties and reactivity. The Level III fugacity model is non-equilibrium, steady-state multimedia fate model that is designed to provide information on environmental partitioning and inter-media transport at the screening level. The multimedia mass balance model requires a series of physical/chemical properties and environmental half-lives as input. Physical properties are used directly by the fugacity model to determine the transport between environmental compartments. These properties used for each are: Henry's Law constant, vapour pressure, melting point, octanol/water partition coefficient and molecular weight (PBT Profiler).

Table 4 gathers physico-chemical properties of statin compounds (Sim-L; Lov-L and Pra-H) and the information about their environmental distribution (estimated by PBT Profiler).

Table 4: Physico-chemical properties of statin compounds and the information about their environmental distribution (estimated by PBT Profiler)

Simvastatin			
		Half life [days]	**Percent in each medium [%]**
Media	water	38	14
	soil	75	70
	sediment	340	16
	air	0.01	0
	Property	**Value**	**Units**
	Molecular Weight	418.58	g mol^{-1}
	Melting Point	483	K
	Vapor Pressure	$1.33 \cdot 10^{-10}$	Pa at 298 K
	Log K_{ow}	4.7	at 298 K
	Water Solubility	0.77	mg L^{-1} at 298 K
	Henry's Law Constant	$2.8 \cdot 10^{-10}$	atm m^{-3} mole at 298 K
	Hydroxyl Radical Reaction Rate Constant	$2.3 \cdot 10^{-10}$	cm^3 $molecule^{-1}$-sec^{-1} at 298 K
	Ozone Reaction Rate Constant	Not Estimated	-
Lovastatin			
		Half life [days]	**Percent in each medium [%]**
Media	water	38	14
	soil	75	70
	sediment	340	16
	air	0.01	0
	Property	**Value**	**Units**
	Molecular Weight	404.55	g mol^{-1}
	Melting Point	473	K
	Vapor Pressure	$1.33 \cdot 10^{-10}$	Pa at 298 K
	Log K_{ow}	4.3	at 298 K
	Water Solubility	2.1	mg L^{-1} at 298 K
	Henry's Law Constant	$2.1 \cdot 10^{-10}$	atm m^{-3} mole at 298 K
	Hydroxyl Radical Reaction Rate Constant	$2.3 \cdot 10^{-10}$	cm^3 $molecule^{-1}$-sec^{-1} at 298 K
	Ozone Reaction Rate Constant	Not Estimated	-
Pravastatin			
		Half life [days]	**Percent in each medium [%]**
Media	water	15	25
	soil	30	75
	sediment	140	0
	air	0.0096	0
	Property	**Value**	**Units**
	Molecular Weight	424.54	g mol^{-1}
	Melting Point	513	K
	Vapor Pressure	$1.73 \cdot 10^{-10}$	Pa at 298 K
	Log K_{ow}	3.1	at 298 K
	Water Solubility	12	mg L^{-1} at 298 K
	Henry's Law Constant	$1 \cdot 10^{-12}$	atm m^{-3} mole at 298 K
	Hydroxyl Radical Reaction Rate Constant	$2.4 \cdot 10^{-10}$	cm^3 $molecule^{-1}$-sec^{-1} at 298 K
	Ozone Reaction Rate Constant	Not Estimated	-

If released to the environment, simvastatin, pravastatin and lovastatin are expected to be found predominantly in soil. The half-life in soil (for simvastatin and lovastatin), 75 days, exceeds the EPA criteria of ≥ 2 months (and ≤ 6 months). Therefore, they are estimated to be persistent in the environment. Their vapor pressure, suggests that they will exist as a gas/particulate mixture in the atmosphere. Since particulates react slower with hydroxyl radicals and ozone (relative to a gas-phase reaction), the atmospheric lifetime of simvastatin, lovastatin and pravastatin is expected to be longer than that predicted by the PBT Profiler. As a result, their distribution in the various environment compartments may be different than that predicted by the PBT Profiler.

The PBT Profiler contains a simple set of algorithms to point out chemicals that may travel through soil into an underground aquifer based on its octanol/water partition coefficient (K_{ow}). It only highlights chemicals that are expected to have the potential to persist in groundwater. If the log K_{ow} of an individual chemical is < 4 and it is found to be persistent in soil or sediment (half-life > 2 months), then the PBT Profiler flags this chemical as one that has the potential to migrate to and be persistent in groundwater (PBT Profiler).

Moreover, the results of this model are calculated at steady state; a condition that may not occur in the environment (on a global scale). Sewage treatment plants are expected to be main point source discharge of statin compounds to the environment. PBT Profiler estimates that only 14 % (simvastatin and lovastatin) and 25 % (pravastatin) of total amount of statin, released to the environment will be found in the aqueous media. Statins might be subjected to partition to sediment and absorption to activated sludge. In the case of its usage as a soil conditioner (which is against the law in Slovenia) we could presume that statins might enter the soil. However, we still expect to find them in aqueous media and sediments, due to their continuous input from sewage treatment plants. We have to keep in mind that PBT Profiler might not reflect real data. One of our further experiments (biodegradability experiments) proofs that estimated half-life of statin, in biotic conditions, is not as foreseen by the PBT Profiler. Nevertheless, this model provides useful information to compare chemicals in a screening-level assessment. It is important to note, however, that the percent in each medium will change as a function of the size of the compartments

chosen, and laboratory investigation has to be performed in order to verify estimated scenarios.

2.3.4. Analytical methods for statin determination in biological and environmental samples

The developed methods published in the literature, enable the analysis of lipid regulators at concentration levels of ng L^{-1} (Hernando et al., 2007; Hernando et al., 2004; Quintana et al., 2004; Zuccato et al., 2005a; Comoretto and Chiron, 2005; Farre et al., 2001; Ahrer et al., 2001).

Quantification of statins and their human metabolites in biological matrix such as blood and urine has been extensively studied (Whigan et al., 1989; Kawabata et al., 2005; Bauer et al., 2005; Carlucci et al., 1992; Grahek et al., 2001; Huclova et al., 2006; Jemal et al., 2000a and 2000b; Zhang et al., 2004; Zhu and Neirinck, 2003). Similar method developments concerning statin residues in environmental samples (natural waters) present more difficulties and has not been widely investigated (Metcalfe and Miao, 2003; Hernando et al., 2007) Table 5 represents available data for statin group of pharmaceuticals in waters from waste water treatment plant and natural waters. Method detection limits for each compartment are already presented in the Table 1.

In spite of statin similar structures analytical methods for their quantitative determination in biological samples have been developed usually individually because of their different solubility, stability, etc. Almost all assays used for the separation of statins are based on either high-performance liquid chromatography (HPLC) or gas chromatography (GC). All HPLC methods for statin are based on reversed phase separation. Most often a UV detector, rarely a fluorescence detector and a mass detector have been used (Erturk et al., 2003).

Simultaneous determination of the lovastatin, simvastatin and pravastatin in plasma using GC with chemical ionization mass spectrometry has been assayed by derivatization with pentafluorobenzyl bromide. In this method the analytes are isolated from plasma by a solid-phase extraction procedure separating the lactone

and acid forms of the drug. Then the lactone is converted to the acid form which is subsequently derivatized by pentafluorobenzyl bromide (Erturk et al., 2003).

Some simple HPLC procedures with UV detection have been published (Carlucci et al., 1992). The HPLC-UV methods, being the sample preparation comparatively less than arduous, still required another reaction step (protein precipitation, centrifugation, etc.) and the chromatographic time is long. A highly sensitive and selective HPLC method using a fluorescent derivatization and column switching has been introduces by Ochiai et al. (1997). In this method, solid phase extraction (SPE) and derivatization procedures have been used, which are time consuming. This method has adequate quantification limits (0.1 ng L^{-1}) for both analytes, and is applied to the determination of simvastatin in plasma after oral administration. Jemal et al. (2000b) have presented LC-MS-MS procedure for determination of both forms of simvastatin in plasma with a positive ion electrospray method after cleaning-up and the extraction of the sample with sample extraction column in line with the analytical column. In this method, a sample preparation procedure has not been used, except the addition of the internal standard solution to the plasma samples prior to analysis by direct injection. The total run per sample was only 2.5 min.

In another work Jemal et al. (2000a) and his research group have presented LC-MS-MS procedure with atmospheric pressure ionization (API) for the simultaneous determination of pravastatin (Pra-H and Pra-L) and its main metabolite (3-α-hydroxy isomeric compound) in human serum. Kawabata et al. (1998) have described a HPLC method with MS detection by atmospheric pressure chemical ionization (APCI) without derivatization procedure. Jemal et al. (1998) have presented two HPLC procedures based on MS-MS detection. In the first method one column (Oasis) has been used for analysis of single analyte (tertamethylbutyl amine salt of Pra-H) in rat plasma. In the second method, a second column (C_{18}) has been added in line with the Oasis column to achieve the needed chromatographic separation. The second method has been applied with slight modifications to the quantitative determination of pravastatin and its isomeric biotransformation product in human, mouse, rat and monkey serum. Zhu and Neirinck (2003) have described a HPLC, with negative ion tandem mass spectrometric method, which provides good specificity and sensitivity with a total run time less than 2 min.

To conclude, most of the analytical methods published in the literature are developed for biological samples. Only scarce methods were implemented for environmental samples so far (Metcalfe and Miao, 2003). However both of them are based on the use of LC-MS systems. The selection of negative ionization (NI) mode has been the starting point for most of acidic pharmaceuticals (pravastatin) and positive-ion (PI) mode for the lactone compounds (i.e. lovastatin, simvastatin and mevastatin) of the statin group. LC separation of lipid regulators using a narrow-bore C_{18} column (2-mm i.d.) and the use of methanol or acetonitrile as organic mobile phase have been the optimal conditions which have enabled a better resolution and shorter retention times. Mobile phase additives for adjusting the pH and/or to enhance ionization, such as ammonium acetate or methylammonium acetate, have been common (Metcalfe and Miao, 2003). The mass analyzers, triple quadrupole or ion trap, have been successfully applied to the analysis of environmental samples, allowing the discrimination against matrix interference and achieving the sufficient sensitivity.

Table 5: LC-MS methods for the determination of statin residues in the environmental samples

Compounds	**Matrix**	**Sample pretreatment**	**Extraction method**	**LC column**	**Mobile phase**	**MS**	**Reference**
Lovastatin, pravastatin, simvastatin	Surface and WW	pH 4.5	SPE/HLB/MeOH	C_{18}	Aq. Methylamine/aq. CH_3COOH/MeCN	LC-ESI (+)-MS/MS	Metcalfe and Miao, 2003
Atorvastatin	Surface and WW	pH 4.0	SPE/HLB/MeOH	C_{18}	Aq. ammonium acetate/MeOH	LC-ESI (+/-)- MS/MS	Metcalfe and Miao, 2003; Metcalfe and Miao, 2002

The fragmentation mechanism of Sim-L and Lov-L was investigated previously by Wang et al. (2001) using both triple quadrupole and ion trap mass spectrometers with instruments parameters as follows:

- A Finnigan (San Jose, CA, USA) LCQ ion trap mass spectrometer equipped with an electrospray ionization interface was used for the MS^n experiments. Collision energies for the MS^n analyses ranged from 15 to 30 % depending on the compounds and/or fragment ions analyzed. The electrospray ionization voltage was 4.5 kV in the positive ion mode.
- For MS/MS experiments on a triple-quadrupole instrument, a PE SCIEX (Concord, Ontario, Canada) API-300 tandem mass spectrometer equipped with a TurboIonSpray interface was used. All MS/MS experiments were carried out in the positive ion mode. The electrospray needle voltage was set at 5.2 kV. Orifice voltages of 0-20 V were used for normal MS/MS experiments and higher orifice voltages of 20-60 V were applied when quasi-MS/MS/MS experiments were performed.

Fig. 3 presents general fragmentation pathway of Sim-L and Lov-L, proposed by Wang et al. (2001).

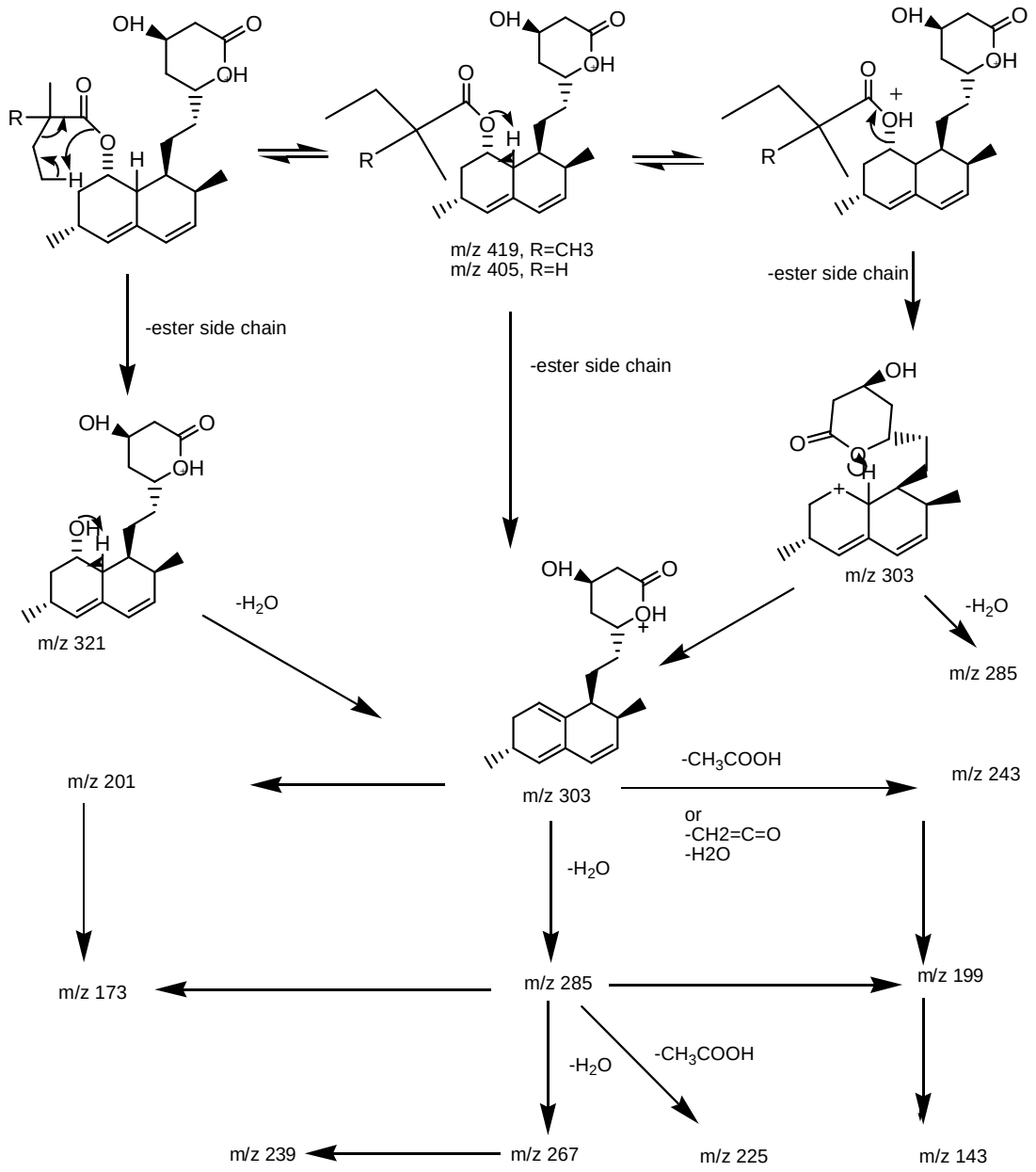


Fig 3: Proposed general fragmentation pathway for Sim-L and Lov-L (Wang et al., 2001)

The major product ions for Sim-L and Lov-L are *m/z* 199, 267, 285, 303 (Fig. 3), and *m/z* 285 corresponds to loss of ester side chain ($C_5H_{10}O_2$) and subsequently H_2O molecule. The product of direct cleavage undergo dehydration to generate the ion at *m/z* 285 or rearrange to the same ion at *m/z* 303 derived from the charge-induced fragmentation process. One of the possible formation pathways for the ion at *m/z* 303 is through the dehydration process from the ion at *m/z* 321 (Wang et al., 2001).

2.4. Methods of statin degradation

To our knowledge degradation of statin compounds in the environment has not been widely investigated, and available data are based mostly on the PBT Profiler, which gives only estimated, not real values about persistence, toxicity and bioaccumulation of lipid regulating agents. Models are able to identify where and when measurable concentrations will occur, but it is absolutely necessary to calibrate and validate the applied models against real data. Due to this fact, we decided to perform different experiments, broadly implemented for pharmaceutical compounds. During the degradation experiments a lot of attention has been paid to formed by-products and their toxicity.

A lot of research has been done in the field of determination of different pharmaceuticals and their by-products within photodegradation studies, including direct-, photosensitized- (photo-Fenton, hydroxyl radicals), and photocatalytic- (semiconductors, i.e. TiO_2) degradation, but to our knowledge studies dedicated to cholesterol-lowering statin drugs degradation and their transformation products are limited to the recently published paper of Radjenovic et al, 2009. He reported on the performances of full-scale conventional activated sludge (CAS) treatment and two pilot-scale membrane bioreactors (MBRs) in eliminating various pharmaceutically active compounds (PhACs) belonging to different therapeutic groups and with diverse physico-chemical properties. Applied two pilot-scale MBRs exhibited enhanced elimination of several pharmaceutical residues (and pravastatin among them), poorly removed by the CAS treatment. Pravastatin mean concentration value in the primary effluent in the WWTP (Terrassa, Spain) was found to be equal to 0.886 μ L^{-1}. The degradation of pravastatin was also enhanced from 60% in CAS to around 85% in MBRs.

III. Research goals

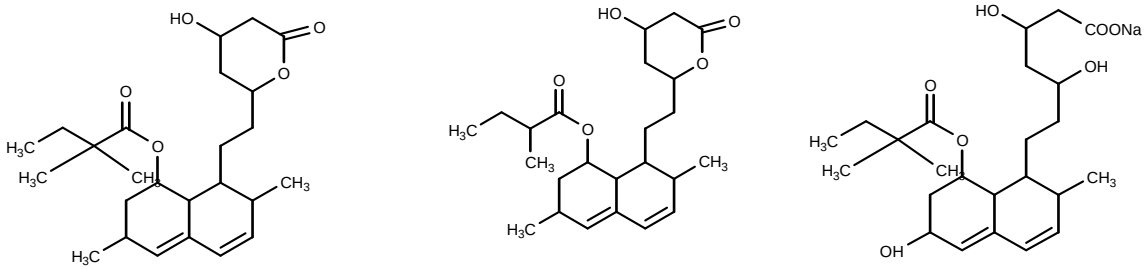
The main goals of research could be divided into three parts:

- development of sensitive and selective analytical methods for the determination of statin compounds in aqueous solutions. Determination studies were combined with stability experiments, since statins are significantly affected by various conditions such as pH, temperature, solvent systems, sun light, etc. Method for statin determination has been implemented for the degradation experiments, where higher concentration (mg L^{-1}) has been applied, which allowed easier monitoring of initial and transformation products, without time-requiring sample preparation
- application of different degradation techniques (ozonation and photocatalytic degradation as two important processes belonging to the class of Advanced Oxidation Methods (AOP'S)), in order to investigate their potential for statin removal in the case of their presence in STP's
- performance of toxicity measurements for standard statin compounds, as well as for by-products, formed during degradation experiments. The evaluation of short term acute toxicity using bioluminescence inhibition test with the marine bacterium *Vibrio fischeri* as the test organism was applied. Additionally, the assessment of statin biodegradability by the application of manometric respirometry test was performed.

IV. Experimental –in general

4.1. Materials and instruments

Simvastatin and lovastatin as lactones (Sim-L; Lov-L), and pravastatin sodium (Pra-H salt) (Fig 4), at least 99.9 % pure, were kindly provided by one pharmaceutical company. No further purification was required. Simvastatin and lovastatin are highly lipophilic compounds, while pravastatin is hydrophilic. Lovastatin and simvastatin are poorly soluble in water, with solubility values ranging from 1.3 - 1.5 µg mL^{-1} at 23 °C. In comparison, pravastatin is hydrophilic, as demonstrated by the greater than 100-fold solubility (0.18 mg mL^{-1}) (Serajuddin et al., 1991).



Simvastatin Lovastatin Pravastatin

Fig 4: Chemical structures of statin

Acetonitrile, acetone and methanol (all of analytical grade) were purchased from Sigma-Aldrich Company Ltd (Gillingham, GB), hydrochloric acid (HCl) from Riedel-de Haen (Hanover, Germany), sodium hydroxide and glacial acetic acid from Merck (Darmstadt, Germany). For all the experiments, if not stated differently, double de-ionized water was prepared through the Milli_Q Plus Ultra-Pure water system (Millipore, USA) and its purity was controlled by its resistivity. The SPE cartridges DSC-C_{18} (1 mL/100 mg and 3 mL/500 mg), STRATA-X (3 mL/60 mg and 3 mL/200 mg), Bond Elute C_8 (1 mL/100 g and 3 mL/200 mg) were purchased from Phenomenex (Torrance, USA) and Varian (Harbor City, USA) for the latter one. For all the LC-MS measurements LC-MS grade solvents: acetonitrile and water Sigma-Aldrich Company Ltd (Gillingham, GB) were used.

Statins were analyzed by several chromatographic techniques and instruments:

- high performance liquid chromatograph with diode-array detector (HP 1100 HPLC-DAD with Luna C_{18} column (4.6 mm x 250 mm, 3 µm) (Phenomenex (Torrance, USA)) was employed for the stability experiments (chapters:4.4.1; 4.4.2; 4.4.3; 4.4.4; 4.4.5; 4.4.6) and all the degradation experiments, in order to monitor the consumption of each statin and formation of the byproducts (chapters: 5.1.2.1; 6.2.3.1; 7.2.3)

- liquid chromatograph with mass spectrometry detector (Waters Alliance 2695 Separations Module), equipped with an ESI source. Terra MS C_{18} column (2.1 mm x 100 mm, 3.5 µm was used for analytical studies, only for some selected irradiated samples (photohydrolysis: chapter: 5.1.2.2); photocatalysed samples (chapter: 6.2.3.1); 12 months sun exposed samples and samples heated in the water bath (chapter: 4.4.5) and ozonated samples (7.2.3).

4.2. Methods

4.2.1. Solid phase extraction

For the best recovery of analytes solid phase extraction was performed according to the literature data (Metcalfe and Miao, 2003). Various concentrations of investigated statins (for each cartridge), flow rates, and amount of applied solvents for elution were tested during optimizing the extraction procedure. 1000 mL of double de-ionized water, spiked with 0.5 mg of each statin was selected as optimum. Time of sample preparation is crucial, since statins are susceptible to hydrolysis. Sample acidification only retards interconversion between lactone and hydroxy acid, but do not eliminate it completely. Therefore, while optimizing extraction procedure time played an important role.

The cartridges were sequentially preconditioned with 6 mL of acetone, 6 mL methanol and 6 mL water (pH 4.5). Thereafter the aqueous samples (1000 mL) were allowed to pass through the cartridges at a rate of approximately 10 mL min^{-1}. After passage of the samples, each sample bottle was rinsed with 10 mL of pH 4.5 water, and the rinse was allowed to flow through the cartridge. The cartridges were dried under the nitrogen stream, and subsequently rinsed using three successive 3 mL aliquots of methanol. Each aliquot of methanol was allowed to go through the cartridge for a minimum of 10 min. The eluates were collected in a 10 mL collection

tube and concentrated to almost dryness with a vacuum centrifuge. Then the samples were reconstituted to 1.0 mL with acetonitrile: acidified water (pH 4.5) (3:2).

Extraction procedure was developed for 1000 mL aqueous samples, in order to obtain approx. 1000 times concentration of the compounds in a sample, allowing determination of statins at the environmental level of ng L^{-1}.

4.2.2. Liquid chromatography (HPLC-DAD)

Aqueous samples (acidified with HCl to pH=4.5 in order to avoid hydrolysis of lactones) were prepared daily. The linearity of the analytical method (calibration curves) was tested using standard mixtures at concentrations between 10 µg L^{-1} – 1.28 mg L^{-1} (double deionized water spiked with the mixture of 3 statins). Correlation coefficients higher than 0.995 were obtained. Additionally, calibration curves were prepared for hydroxy acid forms of statins, by pH adjustment to the value 9, and converting lactones to acids. The same concentration range was applied, and correlation coefficients higher than 0.996 were obtained.

Mobile phase consisted of acetonitrile (mobile phase A) and water (pH 4.5 acidified with glacial acetic acid) (mobile phase B) with the flow rate 1 mL min^{-1}. The gradient programme was as follows: 0 min 15 % A; 4 min 25 % A; 6 min 40 % A; 12 min 100 % A and held until 20^{th} minute. Additional 5 min post-run time (switching to initial conditions) was applied afterwards. The sample injection volume was 50 µL. Absorbance was monitored at 238 nm, which was determined to be the optimum wavelength in preliminary studies performed on a spectrophotometer. The retention times were: 10.3 min for pravastatin (Pra-H), 13.7 min and 13.0 min for lovastatin (Lov-L and Lov-H); 14.3 min and 13.7 min for simvastatin (Sim-L and Sim-H)

(Fig. 5 represents HPLC-DAD chromatogram for freshly prepared samples, where only lactone form of Sim-L and Lov-L were observed).

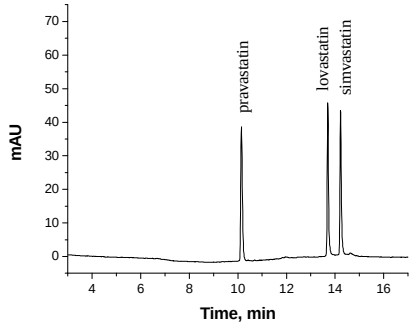


Fig 5: HPLC-DAD chromatogram of pravastatin, lovastatin and simvastatin standard solution

4.2.3. Liquid chromatography - mass spectrometry (LC-MS)

LC-MS studies were carried out with a Waters Alliance 2695 (Milford, USA) High performance liquid chromatography system coupled to a Quattro LC triple quadrupole mass spectrometer (Micromass (Manchester, UK)) equipped with a pneumatically assisted electrospray ionisation source (ESI) in positive ion mode and a Waters photodiode array detector. Instrument offset allowed recording of characteristics absorption bands, but recording of the whole UV absorbance spectra was not possible. The capillary voltage was set to 3.0 kV, while the sampling cone voltage was equal to 35.0 V. Source and desolvation temperature were set to 120.0 °C and 300.0 °C respectively. MS data were acquired over an *m/z* range 50 – 800 at collision energy of 10.0 eV, by MassLynx NT 3.5 Waters system. Mobile phase consisted of acetonitrile (mobile phase A) and water, acidified with acetic acid to pH=4.5 (mobile phase B) with gradient programme as follows: 0 min 5 % A; 15 min 95 % A; 25 min 95 % A, 35 min 5 % A. The flow rate was equal to 0.2 mL min^{-1} and the injection volume was equal to 30 µL.

Our research showed that positive-ion mode is more suitable for all of tested compounds, not only simvastatin and lovastatin, as we assumed before. Zhao et al. (2002) investigated the effects of ammonium and alkylammmonium acetate as mobile phase additives on the ionisation of simvastatin and simvastatin hydroxy acid, and observed that methylammonium acetate could much improve the ion signal intensity of simvastatin. In our study acetic acid was used as a mobile phase additive.

For mass spectrometric studies of standards: lovastatin and simvastatin were received as lactones they were administrated in this form (in fresh samples, pH=7), while pravastatin as sodium salt of the active hydroxy acid. Therefore pravastatin was observed as free acidic form. In order to define fragmentation of statin in hydroxy acid forms pH of the aqueous sample was adjusted with NaOH to the value of 9 (complete conversion to hydroxy acid) or 1 day old sample (pH=7) was analysed, where both forms of statin were present. LC-MS instrument was employed for the measurements, in order to confirm that the molecule we are dealing with is the statin compound in acidic form. Sim-L and Sim-H eluted at 17.5 min and 16.0 min; Lov-L and Lov-H at 16.7 min and 15.3 min, Pra-H at 11.2 min. No Pra-L was found at any time of our research.

Obtained mass spectra were compared with literature data, where Wang et al. (2001) elucidated lactone form of Sim-L and Lov-L, using both triple quadrupole and ion trap mass spectrometers. For the sake of clarity instrument parameters applied by his research group are described in details in the chapter 2.3.4.

4.3. Stability studies

4.3.1. Introduction

Stability research was divided into 4 main parts. Statins were monitored:

- in the presence of natural sun light
- under different solvents ratio and influence of acidic pH
- under increased temperature
- while being purged with a gas

4.3.1.2 Sun light

Aqueous solutions (with the concentration of 10 mg L^{-1} of each statin) were kept in 100 mL SCHOTT DURAN closed, sealed flasks, with their transmittance measured with spectrophotometer Hewlett Packard 8453 UV-VIS. One set of water samples was exposed to the natural environmental conditions (sun light), another set of samples at the same conditions (although protected from the sun with aluminium foil) (no light), and another sample set kept on the laboratory desk, protected from the light, room temperature (as the control samples).

0.5 ml aliquots were collected for the analysis at the time 0, 24 hrs, 48 hrs, 1 week, and further with the time intervals of 1 week (until 60^{th} day). Additionally, after 12 months of sun exposure samples were analysed by HPLC-DAD and LC-MS instrument and their toxicity towards *Vibrio fischeri* bacteria (Hach-Lange (Dusseldorf, Germany)) was evaluated.

There are no specified guidelines how long stability test should be performed, so we follow the guiding principle for biodegradability test, which takes usually 28 days (ISO standard 9408) prolonging the test for 60 days, in order to monitor (within HPLC-DAD instrument) appearing any additional products. Our research involved monitoring of statin behaviour at the conditions, which may appear in the surface waters, like pH equal to 7 or 8, and in specific, acidic conditions, rarely detected in the environment (pH = 4.5 as a result of acid rain, acid mine drainage etc). The exact temperature of sun-exposed samples was not measured, although during the

experiment we discovered that while summer months, samples might have reached much higher temperatures, than those kept on the laboratory desk.

Number of sunny days, minimum, maximum and average daily air temperatures were recorded (7^{th} and 8^{th} monthly bulletin of Environmental Agency of The Republic of Slovenia, 2007; meteorological station in Bilje). Absolute maximum temperature of air was equal to: 38.0 ºC (July and August 2007); minimum absolute air temperature: 7.5 ºC (July and August 2007); average air daily temperature: 22.9 ºC (July 2007) and 22.1 ºC (August 2007). For both months amount of sunny days was equal to 39.

4.3.1.3. Various solvents systems and influence of acidification

Since sample preparation implies presence of different solvents it has been decided to monitor statin stability under diverse solvents ratio systems. Additionally influence of sample acidification on interconversion between lactones and hydroxy acids was investigated. Experiment was performed at 20°C, without presence of direct sun light. The vials were sealed between injections, in order to avoid/reduce the solvent evaporation.

Aqueous samples were prepared by diluting 10 mg of simvastatin or lovastatin in 1 mL of acetonitrile and pravastatin in water (easily soluble) and diluting it with double deionised water to the concentration of 10 mg L^{-1}. The samples of higher acetonitrile content (acetonitrile/water 60/40) were prepared by diluting 10 mg of simvastatin or lovastatin in 1 mL of acetonitrile and pravastatin in water and diluting it in the solvent: acetonitrile/water 60/40 to the concentration of 10 mg L^{-1}. The change of statin concentration was tested at neutral (7) and acidic pH (4.5; obtained by pH adjustment with HCl), to investigate, if addition of acid retards interconversion between lactone and hydroxy acid form, as some researchers noticed (Hwang and Yang, 2006). During the whole experiment a sum of lactone and hydroxy acid form was considered as 100 % of each statin, since we did not observe any additional products appearing within time (HPLC-DAD chromatograms). Compound separation was achieved with HPLC-DAD, with operating conditions described in 4.2.2 chapter.

4.3.1.4. Increased temperature

In order to define statin behavior at increased temperature, aqueous samples (concentration of 10 mg L^{-1}, at pH=7) were heated for 60 minutes in the water bath at 50°C and 80°C degree. After 60 minutes 1 mL sample was collected for the HPLC-DAD analysis (with operational conditions described in the chapter 4.2.2) and compared with sample kept in the room temperature. Supplementary analysis involved LC-MS instrument, with working conditions as in the chapter 4.2.3.

4.4. Results and discussion

4.4.1. Extraction efficiency

Table 6 lists the recoveries of analytes, for all tested cartridges, which ranged from 57±6 % to 82±9 %. Simvastatin and lovastatin were found as Sim-L and Lov-L; pravastatin as Pra-H.

Table 6: Mean recoveries of statin from double-deionised water with different SPE cartridges (%±SD)

Analyte	DSC-C_{18}	Bond Elute C_8	Strata-X
Simvastatin	68±8	64±5	57±6
Lovastatin	82±9	74±5	71±6
Pravastatin	72±4	61±6	67±5

On the basis of obtained results (Table 6) DSC-C_{18} (3 mL/500 mg) cartridges were selected for the sample preparation. There was no difference in extraction efficiency between DSC-C_{18} cartridges: 1 mL/100 mg and 3 mL/500 mg, however 3 mL cartridges allowed higher flow rate (5-10 mL min^{-1} without affecting extraction efficiency), significantly shortening extraction procedure.

The solid phase extraction efficiency of standard solutions, on DSC-C_{18} (3 mL/500 mg) cartridges, together with limits of detection and quantification are presented in the Table 7. Each sample was extracted in triplicates, and additionally diverse amount of solvents was investigated in order to find an optimum for the compound elution. The average for triplicates of spiked water samples was calculated for each compound. Limits of detection and quantification (LOD and LOQ) were defined as the concentration yielding a signal noise ratio of 3:1 and 10:1 respectively.

Table 7: SPE characteristics of water statin solutions on DSC-C_{18} cartridges

	Calibration curve	Recovery %	LOD [ng L^{-1}]	LOQ [ng L^{-1}]
Simvastatin	y = 152.86x – 9.8163 R^2 = 0.9999	68±8	16	53
Lovastatin	y = 157.01x + 22.602 R^2 = 0.9986	82±9	14	46
Pravastatin	y = 134.91x – 20.345 R^2 = 0.9999	72±4	19	63

4.4.2. Statin interconversion in different solvents systems and in acidic media

The interconversion from lactone to hydroxy acid form influences the results of quantification. The problem we had to cope with was the change of lactone concentration, even at room temperature (lactone concentration in the mixture of MeCN/H_2O (60/40, v/v) decreased to 70.6±1.9 % and 70.9±2.3 % (Sim-L and Lov-L) after 1 day storage (Table 8). Addition of CH_3COOH to the solution retards interconversion by lowering lactone concentration to 96.4±2.3 % (simvastatin) and 91.4±2.0 % (lovastatin). Besides, our research revealed that conversion, between two forms of statin, caused by pH adjustment is a reversible process. For pravastatin compound sample acidification (in any of the solvent systems) doesn't seem to have any influence on the concentration, as in the case of two previous drugs. No Pra-L is observed.

Table 8: Interconversion of statin between lactone and hydroxy acid form in different solvents

Time [hours]	**Sim-L content by percentage of initial value [%±SD, n=3]**				**Lov-L content by percentage of initial value [%±SD, n=3]**				**Pra-H content by percentage of initial value [%±SD, n=3]**			
	in MeCN/H_2O (60/40, v/v)	in MeCN/H_2O (60/40, v/v), pH=4.5	in H_2O, pH=7	in H_2O, pH=4.5	in MeCN/H_2O (60/40, v/v)	in MeCN/H_2O (60/40, v/v), pH=4.5	in H_2O, pH=7	in H_2O, pH=4.5	in MeCN/H_2O (60/40, v/v)	in MeCN/H_2O (60/40, v/v), pH=4.5	in H_2O, pH=7	in H_2O, pH=4.5
0	99.2±1.6	99.4±1.2	99.8±1.3	98.8±1.0	99.4±1.1	99.2±1.6	98.9±1.4	99.2±2.0	98.9±2.2	98.6±2.1	99.2±1.4	98.6±1.9
8	86.9±1.6	97.5±2.2	94.3±1.6	96.2±1.3	87.9±2.0	97.8±1.4	94.9±1.6	97.3±1.7c	98.7±1.9	98.9±1.4	99.4±1.2	99.1±1.2
24	70.6±1.9	96.4±2.3	90.1±1.8	94.7±1.1	70.9±2.3	91.4±2.0	90.8±1.4	94.7±2.1	98.4±2.0	98.5±1.8	99.2±1.7	99.0±0.9
48	69.6±2.4	96.2±2.0	88.4±2.1	92.4±1.4	70.2±1.9	87.1±2.1	89.2±1.5	89.9±1.6	97.9±1.7	98.0±1.2	99.0±1.3	98.9±1.0

During 48 hours experiment no degradation products were observed for any of the compounds, by applied HPLC-DAD instrument. However we should keep in mind that this instrument has limited abilities to determine very low concentration levels of transformation products, due to its low sensitivity and selectivity. On the basis of obtained chromatograms, and previously prepared calibration curves we determined that simvastatin and lovastatin were converted to their hydroxy acid forms. Increase of hydroxy acid amount was equal to decrease of lactone. The percentage of conversion is much lower in water samples, especially in acidified ones. Within first 4 hours the decrease of the lactone concentration was not observed at all. According to the finding that acetic acid hinders interconversion of simvastatin and lovastatin, water samples for calibration curves and extraction procedure were prepared daily (acidified for Sim-L and Lov-L).

In the case of pravastatin we did not observe the decrease of Pra-H concentration (in all solvents systems), and we did not notice any degradation products, because they could not be detected by UV-DAD detector of HPLC instrument.

4.4.3. Mass spectrometry

Despite of using only single LC-MS measurement, we obtained clear fragmentation pathway of simvastatin and lovastatin (in both lactone and hydroxy acid forms).
Fig. 6 shows total ion chromatograms together with mass spectrum of Sim-L and Sim-H; Lov-L and Lov-H (1 day old aqueous sample, pH=7), as well as pravastatin, which was determined only as Pra-H. For Lov-H and Lov-L retention times were equal to 15.3 min 16.7 min (Fig. 6 a) and for Sim-H and Sim-L: 16.0 min and 17.4 min (Fig. 6 b) for Sim-L and Sim-H. Pra-H eluted at 11.2 min (Fig. 6 c).

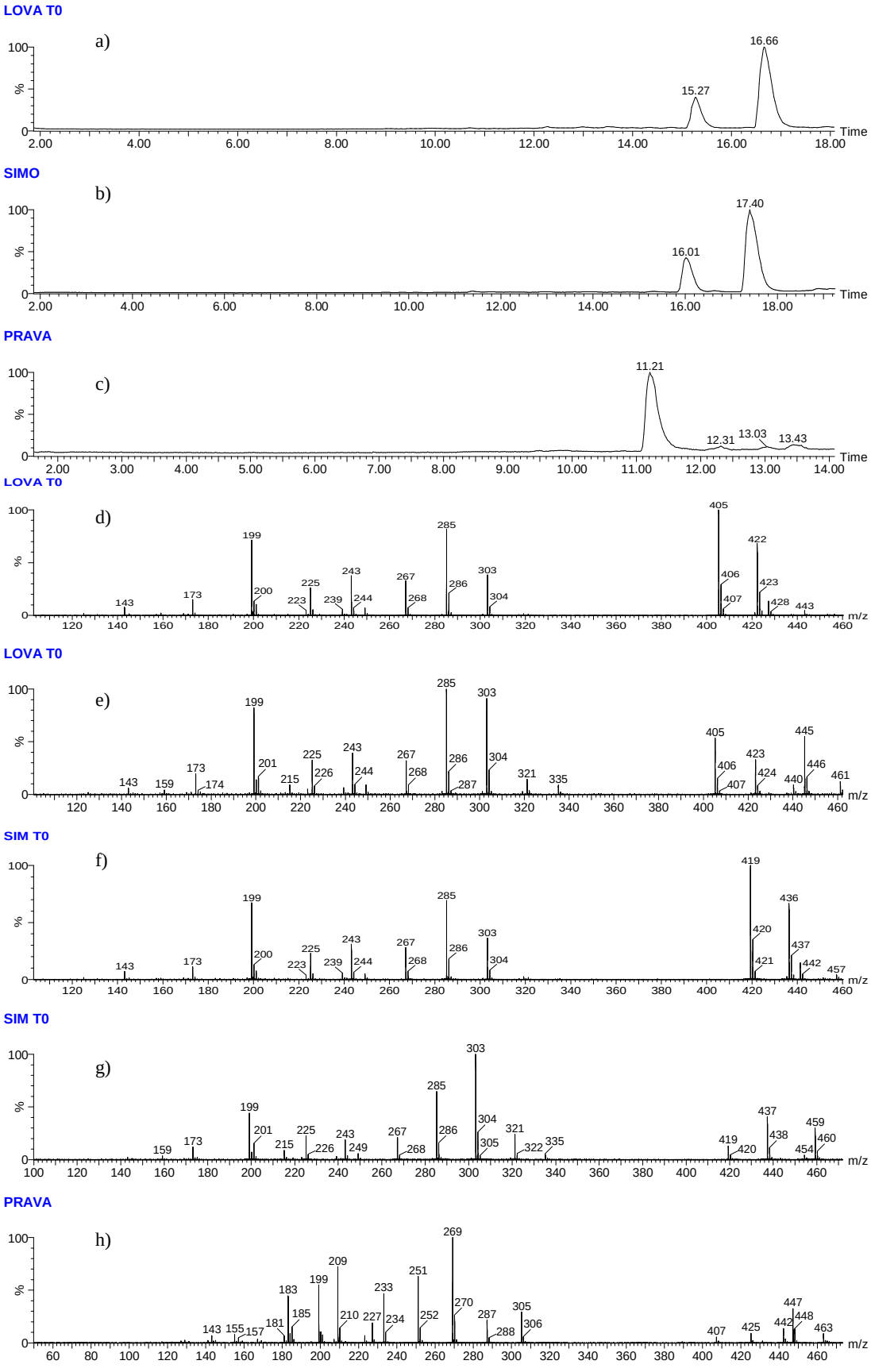


Fig 6: LC-MS total ion chromatograms of lovastatin (a); simvastatin (b); pravastatin (c); together with their mass spectrum: Lov-L (d); Lov-H (e); Sim-L (f); Sim-H (g) and Pra-H (h) in ES positive-ion mode

Lactones fragmentation pathway proposed by Wang et al. (2001), is identical to the one, which we observed for hydroxy acids. The major product ions for simvastatin and lovastatin (both in lactone and hydroxy acid form) are at *m/z* 199, 267, 285, 303 (Fig. 6 d – 6 g). According to the signal intensities and structure-specificities of the product ions, ion of *m/z* 285 is generated by loss of ester side chain ($C_5H_{10}O_2$) and subsequently H_2O molecule (both compounds form identical ion at *m/z* 303 as a proof of a weak –C-O- bond of the ester side chain at the C_8 position). The product of direct cleavage could undergo dehydration to generate the ion at *m/z* 285 or rearrange to the same ion at *m/z* 303 derived from the charge-induced fragmentation process. Another possible formation pathway for the ion at *m/z* 303 was from the ion at *m/z* 321 through the dehydration process.

Values *m/z* 428 (Lov-L) and 446 (Lov-H) found for lovastatin, correspond to the sodium clusters $[M+Na]^+$. The same pattern appears in regard of simvastatin – 442 (Sim-L) and 460 (Sim-H). For the pravastatin compound, in acidic form major product ions are at *m/z* 199, 269, 287. Successive ions at *m/z* 287 and 269 are the result of dehydration, with identical fragmentation pattern as for two other compounds.

4.4.4. Statin stability under natural sun light

We have chosen neutral medium (pH=7, as the most frequent in the environment) to explain statin behaviour under natural sun light. Table 9 shows experimental data for all investigated statin, together with the information about degradation products occurrence (triplicate injection).

Experimental data show that both compounds Sim-L and Lov-L exposed to the sun light are converted to the active hydroxy acid form, and at the same time they start to degrade already from the first day of the test (Fig 7 shows the decrease of total compound concentration as the sum of Sim-L and Sim-H). Parallel experiment, performed in flasks protected from the light (exposed to the sun, but covered in aluminium foil) proved the same compounds behaviour (interconversion and degradation rate, identical formed products), which led us to the conclusion that we did not observe direct photodegradation. In control samples (kept for 60 days on the

laboratory desk) we did not notice any decrease of the total compound concentration, and none of the degradation products were detected by HPLC-DAD.

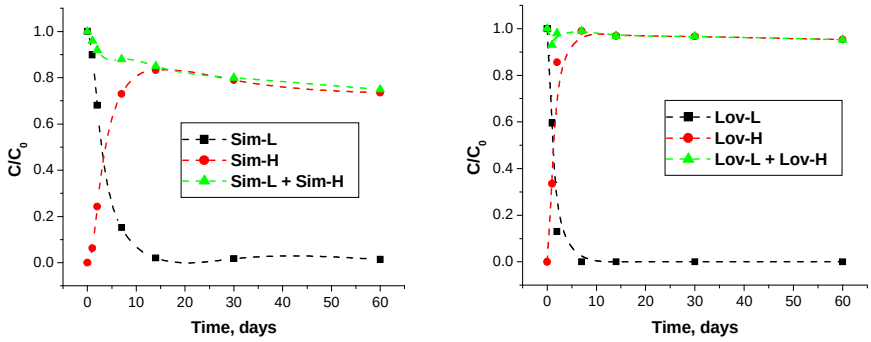


Fig 7: The change of simvastatin and lovastatin concentration during sample exposure to the sun light at the pH = 7

Table 9: The change of statin concentration after sample exposure to the natural sun light (mean values ± SD)

Time	Statin content by percentage of initial value at pH 7 [%±SD, n=3]			Statin content by percentage of initial value at pH 7 [%±SD, n=3]			Statin content by percentage of initial value at pH 7 [%±SD, n=3]		
	Sim-L	Sim-H	Deg prod	Lov-L	Lov-H	Deg prod	Pra-H	Pra-L	Deg prod
0 h	99.8±1.2	≤0.16	nd	99.7±1.1	≤0.14	nd	99.8±0.9	nd	nd
24 h	89.9±1.0	6.3±1.1	nd	59.5±1.0	33.5±1.2	nd	99.3±1.0	nd	nd
48 h	68.0±1.7	24.3±1.6	nd	12.9±2.0	85.6±2.0	nd	99.0±0.7	nd	nd
7 days	15.1±1.3	73.0±1.2	yes	≤0.14	99.0±2.0	nd	96.1±1.0	nd	nd
14 days	2.0±0.6	83.3±1.0	yes	≤0.14	96.9±1.2	yes	91.9±1.1	nd	yes
30 days	1.7±0.5	79.0±1.2	yes	≤0.14	96.7±2.0	yes	89.7±1.2	nd	yes
60 days	1.3±0.7	73.5±2.0	yes	≤0.14	95.3±1.6	yes	88.7±1.4	nd	yes

nd - not detected

Deg prod – degradation product

During 48 hours aqueous sample storage in the laboratory (experiment with different solvents ratio at 20°C – chapter 4.4.2) we have discovered that simvastatin and lovastatin remain in neutral media as Sim-L and Lov-L in 88.4±2.1 % and 89.2±1.5 % respectively (Table 8, aqueous samples). Identical samples, exposed to the sun (and the samples protected from the light by aluminium foil coverage), for the same period of time (48 hours), revealed that conversion occurs much faster – simvastatin exists in 68.0±1.7 % as Sim-L, while lovastatin is remaining as Lov-L only in 12.9±2.0 % (total compound concentration decrease, but degradation

products are still not observed on HPLC-DAD chromatogram) (Table 9). Further sun-exposure of the samples results in additional degradation of the compounds. When simvastatin and lovastatin are exposed to the sun in the acidic medium a much faster degradation is observed than for the samples under identical conditions (sun light), but in neutral media. On contrary, in the basic medium the degradation is retarded in comparison to the samples with pH=7.

After 2 months experiment, in neutral medium, simvastatin and lovastatin reach 74.8±2.7 % and 95.3±1.6 % of the total compound concentration subsequently, mostly as the result of conversion from lactones (Fig. 7, Table 9). Besides, in these conditions first degradation products were detected only from 7^{th} (simvastatin) and 14^{th} (lovastatin) day. At acidic conditions they were formed and detected much earlier (after 48 hours at pH = 4.5, for all three compounds). Concentration of Pra-H in acidic medium decreases to 2.8±0.9 % of initial concentration already within one month. Significant peaks of degradation products are noticed.

Additionally, samples exposed to the sun light for 12 months were injected on HPLC (Fig. 8 – Fig. 10), since we were interested in statin behaviour under sterile conditions. For the first 60 days we observed simvastatin conversion from lactone to hydroxy acid and at the same time simvastatin degradation. After 12 months sun-exposure Sim-H is present only in the control sample, suggesting that in other samples, between 60^{th} day and 12 months hydroxy form was either converted back to lactone and/or further degraded (total compound concentration decreased). Acidic media appear to be an accelerating factor of simvastatin degradation, giving multiple degradation products (Fig. 8), besides the parent compound in Sim-L form. The chromatogram of simvastatin control sample (Fig. 8) revealed the presence of product, eluting before parent compound at 8.4 min. It was not formed in any other sun-exposed samples (regardless of pH) (Fig. 8). At the same time, two simvastatin samples: pH=7 (sun) and pH=7 (no light) showed identical chromatograms, suggesting that the degradation might not be caused by the direct sun light. To support our hypothesis we decided to perform further experiment with sample heating (chapter 4.4.5).

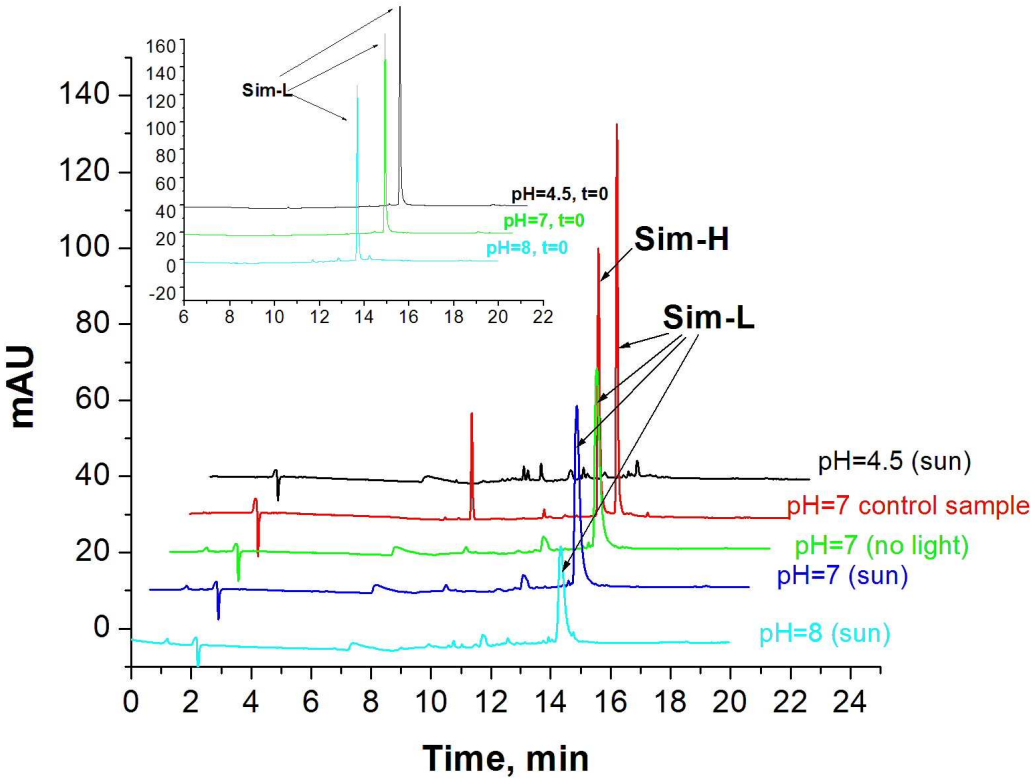


Fig 8: HPLC-DAD chromatograms of simvastatin exposed to the natural sun light for 12 months in solutions of various pH values

For the first 60 days we observed lovastatin conversion from lactone to hydroxy acid and at the same time its degradation (in 60th day Lov-L was not found anymore in the solution). After 12 months of sun-exposure Lov-H is present only in the control sample. In all the other samples lovastatin remains as Lov-L, besides multiple degradation products. We assume that between 60th day and 12 months under the sun light lovastatin hydroxy form was converted back to lactone and/or further degraded (total compound concentration decreased). In the control sample of lovastatin compound we observe its two forms (Lov-L and Lov-H), and no additional peaks on HPLC-DAD chromatogram. Again, as in the case of simvastatin, acidic media accelerates lovastatin degradation, giving various by-products, detected by HPLC-DAD. Lovastatin sample ((pH=7 (sun); and pH=7 (no light)) give congruent chromatograms and amount of remaining product (Fig. 9).

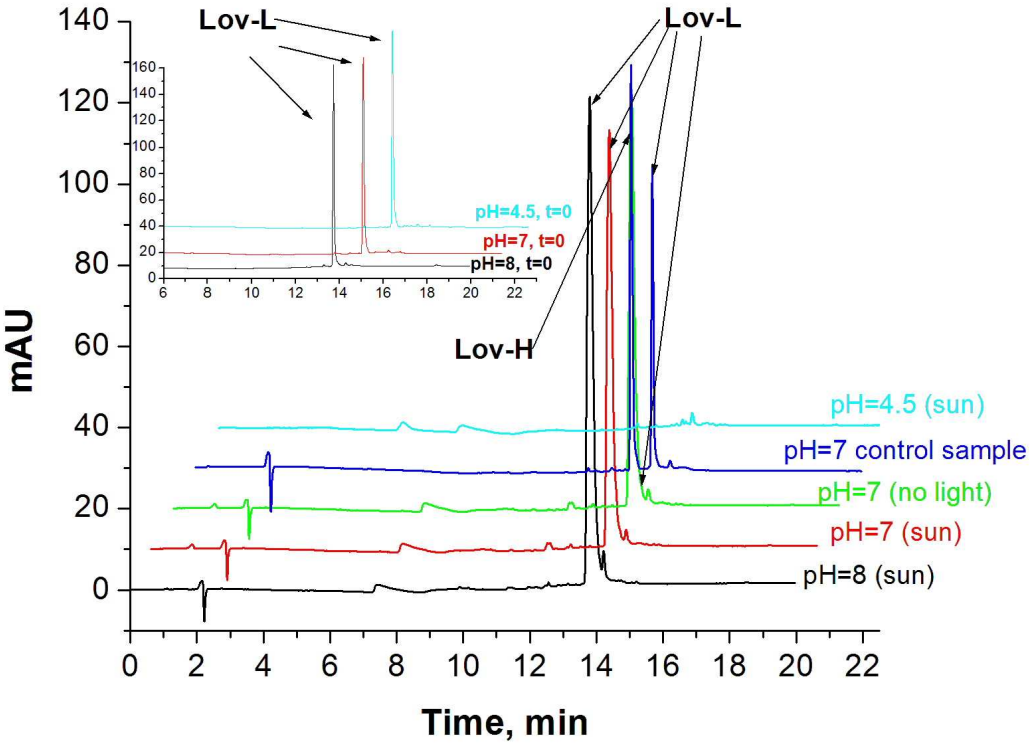


Fig 9: HPLC-DAD chromatograms of lovastatin exposed to the natural sun light for 12 months in solutions of various pH values

Pravastatin is converted to less polar (and/or bigger molecular mass) compounds, eluting later than parent compound. They are formed under all investigated values of pH, and also in control sample. However, in the latter one amount of them is much lower than in samples exposed to the sun. Sample in neutral media (with and without sun) give identical chromatograms. The highest amount and number of degradation products is observed in acidic media (Fig. 10). Hach-Lange (Dusseldorf, Germany) *Vibrio fischeri* bacteria test was employed for toxicity evaluation of formed products for all investigated statin under all investigated pH values, and the results are discussed in details in the chapter: 8.3.2. Toxicity of sun exposed samples.

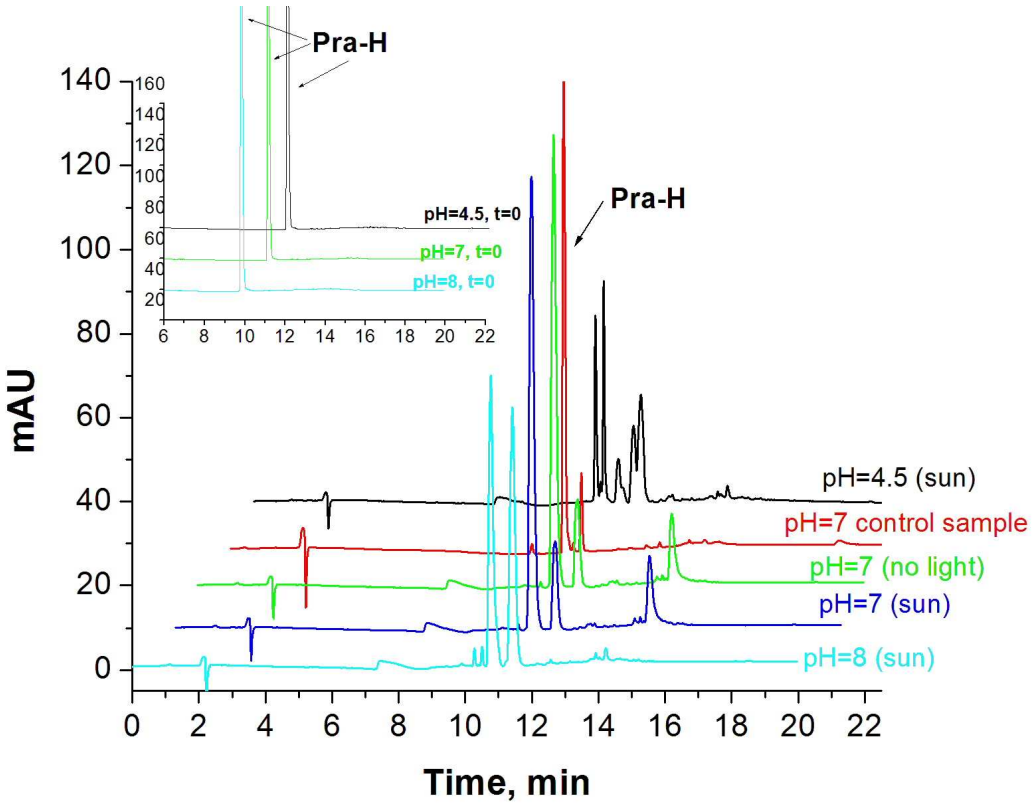


Fig 10: HPLC-DAD chromatograms of pravastatin exposed to the natural sun light for 12 months in solutions of various pH values

4.4.5. Statin stability under increased temperature

Experimental data revealed that after sample heating to the temperature 50°C 52±6 % of simvastatin remains as Sim-L. Only small, insignificant peak of additional product is observed on the HPLC chromatogram. At 80°C 47±2 % of simvastatin remains as Sim-L, 41.4±4 % as Sim-H, and the rest of the compound is most probably converted to more polar products and/or a product with smaller molecular mass (retention time shorter than parent compound, Fig. 11).

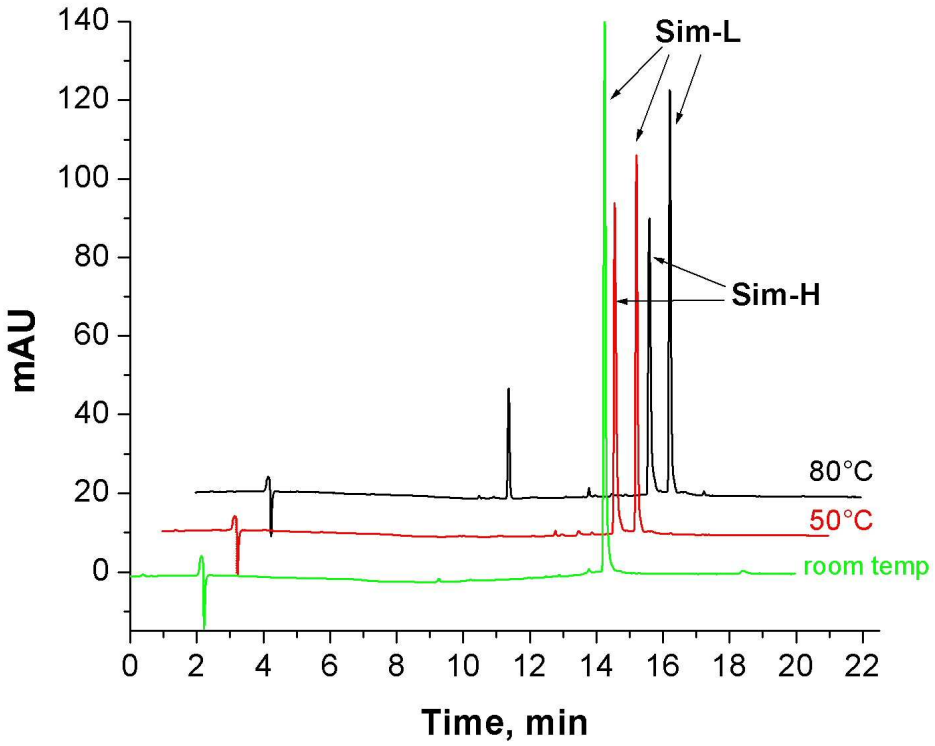


Fig 11: HPLC-DAD chromatogram of simvastatin sample after 60 minutes at room temperature (a); at 50°C (b); at 80°C (c), pH=7

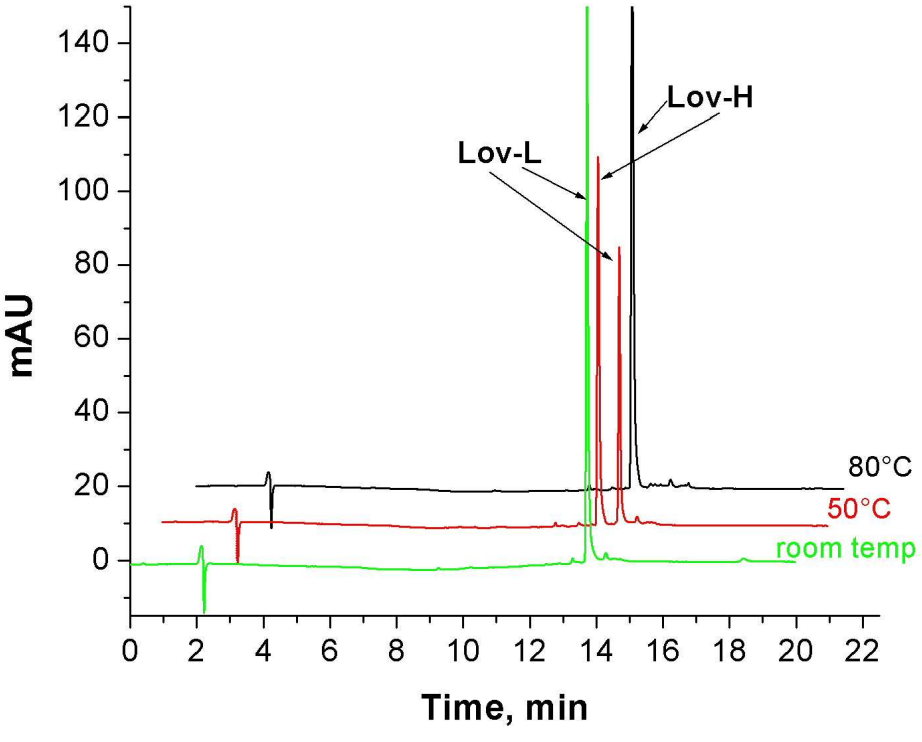


Fig 12: HPLC-DAD chromatogram of lovastatin sample after 60 minutes at room temperature (a); at 50°C (b); at 80° C (c), pH=7

On the basis of HPLC-DAD chromatogram (Fig. 12), and previously prepared calibration curve, we determined that at 50°C lovastatin exists in 61±2 % as hydroxy form and remaining amount exists in lactone form. We did not observe any significant additional by-products on HPLC-DAD chromatograms. After 60 minutes at 80°C almost whole (97±1 %) of lovastatin appears as hydroxy acid. Small peak of additional product can be observed.

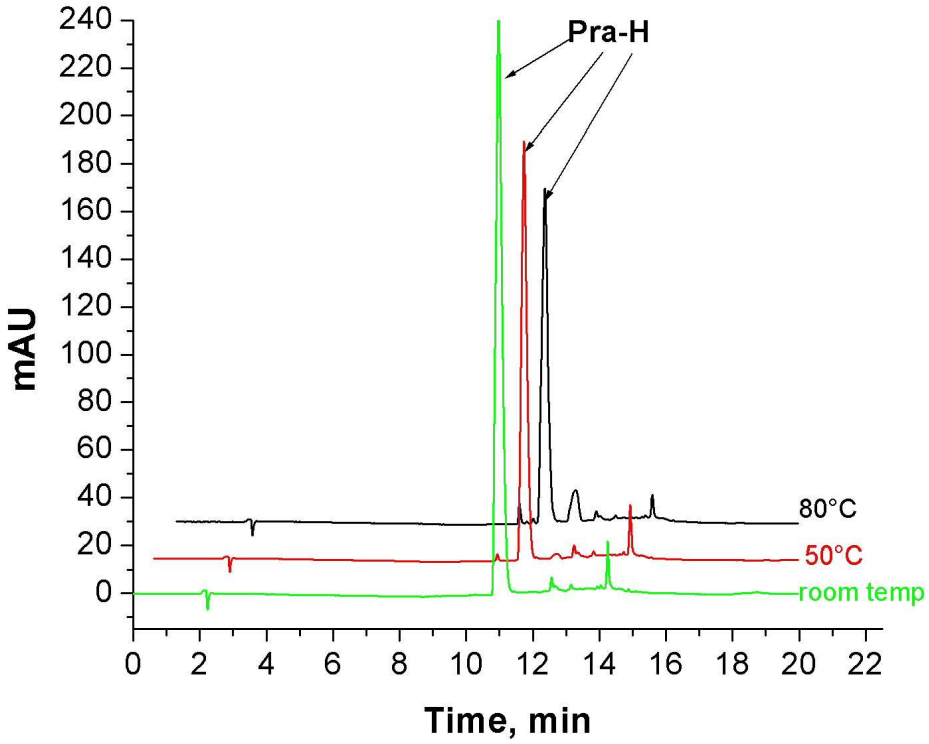


Fig 13: HPLC-DAD chromatogram of pravastatin sample after 60 min at room temperature (a); at 50°C (b); at 80° C (c), pH=7

In the case of pravastatin it can be observed that heating results in the formation of less polar and/or higher molecular mass products (Fig. 13), eluting later than parent compound. The amount of them increases when the temperature is raised to 80° C. Pravastatin concentration decreases to 64.7±3.4 % of initial value, under these experimental conditions.

On the basis of absorbance spectra (HPLC-DAD chromatograms), and supplementary LC-MS analysis (presented for simvastatin compound on the Fig. 14) we proposed that similar products (as in the case of sun-exposed samples) are

formed. This confirms our previous suggestions that statin degradation under sun light is not the result of direct photodegradation. In order to investigate the influence of increased temperature (water bath) and potentially increased temperature of sun exposed samples (as we have noticed, it was much higher than on the laboratory desk) we have chosen results obtained for simvastatin. It allowed us to explain similarities in formed products and their character (Fig. 14).

Fig. 14 a presents LC-MS total ion chromatogram of standard simvastatin sample, sample exposed to the sun light for 12 months (Fig. 14 b) and its mass spectra of product eluting at 10.73 min (Fig. 14 c) with its characteristics absorption bands (Fig. 14 d). On the Fig. 14 e we can see LC-MS total ion chromatogram of sample heated to the temperature 80°C and its mass spectra of product eluting at 10.73 min (Fig 14 f) with its characteristics absorption bands (Fig 14 g).

In both simvastatin samples: exposed to the sun light for 12 months, and heated to 80°C, the product with retention time 10.73 min is present. Mass spectrum (Fig. 14 c and Fig. 14 f) and characteristics absorption bands (Fig. 14 d and Fig. 14 g) suggest that we are dealing with the same compound. However, MS alone is insufficient to identify precisely the structure of obtained transformation products. Only supplementary studies, employing various instruments and techniques (LC-nuclear magnetic resonance, chemical derivatisation, and hydrogen/deuterium-exchange (H/D-exchange) combined with MS), could give us certainty to support our hypothesis. Thus, only on the basis of obtained results, we proposed that some of the products, formed in both samples are identical, suggesting their formation by increased temperature. Our hypothesis is supported by comparison of retention times, mass spectra and characteristics absorption bands of formed product.
Additionally, for both: simvastatin control sample (sun-exposure experiment, Fig. 8 and Fig 14 h) and sample heated to 80°C (Fig. 11 and Fig. 14 e), we have discovered product, eluting before initial simvastatin compound, with similar retention time in both cases (see Fig. 14 e and 14 h). However, comparison of its mass spectra (Fig. 14 f and Fig. 14 j) and characteristics absorption bands (Fig. 14 g and 14 i) suggested that we are not dealing with the same product.

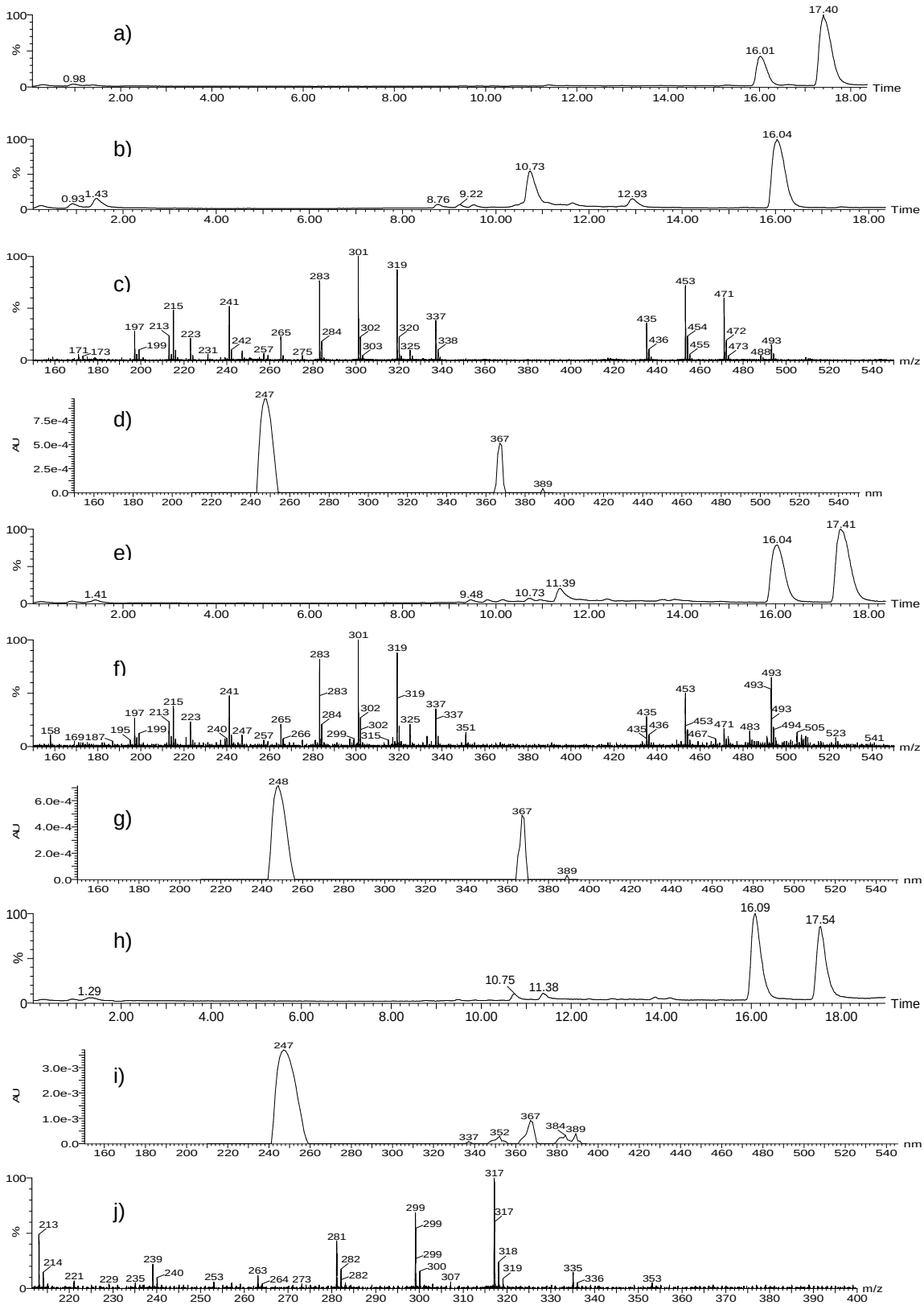


Fig 14: LC-MS total ion chromatogram of standard simvastatin sample (a), sample exposed to the sun light for 12 months (b) and its product mass spectra at 10.73 min (c) with its characteristics absorption bands (d); sample heated to the temperature 80°C (e) and product mass spectra at 10.73 min (f) with its characteristics absorption bands (g). LC-MS total ion chromatogram of control sample (h) (sun exposure experiment), with its characteristics absorption bands (i) and mass spectra (j) of product at 10.75 min

4.4.6. Behaviour of lactones while purging with gas

It has been observed that lactone forms of statins (simvastatin and lovastatin), while purging with air or oxygen are susceptible to evaporation, which is due to their vapour pressure. Simple experiment confirmed our hypothesis. While purging the water sample in the plastic tube, its vapours were collected into another tube filled with 1 mL of de-ionised water and analysed on HPLC-DAD. Statin compound was detected in the second tube, while in the first one its concentration decreased significantly. In order to confirm that only lactone form evaporates so easily, it was converted to hydroxy acid form (by the increase of pH to the value of 9) and the experiment was repeated. No considerable evaporation was observed and the compound, collected from vapours could not be detected by HPLC-DAD. Pravastatin, determined only in hydroxy acid form did not cause any problems, due to insignificant evaporation. Observed evaporation of lactone form had to be considered during photocatalyst degradation studies with TiO_2, when samples were purged with oxygen, before the experiment. After purging, the sample was collected and considered as initial value of the compound, before the degradation test.

4.5. Conclusions

Mass spectrometry confirmed that simvastatin and lovastatin exist in two forms: lactone and hydroxy acid form, and identification of both compounds was based on LC-MS analysis. Lactone forms can be easily converted to their corresponding hydroxy acid forms under different conditions such as high pH, as well as under presence of different solvents. Such investigations had to be done in order to make quantification analysis more accurate and to monitor statin behaviour under various, natural conditions (water samples at natural pH 7; pH 4.5 and pH 8) as well as under laboratory conditions for example during extraction procedure, which involves diverse solvents.

To our knowledge no data, regarding statin stability under natural conditions as well as formation of statin transformation products were available. Our experiments showed that statin exposed to the natural sun light undergo interconversion between two forms, and at the same time they start to degrade (total amount of compound as the sum of lactone and hydroxy acid is decreasing). Samples in flasks, covered with

an aluminium foil (protected from the light) reveal identical HPLC-DAD chromatograms; LC-MS total ion chromatograms; characteristics absorption bands, and mass spectrum of products. The results were compared with the samples heated to 80°C and they allowed us to suggest that increased temperature could be involved in product formation in all cases (sample exposed to the sun, protected from the sun and heated on the water bath). In the control samples those products were not found (different transformation products were present). Despite of the fact that exact temperature of samples was not measured (only maximum, minimum and average air temperature was determined) we have noticed much higher temperature of samples in comparison to control samples, kept on the laboratory desk (20°C). While performing all, mentioned above experiments we observed the decrease of the initial compound concentration and in some cases the appearance of transformation products. However, adsorption to the glass might have contributed to this process as well and has to be considered as one of the reasons of statin's decreased concentration.

V. Photolysis studies

5.1. Statin behaviour under UV light

5.1.1. Experimental

Performed photodegradation studies could be divided into two parts:

- Preliminary irradiation studies with 125 W Cermax Xenon parabolic lamp (the photon irradiance was evaluated by potassium ferrioxalate, and determined to be 2.6×10^{16} photons s^{-1} cm^{-2}).
- Irradiation with low pressure mercury lamp emitting at 254 nm, 310 nm, 365 nm. For the determination of the quantum yields, a parallel 254 nm beam was used. For analytical studies, a cylindrical setup emitting at 254 nm was employed in order to monitor the formation of the by-products and the disappearance kinetics of the parent compound. To avoid sample heating cooling setup was used.

All of the irradiation experiments were performed in a quartz cells and fresh water samples were prepared daily by diluting 10 mg of simvastatin or lovastatin in 1 mL of acetonitrile and pravastatin in water and diluting it with double deionised water to the concentration of 10 mg L^{-1}.

Samples for Xe lamp irradiation were irradiated in a small quartz cell ($10 \times 10 \times 40$ mm), at the distance of 45 cm from the lamp (preventing additional sample heating), and stirred with magnetic stirrer. During the experiment, samples were collected for HPLC analysis after certain period of time (3.5; 7; 15; 30 and 60 min). The experiment was performed at room temperature in the presence of oxygen and in parallel after molecular oxygen removal from the solution (no significant difference was observed).

For the experiment with monochromatic 254 nm Hg light, the photon flux was evaluated by actinometry using potassium ferrioxalate. Its value was found equal to 2.41×10^{14} photons $s^{-1}cm^{-2}$. During the experiment, samples were stirred with the

magnetic stirrer and collected for HPLC analysis after certain period of time (10, 20, 30 and 60 min). After each sample collection a fresh sample was irradiated in order to retain the same sample volume. For analytical studies, first experiments with 254 nm light were performed with 6 Hg lamps (Phillips, 15 W, 438 mm x 26 mm), but in order to monitor degradation products more precisely number of lamps was reduced to 3. Primarily, analytes were separated using HPLC-DAD. Secondly, in order to elucidate transformation products, LC-MS instrument was employed. Operational conditions for both of instruments are described in the chapter 4.2.2 and 4.2.3.

5.1.2. Results and discussion

5.1.2.1. Disappearance of statin compound

The UV absorption spectrum of the three compounds presents a well defined band with a maximum at roughly 238 nm (Fig. 15). Such absorption band corresponds to a π- π * transition owing to the conjugated double bonds. The molar absorption coefficients were evaluated and are 2.40×10^4; 1.98×10^4 and 19.0×10^4 mol^{-1} L cm^{-1} for lovastatin, simvastatin and pravastatin respectively. For the three compounds, the absorption band clearly appeared with a vibrational structure.

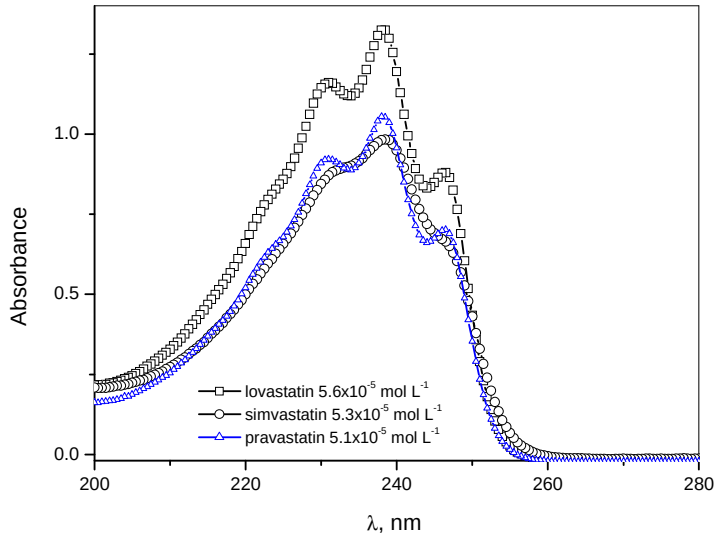


Fig 15: UV absorption spectra for the studied compounds in water-acetonitrile solution

Each sample irradiated with Xenon light for the certain amount of time was collected for the maximum absorbance measurement, in order to obtain preliminary results. (Fig. 16).

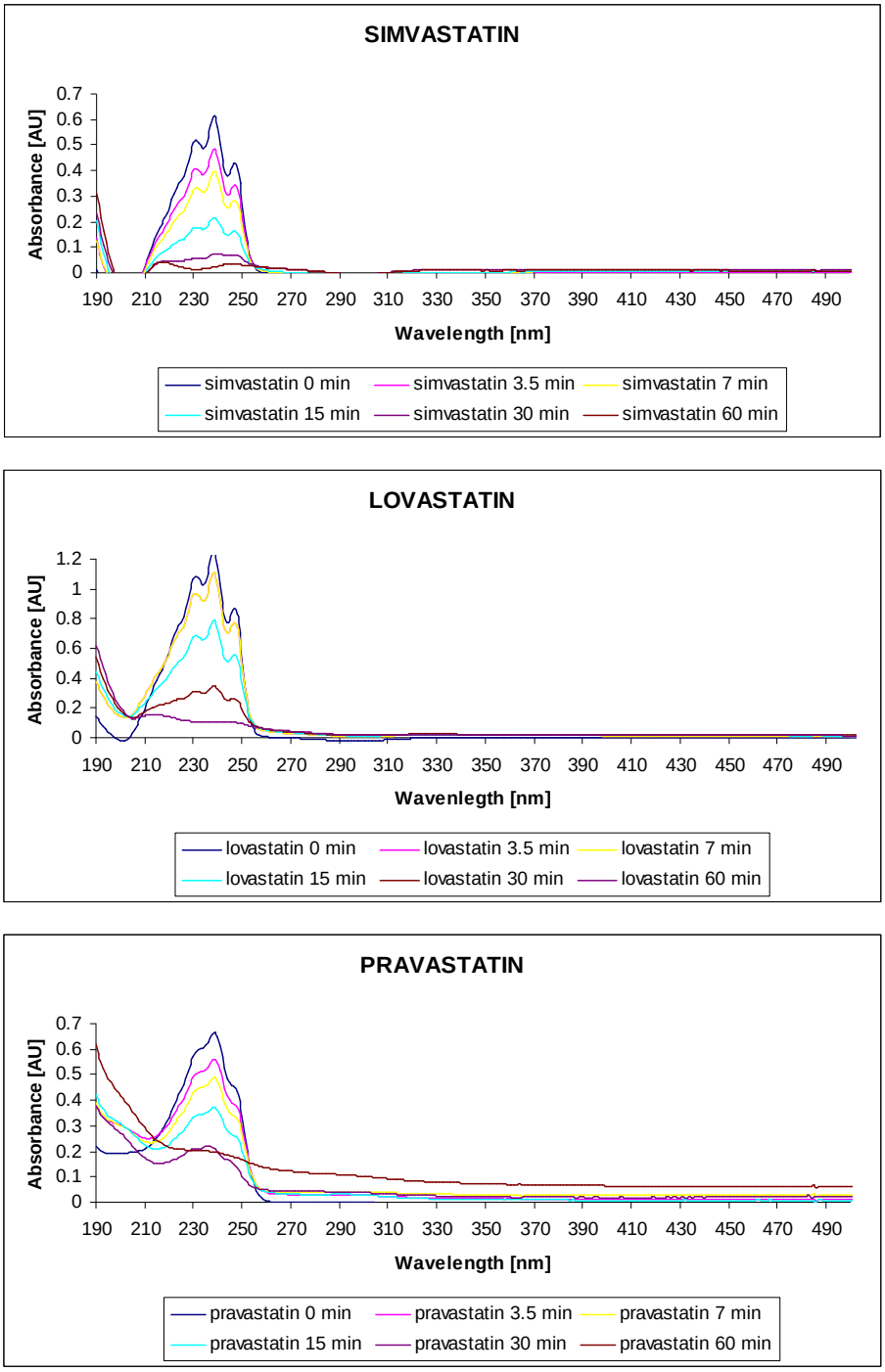


Fig 16: Absorbance spectra of statin samples irradiated with Xenon parabolic light

On the basis of obtained absorbance values we can observe that there is no significant increase of absorbance observed at higher wavelengths due to intermediate degradation products, formed during irradiation experiment. However,

an increase of absorbance, in the range of shorter wavelengths (190 nm) might be attributed to some of the degradation products (the best observed in the case of pravastatin). In order to obtain more detailed results disappearance of initial compound, as well as appearance of photogenerated products was observed by HPLC-DAD instrument (Table 10).

Further experiments involved investigation of pH value influence on the irradiation results (Table 10). After 15 min of irradiation simvastatin and lovastatin degraded faster in the basic medium reaching 5.5±1.2 % (simvastatin) and 1.4±1.1 % (lovastatin) of the initial concentration, which is much lower than in the acidic and neutral medium (see Table 10). This trend is not observed for pravastatin compound, where after 15 min the largest decrease of the concentration was observed in neutral medium (reaching 1.2±1.0 % of the initial concentration). Regardless of diverse degradation kinetics in all investigated pH solutions, all of the statin compounds are degraded by 60th minute of the experiment.

Table 10: Influence of the pH on the degradation during Xe lamp irradiation

Time of irradiation [min	**Simvastatin content by percentage of initial value [%±SD, n=3]**			**Lovastatin content by percentage of initial value [%±SD, n=3]**			**Pravastatin content by percentage of initial value [%±SD, n=3]**		
	pH=7	**pH=4.5**	**pH=8**	**pH=7**	**pH=4.5**	**pH=8**	**pH=7**	**pH=4.5**	**pH=8**
0	98.6±4.4	98.4±3.9	99.6±3.2	99.4±4.9	99.0±3.6	98.9±4.0	98.2±2.7	97.9±4.4	99.0±2.2
3.5	81.9±6.2	78.8±5.5	81.5±6.6	83.7±6.6	85.6±5.9	78.7±6.3	69.8±7.6	52.8±5.8	64.9±5.9
7	55.5±5.0	53.7±7.3	31.2±4.3	70.2±7.2	70.5±8.2	62.6±7.2	44.1±6.9	27.0±3.3	37.8±3.8
15	19.4±2.1	22.1±3.9	5.5±1.2	37.9±4.8	44.1±5.5	1.4±1.1	1.2±1.0	4.3±1.8	9.1±2.2
30	5.5±1.2	1.2±0.7	nd	5.7±2.2	12.6±4.1	0.3±0.6	nd	nd	0.6±0.4
60	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd – not detected

Irradiation products were defined by LC-MS instrument, and toxicity studies for each product were performed in order to define potential danger, which compounds might possess to the aqueous organisms.

Irradiations of solutions at 254 nm with mercury lamp result in degradation at different rate. In order to simplify analysis results, the formation of irradiation products is explained for the irradiation of the solutions performed at 254 nm (monochromatic). Each sample irradiated for a certain period of time (10, 20, 30, 60 min) was analyzed on the HPLC-DAD. The relative change of concentration is presented in the Fig. 17.

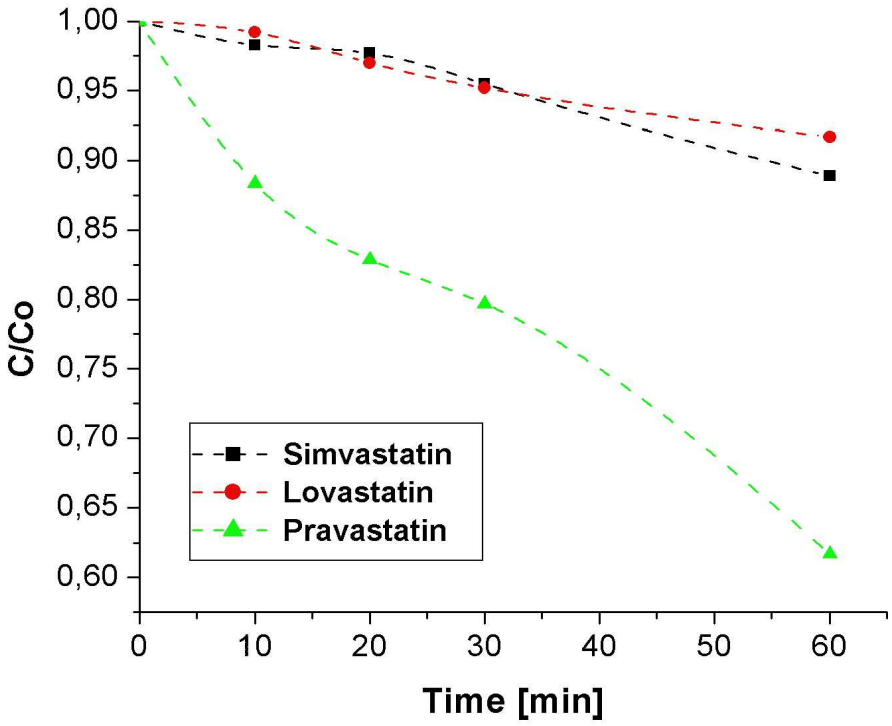


Fig 17: Decrease of statin concentration after sample exposure to the 254 nm Hg light, pH=7

Under our experimental conditions, the obtained results (Fig. 17) show that the most susceptible compound for UV degradation is pravastatin (identical as for Xe lamp irradiation), more likely owing to its high absorbance at the excitation wavelength. After 60 min of exposure to the 254 nm light its concentration decreased to 62.0±3.2 % of initial value. Simvastatin and lovastatin appear to be more resistant under these conditions. After 60 min of experiment the concentration decreased to 88.9±2.9 % and 91.7±2.6 % of initial value for simvastatin and lovastatin respectively.

Table 11: Parameters for spectrophotometric determination of photodegradation with mercury lamp for aqueous solution of simvastatin, lovastatin and pravastatin (254 nm)

	simvastatin	**lovastatin**	**pravastatin**
initial concentration [mg L^{-1}]	21.5	22.8	22.5
$\lambda_{emission}$	254 nm	254 nm	254 nm
Absorbance at 254nm	0.026	0.038	0.037

In order to obtain a better insight about this behaviour, we undertook experiment for the determination of degradation quantum yields. Such values were estimated by employing the following expression:

$$\Phi = (-dC_A/dt)\ (N \cdot l \cdot 10^{-3}) / [I_o(1-10^{-Ao})]$$

where dC/dt (mol L^{-1} s^{-1}) is the slope of the plot concentration as a function of irradiation time (the conversion was limited to less than 10-15 %).
N: Avogadro number
l : optical pathlength (cm)
I_o: photonic flux evaluated to 2.41×10^{14} photons s^{-1} cm^{-2}
and A_o: the initial absorbance at the excitation wavelength.

Under our experimental conditions the disappearance quantum yields by excitation at 254 nm were similar for simvastatin and lovastatin; and about one magnitude higher for pravastatin. They were calculated to be: 0.068, 0.040 and 0.16 for simvastatin, lovastatin and pravastatin, respectively. From the calculated quantum yields we can conclude that pravastatin is the most susceptible to photodegradation at 254 nm, since the quantum yield is the highest one. The rapid degradation of pravastatin can be observed on the Fig. 17, where pravastatin disappears much faster than the two other statins.

It is worth noting that for the three compounds, the quantum yield was shown to be unchanged when molecular oxygen was removed from the solution indicating that the degradation proceed via a short lived excited state, namely the singlet excited state, obtained by excitation into the π - π * band.

The results obtained after sample irradiation at 310 nm, 365 nm are in excellent agreement with our expectations – no degradation was observed, since absorption spectrum of investigated compounds is at the narrow range of 238 nm.

5.1.2.2. Determination of irradiation products

The results, obtained by LC-MS analysis show that during irradiation experiment (both with Xe and Hg lamp) the same irradiation products were obtained. The disappearance rate of parent compound depends on the wavelengths range used in the experiment.

Several degradation products have been determined for the irradiated samples and the photogenerated products are explained on the example of simvastatin and pravastatin. Besides parent compound (Sim-L and Sim-H) additional peaks are detected at 13.7 min, and 14.12 min (Fig 18 a). LC-MS analysis confirms that formation of irradiation products appear through photochemical degradation. The presence of at least 2 isomers (hydroxylated simvastatin) (Fig 18 d) was found. Their occurrence has been proved by extracted mass chromatogram with *m/z* 437 (418+18+1), 459 (418+18+23) (sodium cluster of hydroxylated simvastatin) from total ion chromatogram – Fig 18 d and Fig 18 e. Dihydroxylated derivative has been confirmed by *m/z* 451 (418+32+1) and 489 (418+32+39) (potassium cluster) - Fig 18 f and Fig 18 g. Besides, main fragment ions, detected previously for standard sample (*m/z* 303, 285, 267) are present in the hydroxylated molecule, with an additional mass of 18 (and giving *m/z* 321, 303, 285). Such finding clearly demonstrates that hydrolysis reaction occurred leading to the formation of M+18 products (hydroxylated derivative). The amount of them increases within irradiation time. The fragment ion at *m/z* 321, observed on the total ion chromatogram, indicating a weak –C-O- bond of the ester side-chain at the C-8 position. Its elimination was presumably via either a remote charge-induced fragmentation process or a direct cleavage at C-8 with a neutral loss of $CH_3CH_2CR(CH_3)COOH$. The product of the direct cleavage could undergo dehydration to generate the ion at *m/z* 303 or rearrange to the same ion at *m/z* 321 derived from the charge-induced fragmentation process.

Fig. 18 presents LC-MS total ion chromatogram of irradiated simvastatin sample (a), mass spectrum of Sim-L (b) and Sim-H (c), extracted mass chromatogram at m/z 437 (d) with its mass spectrum (e) and extracted mass chromatogram at m/z 451 (f) with its mass spectrum (g). Table 12 presents proposed simvastatin photogenerated products and main fragment ions.

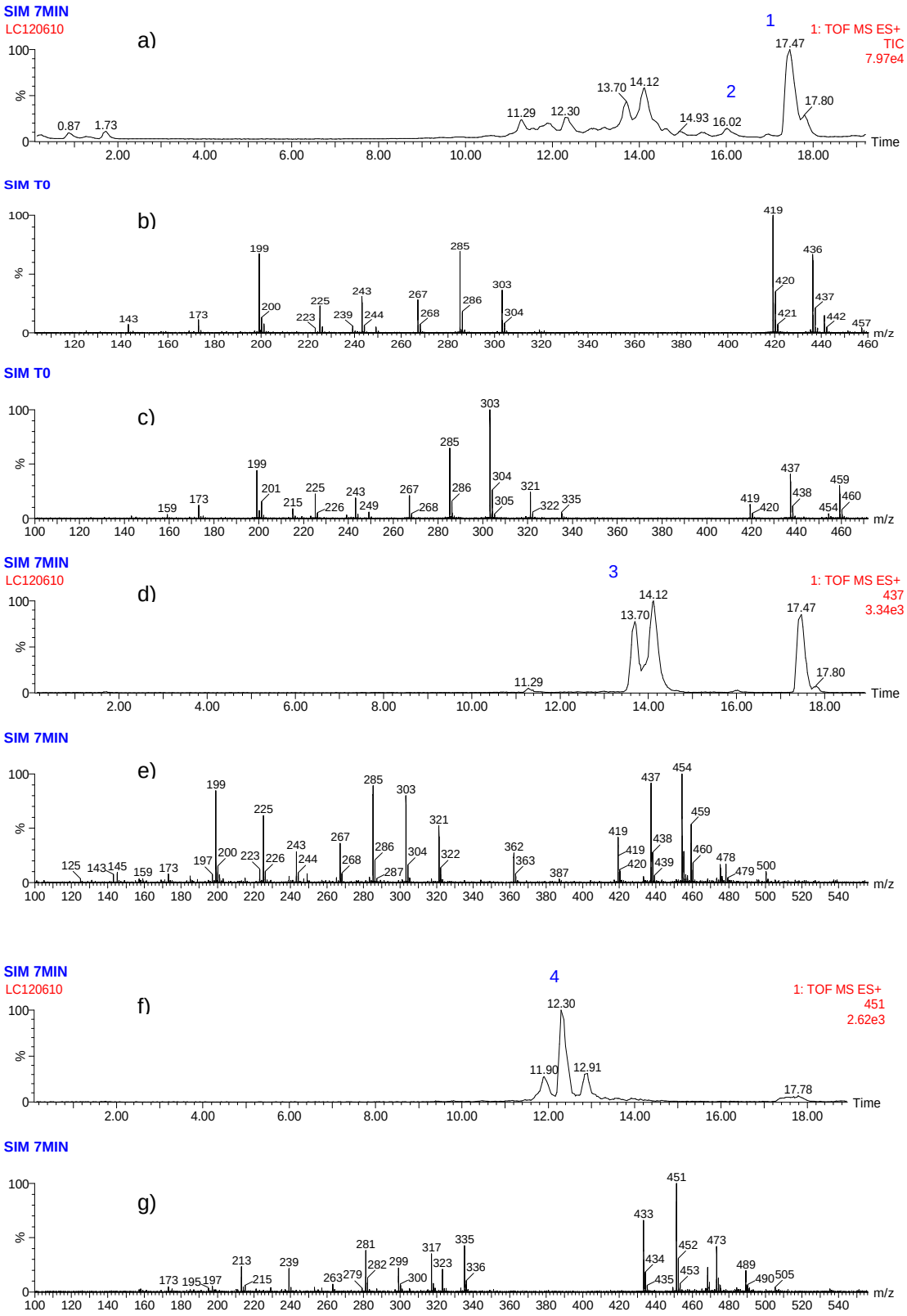
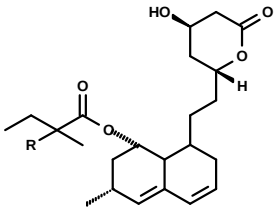
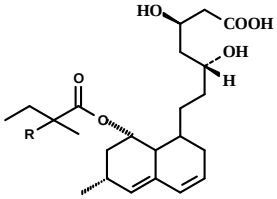
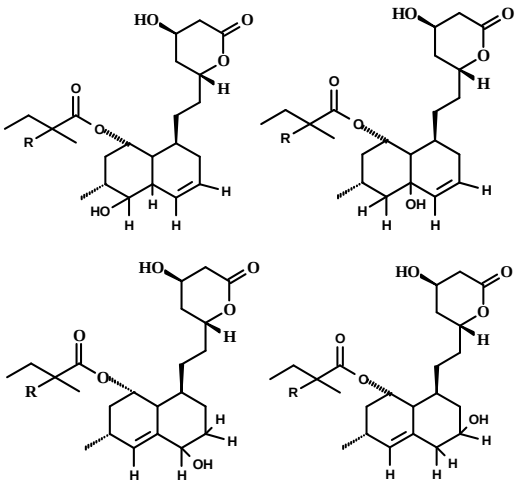
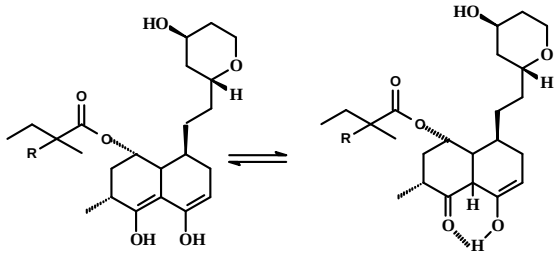


Fig 18: LC-MS total ion chromatogram of irradiated simvastatin sample (a), mass spectrum of Sim-L (b) and Sim-H (c), extracted mass chromatogram at *m/z* 437 (d) with its mass spectrum at 13.7 min (e) and extracted mass chromatogram at *m/z* 451 (f) with its mass spectrum at 12.3 min (g)

It should be pointed out that ions with *m/z* 451 are the result of photohydrolysis with two OH present in the ring, or molecule structure presented in the Table 12, which is

in agreement with *m/z* 451 and 489, attributed to dihydroxylated simvastatin derivative Proposed compounds refer to keto-enol tautomerism - a chemical equilibrium between a keto form (a ketone or an aldehyde) and an enol. The interconversion of the two forms involves the movement of a proton and the shifting of bonding electrons; hence, the isomerism qualifies as tautomerism. Since for simvastatin and lovastatin the same fragmentation pathway has been observed fragmentation of hydrolysed product is presented for simvastatin (Table 12) and pravastatin (Table 13) compounds.

Table 12: LC-MS data in ES positive mode for the simvastatin photogenerated products and their proposed structures

Product[a]	t_{ret}[b]	$[M+H]^+$; main fragment ions	Structure
1	17.5	419, 303, 285, 267, 243, 225, 199	
2	16.0	437, 303, 285, 267, 243, 225, 199	
3	13.7 and 14.12	459, 437, 321, 303, 285, 243	
4	12.3	489, 451, 433, 335, 323, 281	

[a]- The numbers refer to the peaks in LC-MS chromatogram given in Fig. 18

m/z 419 (simvastatin), R=CH_3

[b]- Retention time

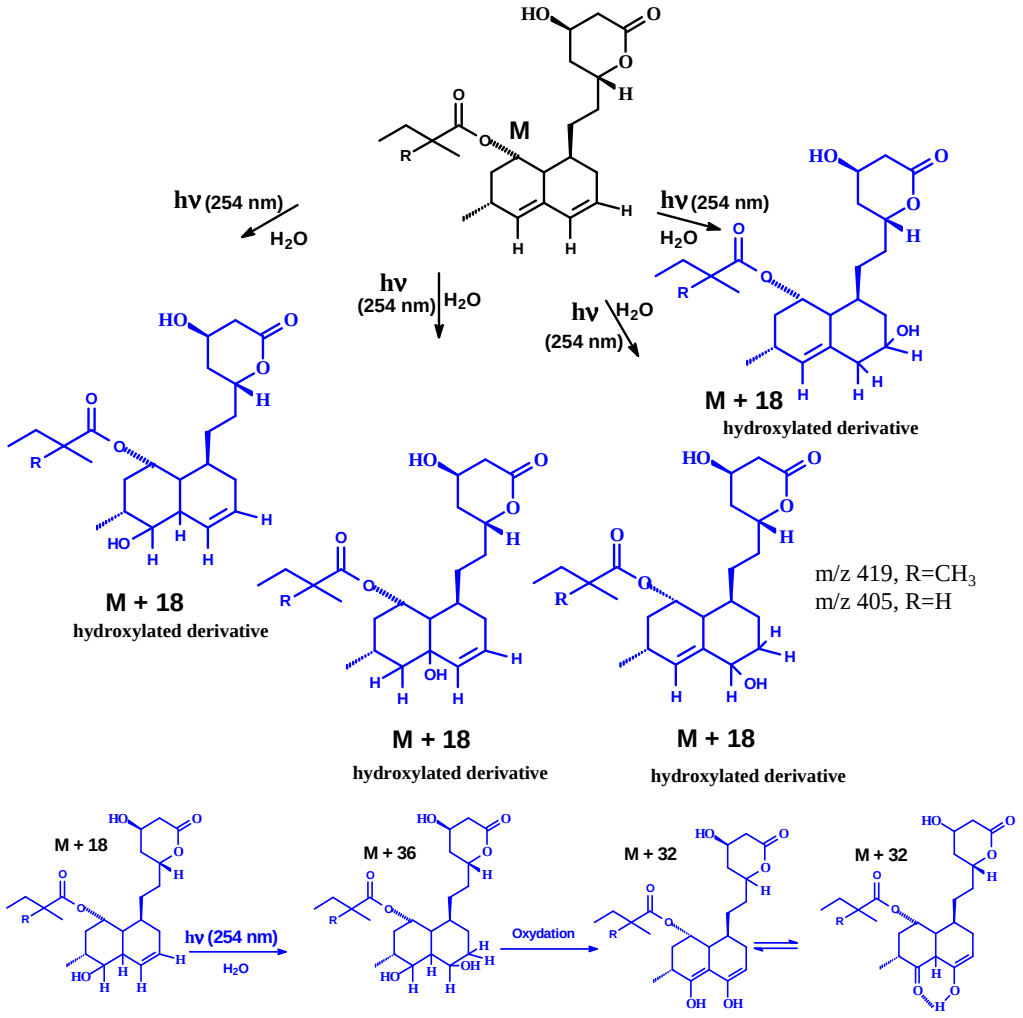


Fig 19: Proposed photohydrolysis pathway of simvastatin and lovastatin compounds

For pravastatin, which exists only in hydroxy acid form similar photohydrolysis pattern has been observed. Table 13 consists of proposed photogenerated products and main fragment ions of pravastatin. Pravastatin elutes at 11.21 min (Fig. 20 a). Extracted chromatogram of irradiated sample and ion at *m/z* 443 (424+18+1) (Fig. 20 c) correspond to hydrolysed structure (different isomers at 8.99 min, 9.58 min, 10.38 min). Such a structure is further oxidized, which results in conjugated double bonds in the ring again. Extracted chromatogram at *m/z* 457 (424+32+1) may be attributed to a keto-enol equilibrium (ret time 10.38 min, Fig 20 c) between two forms.

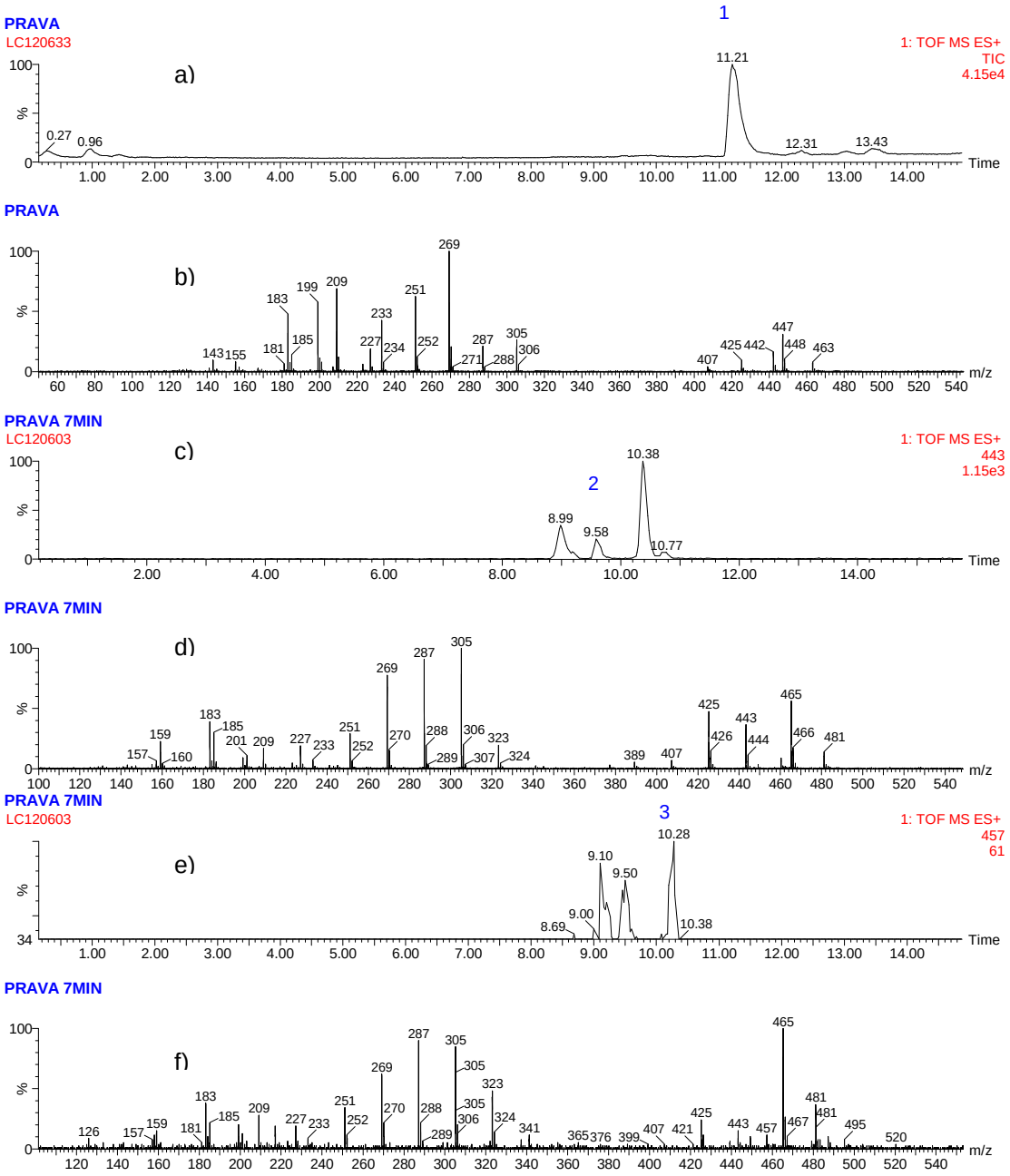
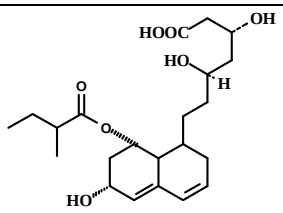
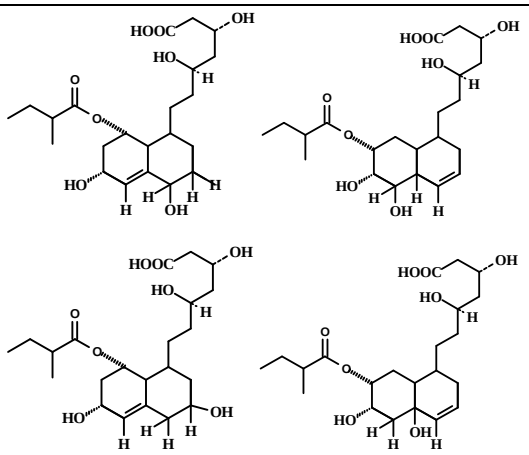
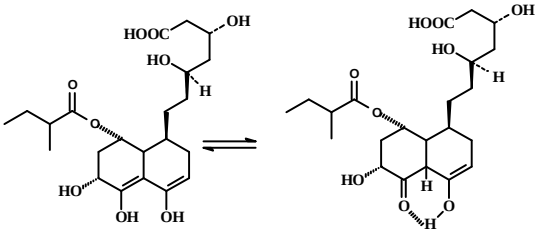


Fig 20: LC-MS total ion chromatogram of irradiated pravastatin sample (a) with its mass spectrum (b); extracted mass chromatogram at *m/z* 443 (c) with its mass spectrum at 10.38 min (d); extracted mass chromatogram at *m/z* 457 (e) with its mass spectrum at 10.28 min (f)

Table 13: LC-MS data in ES positive mode for the pravastatin photogenerated products and their proposed structures

Product[a]	t_{ret}[b]	$[M+H]^+$; main fragment ions	Structure
1	11.2	425, 305, 287, 269, 251, 233, 209, 199	
2	8.99; 9.58 10.38	443, 323, 305, 287, 269, 251, 227	
3	10.28	495, 465, 457, 323, 305, 287, 269, 251, 227	

[a]- The numbers refer to the peaks in LC-MS chromatogram given in Fig. 20

[b]- Retention time

5.2. Conclusions

First photodegradation studies were performed with Xe lamp (polychromatic light within a broad range 260 nm – 1000 nm), latter with Hg lamp emitting monochromatic light (254 nm as well as 365 nm). Maximum absorbance for statin drugs is about 240 nm, so according to our expectations there was no degradation observed with 310 nm and 365 nm light. Irradiation with 254 nm resulted in photohydrolysis, confirmed by the extracted chromatograms on LC-MS instrument.

It is worth mentioning that single, low resolution MS measurement (in applied ESI-MS full scan mode) did not allow to identify the exact position of transformation, and to differentiate isomers, or to provide exact structure of isomers/transformation products. Thus various products are proposed. Multiple transformation products, present in the irradiated sample could suppress ionization complicating product identification. In order to define the exact structure of formed molecules, various analytical and wet chemistry techniques (LC-nuclear magnetic resonance, chemical derivatisation, and hydrogen/deuterium-exchange (H/D-exchange) combined with MS) should be applied.

VI Photocatalysis

6.1. Introduction

The most widely investigated photocatalyst for the degradation of organic pollutants is TiO_2. TiO_2 is remarkably active, cheap, nontoxic and chemically stable over a wide pH range and it is not subject to photo corrosion. In general, the goal of the application of photocatalysis in water treatment is the transformation, deactivation and finally minimization of environmentally persistent compounds or xenobiotics.

In this context it is useful to apply various technologies to purify aqueous civil and industrial effluents containing pharmaceutical substances. Among them, advanced oxidation processes (AOPs) have been the subject of major interest in recent years. These processes are characterized by the formation of OH radicals, which ensure high reactivity and low selectivity, as they are required for the degradation of different pollutants. Heterogeneous photocatalysis represents an example of AOPs capable of achieving a complete oxidation of organic and inorganic species, including also pharmaceutical substances. It takes advantage of some semiconductor solids, which can be used as photocatalysts suspended in the water effluent to be treated, or immobilized on various types of supports. Among the various solids, polycrystalline anatase TiO_2 is largely used because of its low cost and its (photo)stability (Molinari et al., 2006). The aim of this work was to study the photocatalytic degradation of simvastatin, lovastatin and pravastatin cholesterol-lowering statin drugs and to elucidate the photodegradation products.

6.2. Experimental

6.2.1. Materials and instruments

For the preparation of sol-gel derived TiO_2 films tetraethoxysilane (Acros Organics (Geel, Belgium)), ethanol (Riedel-de Haen (Hanover, Germany)), and concentrated (65%) nitric acid (Acros Organics (Geel, Belgium)) and for TiO_2 sol: titanium (IV) isopopoxide (Acros Organics (Geel, Belgium)), ethyl acetoacetate (Riedel-de Haen (Hanover, Germany)), 2-methoxyethanol (Fluka (Buchs, Switzerland)), ND THE

TRIBLOCK COPOLYMER Pluronic F-127 Sigma-Aldrich Company Ltd (Gillingham, GB) were used.

Transparent TiO_2 - anatase films deposited on both sides of SiO_2-precoated soda lime glass slides (175 mm × 12.5 mm × 2 mm) were produced by sol-gel processing route according to Černigoj et al., 2006.

The photocatalytic cell consisted of a DURAN glass tube (240 mm, inner diameter 40 mm), which was closed on the lower side with a glass frit and the valve for purging with oxygen. The effective volume of the glass tube is 250 mL. The spinning basket was made entirely of Teflon and fitted into the photocatalytic cell. Six glass slides with immobilized catalyst were fastened around the axis by the help of two holders. The glass slides and the axis were not joined together. There was a gap of 1.5 mm in between to enable homogenous mixing of the solution an all segments of the cell. The spinning basket with immobilized TiO_2 placed in the glass tube could freely rotate around its axis (Fig. 21) (Černigoj et al., 2007a and Černigoj et al., 2007b).

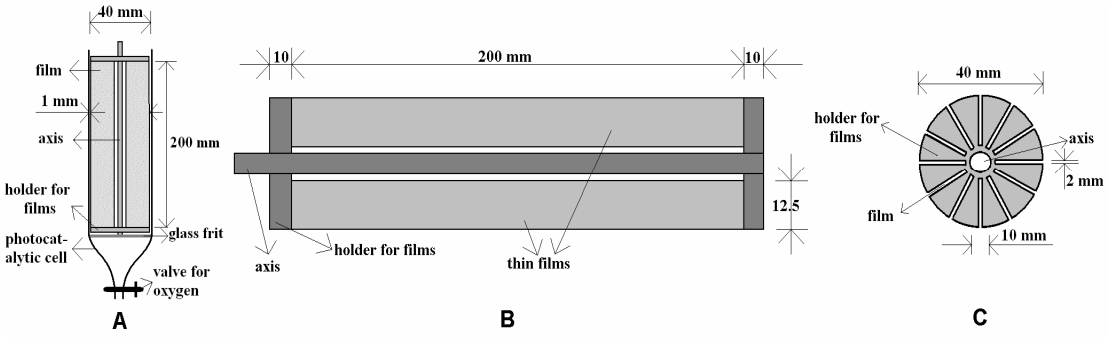


Fig 21: The photocatalytic cell (A), its longitudinal profile with thin films position (B) and Teflon holder with twelve notches for film fastening (C) (Černigoj et al., 2007a)

The photocatalytic activity of the as-prepared TiO_2 films was evaluated in a tailor-made chamber photoreactor using 3 low-pressure mercury fluorescent lamps as a UVA radiation source (CLEO 20 W, 438 mm × 26 mm, Phillips; broad maximum at 355 nm). The photocatalytic cell was put in the centre in-between the lamps. The motor on the top of the reactor rotates the spinning basket with the variable speeds (0-300 rpm).

Mass spectrometry of irradiated samples was performed with LC-MS (Waters Alliance 2695 Separations Module) X Terra MS C_{18} column (2.1 mm × 100 mm, 3.5 µm) with operating conditions identical to those used previously – chapter 4.2.3.

6.2.2. Photocatalytic experiments

Aqueous solution of simvastatin, lovastatin and pravastatin at the concentration of 10 mg L^{-1} were irradiated in the presence of 6 glass slides with immobilized sol-gel derived TiO_2. During the experiment 0.5 mL aliquots were collected for HPLC-DAD analysis (time: 0; 3,5; 7; 15; 30; 60; and 120 min). For kinetic studies (Fig. 22), samples, previously purged with oxygen were selected. For mass spectrometry analysis, samples of 30 min irradiation (previously purged with air, in order to slower degradation in comparison to samples purged with oxygen and to monitor easier transformation products) (simvastatin and lovastatin) and 60 min irradiation (pravastatin) were selected. HPLC-DAD and LC-MS operational conditions were as applied before (chapters: 4.2.2 and 4.2.3). Due to the problems with evaporation of simvastatin and lovastatin compounds in lactone form (explained in details in the chapter 4.4.6) samples were purged with oxygen, in the darkness, for 15 minutes only before the experiment, in order to provide sufficient amount of oxygen. Pravastatin sample has been provided with oxygen, as a source of oxygen during the whole experiment. Blank experiments were performed in the same manner (statins irradiated without TiO_2 glass plates).

6.2.3. Results and discussion

6.2.3.1. Kinetics and product analysis

From the obtained HPLC-DAD chromatograms indicating the disappearance of the starting compound, a first-order degradation plot can be derived (Fig. 22). The degradation half-life times ($t_{1/2}$) were equal to 13.3±2.1 min (simvastatin), 15.2±1.9 min (lovastatin), 6.5±0.4 min (pravastatin) and observed rate constant K_{ob}: 0.0521±0.008 min^{-1}; 0.0456±0.008 min^{-1} and 0.1066±0.0065 min^{-1}; respectively.

The degradation kinetics depends upon experimental conditions (and purging with oxygen is one of them). Complete degradation has been achieved within 60 minutes in the case of pravastatin, provided with oxygen during the whole experiment. Simvastatin and lovastatin, due to their volatility could not be purged all the time during the irradiation experiment. However, we assume that they would degrade faster, as pravastatin, if they were provided with sufficient amount of oxygen for the whole duration of catalysed irradiation. For the heterogeneous photocatalysis with TiO_2 molecular oxygen is required in the solution, being involved in the process of electron scavenging leading to an efficient formation of hydroxyl radicals. Since the simvastatin and lovastatin samples were purged only before the experiment their degradation was achieved only by 120^{th} minute.

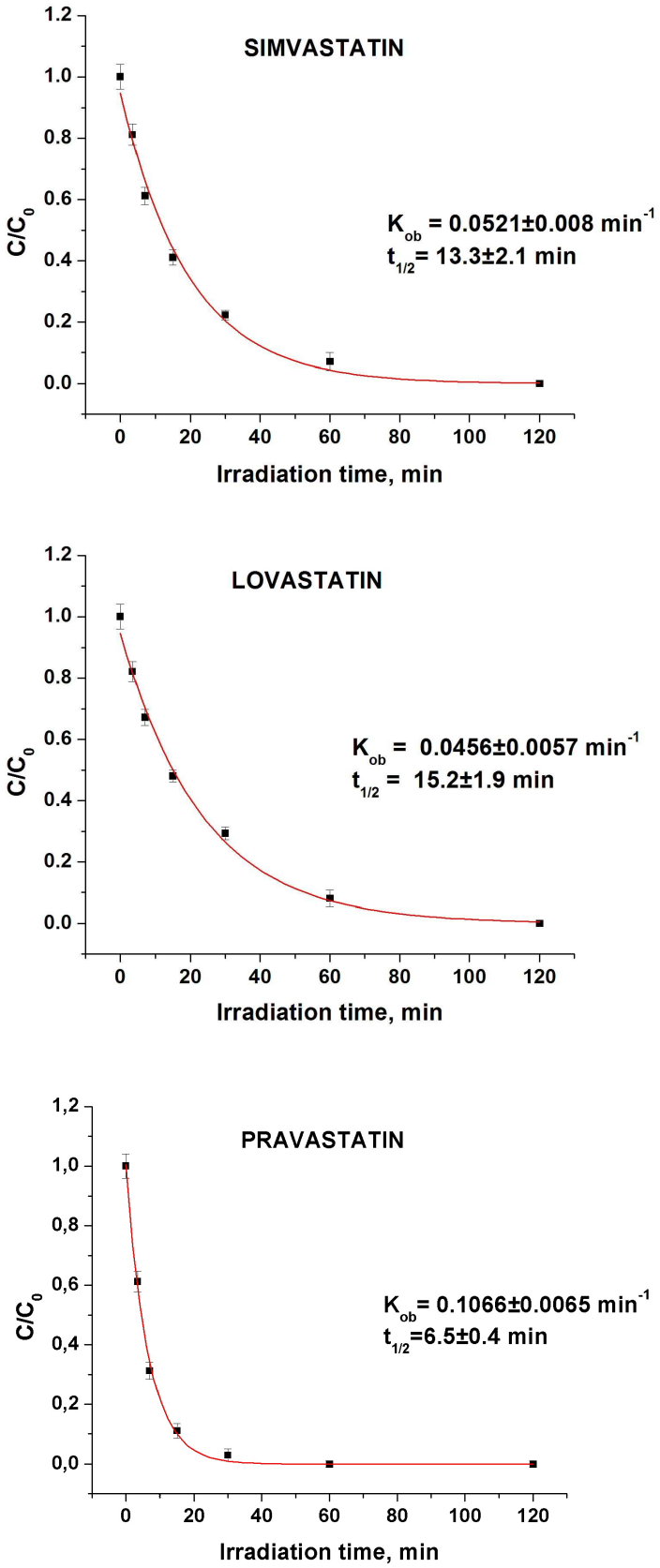


Fig 22: First order degradation curve of statin compounds irradiated in the presence of TiO_2 catalyst

60 min of irradiation results in decrease of the compound concentration to 7.1±2.9 % (simvastatin), 8.2±2.6 % (lovastatin) and for pravastatin in complete degradation.

The LC-MS analysis of all three compounds showed the formation of several irradiation products. As the first example to present photogenerated products we have chosen simvastatin sample (total ion chromatogram is presented on the Fig. 23 a) LC-MS analysis confirms that formation of irradiation products appear through the hydroxyl radical attack on the double bond of the ring (proposed mechanism is suggested and explained in details in the Fig. 24 and Fig. 25). At least two hydroxyl isomers have been confirmed by the extracted chromatogram at *m/z* 435 (418+16+1) - peaks with retention time 10.71 min; 11.36 min. Subsequently the molecule is oxidized and $HO_2^{\bullet}$ radical is eliminated, resulting in conjugated double bond reconstruction (giving the mentioned above hydroxyl isomers, as an intermediate products). Further irradiation in the presence of TiO_2 catalyst results in the same dihydroxysimvastatin derivative product as in the case of photohydrolysis, without a presence of TiO_2 (described in detail in the chapter 5.1.2.2). Besides, main fragment ions, detected previously for standard sample (*m/z* 303, 285, 267) are present in the hydroxylated molecule (Fig. 23 c), with the addition of mass of 16 (giving *m/z* 319, 301, 283). Such finding clearly demonstrates that oxidation reaction occurred leading to the formation of M+16 products (hydroxylated derivatives). The amount of them increases within irradiation time.

Ions with *m/z* 451 (extracted from total ion chromatogram), are the confirmation of proposed dihydroxylated simvastatin structure. Other, major fragments of the irradiated product (dihydroxylated simvastatin) are *m/z* 433, 335, 317 and 281 (see Fig. 23 f).

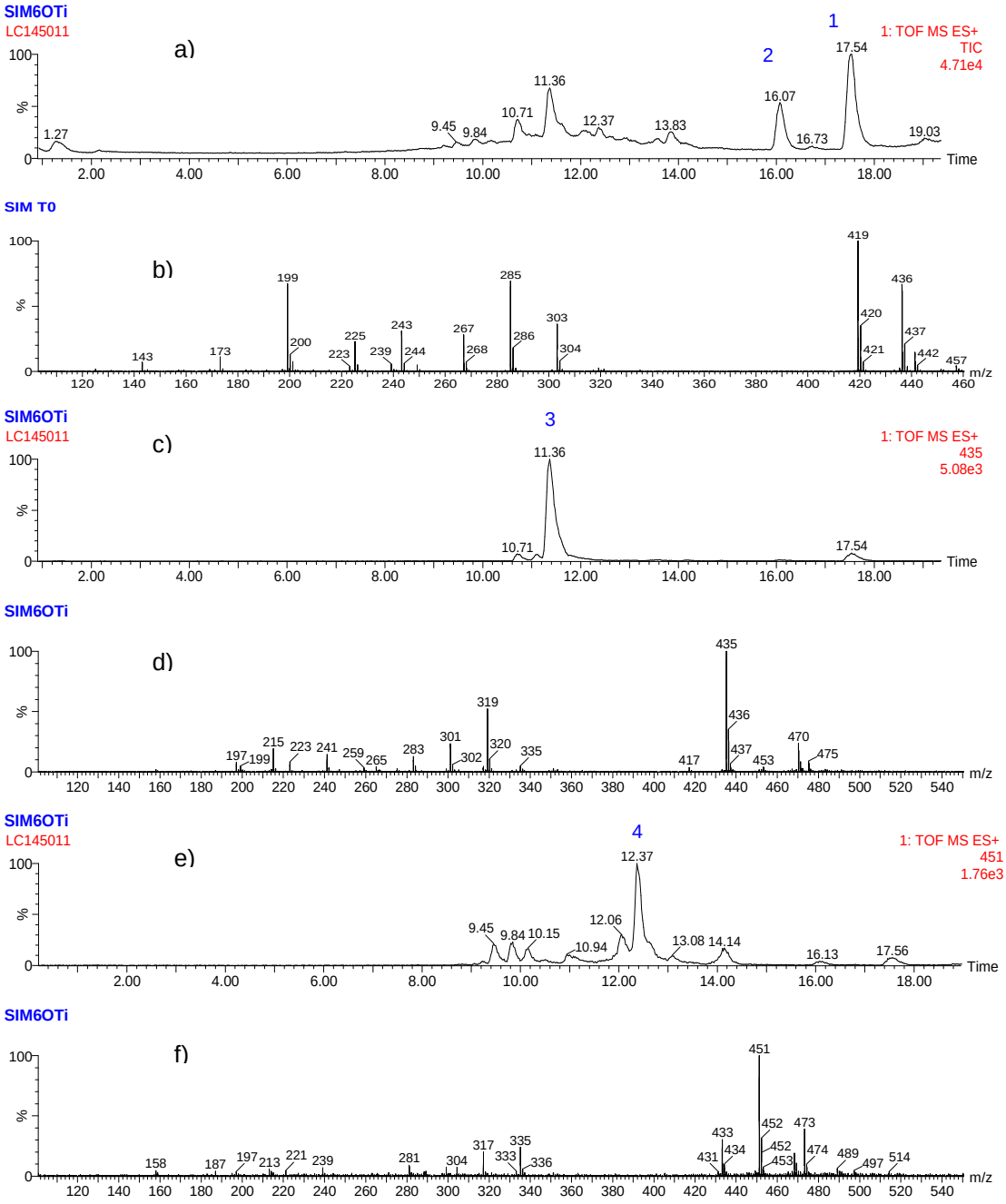


Fig 23: LC-MS total ion chromatogram of irradiated simvastatin sample in the presence of TiO_2 catalyst (a), Sim-L mass spectrum (b), extracted mass chromatogram at *m/z* 435 (c) with its mass spectrum at 11.36 min (d) and extracted mass chromatogram at *m/z* 451 (e) with its mass spectrum at 12.37 min (f)

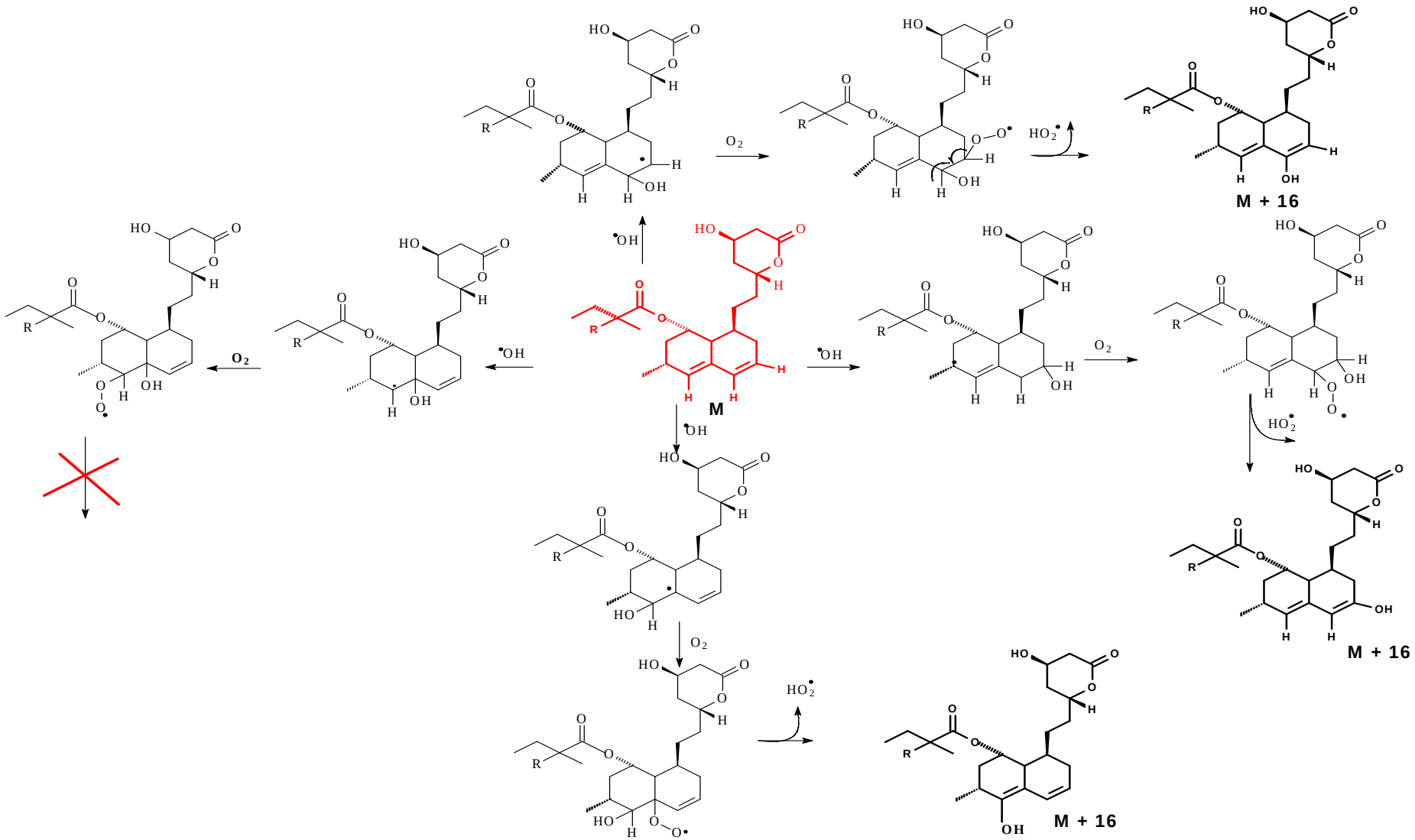


Fig 24: Proposed photodegradation pathway of statin compunds in the presence of TiO_2 catalyst

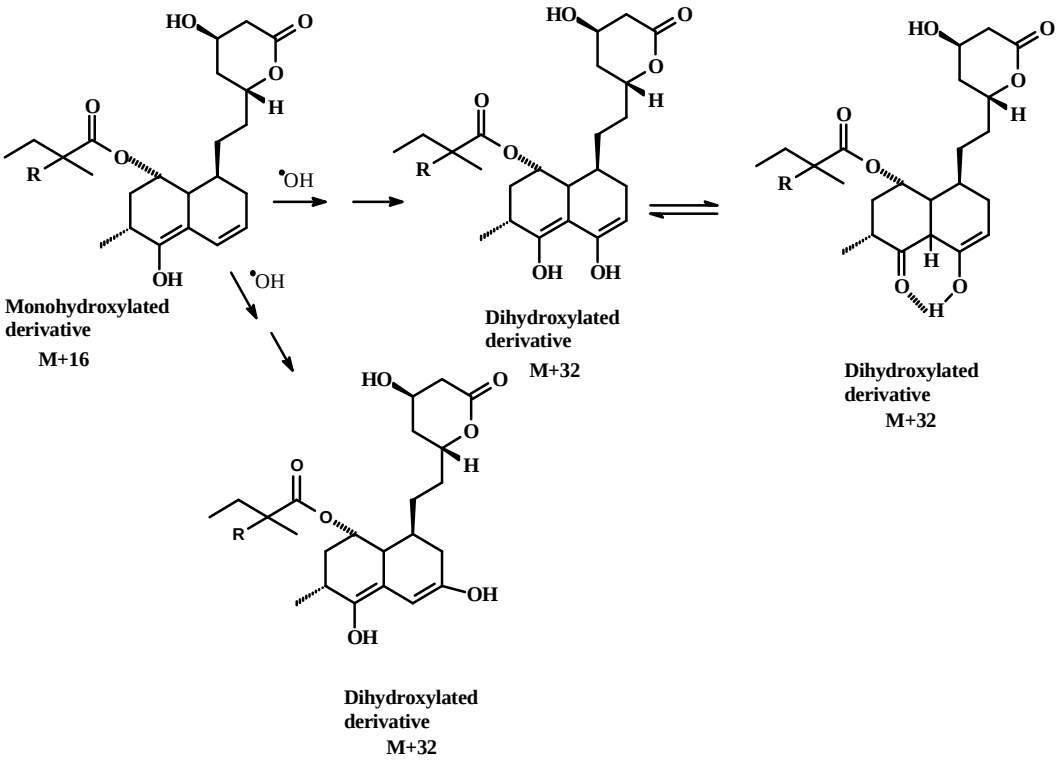
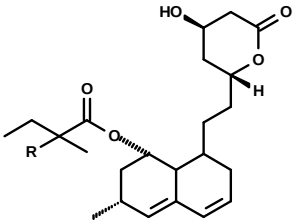
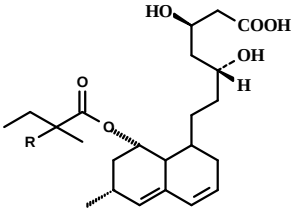
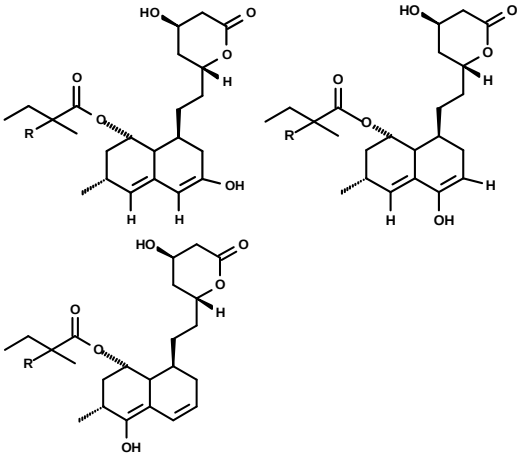
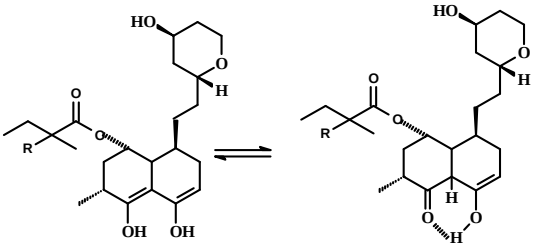


Fig 25: Proposed photodegradated products of statin, irradiated in the presence of TiO_2 catalyst

According to the proposed degradation mechanism (Fig. 24 and Fig. 25) Table 14 consists of suggested photogenerated simvastatin products.

Table 14: LC-MS data in ES positive mode for the simvastatin photogenerated products (in the presence of TiO_2 catalyst), and their proposed structures

Product [a]	t_{ret} [b]	$[M+H]^+$; main fragment ions	Structure
1	17.5	419, 303, 285, 267, 243, 225, 199	
2	16.0	437, 303, 285, 267, 243, 225, 199	
3	10.71 and 11.36	435, 319, 301, 283, 241	
4	12.37	451, 433, 335, 317, 281	

[a]- The numbers refer to the peaks in LC-MS ion chromatogram given in Fig. 23

[b]- Retention time

m/z 419 (simvastatin), R=CH_3

For pravastatin, existing only in hydroxy acid form, similar photodegradation pattern has been observed. Ion with *m/z* 441 corresponds to hydrolysed structure - different

isomers as for simvastatin, (which stands for 424+16+1) (Fig 26 c). However, high background noise and relatively low resolution does not allow separating clearly proposed isomers. The detection at *m/z* 457 (424+32+1), 480 (424+32+23+1), 497 (424+32+39+1) (Fig 26 e and Fig 26 f) is related to the presence of additional two OH groups in the ring or the molecule (and its sodium and potassium clusters).

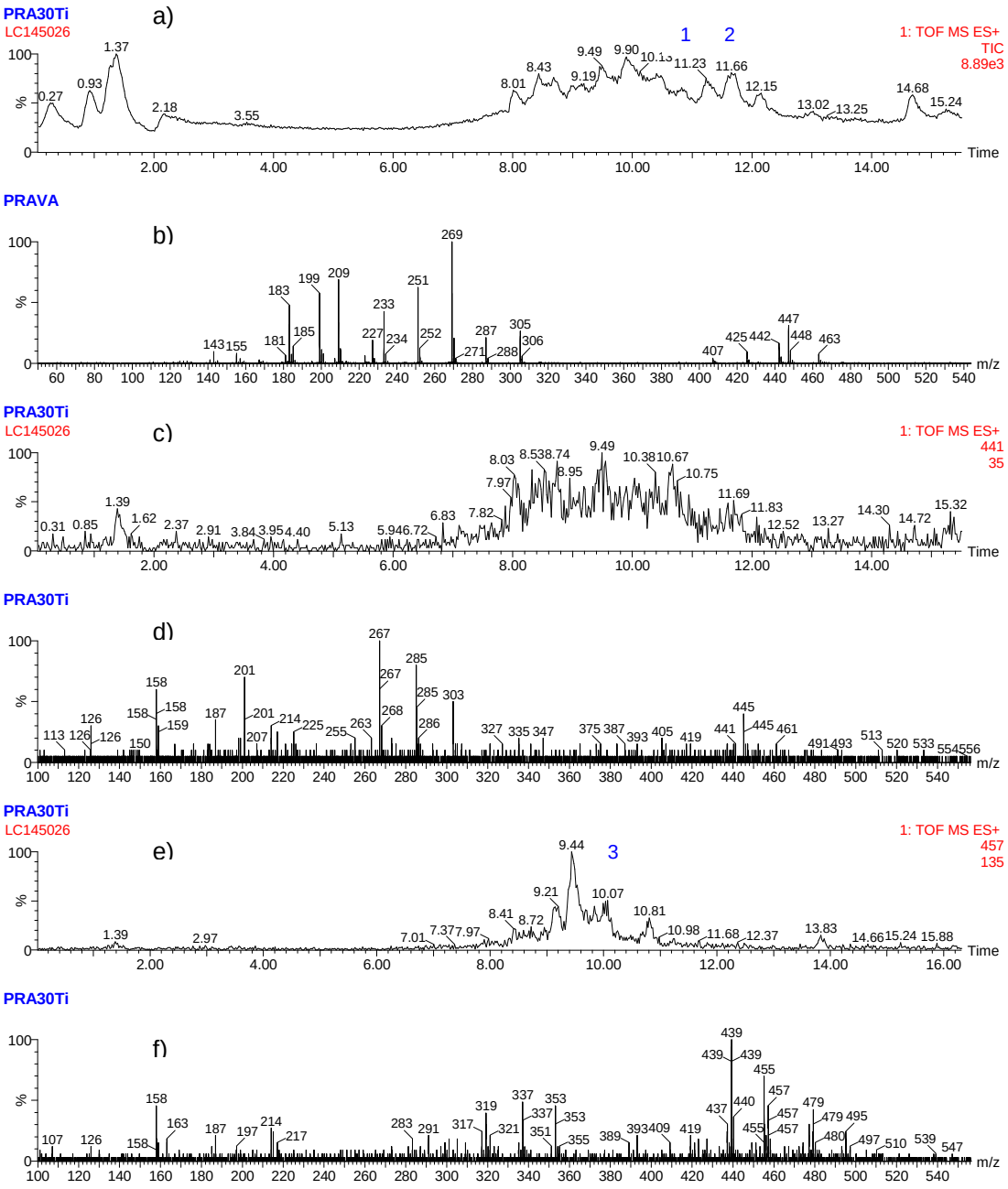
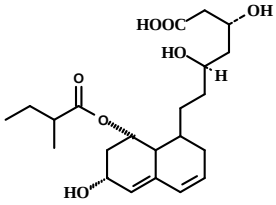
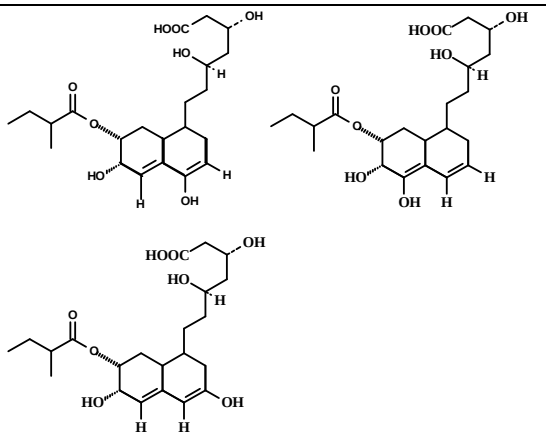
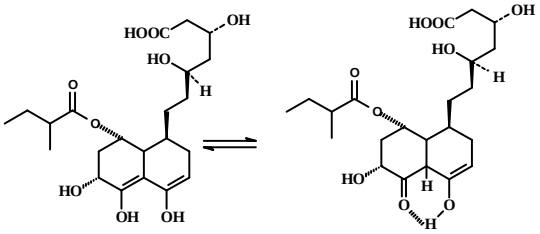


Fig 26: LC-MS total ion chromatogram of irradiated pravastatin sample in the presence of TiO_2 catalyst (a), Pra-H mass spectrum (b), extracted mass chromatogram at *m/z* 441 (c) with its mass spectrum at 11.8 min (d); extracted mass chromatogram at *m/z* 457 (e) with its mass spectrum at 10.1 min (f)

Table 15: LC-MS data in ES positive mode for the pravastatin photogenerated products (in the presence of TiO_2 catalyst), and their proposed structures

Product[a]	t_{ret}[b]	$[M+H]^+$; main fragment ions	Structure
1	11.2	425, 305, 287, 269, 251, 233, 209, 199	
2	11.8	441, 303, 285, 267, 225	
3	10.1	497, 480, 457, 439	

[a]- The numbers refer to the peaks in LC-MS ion chromatogram given in Fig. 26

[b]- Retention time

6.3. Conclusions

On the basis of obtained results, one can conclude that photocatalytic degradation is a complex process, involving various intermediate products (isomers). The faster disappearance rate for pravastatin compound (in comparison to simvastatin and lovastatin), which is most probably due to sample purging with oxygen during the whole irradiation experiment, and sufficient amount of provided oxygen, which is involved in formation of hydroxyl radicals. Within 120 minutes we achieved complete removal of simvastatin and lovastatin compounds and the products of their irradiation (we did not observe any signal of them on LC-MS total ion chromatogram). On the basis of pravastatin behaviour we can assume that the same disappearance would occur for other investigated statins, if we would not have problems with compound evaporation and we would purge simvastatin and lovastatin sample with oxygen during the whole experiment. Additional experiments, where oxygen was replaced with air showed that more time is required to degrade statin compounds, and that oxygen plays an important role in the catalysed degradation. LC-MS analysis confirms that formation of irradiation products appear through the hydroxyl radical attack on the double bond of the ring. At the same time, we discover similarities between intermediate products of photocatalysed reaction and photolysis performed with 254 nm mercury lamp. As hydroxyl radical attacks directly the double bound of the ring, different isomers are formed. Consequently the molecule is oxidized and $HO_2^{\bullet}$ radical is eliminated, resulting in conjugated double bond reconstruction. Further irradiation in the presence of TiO_2 catalyst results in the same dihydroxylated derivative of statin compound.

VII Ozonation

7.1. Introduction

Ozonation is widely recognized to be an effective tool for the removal of recalcitrant chemicals in water. Ozone treatment is one of the existing AOPs and can be used for purification of influents and/or effluents from various industries. A major limitation of the ozonation process is the relatively high cost of ozone generation coupled with the very short half-life time of ozone. Thus, ozone always needs to be generated on site. Moreover, the process efficiency is highly dependent on efficient gas liquid mass transfer, which is quite difficult to achieve. It is now widely accepted that ozone reacts in aqueous solution with various organic and inorganic compounds, either by a direct reaction of molecular ozone or through a radical type reaction involving the hydroxyl radical produced by the ozone decomposition in water.

Ozonation rarely originates complete mineralization to carbon dioxide and water, but leads to partial oxidation sub-products such as organic acids, aldehydes and ketones. The solution pH alters the chemical decomposition of ozone: for example, hydroxyl radicals are formed during ozone decomposition at high pH, while the molecular ozone remains as the main oxidant at low pH.

7.2. Experimental

7.2.1. Materials and instruments

Ozone has been generated using a Pacific Ozone (L11-L24) generator and the inlet oxygen gas flow rate was equal to 10 L h^{-1}. The mass flow of generated ozone was calculated from iodometric titration with a standard thiosulphate solution. Different ozone concentration (40-80) mg L^{-1} were studied in order to optimize the working conditions, which led us to the selection of concentration (46.0±5) mg L^{-1}.

Analytes were separated using HPLC-DAD equipment, which consisted of a HP 1100 series chromatograph, coupled with DAD detector. Luna C_{18} column (4.6 mm i.d. × 250 mm, 3 µm) (Phenomenex, Torrance, CA, USA) and the sample injection

volume was 50 µL. Mobile phase and gradient programme were identical to those applied before (chapter 4.2.2).

For analytical studies LC-MS (Waters Alliance 2695 Separations Module) X Terra MS C_{18} column (2.1 mm × 100 mm, 3.5 µm) has been applied with the identical gradient program and operational conditions as described in the chapter 4.2.3.

7.2.2. Ozonation experiments

Aqueous solution of simvastatin, lovastatin and pravastatin at the concentration of 10 mg L^{-1} were purged with ozone (in 100 mL flask, covered with parafilm, in order to avoid evaporation) for a fixed period of time (2; 5; 10; 15; 20 min) and collected for HPLC-DAD analysis. The solutions were prepared daily by diluting 10 mg of simvastatin or lovastatin in 1 mL of acetonitrile and pravastatin in water and diluting it with double deionised water to the concentration of 10 mg L^{-1}.

After selection of optimum ozone flow (46.0±5) mg L^{-1} various pH values (4.5; 7 and 8.5) were tested to investigate its influence on compound degradation. If at any time of the experiment both forms (lactone and hydroxy acid) of statins appeared in the solution, their sum was considered as the total amount of the compound.

7.2.3. Results and discussion

Due to problems with maintaining constant ozone concentration (it varied by 10-30% within 24 hours, for the same instrumental parameters) presented data refer to the average daily ozone concentration (46±5) mg L^{-1}, for all of the compounds (in order to achieve identical conditions for all three statins). Before and right after ozonation of each drug, the concentration of ozone was measured (iodometric titration). Each sample was analysed on HPLC-DAD instrument, by triplicate injection of the sample (Table 16 – Table 18).

Experimental data show that pH has an influence on the degradation of each statin compound. For simvastatin drug, after 2 minutes of ozonation the concentration reaches 16.4±1.7 % (pH=4.5); 3.8±0.8 % (pH=7) and 10.5±1.0 % (pH=8.5) of initial value, respectively. By the 20th minute of the experiment, regardless of pH, no parent compound can be found in the solution (Table 16).

Table 16: The change of simvastatin concentration during sample ozonation

Time [min]	**The change of simvastatin concentration in different pH media [%±SD, n=3]**		
	pH=4.5	**pH=7**	**pH=8.5**
0	98.8±1.6	100.7±2.0	99.1±1.5
2	16.4±1.7	3.8±0.8	10.5±1.0
5	4.6±0.7	0.7±0.3	nd
10	1.4±0.5	nd	nd
15	1.0±0.4	nd	nd
20	nd	nd	nd
30	nd	nd	nd

nd – not detected

Lovastatin degradation is also affected by the pH. After 2 minutes of the ozonation experiment lovastatin reaches 20.1±1.2 (pH=4.5); 3.2±0.3 (pH=7) of initial value, while in basic medium the parent compound can not be detected. Complete degradation of lovastatin, under acidic conditions is achieved by 15th minute (Table 17).

Table 17: The change of lovastatin concentration during sample ozonation

Time [min]	The change of lovastatin concentration in different pH media [%±SD, n=3]		
	pH=4.5	**pH=7**	**pH=8.5**
0	99.0±2.0	99.3±2.1	100.2±1.4
2	20.1±1.2	3.2±0.3	nd
5	5.1±0.9	1.5±0.4	nd
10	2.0±0.3	1.2±0.5	nd
15	nd	nd	nd
20	nd	nd	nd
30	nd	nd	nd

nd – not detected

Since hydroxyl radicals are formed from ozone decomposition at high pH it has been expected that all of three compounds will degrade faster at the basic pH, which is confirmed by our results only for simvastatin and lovastatin. Literature data (Černigoj et al., 2007b) reveal that high pH values accelerate degradation process.

Pravastatin concentration reaches (16.4±1.1 % (pH=7) and (24.7±1.4) % (pH=8.5) of initial value after 2 min of ozonation (Table 18). At the same time in acidic media, (35.9±1.8) % remains in unchanged Pra-H form, showing that sample acidification retards degradation of pravastatin under experimental conditions. Till 20th minute of the ozonation experiment pravastatin is completely degraded regardless of pH.

Table 18: The change of pravastatin concentration during sample ozonation

Time [min]	The change of pravastatin concentration in different pH media [%±SD, n=3]		
	pH=4.5	**pH=7**	**pH=8.5**
0	99.3±1.3	100.4±1.2	100.7±1.7
2	35.9±1.8	16.4±1.1	24.7±1.4
5	21.6±0.9	3.2±1.1	16.5±1.2
10	12.1±1.2	nd	8.26±0.9
15	5.3±0.6	nd	3.82±0.7
20	nd	nd	nd
30	nd	nd	nd

nd – not detected

LC-MS analysis confirmed that formation of ozonation products appears through the direct attack of hydroxyl radical on one of the double bonds, present in the ring (as it was in the case of irradiation in the presence of TiO_2 catalyst). LC-MS total ion

chromatograms, extracted mass chromatograms and characteristics absorption bands of ozonated samples revealed the presence of M+16 and M+32 products, for all investigated statins. The difference in the mass of major fragments (16) suggests the degradation through hydroxy radicals attack (presence of the additional OH group). M+32 is related to the presence of two, additional OH groups in the statin molecule (difference of 32 in mass of initial compound). Unfortunately, the MS data did not provide more detailed structural information to determine the position of the added hydroxyl group on the statin ring; so that several isomers can be hypothesized for these compounds (vicinity of conjugated double bonds is favourable to the attack of hydroxyl radical).The products formation is proposed as described in the paragraph 6.2.3.1, for catalysed irradiation.

7.2.4. Conclusions

The ozonation process turned out to be a powerful tool for statin degradation, under the applied conditions. All of the three compounds, while ozonated, were completely degraded within 20 minutes of experiment, already at (46±5) mg L^{-1} ozone concentration. For the photocatalysis complete degradation was achieved by 60^{th} minute only for pravastatin.

The efficiency of ozonation depends upon variety of parameters. Changing pH, oxygen and ozone-flow rates would result in different amount of time, required to obtain complete degradation (as we could see for solutions with different pH values and various ozone concentration while method development). Under experimental conditions we have achieved complete degradation of each statin compound.

VIII Toxicity of statin compounds

8.1. Introduction

Since drugs, and statins among them, are considered as environmental contaminants, it is important to identify their degradation products, and whether they retain the activity of the parent molecule to elicit a toxicological effect on non-target organisms in aqueous systems. In the environment multiple reaction products may be more toxic than the parent compound.

The test principle is to compare the bacterial bioluminescence before and after exposure to the toxic compound. Gram negative bacterium *Vibrio fischeri* is used for the test. The luminescence machine is equipped with its own software which allows evaluating the test results automatically. EC_{50} values (half maximal effective concentration), or at least luminescence inhibition are the output. All the output data are evaluated for time intervals of 30 min individually. Based on measured data the EC_{50} value with appropriate confidence interval (95%) is determined (ISO 11348-3).

The aim of the toxicity measurements was to evaluate toxicity of statin standard solutions (EC_{50} calculation) and to determine difference in bioluminescence inhibition between 12 months sun-exposed samples and samples after photolysis experiment in comparison to standard solutions.

8.2. Experimental setup

Toxicity of aqueous samples for each standard statin compound (at the initial concentration of 100 mg L^{-1}) was determined following international standard ISO 11348-3 with LUMIStox produced by Hach-Lange (Dusseldorf, Germany). All the solutions were incubated on LABSONIC®M ultrasonic homogenizer (Sartorius (Goettingen, Germany)) in order to achieve maximum solubility (10 min). The samples were treated with appropriate amount of NaCl (2 g per 100 mL solution) and the pH of each sample was adjusted to the value of 7±0.2. Each standard solution was exposed to luminescent bacteria for 30 minutes at 15±0.2 °C using a temperature-controlled block. Various dilution levels (2, 4, 8, 16, 64, 128, and 256)

were achieved by following ISO 11348-3 standard. The preserved luminescent bacteria were reactivated before the test was started. Their viability was indicated by their natural luminescence. *Vibrio fischeri* was added to each sample in two parallels and luminescence was measured for each cuvette immediately and after 30 minutes of incubation. Whole process was done by stable temperature 15±0.2 °C and in accurate time intervals which was both provided under machine signalization.

Described above method was implemented also for the toxicity evaluation of degradation products (12 months sun-exposed samples and sample after photolysis), with the only exception of dilution levels. Sample were not diluted, but added directly to bacteria. Their luminescence inhibition was compared to the inhibition of standard solutions with the same initial concentration of each statin.

8.3. Results and discussion

8.3.1. Toxicity of standard solutions

On the basis of evaluation of a single test for each standard compound EC_{50} (half maximal effective concentration) could not be calculated, which indicates that standard solutions do not posses toxicity to luminescence bacteria. However, obtained results of luminescence inhibition for various dilution levels were used for further experiments to be compared with results obtained for samples subjected to degradation experiments (chapter 8.3.2; 8.3.3) (Table 19 and Table 20) It should be mentioned that inability of EC_{50} calculation in this example does not exclude statin toxicity. *Vibrio fischeri* are not target organisms for statin compounds so future studies should involve different toxicity experiments, with diverse organisms and end points.

8.3.2. Toxicity of sun-exposed samples

HPLC-DAD chromatograms allowed us to presume that some of the statins (for example in acidic media) forming several, additional products while being exposed to the sun light, might be dangerous to living organisms.

Toxicity of fresh standard solutions was determined, in parallel, in order to be compared with sun-exposed samples. Table 19 represents obtained results.

Table 19: Experimental data of luminescence inhibition of statin standard solutions as well as sun-exposed samples

	Luminescence inhibition of *Vibrio fischeri* [%]			
	Fresh standard sample solution before sun exposure (pH=7)	Sample after 12 months sun exposure at		
		pH=7	pH=8	pH=4.5
Simvastatin	9.0±1.0	20.4±2.1	22.5±3.0	45.5±3.2
Lovastatin	10.4±2.2	16.9±1.2	14.9±1.3	43.2±4.1
Pravastatin	7.1±1.2	12.4±2.2	28.1±2.4	7.8±2.0

The experimental values are scattered owing to the different pH of samples. As soon, as fresh, standard statin solutions did not raise our concerns in regard of toxicity, sun-exposed samples did so. There is a significant difference in luminescence inhibition of samples. The luminescence inhibition reached the highest value (45.5±3.2) % for simvastatin at pH=4.5; (43.2±4.1) % for lovastatin at pH=4.5 and (28.1±2.4) % for pravastatin at basic pH. Obtained results are in agreement with our assumption (on the basis of HPLC-DAD data) that various, additional products might possess higher toxicity towards *Vibrio fischeri* than parent compounds.

To conclude, statins once released to the environment might affect aqueous organisms. Increased temperature, as well as experimental pH values, might also appear in the natural water basins during the summer months, and additional end point organisms should be tested in regard of toxicity of statin drugs.

8.3.3. Toxicity of photolysed samples

Toxicity of photodegradation products (the highest concentration of statin derivatives during sample irradiation) has shown (12.6±3.2) % inhibition for simvastatin, (7.5±2.3) % for lovastatin and (5.1±1.9) % for pravastatin, which is not considered as toxicity towards *Vibrio fischeri.* Table 20 represents experimental results.

Table 20: Experimental data of luminescence inhibition of statin standard solutions as well as irradiated samples

	Luminescence inhibition of *Vibrio fischeri* [%]	
	Standard sample solution before irradiation	Sample after irradiation
Simvastatin	8.9±1.2	12.6±3.2
Lovastatin	11.9±2.2	7.5±2.3
Pravastatin	8.4±1.8	5.1±1.9

These preliminary toxicity investigations are the evidence that during irradiation procedure non toxic (in regard of *Vibrio fisheri* bacteria) compounds are produced, which is very important from the environmental point of view. Further toxicity experiments should involve additional end point organisms.

IX Biodegradability

9.1. Introduction

OECD ready biodegradability test are commonly used to obtain a first characterisation of organic compounds in terms of their susceptibility to microbial degradation and play an important role in the EU environmental classification of chemicals. One of the objectives of our studies was to evaluate biodegradability, following OECD method 301F. Manometric Respirometry Test has been selected for the experiment. This procedure evaluates the ready biodegradability of a test substance by measuring BOD (biochemical oxygen demand). The amount of oxygen uptake by microbial population during biodegradation of the test substance (corrected for uptake by blank inoculum, run in parallel) is expressed as percent ThOD (theoretical oxygen demand - amount of oxygen required to oxidise a compound to its final oxidation products, calculated on the basis of molecule's structure). A value of BOD equal or higher than 60 % ThOD within 28 days is regarded as an evidence of ready biodegradability.

9.2. Materials and instruments

All the chemicals were of analytical grade and were provided by:

- Sigma-Aldrich Company Ltd (Gillingham, GB): potassium dihydrogen orthophosphate (KH_2PO_4); calcium chloride, anhydrous ($CaCl_2$); magnesium sulphate heptahydrate, $MgS0_4 \times 7H_20$
- Riedel-de Haen (Hanover, Germany): iron (III) chloride hexahydrate ($FeCl_3.6H_20$)
- Fluka (Buchs, Switzerland): dipotassium hydrogen orthophosphate (K_2HPO_4); disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4 \times 2H_2O$); ammonium chloride (NH_4Cl)

Manometric respirometry tests were carried out in the OxiTop® IS 12 system (WTW (Weilheim, Germany)), which is based on a manometric respirometry test principle and uses innovative piezo-resitive pressure sensor technology.

9.3. Experimental setup

Biodegradation of each compound was tested in triplicates in bottles containing mineral medium, inoculum (local stream water), and the test substance (each statin). The theoretical oxygen demand (ThOD) was calculated on the basis of elemental composition for each statin compound, according to following formula (OECD, 1992):

$$\text{ThOD} = \frac{16[2c + 1/2(h - cl - 3n) + 3s + 5/2\,p + 1/2na - o]mg/mg}{MW}$$

Where MW= molecular weight

On the basis of calculated ThOD values, the concentration of 50 mg L^{-1} for each statin was selected, giving at least 50-100 ThOD L^{-1}, as the nominal sole source of organic carbon (OECD, 1992).

Stock solutions for mineral medium were prepared using analytical grade reagents following described below procedure (OECD, 1992):

(a) Potassium dihydrogen orthophosphate, KH_2PO_4.....8.50 g
Dipotassium hydrogen orthophosphate, K_2HPO_4……...21.75 g
Disodium hydrogen orthophosphate dihydrate, $Na_2HPO_4 \times 2H_2O$…….33.40 g
Ammonium chloride, NH_4Cl……0.50 g

Dissolve in water and make up to 1 L. The pH of the solution should be 7.4.

(b) Calcium chloride, anhydrous, $CaCl_2$….....27.50 g

Dissolve in water and make up to 1 litre.
(c) Magnesium sulphate heptahydrate, $MgS0_4 \times 7H_20$……...22.50 g

Dissolve in water and make up to 1 litre.

(d) Iron (III) chloride hexahydrate, $FeCl_3.6H_20$……...0.25 g

Dissolve in water and make up to 1 L. If a precipitate forms in a stock solution replace with a freshly made solution. For each biodegradability test mix: 10 mL of solution (a) with 800 mL water, then add 1 mL of solutions (b), (c) and (d) and make up to 1 L with water.

Preliminary biodegradation experiments were performed without pre-concentration of stream water, and the amount of microorganism was rather low. Due to that fact, in further experiment, collected stream water was pre-concentrated 3 times on the centrifuge (10 min, 3000 rpm), and purged with air for 24 hours in order to avoid high blank values. Owing to microbial activity, oxygen was taken from the gas phase of the hermetically sealed reaction vessels, while carbon dioxide released from respiration was absorbed by NaOH in a small tube and the resulting reduction in air pressure inside the closed system was measured. A biotic control test (blank) and a positive control test, containing CH_3COONa, which is known to be easily biodegradable, were also used. The tests were carried out in thermostatically controlled conditions (temperature 20°C), and the solutions were stirred to provide the biomass in the liquid phase with sufficient dissolved oxygen. From the results obtained the BOD can be directly calculated in mg L^{-1}.

Due to low solubility of two statin compounds (simvastatin and lovastatin) in water, stock solutions for the biodegradation test were prepared by weighting and dissolving appropriate amount of drugs directly in mineral media (as it is suggested in general procedures and preparations of OECD (1992) guidelines for testing of chemicals, for substance, with water solubility < 1g L^{-1}). River water for the experiment was taken from the local stream, and pre-concentrated in order to provide sufficient amount of micro organisms

To investigate biodegradation of statin compounds, three OxiTop® flasks containing 50 mg L^{-1} (triplicates for each statin), inoculum and mineral medium were used. Furthermore, a positive control test, containing 260 mg L^{-1} CH_3COONa and a biotic control test (blank), were also used. Percentage biodegradation was calculated according to the equation:

$$Degradation\ (\%) = (BOD - BOD_{blank})/\ ThOD$$

Where BOD is a biochemical oxygen demand of the test compound (mg L^{-1}), BOD_{blank} is biochemical oxygen demand of the mineral medium to which the inoculum was added (mg L^{-1}) and ThOD is theoretical oxygen demand (mg) – the total amount of oxygen required to oxidise a chemical completely.

9.4. Results and discussion

Simvastatin, lovastatin and pravastatin were aerobically biodegraded in the three replicates at a rate equal to 6.2±0.9 %; 12.4±1.8 % and 13.9±1.2 % subsequently. Positive control (sodium acetate) was degraded in 89.7±5.3 % by the day 28th, which indicated the activity of the microbial seed. Fig. 25 represents experimental data of biodegradation test, potentially indicating statin environmental persistence.

Obtained results signify that microbial biomass used in respirometric tests was viable, all tests were valid according to OECD (1992) protocol and satisfactory reproducibility of the method was achieved.

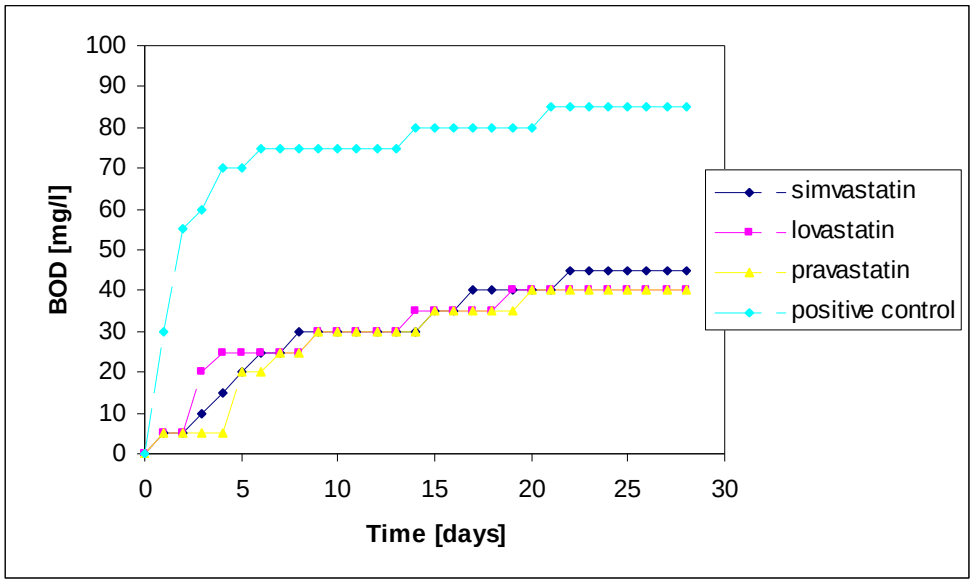


Fig 27: Experimental data of biodegradation test of statin compounds within 28 days

Although the partial elimination of tested statin compounds does not meet strict definition of ready biodegradability according to OECD (1992) it is possible that a faster process and efficient biodegradation could be achieved in the presence of high concentration of acclimatized microorganisms in a WWTP. Further simulation test

should be performed in the future, investigating biodegradation of these compounds under different operating conditions. Due to our knowledge there were no biodegradability test performed on the statin drugs. In order to simulate conditions, appearing in natural waters we decided to perform the test with stream water as a source of inoculum. It should be emphasized that our results do not reflect fate of statin compounds in activated sludge systems. Supplementary studies should cover this topic.

Manometric respiratory test (OECD, 1992) is advantageous because it is a direct biological parameter of aerobic degradation in contrast to other ready biodegradability test using dissolve organic carbon removal (DOC). Obtained results prove cholesterol lowering statin drugs to be persistent in the natural waters. Only a BOD value equal or higher than 60% of theoretical oxygen demand (ThOD), obtained within 28 days, is regarded as evidence of ready biodegradability. In order to simulate natural conditions experiments were performed with the local stream water, so the presence of microorganisms was relatively low in comparison to tests with activated sludge.

IX. Conclusions – general

- One of the goals of our research was to investigate how statin drugs are affected by various parameters such as diverse pH, time of sample storage, solvent systems and presence of sun light. Some of them could have had important influence on quantification studies, since on the daily basis we have had only access to simple instrument, such as HPLC-DAD, with lower sensitivity and selectivity than sophisticated LC-MS instrument. Thus it was of great importance to develop a method, which could possibly allow us to determine statins in aqueous samples at the concentration of ng L^{-1} (environmental concentration level, in the case we decide to investigate natural waters), and for the further degradation experiments, where we worked with higher concentrations (mg L^{-1}), without time-consuming sample preparation (just direct injection on the HPLC-DAD). Hence, developed method of statin determination was a tool for further degradation experiments. Our work was not dedicated to statin determination in environmental samples (Slovenia), although we are aware that such work has to be done in the future, in order to verify statins occurrence in natural waters of Europe.
- Published so far mass spectrometric studies of statin compounds (Wang et al., 2001; Metcalfe and Miao, 2003) refer only to lactone forms. We succeeded, additionally in the determination of hydroxy acids fragmentation, although only single MS measurements were performed. We observed that lactone forms can be easily converted to their corresponding hydroxy acid forms under different conditions such as high pH, while sample storage even at the room temperature, as well as under presence of different solvent systems. One of our discoveries is the fact that pravastatin is not influenced by pH at all, and was found only in hydroxy acid form, regardless of pH.
- Our results regarding statins exposed to the natural sun light show that compounds (simvastatin and lovastatin) undergo interconversion between two forms, and at the same time they start to degrade. Pravastatin, existing only in acidic form show the highest stability in acidic media among all investigated compounds, under experimental conditions. However it is worth mentioning

that samples, exposed to the same conditions, protected from the light, revealed equal mass and characteristics absorption bands, pointing out that we might be dealing not with the direct photodegradation, but the influence of increased temperature (in comparison to control samples, kept at the ambient temperature 20°C, where those products were not detected). Thus, single MS turned out not to be a sufficient measurement to conclude with 100 % certainty identity of formed transformation products. Additional analysis would be required (LC-nuclear magnetic resonance, chemical derivatisation, and hydrogen/deuterium-exchange (H/D-exchange) combined with MS), to support with confidence our hypothesis. Unfortunately, the MS data did not provide more detailed structural information to determine the position of the added hydroxyl group on the statin ring, so that several isomers can be hypothesized for this compound. The simvastatin and lovastatin samples were the most stable, when pH was equal to 7. After 12 months sun exposure the toxicity samples with *Vibrio fischeri* organisms was measured. The bioluminescence inhibition of bacteria has been significantly higher in comparison to fresh, standard solutions, but still below EC_{50} value. It raised our concerns, pointing out statins as potential environmental contaminants and threat to aqueous organisms.

- Photostability of three investigated by us drugs hasn't been determined so far. We succeeded (for the first time) in the evaluation of quantum yields for simvastatin, lovastatin and pravastatin. For the determination of the quantum yields, a parallel 254 nm beam was used. For analytical studies, a cylindrical setup emitting at 254 nm was employed in order to monitor the formation of the by-products and the disappearance kinetics of the parent compound. Irradiation with 254 nm resulted in photohydrolysis, confirmed by the total ion chromatograms and extracted mass spectra (LC-MS instrument).
- Photocatalytic degradation of statin compounds revealed that it is a complex process, involving various intermediate products (isomers). The faster disappearance rate for pravastatin compound (in comparison to simvastatin and lovastatin) is most probably due to the sample purging with oxygen during the whole irradiation experiment, and sufficient amount of provided oxygen, which is involved in formation of hydroxyl radicals. Pravastatin was degraded completely by 60^{th} minute of irradiation experiment. Within 120

minutes we achieved complete removal of simvastatin and lovastatin compounds and the products of their irradiation (we did not observe any signal of them on LC-MS total ion chromatogram). LC-MS analysis of all three compounds showed the formation of several irradiation products, as the result of hydroxyl radicals' oxidation, which takes place by the hydroxyl radical attack on the double bond of the ring. At the same time, we discover similarities between intermediate products of photocatalysed reaction and photolysis performed with 254 nm mercury lamp. As hydroxyl radical attacks directly the double bound of the ring, different hydroxyl isomers are formed. Consequently the molecule is oxidized and $HO_2^{\bullet}$ radical is eliminated, resulting in conjugated double bond reconstruction. Further irradiation in the presence of TiO_2 catalyst results in the same intermediate products as in the case of photolysis, completely degraded by 60^{th} minute in the case of pravastatin.

- Our results proof that the ozonation process seems to be a powerful tool for statin removal from the environment, in the case of their presence in STP's. All of the three compounds were completely degraded within 20 minutes of experiment, already at (46 ± 5) mg L^{-1} ozone concentration, obtained by using available equipment. The efficiency of ozonation depends upon variety of parameters. Changing pH, oxygen and ozone-flow rates would result in different kinetics, as we could observe while method development changing ozone concentrations, and diverse values of applied pH. Despite of different mechanism of product formation during photolysis, photocatalysis and ozonation process (the first one is a photohydrolysis, the latter ones involve hydroxyl radicals leading to photocatalytic degradation) the same dihydroxylated derivatives were observed (conclusion based on comparing LC-MS total ion chromatograms, retentions times of formed products, their mass spectra and characteristics absorption bands). Supplementary toxicity studies showed that those transformation products are non toxic (although there was only one group of tested organisms – *Vibrio fischeri* bacteria). Additional organisms should be involved in toxicity test, in order to exclude, with certainty, toxicity of those products.
- On the basis of experimental data we can conclude that neither standard solutions, nor derivates of the parent compounds after sample irradiation

(with and without a catalyst) show toxicity towards *Vibrio fischeri* bacteria. These preliminary toxicity investigations are evidence that during irradiation procedure non toxic (in regard of *Vibrio fisheri* bacteria) compounds are produced, which is very important from the environmental point of view. Further toxicity experiments should involve additional end point organisms.

- We applied some of the novel experiments (such as evaluation of ready biodegradability), never performed for statin drugs. Biodegradability tests proved that simvastatin, lovastatin and pravastatin were aerobically degraded at a rate equal to 6.25±0.92 %; 12.44±1.8 % and 13.95±1.20 % subsequently. Obtained results prove cholesterol lowering statin drugs to be persistent in the natural waters, which is one of the scientific outcome of this work. Only a BOD value equal or higher than 60 % of theoretical oxygen demand (ThOD), obtained within 28 days, is regarded as evidence of ready biodegradability. It is worth mentioning that our results do not reflect fate of statin compounds in activated sludge systems. So far, statin drugs were not tested at all in regard of their susceptibility to microbial degradation. Supplementary studies should cover this topic, and activated sludge systems should be involved in the tests, in order to verify statin biodegradability in the presence of higher amount of microorganisms.

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