‘Investigations on drug related compounds using online photoreactor HPLC hyphenation and complementary mass spectrometric characterization illustrated by ketoprofen and related benzophenone compounds’

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Research is to see what everybody else has seen and to think what nobody else has thought

Albert Szent-Györgyi

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Correction does much, but encouragement does more.

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The choice of a young person depends on his inclination, but also on the chance to meet a great teacher.

La scelta di un giovane dipende dalla sua inclinazione, ma anche dalla fortuna di incontrare un grande maestro

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"A prudent man should always follow in the footsteps of great men and imitate those who have been outstanding."

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Coming together is a beginning keeping together is progress working together is success.

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A real friend is one who walks in when the rest of the world walks out.

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We Cannot Direct the Wind, but We Can Adjust the Sails.

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A good golfer has the determination to win and the patience to wait for the breaks.

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All performance all the time, thanks for your help.

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"I get by with a little help from my friends."

The Beatles

Happy to work with you.

Special thanks for my family, my colleges and friends, your support makes this work achievement possible

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Abbreviations

Δ*m/z*	Mass accuracy
AOP	Advanced oxidation process
AOS	Active oxygen species
API	Active pharmaceutical ingredient
BP	Benzophenone
CF	Common fragment
CHX	Cyclohexane
CID	Collision-induced dissociation
D0	Ground state of the molecule
1D	Excited singlet state of the molecule
3D	Excited triplet state of the molecule
DC	Direct current
DOM	Dissolved organic matte
EI	Electron ionization
Ph. Eur	European Pharmacopeia
ESI	Electrospray ionization
ESI-	Negative electrospray ionization
ESI+	Positive electrospray ionization
eV	Electron volt
GC	Gas Chromatography
HPLC	High performance liquid chromatography
IC	Internal conversion
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IR	Infrared radiation
ISC	Intersystem crossing
LE-EI	Low energy of electron ionization
m/z	Mass per charge
$M^{+\bullet}$	Molecular ion (radical cation)
MS	Mass spectrometry
MS(/MS)	Tandem mass spectrometry

MSD	Mass selective detector
MSTFA	N-Methyl-N-trimethylsilyl trifluoroacetamide
NMR	Nuclear magnetic resonance
NSAID	Non-steroidal anti-inflammatory drug
ppm	Parts per million
PUVA	Psoralen and Ultraviolet light therapy
Q	Quadrupole
QC	Quality control
QTOF	Quadrupole time-of-flight
R	Alkyl moiety
RF	Radio frequency current
RT	Retention time
SPE	Solid phase extraction
SPP	Superficially porous particle
Std	
TMBE	t-Butyl methyl ether
TMCS	Chlorotrimethylsilane
UHPLC	Ultra high performance Liquid Chromatography
UVA	Ultra violet A light (λ = 315–400 nm)
UVB	Ultra violet B light (λ = 290–315 nm)
UVC	Ultra violet C light (λ = 180–290 nm)

1 Introduction and aim of the project

The importance of investigations on drug related compounds are on a notable rise during the recent years. These compounds may be considered in various fields, including early stage drug development, metabolism studies and environmental analyses.

One route for generation of such compounds is the interaction of photoirradiation with drug substances, which may lead to decreasing effective concentration of the drug substance and formation of photoproducts. The generated photoproducts may be associated with biological adverse effects as well as ecotoxicological aspects. Thus, pharmaceutical companies are requested by regulatory agencies to provide data on drug photochemical properties and sequential potential toxicological effects, which are demanding tasks, requiring animal testing and being time and cost consuming.

In this work, investigations on the photochemical stability of pharmaceutical substances as well as mass spectral characterization of drug related compounds will be discussed using ketoprofen and its related benzophenone impurities including those listed in the European Pharmacopeia (Ph. Eur. 9.0) as model compounds.

The goal of this project is to establish a modernized method for easy and fast photostability testing using an online reactor LC hyphenation and to develop a complementary approach that comprises recent mass spectrometric techniques and isotopic labelling for structural elucidation of drug related compounds.

The established photostability assay and accompanying mass spectrometric approach provide the ability to perform kinetic studies, detect the photoproducts of the active pharmaceutical ingredient (API) *in vitro* and assist the photosensitivity tests of API *in vivo* . To narrow the gap between drug photodegradation and formed compounds including intermediate products, fast identification has to be realized with the capability to isolate resulting substances in order to offer them as reference standards for further confirmation and phototoxicity tests.

Structural elucidation of drug related compounds including those previously formed in the context of the work and defined as photoproducts should be feasible by establishing an universal approach based on recent mass spectrometric instrumentation, isotopic labelling and fragment ion recognition. A scheme of the planned work stages is shown in figure 1 and figure 2.

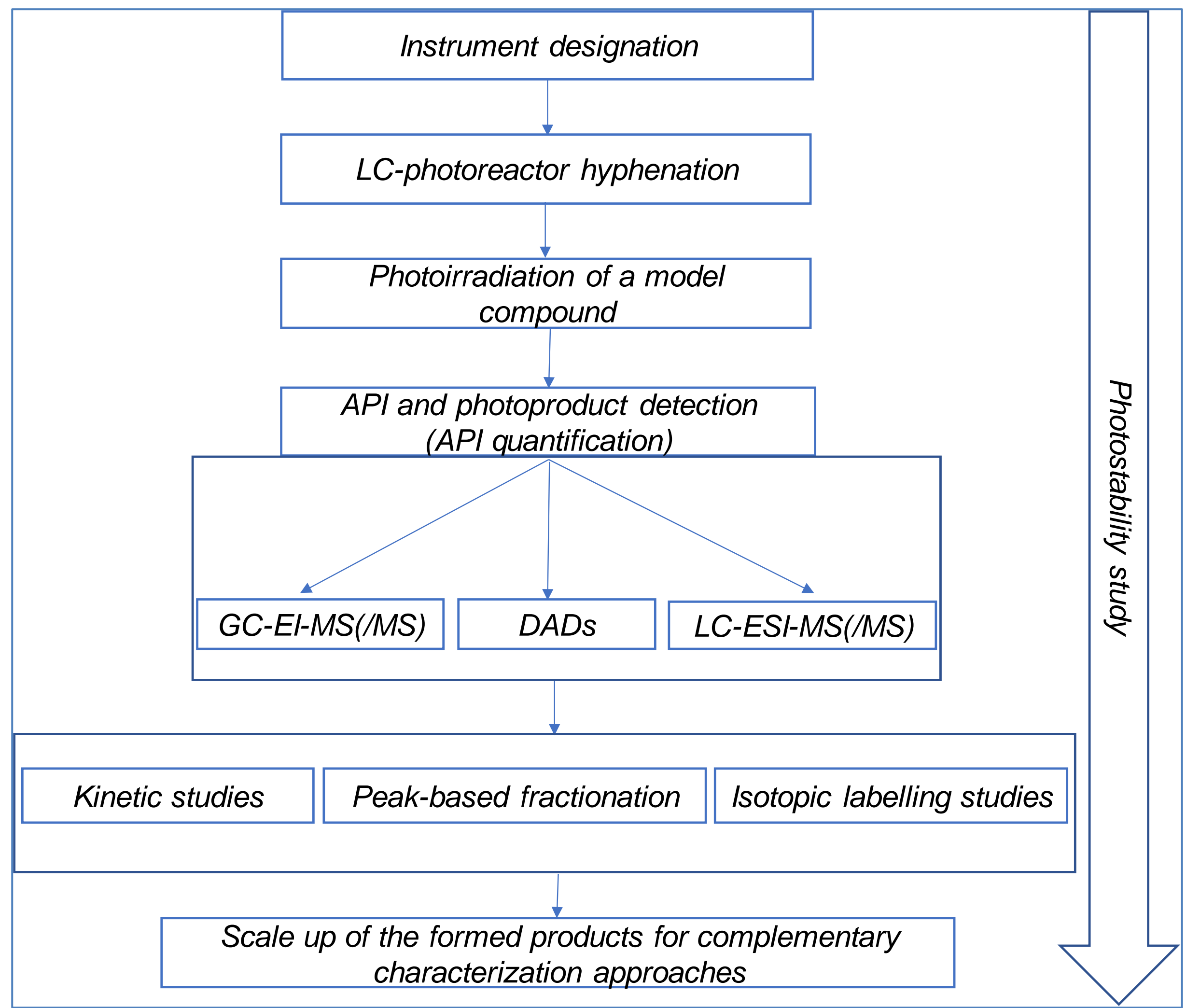


Figure 1 Scheme shows the stages of the planned work during the photostability study and isolation of the photoproducts

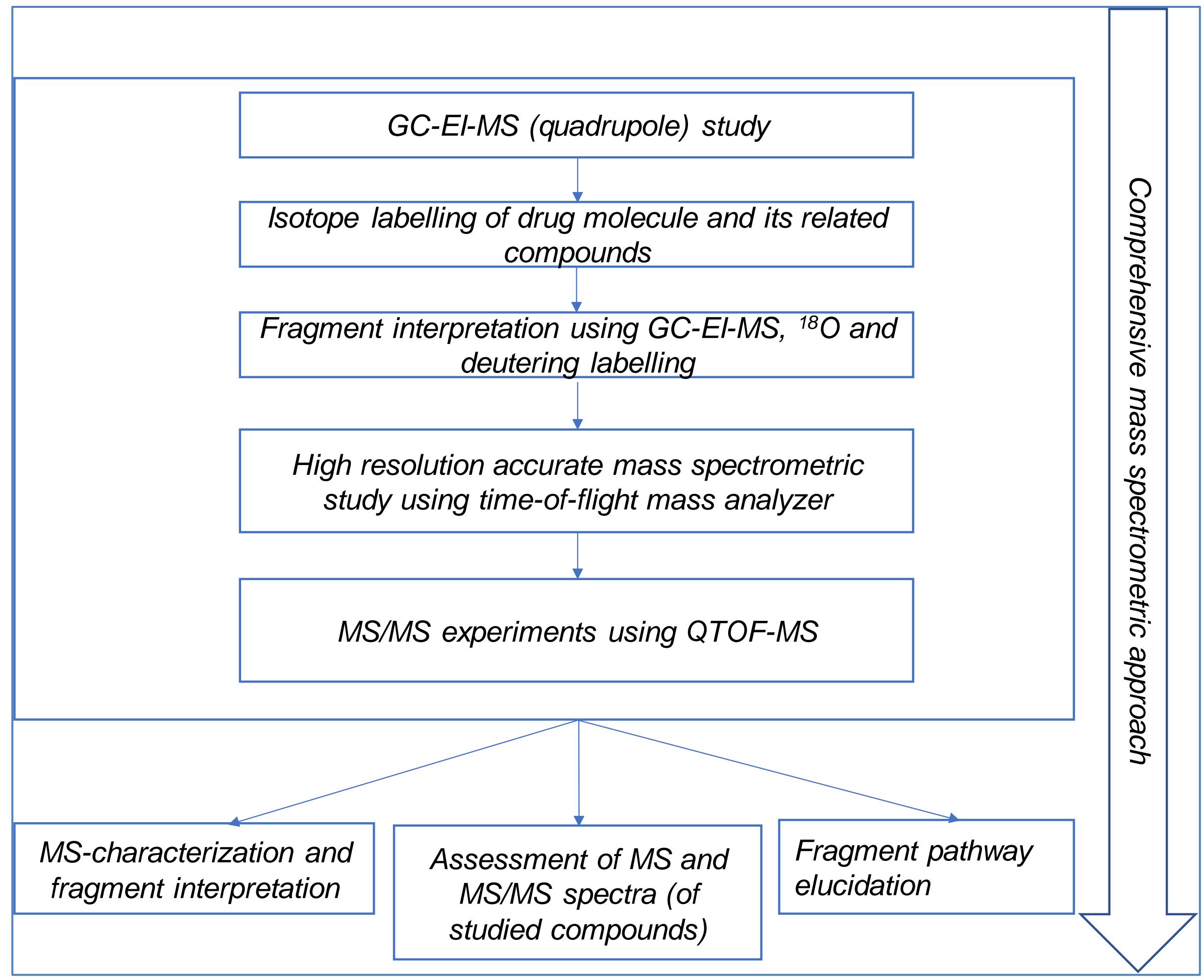


Figure 2 Scheme shows the stages of the planned work in the complementary mass spectrometric characterization

2 Theoretical Background

2.1 Photostability of drug substances

Photosynthesis in plants and vitamin D production in mammals demonstrate the essential role of sunlight for life on Earth. Nevertheless, sunlight may also lead to skin aging, cancer and photoderamtoses [1]. Related to this, photosensitivity is reported to make (8%) of unwanted side effects of drugs [2].

Thus, photochemical properties of drug substances and their associated phototoxicity received increasing attention and are reflected in many recently published studies [3-13]. The European Pharmacopeia lists more than 250 substances as photolabile [14]. Upon photodecomposition, the effective concentration of the API can be decreased and photoproducts may be generated which can lead to considerable amounts of adverse effects when administered. Furthermore, phototoxic products may also be generated in the body at the irradiation after drug administration [15, 16]. These resulting photoproducts may have an influence on the macromolecules that make up the normal cell and can lead to DNA damage, lipid peroxidation as well as reaction with proteins. As a final consequence cell damage may occur [17, 18].

Moreover, long irradiation of pharmaceuticals and their metabolites in surface water or during wastewater treatment results in worldwide problems with environment pollution [19, 20]. The transformation products may then be more toxic than their precursor compound [21, 22]. These problems have increased the concern about ecotoxicological aspects of photodegradation of drug substances.

Therefore, knowledge about photochemical properties of API is a core stone in the drug life cycle from the early stage of drug development, through the drug supply chain, including administration and metabolism to the wastewater treatment.

2.1.1 The photochemical process and photosensitizing reactions

The photochemical process relies on the absorption spectrum of the drug molecule as can be deduced from the basic law of photochemical absorption by Grotthus and Draper which states that no photochemical (or subsequent photobiological) reaction can occur unless electromagnetic radiation is absorbed [14]. Due to retention of shorter wavelengths by the ozone layer direct photodegradation via sunlight can occur only, if the substance absorbs radiation $\lambda > 290$ nm. However, degradation of photolabile compounds besides this limitation is also possible if artificial light sources such as low pressure mercury lamps are used.
The energy-level diagram in figure 3 outlines the photochemical process. After absorption of electromagnetic radiation the process starts with excitation of a drug molecule from its ground state (D_0) to the excited state (^1D) in which a valence electron moves to the first available outer shell corresponding to the first singlet state.

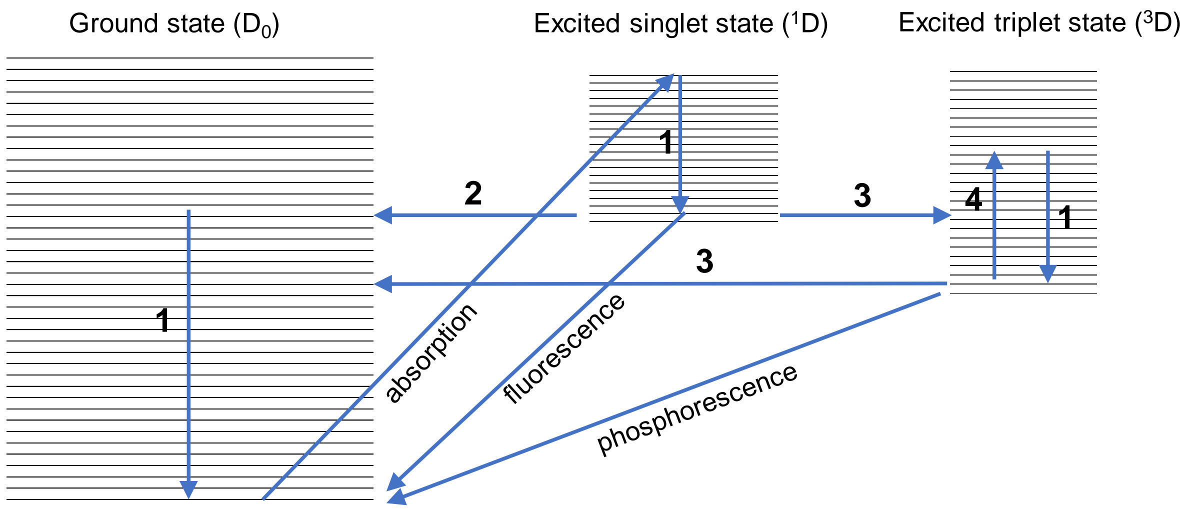


Figure 3 Energy levels of molecules showing transitions after irradiation absorption based on Tonnesen [14]: 1. vibrational relaxation; 2. internal conversion; 3. intersystem crossing; 4. thermal activation

As the excited state (^1D) is less stable than the ground state (D_0), usually rapid deactivation occurs. This deactivation comprises different competing physical processes and can be achieved via internal conversion (IC), which is a non-radiative transition between states of like multiplicity, or by photon emission (fluorescence). Photoionization (removal of an electron from a molecule) as well as vibrational relaxation may also be included.
The odds of an excited singlet state molecule interacting with neighbouring molecules is limited as the lifetime of the mentioned state is generally restricted in nanoseconds. However, intersystem crossing (ISC) may occur from the excited singlet state to an excited triplet state

(electron spin parallel). Due to its relatively longer lifetime the triplet state of the molecule has much higher likelihood to interact with other molecules. Furthermore, the excited molecule has the ability to form a complex with other species including the ground state of the drug molecule itself. Depending on the ability of the excited state to donate or accept electrons from ground state molecules, an electron transfer leading to a chemical reaction may occur as oxidative or reductive reaction. A pair of radical ions can be then produced that is able to react further with oxygen. As a final consequence superoxide anions can be formed. If the drug molecule is an olefin, for example, a dioxetane can be formed which further dissociates to yield ketones.
Generally, a transfer of reactivity to a species other than that absorbing the radiation (D_0) is known as photosensitization reaction, which follow one of two different reaction pathways: The first type reactions or free radical reactions (type 1) involve electron transfer, including simultaneous transfer of a proton corresponding to the transfer of a hydrogen atom (Equation 1 to Equation 3), whereas type 2 reactions include formation of excited singlet oxygen by energy transfer with spin conservation (Equation 5). These processes have a competitive fashion and their distribution depends on sensitizer, solvent, substrate, oxygen concentration, and affinity of sensitizer and substrate.

Free radical formation	$^3D + AH \longrightarrow D_0 + A^{\bullet} + H^+$	1
Or	$^3D + AH \longrightarrow DH + A^{\bullet}$	2
Or	$^3D + AH \longrightarrow D_0 + A^{-} + H^+$	3
(Type 1 photo-oxidation)	$A^{\bullet}\ (A^{\bullet -}) + O_2 \longrightarrow AO_2^{\bullet}\ (\text{or } AO_2^{\bullet -})$	4
Energy Transfer to oxygen:	$^3D + {}^3O_2 \longrightarrow D_0 + {}^1O_2$	5
Oxidation by singlet oxygen (type 2 photo-oxidation)	$^1O_2 + AH \longrightarrow AOOH$	6

The radicals formed from the type 1 mechanism may undergo further reactions. While these reactions in absence of oxygen (non-photodynamic reactions) means recombination, dimerization or disproportionation, molecular oxygen (when it is available in sufficient concentration) is rapidly added to the radical (photodynamic reactions). The formed peroxy radical will then stabilize itself by proton abstraction from neighboring molecules. This mechanism of interaction can be performed as chain reaction, which has the most damaging potential on biological systems [23].
In addition to the free radicals or singlet oxygen formation in type 1 and type 2 reactions, several short-lived species such as hydroxy radicals and superoxide radicals are generated whom together with singlet oxygen are termed reactive oxygen species and play a critical role in photosensitization reactions.

2.1.2 Photodermatoses

Sunlight specially its UV component can induce or exacerbate a wide variety of skin disorders known as photodermatoses [24, 25]. They may be divided into primary and secondary types as illustrated in figure 4. Primary photodermatoses are induced by photosensitizing substances and for their occurrence electromagnetic radiation is considered as critical pathogenic factor, whereas secondary photodermatoses have another genesis in addition to sunlight induction (altogether) and they are expressed as systemic diseases, metabolic disorders, or disorders of DNA repair [26].

Primary photodermatoses can be further subdivided into primary idiopathic photodermatoses, which occur with unknown etiology, and photosensitivity reactions associated with known photosensitizers. Comparably, Photosensitivity reactions with known photosensitizers can be further divided into photoallergic and phototoxic reactions (figure 4).

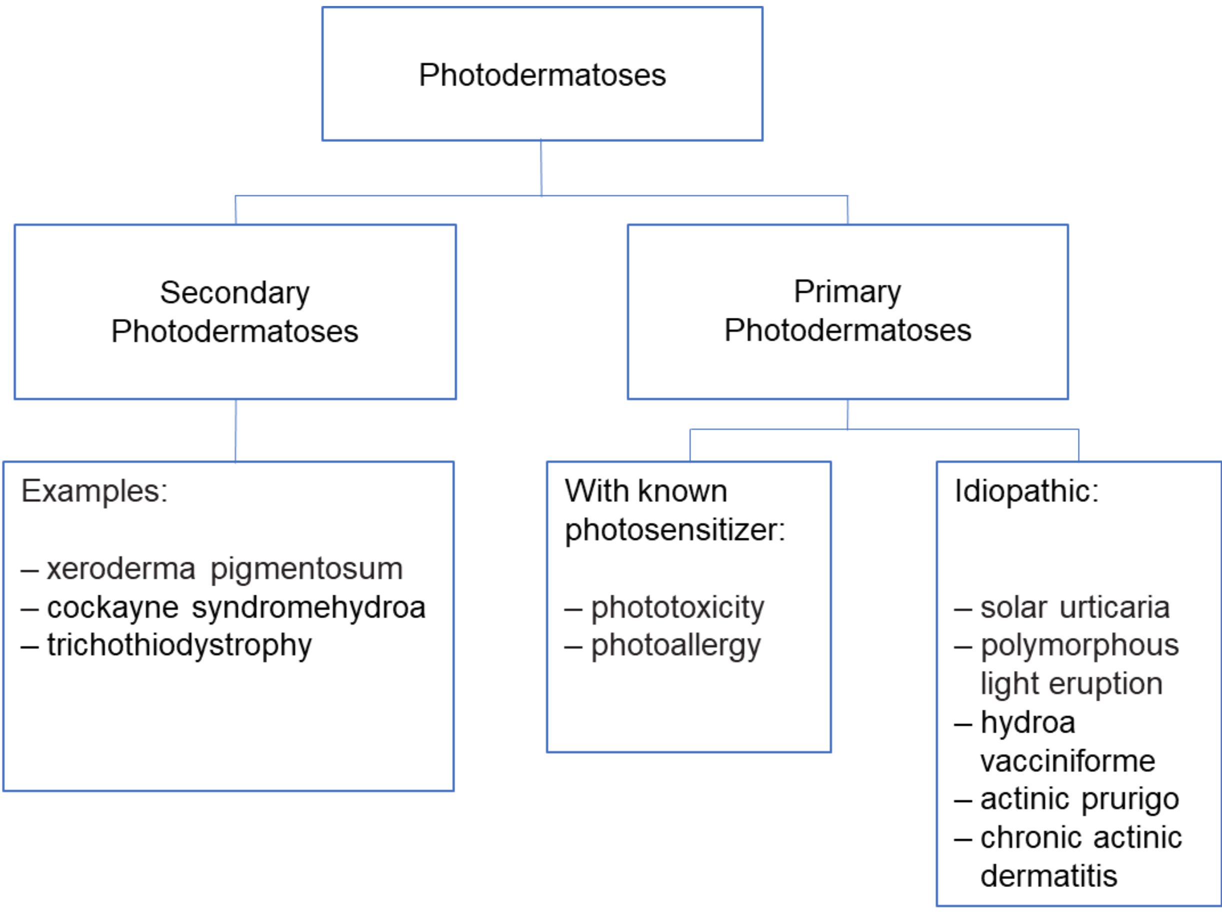


Figure 4 Classification of photodermatoses based on Lehmann and Schwarz [26]

2.1.3 Photosensitivity induced by drug substances

A photosensitive reaction induced by chemicals is illustrated in figure 5. It is defined as development of cutaneous disease due to the interaction of a given chemical agent (photosensitizer) with sunlight [27]. This photosensitivity type can be divided in several categories:

- Phototoxic reaction: an acute toxic response that occurs after exposure of the skin to new chemical entities and subsequent exposure to light/UV radiation. It can also be induced by skin irradiation after systemic administration of a chemical. When the phototoxic reactions are induced by chemicals within 0–72 hours after exposure to light/UV radiation they are generally named as photoirritation [28].
- In contrast to phototoxic reactions, photoallergy is an acquired immunological reactivity, which requires multiple exposures of photosensitizer and light /UV radiation. An induction period of one or two weeks is required before skin reactivity can be demonstrated [28].
- Photogenotoxicity is a genotoxic response, which is observed after exposure to a chemical (a photosensitizer) and a (non)-genotoxic dose of light/UV resulting in formation of genotoxic species.
- Photocarcinogenicity represents a possible transformation, caused by visible or UV radiation of a compound resulting in formation of carcinogenic species.

Among photosensitivity reactions, phototoxicity is reported to be the most common adverse effect and can appear in any patient after enough irradiation doses and sufficient concentration of the photosensitizer. The clinical manifestations of phototoxicity are similar to sunburn and include erythema, edema and severe pigmentation.

In contrast to phototoxicity, photoallergy is relatively uncommon as it occurs provided that a specific sensitization has been acquired. However, photoallergy requires lower doses of both photosensitizer and radiation energy than phototoxicity. The acute clinical expressions are restricted to light exposed area of the skin and there are signs of allergic contact dermatitis with erythemas, papular vesicles, and rarely blisters [26].

Two major pathways can be considered by which photosensitizing drugs may produce a phototoxic skin response involving either the excited triplet state or free radical entities. Most of the macromolecules that make up the normal cell are susceptible to oxidative degradation of the type occurring in photodynamic reactions. Lipids in the cell membranes can react at their unsaturated sites through both free radical (type 1) and singlet oxygen (type 2) mediated processes. As final consequences, these result in splitting of the lipid molecule into shorter fatty acids and a disruption of the cellular membrane. Proteins are oxidized specially via their amino acid side chains. The two most susceptible amino acids are histidine and tryptophan

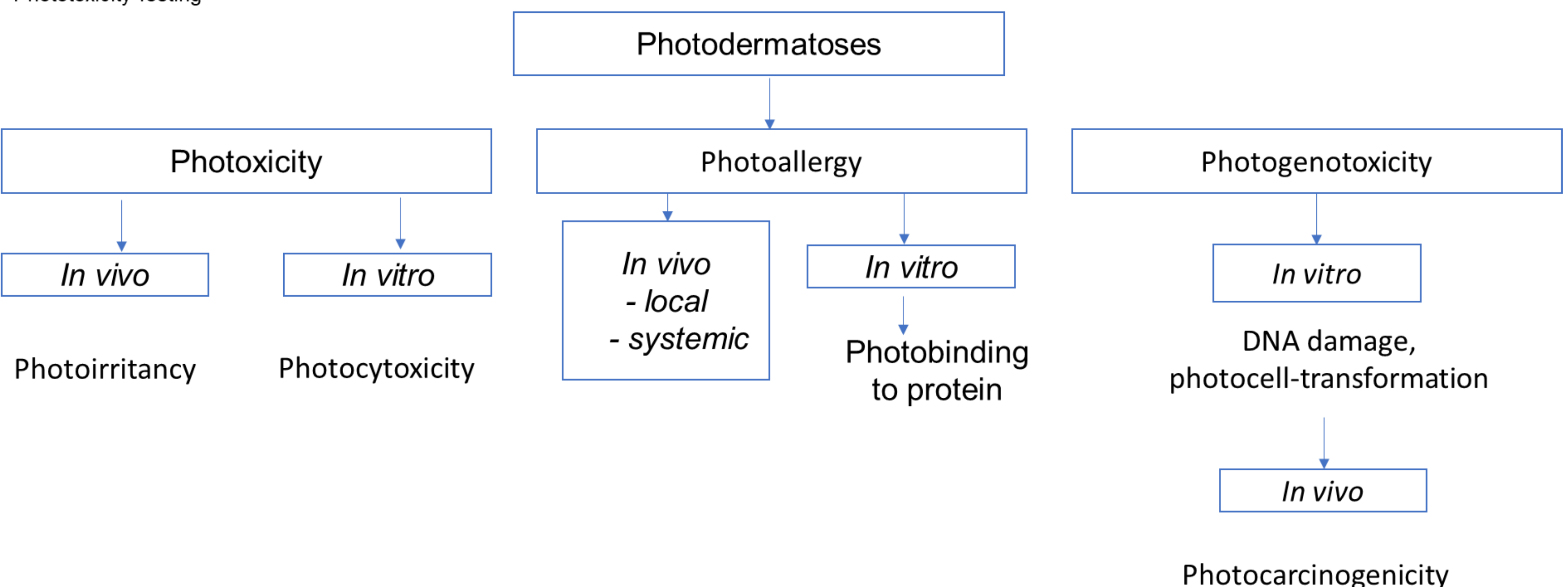
The imidazole group of histidine is a specific substrate for singlet oxygen (type 2) while the indole group of tryptophan experiences both type 1 and type 2 reactions.

UVA is able to penetrate further and has the potential to cause photoreactions deeper in the skin than shortwave limit of sunlight (UVB), which can only reach the viable layers of the epidermis [29]. In spite of its relatively weak absorption by proteins and nucleic acids, epidemiological studies suggest UVA to play an important role in skin cancers. This is caused via photosensitisers such as pharmaceuticals which absorb photons and generate excited molecules [30]. Those molecules are then able to react with DNA and proteins either through an electron transfer (type 1 mechanism) or by reaction between DNA and singlet-oxygen to form 8-oxoguanine (type 2 mechanism). Type 2 mechanism may also be initiated through formation of a superoxide anion radical which undergoes disproportionation reaction, yielding hydrogen peroxide and reactive oxygen species.

Drugs, which are reported frequently to give rise to some form of photosensitivity responses show a wide range of pharmacological activities and different chemical structures. The list of these drugs includes sulfonamides, tetracyclines, quinolones, sulfonylureas, thiazides, phenothiazines, furocoumarins and nonsteroidal anti-inflammatory drugs (NSAIDs). Photosensitizing chemicals usually have a low molecular weight (200 to 500 Daltons) and a planar, tricyclic, or polycyclic configuration, often with heteroatoms in their structures enabling resonance stabilisation. All of them absorb ultraviolet and/or visible radiation, regarded as an essential characteristic for a chemical to be considered as a photosensitizer.

Photodegradation and free radical formation have on the other hand numerous advantages. Besides the beneficial conversion of 7-dehydrocholesterol to vitamin D3 in skin via UV radiation, PUVA therapy, a combination of psoralens and UVA light, is well established in the treatment of psoriasis. Light-activated liposomes or prodrugs may also be used as new drug delivery systems [31].

Figure 5 Photobiological effects of chemicals based on the second ECVAM Workshop of Phototoxicity Testing



2.1.4 Ecotoxicological aspects of drug photodegradation

Pharmaceuticals as well as other chemicals can reach the environment by various routes such as excretion, wastewater effluents, industrial waste from manufacturing, or agriculture [32, 33]. Many drug substances have been detected in natural water including lakes and rivers, high concentrations of some of these drugs have been frequently identified in influent and effluent of urban wastewater as well as in hospital effluents [34, 35]. Moreover, after drug administration the formed metabolites are excreted and should be considered as environmental pollutants. Conventional wastewater treatment which consists of two parts, physio-chemical (primary treatment) and biological (secondary treatment), is not often effective enough to remove these pharmaceuticals [36].

Here, positive and negative environmental aspects can be seen regarding the interaction of drug substances with visible and UV radiation. Environmental pollution problems caused by the emission of certain pharmaceuticals and their metabolites into the water environment were recognized [37-39]. The resulting transformation products may pose then greater risks than their parent compounds [40].

These problems have increased the concern about ecotoxicological effects on aquatic organisms and potential for the appearance of drug-resistant bacteria or viruses in the water environment [41-45]. Many pharmaceuticals were previously known to undergo direct sunlight photolysis and transformation in surface waters; however, the synergistic increase in toxicity caused by photo-by-products cannot be ignored. The sunlight can also alter dissolved organic matter (DOM) either directly leading to free radical formation or indirectly via residues of photosensitizes including pharmaceuticals [46].

Residues of azole fungicides, for example, can undergo photolysis under UV irradiation in wastewater. Thereby, products may cause toxic effects on aquatic organisms such as algae and fish [47-49]. Ibuprofen, in its turn, was found to cause histopathological changes (microscopic anatomical changes in diseased tissue) in the gills and kidneys of fish after long UV exposure [50]. However, modern wastewater treatment methods among others include advanced oxidation processes (AOPs) which are based on formation of active oxygen species (AOS). AOPs can be performed with or without radiation. Solar radiation itself may be applied during such treatment processes with pharmaceuticals absorbing irradiation over 290 nm. Furthermore direct photodegradation using UVC-radiation of other compounds can be performed and was reported by several publications [51-53]. Some wastewater treatment methods realized the use of a combination of UV radiation and hydrogen peroxide (H_2O_2). In this case hydroxy radicals are produced, which substantially increase the degradation rate of targeted compounds in comparison to UV alone, thereby enhancing the removal of lots of pharmaceuticals although some of them can resist such a treatment [54]. Another example of

irradiation usage in modern wastewater treatment is the combination of Fenton process (Eq. 7 and 10) with UV, which regenerates Fe^{2+} from the formed aqua-complexes during the Fenton process (Eq. 9). Furthermore, the yield of hydroxy radicals is increased through direct H_2O_2 irradiation (Eq. 10) [55].

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{-}HO^{\bullet} \qquad 7$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + HO_2^{\bullet} + H^{+} \qquad 8$$

$$[(Fe\ (H_2O)_5(OH)]^{2+} + h\nu \longrightarrow Fe^{+2} + H^{+} + HO^{\bullet} \qquad 9$$

$$H_2O_2 + h\nu \longrightarrow 2HO^{\bullet} \qquad 10$$

As mentioned previously, elimination of drug molecules via photoirradiation may result in compounds that can be more toxic than origin compounds. Therefore, further investigation on the resulting photoproducts via sunlight or after some modern waste water treatments should be considered in order to elucidate their structures and possible phototoxicity.

2.1.5 Analytical photodegradation studies of drug substances

The application of the ICH Harmonized Tripartite Guideline on Photostability Testing of New Drug Substances and Products (ICH Q1B) started in Europe in 1996 and in the US and Japan in 1997. Investigations on photochemical stability of pharmaceutical substances are required for new drug licensing since 1998 in the US, the European Union, Japan, and Canada [56].

The (ICH Q1B) guideline divides the photostability tests in forced degradation testing and confirmatory testing. According to the guideline, confirmatory studies should provide the information necessary for handling, packaging, and labelling. For confirmatory testing the overall illumination of the sample is defined to be not less than 1.2 million lux hours and integrated near ultraviolet energy should not be less than 200 watt Wh/m^2.

The purpose of forced degradation testing studies is to evaluate the overall photosensitivity of the material for method development purposes and/or photodegradation pathway elucidation. The design of forced degradation experiments on drug substances is left to the applicant's discretion.

It should be noted that many drug substances are exposed to light for long durations such as infusion solutions which are often stored in transparent bottles. This issue may not be perfectly covered by Q1B guideline. The guideline may also not cover all steps in the drug supply chain, for example dilution and reconstitution before administration. Therefore, a second exposure time for confirmatory studies with 6 million lux hours instead of 1.2 million lux hours was proposed by Baertschi et al. [57].

In order to detect the photoproducts long exposure periods in photostability chambers and several preparative steps during the entire study are usually required. This may affect the whole result especially the identification of the photoproducts and intermediate products as well as the investigation of the chemical background of the initiated phototoxic compounds. Furthermore, Q1B guideline covers neither photoproducts generated *in vivo* under sunlight exposure of the skin nor those generated in surface water.

For the separation of resulting products, chromatographic methods are commonly used in the context of photodegradation of pharmaceuticals. High performance liquid chromatography (HPLC) is often the method of choice, whereas gas chromatography (GC) may also be used after transformation into volatile status for limited volatile substances.

During such studies, a variety of detectors is implemented after appropriate separation techniques. Conventional UV/VIS detection shows its limitation regarding structure elucidation of the resulting photoproducts and is therefore, suitable for quantitative analysis or for identification by retention time only. Ultra-high-performance liquid chromatography coupled with diode array detector (DAD) can improve the detection and shorten the time required for separation on photodegradation studies than HPLC-DAD and enable measuring of a wide

variety of absorbing substances present in the samples. Mass spectrometry (MS) demonstrates its potential as a powerful tool for photoproduct detection as well as structural elucidation. NMR and IR are also recommended. However, they are both much less sensitive than MS. In case of MS techniques, matrix effect problems should be considered especially with environmental samples and electron spray ionization sources. Such obstacles may be overcome by appropriate sample preparation [58, 59]. According to a recent study by Trawiński and Skibiński on photodegradation of psychotropic drugs, chromatographic and mass spectrometric methods are the most often applied (figure 6) [22]. For faster separation and sequential detection, photochemical derivatization can be applied as the simplest derivatization method in liquid chromatography. However, it is used least often and generally unfamiliar to analytical chemists. This can attribute to the operating problems emerged in the early years, which limit the routine use of such instrumentation, including among others leaking and reactor coil heating [60, 61].

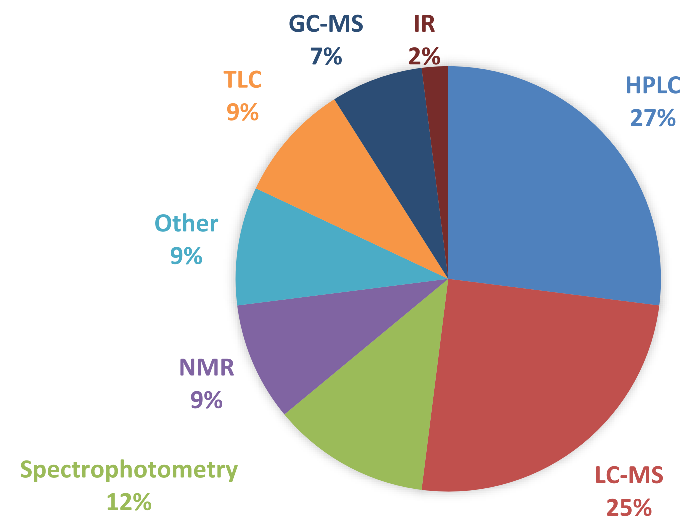


Figure 6 Analytical methods used in photodegradation studies on psychotropic pharmaceuticals [22]

2.2 The role of mass spectrometry in the identification of unknown drug related compounds

Mass spectrometry plays an important role as one of the most edifying techniques used to identify pharmaceutical substances and enhance the detection of both process and degradation-related compounds [62-65]. In contrast to the active pharmaceutical ingredient (API), impurities and transformation products provide no benefits to the patients and convey only risks [65-67]. Hyphenation with appropriate chromatographic separation technologies and the recent development in software and instrumentation have improved the potential of MS for structural characterization of unknow analytes. However, confidence in these MS-identifications varies between studies and substances, since it is not always possible or even meaningful to synthesize each substance or confirm them via other methods (e.g., nuclear magnetic resonance) [68]. In order to reduce time consumed in identification of unknown substances, fragment ions and isotopic labelling accompanied with different derivatization methods may help to further substantiate structural proposals. Thus, assessing MS and MS/MS spectra libraries and associated interpretation of the emerged fragment ions for drug molecules and their related compounds are of core importance.

Through this work, a universal approach is introduced to identify the fragment ions of ketoprofen and its related benzophenone derivatives as model compounds including those detected as photoproducts.

2.2.1 The utilized mass spectrometric techniques

Single quadrupole mass spectrometry (Q) and Quadrupole-Time-of-Flight mass spectrometry (QTOF-MS) were used for the identification of the analytes. Single quadrupole provides several advantages including its relatively low cost and small size. However, it demonstrates limited capability in terms of mass range and resolving power [69]. Further inspection on the analytes was achieved by utilizing QTOF-MS which is composed of a quadrupole mass filter (Q1) and collision cell (hexapole) coupled with a time of flight mass analyzer (TOF) that is geometrically aligned in the orthogonal configuration with respect to quadrupole mass filter and the collision cell as depicted in figure 7.

QTOF-MS provides both one stage MS and (tandem) MS/MS spectra. During MS mode quadrupole Q1 as well as hexapole are operated in radio frequency (RF) only mode. This means that the direct current component (DC) of the applied voltages is removed. Thus, all ions are focused around the central axis and allowed to be transmitted to the TOF.

The separation methodology used in TOF is relatively straightforward and simple. The ions are subjected to an accelerated voltage and obtain ideally the same kinetic energy before they draft or fly down an evacuated tube of a set length. The speed at which the ions fly down the tube correlates with their mass and charge. Provided that all ions start their flight at the same time, the lighter ions will arrive earlier at the detector than the heavier ones. However, due to the distribution of the acquired kinetic energies in the accelerating voltage, not all ions of the same *m/z* values reach the detector simultaneously. Therefore, linear TOF instrumentation may influence the resolving power of the analyzer especially for ions of high masses. This drawback is solved to a large extent through the use of a reflectron device mounted at the top of the TOF tower as displayed in figure 7. The reflectron acts as an ion mirror that compensates for the kinetic energy differences between the isobaric ions. Therefore, ions with higher kinetic energy will penetrate deeper into the ion mirror. Moreover, the reflectron doubles the flight path and improves the resolving power by increasing the time interval between the ions of different *m/z*. TOF analyzer is favorable over quadrupole in qualitative analysis as it may provide higher resolution and accurate mass measurements.

The QTOF-MS can also be used in MS/MS mode which is based on the separation of the ions with certain *m/z* values (precursor ions) by the quadrupole mass filter (Q1) followed by collision-induced dissociation (CID) in the collision cell. Here, the precursor ions undergo collision with a stream of inert gas (e.g., nitrogen) of a specific energy level in order to produce product ions which are finally separated by the TOF analyzer.

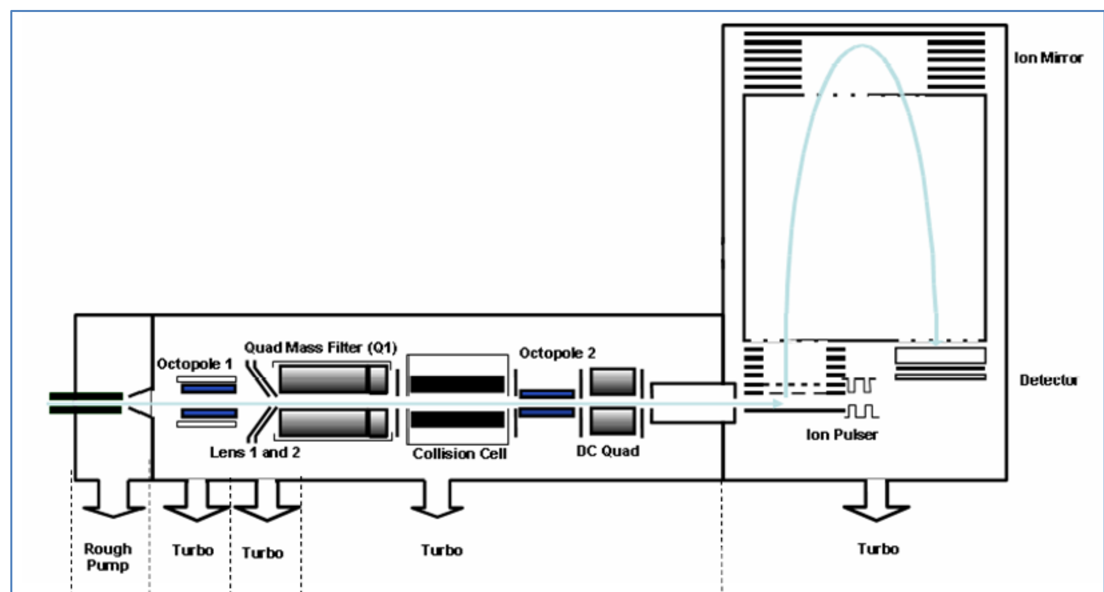


Figure 7 Schematic design of Q-TOF mass analyzer (courtesy of Agilent Technologies)

Different ionization techniques have been used depending on the chromatographic hyphenation. Electrospray ionization (ESI) was applied in combination with LC, whereas electron ionization (EI) was realized after gas chromatographic separation. EI is usually performed at 70 eV since this energy gives the highest ion yield (figure 8). In contrast to EI, it is common in ESI to vary the capillary voltage (typically 2 to 6 kV).

In addition to the conventional EI at 70 eV for classical fragment libraries, lower energies (LE) were also applied using an Agilent 7250 GC-QTOF-MS. This instrument is capable of LE-EI (12-20 eV) measurements, due to its optimized source configurations, lenses, magnets and tuning. In a recent study, Polet et al. [70] reported that an ionization energy of 15-18 eV was optimal in terms of sensitivity of the analyses derived by reduced background.

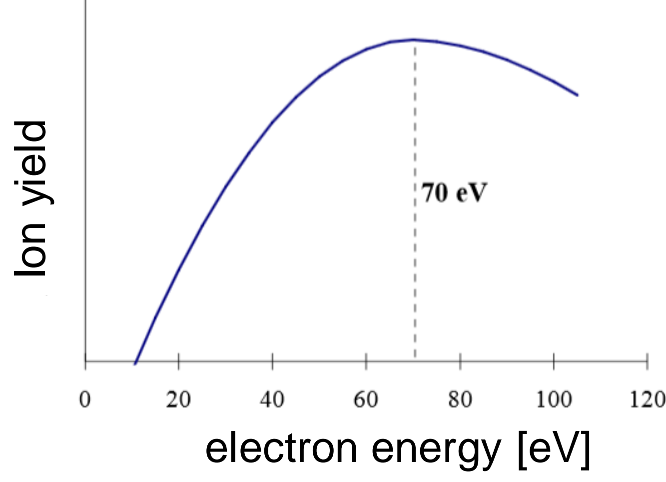


Figure 8 Diagram showing the ionization efficiency (ion yield) in EI as a function of energy, adapted from Budzikiewicz H et.al.[71]

2.2.2 Ketoprofen

Ketoprofen, 2-(3-benzoylphenyl) propanoic acid, is a non-steroidal anti-inflammatory drug (NSAID) widely used in the management and treatment of inflammatory diseases, pain relief, and chronic arthritis and well known to act as a photosensitizer which has been proved *in vitro* as well as *in vivo* [72, 73]. Photoirradiation of ketoprofen was used as model reaction in this project for photostability studying. Ketoprofen is a benzophenone derivative, which is part of some other nonsteroidal anti-inflammatory drugs (NSAIDs) as well as the antiarrhythmic drug amiodarone and lipid lowering agents such as fenofibrate. All of these drugs cause photosensitive skin reactions as adverse effects [74].

2.2.3 Confidence levels for mass spectrometric identifications

Confidence levels for mass spectrometric identifications are required in order to overcome the lack of reference standards and to clarify the variations in confidence derived from different studies. According to the Metabolomics Standards Initiative (MSI), four levels of metabolite identifications can be found in published metabolomics literature [75], which include: 1. identified compounds; 2. putatively annotated compounds (e.g. without chemical reference standards, based upon spectral similarity with public/commercial spectral libraries and/or physiochemical properties); 3. putatively characterized compound classes (e.g. based upon spectral similarity with known compounds of a chemical class); 4. unknown compounds, although unidentified or unclassified these metabolites can be differentiated and quantified based upon spectral data. Jeon et al. suggested a framework to clarify these confidence levels for specific investigations on biotransformation pathways of biocides and pharmaceuticals in freshwater crustaceans [76]. As many cases were found to fit “in between” these proposed levels, additional levels were adapted by Schymanski et al. [68]. These levels are illustrated in figure 9 and can be described as follows:

- Confirmed structure (level 1) where measurement of a reference standard has been used to confirm the proposed structure by MS, MS/MS and retention time matching (RT). If possible, an orthogonal method should also be realized.
- Probable structure (Level 2) where an exact structure was proposed using different evidence and can be further subdivided into library (level 2a) and diagnostic (level 2b). Level 2a involves matching literature or library spectrum data where the spectrum-structure match is unambiguous. Level 2b represents the case where no other structure fits experimental data. Therefore, diagnostic MS/MS fragments and/or ionization behaviors, parent compound information and the experimental context can compensate for the absence of reference standard or literature information. This break down into (a) and (b) is useful for research purposes, whereas for practical reporting probable structure (level 2) may often be sufficient to indicate the confidence in the candidate.
- Tentative or putative candidate(s) (Level 3) represent(s) the case where evidence exists for possible structure(s), but there is insufficient information for one exact structure only. For example, the cases where the position of certain substituents could not be clarified.
- Unequivocal molecular formula (Level 4) which indicates that a formula can unambiguously assigned using the spectral information. However, insufficient evidence exists to propose possible structure.

- Exact mass (level 5) where the exact mass (*m/z)* can be measured in a sample, but neither a structure nor a formula can be assigned due to lack of information. Level 5 can be beneficial for specific investigations where screening or nontarget methods allow tracing of certain masses. However, level 5 should be limited to a few masses of specific interest.

As can be deduced from this classification, structure identification is feasible with levels 1 and 2, whereas levels 3 to 5 can be considered for assignment of substance class, formula or mass of interest.

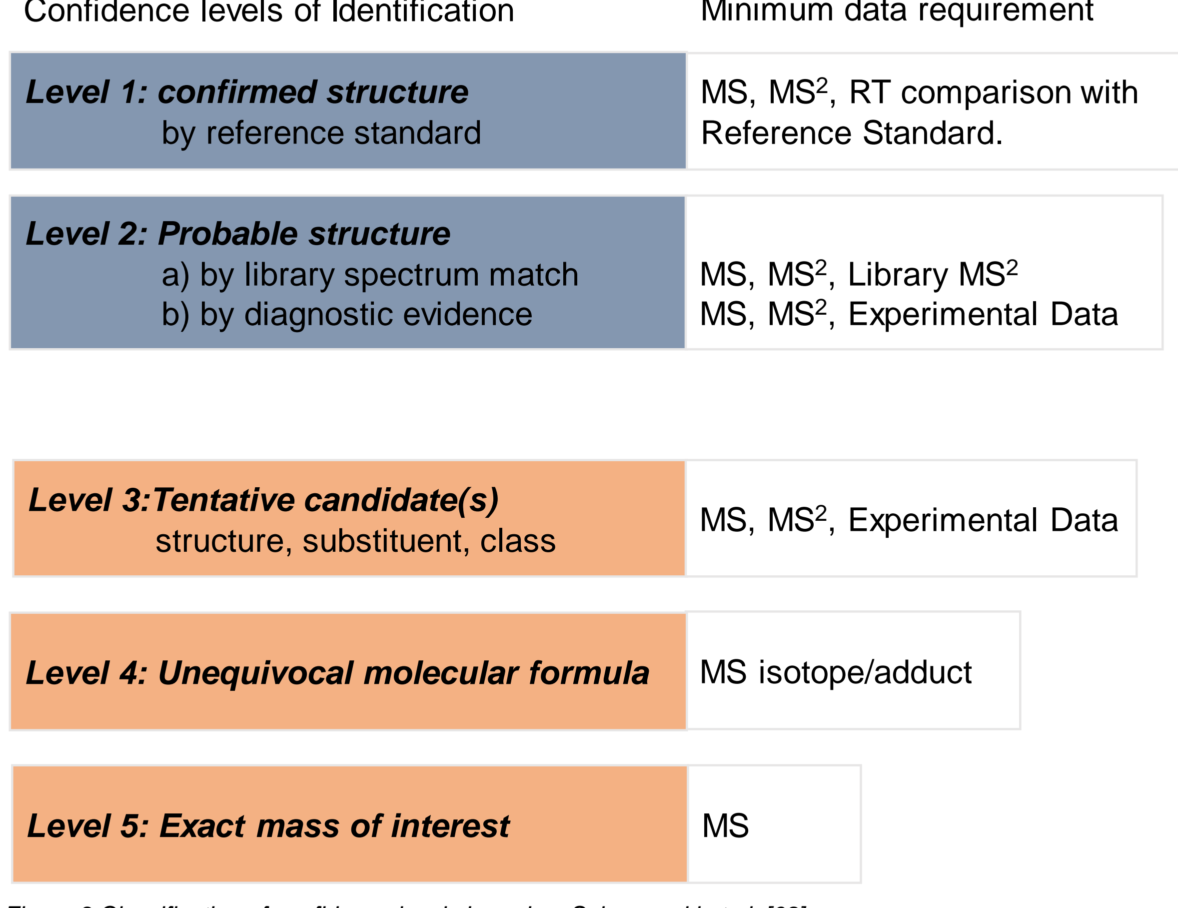


Figure 9 Classification of confidence levels based on Schymanski et al. [68]

3 Project highlights

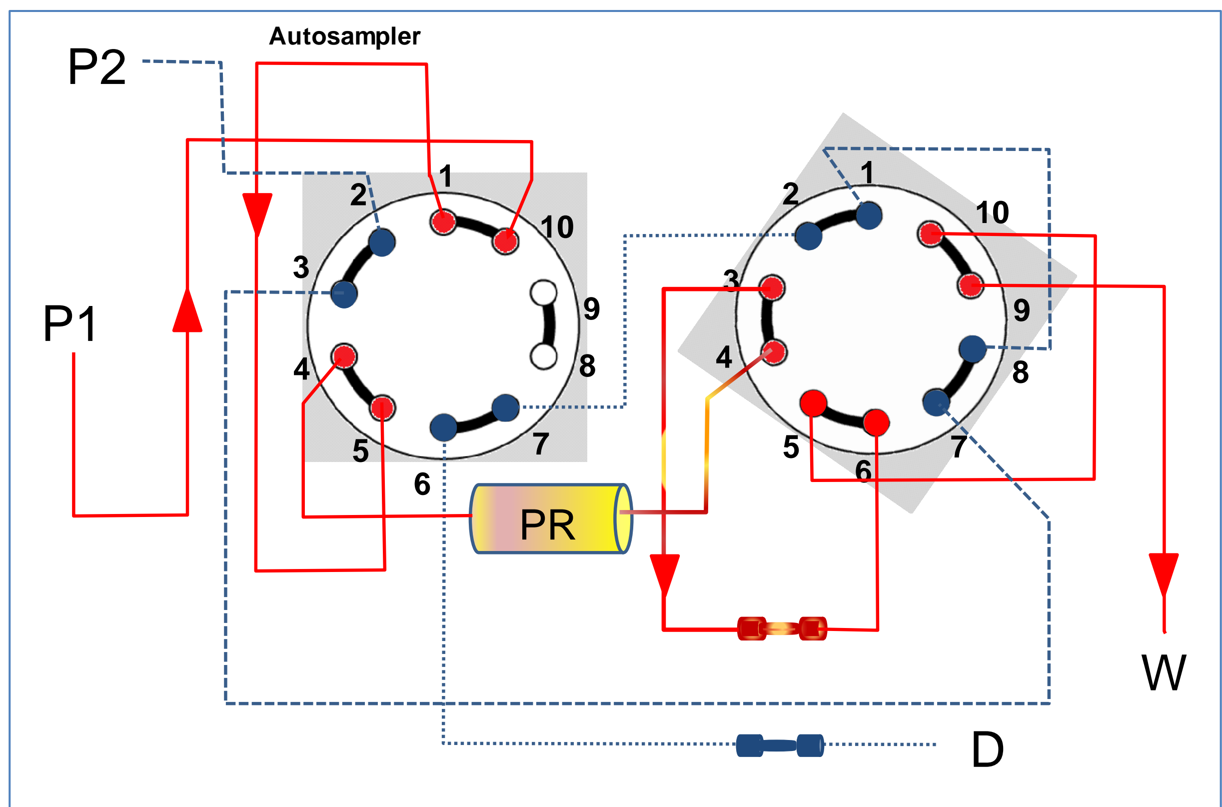
A comprehensive strategy for both photostability testing and structural characterization of photoproducts as well as other drug impurities was established utilizing ketoprofen as a model compound. Photoproducts were generated by a tailored online reactor HPLC hyphenation and identified by mass spectrometry (manuscript 1). Subsequent fragmentation elucidation of these photoproducts and related compounds was achieved using isotopic labelling and recent mass spectrometric instrumentation (manuscript 2).

The online reactor HPLC hyphenation is equipped with an exchangeable pen light source and online-solid phase extraction (SPE). This instrumental design includes an integrated flush pump used to load the drug substance into the photoreactor where irradiation takes place.

A schematic display of the flow configuration is shown in figure 10. Following irradiation, the remaining drug substance and its photoproducts are trapped on SPE cartridge. Subsequently the quaternary pump is used to backflush the analytes out of the cartridge onto the analytical column.

Separation of the analytes can be accomplished on superficially porous particle (SPP) columns. The use of such columns offers several advantages over the conventional fully porous columns especially during the method development phase. As the solid core does not participate in the diffusion (which only occurs on the porous outer shell), the efficiency of the separation is increased compared to the porous columns of the same size. This is not achieved at the expense of the resulted backpressure, which remains at relatively low limits. As a consequence, the flow rate can be increased or another system setup may then be realized using the photoreactor directly in front of the separation column.

As can be deduced from the schematic display (Figure 10), the analytes with their accompanied photoproducts can be identified using different detectors such as DAD or mass spectrometry. Possible offline/online hyphenation with different mass spectrometric techniques can offer both optimum detection as well as structural characterization of the resulting substances. Peak-based fractionation and scale up processes may also be realized in order to generate reference material for further structural confirmation with NMR or toxicity studies. Using this experimental design, kinetic studies of ketoprofen were easily performed with qualitative and quantitative perspectives combined into one experimental system and several photoproducts were additionally detected. Both methylation and trimethylsilylation after peak based fractionation were used for EI-MS characterization of the analytes.



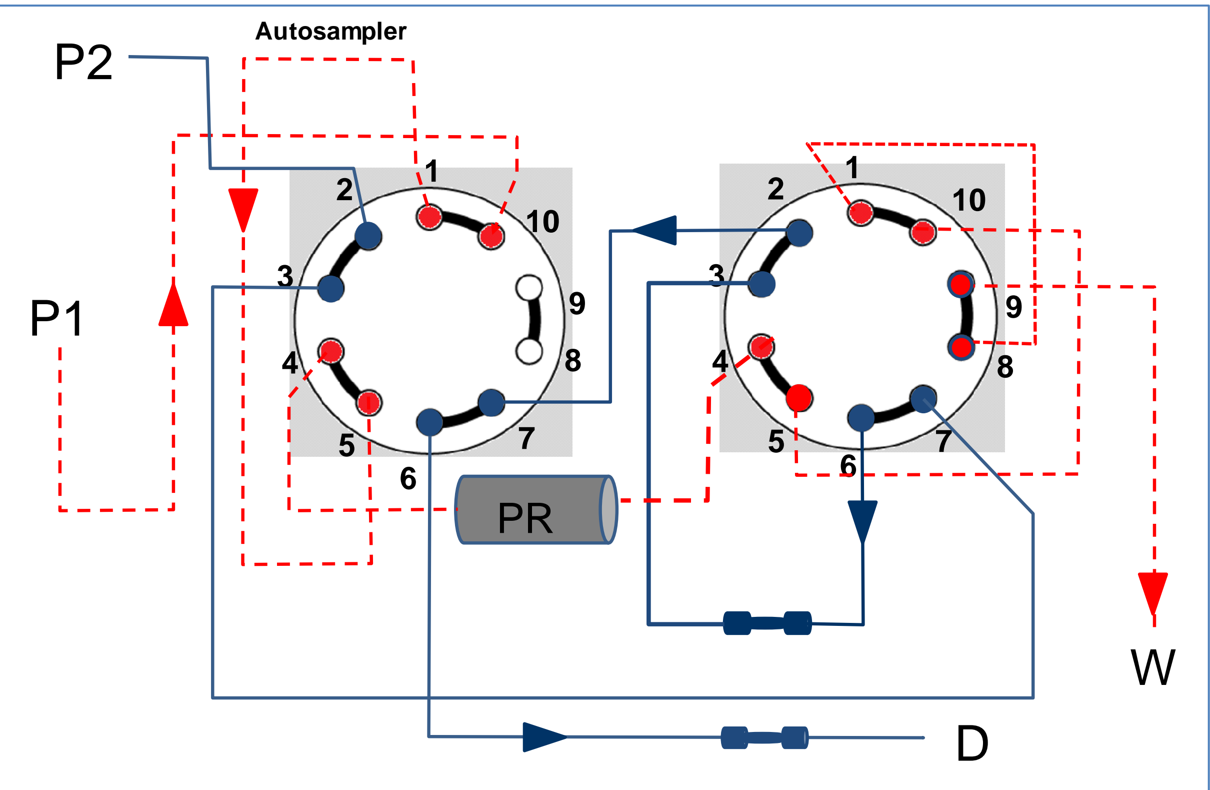


Figure 10 Schematic display of flow configuration of the online photostability experiments: PR: photoreactor; W: waste; P1: FlexCube pump; P2: quaternary pump; SPE: solid phase extraction; D: detector.(Irradiation and trapping: upper, elution and analytical separation: lower)

Substantiation was achieved by high resolution accurate mass spectrometry (QTOF-MS). Further mass spectrometric investigation was obtained by isotopic labelling, as deuterated photoproducts were derived via photolysis experiments.

1-(3-benzoylphenyl) ethenone (BP05, keto 2), 3-(1-hydroxyethyl) benzophenone (BP06, keto 1) and ethylbenzophenone (BP08, keto 3) were assigned as photproducts with the highest level of confidence (level 1) according to Schymanski et al [68]. This assignment allowed for generation of site specific isotopes of the respective compounds. In addition to structure confirmation, the resulted photoproducts can be used as internal standard for future analysis or for toxicological tests. Two other photoproducts were tentatively assigned to hydroxyethylphenyl-hydroxyphenyl-methanol (keto 4) and vinyl-benzophenone (keto 5) based on their mass spectral data . The both are classified as level 3 according to Schymanski et al. Fragment explanation of ketoprofen and its derived photoproducts were deduced from ESI-MS(/MS) experiments. Fragments of ketoprofen attributed to the loss of formic acid and benzene were successfully identified with high accuracy and confirmed by analysis of deuterated ketoprofen (ketoprofen-d_3). Cleavage of phenyl moiety was characterized as common loss for (BP05, keto 2), (BP06, keto 1) and (BP08, keto 3) in their product ion spectra and substantiated from the corresponding product ions of ketoprofen-d_3 derived isotopologues. Through this study, the online photoreactor-SPE-HPLC system and the concomitant mass spectrometric identification showed their potential for fast and easy photostability testing. Data achieved in the traditional photochamber system and the online photoreactor HPLC hyphenation system are qualitatively similar. The online reactor HPLC hyphenation may also be used for the simulation of photoreactions *in vivo* as irradiation of mixtures may be also realized in the photoreactor to mimic the biological situation. Therefore, this system may be beneficial to reduce time consuming *in vitro* experiments and animal trials.

All of the assigned photoproducts were found to be benzophenone derivatives. Due to their common use and spread in the environment from several sources [77, 78], benzophenone derivatives are considered in many chromatographic and mass spectrometric investigations, where structural elucidation is required. However, proper structure confirmation may be challenged by various factors including, scantiness of the compounds and lack of reference standards. An established flow scheme proposed in this project showed its ability to track ketoprofen as a model compound together with seven of its related benzophenone impurities including those listed in the European Pharmacopeia (Ph. Eur. 9.0) and identify their chemical background. The established workflow incorporates isotopic labelling in addition to various mass spectrometric techniques. Two different deuteration procedures were successfully established in order to obtain site specific d_x-derivatives. These included generation of deuterated photoproducts using BP01-d_3 as starting material via photolysis experiments and/or introduction of d_9-TMS groups into the compounds where applicable. ^{18}O-Labelling was used

to further substantiate fragment structure proposals. Tiered mass spectrometric approach was implemented with different ionization energies, low resolution as well as high resolution accurate mass analyses. This helped to substantiate rationales for structure assignments. Moreover, MS/MS measurements were well integrated in the flow scheme to further elucidate fragmentation pathways.

Surprizing findings concerning mass spectral fragments of the analytes undermined common textbook knowledge on fragmentation reactions in EI-MS. This is ably illustrated, for instance by $[M\text{-}31]^+$ fragment ion (CF2) in the studied group, which included arylpropionic acid derivatives of ketoprofen (BP01), (3-benzoylphenyl) acetic acid (BP02), (2RS)-2-[3-(4-methylbenzoyl)phenyl] propanoic acid (BP03) and (2RS)-2-(3-benzoylphenyl) propanamide (BP04). In this case CF2 cannot be assigned to a loss of $[OCH_3]^\bullet$ from the molecular ion, but as consecutive losses of methyl radical and methane ($[M\text{-}C_2H_7]^+$). Figure 11 illustrates an example of the progressive application of the flow scheme for fragment ion interpretation of ($[M\text{-}31]^+$) of BP-01-TMS (ketoprofen). In line with these findings, (1-(3-benzoylphenyl) ethenone) derivative (BP05-TMS) and 3-(1-hydroxyethyl)benzophenone) derivative (BP06-TMS) also show fragment ion $[M\text{-}31]^+$.

Another example of the importance of the isotopic labelling study and the tiered mass spectrometric approach is obviously shown in the mass spectrum of non-silylated benzophenone derivative of 3-ethylbenzophenone (BP08) exemplified by fragment ion *m/z* 105 (figure 12). As site specific labelling could not be achieved by TMS derivatives of non-silylated compounds, the online photoreactor HPLC hyphenation was used to generate the isotopologues for further confirmation.

Based on these findings, the comprehensive strategy of the online photostability testing and mass spectrometric characterization offers an effective perspective to track drug related compounds and elucidate their structures.

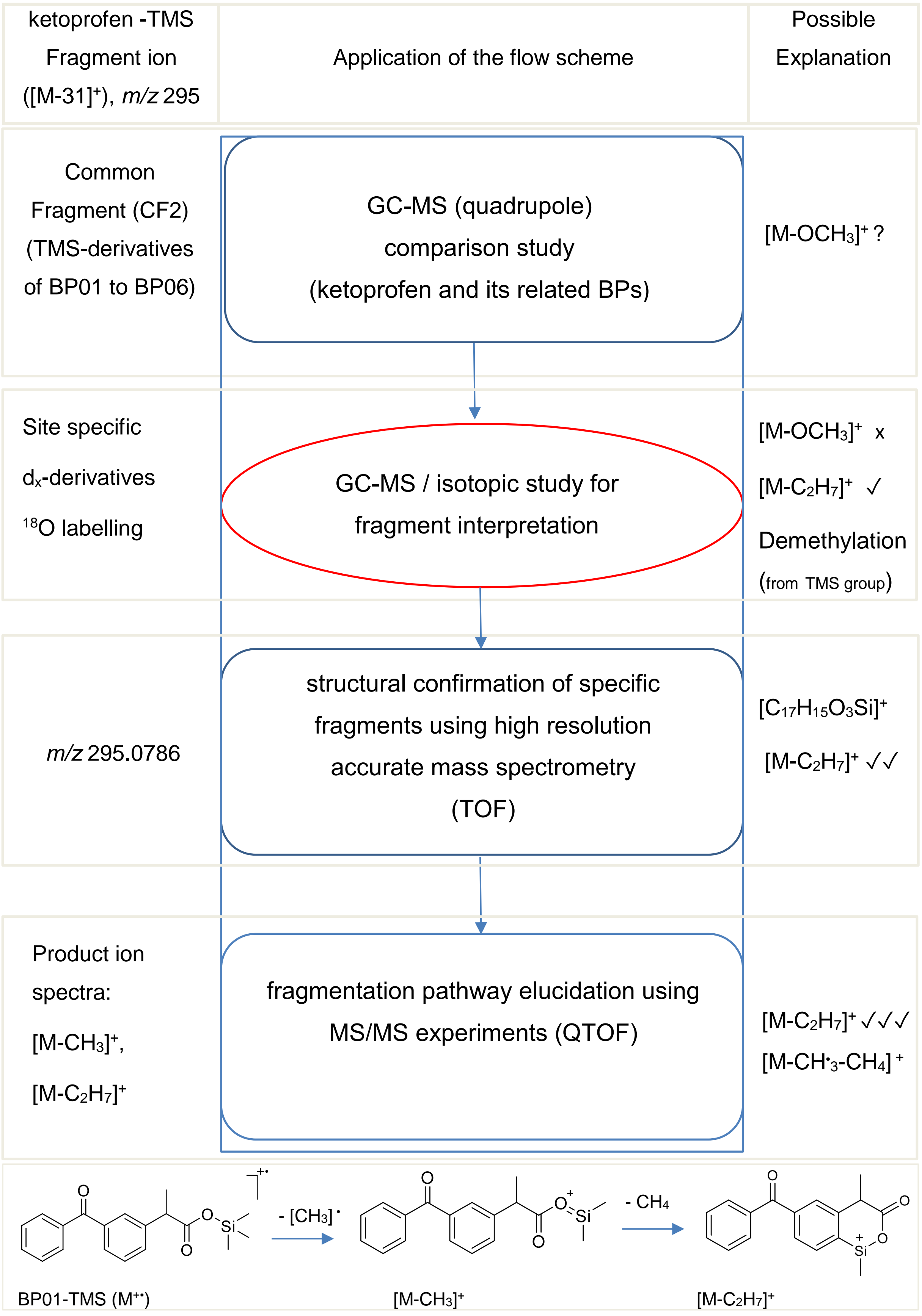


Figure 11: Example showing the application of flow scheme including isotopic labelling study and the tiered mass spectrometric approach for fragment ion interpretation of ([M-31]$^+$) of BP-01-TMS (ketoprofen). Same strategy (manuscript 2) was applied for all studied benzophenone TMS -derivatives (BP01-TMS to BP06-TMS).

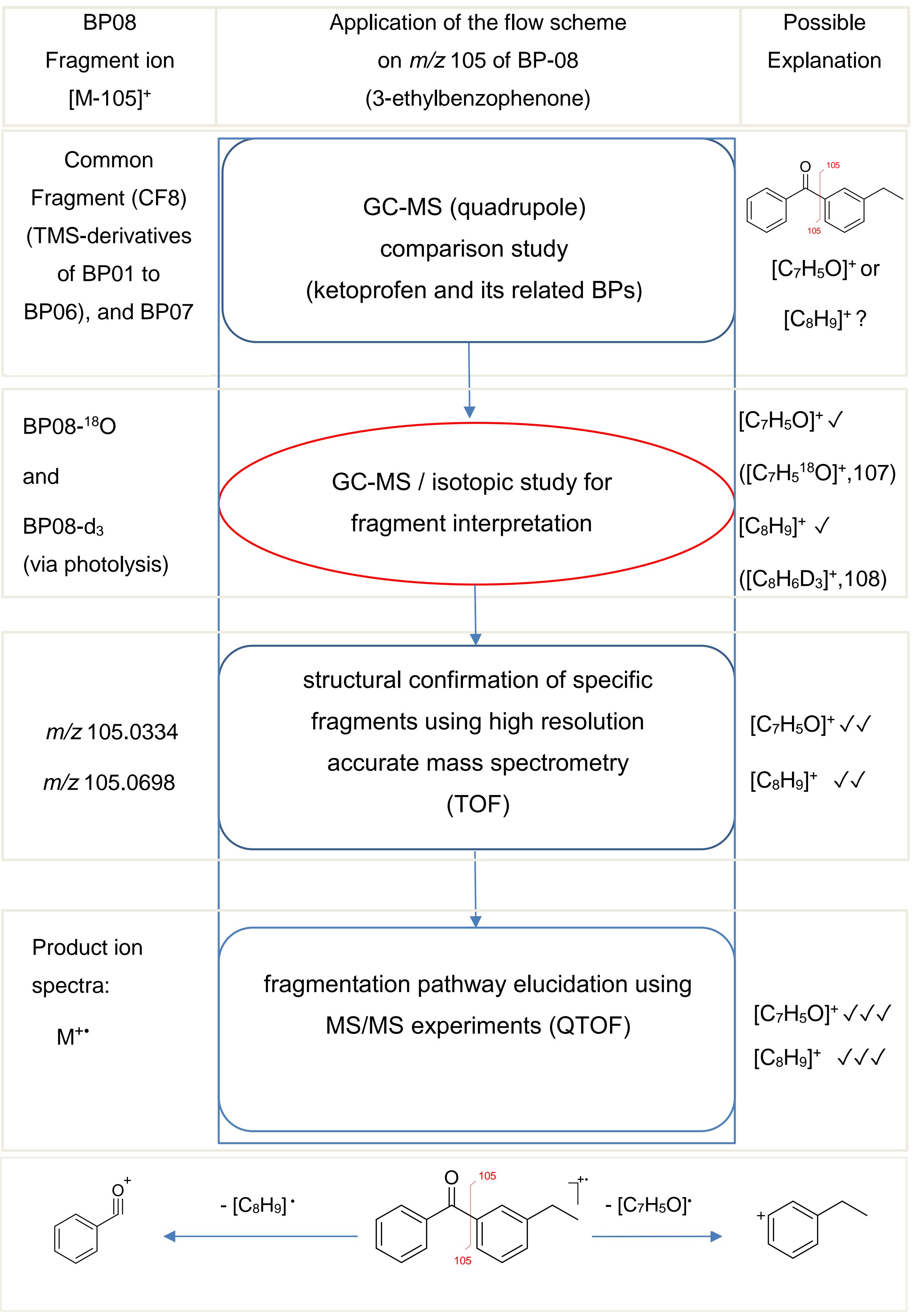


Figure 12: Example showing the application of flow scheme including the isotopic labelling study and the tiered mass spectrometric approach for fragment ion interpretation of $[M-105]^+$ of BP08 (3-ethylbenzophenone)

4 Publications

4.1 Manuscript NO. 1

“Photostability testing using online reactor HPLC hyphenation and mass spectrometric compound identification illustrated by ketoprofen as model compound”

Jaber Assaf, Diego Zulkiewicz Gomes, Bernhard Wuest, Maria Kristina Parr

Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 414–422
https://doi.org/10.1016/j.jpba.2017.07.006

Investigations on the photochemical stability of pharmaceutical substances are mandatory in drug devel-opment and licensing as photo-induced degradation of an active pharmaceutical ingredient (API) may not only lead to decreased API concentrations but also to toxic or reactive products. Thus, the US Food and Drug Administration (FDA) and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) issued Guidance for Industry Q1B “Photostability Testing of New Drug Substances and Products” for testing of pure but also packed drugs. However, photoproducts are also known to be generated in vivo under sunlight exposure of the skin and lead to considerable amounts of adverse drug effects. Herein we present an alternative system that may be used for photostability testing mimicking both sit-uations. It combines a tailored photoreactor with an exchangeable pen light source and a modified HPLC system with online-SPE. Identification of photoproducts may be performed using mass spectrometry. The potential of accurate mass spectrometry as a tool for identification of photoproducts was demonstrated as well. A comparison of the online photoreactor system and the traditional photochamber irradiation was performed using ketoprofen for proof of concept. In both designs acetylbenzophenone and ethylben-zophenone were detected as main photoproducts. The new device allows for fast and easy photostability studies that may help to reduce time consuming in vitro experiments and animal trials. Using state of the art instruments kinetic studies could also easily be performed with qualitative and quantitative per-spectives combined into one experimental design with only very low amounts of API needed. This may be useful in early drug development, where only small amounts of API are available. Scale-up may also be easily realized for the generation of reference material for quantification and quality control (QC) processes as well as for toxicity testing.

4.2 Manuscript NO. 2

Reconsidering mass spectrometric fragmentation in EI-MS- New insights from recent instrumentation and isotopic labelling exemplified by ketoprofen and related compounds

Jaber Assaf , Annette S. Kollmeier, Christian Müller , Maria Kristina Parr
https://doi.org/10.1002/rcm.8313

Rationale: In various fields of chemical analyses, structurally unknown analytes are considered. Proper structure confirmation may be challenged by the low amounts of analytes that are available e.g. in early stage drug development, in metabolism studies, in toxicology or in environmental analyses. In these cases, mass spectrometric techniques are often used to build up structure proposals for these unknowns. Fragmentation reactions in mass spectrometry are known to follow definite pathways that may help to assign structure elements by fragment ion recognition. This work illustrates an investigation of fragmentation reactions for gas chromatography electron ionization mass spectrometric characterization of benzophenone derivatives using the analgesic drug ketoprofen and seven of its related compounds as model compunds.

Methods: Deuteration and ^{18}O-labelling experiments along with high-resolution accurate mass and tandem mass spectrometry were used to further elucidate fragmentation pathways and to substantiate rationales for structure assignments. Low energy ionization was investigated to increase confidence in the identity of the molecular ion.

Results: The high-resolution mass analyses yielded unexpected differences that led to reconsideration of the proposals. Site-specific isotopic labelling helped to directly trace back fragment ions to their respective structure elements. The proposed fragmentation pathways were substantiated by MS/MS experiments.

Conclusion: The described method may offer a perspective to increase the level of confidence in unknown analyses, where reference material is not (yet) available.

5 Summary and Outlook

Here a comprehensive strategy for both photostability testing of drug substances and a complementary structural characterisation approach for drug related compounds, including photoproducts was established and tested.

The photostability studies were performed using a tailored online photoreactor-SPE-LC system and its potential was demonstrated by irradiation of Ketoprofen as a model compound. The new devise allows fast and easy photostability testing that may help to reduce time and animal trials. Using this state of the art setup, kinetic studies can easily be performed with qualitative and quantitative perspectives combined into one experimental design with only very low amounts of pharmaceutical substance needed. The system allows online coupling using ESI-MS or offline EI-mass spectrometric characterization of photoproducts after fractionation. Moreover, scale-up process can be realized for structure confirmation and can be used for further toxicity tests and as reference material for product quantification. Deuterated photoproducts were derived via photolysis experiments and used for further inspections on structural proposals.

In order to increase the confidence level of MS-characterization of unknown compounds, a tiered mass spectrometric approach was suggested through this work for EI-mass spectrometric characterization of ketoprofen and its related benzophenone derivatives including those identified as photoproducts. In addition to high resolution accurate mass measurements derived from recent mass spectrometric instrumentation, the established approach comprises isotopic labelling together with tandem mass spectrometry.

The isotopic labelling was performed by both deuteration and ^{18}O labelling using different methods in order to produce compounds that contain deuterium or oxygen-^{18}O at definite positions. The suggested approach demonstrated its potential as a powerful tool for mass spectrometric characterization of benzophenone derivatives and fragment ion recognition. Upon these promising results, it can be used to increase the level of confidence in the identification of unknown analytes, where reference material is not (yet) available.

The new photostability setup testing together with the established mass spectrometric approach can be beneficial for the simulation of photoreactions *in vivo*. This could be achieved by irradiation of mixtures to mimic the biological situation and identify the resulted compounds.. A similar strategy may also be used for other stability testing utilizing a slightly different reactor design, in which various factors affecting drug substances such as temperature and environmental conditions can be investigated. Structure elucidation as well as generation of reference materials upon these stability testing can then be realized.

5. Zusammenfassung und Ausblick

eine umfassende Strategie wurde hier für die Prüfung der Photostabilität von Arzneistoffen und ein komplementärer Ansatz zur strukturellen Charakterisierung von Arzneimittelwirkstoffen einschließlich Photoprodukten etabliert und getestet.

Die Photostabilitätsstudien wurden unter Verwendung eines entwickelten Online-Photoreaktor-SPE-LC-Systems durchgeführt, und sein Potential wurde durch Bestrahlung von Ketoprofen als Modellverbindung gezeigt. Das neue Gerät ermöglicht schnelle und einfache Photostabilitätstests, die Zeit und Tierversuche reduzieren können. Unter Verwendung dieses Stands der Technik System konnten kinetische Studien leicht mit qualitativen und quantitativen Perspektiven durchgeführt werden, die in einem experimentellen Design mit nur sehr geringen benötigten Mengen an pharmazeutischer Substanz kombiniert wurden

Das System ermöglicht die Online-Kopplung mit ESI-MS oder die Offline-EI-Massenspektrometrie-Charakterisierung von Photoprodukten nach der Fraktionierung. Darüber hinaus kann der Scale-up-Prozess zur Strukturbestätigung realisiert und für weitere Toxizitätstests sowie als Referenzmaterial zur Produktquantifizierung genutzt werden.

Deuterierte Photoprodukte wurden mittels Photolyseexperimenten gewonnen und für weitere Untersuchungen zu Strukturvorschlägen verwendet. Um das Konfidenzniveau der MS-Charakterisierung unbekannter Verbindungen zu erhöhen, wurde in dieser Arbeit ein gestufter massenspektrometrischer Ansatz für die EI-massenspektrometrische Charakterisierung von Ketoprofen und seinen verwandten Benzophenonderivaten, einschließlich solcher, die als Photoprodukte identifiziert wurden, vorgeschlagen. Zusätzlich zu hochauflösenden akkurate Massenmessungen, die aus neueren massenspektrometrischen Instrumenten realisiert wurden, umfasst der etablierte Ansatz die Isotopenmarkierung zusammen mit der Tandem-Massenspektrometrie.

Die Isotopenmarkierung wurde sowohl mit Deuterierung als auch mit ^{18}O-Markierung unter Verwendung verschiedener Methoden durchgeführt, um Verbindungen herzustellen, die Deuterium oder Sauerstoff-^{18}O an bestimmten Positionen enthalten. Der vorgeschlagene Ansatz zeigte sein Potenzial als ein leistungsfähiges Werkzeug für die massenspektrometrische Charakterisierung von Benzophenonderivaten und die Fragment-Ionen identifizierung. Nach diesen vielversprechenden Ergebnissen kann der Ansatz verwendet werden, um das Vertrauen in die Identifizierung von unbekannten Analyten zu erhöhen, wo Referenzmaterial (noch) nicht verfügbar ist.

Das neue Photostabilitätstestverfahren zusammen mit dem etablierten massenspektrometrischen Ansatz kann für die Simulation von Photoreaktionen *in vivo* von Vorteil sein. Dies könnte durch Bestrahlung von Gemischen erreicht werden, um die biologische Situation nachzuahmen und die resultierenden Verbindungen zu identifizieren. Eine ähnliche Strategie kann auch für andere Stabilitätstests verwendet werden, die ein etwas

anderes Reaktordesign verwenden, bei dem verschiedene Faktoren untersucht werden können, die Arzneimittelsubstanzen wie Temperatur und Umweltbedingungen beeinflussen. Die Strukturaufklärung sowie die Erzeugung von Referenzmaterialien bei diesen Stabilitätstests kann dann realisiert werden.

6 Declaration of own contribution

In the following ,the author`s contribution to the individual publications, which are used in this cumulative work,

Manuscript number 1:

- Conception and design of the experiments for photodegradation study
- Generation of photoproducts and isotopologues and mass spectrometric analysis
- Evaluation of the corresponding data in cooperation with the co-authors
- Manuscript preparation in cooperation with the co-others

Manuscript number 2:

- Conception and design of the study with tiered mass spectrometric approach
- MS experiments for ketoprofen and related benzophenone compounds in cooperation with co-author
- MS(/MS) experiments for ketoprofen and related photoproducts
- Evaluation of the corresponding data in cooperation with the co-authors
- Manuscript preparation in cooperation with the co-authors

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9 Curriculum Vitae

10 Relevant presentations and posters in International conferences

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Photostability testing of drug substances: principles and beyond

King's College London (KCL) – Freie Universität Berlin (FUB) Joint Research Workshop

King's College London, London, UK, 26th to 29th March 2018

Assaf, J, J.F. Joseph, A.S. Kollmeier, D.Z. Gomes, B. Wuest, P. Gautschi, M.K. Parr

Mass spectrometric characterization of ketoprofen impurities

27th International Symposium on Pharmaceutical and Biomedical Analysis (PBA 2016), Guangzhou, China, 13th to 16th, November 2016

Assaf, J, Diego Zulkiewicz Gomes, Bernhard Wuest, Maria Kristina Parr,

Study of photodegradation of ketoprofen utilizing photoreactor-HPLC-DAD, HPLC-MS/MS and isotopic Labeling.

44th international symposium on high performance liquid phase separations and related techniques, San Francisco, CA, USA, , 19th to 24th, June 2016.

Assaf, J.; Zulkiewicz Gomes, D.; Schulze, T.; Wuest, B.; Parr, M.K.

Identification of photodegradation products of ketoprofen utilizing online photoreactor-HPLC, GC/MS and isotopic labeling

DPhG Jahrestagung 2015 / Annual meeting, Düsseldorf, Germany, 23th to 25th September 2015.

Assaf, J; Diego Zulkiewicz Gomes; Thomas Schulze; Bernhard Wuest; Maria Kristina Parr

Study of photodegradation of ketoprofen

ANAKON 2015, Gesellschaft Deutscher Chemiker (German Chemical Society), Graz, Austria, 23th to 26th March 2015.

Diego Zulkiewicz Gomes; Jaber Assaf; Thomas Schulze; Bernhard Wuest; Maria Kristina Parr

Online photostability study of pharmaceutical substances

48. Jahrestagung der Deutschen Gesellschaft für Massenspektrometrie (DGM), Wuppertal, Germany, *1st to 4th March 2015.*