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MASTER THESIS

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Evaluation of parameters influencing medical device extraction for testing in *in vitro* skin sensitization assays

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Abstract

With about 15-20 % of the general population every fifth person suffers from allergic contact dermatitis (ACD) making it one of the most prevalent immunologically mediated diseases.
Since medical devices are products intending to directly contact the human body they may be a potential cause of allergic contact dermatitis. Therefore, they need to be tested upon their skin compatibility in order to ensure that the product is safe and does not cause any endogenous cellular defence responses in the patient.

Whereas traditionally these tests are performed *in vivo* using mice or guinea pigs the growing medical device market and an increased ethical and political pressure encourage the development towards the "3Rs principle", meaning Replacement, Reduction and Refinement of animal experiments.
Today, reliable and sensitive *in vitro* methods are available that allow evaluating the skin sensitizing potential of substances. One of them is the NanoSens assay addressed in this thesis.
It is a cell-based reporter gene assay based on the Nrf2-ARE pathway which is involved in the dermal sensitization process. The NanoSens assay succeeds as the first method of its kind to use extracts of medical devices instead of pure chemicals as test substance.
The sample extraction process has to be performed according to DIN ISO 10993-12:2012, a norm used for medical device extraction which still leaves plenty of scope regarding several conditions and procedures.

In the context of this work the influence of certain extraction parameters on the final sample extract were examined. For this purpose reporter substances were incorporated into common medical device materials. By varying single parameters during sample extraction the main influencing factors could be revealed.
Whereas the rotation speed of the shaker during extraction does not influence the amount of leached substances, parameters such as the used vessel, the solvent or the surface roughness of the sample after cutting indeed have an impact on the amount of sensitizers leached from material into extract. Further, foil-like materials should be extracted by ensuring that the whole sample surface is exposed to the solvent at any time of the extraction process.

In summary, the sample extraction is a crucial process that definitely influences the quality of the extract and hence the result of the assay. Within this work the extraction process was optimized and critical steps were identified.

Kurzfassung

Mit etwa 15-20 % der Gesamtbevölkerung leidet fast jeder Fünfte unter allergischer Kontaktdermatitis (AKD), einer der häufigsten immunologisch bedingten Krankheiten.
Da Medizinprodukte dafür vorgesehen sind, bei ihrer Anwendung direkt die menschliche Haut zu berühren, besteht die Gefahr allergische Kontaktdermatitis im Patienten auszulösen. Daher ist es wichtig, Medizinprodukte auf ihre Hautverträglichkeit zu testen um ihre Sicherheit zu beurteilen und zu verhindern, dass endogene zelluläre Abwehrreaktionen im Körper hervorgerufen werden.

Während diese Test traditionellerweise *in vivo* mit Mäusen oder Meerschweinchen durchgeführt werden, fordert der wachsende Medizinproduktemarkt sowie der zunehmende ethische und politische Druck die Entwicklung in Richtung „3R Prinzip“. Dies steht für „Replacement“ (Vermeidung), „Reduction“ (Verringerung) und „Refinement“ (Verbesserung) bezogen auf Tierversuche.
Heute stehen bereits zuverlässige und sensitive *in vitro* Methoden zur Verfügung, die eine Detektion des hausensibilisierende Potential von Substanzen ermöglichen. Eine davon ist der NanoSens Assay der in dieser Arbeit behandelt wird. Es handelt sich hierbei um einen zellbasierten Reportergen Assay auf Basis des Nrf2-ARE Signalwegs, welcher in den dermalen Sensibilisierungsprozess involviert ist. Als erste Methode ihrer Art ist es mit dem NanoSens Assay möglich, Extrakte von Medizinprodukten anstatt Reinsubstanzen zu testen. Der Probenextraktionsprozess muss entsprechend der Norm für die Extraktion von Medizinprodukten DIN ISO 10993-12:2012 erfolgen, welche aber einiges an Spielraum bezüglich mancher Bedingungen und Vorgehensweisen freilässt.

Im Rahmen dieser Arbeit wurde der Einfluss von bestimmten Extraktionsparametern auf den resultierenden Probenextrakt ermittelt. Dazu wurden Reportersubstanzen in häufig verwendete Medizinproduktmaterialien eingearbeitet. Durch Variieren einzelner Parameter während der Probenextraktion konnten die Haupteinflussfaktoren ermittelt werden.
Während die Rotationsgeschwindigkeit des Schüttlers bei der Extraktion keinen Einfluss auf die herausgelöste Menge an Reportersubstanz hat, spielen Parameter wie das Extraktionsgefäß, das Lösungsmittel oder die Rauigkeit der Probenschnittfläche sehr wohl eine entscheidende Rolle.
Des Weiteren sollte bei der Extraktion folienähnlicher Materialien darauf geachtet werden, dass die gesamte Oberfläche während der 72-stündigen Extraktionszeit mit dem Lösungsmittel in Kontakt steht.
Zusammenfassend kann gesagt werden, dass die Probenextraktion einen maßgebender Prozess darstellt, der definitiv die Qualität des resultierenden Extraktes und damit auch das Ergebnis des Assays beeinflusst. Im Zuge dieser Arbeit wurde der Extraktionsprozess optimiert und kritische Schritte identifiziert.

Acknowledgement

I would like to take this opportunity to express my deepest gratitude to all those who gave me the possibility to complete this thesis and supported me during this time.

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Last but not least I would like to thank my family and friends for all the support during this time.
I especially want to express my gratitude to my parents who not only made my studies financially possible but also provided me with unfailing support and continuous encouragement throughout my whole study time.
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Abbreviation list

ARE	Antioxidant response element
ACN	Acetonitrile
CAH	Cinnamic aldehyde
DMSO	Dimethylsulfoxid
DPRA	Direct peptide activity assay
EC	Effective concentration
ECVAM	European Centre for the Validation of Alternative Methods
Eppi	Eppendorf tube
EtOH	Ethanol
FCS	Fetal calf serum
FI	Fold induction
FDA	Food and Drug Administration
GPMT	Guinea Pig Maximization Test
HAc	Acetic acid
h-CLAT	Human Cell Line Activation Test
ISO	International Organization for Standardisation
KEAP1	Kelch-like ECH-associated protein 1
LC	Langerhans cells
LLNA	Murine Local Lymph Node Assay
MB	Methylene blue
Med	Culture medium
MHC	Major histocompatibility complex
NaCl	Sodium Chloride
Nrf2	Nuclear factor erythroid 2–related factor 2
OECD	Organisation for Economic Cooperation and Development
PBS	Phosphate buffered saline
RO	Reverse osmosis
SI	Sudan I
T'g	Glass transition temperature
THF	Tetrahydrofuran
TPU	Thermoplastic polyurethane

1 Introduction

During the last few decades the healthcare science and particularly the medical technology sector have gained rising technological advances.

Their continuous aim to invent ground-breaking and innovative technical solutions for a wide range of medical problems and applications repeatedly confronts engineers with unexpected limitations. Speaking of medical devices, not every material is suitable for the direct application on the human body, as endogenous cellular defence mechanisms may be the result.

However, special medical applications often demand the development of new materials with certain desired properties. As a result the medical device industry is often faced with new challenges in finding the optimal compromise between skin compatibility and mechanical properties. Materials are required that are stable and, depending on the application, more or less flexible or stiff but in any case toxicologically harmless.

Since medical devices intend to come into direct contact with human skin, they have to be tested upon their skin sensitizing potential and thus their ability to cause contact allergies.

In around 15-20% of the general population the exposure to at least one specific allergen causes allergic contact dermatitis (ACD) which can further lead to inflammatory skin diseases or an increased susceptibility (Peiser et al., 2012).

For the identification of new skin sensitizers and the biocompatibility assessment of materials traditionally rodents like guinea pigs and mice serve as test models. However, the growing medical device market as well as an increasing political and ethical pressure drive the development of animal experiment free alternatives.

The NanoSens Assay treated in this thesis is one of those novel *in-vitro* cell-based detection assays.

A medical device needs to be extracted in order to enable its skin sensitization assessment with bioassays such as the NanoSens. Therefore, the DIN ISO 10993-12:2012 provides requirements and guidelines for sample preparation. However, this norm does not exactly specify the whole extraction procedure although some of those undefined parameters may potentially influence the final result of the assay.

The following subchapters focus the human skin, its sensitization reaction as well as methods to detect the skin sensitizing potential of materials and substances. Furthermore, the laws and processes behind the preceding extraction procedure of the materials tested are outlined.

1.1 The human skin

The skin comprises about 15 % of the total body weight of an adult human and is therefore our largest organ. It is a complex arrangement of different cell types all contributing to a multitude of functions that are essential for an organism. As the outermost barrier of the body especially protection at micro and macro level is performed effectively. The skin is organized in three different layers which are described more closely below (Kanitakis, 2002).

1.1.1 Structure of the skin

The skin is composed of three main layers, namely the epidermis, the dermis and the hypodermis or subcutaneous tissue as illustrated in Figure 1.

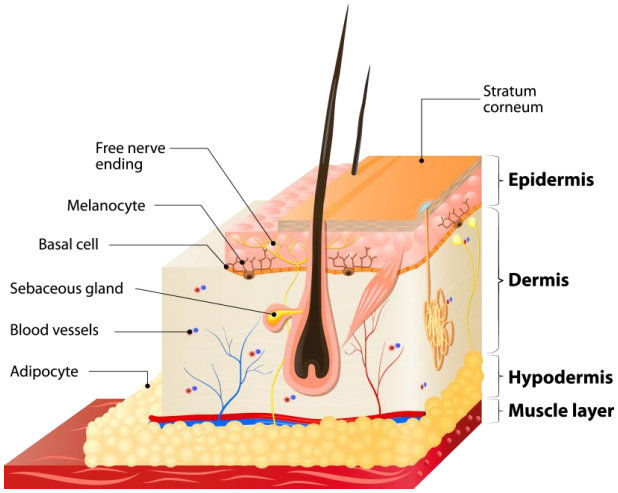


Figure 1: Schematic cross-sectional enlargement of human skin tissue. Source: https://vesiderm.com/product-technology/; accessed on 22.09.2018

Epidermis

The outermost barrier, the epidermis consists of about 80 % of keratinocytes. This layer is continually renewing itself by a process called keratinisation. These cells synthesize keratin, a fibrous intermediate filament forming an alpha-helical coil that acts as part of the cytoskeleton in order to prevent mechanical cellular damage (Kolarsick, Kolarsick, & Goodwin, 2011).

As demonstrated in Figure 2, the epidermis is further made up of four (in some tissues also five) different layers.

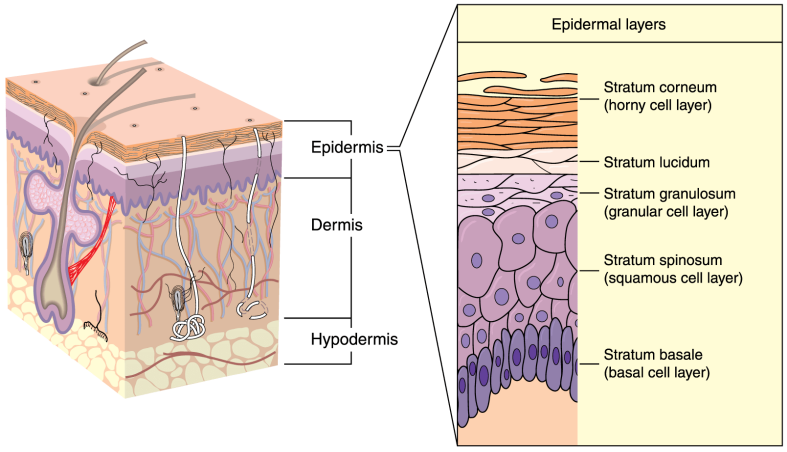


Figure 2: Schematic cross-sectional enlargement of epidermal skin layers.
Source: https://vesiderm.com/product-technology/; accessed on 22.09.2018

In the lowest layer, the basal layer (*stratum basale*) the keratinocytes are formed. These cell continuously proliferate and migrate upwards, passing the squamous or prickle layer (*stratum spinosum*), the granular layer (*stratum granulosum*), in some tissues also the clear layer (*stratum lucidum*) until their final destination, the horny layer (*stratum corneum*) is reached.

During the migration process the keratinocytes differentiate and then die, resulting in tightly-linked and protein-filled corneocytes covered by a rigid protein envelope. These corneocytes are fully differentiated keratinocytes forming the outermost horny layer by a stack of about 12-16 cells (Vesiderm, 2017).

During keratinocytes differentiation the keratin filaments are aggregated by filaggrin into tight bundles triggering a cellular collapse and resulting in the characteristic flattened shape of corneocytes. Keratin and filaggrin comprise 80 – 90 % of the total mammalian epidermal protein mass (Proksch, Brandner, & Jensen, 2008).

In addition, the following two cell types are also importantly present in the epidermis. One of them is the melanocytes, synthesizing melanin pigment granules. These cells then transfer so called melanosomes to the epidermal keratinocytes where they protect the skin from UV light and are responsible for the skin color.

Another important epidermal cell type are the Langerhans cells. As dendritic and antigen-presenting cells of the skin they have a major function as immunologic barrier and therefore also participate in contact allergy (Wickett & Visscher, 2006).

Dermis

The elastic and tension-proof dermis is the connective tissue component of the skin protecting the body from mechanical injuries, binding water and supporting thermal regulation and sensual stimulation. It interacts with the epidermis located above, especially during morphogenesis of epidermal appendages and through dermal-epidermal junctions (DEJ), the acellular zone between the two layers. Collaboration of dermis and epidermis also occurs during wound healing where the concerned skin area is repaired and remodelled (Freinkel & Woodley, 2001).

The dermis is characterized by its special structure primarily consisting of collagen and elastin fibres being embedded into the extracellular matrix. This highly branched network of macromolecules gives the typical strength and elasticity at the same time. Visible scars on the skin are formed as a result of damage to the dermis (Romero, 2017).

As indicated in Figure 1 there are also some additional structures located in the dermis making it the most functional skin layer. For example blood vessels, passing through this dense meshwork allow efficient nutrient supply and waste removal of the proliferating cells in the basal layer. Dilating the vessels results in an increased heat loss to the surface and so prevents the body from overheating. Narrowing them in return, reverses this process (myDr, 2015) .
Neurovascular bundles located in the dermis contain a high number of nerve fibres transmitting pain, temperature and itch sensation (Kolarsick et al., 2011) .
Nearly the whole area of the skin is studded with hair follicles embedded into the dermis. Base of each follicle is a continuously dividing cell layer that pushes overlying cells inside this follicle upwards. The hair shaft that is visible above the skin is formed when these cells become keratinised and die, similar to the corneocytes in the epidermis.
Further, sweat glands as body temperature regulating tool and the sebum (oil) producing sebaceous glands are as well embedded into the dermis (myDr, 2015).

In general, the dermis is much less cellular than the outermost one. However, the primary occurring cell type is the mesenchymal derived fibroblast that migrates through the tissue. This highly diverse cell type synthesizes and degrades fibrous and non-fibrous connective tissue matrix proteins and as well numerous soluble factors (Freinkel & Woodley, 2001) .

Hypodermis

The deepest layer of the skin mainly consists of adipocytes (fat cells) and collagen and serves as storehouse of energy. In addition, it insulates the body and acts as natural shock absorber to prevent mechanical damage (DesiMD, 2013).

The thickness of the hypodermis strongly depends on the skin site and the overall body fat level of the individual person. The subcutaneous tissue can also be considered an endocrine organ since the hormone leptin is secreted by the adipocytes regulating fat mass by providing a long-term feedback signal (Kolarsick et al., 2011).

1.1.2 Functions of the skin

The skin is the most extraordinary organ of our body in terms of function. Its most essential tasks are the protection of the organism from desiccation and other environmental hazards by simultaneously allowing continuous communication with its surrounding. As the first barrier to the outside world the skin protects the body from injuries, prevents body fluids from escaping and external ones from penetrating inside (Montagna, 2012). Especially microorganisms are shielded effectively from our body's interior by the dry and acidic milieu of the skin surface. Another basic function that should not be underestimated is the thermoregulation of the epidermis in order to maintain our constant body temperature of 37 °C. Mechanical support is provided by a subcutaneous fat layer acting as protective pad against external shocks and forces. Finally also the skin's sensory function fulfils a protective role against parasites as it puts us on alert after recognizing tickling or itching.

The skin also acts as an immune organ with its antigen-presenting cells, the so called Langerhans cells, and memory-lymphocytes that contribute to our innate and adaptive immunity (Fritsch, 2004).

However, since the skin is an excellent organ to demonstrate sensitization and to explore biologic antibody activities it has been used for years to study allergic and immunologic responses. (Montagna, 2012).

1.2 Skin sensitisation

Being the prerequisite event in the development of allergic contact dermatitis (ACD), skin sensitization is a key parameter in both hazard and risk assessments. With an estimated percentage of 15 – 20 %, nearly every fifth person will be sensitized by at least one specific allergen. (Urbisch et al., 2015) Allergic contact dermatitis is today one of the most important occupation-related skin diseases (Martin, 2015).

According to Europe's Globally Harmonizes System for Classification and Labelling of Chemicals (GHS) a skin sensitizer is a substance that will lead to an allergic response following skin contact (Coleman et al., 2015). Basically, skin sensitizing molecules are small reactive chemicals with the potential of covalently modifying skin proteins. After binding the proteins they are recognized as foreign substances by the immune system (Emter, Ellis, & Natsch, 2010).

Allergic contact dermatitis (ACD) which is also classified as a delayed or type IV hypersensitivity reaction is caused after the repetitive exposition to a skin sensitizing molecule in course of an immune reaction followed by the formation of specifically reacting T-lymphocytes or antibodies, such as IgE (Merk, Baron, Neis, Obrigkeit, & Karlberg, 2007).

The detailed sensitization process is explained in the following subchapter.

1.2.1 Adverse Outcome pathway

Skin sensitization is a complex process involving lots of different biological and chemical components. The Adverse Outcome Pathway (AOP) is described by the Organisation for Economic Co-operation and Development (OECD) and summarizes the four key events including their biological mechanisms that occur during skin sensitization. Figure 3 shows a schematic illustration of the AOP starting from the causing chemical substance to the organism's response.

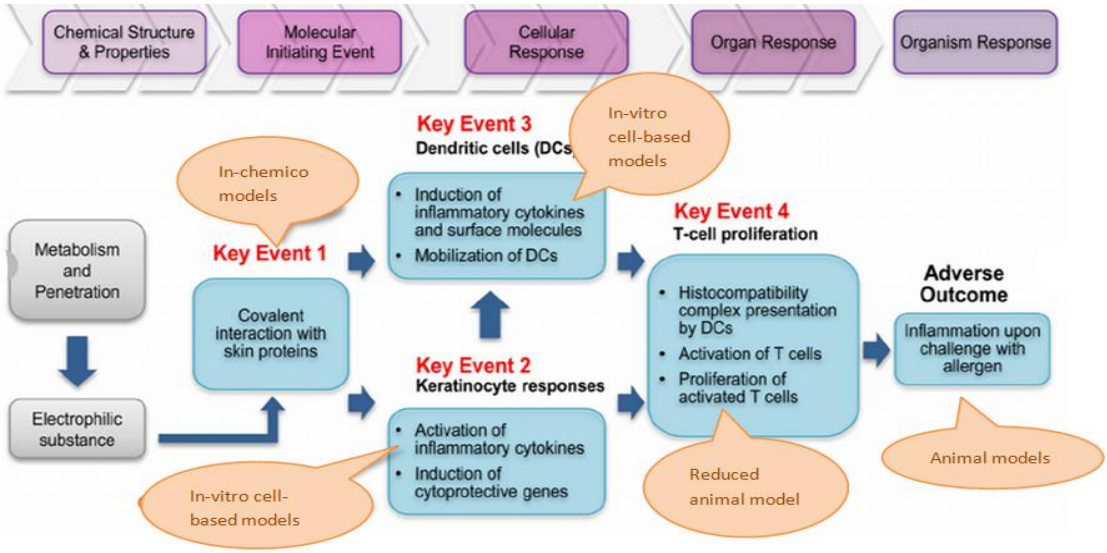


Figure 3: Adverse Outcome pathway with key events and respective detection methods. (OECD, 2012)

The AOP starts with the induction phase in which innate pathways are activated, similar so the innate responses to pathogens (Emter et al., 2010).

Most contact allergens are small, electrophilic and chemically reactive molecules with a molecular weight less than 500 Da. When such a molecule comes into direct contact to the skin and enters the epidermis through penetration it can bind to a skin protein forming a hapten. This is a small electrophilic molecule with the ability of eliciting an immune response when attached to a large carrier such as a protein. Sensitizing organic compounds are able to covalently bind to nucleophilic proteins groups, such as thiol, amino, and hydroxyl groups. Metal ions in contrast, such as nickel cations form stable metal–protein chelate complexes. This process is referred to as key event 1 or Molecular Initiation Event (MIE) (Rustemeyer, M. W. van Hoogstraten, E. von Blomberg, & Scheper, 2006a).

Key event 2 addresses the interaction of the protein-hapten conjugate with cell surface proteins and the subsequent activation of signalling pathways. These pathways include responses of keratinocytes and dendritic cells to the skin sensitizing substance. As a result inflammatory cytokines are activated and cytoprotective genes induced.

In key event 3 Langerhans cells (LC), a sort of immature epidermal dendritic cells come into play. They recognize the protein-hapten complex and take it up by endocytosis. Afterwards the dendritic cells migrate from the epidermis to the dermis and further to the local lymph nodes where the hapten (antigen) is presented to T-lymphocytes via a major histocompatibility complex molecule (MHC). Additionally, during this dendritic cell maturation inflammatory cytokines and surface proteins are induced.

During key event 4 the T-lymphocytes recognize the presented peptide antigen and if it is a foreign peptide, the cell will be activated to form a memory T-cell. This event is followed by an enhanced proliferation, a so called clonal expansion of these allergen responsive cells (McKim, Keller, & Gorski, 2012; OECD, 2012).

The induction phase with its four key events as described above is followed by the so called elicitation phase which is initiated by a renewed allergen contact. Since the activation threshold is lowered during a repeated exposition, hapten-specific T-cells are triggered by haptenized keratinocytes and LC resulting in the production of proinflammatory cytokines and chemokines. This signal secretion further recruits inflammatory cells resulting in the gradual development of an eczematous reaction that reaches its maximum within 18 – 48 h. This response of the organism is the final phase or rather the adverse outcome of this complex pathway and the trigger for the disease allergic contact dermatitis (Rustemeyer, M. W. van Hoogstraten, E. von Blomberg, & Scheper, 2006b).

1.3 Methods detecting skin sensitization

As skin sensitization is the first step in the development of the nowadays frequently appearing allergic contact dermatitis, methods detecting suspicious triggers have gained more attention during the last decades (Urbisch et al., 2015a).

With the expanding need of medical devices, encompassing various products including hearing devices, prostheses, catheters, blood bags, surgical instruments, gloves and also new frontiers like tissue engineering products, the medical device market is poised to grow dramatically (Kerecman Myers et al., 2017).

In many cases those products are made of special materials that fulfil the properties that are desired for their specific application or function. Mostly certain kinds of plastics are used since they combine high tensile strength with elasticity, or other desired mechanical properties.

However, since a medical device intends to directly contact the human skin or even may be implanted into the body it needs to be safe for the patient, meaning that it has to be biologically compatible with tissues. Biocompatibility testing of materials aims to guarantee the maximum benefit with the minimum risk for the patient (Pizzoferrato et al., 1994).

Usually biocompatibility evaluation includes at least testing for cytotoxicity, irritation and sensitization whereas the latter one allows investigating the risk of allergic responses to devices or their leachates.

In general, different methods are available that allow detection of the skin sensitizing potential of a chemical substance. Commonly these tests are performed in mice or guinea pigs using pertinent routes and exposure conditions (Bernard, Jubeli, Pungente, & Yagoubi, 2018).

However, over the past decade, concerns about animal welfare and political pressure in the industrialized world have prompted the search for alternative test methods. This search was significantly supported by the European Union as the driving force behind it (Coleman et al., 2015).

Even though the fact that *in vitro* testing of medical devices can be more effective than *in vivo* testing, both in terms of cost and time, unfortunately their acceptance has not kept pace with today's technology. While regulators already accept some *in vitro* tests such as cytotoxicity and gene toxicity, many sensitization and irritation tests still rely on animals. So there is an urgent need to boost confidence and promote conviction of the enormous potential of alternative medical device testing methods (Kerecman Myers et al., 2017).

1.3.1 *In vivo* methods

The following subchapters describe the two most common *in vivo* methods used to detect skin sensitization. Both of them do not address any key event of the adverse outcome pathway but the final phase being the organism's response as marked in figure 3.

Guinea Pig Maximization Test (GPMT)

The albino guinea pig has traditionally been the animal of choice in terms of testing chemicals for skin sensitization. The Guinea pig maximization test developed in 1969 by Magnusson and Klingman is widely accepted by regulatory authorities because of its reliable detection of a wide variety of potential human contact allergens (Basketter & Scholes, 1992; Magnusson & Kligman, 1970).
A minimum of ten guinea pigs is used in the treatment group and at least five ones in the control group. The assay starts with the induction phase in which test solutions are injected intradermally along with an adjuvant in order to enhance the immune reaction of the animals.
One week later the animals are exposed to a lower concentration of the test substance and the resulting allergic reaction, if any, is measured optically. The test substance is considered positive if at least 15 % of the guinea pigs show a reaction (Andersen & Maibach, 1985; OECD, 1992).

Local Lymph Node Assay (LLNA)

Developed in 1989 and accepted by the OECD in 2002, the murine local lymph node assay (LLNA) allows identification of chemicals with the potential of causing skin sensitization. As the first test to pass through the formal regulatory validation process established in the USA under the approval of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) this method was proven an advantageous alternative to the guinea pig tests with respect to animal welfare (Basketter et al., 2002).
It is based upon immunological events stimulated by chemical substances in the induction phase of sensitization. In contrast to the GMPT, elicitation of challenge-induced dermal hypersensitivity reactions as well as the use of adjuvants are not necessary.
Dilutions of test substances are applied daily on the rear side of the murine ear on three consecutive days, including both positive and negative controls. After two days without any treatment proliferation of the lymphocytes in the lymph nodes draining from the affected site is evaluated since it is proportional to the dose and potency of the applied allergen. For this purpose tritiated (^{3}H)-methyl thymidine as a tracer is injected into the tail vein for lymphocyte incorporation.
The mice are euthanized before removing and analysing their lymph nodes. Incorporation of the radioactively labelled tracing substance is measured by ß-szintillation counting as disintegrations per

minute (DPM). Finally the stimulation index (SI) is determined as ratio of tracer incorporated into the lymph nodes from dosed animals compared to those of control animals (OECD, 2010).

1.3.2 *In vitro* methods

As already mentioned, the growing medical device market as well an increased ethical and political demand advance the replacement, reduction, and refinement of the use of animals in bioassays. This approach is additionally supported by the fact that actually available *in vitro* methods as alternatives to the controversial *in vivo* tests enable to save both money and time and promise high assay sensitivity (Kerecman Myers et al., 2017).

The following subchapter describe three alternative test methods for skin sensitization including their key event they address.

Direct Peptide Reactivity Assay (DPRA)

The Direct Peptide Reactivity Assay (DPRA) developed by Gerberick et al. (2004) is a straightforward approach to predict the sensitization potential of chemicals. As an *in chemico* method, it addresses key event 1 of the adverse outcome pathway for skin sensitization.

The DPRA determines the reactivity of a chemical substance towards synthetic cysteine (C)- and lysine (K)-containing peptides as a measure for the compound's potential to haptenize proteins and peptides *in vivo*.

The test substances are incubated with those peptides in ratios of 1:10 (for C-peptide) or 1:50 (for K-peptide) for 24 h at room temperature whereby potential skin sensitizers may bind to the cysteine or rather lysine residues due to their electrophilic properties. After that time the remaining non-depleted peptide concentration is determined by high performance liquid chromatography using UV light detection (HPLC/UV) (Gerberick, 2004).

NanoSens Assay –Nrf2-ARE pathway

This assay is subject of this thesis as it was used for evaluating and comparing the differently prepared medical device extracts.

The NanoSens is a cell-based reporter gene assay allowing the identification of skin sensitizing substances by modelling the second key event of the adverse outcome pathway (AOP).
It is based on the electrophilic properties of haptens allowing them to covalently bind to proteins of the skin and turning them immunogenic. The assay makes use of the Nrf2-ARE pathway which is shown graphically in figure 4.
Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor regulating cellular defence mechanisms as a response to oxidative stress through electrophilic molecules.
Normally Nrf2 is located exclusively in the cytoplasm where it is bound to Kelch-like ECH-associated protein 1 (KEAP1). However, electrophilic skin sensitizers act as inducers of this pathway leading to interactions with several critical cysteine residues in KEAP1. This in return causes conformational changes and a subsequent dissociation of Nrf2 from the complex. The now released transcription factor migrates from the cytoplasm to the nucleus where it binds together with a cofactor to antioxidant response elements (AREs). As these *cis*-acting enhancers are located in the promoter region of cytoprotective genes they initiate the cellular defence response.
The NanoSens Assay as *in vitro* method makes use of this pathway by detecting and semi-quantifying this response caused by skin sensitizing chemicals. For this purpose, a detection cell line has been created by incorporating multiple ARE sequences upstream of a promoter controlling the activity of the inserted luciferase reporter gene).
A schematic illustration of the transfected vector is provided by Figure 4.
The defence gene was replaced by luciferase genes enabling the detection of this pathway by using a luminometer (Glück, Riegel, Ettenberger-Bornberg, & Czerny, 2015).

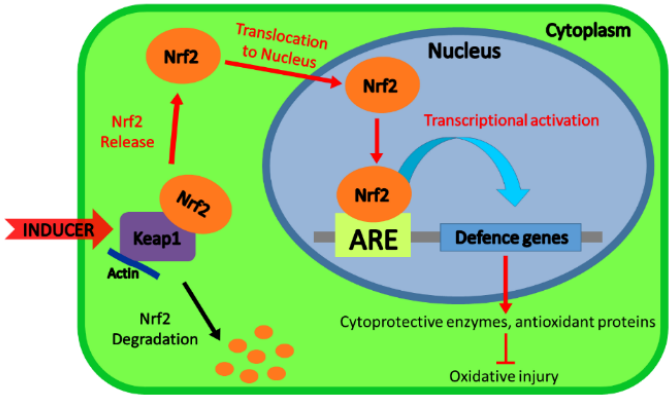


Figure 4: Regulation of Nrf2.
(Glück, Riegel, Ettenberger-Bornberg, & Czerny, 2015)

Human Cell Line Activation Test (hCLAT)

The human Cell Line Activation Test (h-CLAT) is a cell-based assay which has been recommended as a useful tool in an integrated testing strategy (see below) for identification of skin sensitizers by the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM).

The assay has been validated and adopted by the OECD (TG 442E) for contributing to the evaluation of the skin sensitisation hazard potential of chemicals.

Using the human leukemia cell line THP-1 this method addresses the third key event of the AOP for skin sensitization. Cells that are exposed to a sensitizing substance are characterized by changes in the expression pattern of cell surface markers which are involved in dendritic cell (DC) activation, such as CD86 and CD54. Therefore, after treatment of the cells with the test chemical, the expression levels of CD86 and CD54 are measured by flow cytometry and then used for discriminating between skin sensitizers and non-sensitizers (Ashikaga et al., 2006; OECD, 2014).

Integrated Testing Strategy (ITS)

As sensitization reactions involve a complex sequence of events many investigators fear that no single *in vitro* assay can accurately identify all human sensitizers. Therefore, guided by the OECD's adverse outcome pathway (AOP) the European Centre for the Validation of Alternative Methods (ECVAM) has been pursuing an integrated testing strategy (ITS) aiming to combine *in vitro*, *in chemico*, and *in silico* methods in order to create a highly accurate predictive model (Urbisch et al., 2015b).

For this purpose several studies have been conducted on establishing a test battery including at least three different non-animal skin sensitisation assays all addressing various key events of the AOP.

As an example, Bauch et al. (2010) developed a "weight of evidence" based approach in which 2 out of 3 *in vitro* methods decide the end result of the safety assessment. The test battery, illustrated exemplary in Figure 5, addresses key event 1 with the DPRA, key event 2 with the KeratinoSens (ARE-Nrf2 pathway like NanoSens) and key event 3 with the human Cell Line Activation Test (hCLAT) (Bauch et al., 2012).

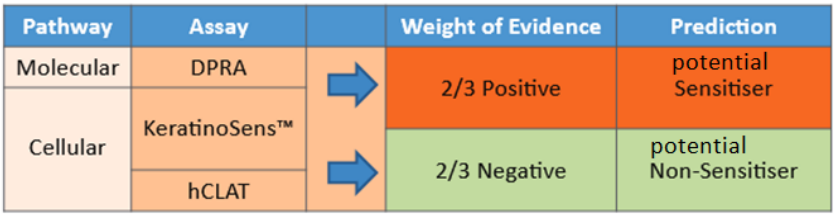


Figure 5: Skin sensitisation weight-of-evidence decision tree.
(Kidd, Rothwell, Inns, Dreher, & Henderson, 2017)

1.4 Testing of medical devices - Extraction

Medical devices have the potential to leach substances during their application which may directly contact the human body. Since medical device materials are often composed of a complex composition of various chemicals and additives, it is not uncommon that some of them may interact with proteins and cells of the organism.

Therefore, appropriate biocompatibility risk assessment of medical devices is crucial, especially for those made of novel and unknown materials (Przygoda, 2017).

A biomaterial is per definition a nonviable material used in a medical device intending to interact with biological systems to evaluate, treat, augment, or replace any tissue, organ or function of the body.

Biocompatibility in return describes the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable or systemic effects in the recipient (Black, 2005).

However, skin sensitization which is central topic of this thesis is one of the three main endpoints of biocompatibility, along with skin irritation and cytotoxicity.

Testing of solid samples with *in vitro* methods is not possible as all cells are cultivated in liquids. To enable testing of solid medical devices, they have to be extracted.

As the result of the bioassay depends on the sample extract applied, the extraction process itself is crucial for assessment of medical devices.

The following section discusses the mechanisms involved in the solid-liquid extraction or rather leaching process which the samples undergo during their preparation process prior to be tested in the assay.

1.4.1 Leaching process and its mechanisms behind

Solid-liquid extraction which is also known as “leaching”, means the removal of soluble components from a solid matrix and can be described by the a very simple equation:

Sample material (Leachee) + Extraction agent (Leachant) → Extract (Leachate)

Several different leaching methods are available. However, the so called batch leaching is used for medical device extraction. For this purpose the sample is placed in a given volume of extraction agent for a certain period of time. Usually some type of agitation or shaking is performed to ensure a balance of the concentration and avoid the development of a concentration gradient. During this process solutes diffuse from inside the solid matrix into the surrounding solvent. At the end of the extraction period, the liquid is removed and analysed (Kim et al., 2006).

Leaching of biomaterials

Taking an implant biomaterial as example, the simplest form of interaction that can occur is the transfer of substances across the material – tissue interface in absence of reaction.
A migration of fluids and ions from the tissue into the biomaterial results in swelling of the fully dense material due to conservation of volume. Even if the material does not take up fluid, it may absorb components or solutes from the surrounding fluid phase.
In contrast, leaching occurs if the fluid migrates back into the tissue or if a component or solute of the biomaterial dissolves in the fluid phase of the tissue resulting in material porosity.
These two effects may extensively influence the behaviour of materials, even if no obvious shape changes or externally applied mechanical stress are present (Black, 2005).

Figure 6 shows a schematic drawing of the micromolecular processes occurring during leaching of a solid material in a liquid solvent.

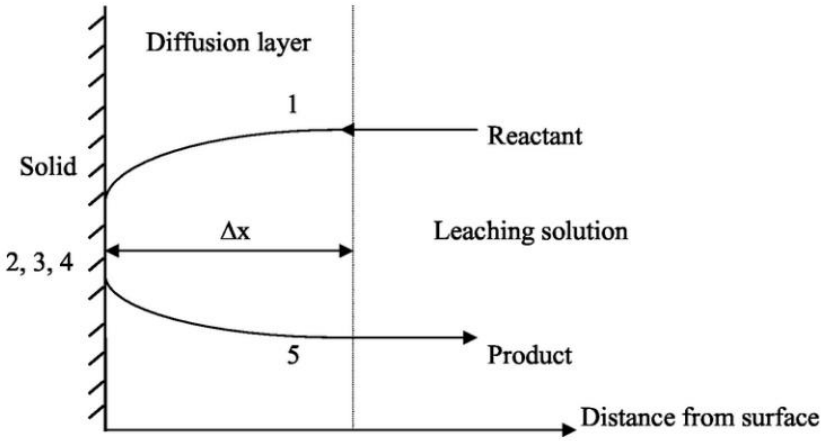


Figure 6: Schematic sketch of the leaching process; Leachable concentration (x-axis) vs. distance from the surface (y-axis)
Source: http://wiki.biomine.skelleftea.se/biomine/leaching/letheo_02.htm (accessed on 02.10.2018)

1.) Diffusion of the reactant through the diffusion layer (Δx)
2.) Adsorption of the reactant on the solid
3.) Chemical reaction between the reactant and the solid
4.) Desorption of the product from the solid
5.) Diffusion of the leaching product through the diffusion layer (Δx)

The principles behind these swelling and leaching processes underlie the mechanisms of diffusion described by Fick's law.

Formula 1: Fick's first Law of Diffusion in isotropic materials

$$J = -DA\,\frac{\partial C}{\partial x}$$

J = Diffusion flux or amount of substance diffusing through a surface per time unit
D = diffusion coefficient
A = surface area
$\frac{\partial C}{\partial x}$= concentration gradient

According to Formula 1 the leaching rate depends on the area of the solid leaching sample, the diffusion coefficient and the concentration gradient which in turn is dependent on the thickness of the diffusion layer (Δx) (BioMinE Integrated Project, 2006).
To avoid a concentration gradient several standards describing extraction propose shaking of the sample in the solvent.

Types of diffusion

1) Volume diffusion : through the bulk of the material
2) Grain boundary diffusion: only along the grain boundaries
3) Surface diffusion: along the surface of a phase

(Roshni, 2018)

With Q being the energy of activation of the diffusion process and D_0 being the intrinsic diffusion coefficient, the following expression arranges these three types in an order according to their magnitude.

$Q_{volume} > Q_{grain\ boundary} > Q_{surface}$ [4:3:2] or [4:2:1]

$D_{0_{surface}} > D_{0_{grain\ boundary}} > D_{volume}$

These equations visualize that surface diffusion is favoured at typical biological temperatures within a given substance diffusing through a given biomaterial (Black, 2005).

Therefore, extraction of medical devices underlies the surface diffusion. Furthermore, only the surface of the device gets in contact with the patient and is therefore more relevant for assessment of biocompatibility.

1.5 Aim of this thesis

The NanoSens Assay had already been proven a reliable and robust *in-vitro* test method for evaluating the skin sensitizing potential of medical devices. It pursues the objective of reducing or even replacing the use of animal tests being the state-of-the-art tool for dermal sensitization assessment. Previous studies revealed a remarkably high sensitivity of this cell based reporter gene assay since it is able to detect a large number of sensitizers at concentrations more than 1000 times lower compared to the LLNA being the most common standard animal test (Mertl et al., 2015).

However, medical devices intending to undergo the NanoSens skin sensitization test have to be extracted in order to obtain a solution enriched in the contained low molecular substances suspected to be potential sensitizers. The extraction process of medical devices has to be performed according to DIN ISO 10993-12:2012 specifying requirements and guidance for sample preparation of biological evaluation methods. However, this framework still leaves plenty of scope regarding several conditions since it does not make exact stipulations about each step of the extraction procedure.

The aim of this thesis is to determine the impact of varied parameters in conjunction with the extraction process on the quality (properties) of the final extract. It should be evaluated which parameters cause significant variations in the amount of low molecular substances migrating out of the tested material and which ones do not affect the final extract and thus can be chosen freely.

Parameters which were suspected to be an influencing factor in the extraction process were the medical device material, the roughness and thickness of the sample's cutting surface, the extraction vessel, the rotation speed during shaking, the temperature and the extraction agent. Moreover, the elapsed time is monitored in which the concentration in the extract does not increase anymore. To optimize extraction of thin film-like materials, differences were measured if foils stick together or if they are extracted without direct contact.

Furthermore it was evaluated whether a possible saturation effect took place which would result in distortion of the ascertained results.

In order to monitor this varying migration behaviour cinnamic aldehyde, a model substance for skin sensitization was incorporated into different and commonly used medical device materials. These spiked samples were subsequently prepared for the NanoSens Assay like a real medical devices being tested. Altering single parameters of the sample preparation and extraction process allowed drawing conclusions about crucial influential factors.

In a further experiment the same medical device materials were spiked with prior selected azo dyes characterized by different polarity behaviours and mimicking potential skin sensitizers. By submitting these coloured samples as well to a parameter-varied sample preparation procedure, information about the success and strength of the extraction process could be obtained. Evaluation of the

amount of dye substance migrating from material into extract was then performed photometrically. After generating a calibration curve by absorption measurement of a dilution series of a known stock solution, quantification of the dye substance extracted from the respective medical device material was accomplished.

The selection of the solvent used as extraction agent was one of the major parameters. With regard to this, a further aim was to prove that DMSO and acetonitrile used for NanoSens Assay samples by default are stronger extraction agents for those potential sensitizers than olive oil which is usually used in animal tests. Additionally, the efficiency of ethanol, isopropanol, water, PBS and physiological saline solution as extraction agent were compared.

2 Material

This chapter contains all the materials, chemicals and the equipment used in course of this thesis.

2.1 Material and equipment

Table 1 shows all the used equipment and technical devices including their manufacturer.

Table 1: Used equipment and technical devices

Equipment	Manufacturer
Multichannelpipette Xplorer (50 – 1200 µL)	Eppendorf (Germany)
Pipetboy with accumulator	Integra Biosciences (USA)
Tissue culture flask 75 cm^2; treated, vented cap, sterile	VWR (USA)
Microscope – Eclipse TS100	Nikon (Japan)
Hemocytometer Neubauer	LaborOptik (GB)
Multilabel plate counter- 1420 Victor3TM	Perkin Elmer (USA)
Lambda35 UV/VIS Sprectrometer	Perkin Elmer (USA)
Laminar Flow – Biosafe 7-130; sterile	Ehret (Germany)
Ultrapure water system – Reference A+	Merck (Germany)
-80°C Freezer- Igloo	Telstar technologies (Spain)
Autoclave – Varioklav 400 E	HP Medizintechnik (Germany)
CO^2-Incubator (New Brunswick – Galaxy 170 R)	Eppendorf (Germany)
Vortex Genius 3	IKA (Germany)
Water bath – 1002	GFL (Germany)
analytical balance – A200S	Sartorious analytic (Germany)
Analytical balance – GJ-4100-2M	Kern & Sohn (Germany)
Analytical balance – XS205 Dual Range	Mettler -Toledo (USA)
Incubator shaker- Laboshake THL 500	Gerhardt (Germany)
Nitrogen Dewar GT11	Air Liquide (France)
Warming cabinet	Binder (Germany)
Perthometer MarSurf 300	Mahr (Germany)

Table 2 lists all the used disposable material.

Table 2: Used Disposables

Disposables	Supplier
1.8 mL cryo tube vials, sterile	Nunc (Denmark)
2 mL pipette, sterile	Nerbe plus (Germany)
5 mL pipette, sterile	Nerbe plus (Germany)
10 mL pipette, sterile	Nerbe plus (Germany)
25 mL pipette, sterile	Nerbe plus (Germany)
2 mL Eppendorf tubes	Eppendorf (Germany)
50 mL polypropylene falcons, sterile	Sterilin (UK)
15 mL polypropylene falcons, sterile	Sterilin (UK)
75 cm2 cell culture flasks	VWR (USA)
96-well white tissue culture plate; polystyrene, flat bottom	Perkin Elmer (USA)
96-Well microtiter plates, untreated, 300 µl	Nunc™ (Denmark)
Pipette tips 20 - 200 µL	VWR (USA)
Pipette tips Diamond Tip 0.1-20 µL	Gilson (USA)
Pipette tips epT.I.P. 50-1250 µL	Eppendorf (Germany)
Reagent reservoirs	VWR (USA)
Reagent reservoirs, sterile	VWR (USA)
Weighing dishes	VWR (USA)
Disposable cuvettes Macro 4ml, visible range	VWR (USA)
20 ml glass vials G075Y-27/057-H (sample extraction)	Infochroma (Switzerland)
Petri dishes 100 x 15 mm	VWR (USA)
Disposable cell spreader	VWR (USA)

2.2 Chemicals

The cell culture media and supplements used for cultivation of the human cell line are listed below in Table 3. In the last two lines, the detection kits for the NanoSens Assay is stated.

Table 3: Used media and supplements

Cell culture media and supplements	CAS. №	Catalogue №	Supplier
0,25 % Trypsin/EDTA with phenol red	n.a.	170223	Gibco (UK)
D-MEM/High Glucose with L-glutamin and natrium pyruvate - Hyclone	n.a.	SH30243.01	Gibco (UK)
Dimethylsulfoxid	67-68-5	20-139	Merck (Germany)
Dulbeccos´s phosphate buffered saline (Ca2+/Mg2+)	n.a.	14190144	Gibco (UK)
Fetal calf serum	n.a.	16000044	Gibco (UK)
MEM NEAA: Minimum Essential Medium Non –Essential Amino Acids	n.a.	X0557-100	Biowest (France)
DMEM, high glucose, no glutamine, no phenol red (used as extraction solvent)	n.a.	31053028	Gibco (UK)
Penicillin/Streptomycin (10.000 U/mL)	n.a.	P8833	GE Heathcare Hyclone (USA)
Puromycin Dihydrochlorid	58-58-2	P8833	Sigma Aldrich (USA)
Steady-Glo® Luciferase Assay System	n.a.	E2550	Promega (USA)
Nano-Glo® Luciferase Assay System	n.a.	N1110	Promega (USA)

All the used chemicals, solvents and dyes, as well as the components for the silicone and epoxy resin are listed below in Table 4.

Table 4: Chemicals and dyes

Silicone components	**Lot №**	**Catalogue №**	**Supplier**
SILPURAN ® 2400 A	SR036475 SR046129	60063036	Wacker (Germany)
SILPURAN ® 2400 B	SR036475 SR046129	60063036	Wacker(Germany)
Epoxy resin components			
EPINAL epoxy resin systems – Resin component	18.77.73-A1.00.007	IR 77.73-A1.00	bto-epoxy GmbH (Austria)
EPINAL epoxy resin systems – Hardener component	18.77.73-B1.00.007	IH 77.73-B1.00	bto-epoxy GmbH (Austria)
Chemicals and dyes	**CAS. №**	**Catalogue №**	**Supplier**
Ethanol (≥99.9 %)	64-17-5	200-578-6	Merck (Germany)
Acetonitrile	75-05-8	9017	J.T. Baker (USA)
Dimethylsulfoxid	67-68-5	20-139	Merck (Germany)
Acetone (≥99.8%)	67-64-1	200-662-2	Merck (Germany)
Isopropanol	67-63-0	200-661-7	Merck (Germany)
Sesame Oil	8008-74-0	232-370-6	Sigma Aldrich (USA)
Olive Oil	n.a.	n.a.	Spar AG (Austria)
n-Hexane	110-54-3	203-777-6	Merck (Germany)
Acetic acid (99.8 – 100.5 %)	64-19-7	6755.1	Carl Roth (Germany)
Sodium chloride	7647-14-5	746398	Sigma Aldrich (USA)
Cinnamic aldehyde	104-55-2	W228613	Sigma Aldrich (USA)
Sudan I	842-07-9	103624	Sigma Aldrich (USA)
Methylene blue	122965-43-9	M9140	Sigma Aldrich (USA)

Table 5 lists some of the medical device samples which were extracted and tested by means of the NanoSens Assay.

Table 5: Medical device samples tested by means of the NanoSens Assay

Sample №	Type of device	Material
1	Hearing device	Acrylic resin, transparent
2	Hearing device	Silicone with coating 2 and colour (yellow, purple)
3	Plastics for denistry device	FKM, VMQ, Silicone
4	Anitmicrobial Spray	Liquid
5	implant-prototype	epoxy resin
6	Implant	Silicone
7	Implant	Silicone
8	Needle	Stainless steel
9	Implant-prototype	Epoxy resin
10	Implant-prototype	Epoxy resin
11	Implant-prototype	Epoxy resin
12	Infrared device	Plastics
13	Implant-prototype	Carbone fibre
14	Implant	PE and PP
15	Gloves	Nitrile, purple
16	Goves	Nitrile, blue
17	Gloves	Latex, white
18	Gloves	Latex, green
19	Laboratory device	Thermoplast
20	Hearing device	DLPA Acrylate
21	Hearing device	DLPA Acrylate
22	Hearing device	TPU
23	Prosthesis	Coating
24	Prosthesis	TPE, PC
25	Suture	Silicone
26	Suture	Silicone
27	Suture	PES
28	Active coal	Coal
29	Active coal	Coal
30	Dentrist device	Glass and Metals: X4613Cr, CuZn37
31	Hearing device	Silicon parts (grey)
32	Hearing device	ABS
33	Hearing device	Rubber
34	Hearing device	TPE-A
35	Hearing device	TPE-A, brown
36	Hearing device	Silicon parts (grey)
37	Hearing device	TPE-A
38	Menstrual cup	Latex
39	Menstrual cup	TPE
40	Menstrual cup	Silicone

2.3 Cell line

The reporter cell line used for the NanoSens Assay is called MDA-AREbn2. It was created by stable integration of the vector pGVL8 4xARE into the two LTR regions of the mammary gland adenocarcinoma cell line MDA-MB468 via PiggyBac transposon system.

Figure 7 shows a schematic drawing of the used vector.

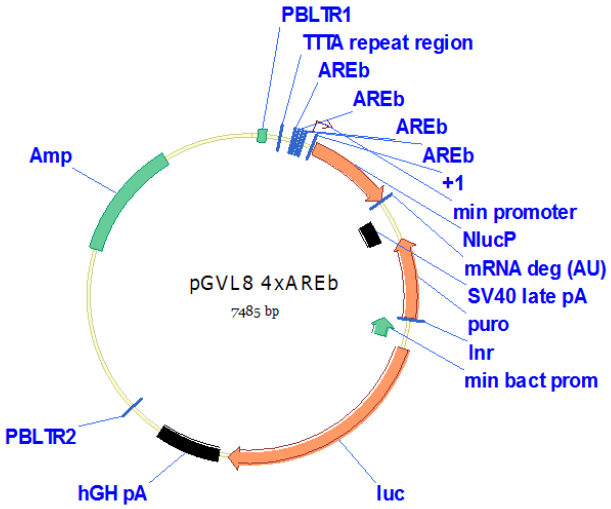


Figure 7: Vector pGVL8 4xARE of MDA-AREbn2 (Glück et al., 2015)

The transfection was performed by the working group of Thomas Czerny at the FH Campus Vienna.

The gene construct contains four AREb sequences that are located upstream of a minimal artificial promoter controlling the activity of the reporter gene luciferase NanoLuc® from Promega. This NlucP sequence serves as quantification tool for the sensitization reaction. Furthermore the vector contains a constitutively expressed firefly luciferase gene from Promega (luc), enabling the measurement of the cellular viability in the NanoSens Assay. Selection of successfully transfected cells can be carried out via puromycin, since the integrated gene construct contains the puro sequence as selection marker (Glück et al., 2015).

2.3.1 Preparation of the cell culture media

The following cultivation medium was used for passaging the cells and also served as diluting agent for the cell suspension when a NanoSens Assay was performed.

A 500 ml bottle of D-MEM/High Glucose solution was opened under sterile conditions in the laminar flow workbench. This basis medium was completed by the addition of 5 ml Minimum Essential Medium Non-Essential Amino Acids, as well as 5 ml Penicillin and Streptomycin (Pen.: 10000 U/ml, Strep.: 10000 µg/ml). Furthermore, 560 µl Puromycin (1.67 mg/mL) and 50 mL of fetal calf serum were added. After completion the medium was stored in the fridge and could be used for one month.

2.3.2 Preparation of CAH-spike solution

The 3.2 mM cinnamic aldehyde solution, used to spike the sample extracts was prepared by a dilution of a 200 mM stock solution. To achieve this concentration, a mass between 350 and 700 mg CAH (purity: 95%) was weighted into a glass vial and after calculating the required volume of solvent by Formula 2, the determined amount of DMSO was added. The resulting stock solution was stored in the fridge for about four months.

Formula 2: Calculation of the solvent volume for solid and liquid pure substances

$$V_{solvent} = 5 * (\frac{purity * mass\ [mg]}{molar\ mass\ \left[\frac{mg}{mmol}\right]} - \frac{mass\ [mg]}{1000})$$

To achieve the CAH working concentration of 3.2 mM, a certain volume of 200 mM stock solution was diluted with a certain volume of PBS according to Formula 3.

Formula 3: Calculation for diluting the stock solution

$$c1 * V1 = c2 * V2$$

c1 = concentration of solution 1 [mM]
c2 = concentration of solution 2 [mM]
V1 = Volume of solution 1 [ml]
V2 = Volume of solution 2 [ml]

3 Methods

3.1 Cell culture work

The following subchapters describe the routine work which was carried out to culture the MDA-AREbn2 cell line. The conditions during incubation were 37 °C with addition of 5 % CO_2 .

3.1.1 Cultivation of the cell line MDA-AREbn2

The cell line MDA-AREbn2 was cultivated in DMEM high glucose medium, completed by additional supplements as described in 2.3.1.

Thawing of cells

The cells used for the NanoSens Assay were stored in cryo vials surrounded by liquid nitrogen at a temperature of -196 °C. After taking out a frozen vial from the dewar it is placed into a 37 °C preheated water bath for some minutes. When the cell suspension was completely thawed, the vial was inverted ten times in order to obtain homogenization.

Under sterile conditions in the lamina flow workbench 1 ml of this cell suspension was transferred into a 75 cm^3 tissue culture flask containing 20 mL of the cultivation medium. For this step, a 2 ml pipette was used to minimize shear forces.

Afterwards the cells were incubated for 24 h in the CO_2 – incubator at 37 °C to attach to the culture flask before the medium was changed.

The following two weeks the cells were passaged when the surface was covered to 80 % in order to maintain their exponential growth. This state was reached usually once or twice a week.

After this equilibration time the cells could be used for the NanoSens Assay.

Passaging of cells

The cell line MDA-ARElbn2 was passaged at a confluence of 80 - 90 %.

For this purpose the medium in the culture flask was discarded before cells were washed with 10 ml Dulbecco's phosphate buffered saline (DPBS).

In order to detach the adherently growing cells from their surface, 1 ml of 0.25 % trypsin-EDTA with phenol red was added and distributed evenly throughout the whole flask. This enzymatic reaction step was carried out in the CO_2- incubator at 37 °C and took about seven minutes.

After that time detachment of the cells was supported by tapping vigorously the side of the flask. The success of the trypsinization process step was then examined under the microscope and could be indicated through smear formation in the flask. Addition of 4 ml culture medium then stopped the enzymatic reaction. This volume was then used to carefully rinse the inner surface of the flask in order to flush as many cells as possible from the plastic wall into the suspension. Pipetting up and down several times ensured the formation of a homogenous suspension without major cell clumps.

Afterwards an aliquot was transferred into a new flask containing 20 mL of fresh medium. The volume of this aliquot depended on the confluence of the cells but usually a split of 1:2 to 1:5 was carried out. The cells were then cultivated at 37 °C in the CO_2- incubator until their next passage.

Freezing of cells

Cell aliquots can be frozen between their second and fifth passage for later use.

For this purpose, cryo vials were precooled to -20 °C about 24 h prior to the freezing process.

The freezing medium consisted of 50 ml DMEM complemented with 20% FCS and 10 % highly pure DMSO. In total, three to five culture flasks with a cellular coverage of 80 - 90 % were needed.

First the culture medium was removed before 1 ml of 0.25 % trypsin – EDTA with phenol red was applied and distributed evenly throughout the whole flask. During the following incubation time of about seven minutes at 37 °C in the CO_2- incubator, the enzyme caused detachment of the cells from the plastic surface. This catalytic reaction was then stopped by the addition of 4 ml culture medium.

By pipetting the medium up and down and rinsing it over the inner flask surface, a homogenous cell suspension could be obtained.

In order to achieve a high cell density the full 5 mL of this suspension were transferred into the second culture flask by simultaneously stopping the trypsinization process there.

As in the previous step the cells were rinsed from the surface and transferred to the next culture flask. This procedure was repeated until the cells from all flasks were combined in one suspension.

The resulting suspension was then transferred into a centrifuge tube of which an aliquot of 200 µl was taken for the subsequent cell count procedure to examine the number of cells per ml as described below.

Afterwards, the cell suspension was centrifuged at 125 g for five minutes. While the supernatant was then discarded, the cell pellet was resuspended in the freezing medium to reach a final concentration of 10^6 cells per mL.

By using a 2 ml pipette 1 mL the cell suspension was aliquoted into a precooled cryo vial. These vials were then stored at -80 °C before transferring them into the liquid nitrogen dewar about one to three days later.

Determination of cell number

Knowing the average cell density of a suspension is essential when preparing a dilution in order to seed cells into microtiter plates. This determination of the cell number was performed by using a Neubauer chamber shown in Figure 8.

It is a specialized microscope slide in which two grids are engraved, each one forming a counting chamber.

The cover glass was fixed on the slide with 95 % ethanol and about 10 µl of the homogenous cell suspension were pipetted into the narrow aperture between the glass slides. Capillary forces drive the fluid into the chamber consisting of nine small squares with inner dimensions of 1 x 1 x 0.1 mm which therefore comprise a volume of 0.1 µl each.

After counting the cell number in four large squares under the microscope and calculating the mean, the cell density [cells/ml] was determined by multiplying this value by 10^4.

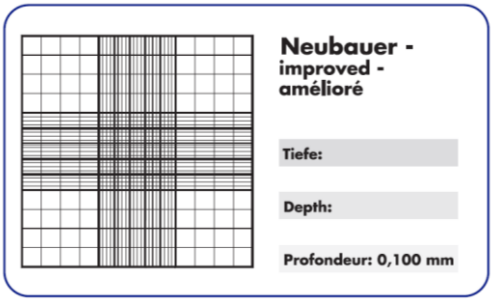


Figure 8: Neubauer chamber for cell counting

3.2 NanoSens Assay

The NanoSens Assay was performed using the reporter cell line MDA-AREbn2 described in 2.3. One assay run could be completed within two days after the cells have been passaged at least twice.
In the following, the preparation of the 96 well plates, the assay performance as well as the measurement of cell viability and fold induction are stated.

3.2.1 Seeding of the plates

The first day was devoted to the preparation of the 96 well plates which are the reaction chambers of the assay. One plate provides space for two extracts. Most of the medical device samples were tested in duplicate per extraction agent therefore usually one plate was needed for each sample and solvent. All preparation steps except the microscopical cell counting were carried out in the laminar flow workbench.

The total volume of cell suspension needed for one assay run was calculated using the following Formula 4 complemented by a safety factor.

Formula 4: Calculation of the needed cell suspension volume for seeding

$$V_{total} = 100\ \mu l * 96 \frac{wells}{plate} * number\ of\ plates * 1.2$$

The cells were harvested at a confluence of 80-90 % before their density was determined by using a Neubauer chamber as described in 3.1.1.4.
Formula 5 shows the calculation of the conversion factor used to achieve the required cell concentration. The mean value $\overline{(x)}$ obtained by counting four squares of the Neubauer chamber was inserted into the equation.

Formula 5: Calculation of the conversion factor for cell number adjustment

$$Conversion\ factor = \frac{10^5\ cells/ml}{\bar{x} * 10^4\ cells/ml} = \frac{1}{\bar{x} * 0{,}1}$$

The cell suspension was then diluted aiming the total volume (V_{total}) with a final concentration of 10^5 cells per ml. The calculation of the needed initial cell suspension volume obtained after cell harvest is demonstrated in Formula 6.

Formula 6: Calculation of initial cell suspension volume

$$V_{initial\ cell\ susp.} = \frac{V_{total}}{\bar{x} * 0{,}1}$$

Dilution was performed by adding culture medium (V_{medium}) whereby subtraction of the initial cell suspension volume ($V_{initial\ cell\ susp.}$) from the total one (V_{total}) gives the required volume of diluent. (Formula 7)

Formula 7: Calculation of the required volume of diluent

$$V_{medium} = V_{total} - V_{initial\ cell\ susp.}$$

Afterwards the 96 well plates were seeded by pipetting 100 µl into each well, except for well H12 serving as blank. Subsequently the plates were incubated for 24 h ± 1 h at 37 °C in the CO_2 incubator in order to give the cell enough time to attach to the bottom of the wells.

3.2.2 Dilution of the extracts

After extracting the samples according to chapter 3.3 the resulting extracts were stored in the fridge until their later use. In the meantime, during incubation of the cell culture plates the extract dilutions were prepared.

Each sample was tested in six dilution steps.

For PBS extracts the series started with a dilution factor of 1:50 continued by five 1:2 dilution steps resulting in the lowest concentrated 1:600 dilution. To this end, five Eppendorf tubes (hereafter called “eppis”) were filled with 500 µl of PBS with 1 % DMSO and a further one with 980 µl of the same diluent. The latter one was supplemented by 20 µl of the extract to result in the 1:50 dilution as starting point. Afterwards a 1:2 dilution series was created by continuously pipetting 500 µl of the prior prepared dilution into the next eppi containing the same amount of diluent. In the last dilution step 500 µl of the liquid was discarded to end up with the same volume in each eppi. This was important to achieve the same concentration of spike per sample in a later step.

DMSO extract dilutions ranged from 1:100 to 1:3200 as prior experiments demonstrated the highest cell-tolerated DMSO concentration of 1 %.

The 1:100 starting dilution was prepared by adding 10 µl DMSO extract to an eppi containing 990 µl of pure PBS. The following steps were identical to the dilution of PBS extracts.

After the incubation time of 24 h the medium was removed from the 96-well plates and each cell containing well was covered with 50 µl of respective dilution. In order to end up in a three-fold determination each extract dilution was applied onto three wells. The remaining sample volume was then spiked with cinnamic aldehyde (CAH) which has already been proven a skin sensitizing substance. Testing of spiked extract dilutions on the 96-well plates is important to verify whether the NanoSens Assay is also able to detect positive substances in negative samples to exclude false negative results.

For this purpose a prior calculated volume of the 3.2 mM CAH spike solution (preparation see 2.3.2) was added to each extract dilution in order to result in a CAH concentration of 32 µM by performing a 1:100 dilution.

Afterwards, 50 µl of this spiked extracts were pipetted in three-fold determination into the plate. The blank for the NanoSens Assay was made up of the extraction agent incubated without sample but under the same conditions as the sample extracts. It was diluted 1:100 with PBS and again a final DMSO concentration of 1 %.

As positive control 50 µl of PBS with 1 % DMSO supplemented with 1 % CAH (≙ 32 µM) were applied in five wells of the NanoSens plate. Six wells were further inoculated with each 50 µl of PBS with 1 % DMSO serving as additional negative control to the blank.

Figure 9 demonstrates the pipetting scheme of the NanoSens plates. The rows A-C contain the extract dilutions whereas rows E-G are intended for the spiked ones. Each half plate is reserved for one extract. Well H12 does not contain any cells.

After each well was filled with its respective dilution the plates were covered with a sealing film before incubating them for 6 h (± 0.5 h) at 37 °C in the CO_2 incubator.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1:1600	1:800	1:400	1:200	1:100	1:50	1:1600	1:800	1:400	1:200	1:100	1:50
B	1:1600	1:800	1:400	1:200	1:100	1:50	1:1600	1:800	1:400	1:200	1:100	1:50
C	1:1600	1:800	1:400	1:200	1:100	1:50	1:1600	1:800	1:400	1:200	1:100	1:50
D	Blank						Blank + spike (32 µM CAH)					
E	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$
F	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$
G	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$
H	PBS + 1 % DMSO						Positive control (32 µM CAH)					-

	1	2	3	4	5	6	7	8	9	10	11	12
A	1:3200	1:1600	1:800	1:400	1:200	1:1000	1:3200	1:1600	1:800	1:400	1:200	1:1000
B	1:3200	1:1600	1:800	1:400	1:200	1:1000	1:3200	1:1600	1:800	1:400	1:200	1:1000
C	1:3200	1:1600	1:800	1:400	1:200	1:1000	1:3200	1:1600	1:800	1:400	1:200	1:1000
D	Blank						Blank + spike (32 µM CAH)					
E	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$
F	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$
G	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$	1:1600$^+$	1:800$^+$	1:400$^+$	1:200$^+$	1:100$^+$	1:50$^+$
H	PBS + 1 % DMSO						Positive control (32 µM CAH)					-

Figure 9: Pipetting scheme of NanoSens plates. At the top for PBS- and at the bottom for DMSO-extracts

3.2.3 Measuring of viability

After the reaction time of six hours the plates were ready for the measurement of activity. First the viability of the cells was determined by adding the Steady-Glo® solution. This step delivers luciferin being the substrate for the constitutively expressed firefly luciferase resulting in a mono-oxygenation reaction in presence of Mg^{2+}, ATP and molecular oxygen. A further product besides oxyluciferin is light which is emitted during this enzymatic step and can be detected via luminescence measurement.

Formula 8 and Formula ***9*** demonstrate the calculation of the volume of buffer and mass of GloMix powder that is needed to prepare the Steady-Glo® solution.

Formula 8: Calculation of the volume of Steady-Glo® buffer required [ml]

$$V_b = 0.6 \frac{ml}{plate} * number\ of\ plates$$

Formula 9: Calculation of the mass of GloMix substrate needed [g]

$$m_{GloMix} = 0.01541 \frac{g}{ml} * V_b$$

The required amount of GloMix was weighted using an analytical balance and afterwards dissolved in the determined volume of buffer. This solution was then filled up with a certain volume (Formula 10) of ultrapure water to obtain the viability measurement solution.

Formula 10: Calculation of the volume of ultrapure water needed for the measurement solution

$$V_{H_2O} = 5.4 \frac{ml}{plate} * number\ of\ plates$$

In the next step 50 µl of this measurement solution were added onto each well before incubating the plates for five minutes on the shaker at 300 rpm and room temperature. Subsequently luminescence measurement was performed on the multilabel plate reader using the program Walluc 4500. The total emitted light provided information about the viability of the cells.

3.2.4 Measuring of induction

The second measurement of the dual luciferase assay was the determination of the sensitization by quantifying the induction of the NanoLuc® luciferase gene.
For this purpose a measurement solution was prepared by combining two liquid compounds of the NanoLuc® test kit followed by a 1:10 dilution step with ultrapure water according to Table 6.

Table 6: Volumes required for the induction measurement solution

	Volume [ml/plate]
$V_{substrate}$	0.012
V_{buffer}	0.588
V_{H2O}	5.4

Afterwards 50 µl of this solution was pipetted in each well of the NanoSens plates before incubating them again for five minutes on the shaker at 600 rpm and room temperature.
The multilabel plate reader measured subsequently the emitted light of the luminescence reaction.

3.2.5 Evaluation

In order to classify the tested samples as sensitizing or non-sensitizing it is indispensable to define thresholds and acceptance criteria. The following lines demonstrate the way how the measured viability and induction data were evaluated in more detail.

The most significant value to assess the degree of sensitization of the cells is the fold induction (FI). It was calculated as the ratio between the relative light intensity of the sample to that of untreated PBS with 1 % DMSO being the negative control. A sample, causing an fold induction greater than 2 was classified as positive, meaning that it activates the ARE-Nrf2 pathway in a significant manner.
Conversely, the luciferase activity induction value of used blanks and all negative controls, including that of the pure extraction agent must never exceed this threshold of 2 in order to consider the test as valid. Hence, it follows that the positive control containing 32 µM of the skin sensitizer CAH must show a fold induction greater than 2.
Extracts and chemicals were therefore categorized as skin sensitizers if the EC2 (effective concentration) exceeded the threshold of 2 and a clear dose-response was obtained. This means a signal intensity that increases continuously and ideally linearly with increasing extract concentration until toxic effects of the cells are noticeable.
However, a minimum of 70 % cell viability has to be given in order to make a statement about the sensitization potential of a sample.

The sample extracts which have been applied on the 96-well plates after spiking them to a final CAH-concentration of 32 µM should exclude false negative results caused by effects of the sample matrix.
The FI value of these spiked sample dilutions should therefore achieve a similar overall value above 2.
The lowest ineffective dilution (LID) corresponds to the lowest concentrated dilution stage that reaches a fold induction greater than 2. A sample resulting in a LID ≤ 0.005 was classified as „sensitizing" due to the fact that the internal threshold of a non-sensitizing sample is 0.01.

3.3 Extraction of medical devices for the NanoSens Assay

The NanoSens assay succeeds as the first method of its kind to use extracts of medical devices instead of pure chemicals as test substance.

Therefore, in order of evaluating the skin sensitizing potential of a medical device, it has to be extracted according to DIN ISO 10993-12:2012. This norm describes the defined standards for sample preparation and reference materials, but still leaves some space for the choice of extraction parameters.

3.3.1 Regulations for the extraction procedure prescribed by the norm

This norm addresses regulations for the preparation of sample extracts. The specifications and conditions defined in this section are described in the following three subchapters.

Extraction vessels

Extraction has to be performed in clean, chemically inert and closed vessels with minimum dead space. To prevent distortion of the extracts by the vessel, the use of borosilicate glass vials with inert sealing is proposed. Alternatively other inert extraction vessels can be used.

Extraction conditions and procedures

The extraction has to be performed according to one of the following temperature and incubation time conditions.

a) (37 ± 1) °C for (72 ± 2) h;
b) (50 ± 2) °C for (72 ± 2) h;
c) (70 ± 2) °C for (24 ± 2) h;
d) (121 ± 2) °C for (1 ± 0.1) h;

Extraction should be performed by circulation or movement, otherwise it has to be documented and justified. The ratio between sample surface and volume of extraction agent has to be chosen according to Table 7.

Table 7: Defined extraction standards for the biological evaluation of medical devices and materials (DIN ISO 10993-12:2012)

Thickness [mm] or shape of solid samples	Extraction ratio (surface area or mass/volume) ± 10 %	Examples of materials
< 0.5	6 cm^2/ml	Films, metal sheets, tube walls
0.5 – 1.0	3 cm^2/ml	Tube walls, plates, small injection moulded parts
> 1.0	3 cm^2/ml	Large injection moulded parts
> 1.0	1.25 cm^2/ml	Elastomeric seals
Irregular solid samples	0.2 g/ml	Powders, beads, foam, non-adsorbable injection moulded parts
Irregular porous samples (materials with low density)	0.1 g/ml	Membranes, textiles

Extraction agents

The use of polar and non-polar solvents is mandatory. As examples for polar extraction agents water, physiological saline solution or culture medium without FCS are mentioned. A non-polar one could be for example freshly refined oil such as sesame oil or other organic solvents including alcohols, hexane, acetonitrile and others.

3.4 Comparison of extraction parameters

The main aim of this thesis was to determine the impact of varied parameters in conjunction with the extraction process on the quality and properties of the final extract.
For this purpose reporter substances were incorporated into commonly used medical device materials as described below in chapter 3.5.
These supplemented materials served as basis for the following extraction experiments. By varying certain parameters during sample extraction listed in 3.4.1 the main influencing factors could be revealed.

The materials used for these experiments were medical silicone, epoxy resin, TPU polymer and acrylic resin.
The extraction experiments in this thesis focussed on two main parts. On one hand, medical device materials were spiked with a proven skin sensitizing substance before evaluating the resulting extracts in the NanoSens Assay. In the second part, relevant materials were spiked with azo dyes. Afterwards the extracts, obtained from those coloured samples were then compared photometrically.

Biochemical evaluation by the *in-vitro* NanoSens Assay

In the first part the portioned silicone and epoxy resin samples spiked with the skin sensitizer cinnamic aldehyde as described in 3.4.1 were exposed to a parameter-varied extraction process.
For this purpose individual samples underwent the usually applied standard extraction procedure (see 3.2.2) whereas further ones were extracted by varying single parameters of the process.
In order to assess the effect of certain extraction conditions the resulting extracts were then tested in the NanoSens Assay as described in 3.3.

Photometric evaluation

The second part dealt with the silicone, epoxy resin, TPU polymer and acrylic resin spiked with Methylene blue and Sudan I (see 3.4.2) aiming to mimic potential skin sensitizing substances.
These samples were then treated analogous to the CAH-spiked samples described in the previous paragraph. Afterwards, the coloured extracts resulting from the standard- and the modified extraction process were measured photometrically at the absorption maximum of the respective dye. The intensity of the coloured fluid was evaluated as a direct measurement of the amount of potential skin sensitizer migrated into solution.

3.4.1 Extraction parameters

This chapter contains a detailed description of all parameters which were tested and varied in order to determine the major influence factors that affect the amount of sensitizing substance extracted out of the respective medical device materials.

All extraction experiments were performed in double determination. The total extraction time for all experiments was constantly 72 h according to DIN ISO 10993-12:2012.

Table 8 summarizes all tested parameters with indication of its respective method of evaluation.

Table 8: Summary of the tested parameters with their used evaluation method

Tested parameter	**Evaluation method**	
	NanoSens Assay (CAH spike)	**Photometer** (dye spike)
Rotation speed of shaker	x	
Extraction vessel	x	
Extraction agent		x
Medical device material		
➢ Medical silicone	x	x
➢ Epoxy resin	x	x
➢ TPU polymer		x
➢ 3D printed acrylic		x
Sample cutting surface	x	x
Foil extraction		x
Time course	x	x
Temperature		x

Standard procedure

1A This sample served as a reference as it has been extracted according to the standard procedure used for the NanoSens Assay described in 3.2.2. This implies that the sample was cut by creating a smooth cutting edge before extracting it in an inert glass vial (20 ml) in the respective volume (see table 6) of PBS and DMSO. Shaking was performed at a rotation speed of 110 rpm at 37 °C for 72 h.

Rotation speed of shaker

1B Sample incubated in a shaker at 175 rpm

1C Sample incubated without shaking

Extraction vessel

1D Standard glass vial (20 ml) incubated in horizontal orientation

1E Centrifugation tube (15 ml)

1F Centrifugation tube (50 ml)

1G Long glass vial (40 ml) incubated in horizontal orientation

1H Standard glass vial (20 ml) incubated with Eppendorf tube

Figure 10 shows a schematic drawing of the extraction vessels which were used and compared.

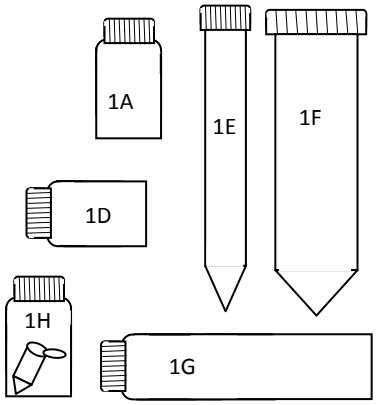


Figure 10: Schematic drawing of the compared extraction vessels

Extraction agents

The following set of polar and non-polar solvents was tested and compared for their use as extraction agent and is arranged according to increasing polarity.

- 0.9 % NaCl
- RO-H_2O
- 3 % Acetic acid (HAc)
- Phosphate buffered saline (PBS)
- Culture medium
- Culture medium with FCS
- 20 % ethanol
- 50 % ethanol
- 95 % ethanol
- Isopropanol (IPA)
- Acetonitrile (ACN)
- Dimethylsulfoxid (DMSO)
- Acetone/Olive oil - 4:1 (v/v)
- Olive oil

Sample cutting surface

2A The thick-layered sample was cut into pieces with a rough cutting edge by using a Stanley knife. All the other conditions were kept constant.

Saturation effect

Samples were extracted in both, the 1-fold and the 5-fold volume with regard to the prescription according to Table 7. This was performed to examine whether a saturation effect takes place when extracting a material in a certain solvent. Excluding that suspicion was important to prevent limitation of the chemical migration and to ensure reliability of these experiments.

Foil extraction

In this experiment the extract of a small piece of spiked silicone foil was compared to that of a large piece crumpled tightly into a vial as illustrated in Figure 11. The aim was to determine whether a packed bundle of foil is less accessible to the surrounding extraction agent than a planar sample.

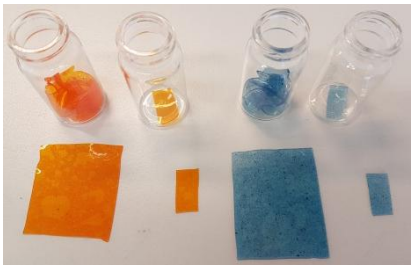


Figure 11: Dye-spiked silicone foil.

Time course

The extraction process was monitored photometricall by hourly taking an aliquot of the coloured solution and measuring its absorption intensity afterwards.

Additionally CAH-spiked silikone samples were evaluated in the NanoSens skin sensitization assay at regular intervals to reveal the degradation speed of the contained sensitizer in the sample.

Materials

Medical device materials were spiked with a sensitizer and azo dyes according to chapter 3.4.

Temperature

The dye-spiked samples were extracted by adjusting the incubation temperature to 50 °C.

3.5 Preparation of spiked materials

In order to evaluate influences of the tested extraction parameters (stated in 3.4.1) on the amount of sensitizers and other low molecular substances migrating out of the material, medical silicone, epoxy resin, TPU polymer and acrylic resin were spiked on one hand with skin sensitizers and on the other hand with azo dyes. The spiking procedures are described in the following subchapters.

3.5.1 Spiking with sensitizers

For this experiment silicone and resin were spiked with 0.1 % of the sensitizer cinnamic aldehyde. This concentration had been shown in a previous thesis to be most suitable since it does not prevent the curing process of the materials and can be detected in the NanoSens Assay (Auer, 2017).
The pure substance cinnamic aldehyde which was as well used as positive control in the NanoSens Assay was chosen as spiking reagent. It has been hypothesized as a "prohapten" that can be metabolically activated to the protein-reactive and skin sensitizing cinnamaldehyde
(Smith, Moore, Elahi, Smart, & Hotchkiss, 2000).

Spiking of silicone

The medical silicone used for the extraction experiments was SILPURAN 2400® from Wacker Chemie GmbH. It is composed of two components, whereas one of them contains a platinum catalyst ensuring the start of the polymerisation reaction in order to form a solid material. Table 9 lists some properties of the used silicone.

Table 9: Selected properties of cured SILPURAN 2400® by Wacker Chemie GmbH

Property	Inspection method	Value
Hardness Shore A	ISO 868	7
Hardness Shore 00	ASTM 2240/ Type 00	55
Tensile strength	ISO 37	2.00 N/mm^2
Elongation at break	ISO 37	600 %
Tear strength	ASTM D 624 B	3.0 N/mm
Test for sensitization via LLNA According to ISO 10993-10	Test of extractables from elastomer in acetone/olive oil	No sensitization

The raw components A and B had to be mixed in a ratio of 1:1. First the required mass of cinnamic aldehyde, needed to end up with a concentration of 0.1 % was weighted into a beaker glass. This was then followed by the addition of the appropriate mass of both silicone components.
After stirring the mixture with a disposable cell spreader to ensure a homogenous distribution of the CAH, it was poured into petri dishes by producing both thin and also thick layers of silicone. After a curing time of 24 h the samples were ready for use. The silicone layers shown in Figure 12 were then cut into small pieces of defined size in order to allow extraction with the desired surface-volume ratio. The thin-layered samples shown on the left hand side of Figure 12 were cut by using a scissors to create a smooth edge. Those illustrated on the right hand were processed with a Stanley knife enabling to get thick pieces with a rough cutting edge.

Figure 12: CAH-spiked silicone samples before cutting; left: thin layer for smooth cutting surface; right: thick layer for rough cutting surface

Spiking of epoxy resin

The two-component epoxy resin used was the system IR 77.73 provided by bto-epoxy GmbH. The following Table 10 lists some properties of the cured material.

Table 10: Selected properties of cured epoxy resin by bto-epoxy GmbH

Property	Cured resin IR 77.73
Density [g/cm^3]	1.000 – 1.200
Viscosity [mPas]	1.500 - 3.500
Mixing ratio	100:21
T'g after tempering	102

The spiking procedure of the resin was performed analogous to that of the silicone with the exception of the different mixing ratio of 100:21. Furthermore a part of the mixture was poured into petri dishes as well while the rest was filled directly into the extraction vessels as it was not possible to strip away thin epoxy resin layers from a surface without destroying them.

After the curing time of about 24 h the spiked epoxy resin samples were ready for use (Figure 13). The thick layers which were poured into petri dishes were then cut with a mechanical device whereas those, directly filled into the vessels could be exposed directly to the extraction agent.

Figure 13: CAH-spiked epoxy resin samples before cutting; left: thick layer for rough cutting surface; right: thin layer directly poured into extraction vessels

3.5.2 Spiking with azo dye

For the second part of the extraction experiments two azo dyes have been chosen.

The two dyes used show opposite polarity behaviour enabling to cover a wide range of possible sensitizers. Molecules that bind covalently to skin proteins and therefore may trigger the AOP (see 1.2.1) before resulting in an allergic reaction usually are substances with low molecular weight. Due to their small size such chemicals are also very likely to migrate out of a material when it comes into direct contact with a favourable liquid, e.g. sweat on the skin.

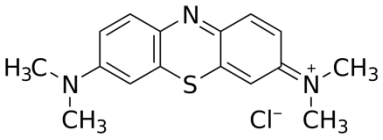
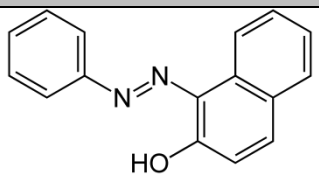
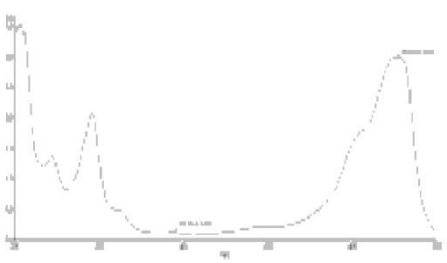
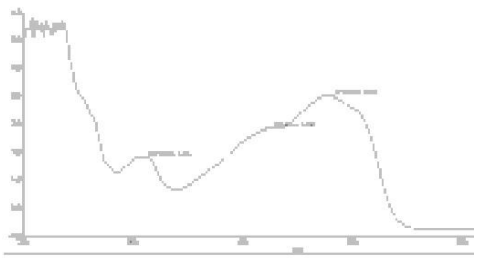
Methylene blue and Sudan I are low molecular dyes mimicking potential skin sensitizers and were therefore used as reporter substances during medical device extraction.

Furthermore, Kgzuka et al., (1980) revealed that Sudan I is a potent sensitizer with the ability of causing contact sensitivity. Evaluation in this study was performed by patch testing which is a non-invasive skin test for identifying possible skin allergens.

Methylene blue is a common dye used in medical devices.

Table 11 lists some selected properties of the used azo dyes.

Table 11: Selected properties of the used azo dyes (data and figures from wikipedia.org)

	Methylene blue (MB)	**Sudan I (SI)**
Structure		
Chemical formula	$C_{16}H_{18}ClN_3S$	$C_{16}H_{12}N_2O$
Molar mass	319.85 g/mol	248.28 g/mol
Solubility in H_2O	50 g/L (20 °C)	0.5 g/L (30 °C)
Solubility in EtOH	10 g/L (20°C)	Soluble (no values available)
	More soluble in polar solvents	More soluble in non-polar solvents
Absorption max.	664 nm	418/ 476 nm
Absorption spectrum		

Spiking of silicone

The required mass of dye was weighted into a beaker glass before about 1 ml of solvent was added to support dissolving of the substance. (Water for MB, Ethanol for Sudan I). The further spiking and cutting procedure was performed analogous to that of the CAH-silicone in 3.5.1 with the exception of using 0.1 % dye instead of 0.1 % CAH. The resulting coloured silicone samples can be seen in Figure 14.

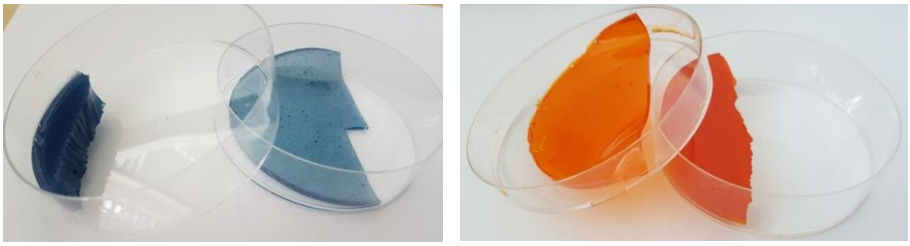


Figure 14: Dyed silicone samples before cutting; thin layers for smooth and thick layers for rough cutting surface; left: Methylene blue; right: Sudan I

Spiking of epoxy resin

The spiking and cutting procedure was analogous to that of the CAH-epoxy resin in 3.4.1. with the exception of using 1 % dye instead of 0.1 % CAH which was again dissolved in 1 ml solvent prior to the addition of the two resin components.

Furthermore, after pouring the liquid and coloured epoxy resin into petri dishes and extraction vessels the samples underwent an additional hardening step. For this purpose the resin layers in the petri dishes were cut into pieces before exposing them together with the resin-containing vessels to the heat cabinet for 3 h at 80 °C followed by 4h at 140 °C. This tempering process had been shown before to be necessary in order to prevent dissolving of the cured epoxy resin when exposing it to DMSO for several hours. Figure 15 illustrates the coloured and tempered resin samples.

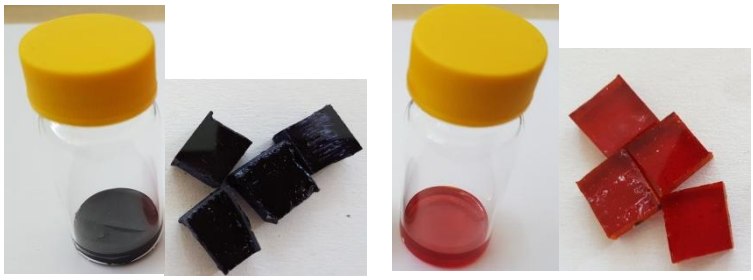


Figure 15: Dyed epoxy resin samples after cutting and tempering; thin layers in vial for smooth, and thick pieces for rough cutting surface; left: Methylene blue; right: Sudan I

Spiking of TPU polymer

Methylene blue and Sudan I were further incorporated in a polymer called thermoplastic polyurethane (TPU). This procedure and the resulting samples were kindly performed and provided by Sonova AG, a Swiss company specialized for the development and production of innovative hearing instruments.

The production of the spiked TPU started by dissolving the polymer in Tetrahydrofuran (THF) at 40°C before 1 wt% of the respective dye was added. The coloured material was then evaporated in a petri dish at room temperature for 24 h and can be seen in Figure 16. The resulting foils were cut in smaller pieces and used for extraction experiments by using different solvents as described in a following chapter.

Figure 16: Dye-spiked TPU polymer samples provided by Sonova

Spiking of 3D printed acrylic shell

Sonova AG as well prepared and provided a second commonly used medical device material spiked with the two azo dyes. The used acrylic is UV curing, normally transparent and has been 3D-printed into the shape of hearing device shells.

For the preparation of the acrylic shells the respective dye was mixed into the liquid acrylic resin before the 3D printing process was started. The dye concentrations which were feasible were 0.13 wt% for Methylene blue and 0.123 wt% for Sudan I. The shells shown in Figure 17 were used directly for extraction experiments.

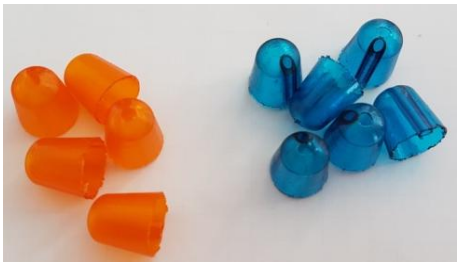


Figure 17: Dye-spiked and 3D-printed acrylic shells provided by Sonova

3.6 Measurement of surface roughness

Some of the silicone and resin samples as shown in Figure 14 and Figure 15 were cut by creating a rough cutting surface. This allowed to evaluate the impact of the roughness of the sample's cutting surface on the leaching behaviour.

In order to get estimation on the magnitude of this roughness the samples underwent the profile method according to ÖNORM EN ISO 4287. For this purpose a perthometer was used to measure a section of 12.5 mm of each sample.

3.7 Trypan blue mixing experiment

This experiment aimed to reveal the time a given substance needs to be completely mixed with its surrounded solvent. Furthermore, the influence of the used vessel and the shaking velocity during the mixing process were examined. For this purpose 3 µl of trypan blue were dropped into 3 ml PBS prepared in the respective vessel as illustrated in Figure 18.

Subsequently the time was measured until the liquid was coloured homogenously in order to be able to make an additional statement on the influence of extraction vessel and rotation speed.

Figure 18: Setup of the mixing experiment after dropping trypan blue into the solvent

3.8 Construction of a dye calibration curve

In order to determine the dye concentration in the resulting extracts when measuring the absorption intensities a calibration curve has been constructed. For this purpose a 1 mg/ml stock solution of Sudan I and Methylene blue was prepared by dissolving the respective dye in an appropriate solvent. (Sudan I in 95 % ethanol, Methylene blue in RO-water)

The stock solutions were serially diluted down to 0.001 mg/ml. After photometric measurement of those dilution steps a calibration curve was created and the linear range determined.

Absorption measurement was performed at 490 nm for Sudan I and 650 nm for Methylene blue.

4 Results

Through evaluating and comparing different extraction parameters that may have an impact on the finally obtaining extract of a medical device, it was aimed to reveal the optimized extraction process for samples tested in the NanoSens skin sensitization assay.

The resulting sample extracts obtained by varying those parameters which are summarized in Table 8, were evaluated by using two different approaches.

After the respective extraction process, cinnamic aldehyde spiked materials were evaluated by the NanoSens Assay itself and dye spiked materials in turn photometrically by absorption measurement. In order to allow quantification of the dye leached from its sample matrix the calibration curve shown in chapter 4.1 was generated.

Further, the influence of shaking during extraction was additionally assessed in the trypan blue mixing experiment described in 3.7.

4.1 Calibration of the dye solutions

The dilution series for each of the two reporter azo dyes ranging from 0.001 to 1 mg/ml was measured photometrically on the respective absorption maximum and is shown in Figure 19.

The resulting curve allows determination of the linear measurement range of the photometer for each dye which is stated in Table 12. Each extract was diluted until its absorption was in a linear range. Table 12 further contains the two resulting calibration curves used to calculate the dye concentrations of the extracts.

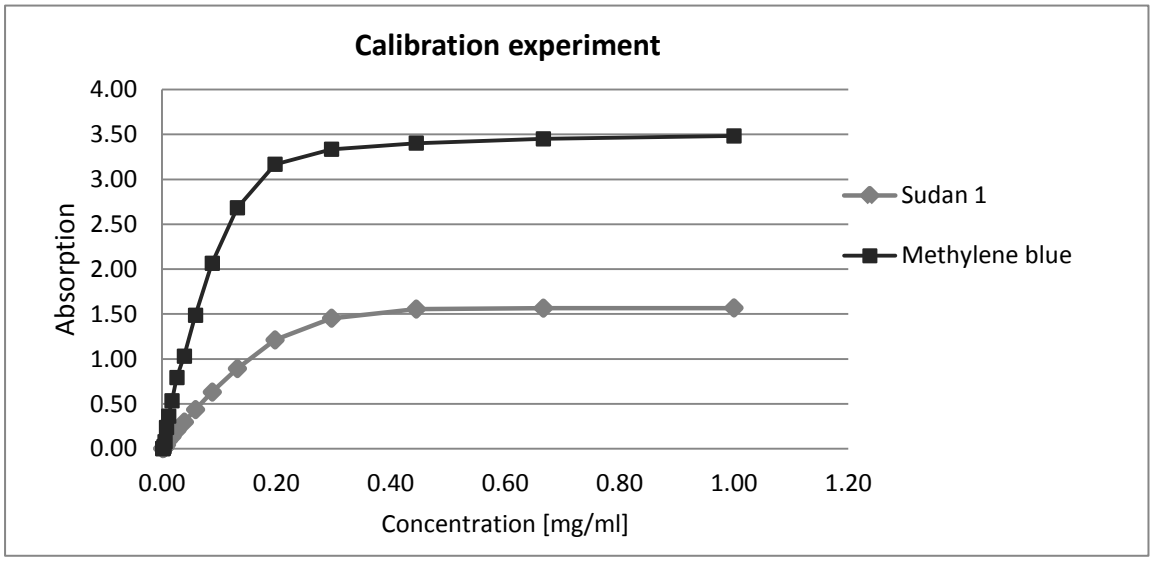


Figure 19: Measured absorption values of the dye dilution series for determining the linear range of the photometer. Standard deviations are indicated.

Table 12: Linear range of photometric dye absorption measurement and resulting calibration equations

Azo dye	Linear concentration range [mg/ml]	Linear absorption range	Calibration equation
Methylene blue	0.002 - 0.132	0.006 - 2.682	y = 21,216x + 0,0912
Sudan 1	0.001 - 0.198	0.001 - 1.21	y = 6,4165x + 0,0124

4.2 Exclusion of saturation effects

This experiment was performed to exclude the suspicion of an eventually occurring saturation effect. Saturation would result in a limitation of the leaching extent of the dye-spiked samples implying distortion of the ascertained results.

The dark blue bars in Figure 20 and Figure 21 show the concentration of the extract when using the prescribed volume according to the norm whereas the light blue ones indicate the concentration when the 5-fold volume is used. The grey bars indicate the concentration corrected by the factor 5 in order to be able to compare the values with those obtained through the standard extraction volume as prescribed and stated in Table 7.

Both figures show that the peak heights of the grey bars are similar to those of the dark coloured ones. The same experiment was also performed with the epoxy resin samples with similar results that are not shown.

It can therefore be said that no saturation effect takes place when extracting a biomaterial in any of the tested solvents and thus reliability of the following experiments can be confirmed.

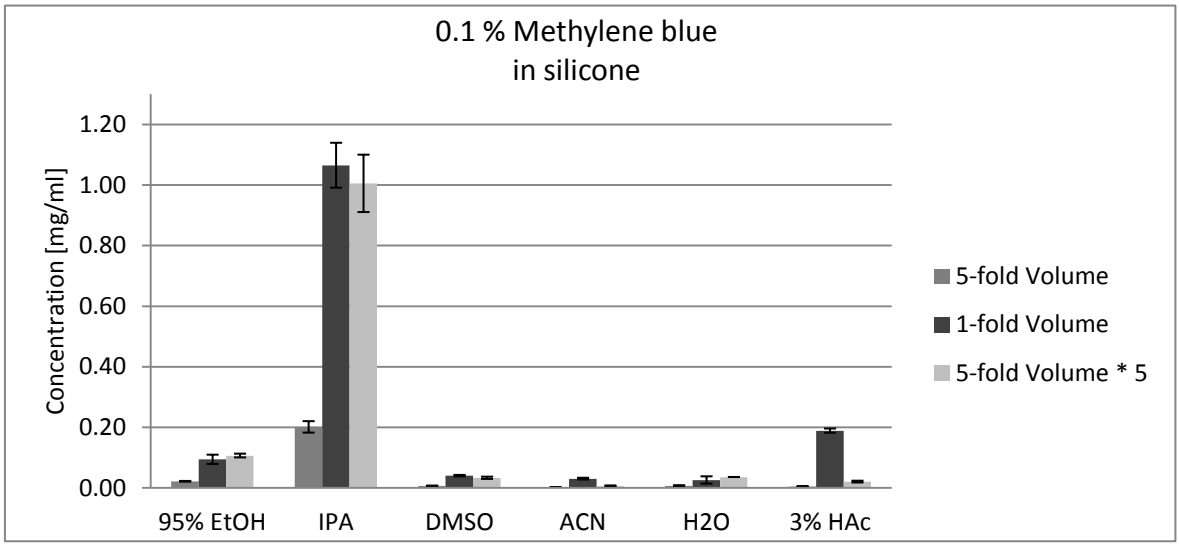


Figure 20: Evaluation of a possible saturation effect during Methylene blue spiked silicone extraction: Comparison of extracts extracted in 5-fold volume instead of prescribed volume

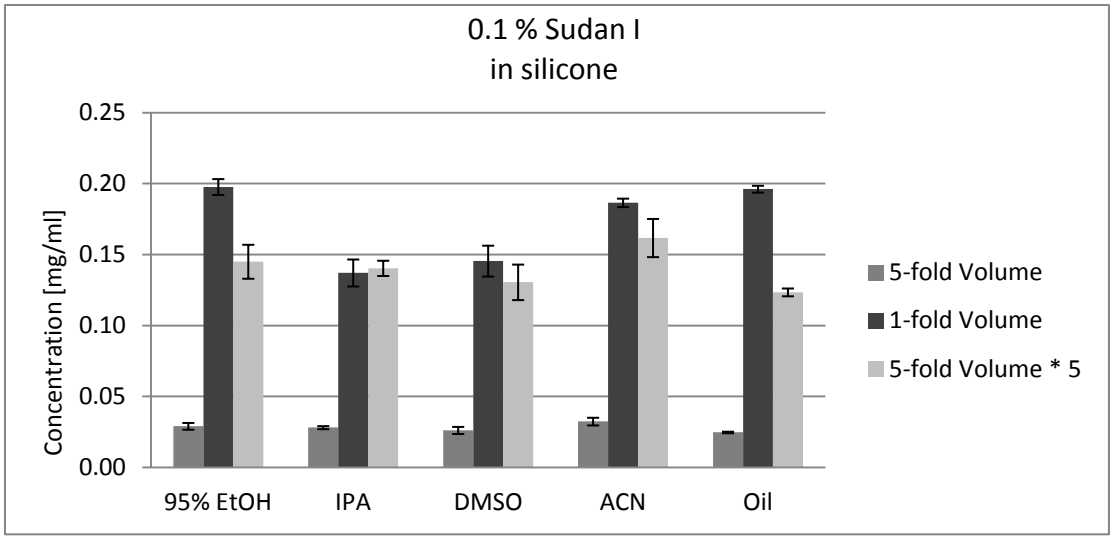


Figure 21: Evaluation of a possible saturation effect during Sudan I spiked silicone extraction: Comparison of extracts extracted in 5-fold volume instead of prescribed volume

4.3 Evaluation of extraction parameters

The following subchapters detailly discuss each parameter and its influence on the amount of substance migrating from the material.

4.3.1 Rotation speed of shaker

The DIN ISO 10993-12:2012 specifies requirements and guidelines for sample preparation including a kind of movement such as shaking or agitation during the extraction process.
This experiment aims to reveal whether differential amounts of skin sensitizer are leached from the medical silicone into the PBS as solvent when the rotation speed of the shaker during extraction is varied.

Evaluation by NanoSens

According to Table 13 and comparing the mean induction values, it can be seen that the amount of CAH in the extract is not affected by increasing or decreasing the shaking velocity. This observation is valid for both, silicone and epoxy resin samples. Surprisingly, even if the sample is incubated for the prescribed 72 hours at 37 °C without any movement, the resulting extract does not show a lower concentration in sensitizer compared to those samples incubated under the same conditions but with moderate to high rotation speed.

Table 13: Mean fold induction values measured in NanoSens Assay by varying the rotation speed of the shaker

Silicone		
Rotation sped (rpm)	**Fold induction**	
	Dilution factor of extract	
	1:100	**1:50**
0	1.61	2.76
110	1.55	2.67
175	1.67	2.78

Evaluation by trypan blue mixing experiment

Table 14 presents the mixing time passed between pipetting a drop of trypan blue into the extraction vessel containing PBS and obtaining an evenly coloured fluid. The time needed to result in a homogenous solution can obviously be greatly shortened by increasing the rotation speed of the shaker. However, even if the vessel is not moved at all, not more than 25 minutes pass until homogenization. This result is consistent with that revealed by the NanoSens test.

Table 14: Measure times needed to completely mix a drop of dye into a vessel containing buffer

Standard glass vial (1A)		**Centrifuge tube 50ml** (1F)		**Centrifuge tube 15ml** (1E)	
Rotation sped (rpm)	Mixing time	**Rotation sped** (rpm)	Mixing time	**Rotation sped** (rpm)	Mixing time
0	25 min	0	25 min	0	20 min
50	12 min				
100	20 sec	100	8 min	100	12 min
150	2 sec				

4.3.2 Extraction vessel

Since the only prescription of the norm regarding extraction vessel is that they should be clean, chemically inert and closed, there is still plenty of scope in the choice. As shown in Figure 10, different potential extraction vessels were tested and compared.

Evaluation by NanoSens

As the values in Table 15 demonstrate, differences can be detected when comparing the amount of cinnamic aldehyde leaching from the spiked silicone when performing the extraction process in different vessel. By using the standard glass vial (1A) the highest FI value was obtained. This outcome is gratifying as these kinds of vessels are commonly used during extraction. Consequently, the results confirm them to be effectively supportive in leaching out as much substance as possible from the biomaterial.

Less substance is able to migrate out when the vial is positioned horizontally (1D). The extra-long glass vial (1G) in horizontal position reaches the lowest fold induction value of the extracts in this experiment.

The results of using the centrifuge tubes 1E and 1F show that the extraction efficiency is also decreased significantly when performing it in a plastic vessel. This suspicion was also confirmed by placing a plastic Eppendorf tube in a standard glass vial during extraction (1H), since the obtained fold induction of the resulting extract is significantly lower compared to the standard vessel (1A).

Table 15: Mean fold induction values measured in NanoSens Assay to compare different extraction vessels

Silicone		
Extraction vessel (Figure 22)	**Fold induction**	
	Dilution factor of extract	
	1:100	**1:50**
1A	1.55	2.68
1D	1.53	2.04
1E	1.35	1.51
1F	1.26	1.63
1G	0.88	1.40
1H	1.23	1.85

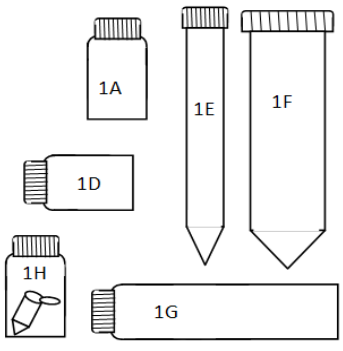


Figure 22: Compared extraction vessels

4.3.3 Extraction agent

The DIN ISO 10993-12:2012 prescribes the use of both polar and non-polar extraction agents as vehicle but does not stipulate specific ones. For the still commonly performed murine Local Lymph Node Assay (LLNA) the OECD recommends acetone/oil 4:1 (v/v) since this solvent is known to elicit a consistent response. Further, pure olive oil as vehicle is often used in the Guinea Pig Maximization Test (GPMT).

However, in the NanoSens assay which is the focussed method of this thesis, PBS and DMSO take over the role of the polar and non-polar extraction agent for medical device testing.

One central aspect of this study is to prove sensitivity of *in vitro* assays compared to *in vivo* animal test methods and therefore the choice of solvent is crucial. A number of solvents have been used as sample extraction agent.

As the amount of sensitizer migrating out during the extraction process strongly depends on the solubility behaviour of the respective substance, both a lipophilic and a hydrophilic reporter substance were used in the following comparison experiment.

Evaluation by absorption measurement of extracts

This experiment aimed to reveal the strength of different solvents when they are used as extraction agents for four commonly used medical device materials

Figure 23 shows the results of the extraction agent comparison of 14 different solvents. Sudan I (SI) mimics lipophilic or rather hydrophobic skin sensitizers whereas Methylene blue (MB) assumes the role of the hydrophilic ones.

Focussing first on the Sudan I peaks it seems like either no dye or a relatively high concentration of dye migrates from the silicone into the respective solvent. Whereas obligatory polar liquids such as water, PBS and acetic acid (HAc) do not interact with the lipophilic dye in the medical silicone at all, less polar ones seem to serve as proper extraction agents in this case.

The most eye-catching blue peak in Figure 23 indicates the exceptionally high solubility of Methylene blue in isopropanol (IPA) when extracted from medical silicone. Despite the fact that solvents like water, PBS or 3% acetic acid (HAc) are much more polar and thus more similar to this dye, IPA seems to be the strongest extraction agent for such polar substances like Methylene blue in a silicone matrix.

Due to this projecting peak, almost no difference can be observed when comparing the other 13 solvents. Therefore, Figure 24 lacking IPA allows a more detailed insight into these results.

PBS which is used as standard polar extraction agent does not leach higher amounts of substance from the silicone than water. The values of 50 % and 20 % ethanol are almost the same whereas 95% ethanol seems to be four times stronger as extraction agent in this case. In this particular experiment, alcoholic and acidic (HAc) solvents appear to act as most reliable leachants. The solvent mixture of acetone/oil 4:1 (v/v) seems to be a proper extraction agent both the lipophilic and the hydrophilic reporter substances.

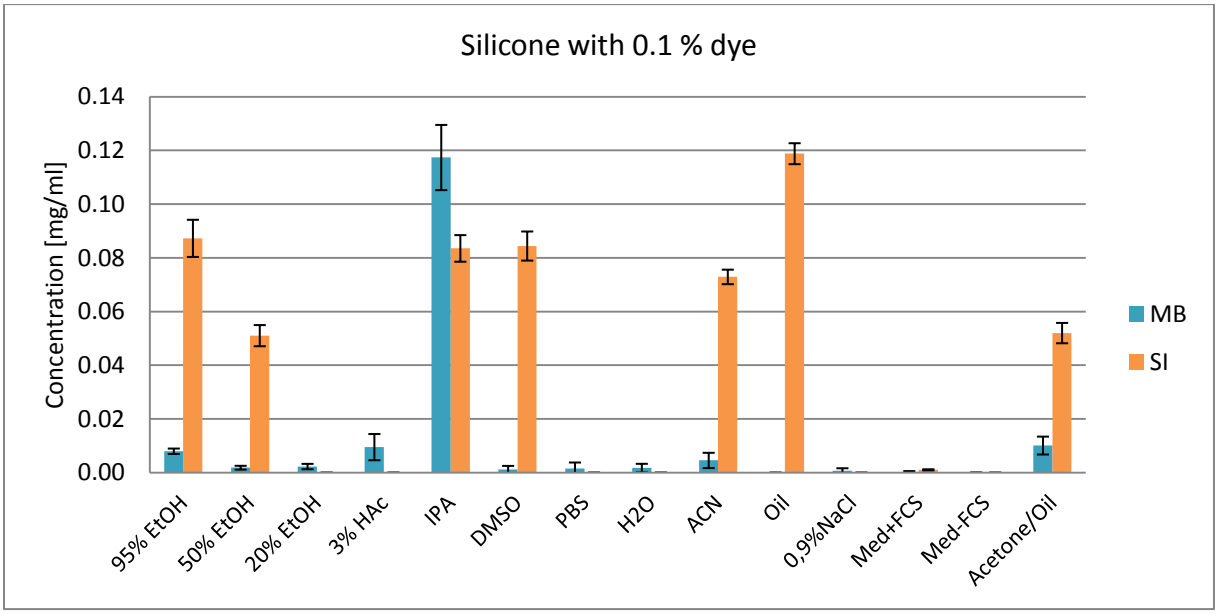


Figure 23: Concentrations of Sudan I (SI) and Methylene blue (MB) leached from the dye-spiked silicone samples into the respective extraction agent

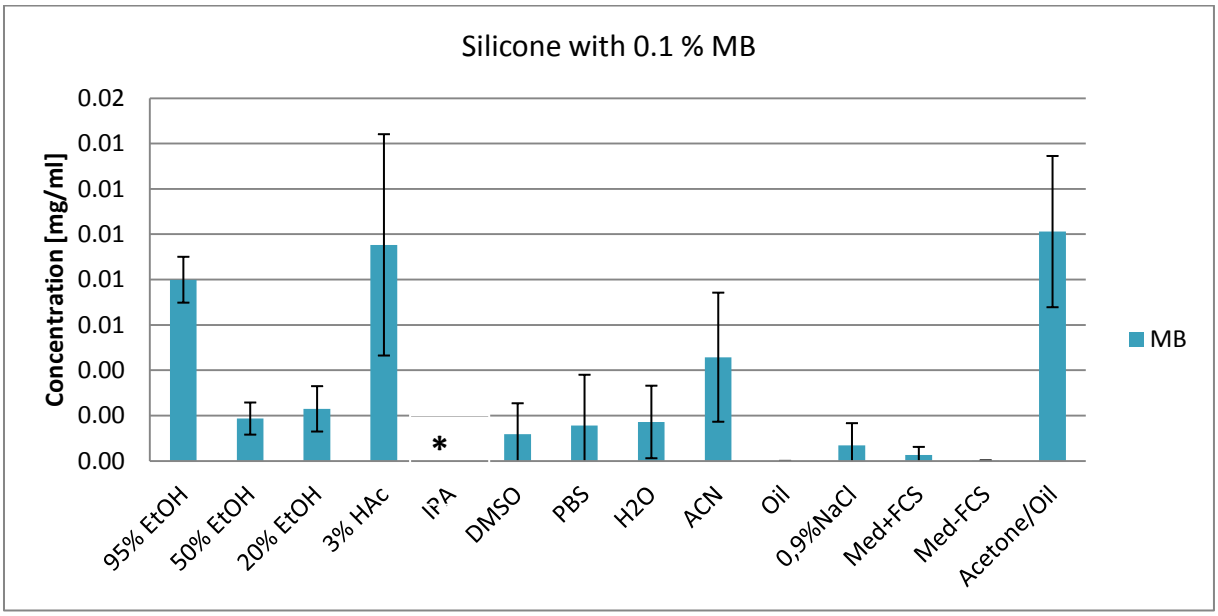


Figure 24: Concentrations of Methylene blue (MB) leached from the dye-spiked silicone samples into the respective extraction agent. (*)The bar for IPA was removed in this graphic.

Figure 25 presents the resulting extraction agent data with the dye-spiked epoxy resin. Since epoxy resin is different to silicone, their properties during extraction differ as well. The hydrophilic Methylene blue is leached stronger from its matrix, whereby water seems to be a stronger solvent for the material than PBS. Regarding Sudan I it can be seen that the peak pattern is similar to that of silicone with one exception. Oil does not interact at all with the epoxy resin.

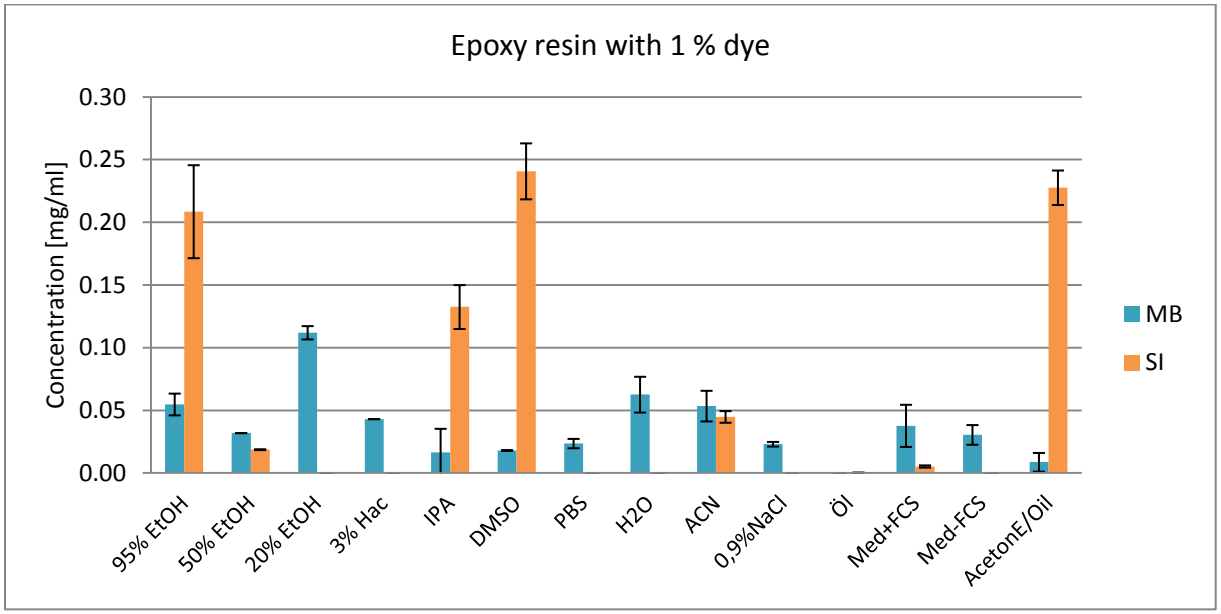


Figure 25: Concentrations of Sudan I (SI) and Methylene blue (MB) leached from the dye-spiked epoxy resin samples into the respective extraction agent

Thermoplastic polyurethane (TPU) is commonly used for healthcare applications being appreciated for its properties between plastic and rubber. According to Figure 26 its accessibility to the tested extraction solvents and the subsequent release of the embedded reporter dyes distinguishes a lot from the previously mentioned biomaterials. Methylene blue seems to be leached well by almost all solvents whereas Sudan I does not leach into PBS, water, acetic acid and physiological saline solution.

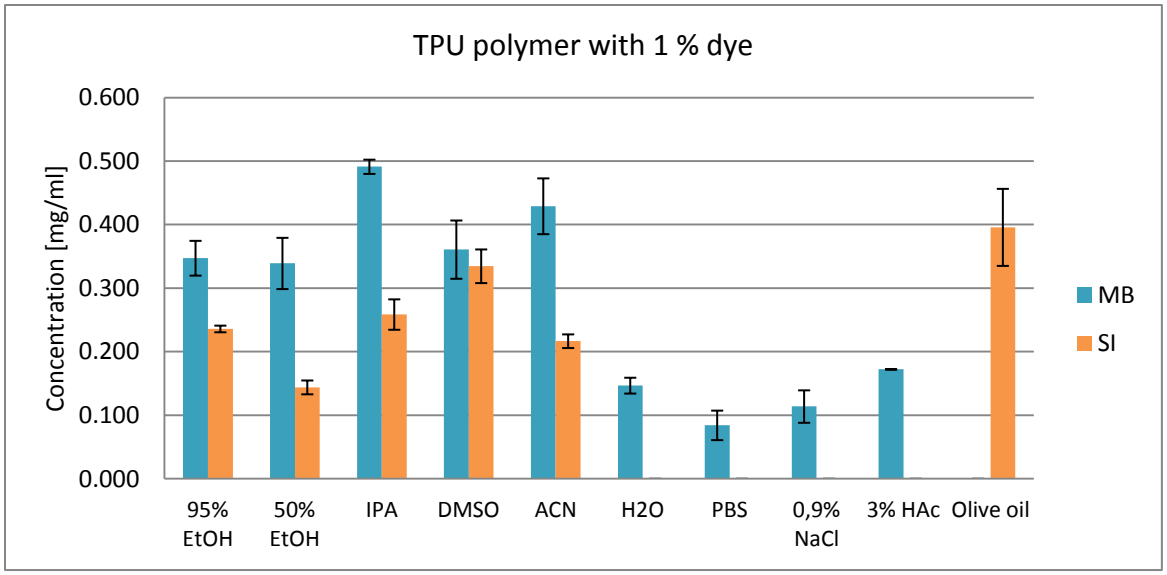


Figure 26: Concentrations of Sudan I (SI) and Methylene blue (MB) leached from the dye-spiked TPU polymer samples into the respective extraction agent

Acrylic behaves again completely different compared to silicone, epoxy resin and TPU polymer when exposing it to certain solvents in order to leach incorporated substances. As Figure 27 reveals, only ethanol, IPA, DMSO, acetonitrile (ACN) and to some extent PBS are capable of leaching Methylene blue from its matrix. The same results are obtained for Sudan I.

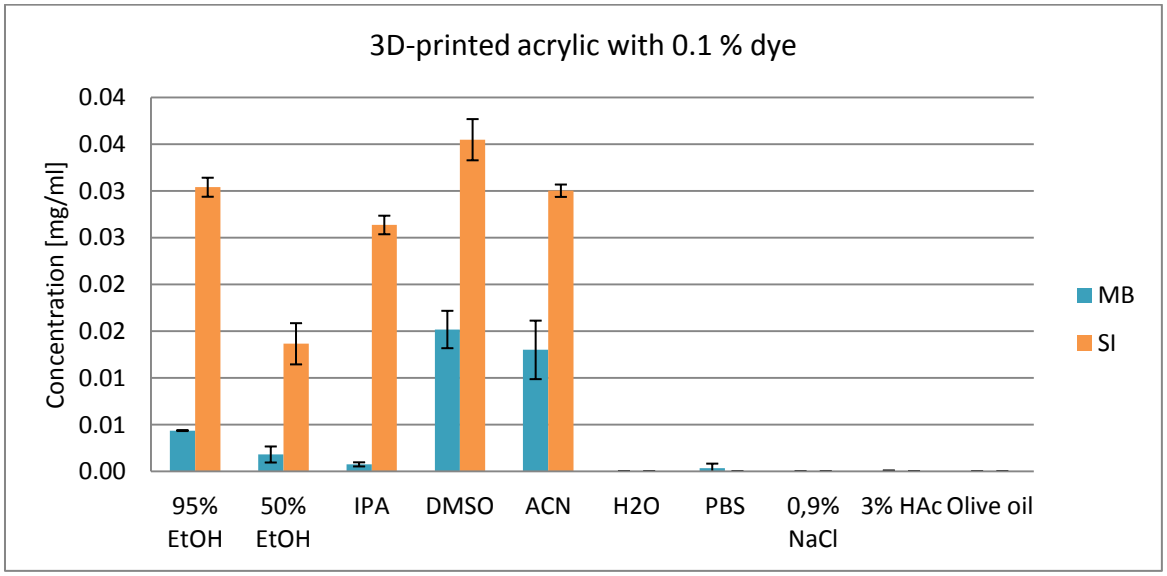


Figure 27: Concentrations of Methylene blue (MB) leached from the dye-spiked 3D-printed acrylic samples into the respective extraction agent

Table 16 and Table 17 summarize the dye concentration values in the extracts obtained by comparing four different medical device materials and ten to 14 different solvents.

Table 16: Concentrations of Methylene blue including standard deviation [mg/ml] leached from different materials by different solvents into the extract. (n.t = not tested)

Methylene blue				
	Silicone	**Epoxy resin**	**TPU polymer**	**Acrylic resin**
95% EtOH	0.01±0	0.05±0.01	0.35± 0.03	0±0
50% EtOH	0±0	0.03±0	0.34±0.04	0±0
20% EtOH	0±0	0.11±0.01	n.t.	n.t
3% HAc	0.01±0	0.04±0	0.17±0	0±0
IPA	0.12±0.01	0.02±0.02	0.49±0.01	0±0
DMSO	0±0	0.02±0	0.36±0.05	0.02±0
PBS	0±0	0.02±0	0.08±0.02	0±0
H2O	0±0	0.06±0.01	0.15±0.01	0±0
ACN	0±0	0.05±0.01	0.43±0.04	0.01±0
Oil	0±0	0±0	0±0	0±0
0.9%NaCl	0±0	0.02±0	0.11±0.03	0±0
Med+FCS	0±0	0.04±0.02	n.t	n.t
Med-FCS	0±0	0.03±0.01	n.t	n.t
Acetone/Oil	0.01±0	0.01±0.01	n.t	n.t

Table 17: Concentrations of Sudan I including standard deviation [mg/ml] leached from different materials by different solvents into the extract. (n.t = not tested)

Sudan I				
	Silicone	**Epoxy resin**	**TPU polymer**	**Acrylic resin**
95% EtOH	0.09±0.01	0.21±0.04	0.24±0.01	0.03±0
50% EtOH	0.05±0	0.02±0	0.14±0.01	0.01±0
20% EtOH	0±0	0±0	n.t	n.t
3% Hac	0±0	0±0	0±0	0±0
IPA	0.08±0	0.13±0.02	0.26±0.02	0.03±0
DMSO	0.08±0.01	0.24±0.02	0.33±0.03	0.04±0
PBS	0±0	0±0	0±0	0±0
H2O	0±0	0±0	0±0	0±0
ACN	0.07±0	0.04±0	0.22±0.01	0.03±0
Öl	0.12±0	0±0	0.4±0.06	0±0
0.9%NaCl	0±0	0±0	0±0	0±0
Med+FCS	0±0	0,01±0	n.t	n.t
Med-FCS	0±0	0±0	n.t	n.t
Acetone/Oil	0.05±0	0.23±0.01	n.t	n.t

4.3.4 Medical device material

The following Figure 28 and Figure 29 summarize the results of the extraction agent comparison by focussing on the four tested biomaterials. The values shown have been adjusted to a dye concentration of 0.1 % in each material.

Figure 28 shows the hydrophilic Methylene blue leached from the respective material in the different solvents. As already noticed the water soluble dye is extracted extraordinarily well from the medical silicone by IPA. Further, all the other solvents except IPA seem to leach relatively low dye concentrations from silicone.
Looking at Figure 29 concerning Sudan I, silicone as well reveals to be the most leachable material of this experiment exceeding by far all the other tested materials in the amount of dye leached into each solvent.

The stiff and brittle epoxy resin in turn is accessed by all tested extraction agents in relatively low amounts when focussing on Methylene blue. As presumed, oil with its strong hydrophobicity does not dissolve the hydrophilic dye from any tested material at all. The Sudan I is leached from the epoxy resin in amounts similar to the TPU polymer when looking at 95 % ethanol, IPA and DMSO. However, surprisingly oil does also not dissolve any of the hydrophobic dye Sudan I from the epoxy resin, but from the TPU polymer.

The TPU polymer seems to be a material which is easy migrateable for water soluble compounds. Except for IPA and silicone, the polymer is extracted by all solvents much stronger than the other materials tested.

Both Figure 28 and Figure 29 indicate that acrylic resin is a relatively inert material compared to the others, since components embedded into its matrix have difficulties in migrating out the material by extraction.
However, the hydrophilic dye is leached well by ACN, DMSO and ethanol. The hydrophobic one in turn is extracted by the same solvents and additionally by IPA.

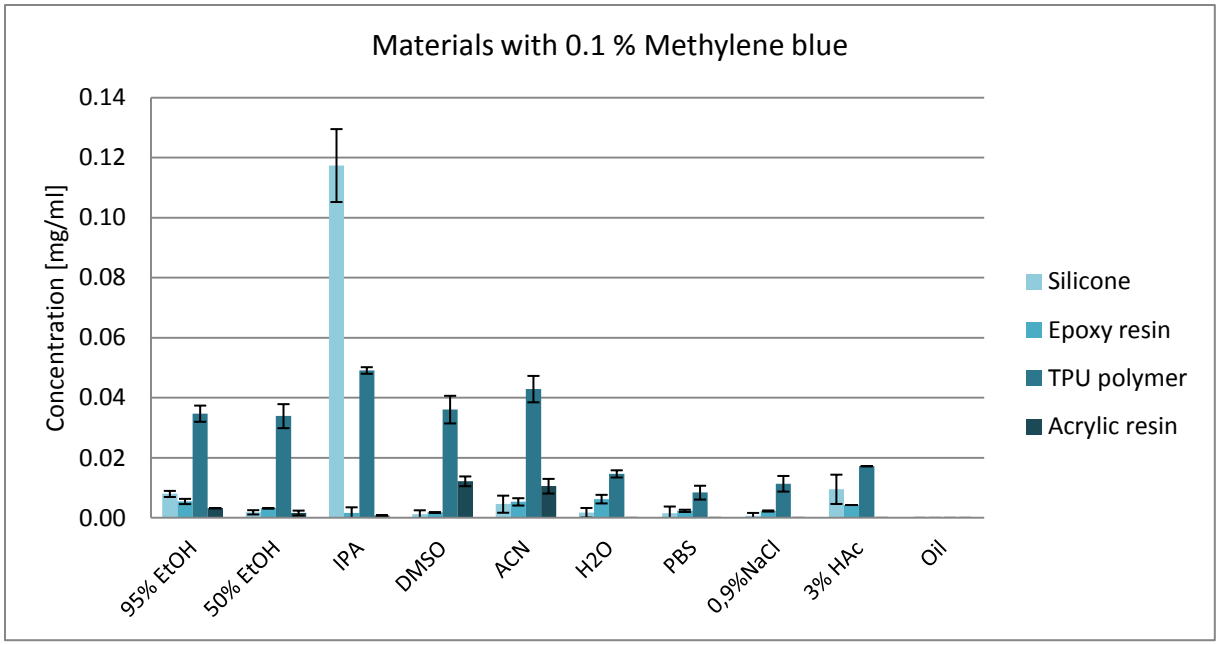


Figure 28: Comparison of four different biomaterials spiked with Methylene blue (0.1%) and extracted in different solvents. Small figure contains all peaks, main figure without silicone in IPA

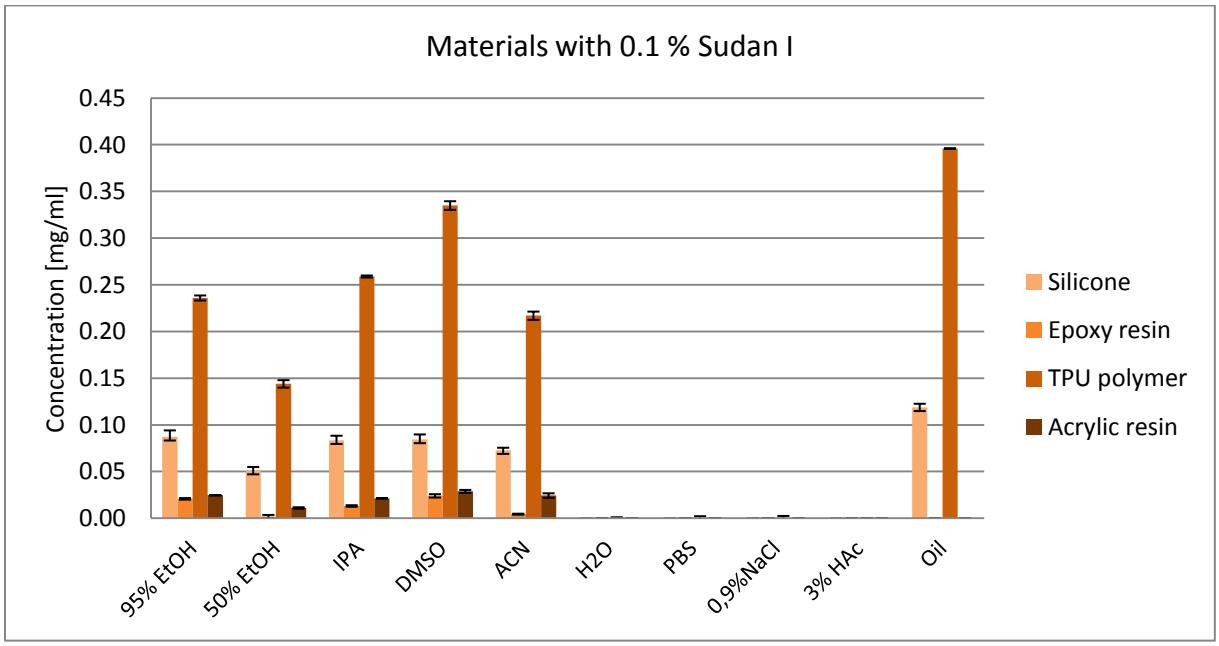


Figure 29: Comparison of four different biomaterials spiked with Sudan I (0.1%) and extracted in different solvents

Figure 30 shows the leached dye-spiked samples after the extraction process performed at 37 °C for 72 h on the shaker (110 rpm). The rectangles consist of TPU polymer whereas 3D-printed shells are made of acrylic resin.

The TPU polymer samples extracted in DMSO are not shown on the picture since they are not stable in this solvent and dissolved during extraction.

The colours of the samples are consistent with the results obtained by measuring the absorption of the resulting extracts. For example all acrylic shells extracted in a polar solvent are nearly unaffected while those treated with non-polar agents have been partially discoloured.

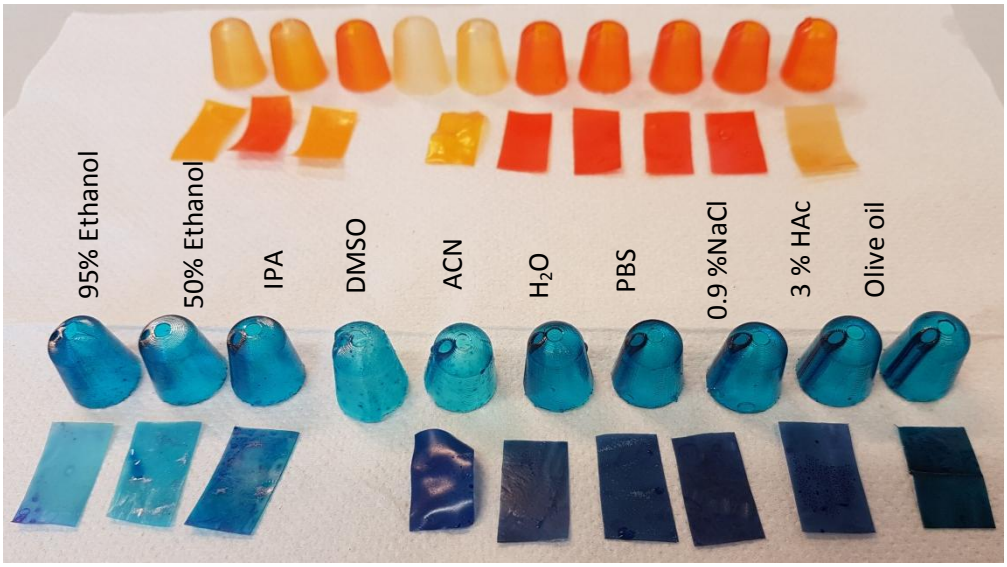


Figure 30: TPU polymer and 3D-printed acrylic shells after extraction with one of 10 solvents.

Concluding the results DMSO, IPA and 95 % ethanol are the extraction agents leaching the highest amounts of both hydrophilic and hydrophobic substance from all tested biomaterials.

Further, extraction not only depends on the characteristics of the extractant but also on the material.

4.3.5 Sample cutting surface

The DIN ISO 10993-12:2012 norm for medical device preparation prior to biological testing suggests cutting samples into pieces of appropriate size if they cannot be tested as a whole. However, it does not emphasize to keep the cutting surface as small and smooth as possible.

Evaluation by NanoSens

Table 18 reveals the impact of the way a material is cut on its accessibility to a polar (PBS) and a non-polar (DMSO) extraction solvent. Regarding the medical silicone it is shown that both extraction agents PBS and DMSO leach higher amounts of sensitizers from the sample matrix when it is cut by creating a rough surface instead of a smooth one. In case of DMSO the FI values between both cutting surface variants even differ by factor 2.

Table 18: Mean FI values obtained by comparing the influence of sample cutting surfaces in the NanoSens. (n.t.= not tested)

		Silicone	
	Cutting surface	Fold induction	
		Dilution factor of extract	
		1:100	**1:50**
PBS	smooth	1.36	2.70
	rough	1.94	3.24
DMSO	smooth	2.86	n.t.
	rough	5.68	n.t.

Result of the surface roughness measurement

Due to the measured roughness values listed in Table 19 it can be seen that the surface of the respective material is magnified significantly after cutting compared to the intact cast surface.
This observation is valid for both silicone and epoxy resin, whereby in case of the resin the difference between both surfaces is much higher than that of the silicone samples. This may be a result of the higher hardness of the resin which makes it more brittle especially during cutting.

Table 19: Measured roughness determined by perthometer

Sample	R_a [µm]	R_z [µm]
Silicone: cast surface	0.40	2.00
Silicone: cutting surface	4.24	30.39
Epoxy resin: cast surface	0.17	0.97
Epoxy resin: cutting surface	4.90	29.71

R_a: arithmetic mean roughness over the whole measuring section

R_z: mean roughness calculated from single consecutive measuring sections

Evaluation by absorption measurement of extracts

Figure 31 and Figure 32 visualize the difference in the leaching extent of a medical device sample depending on the quality of its cutting surface.

The results coincident with those obtained from the NanoSens tests above. A sample which is cut by creating a rough cutting surface leads to a significantly higher release of low-molecular substances such as for example skin sensitizers. This is the consequence of the larger micro-surface area that is generated leading to a shift in the surface/volume ratio of extraction agent. As a result the obtaining extracts are higher concentrated than the ones with smooth surface.

Both tested materials silicone and epoxy resin conform this theory. However, this effect seems to affect Methylene blue samples more than Sudan I samples.

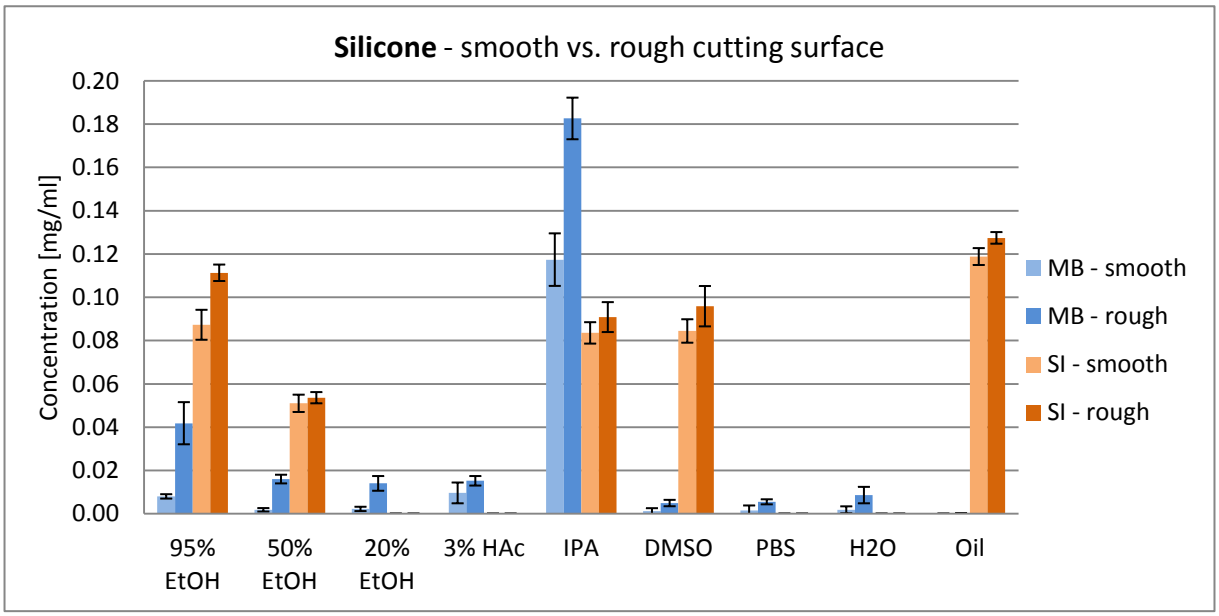


Figure 31: Comparison of the influence of smooth and rough cutting surface of silicone samples

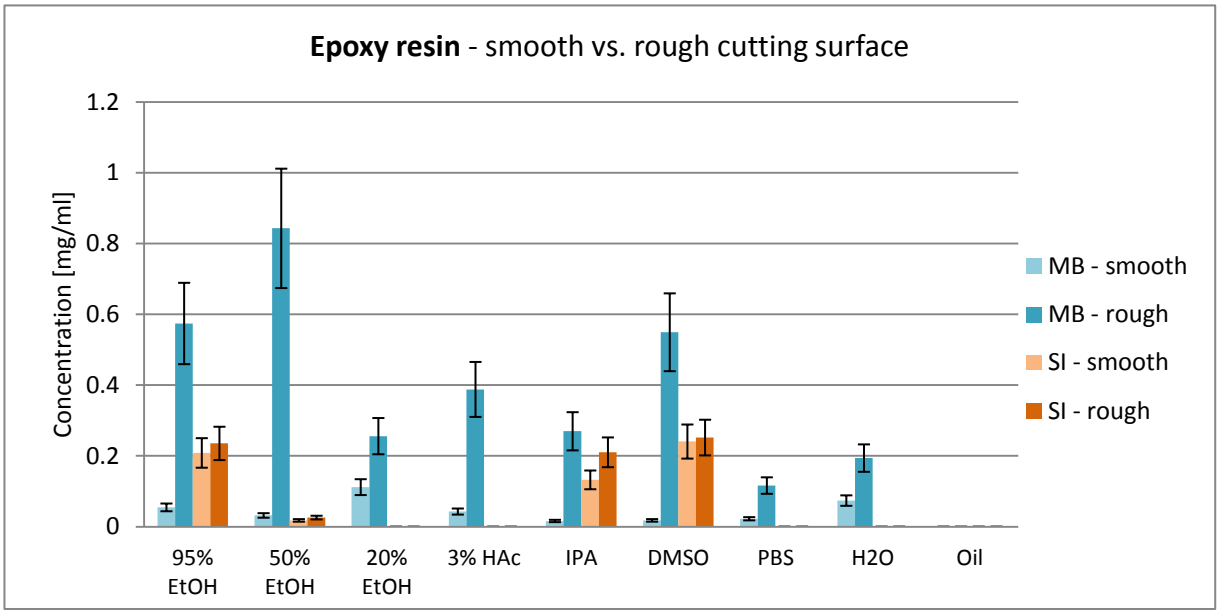


Figure 32: Comparison of the influence of smooth and rough cutting surface of epoxy resin samples

4.3.6 Foil extraction

In order to investigate the extraction of thin film-like materials, differences were measured if foils sticking together in the solvent make a difference compared to plane foils.

Evaluation by absorption measurement of extracts

This experiment aimed to determine whether a packed bundle of foil is less accessible to the surrounding extraction agent than a planar piece. For this purpose the extract obtained from a small piece of spiked silicone foil was compared to that of a large piece crumpled tightly into a vial.

Figure 33 reveals a significant difference between both sample preparation procedures for both tested solvents, water and 95 % ethanol. According to these results the hydrophilic dye is able to migrate in much higher concentrations from the thin silicone foil when it is extracted planar without any folding or wrapping of the material. Especially using the polar solvent water the difference is enormously high. In this experiment the absorption values differ by the factor 7 just by the way of packing the sample into the vial.

Looking at Figure 34 obviously the amount of dye leached by a wrapped foil is not significantly lower than by a planar piece of foil. This observation was made for both solvents isopropanol and 95 % ethanol.

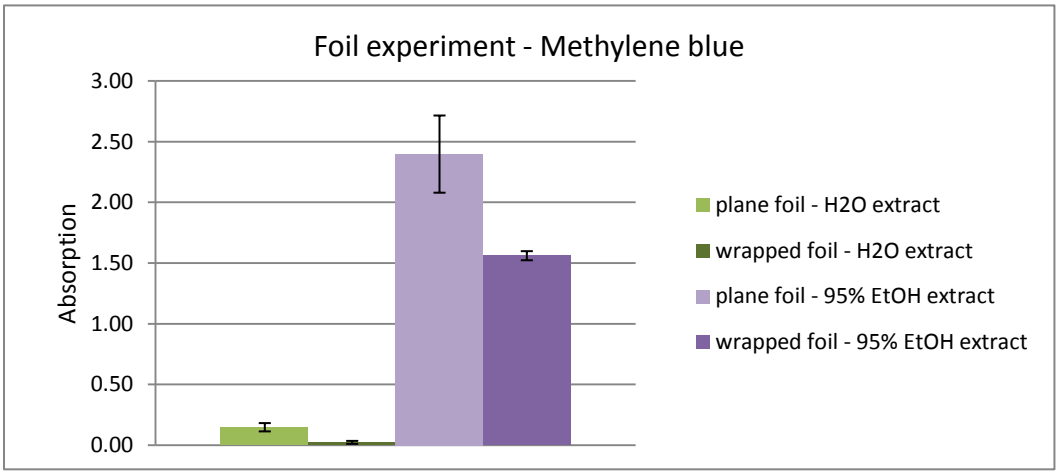


Figure 33: Comparison of Methylene blue extracts from spiked silicone obtained by wrapping a piece of foil during the extraction process instead of ensuring a completely planar surface

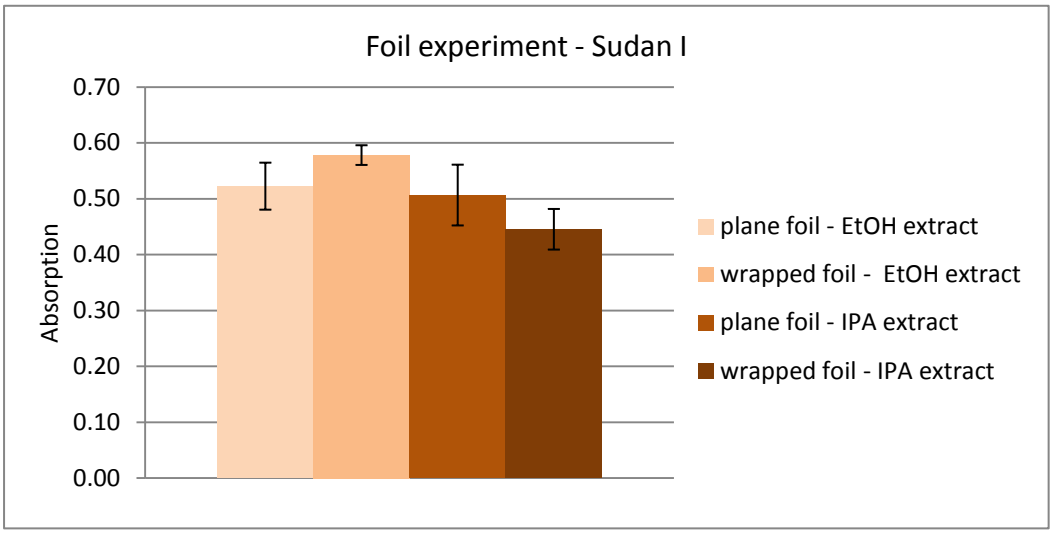


Figure 34: Comparison of Sudan I extracts from spiked silicone obtained by wrapping a piece of foil during the extraction process instead of ensuring a completely planar surface

4.3.7 Time course

The time course evaluation was conducted by performing two different experiments as described below.

Evaluation by NanoSens

The first one aimed to reveal the degradation speed of sensitizing chemicals contained in medical device materials. For this purpose CAH-spiked silicone samples were evaluated in the NanoSens skin sensitization assay in regular intervals of storage times.

Figure 35 indicates the fold induction values of the 1:100 diluted CAH-spiked silicone extracts determined in the NanoSens assay.

On day 0 the silicone was prepared by adding CAH into the raw components and pouring it into petri dishes. Afterwards, the experiment was run over a time span of 53 days in which a total of six sampling steps were performed, which are marked in Figure 35. For this purpose the respective silicone samples were prepared by cutting them smoothly as well as roughly and extracting them in PBS and DMSO.

The diagram shows that the fold induction values of all four silicone samples significantly decrease over the first month. This effect is particularly observable for those samples extracted in DMSO. Moreover, DMSO seems to leach higher amounts of cinnamic aldehyde from the medical silicone since the initial fold induction values are more than twice as high as those compared to the PBS extracts. However, the figure also clearly shows again the influence of the sample's cutting surface. This effect is high in case of the DMSO extracts since the FI values are approximately twice as high when the sample has been cut by generating a rough surface instead of a smooth one. This impact is apparently much lower for PBS extracts but still present.

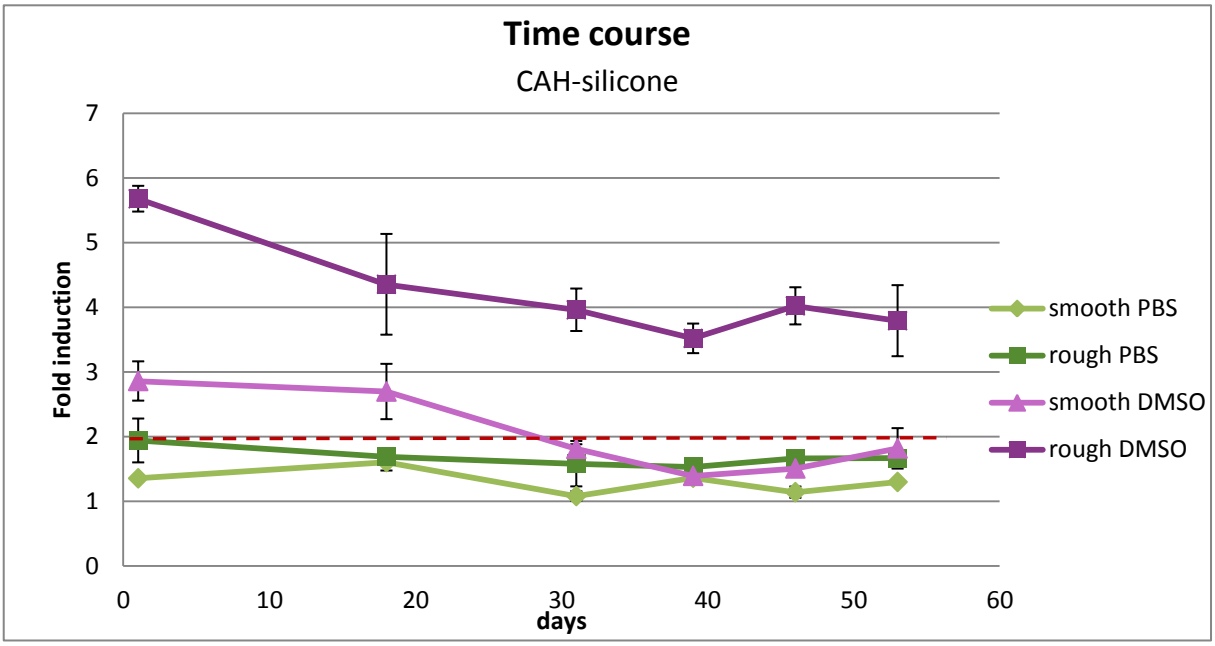


Figure 35: Fold induction values of CAH-spiked silicone extracts (1:100 dilution) between 1 and 53 hours after sample cutting. The red line marks the FI threshold (2) which classifies a sample as positive

Evaluation by absorption measurement of extracts

The second time course experiment aimed to visualize the 72-hour extraction by focussing on the progress of the material leaching process.

For this purpose, aliquots of the dye-spiked biomaterial extracts were taken hourly for the first 14 h and afterwards in larger intervals of 5-10 h before determining the dye concentration. Each bar indicates the measured dye concentration in the extract at one sampling step.

Figure 36 and Figure 37 represent the time courses during the extraction process of silicone and epoxy resin for Methylene blue. As already noticed in previous experiments, the hydrophilic dye is leached by much more different solvents from the epoxy resin compared to the silicone. However, considering the time course it can be seen that the first rise in most cases is relatively steep.

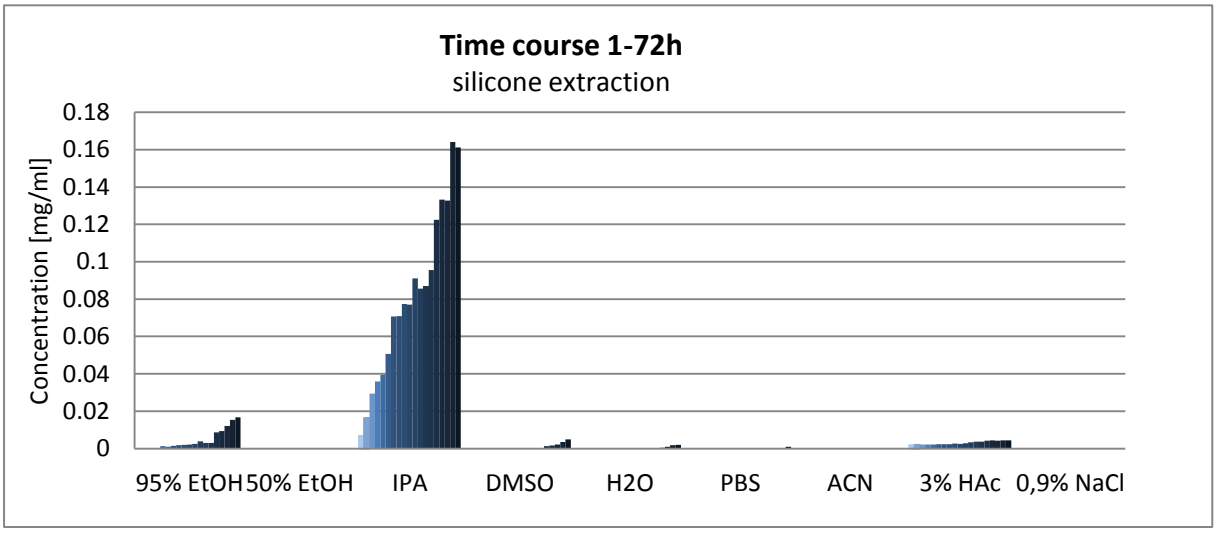


Figure 36: Time course of the extraction process of Methylene blue-spiked silicone (0.1% MB.) Each bar represents the actual dye concentration in the extract at the respective sampling step. The darker the bar, the later the time of sampling and measurement.

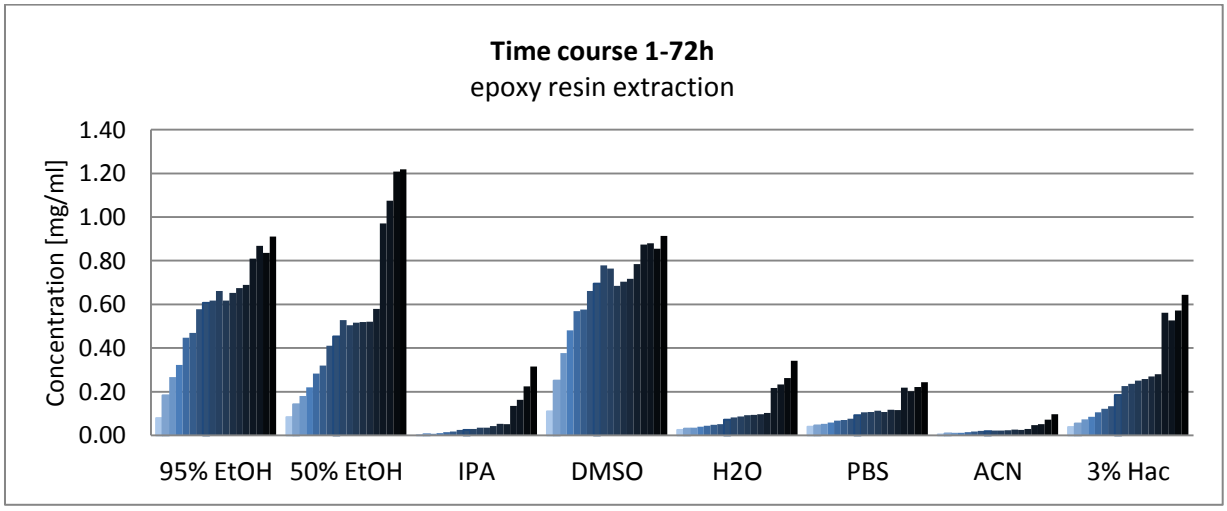


Figure 37: Time course of the extraction process of Methylene blue-spiked epoxy resin (1% MB)

Figure 38 and Figure *39* show the same results for the hydrophobic dye Sudan I. It can be noticed that the leaching behaviour in this case is different, at least for the silicone.

Figure 39 demonstrates that the slope of the dye concentration leached by most of the tested solvents is extremely high in the first 4 hours and then flattens off rapidly.

The lipophilic dye is leached from the silicone by a given solvent either not at all or relatively fast, depending on its polarity.

This behaviour seems to be different for Sudan I incorporated into epoxy resin. In this case the leaching process is distributed relatively evenly over the whole extraction period.

However, in general it can be said that at a temperature of 37 °C the prescribed extraction time of 72 hours is definitely needed in order to ensure a proper chemical leaching of each tested biomaterial.

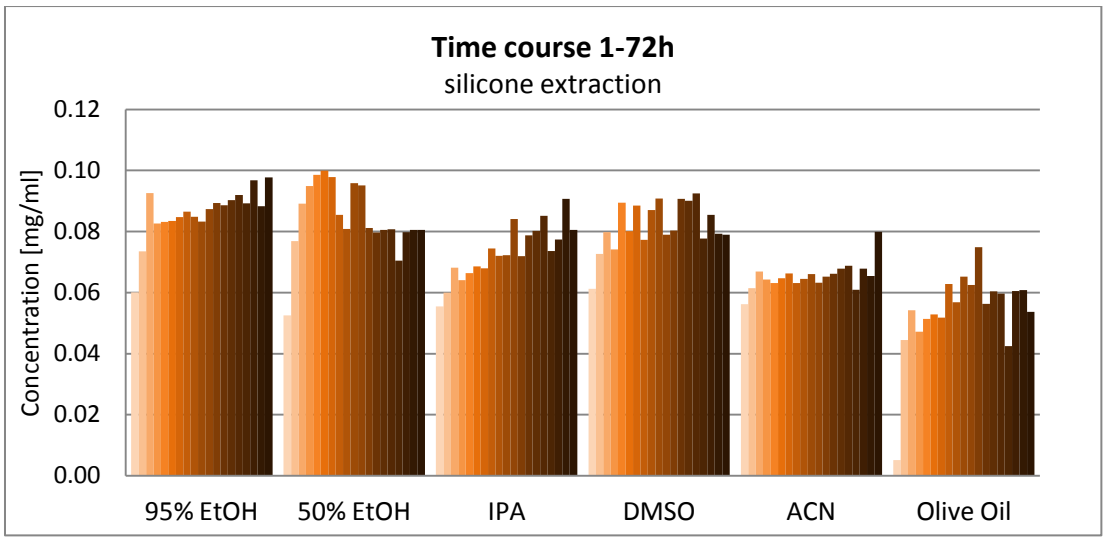


Figure 38: Time course of the extraction process of Sudan I-spiked silicone (0.1% SI)
Each bar represents the actual dye concentration in the extract at the respective sampling step. The darker the bar, the later the time of sampling and measurement.

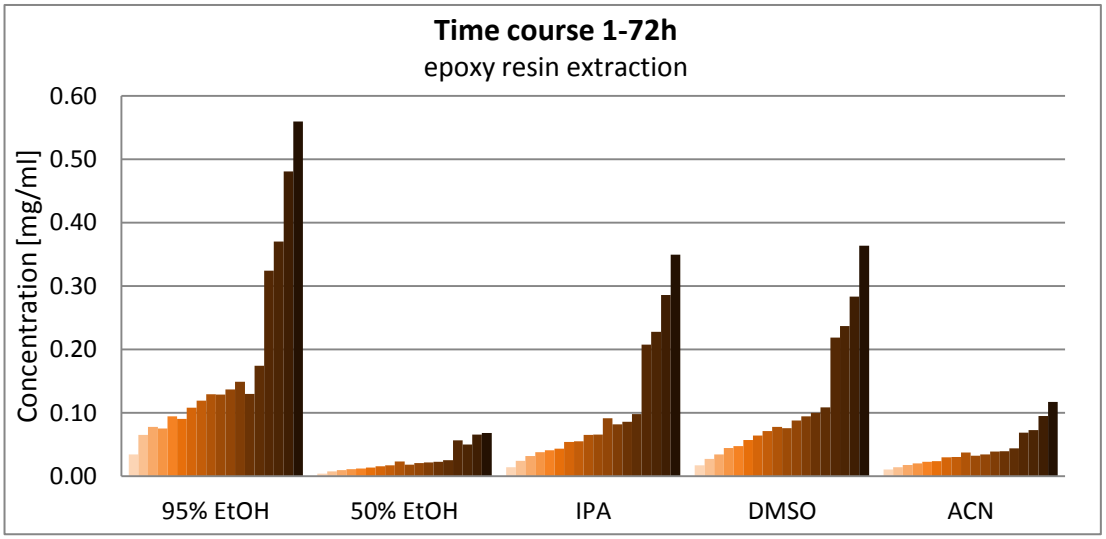


Figure 39: Time course of the extraction process of Sudan I-spiked epoxy resin (1% SI)

4.3.8 Temperature

The norm prescribes an extraction temperature of either 37 °C or 50 °C for the time span of 72 h. Since these two conditions leave quite a margin both temperatures were compared in the experiment described below.

Evaluation by absorption measurement of extracts

The following three diagrams demonstrate a direct comparison of the dye concentrations in the extracts obtained by adjusting the incubation temperature to either 37 °C or 50 °C. This experiment was performed using the dye-spiked silicone, TPU polymer and acrylic resin samples.

According to Figure 40 surprisingly only 95 % ethanol and IPA for Methylene blue and 50 % ethanol for Sudan I show a higher migration of the respective dye from the silicone into the extract when the temperature is higher (50 °C). This observation is also valid for the TPU polymer indicated in Figure 41 since on average more substance is leached from the material at a temperature of 37 °C compared to 50 °C.

Comparing the results of both incubation temperatures for acrylic resin samples in Figure 42 it can be seen that in case of Sudan I almost no difference can be noted whereas Methylene blue migrates on average in higher amounts from the acrylic resin matrix at the higher temperature (50 °C).

A similar outcome was expected for all tested material since the molecular motion increases with higher temperature and therefore a stronger migration of dye into extract was assumed.

Additional experiments were performed to investigate the reason for the unexpected results of silicone and TPU polymer.

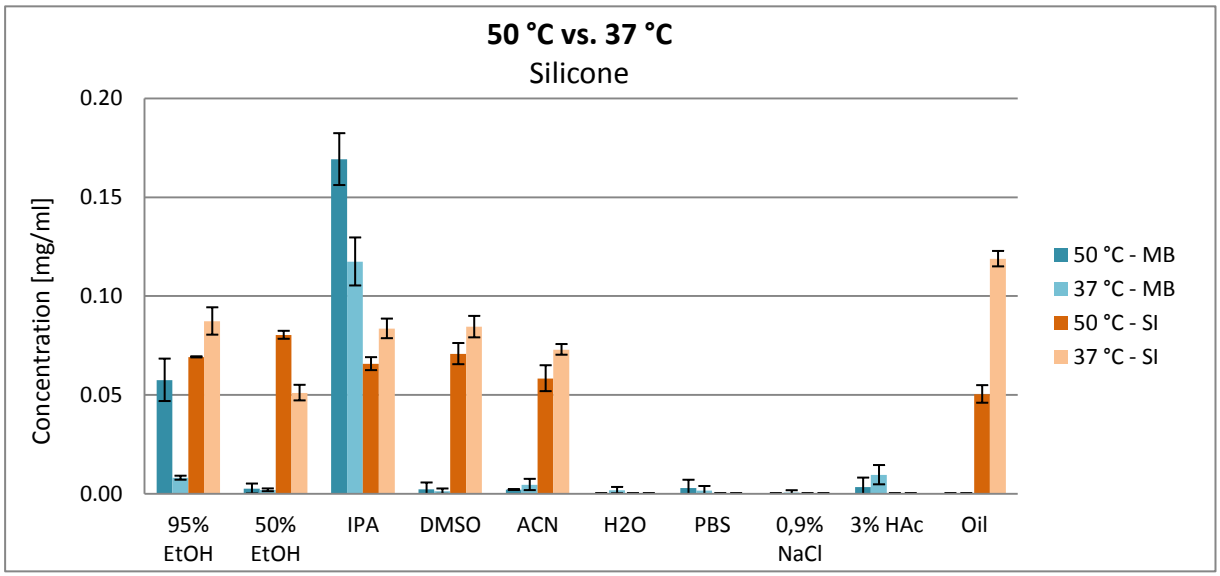


Figure 40: Comparison of dye concentrations leached from spiked silicone samples into extract depending on incubation temperature. The experiment at 50 °C was performed about three weeks after that at 37 °C.

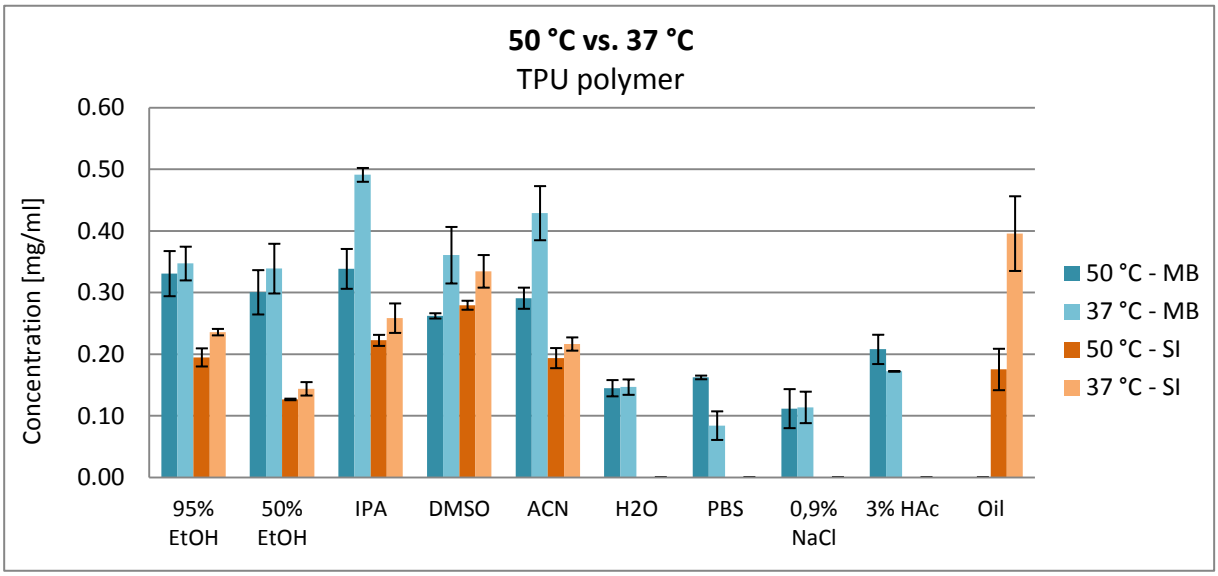


Figure 41: Comparison of dye concentrations leached from spiked TPU polymer samples into extract depending on incubation temperature. The experiment at 50 °C was performed about three weeks after that at 37 °C.

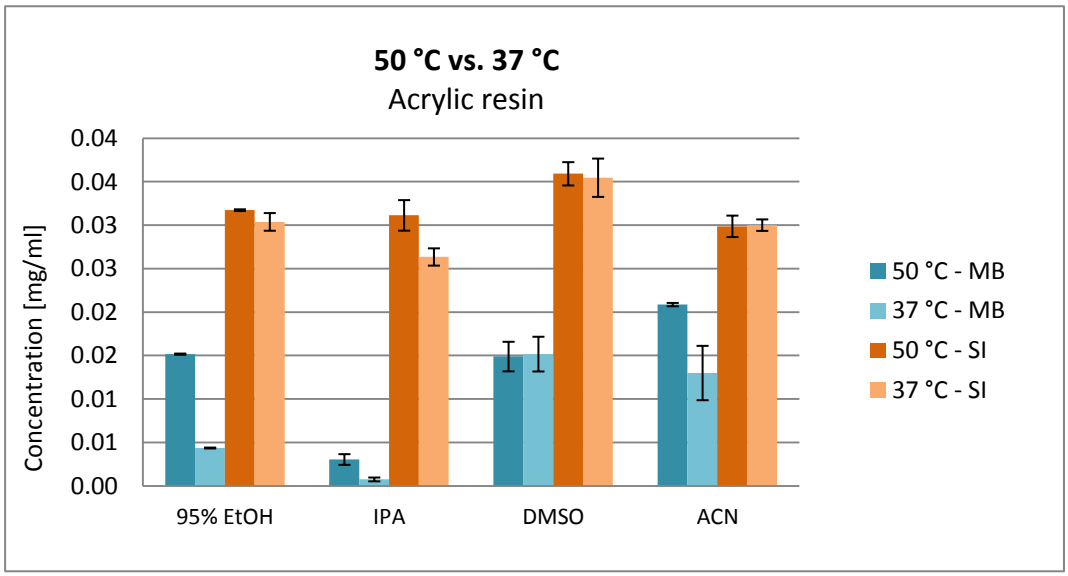


Figure 42: Comparison of dye concentrations leached from spiked acrylic resin samples into extract depending on incubation temperature. The experiment at 50 °C was performed about three weeks after that at 37 °C.

Especially the increased Sudan I concentration leached at 50 °C from the silicone and TPU polymer samples compared to the lower temperature of 37 °C are particularly striking.

As one suspicion for this observation was the occurrence of chemical degradation reactions of the oil that may possibly occur at higher temperatures and that would lead to an absorption shift at altered pH-values, an additional experiment was carried out to exclude this theory. For this purpose, the dye-spiked materials were incubated in oil at 37 °C and 50 °C. Measuring the pH-values of these oil extracts showed no difference, so this theory can be excluded.

As the experiments at 50 °C were performed some weeks after those at 37 °C, a part of that experiment was repeated by starting the extraction for both compared temperatures simultaneously. For this purpose the dye-spiked silicone and TPU polymer samples were stored for four days in IPA and oil at 37 °C and 50 °C and afterwards the resulting extracts were compared by measuring their absorption.

The results in Figure 43 for Methylene blue show that the higher temperature either has no influence or favours the amount of dye leaching from the tested materials.

The migration behaviour of Sudan I from silicone is not affected by the temperature. Methylene blue leaching from TPU polymer is slightly favoured by an increased temperature.

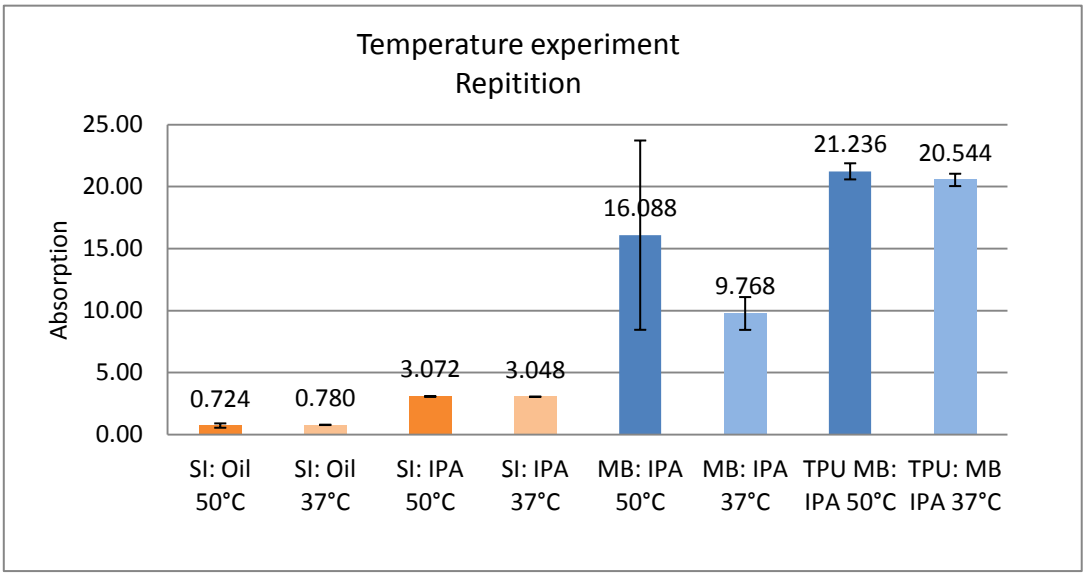


Figure 43: Comparison of absorption values after storing the dye-spiked materials at either 37 °C or 50 °C. The first six bars from the left indicate silicone sample extracts, the right bars those of TPU samples.

4.4 Evaluation of NanoSens using the optimized extraction procedure

The results of the parameters influencing medical device extraction for testing in *in vitro* skin sensitization assays lead to the development of an optimized extraction procedure.

Table 20 lists all tested parameters with their optimized outcome.

Table 20: Summary of the optimized extraction procedure.

Parameter	Optimized procedure
Rotation speed of shaker	110 rpm
Extraction vessel	Glass vial with teflon cap
Extraction agent	Polar: PBS Non-polar: DMSO
Sample cutting surface	Not cutting surface, if not possible cutting as smooth as possible
Foil-like materials	Ensuring complete covering of whole sample surface with solvent
Time after sample preparation	As short as possible
Temperature	37 °C/ 50 °C *

*see discussion

4.4.1 Exemplary results for medical devices tested in NanoSens

The optimized sample extraction starts started by cutting the medical device into pieces of appropriate size when it is not possible to extract the whole device in one. Cutting is performed by generating a surface that is as smooth as possible to represent the surface of intended application. This preparation step should be performed shortly before the subsequent extraction.

The samples are placed into glass vials with teflon caps and the prescribed solvent volume according to Table 7 is added. For polar extracts PBS and for non-polar ones DMSO is used.

The vials containing the samples are incubated on a shaker at 37 °C/50 °C for 72 hours by adjusting the rotation speed to 110 rpm.

After this time the samples are removed and the extracts stored in the fridge until their use.

The following Table 21 lists the results of selected medical device sample extracts (PBS and DMSO) with cell viability and FI values including standard deviations. Further, the table shows whether the CAH-spike of the respective sample extract achieved a FI above 2 and matrix effects interfering with the test system can be ruled out.

Among these 40 tested medical devices six show cytotoxic effects and twelve (30 %) exceed the fold induction of 2 which classifies them as potential sensitizers. In three cases the FI of the spike did not reach the threshold of 2.

In general, it can be seen that by using the non-polar DMSO as solvent more samples have been identified as potentially sensitizing than by using the polar PBS. Additionally DMSO extracts led more often to cytotoxic effects compared to PBS extracts.

Table 21: Results of selected medical devices tested in NanoSens assay

		PBS extract				**DMSO extract**				**Classification: sensitizing potential**
Type of device	**Material**	**Viability [%] mean ±** standard deviation	**FI mean ±** standard deviation	**FI (Spike) < 2** (yes/no)		**Viability [%] mean ±** standard deviation	**FI mean ±** standard deviation	**Spike < 2** (yes/no)		
Hearing device	Acrylic resin, transparent	92 ± 12	2.88 ± 0.55	yes		67 ± 10	0,52 ± 0.05	no		yes
Hearing device	Silicone with coating and colour	103 ± 2	3.95 ± 0	yes		116 ± 10	1,39 ± 0.07	yes		yes
Plastics for denistry device	FKM, VMQ, Silicone	99 ± 9	1.09 ± 0.07	yes		51 ± 2	3.3 ± 0.39	yes		yes
Anitmicrobial Spray	Liquid	82 ± 13	1.08 ± 0.13	yes		52 ± 7	1.34 ± 0.4	yes		no
Implant-prototype	Epoxy resin	115 ± 13	1.25 ± 0.19	yes		121 ± 10	0.87 ± 0.15	yes		no
Implant	Silicone	117 ± 12	1.22 ± 0.1	yes		102 ± 14	2.98 ± 0.33	yes		yes
Implant	Silicone	119 ± 13	1.26 ± 0.12	yes		93 ± 5	1.39 ± 0.17	yes		no
Needle	Stainless steel	88 ± 16	1.24 ± 0.26	yes		104 ± 13	1.17 ± 0.11	yes		no
Implant-prototype	Epoxy resin	91 ± 2	0.68 ± 0.08	yes		126 ± 18	1.27 ± 0.08	yes		no
Implant-prototype	Epoxy resin	78 ± 7	0.84 ± 0.1	yes		129 ± 4	1.15 ± 0.03	yes		no
Implant-prototype	Epoxy resin	94 ± 6	0.99 ± 0.12	yes		82 ± 9	0.95 ± 0.14	yes		no
Infrared device	Plastics	100 ± 6	1.24 ± 0.1	yes		76 ± 7	1.06 ± 0.04	yes		no

Implant-prototype	Carbone fibre	122 ± 6	1.39 ± 0.15	yes		83 ± 19	0.81 ± 0.12	yes		no
Implant	PE and PP	100 ± 15	1.01 ± 0.09	yes		102 ± 10	1.26 ± 0.16	yes		no
Gloves	Nitrile, purple	93 ± 10	1.19 ± 0.01	yes		31 ± 8	0.79 ± 0.04	no		no
Gloves	Nitrile, blue	108 ± 14	1.66 ± 0.13	yes		5 ± 6	0.67 ± 0.01	no		no
Gloves	Latex, white	91 ± 10	1.35 ± 0.28	yes		113 ± 7	2.78 ± 0.2	yes		yes
Gloves	Latex, green	75 ± 2	3.54 ± 0.3	yes		84 ± 17	1.38 ± 0.4	yes		yes
Laboratory device	Thermoplast	102 ± 7	1.28 ± 0.3	yes		99 ± 8	1.69 ± 0.13	yes		no
Hearing device	DLPA Acrylate	114 ± 12	1.25 ± 0.11	yes		95 ± 11	3.06 ± 0.57	yes		yes
Hearing device	DLPA Acrylate	86 ± 7	1.65 ± 0.14	yes		129 ± 9	2.31 ± 0.18	yes		yes
Hearing device	TPU	91 ± 16	1.05 ± 0.31	yes		83 ± 17	0.87 ± 0.12	yes		no
Prothesis	Coating	80 ± 14	0.97 ± 0.17	yes		91 ± 3	1.03 ± 0.1	yes		no
Prothesis	TPE, PC	114 ± 14	1.1 ± 0.06	yes		109 ± 6	3.85 ± 0.31	yes		yes
Suture	Silicone	110 ± 7	0.89 ± 0.11	yes		112 ± 13	1.17 ± 0.25	yes		no
Suture	Silicone	81 ± 12	1.04 ± 0.13	yes		88 ± 13	0.81 ± 0.09	yes		no
Suture	PES	90 ± 11	0.66 ± 0.34	yes		124 ± 14	1.12 ± 0.17	yes		no
Active coal	Coal	62 ± 8	0.67 ± 0.14	yes		92 ± 8	0.68 ± 0.19	yes		no
Active coal	Coal	82 ± 9	0.77 ± 0.13	yes		92 ± 12	0.76 ± 0.15	yes		no
Dentrist device	Glass and Metals: X4613Cr, CuZn37	81 ± 9	1.17 ± 0.02	yes		73 ± 5	2.76 ± 0.12	yes		no
Hearing device	Silicon parts (grey)	78 ± 3	0.76 ± 0.06	yes		78 ± 5	0.6 ± 0.06	yes		no
Hearing device	ABS	89 ± 3	0.8 ± 0.05	yes		87 ± 6	0.96 ± 0.41	yes		no
Hearing device	Rubber	95 ± 4	0.84 ± 0.08	yes		98 ± 10	2.03 ± 0.08	yes		yes
Hearing device	TPE-A	107 ± 6	0.89 ± 0.1	yes		90 ± 5	0.94 ± 0.08	yes		no
Hearing device	TPE-A, brown	103 ± 7	1.73 ± 0.07	yes		91 ± 10	1.02 ± 0.12	yes		no
Hearing device	Silicon parts (grey)	88 ± 12	0.7 ± 0.11	yes		92 ± 10	0.92 ± 0.13	yes		no
Hearing device	TPE-A	101 ± 9	1.04 ± 0.03	yes		109 ± 10	1.09 ± 0.11	yes		no
Menstrual cup	Latex	89 ± 10	0.88 ± 0.24	yes		71 ± 12	2.46 ± 0.23	yes		yes
Menstrual cup	TPE	102 ± 6	1.01 ± 0.14	yes		98 ± 12	1.29 ± 0.13	yes		no
Menstrual cup	Silicone	82 ± 8	1.07 ± 0.04	yes		111 ± 12	1.09 ± 0.2	yes		no

5 Discussion

The aim of this work was to determine the parameters that influence medical device extraction prior to testing in *in vitro* skin sensitization assays. The obtained results finally led to the development of an optimized sample preparation procedure in accordance with DIN ISO 10993-12:2012.
A detailed discussion of the results obtained from the parameter comparison is described below.

Rotation speed

Theoretically the film theory of mass transfer is favoured by shaking since it ensures a continuous establishment of equilibrium in the extraction vessel leading to a more rapid leaching process. However, this is only true for short time periods. Over the sample extraction time of 72 hours the rotation speed of the shaker applied does not influence the final substance concentration migrated into the extract. Since the DIN ISO 10993-12:2012 prescribes any kind of movement during sample extraction a moderate rotation speed of 110 rpm will be applied.
Sampath discovered in his study a gradually increasing phenolic antioxidant content extracted from the leaves of *Polyalthia longifolia* with a rising shaking speed (Sampath M, 2013). However, the total extraction time was only 90 minutes therefore Sampath's results are similar to those obtained in this thesis.
Apart from the extraction time dependence on the influence of shaking also the concentration of leachables in the tested sample could play a role. For instance, the plant leaves extracted in the study of Sampath are much more complex and enriched in low molecular substances than the dye-spiked materials tested in this work. Probably an influence of the rotation speed of the shaker on the amount of leached dye in the extracts would have been noticed if the extracted materials were as well high concentrated.

Extraction vessel

The results revealed that glass vials are the ideal vessels for sample extraction. They have been shown to be completely inert and not to adsorb part of the leached substances in the extract by interacting or binding to them like plastic vessels.
These findings are consistent with the data published by Asbjørg Solberg Christophersen who detected that the low molecular chemical THC dissolved in blood samples binds to plastic surfaces during storage leading to a significant lower THC concentration compared to blood samples stored in glass vials (Christophersen, 1986).

Extraction agents and materials

According to the norm the use of both a polar and a non-polar extraction agent is mandatory.

Focussing first on the results of the comparison of non-polar solvents it can be seen that DMSO, 95 % ethanol and IPA were able to leach on average the highest substance amount from most tested biomaterials. However, using ethanol or IPA as extraction agent for a subsequent bioassay would provoke the need of vaporizing the sample extract prior to its application onto the cells because otherwise the cells would die. In this vaporisation step volatile substances eventually existing in the medical device may be lost leading to a distortion of the final assay result.

Durling et al. noticed in their extraction experiments a loss of volatile substances in extracts due to vaporization (Durling et al., 2007).

DMSO can be applied onto mammalian cells if the concentration does not exceed 1 %. Further, as visible in the summarized diagrams (Figure 28 Figure 29) one can say that DMSO is the stronger extraction agent compared to oil used in *in vivo* assays. Therefore, DMSO will to be the non-polar solvent of choice for the *in vitro* skin sensitization assay NanoSens.

Focussing now on the polar extraction agents it can be seen that 3 % acetic acid was able to leach the highest substance amounts from most materials. However, since this solvent would as well lead to cytotoxicity it can be excluded.

Ultrapure water turned out as second strongest polar solvent in this test. Nevertheless, it is also not suitable to directly apply it onto the cells. It would have to be diluted and this additional step does not justify its small lead over PBS as extraction agent. PBS turned out as proper polar extraction agent for medical devices and additionally it is compatible with mammalian cells making it the polar solvent of choice for the NanoSens assay.

However, it is impossible to ignore the extraordinary high concentration of Methylene blue leached from the silicone when IPA is used as extraction agent. Xu et al. obtained similar results when evaluating the effect of different cleaners on the colour stability of silicone rubbers used for maxillofacial prosthesis. In their experiments IPA caused the most severe fading of different tested solvents (Xu, Jiang, Zhang, Liang, & Li, 2011).

Further, Emel et al. revealed IPA to be an unusual, powerful solvent for the preparation of silicone-urea copolymers (Yilgor, Ekin Atilla, Ekin, Kurt, & Yilgor, 2003).

Also remarkable is the high extraction efficiency of Sudan I from TPU polymers by Oil. Alves et al. also emphasize the hydrophobicity of thermoplastic polyurethane in their study which aims to increase its hydrophilicity in order to enlarge their application range as biomaterials (Alves et al., 2009).

According to the results the interaction between solvent and material is a stronger decision-making factor than the interaction between solvent and migrating substance. No general statement can be made about the efficacy of a certain extraction agent for a given hydrophilic or lipophilic substance. The respective material that serves as matrix for the leachable is crucial for the extraction outcome. Polymeric biomaterials are highly diverse in their properties and thus it is very difficult to predict how they will behave with respect to the leachability in combination with a certain solvent.

Sample cutting surface

The results confirm the theory that higher amounts of sensitizers are leached from a material if the cutting surface is rough compared to a smooth one. The fold induction values obtained from the CAH-spiked samples are clearly higher if their cutting surface has a rough texture.

Considering the dye-spiked samples, it can be seen that depending on the roughness of the sample's cutting edge, the resulting extracts are coloured darker or lighter. Both tested materials silicone and epoxy resin confirm this observation, whereby this difference is even clearer for the epoxy resin.

Comparing the values of the perthometer the arithmetic mean roughness reveals a ten-fold increased surface if the silicone sample is cut roughly. Even more extreme is this difference for the brittle epoxy resin with a 28-fold increase.

Looking at Figure 31 visualizing the amounts of dye migrating from the material into the extract depending on the surface roughness of the sample it can be noticed that for selected solvents the differential factor is very similar to that obtained from the perthometer. For example in the case of DMSO a 30-fold increased Methylene blue dye concentration in the extract is observable. Also for 50 % ethanol this factor is with 26 very similar to the 28-fold increased arithmetic mean roughness.

Therefore, when cutting a sample it is important to pay attention on generating a smooth cutting surface since this is more comparable with the whole sample and strongly influences the extract.

The same effects have been detected by Buckwalter et al. who investigated the effect of surface roughness on glass leaching (Buckwalter, Pederson, & McVay, 1982).

Buckwalter et al. who investigated the effect of surface roughness on glass leaching noted the same effect since a higher roughness of the material increased its leaching extent. (Buckwalter, Pederson, & McVay, 1982).

Foil experiment

According to the results obtained from the dye spiked samples it can be noticed that less substance is migrated from a thin foil-like material when it is packed as a bundle into the extraction vessel compared to a planar piece of foil. However, this observation is only valid for the hydrophilic Methylene blue and not for the lipophilic Sudan I.

Reflecting these results, during sample preparation of foil-like materials it should be considered to ensure that the whole sample surface is exposed to the solvent at any time of the extraction process.

Time course

The decreasing fold induction values obtained when the sensitizing substance CAH is stored a certain period of time after cutting before testing show that substance loss might occur during sample storage.

Materials are able to change over the lifetime of a medical device. Therefore, performance of periodical tests is recommended. ISO 10993 addresses this "problem" by demanding testing at the beginning and at the end of the lifetime of a high risk medical device.

The results of the dye-spiked samples show that the most substance is leached in the first few hours. Nevertheless, the dye concentrations in the extracts get higher and the prescribed extraction time 72 hours is reasonable since no early saturation effect can be noticed except for Sudan I in silicone.

Temperature

The first experiments revealed that most solvents leach higher amounts of substance from silicone and TPU polymer at a temperature of 37 °C compared to 50 °C.

Especially the increased Sudan I concentration leached at 50 °C from the silicone and TPU polymer samples compared to the lower temperature of 37 °C are particularly striking.

One suspicion for this observation was the occurrence of chemical degradation reactions of the oil that may possibly occur at higher temperatures and that would lead to an absorption shift at altered pH-values. To exclude this suspicion, dye-spiked materials were incubated in oil at 37 °C and 50 °C. Measuring the pH-values of these oil extracts showed no difference, so this theory can be excluded.

A clear declaration for the observation of the higher Methylene blue concentrations in the silicone and TPU polymer extracts of most of the tested solvents as indicated in Figure 40 and Figure 41 cannot be made. However, the experiments at 50 °C were performed some weeks after those at

37 °C. It is possible that post-curing of the dye-spiked silicone and TPU polymer samples occurred during this time gap resulting in a stronger cross-linking of the dye in the material matrix.
To further investigate this suspicion a part of that experiment was repeated by starting the extraction for both compared temperatures simultaneously.
The outcome of the repeated experiment encourages the hypothesis that the polymeric materials undergo a post-curing process after storing them for several weeks.
Obviously the actual time of testing silicone and TPU polymer influences the leaching behaviour at different temperatures and further experiments could not be performed due to a lack of sufficient sample material. Therefore, the temperature results have to be scrutinized.

The norm allows choosing an extraction temperature of either 37 °C or 50 °C. Therefore, each institution has to decide individually whether a certain sample should be tested after extraction under real application conditions (37 °C) or under extreme conditions (50 °C) aiming to leach as much substance as possible from the material.

In the United States the Food and Drug Administration (FDA) regulates medical devices in conjunction with ISO 10993. FDA guidelines largely agree with ISO 10993 regulations and ISO test results are generally acceptable for applications in the United States.
In the “FDA Blue Book Memorandum # G95-1” the FDA describes additional evaluation requirements regarding the biocompatibility evaluation of medical devices.
For example, it states that prolonged contact devices and permanent implants should be extracted at 50 °C since that extract would represent the chemicals that may leach out over the use in a more realistic way. According to this guideline in some cases, temperatures above 37 °C would result in degradants that may not occur in clinical use and may result in toxicities not representative of the final product. Therefore, the used extraction conditions should be justified.
However, despite the major goal of an international harmonization of biocompatibility testing there are still regional differences in the applicable regulations.

In summary, this thesis revealed the main parameters that influence the extraction of medical devices intending to undergo skin sensitization assays. It could be seen that sample extraction is a crucial process that definitely influences the quality of the extract and hence the result of the assay.
Within this work the extraction was optimized successfully and critical steps could be identified.

6 Literature

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