DISERTAČNÍ PRÁCE

Univerzita Palackého v Olomouci

Lékařská fakulta

Doktorský studijní program: Lékařská biofyzika

Studium genotoxicity u ingrediencí spotřebních výrobků

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Prohlášení

Prohlašuji, že jsem svoji disertační práci vypracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu.

Olomouc, 2023

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Mgr. Jan Chrz

Poděkování

Rád bych poděkoval své školitelce Prof. RNDr. Haně Kolářové, CSc. za ochotu a cenné rady během mého doktorského studia a za příležitost spolupracovat s katedrou Lékařské biofyziky.

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Tato práce vznikla za podpory Ministerstva zdravotnictví České republiky – RVO („Státní zdravotní ústav, IČ: 75010330“) a projektu ERDS/ESF – „Mezinárodní konkurenceschopnost SZÚ ve výzkumu, vývoji a vzdělávání v alternativních toxikologických metodách“ (č. VZ.02.1.01/0.0/0.0/16_019/0000860).

Abstrakt

Tato disertační práce se zaměřuje na systematické hodnocení genotoxicity vybraných chemických látek a spotřebních výrobků za využití toxikologických metod *in vitro*, konkrétně *in vitro* testu savčích chromozomových aberací a alkalického *in vitro* kometového testu na savčích buňkách.

V první části práce bylo portfolio *in vitro* metod Oddělení alternativních toxikologických metod Státního zdravotního ústavu doplněno o nové přístupy a již zavedené metody byly inovativně optimalizovány. Jednotlivé metody byly následně kombinovány za účelem získání komplexního pohledu na genotoxický potenciál testovaných látek a ingrediencí spotřebních výrobků.

Výsledky testů zvýšily porozumění mechanismům genotoxicity v kontextu využitých *in vitro* metod. Výsledky této disertační práce poskytly důležité informace o genotoxickém potenciálu vybraných látek a parfémů, což má význam v oblasti ochrany spotřebitele. Jednotlivé části v závěru práce diskutují dosažené výsledky v oblasti testování genotoxicity, včetně potenciálního začlenění kombinací *in vitro* metod do regulativního procesu za účelem posílení bezpečnosti a ochrany spotřebitele o nové přístupy bez použití laboratorních zvířat.

Tato disertační práce přispívá k rozvoji metod pro hodnocení genotoxicity a zvyšuje povědomí o bezpečnosti chemických látek a parfémů v kontextu spotřebních výrobků. Její výsledky mají důležité implikace pro oblast regulace, výzkumu a výroby a mohou sloužit jako podklad pro další diskuse o bezpečnosti spotřebních výrobků na trhu.

Klíčová slova: genotoxicita/mutagenita, alternativní toxikologické testování, *in vitro* metody, testovací baterie metod, chromozomové aberace, alkalický *in vitro* kometový test, *in vitro* test savčích chromozomových aberací, spotřební výrobky, ingredience, konzervanty

Abstract

This doctoral thesis is focused on systematic evaluation of genotoxicity of selected chemical substances and consumer products using *in vitro* toxicological methods, specifically the *In vitro* Mammalian Chromosome Aberration Test and Alkaline *In vitro* Comet Assay. In the first part of the study, these *in vitro* methods were implemented at the Unit for Alternative Toxicological Methods at the National Institute of Public Health, systematically optimized, and selected for further assessment. The methods were subsequently combined to obtain a comprehensive view of the genotoxic potential of the tested substances and consumer products.

The results of the tests enhanced the understanding of genotoxicity mechanisms in the context of the employed *in vitro* methods. The findings of this doctoral thesis provide crucial information on the genotoxic potential of selected substances and perfumes, which holds significant importance in the field of consumer protection. The discussion in the conclusion of the thesis proposes directions for further research in the field of genotoxicity testing, including the potential integration of *in vitro* methods into the regulatory process and the enhancement of safety and consumer protection due to novel approaches.

This doctoral thesis contributes to the development of methods for evaluating genotoxicity and increases awareness of the safety of chemical substances and perfumes in the context of consumer products. Its results have important implications for regulation, research, and manufacturing, serving as a basis for further discussions on the safety of consumer products on the market.

Key words: genotoxicity/mutagenicity, alternative toxicological testing, *in vitro* methods, testing battery of methods, chromosome aberrations, Alkaline *in vitro* Comet Assay, *In vitro* Mammalian Chromosome Aberration Test, consumer products, ingredients, preservatives

Cíle práce

Tato disertační práce je zaměřená na využití *in vitro* metod pro testování genotoxicity chemických látek a spotřebních výrobků. Primárním cílem doktorského projektu bylo přispět k pochopení, zdokonalení a rozvoji těchto metod.

Hlavním cílem této disertační práce bylo systematicky ověřovat, optimalizovat i nově zavést do laboratorní praxe Oddělení alternativních toxikologických metod Státního zdravotního ústavu toxikologické metody bez použití laboratorních zvířat vhodné pro identifikaci genotoxicity chemických látek, směsí i spotřebních výrobků, aplikovat tyto metody v kombinaci na testování genotoxicity chemických látek, využívaných jako konzervanty v kosmetických přípravcích, a dále ověřit využití vybraných metod při testování finálních kosmetických přípravků s podobným složením.

Dalšími cíli doktorského projektu bylo zkoumat mechanismy genotoxicity, ověřit využitelnost zavedených metod, zhodnotit citlivost a specifičnost vybraných metod, diskutovat jejich aplikovatelnost, navrhnout metodické zlepšení k jejich optimalizaci a jejich vhodné kombinace pro zlepšení sledování potenciálu genotoxicity *in vitro* u vybraných chemických látek a spotřebních výrobků významných v oblasti ochrany veřejného zdraví a ochrany spotřebitelů. V průběhu doktorského projektu byly cíle práce průběžně doplňovány dle zjištěných výsledků.

Nedílnou součástí doktorského projektu bylo posouzení, zda výsledky testů odpovídají bezpečnostním standardům pro daný typ výrobku a návrhy strategií *in vitro* pro hodnocení genotoxicity, které mohou být aplikovány ve výzkumu a praxi.

V rámci grantového projektu ERDS/ESF "Mezinárodní konkurenceschopnost SZÚ ve výzkumu, vývoji a vzdělávání v alternativních toxikologických metodách" (č. VZ.02.1.01/0.0/0.0/16_019/0000860) byly z hlediska bezpečnosti genotoxicity ověřeny vybrané chemické látky a kosmetické přípravky několika metodami vhodnými pro detekci různých koncových bodů genotoxicity jako závažného toxikologického účinku.

Obsah

Seznam zkratek

3R	Replacement, Reduction and Refinement
ADME	Absorpce, distribuce, metabolismus, exkrece
AOP	Adverse Outcome Pathway
ATCC	American Type Culture Collection
BP	Butylparaben
CA	Chromosome Aberration Test
CAS	Chemical Abstracts Service
CCD	Charge-Coupled Device Camera (zařízení s vázanými náboji)
CMT	Comet assay (test)
CosIng	Cosmetic ingredient database
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxid
DNA	Deoxyribonukleová kyselina
DPRA	Direct Peptide Reactivity Assay
EC	European Commission
ECACC	European Collection of Authenticated Cell Cultures
ECVAM	European Center for Validation of Alternative Methods
EdC	Eau de cologne
EdP	Eau de parfum
EdT	Eau de toilette
EDTA	Etylendiamintetraoctová kyselina
EFSA	European Food Safety Authority
ECHA	European Chemical Agency
EP	Ethylparaben
EPAA	European Partnership for Alternative Approaches to Animal Testing
ES	Směrnice Evropského parlamentu a Rady
EU	European Union
FBS	Fetal bovine serum

FISH	Fluorescenční *in situ* hybridizace
HMP	High Melting Point (agarose)
CHO	Chinese hamster ovary cells
ICCVAM	Interagency Coordinating Commitee on the Validation of Alternative Methods
INCI	International Nomenclature of Cosmetic Ingredients
IUPAC	International Union of Pure and Applied Chemistry
LMP	Low Melting Point (agarose)
MAS	Metabolic Activation System
MN	Micronucleus Test
MP	Methylparaben
MTT	Kolorimetrický test pro měření cytotoxicity s využitím barviva 3-[4,5-dimetylthiazyol-2-yl]-2,5 difenyl tetrazolium bromid
NOAEL	No Observed Adverse Effect Level
NR	Neutral Red
NRU	Neutral Red Uptake (3T3 NRU Cytotoxicity Assay)
OECD	Organisation for Economic Co-operation and Development
OTM	Olive Tail Moment
P	Parfum
p53	Jaderný protein, transkripční faktor
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinine
PHBA	Kyselina p-hydroxybenzoová
PP	Propylparaben
S9	Aktivační metabolická směs z jaterního homogenátu savců
SCCP	Scientific Committee on Consumer Products
SCCS	Scientific Committee on Consumer Safety
SCF	Scientific Committee on Food
SCGE	Single cell gel electrophoresis
SD	Standard deviation

SZÚ	Státní zdravotní ústav
TCC	Triclocarban
TCS	Triclosan
TG	Test Guideline
US EPA	United States Environmental Protection Agency
UV	Ultrafialové záření
YES/YAS	Yeast Estrogen Screen/Yeast Androgen Screen

Literární úvod a přehled problematiky

Genotoxicita, jakožto klíčový aspekt v oblasti toxikologie, představuje komplexní téma, které zasahuje do široké škály biologických procesů a má významný dopad na lidské zdraví a životní prostředí. Termín "genotoxicita" označuje schopnost látek způsobit poškození genetické informace v buňkách, což může mít různé důsledky, včetně mutací, chromozomových aberací a karcinogeneze.

V současném pracovním a životním prostředí, zatíženém neustále rostoucím počtem chemických látek, je posouzení genotoxických účinků stále naléhavějším úkolem v oblasti ochrany veřejného zdraví, bezpečnosti spotřebitelů, pracovního lékařství a ochrany životního prostředí. Různé látky, ať už přirozené nebo antropogenní, mohou vyvolat genotoxické účinky a představovat potenciální riziko pro organismy a ekosystémy.

Výzkum v oblasti genotoxicity neustále poskytuje nové poznatky o mechanismech, skutečných dopadech a přístupech k hodnocení genotoxických rizik. Genotoxické účinky se mohou projevovat na různých úrovních biologické organizace, od molekulárních změn v DNA až po cytogenetické poruchy a vývoj různých onemocnění, včetně onkologických.

Etika a odpovědnost ve vědeckém výzkumu se stávají klíčovými principy, zejména pokud jde o testování a hodnocení bezpečnosti chemických látek v kosmetických přípravcích. Nařízením Evropské unie (EC, 2009), které se týká kosmetiky, bylo zakázáno testování kosmetických ingrediencí a konečných produktů na zvířatech, což zdůrazňuje potřebu hledat alternativní přístupy pro posouzení toxicity a bezpečnosti (Beken *et al.*, 2016). Testování chemických látek, které vyvolávají toxikologické obavy, na lidských dobrovolnících pro koncové body toxicity, jako je genotoxicita, mutagenita a karcinogenita, je eticky nepřijatelným postupem. V tomto kontextu se uplatňují toxikologické metody *in vitro* jako klíčový nástroj pro posouzení bezpečnosti a rizik spojených s používáním chemických látek ve spotřebních výrobcích, včetně kosmetických přípravků. Nedávno byly navrženy vhodné kombinace *in vitro* metod, které využívají testovací systémy lidského původu, jako jsou lidské geny, receptory, proteiny, buňky a tkáně (OECD, 2017). Tyto *in vitro* metody nejenže poskytují spolehlivá a vědecky relevantní data, ale také umožňují efektivní hodnocení genotoxicity bez použití zvířat.

Genotoxicita je jeden z nejkritičtějších toxikologických koncových bodů a má velký význam pro veřejné zdraví, bezpečnost spotřebitelů a ochranu životního prostředí. Etický základ vědeckého výzkumu je dále posílen principy 3R (Replacement, Reduction and Refinement) definovanými Russellem a Burchem v roce 1959. Tyto principy jsou pevně zakotveny ve Směrnici Evropského parlamentu a Rady EU 2010/63/EU o ochraně zvířat používaných pro vědecké účely, která podporuje hledání alternativních metod k testování na zvířatech (Směrnice 2010/63). I přes významný pokrok ve vývoji, validaci a používání alternativních metod bez použití zvířat, komunita v oblasti regulace tradičně preferuje přístupy založené na zvířatech (Combes & Balls, 2014). Obtíže spojené s hodnocením genotoxicity *in vitro*, včetně fyziologického pozadí indukce a propojení výsledků *in vitro* s podmínkami *in vivo*, vedou k nízké akceptaci *in vitro* údajů.

Přestože některé testy *in vitro* byly již přijaty pro regulační účely, jako je test savčích chromozomových aberací (OECD TG 473), test mikrojader na savčích buňkách (OECD TG 487) a bakteriální Amesův test (OECD TG 471), stále existuje potřeba zdokonalení a dalšího výzkumu. Kombinace více validovaných *in vitro* testů s dostatečnou senzitivitou a specificitou se ukazuje jako vhodný přístup pro posouzení genotoxicity chemických látek a spotřebních výrobků. Jednotlivé metody *in vitro* mohou vykazovat specifické výhody i omezení, ale jejich kombinace může zvýšit citlivost hodnocení bezpečnosti (Bhagat, 2018). Dosud však žádný samostatně použitý test *in vitro* neumožňuje detekci široké škály specifických změn ve struktuře DNA projevujících se jako nežádoucí účinky spojené buď s genotoxicitou, nebo mutagenitou (Nesslany, 2017). Proto se aktuálně doporučuje používat kombinace 2 nebo 3 validovaných *in vitro* testů s dostatečnou senzitivitou a specificitou. Chemické látky mohou být vyhodnoceny jako nevykazující genotoxický potenciál, pokud jsou všechny použité koncové body *in vitro* jasně negativní, zatímco alespoň dva koncové body *in vitro* vykazující pozitivní výsledky mohou genotoxický potenciál předpovídat (EFSA, 2011).

V současné době jsou mezinárodní pracovní skupiny a organizace, včetně OECD, EURL ECVAM, EPAA a ICCVAM, aktivně zapojeny do podpory a validace alternativních toxikologických metod. Hledají se další údaje k porozumění toxikologickým mechanismům a vlastnostem chemických látek, včetně genotoxicity, s přesahem do problematiky ADME - absorpce, distribuce, metabolismu a vylučování.

Vzhledem k neustálému pokroku v oblasti vývoje nových metodologií lze očekávat, že budou k dispozici vysoce efektivní hodnotící metody a jejich kombinace *in vitro*, které podpoří jejich širší využití a poskytnou relevantní informace pro lidské zdraví.

Celosvětově se názory a doporučení v oblasti testování genotoxicity promítají do legislativních dokumentů a směrnic, jako jsou Nařízení Rady (ES) č. 440/2008, OECD Overview of Genetic Toxicology Test Guidelines (OECD, 2017), zpráva agentury ECHA o přístupech bez použití zvířat (ECHA, 2017) a vědecké stanovisko úřadu EFSA ke strategiím testování genotoxicity (EFSA, 2018). Tyto dokumenty poskytují rámec pro regulační hodnocení a zajišťují postupný přechod od tradičních postupů založených na zvířatech k moderním, etickým a vědecky relevantním přístupům bez jejich použití.

Tato disertační práce se bude podrobně zabývat problematikou genotoxicity ingrediencí spotřebních výrobků, chemických konzervačních látek používaných v kosmetice a souvislostmi s legislativou, etickými aspekty, vývojem alternativních metod a jejich praktickou aplikací.

Cílem práce je zkoumat genotoxické účinky, které chemické látky mohou vyvolat, a to s důrazem na mechanismy působení a toxikologické metody *in vitro*. Kromě toho práce poskytne komplexní přehled současné situace a trendy v oblasti alternativních metod pro hodnocení genotoxicity, které nabízejí efektivní přístupy bez použití laboratorních zvířat.

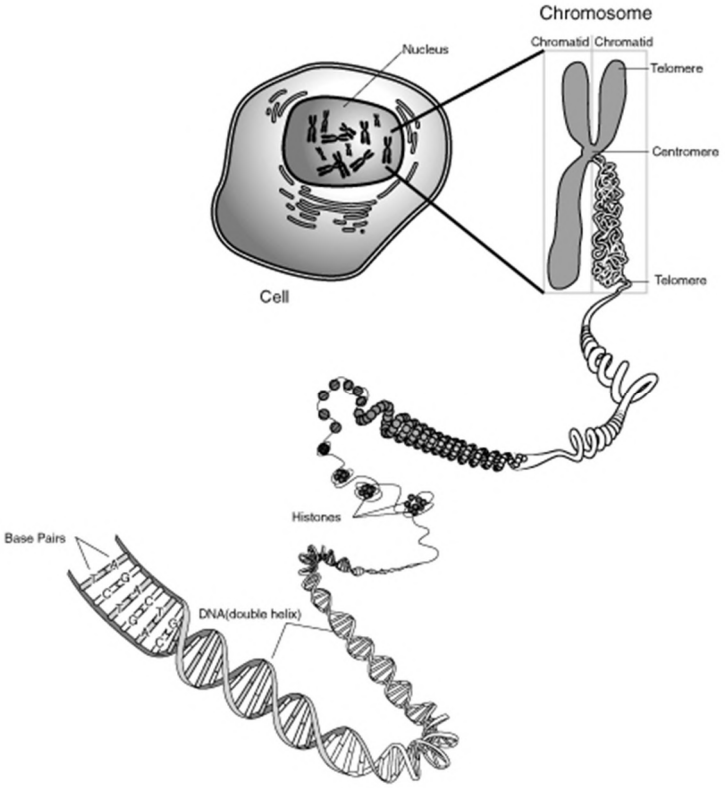
Hlubší porozumění genotoxickým účinkům, implementace moderních a etických postupů hodnocení genotoxicity, které reflektují aktuální vědecký vývoj a normy v oblasti toxikologie, může poskytnout základ pro vývoj bezpečnějších chemických látek a lepší ochranu lidského zdraví a životního prostředí.

2 Teoretická část

2.1 Genotoxické účinky

Deoxyribonukleová kyselina, zkráceně DNA, je základní molekulou genetické informace ve všech živých organismech. Tato dvojitě šroubovicová molekula nese genetický kód, který určuje vývoj, fungování a dědičné vlastnosti organismů. DNA se skládá ze dvou dlouhých řetězců nukleotidů, které jsou propojeny spojovacími můstky tvořenými páry bází (adenin s thyminem, guanin s cytosinem). Tato komplexní struktura DNA tvoří základ pro dědičnost a přenáší genetickou informaci z jedné generace na druhou.

Chromozomy jsou struktury nesoucí genetickou informaci v buňkách živých organismů (Obrázek 1). V lidském těle se nachází v jádře buněk. Každý zdravý člověk má ve svých buňkách 46 chromozomů uspořádaných do 23 párů. Tyto chromozomy obsahují segmenty DNA - geny, které určují různé vlastnosti a charakteristiky jedince. Během buněčného dělení se chromozomy kopírují a přenášejí na další buněčné populace, což zajišťuje předání genetické informace z jedné buňky na další. Studium struktury a funkce chromozomů je klíčové pro pochopení genetiky a dědičnosti v organismech.



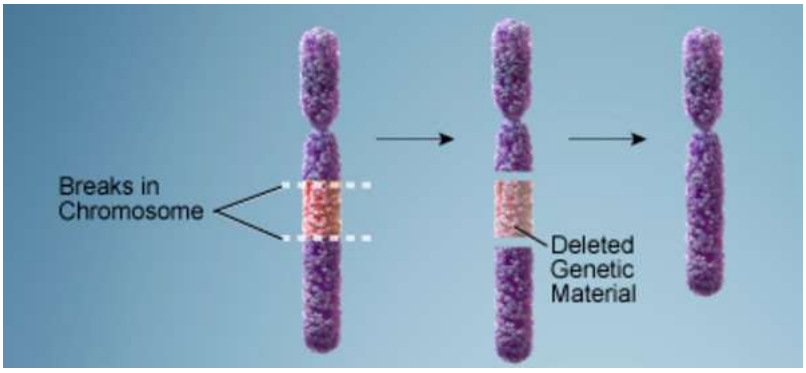
Obr. 1: Stavba chromozomu (převzato z: http://www.genetika-biologie.cz/chromozomy)

Genotoxicitou se v toxikologii a genetice označuje schopnost chemických látek, fyzikálních faktorů nebo biologických agens (jako jsou viry) způsobovat poškození genetické informace v buňkách organismu. Genotoxické látky mohou způsobit změny v DNA, což může mít různé biologické důsledky, včetně mutací, chromozomových aberací a genetických abnormalit, které mohou vést k aktivaci onkogenů a deaktivaci tumor supresorových genů. Tyto procesy prokazatelně zvyšují riziko rozvoje rakoviny nebo jiných degenerativních onemocnění (Očadlíková *et al.*, 2007). Genotoxicita může postihnout buňky buď přímo, nebo nepřímo. Přímé genotoxické vlivy působí na DNA a mohou způsobit její fyzikální nebo chemickou změnu. Nepřímé genotoxické vlivy mohou vykazovat látky, které mají genotoxický účinek až po metabolické přeměně v buňce. Znalosti z tohoto oboru jsou využívány při biologickém monitorování v oblasti prevence nádorových onemocnění nejen u osob profesionálně exponovaných mutagenním a karcinogenním látkám, ale i u osob ovlivněných těmito látkami z životního prostředí (Rössner, 1996).

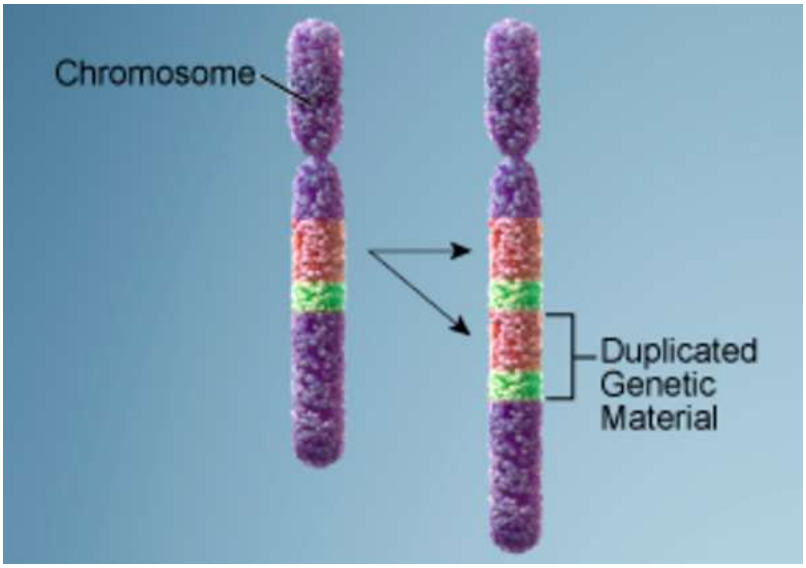
Hodnocení genotoxicity je důležitým krokem v procesu hodnocení bezpečnosti chemických látek, spotřebních výrobků, léčiv, potravin a dalších látek, kterým jsou organismy potenciálně exponovány. Identifikace genotoxických látek a pochopení mechanismů, jimiž působí, přispívá k ochraně veřejného zdraví a životního prostředí.

Přímé mutageny mají schopnost přímo působit na DNA a způsobovat její poškození. Příkladem jsou alkylační činidla, která mohou měnit strukturu bází v DNA. Ionizující záření, jako je rentgenové záření a gama záření, mohou přímo interagovat s DNA, způsobovat zlomy nebo přímé změny v nukleotidové sekvenci. Nepřímé mutageny samy o sobě nejsou mutageny, ale mohou být metabolizovány v buňce na mutagenní sloučeniny. Příkladem mohou být prekurzory aromatických aminů. K napodobení situace *in vivo* se doporučuje zařazovat při testování *in vitro* metabolický aktivační systém (MAS) (Tice *et al.*, 2000; Nesslany, 2017). Nejběžnějším systémem používaným pro metabolickou aktivaci je frakce S9, připravovaná tradičně z jater hlodavců, ale v současnosti jsou komerčně dostupné i lidské frakce S9 (EFSA, 2011; Hong *et al.*, 2018). Ultrafialové (UV) záření ze slunečního světla nepřímo působí na DNA tím, že vytváří tzv. pyrimidinové dimery, což jsou strukturální změny v DNA.

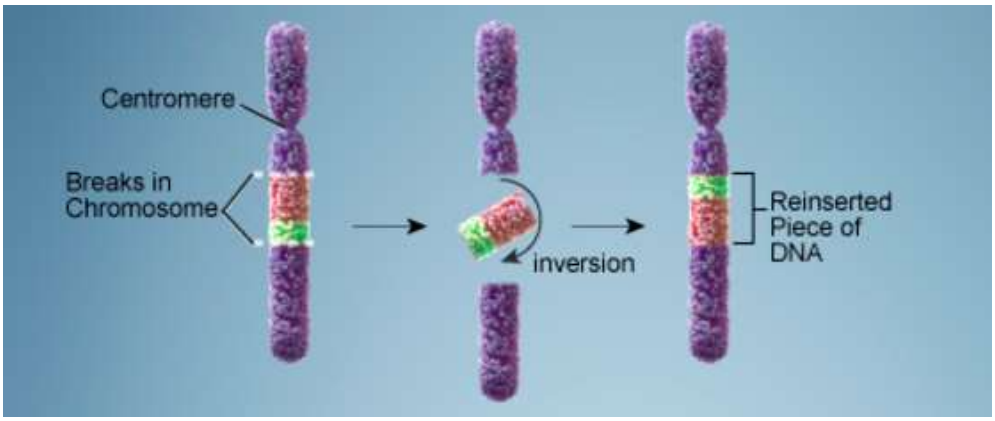
Chromozomové poškození může nastat při působení mutagenů, což jsou látky nebo faktory schopné způsobit genetické změny v DNA. Důsledky poškození chromozomů jsou mutace, což jsou trvalé genetické změny v nukleotidové sekvenci. Přímo nebo nepřímo způsobené poškození chromozomů může vést k abnormalitám v jejich struktuře. Sem patří mutace způsobené delecí (Obrázek 2), duplikací (Obrázek 3), inverzí (Obrázek 4), translokací (Obrázek 5) nebo vznikem prstenčitého chromozomu (ring) (Obrázek 6), projevující se jako chromozomové aberace. Některé mutageny mohou způsobit genetické změny, které následně vedou k nekontrolovanému bujení buněk, což je charakteristické pro nádorový růst. Studium genotoxicity a pochopení mechanismů, které vedou k poškození chromozomů, je klíčové pro posouzení bezpečnosti látek a prevenci rizik spojených s expozicí mutagenům.



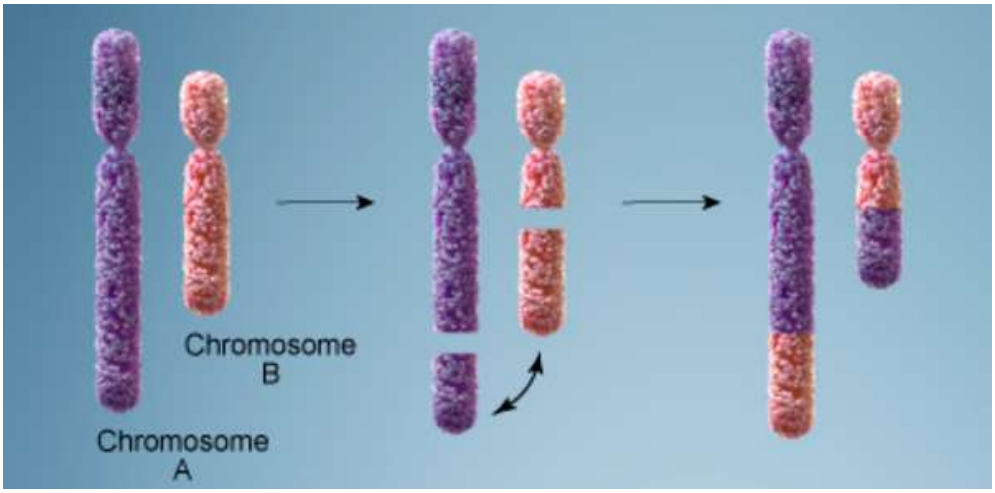
Obr. 2: Schématický nákres delece (převzato z https://medlineplus.gov/genetics/understanding/mutationsanddisorders/structuralchanges/)



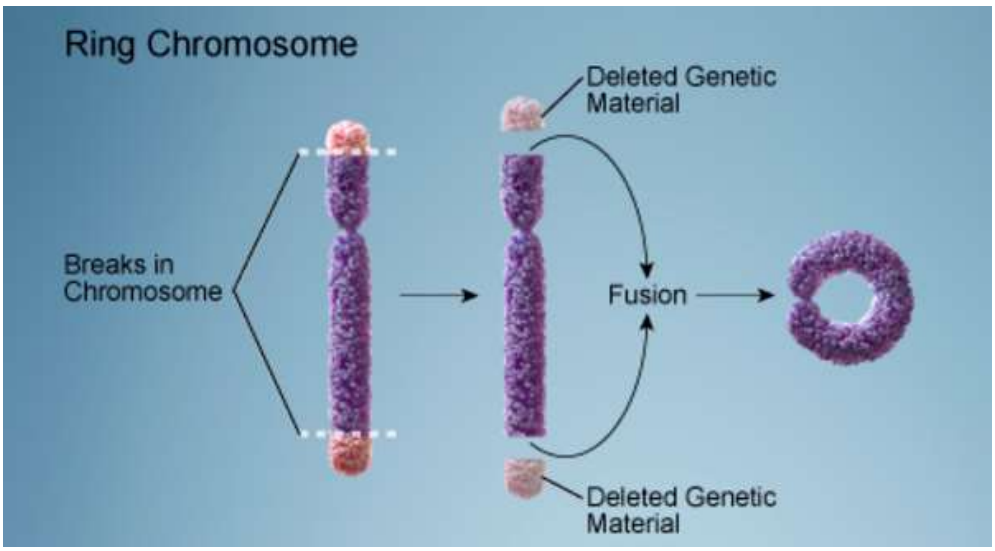
Obr. 3: Schématický nákres duplikace (převzato z https://medlineplus.gov/genetics/understanding/mutationsanddisorders/structuralchanges/)



Obr. 4: Schématický nákres inverze (převzato z https://medlineplus.gov/genetics/understanding/mutationsanddisorders/structuralchanges/)



Obr. 5: Schématický nákres translokace (převzato z https://medlineplus.gov/genetics/understanding/mutationsanddisorders/structuralchanges/)



Obr. 6: Schématický nákres prstenčitého chromozomu (ring) (převzato z https://medlineplus.gov/genetics/understanding/mutationsanddisorders/structuralchanges/)

2.2 Dostupné *in vitro* přístupy pro testování genotoxicity

Testovací baterie navržená pro počáteční screening genotoxického potenciálu by měla umožnit detekci tří důležitých genotoxických koncových bodů, tj. genových mutací, strukturálních chromozomálních aberací (klastogenity) a numerických změn v počtu chromozomů (aneuploidie) (EFSA, 2011). Použití *in vitro* systémů je obecně považováno za užitečné a je zahrnuto do aktualizovaných Směrnic OECD pro testování genotoxicity, jako například OECD TG 489 (*In vivo* savčí alkalický kometový test).

Nejběžněji používané metody hodnocení genotoxického potenciálu látek *in vivo* s možným zahrnutím testovacích systémů *in vitro* jsou uvedeny níže na základě jejich hlavního koncového bodu genotoxicity:

Genové mutace:

- Testy genových mutací somatických a zárodečných buněk transgenních hlodavců (OECD TG 488).

Poškození chromozomů:

- Test mikrojader savčích erytrocytů (CMT) (OECD TG 474),
- Test chromozomových aberací kostní dřeně savců (CA) (OECD TG 475).

Primární poškození DNA:

- Alkalický *in vivo* kometový test na savčích buňkách (CMT) (OECD TG 489)

Mutace:

- Test bakteriální reverzní mutace u *Salmonella typhimurium* a *Escherichia coli* (Amesův test) (OECD TG 471),
- *In vitro* test mutace genu na savčích buňkách (OECD TG 476),
- *In vitro* testy genových mutací s použitím *tk* lokusu (OECD TG 490).

Chromozomové aberace:

- *In vitro* test savčích chromozomových aberací (CA) (OECD TG 473),
- *In vitro* test mikrojader savčích buněk (MN) (OECD TG 487) (klastogenita),
- *In vitro* alkalický kometový test na savčích buňkách (CMT) (prováděný na testovacím systému *in vitro*, v souladu s OECD TG 489) (chromozomální zlomy).

2.3 Kombinace metod doporučených pro testování genotoxicity / mutagenity / karcinogenity

Kombinace testů pro screening karcinogenity byla navržena již v roce 1974 (Stoltz *et al.*, 1974). V nedávné době bylo v odborných publikacích zmíněno, že kombinace Amesova testu, *in vivo* testu mikrojader (MN), *in vivo* testu chromozomových aberací (CA) a kometového testu (CMT) zvyšuje citlivost testovací baterie navržené pro detekci karcinogenů (Kang *et al.*, 2013a; Kim & Margolin 1999; Morita *et al.*, 2016). Alternativně se také uvádí, že kombinace Amesova testu a *in vivo* MN, nebo kombinace testů MN a CMT vykazují slibnou citlivost (Shelby & Zeiger 1990; Recio *et al.*, 2010; Vasquez, 2010; Kang *et al.*, 2013a). Signifikantní citlivost byla popsána při použití kombinace *in vivo* CMT (90 %) a *in vitro* CMT (86,9 %), *in vivo* MN + *in vivo* CA (88,6 %), *in vivo* MN + *in vivo* CMT (92,5 %), *in vitro* CMT + *in vivo* MN (95,6 %) (Bhagat, 2018; Kimura *et al.*, 2013). Nejvyšší citlivost byla pozorována u kombinace *in vivo* CMT + *in vivo* CA (96,7 %) (Bhagat, 2018; Madrigal-Bujaidar *et al.*, 2008; Pfuhler *et al.*, 2009; Bowen *et al.*, 2011). Obecný vývoj toxikologického testování, prováděného podle principů 3R, umožňuje zahrnutí výhradně *in vitro* koncových bodů ve vhodných kombinacích do testovacích baterií bez použití laboratorních zvířat. Nelze očekávat, že by jediný samostatný *in vitro* test předpovídal všechny klíčové události, které mohou potenciálně vést k přetrvávající genotoxicitě a/nebo mutagenitě. Proto by měla kombinace *in vitro* testů zahrnovat testy s dostatečnou citlivostí (aby se zabránilo falešně negativním výsledkům) a specificitou (aby se předešlo falešně pozitivním výsledkům). Standardní baterie *in vitro* genotoxických testů, doporučená pro regulační účely, by měla zahrnovat 2 nebo 3 standardizované či validované metody s alespoň jedním testem na bakteriích a jedním testem na buněčných kulturách (Nesslany, 2017).

V případě dermální expozice se důrazně doporučují exkluzivní přístupy *in vitro*, neboť jsou jedinými dostupnými testovacími přístupy pro testování kosmetiky a kosmetických přísad. V případě kosmetických ingrediencí by hodnocení potenciálu mutagenity, které bude připojeno k nařízení (ES) č. 1223/2009, mělo zahrnovat testy poskytující informace o třech genotoxických koncových bodech, tj. mutagenitu na genové úrovni, poškození chromozomů a/nebo přestavby (klastogenitu) a numerické chromozomové aberace (aneuploidii).

Evropský vědecký výbor pro bezpečnost spotřebitelů doporučil dva testy pro základní testování kosmetických ingrediencí:

- Bakteriální test reverzních mutací (OECD 471) jako test zahrnující genové mutace,
- *In vitro* test mikrojader (OECD 487) jako test na strukturální chromozomové aberace (klastogenicita) i numerické chromozomové aberace (aneugenicita).

Potvrzující nebo podpůrné důkazy lze získat pomocí testů detekujících primární poškození DNA, jako je např. alkalický kometový test (Kirkland *et al.*, 2011).

2.4 Legislativní problematika testování genotoxicity / mutagenity / karcinogenity u kosmetických ingrediencí a kosmetických přípravků

Testování kosmetických ingrediencí a finálních kosmetických přípravků na zvířatech je v Evropské unii zakázáno pro všechny toxikologické účely od roku 2013. Nařízení Evropského parlamentu a Rady (ES) č. 1223/2009 ze dne 30. listopadu 2009 o kosmetických přípravcích (dále jen „Nařízení“) poskytuje seznam ingrediencí zakázaných pro použití v kosmetice (Příloha II) nebo schválených/povolených pouze pro omezené použití (Přílohy III, IV, V, VI). Seznam kosmetických ingrediencí s dostatečně popsanými toxikologickými vlastnostmi není k dispozici a v souladu s Nařízením mají osoby odpovědné za uvádění kosmetického přípravku na trh v EU povoleno použít konkrétní ingredienci, pokud bylo provedeno hodnocení bezpečnosti podle Přílohy I, které musí být k dispozici na vyžádání regulačním orgánům.

V obecné rovině představují finální kosmetické přípravky směsi mnoha ingrediencí. Předmětem hodnocení bezpečnosti je přítomnost a toxikologické vlastnosti každé individuální ingredience. Některé ingredience však mohou být složeny také z mnoha individuálních složek původem z přírodních a biologických extraktů nebo biologických tekutin. Toxikologické vlastnosti kombinované směsi, jako je kosmetický přípravek, závisí nejen na vlastnostech jednotlivých složek, ale také na jejich kombinovaných interakcích, i když jsou jednotlivé složky použité v koncentracích, které je možno považovat za bezpečné, a dále na biodostupnosti, typu a frekvenci expozice. Biologické účinky kosmetického přípravku může ovlivnit mnoho faktorů, jako je povaha výroby, biologická variabilita, nestandardizovaná kvalita, stabilita nebo různý původ přírodních složek (například přírodních extraktů, rostlinných ingrediencí, esenciálních olejů, biologických tekutin atd.). Přírodní produkty mohou vykazovat variabilitu mezi jednotlivými šaržemi v závislosti na ročním období, původu rostlin atd. Použití nepřímých distribučních kanálů může bohužel umožnit vstup nekvalitních výrobků na maloobchodní trh. Podrobné informace o mnoha chemických látkách jsou veřejně dostupné v Registračních složkách na webových stránkách Evropské agentury pro chemické látky (ECHA). Seznam kosmetických ingrediencí je uveden v kosmetické nomenklatuře podle INCI (tj. Mezinárodní nomenklatura kosmetických ingrediencí), kterou lze vyhledávat podle názvu nebo čísla v evropské databázi CosIng (Databáze kosmetických ingrediencí, https://ec.europa.eu/growth/ sectors/cosmetics/cosing_en).

Na obalu kosmetického přípravku jsou jednotlivé složky seřazeny sestupně podle jejich procentuálního rozložení ve finálním objemu (např. hmotnost/hmotnost nebo objem/objem podle typu pevného nebo kapalného kosmetického přípravku). Většina toxikologických údajů o chemických látkách byla historicky generována na *in vivo* modelech a ve studiích, které nemusí být plně relevantní pro člověka, zejména v oblasti toxikokinetiky a toxikodynamiky, metabolismu, systémové toxicity, ale i genotoxicity, mutagenity nebo karcinogeneze.

3 Experimentální část

Cílem experimentální části doktorského projektu bylo zhodnotit genotoxický potenciál vybraných látek prostřednictvím baterie *in vitro* toxikologických metod bez použití laboratorních zvířat, co nejvíce relevantních k člověku. Výzkum reaguje na výzvu Evropské komise k poskytnutí údajů screeningu možného genotoxického potenciálu vybraných látek. Zvláštní pozornost byla věnována *in vitro* alkalickému kometovému testu (CMT), prováděnému na různých buněčných liniích, a *in vitro* testu savčích chromozomových aberací (CA) na lidských periferních lymfocytech podle metodiky OECD TG 473. V rámci *in vitro* kometového testu byly zvoleny buněčné linie HaCat a SVK14, což jsou nenádorové lidské keratinocyty, a myší embryonální fibroblasty NIH/3T3. Pro test chromozomových aberací byly použity lymfocyty periferní krve od zdravého dárce (zdravý nekuřák, nepijící alkohol, který nebyl vystaven záření, infekci nebo medikaci méně než jeden měsíc před datem odběru). Selekce buněčných linií byla motivována potřebou získat komplexní a srovnatelná data při hodnocení genotoxického potenciálu látek na různých typech buněk s potenciálně různými regulačními mechanismy. Tato experimentální část disertační práce nám umožnila aplikovat a optimalizovat *in vitro* toxikologické metody na konkrétních buněčných modelech, a tím získat relevantní data k posouzení genotoxického potenciálu testovaných látek. Metodologie a výsledky byly podrobně diskutovány a interpretovány, s cílem přispět k lepšímu pochopení genotoxických účinků chemických látek a spotřebních výrobků.

3.1 Metodika

3.1.1 *In vitro* test savčích chromozomových aberací

In vitro test savčích chromozomových aberací je krátkodobý *in vitro* test mutagenity umožňující detekci chromozomových abnormalit (strukturálních a numerických aberací) v savčích (lidských) buňkách pomocí optického mikroskopu (Rössner, 1996). Lze použít jak kultury stabilizovaných buněčných linií, tak primárních buněk, např. buňky čínského křečka nebo lidské lymfocyty. Po expozici testované látce s použitím i bez použití vhodného metabolického aktivačního systému (detekce přímých i nepřímých mutagenů) se na buněčné kultury působí mitotickým jedem, např. kolchicinem, aby došlo ke kumulaci dělících se buněk (C-metafáze). Buňky jsou následně laboratorně zpracovány a jsou z nich připraveny mikroskopické preparáty, které jsou obarveny

vhodným barvivem. Dobře rozprostřené metafázické buňky jsou analyzovány na přítomnost chromozomových aberací.

Metoda slouží k biologickému monitorování profesionálně exponovaných pracovníků mutagenním nebo karcinogenním látkám (např. zaměstnanci laboratoří pracující s cytostatiky), nebo se používá pro testování chemických látek z hlediska jejich genotoxicity, a právě tuto modifikaci testu jsme použili pro studium genotoxicity u ingrediencí spotřebních výrobků. Poškození genetického materiálu buňky (DNA), analyzované jako chromozomové aberace, je projevem biologického efektu genotoxických faktorů (Rössner, 1996; Očadlíková *et al.*, 2007).

3.1.1.1 Lidské periferní lymfocyty

Lymfocyty patří mezi bílé krvinky – leukocyty, tedy buňky vykonávající v organismu imunitní funkce, a řadí se k agranulocytům (společně s monocyty a makrofágy). V periferním oběhu převládají malé lymfocyty, které mají průměr 6 – 8 μm.

Z celkového množství cirkulujících leukocytů periferní krve tvoří lymfocyty 20 – 45 % a jsou v nich obsaženy mitochondrie i proteosyntetický aparát. Jádro, které má kulovitý tvar a prakticky vyplňuje celou buňku, se intenzivně barví (Kočárek *et al.*, 2010).

Lymfocyty vznikají z lymfoidní pluripotentní buňky v kostní dřeni a jejich diferenciace se uskutečňuje v primárních imunitních orgánech: thymu a kostní dřeni, podle toho názvy T a B lymfocyty. Ve světelném mikroskopu nelze rozdíl mezi těmito buňkami rozeznat. Většina lymfocytů žije několik měsíců. Výjimkou jsou pouze tzv. paměťové buňky, které vznikají z T lymfocytů nebo B lymfocytů, ty jsou schopny přežívat po celý život jedince (Penka *et al.*, 2001).

3.1.1.2 Kultivace krve

Lymfocyty periferní krve od zdravého dárce byly kultivovány v kultivačním médiu RPMI-1640, které obsahuje L-glutamin, $NaHCO_3$ (vše od Sigma-Aldrich), a je doplněno 10% tepelně inaktivovaným bovinním sérem (Bioveta, Česká republika) a stimulátorem buněčného dělení phytohaemagglutininem (PHA) při 37 °C. Proces kultivace probíhá v 12,5 cm^2 lahvích pro tkáňové kultury Falcon.

Test prováděný za podmínek *in vitro* vyžaduje použití exogenního zdroje metabolického aktivačního systému (MAS), byly tedy paralelně provedeny dvě verze testu (tj. s nebo bez metabolické aktivační stimulace), podle testovacích pokynů OECD TG 473. Jako exogenní zdroj MAS byla použita kofaktorem doplněná postmitochondriální frakce S9, připravená z jater hlodavců (potkani Wistar) (příprava Státní zdravotní ústav, Projekt pokusů č. MZDR 37519/20019-4/OVZ).

Čtyřicet osm hodin po zahájení kultivace byla k buňkám přidána testovaná látka na dobu 4 hodin (s MAS a bez MAS). Současně jsou v experimentu aplikovány pozitivní kontroly thio-TEPA 10^{-6}M (Sigma-Aldrich), přímý mutagen bez MAS, a cyklofosfamid 10^{-4}M, nepřímý mutagen s MAS (Cytoxan, Bristol-Myers Squibb, USA). Jako negativní kontrola bylo použito kultivační médium.

Po 4 hodinách byla kultura dvakrát promyta médiem RPMI-1640 bez séra, aby se odstranila testovaná látka. Následně byly buňky opět nasazeny do kultivačních lahví s kompletním kultivačním médiem a kultivovány po dobu 24 hodin při 37 °C. Dvě hodiny před ukončením kultivace byl ke vzorkům přidán kolchicin (Sigma-Aldrich) jako látka zastavující buněčné dělení v metafázi.

Pro případ negativního výsledku (v přítomnosti i za absence MAS) probíhá kontinuální experiment bez použití metabolické aktivace a testovaná látka je ke kultuře aplikována na interval 1,5 násobku normálního buněčného cyklu, což odpovídá době přibližně 26 hodin.

Dvě hodiny před ukončením kultivace byl ke vzorkům opět přidán kolchicin (Sigma-Aldrich) jako látka zastavující buněčné dělení v metafázi.

3.1.1.3 Zpracování krve

Po procesu kultivace, jehož celková doba byla 76 hodin (v případě druhého testu 74 hodin, neboť se testovaná látka inkubuje s kulturou 26 hodin), byly buňky přelity do plastových zkumavek a zpracovány odděleně pomocí hypotonie, fixace a barvení 5% Giemsa-Romanowski (Hungerford, 1965).

Hypotonie

Zkumavky s buněčnou kulturou byly centrifugovány 3 minuty při 2000 otáčkách/min. Supernatant byl odstraněn a k buňkám byl na dobu 10 minut přidán hypotonický roztok, 0,55% KCl v deionizované vodě, teplota 37 °C. Tento proces probíhá při pokojové teplotě a slouží k odstranění erytrocytů z kultury.

1. fixační roztok – Ibrainův roztok

Ibrainův roztok se aplikuje na buněčnou kulturu jako první fixace a je tvořen deionizovanou vodou, kyselinou octovou a metanolem. Po přilití fixačního roztoku (4 °C) k buňkám byla provedena centrifugace (3 minuty; 2000 otáček/min). Slití supernatantu a důkladná resuspendace sedimentu byla provedena po každé centrifugaci.

Ibrainův fixační roztok:

Látka / roztok	**Množství**	**Příprava, skladovací podmínky**
Deionizovaná voda	92 ml	Látky se smíchají a výsledný roztok je vychlazen na 4 °C. Je určený k přímému použití. Neuchovává se.
Kyselina octová	5 ml	
Metanol	3 ml	

2. fixační roztok

Jako druhý fixační roztok byl použit vychlazený metanol (4 ºC) a po jeho aplikaci byla opět provedena centrifugace po dobu 3 minut při 2000 otáčkách/min.

3. fixační roztok

Poslední, tedy 3. fixační roztok (4 °C) je tvořen metanolem a kyselinou octovou (3:1). Látky byly smíchány v potřebném množství dle předepsaného poměru, výsledný roztok je určen k přímému použití a neuchovává se. Po přilití k buněčné kultuře byla provedena centrifugace (3 minuty, 2000 otáček/min).

3. fixační roztok:

Látka / roztok	Množství	Příprava, skladovací podmínky
Metanol	3 díly	Látky se smíchají a výsledný roztok je vychlazen na 4 °C. Je určený k přímému použití. Neuchovává se.
Kyselina octová	1 díl	

3.1.1.4 Aplikace výsledné suspenze

Předmytá podložní skla, na která se kape výsledná buněčná suspenze, byla uchovávána v chromsírové směsi. Před použitím byla sklíčka jednotlivě promyta pod tekoucí vodou, naložena do destilované vody a vychlazena v chladničce (4 °C).

Po slití supernatantu ze zkumavek je nutné sediment opět dobře promíchat pipetou a nakapat 4 – 6 kapek na mokré, vychlazené podložní sklo. Od každé kultury byla takto připravena 2 podložní sklíčka.

3.1.1.5 Barvení preparátů

Sušení preparátů probíhá volně na vzduchu a poté jsou obarveny 5% roztokem Giemsa – Romanowski (Merck).

Složení barvicího roztoku:

Látka / roztok	Množství	Příprava, skladovací podmínky
Destilovaná voda	80 ml	Látky se smíchají a výsledný barvicí roztok je určený k přímému použití. Neuchovává se.
Sörensenův pufr, 4 °C	15 ml	
Giemsa – Romanowski	5 ml	

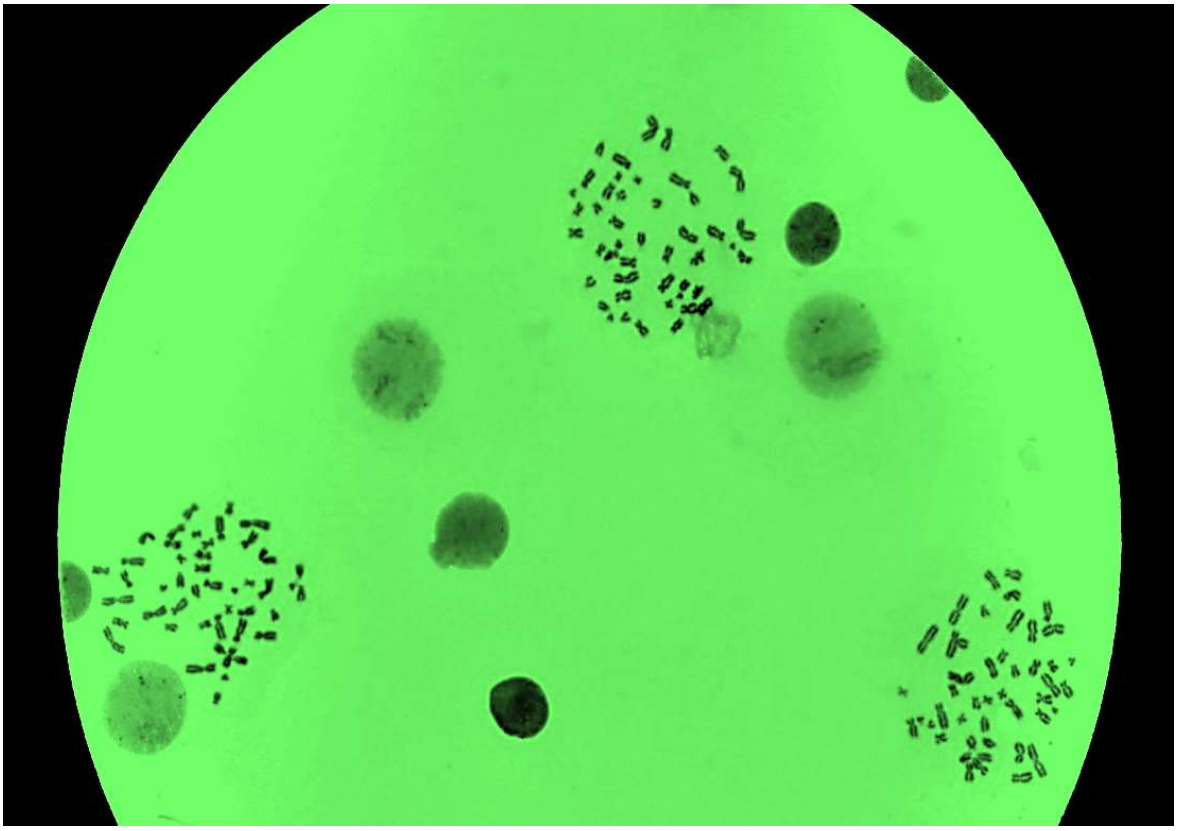
Označená podložní sklíčka byla naskládána do skleněných kyvet a až po okraj přelita barvicím roztokem. Proces barvení trvá 5 minut, následně byly preparáty důkladně opláchnuty pod tekoucí vodou.

Sörensenův pufr (pH 6,8) složení:

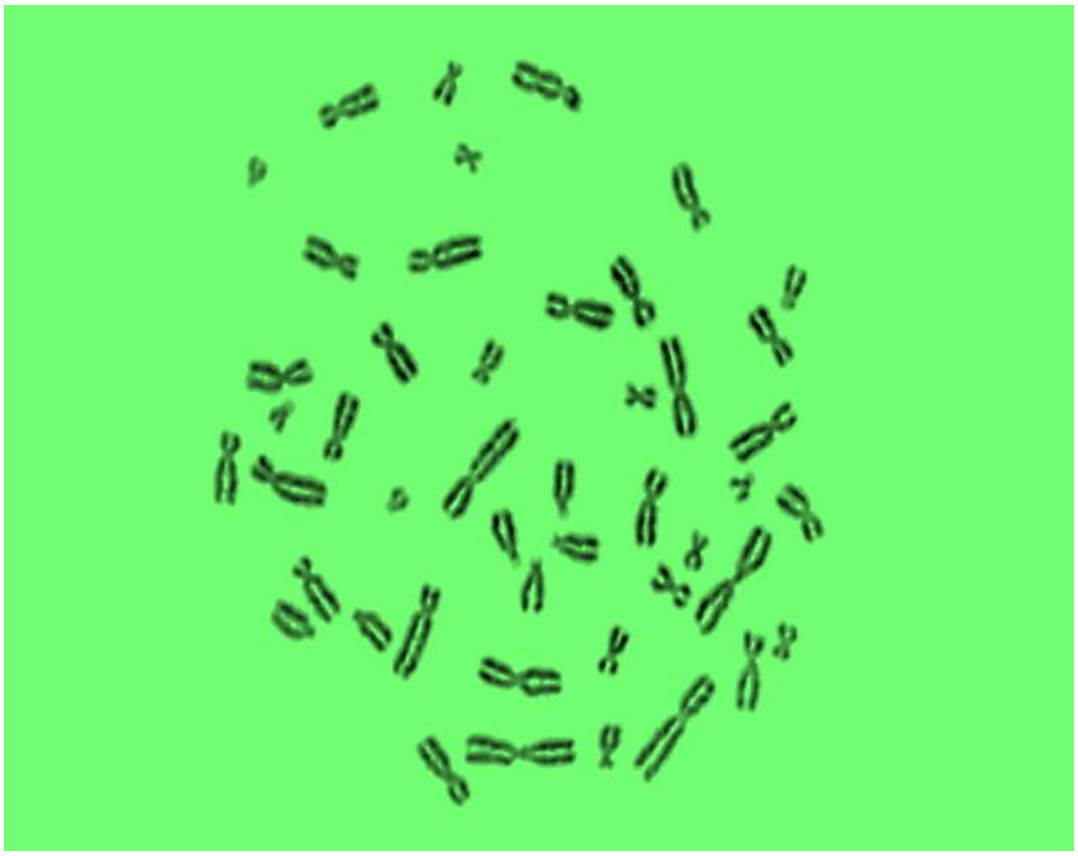
Látka / roztok	Množství	Příprava, skladovací podmínky
roztok A	1,376 g KH_2PO_4 / 400 ml deionizované vody	Roztoky se smíchají a uchovávají se při 4 °C v chladničce.
roztok B	5,52 g Na_2HPO_4 . 12 H_2O / 600 ml deionizované vody	

3.1.1.6 Mikroskopická analýza

Buňky nacházející se v metafázi buněčného cyklu (alespoň 200 dobře rozprostřených metafází se 46 ± 2 centromerami, viz Obrázek 7) byly mikroskopicky analyzovány na přítomnost chromozomových a chromatidových aberací, při tisícinásobném zvětšení a za použití imerzního oleje pro fluorescenci, jak znázorňuje Obrázek 8.



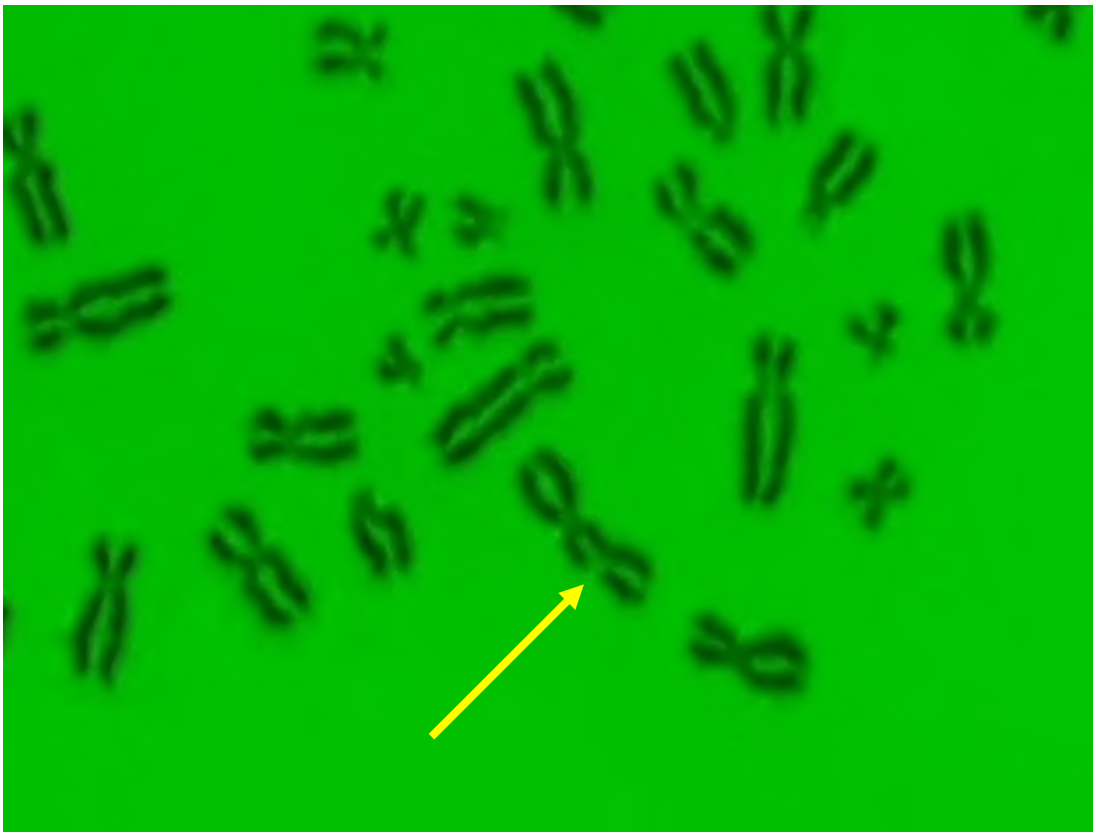
Obr. 7: Dobře rozprostřené metafáze na preparátu při stonásobném zvětšení (fotografie autora)



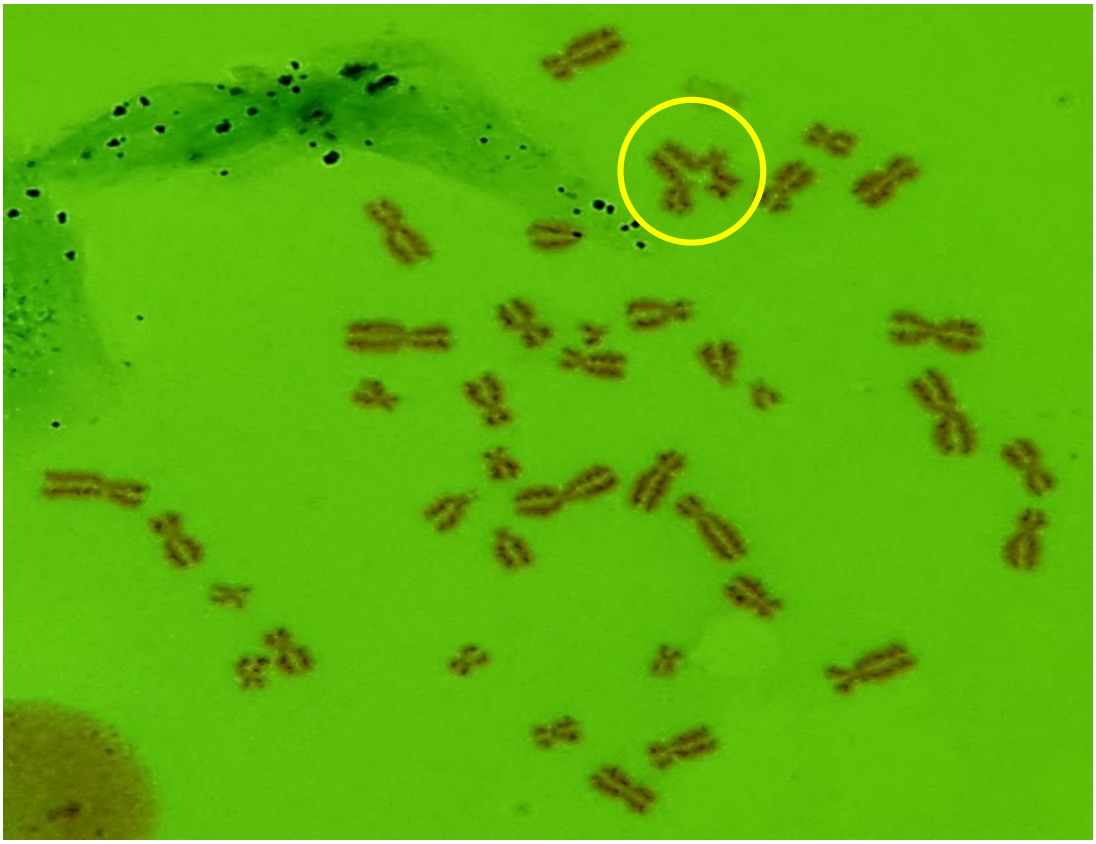
Obr. 8: Metafáze při tisícinásobném zvětšení (fotografie autora)

Posuzují se následující jevy:

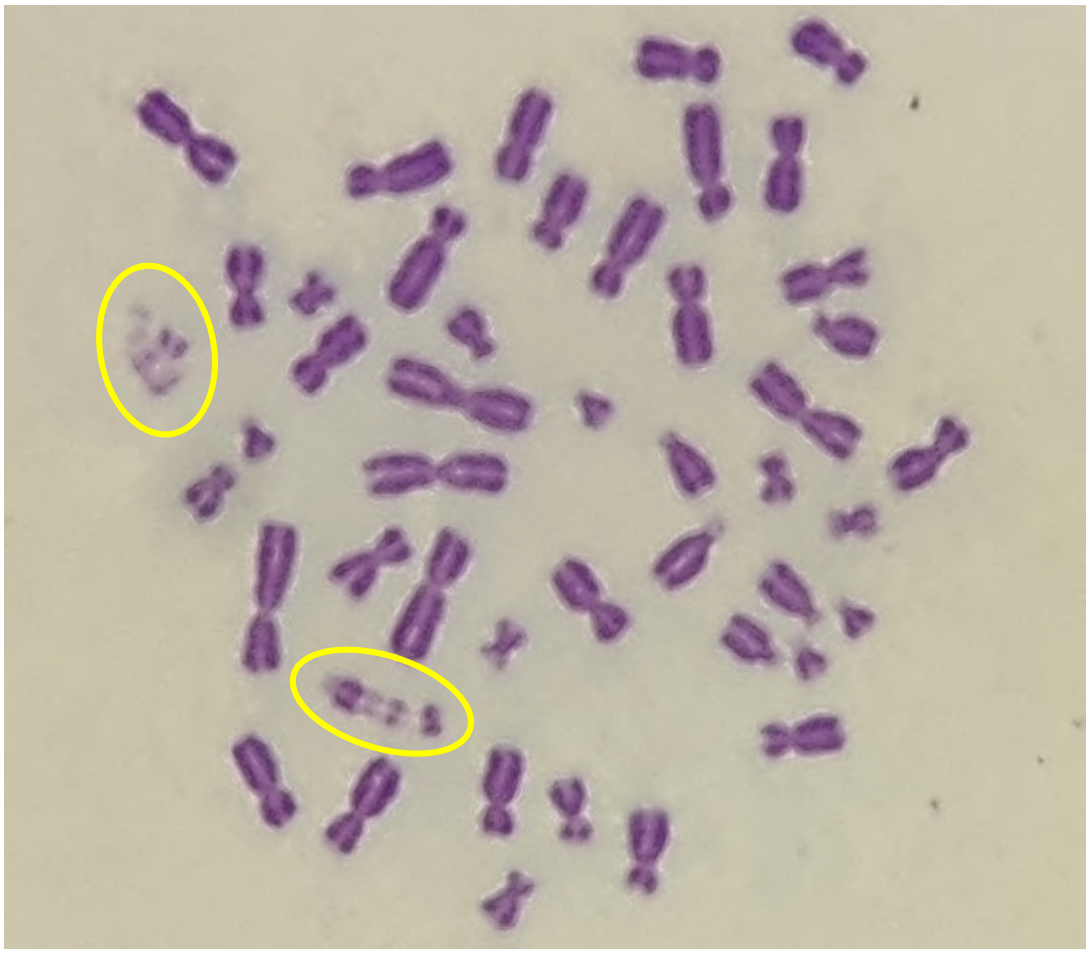
- Přerušení celistvosti jedné nebo obou chromatid, přičemž je mezera přerušené chromatidy stejná nebo menší než je její šířka, se nazývá **Gap**. Gapy byly zaznamenány samostatně a nebyly zahrnuty do celkové frekvence aberací.
- Jako **chromatidový zlom** je hodnoceno porušení kontinuity jedné nebo obou chromatid, pokud je mezera přerušené chromatidy větší než její šířka (Obrázek 9), nebo pokud je fragment dislokovaný mimo osu chromatidy. Chromatidové zlomy mohou být jednoduché nebo dvojité.
- **Chromatidové výměny** (symetrické, asymetrické), jak dokládá Obrázek 10.
- **Chromozomové výměny:**
  - dicentrický chromozom,
  - translokace,
  - prsténčitý chromozom (ring).
- **Fragmentace**, neboli rozpad celého chromozomu (Obrázek 11).



Obr. 9: Jednoduchý chromatidový zlom při tisícinásobném zvětšení (fotografie autora)



Obr. 10: Asymetrická chromatidová výměna při tisícinásobném zvětšení (fotografie autora)



Obr. 11: Fragmentace chromozomů při tisícinásobném zvětšení (fotografie autora)

Bylo hodnoceno procento buněk se strukturálními chromozomálními aberacemi. Alespoň dvojnásobné zvýšení počtu aberantních buněk ve srovnání s negativní kontrolou bylo kritériem pro stanovení pozitivního výsledku včetně zvýšení počtu buněk s chromozomálními aberacemi souvisejícího s koncentrací testované látky.

- **Endoreduplikace**, neboli jev, kdy homologní chromozomy leží těsně u sebe, se zaznamenává, ale při vyhodnocení se nepočítá do aberací.
- Dále se pouze zaznamenávají případné **polyploidní buňky**, u kterých je počet centromer vyšší než 3 a vícenásobek haploidní sady (23 chromozomů), nebo **aneuploidní buňky**, kde je vyšší nebo nižší počet chromozomů než 46.

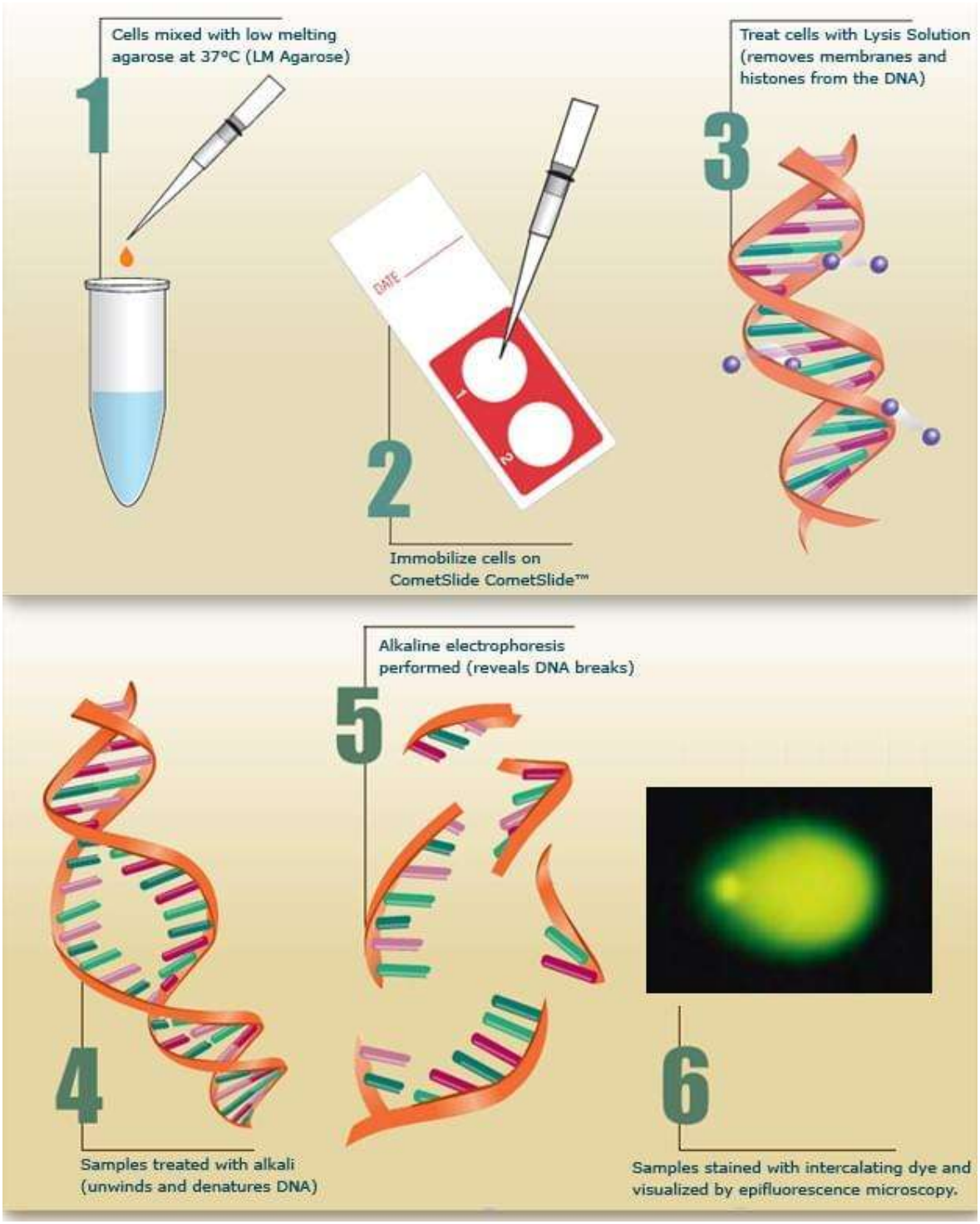
Ačkoliv je účelem testu odhalování strukturálních chromozomových aberací, je rovněž důležité zaznamenat případné polyploidie či endoreduplikace, a zároveň je také nutné evidovat zjištěnou cytotoxicitu v experimentálních i kontrolních kulturách.

Neanalyzují se:

- špatně obarvené či přebarvené metafáze,
- metafáze s nedostatečně oddělenými chromatidami,
- prometafázické chromozomy,
- pozdní metafáze (když jsou chromatidy v centromeře od sebe odděleny),
- nedostatečně rozprostřené chromozomy v metafázích (překrývání chromozomů),
- splývající metafáze (sloučení chromozomů z více metafází),
- mechanické poškození chromozomů/metafází (vzniklé při zpracování vzorků nebo poškrábáním nátěru),
- metafáze, které mají jiný počet centromer než 46 ± 2 (aneuploidie se pouze zaznamená, jev lze spolehlivě odlišit jen metodou FISH).

3.1.2 Alkalický kometový test *in vitro*

Kometový test neboli alkalická varianta jednobuněčné gelové elektroforézy (alkaline Single Cell Gel Electrophoresis – SCGE) je citlivá technika, umožňující detekci fragmentů DNA na úrovni jednotlivých buněk (Obrázek 12). Principem metody je odlišná migrace DNA a jejích fragmentů v elektrickém poli. Buňky jsou po inkubaci s testovanou látkou podrobeny působení lyzačního roztoku, jehož vlivem dochází k odstranění membrán a většiny proteinů z buněk. V alkalickém prostředí nastává denaturace DNA, která se při následné elektroforéze uvolňuje z jádra a jako anion migruje ke kladné elektrodě. Čím větší je počet zlomů v DNA, tím větší množství jí vycestuje z jádra. Po vhodném obarvení fluorescenční sondou připomínají sledované objekty komety (Obrázek 13 a 14), které obsahují neporušenou DNA v hlavě a poškozenou DNA v ocasu. Na mikroskopickém preparátu lze množství zlomů kvantifikovat pomocí softwaru CometScore (Obrázek 15) a následně statisticky vyhodnotit. Mezi nejčastějšími parametry, které jsou vyhodnocovány, je Olive tail moment (OTM), procentuální podíl DNA v hlavě komety nebo v jejím ocasu. Metoda byla použita na bázi OECD TG 489 s modifikacemi pro *in vitro* test, tj. s využitím lidských nenádorových buněčných linií odvozených od lidských keratinocytů (HaCaT a SVK14 zakoupených v ATCC, USA (Jiravová *et al.*, 2016) a myších embryonálních fibroblastů NIH/3T3 (ECACC, UK).



Obr. 12: Schematické znázornění průběhu experimentu s využitím metody kometové analýzy.(převzato z https://www.creative-bioarray.com/Services/In-Vitro-Comet-Assay.htm)

3.1.2.1 Příprava podložních sklíček

Prvním krokem byla příprava mikroskopických podložních sklíček, která byla potažena vrstvou 1% HMP agarózy (agaróza s vysokým bodem tání) v destilované vodě a poté byla umístěna do sušárny, kde byla ponechána po dobu 30 min při teplotě 60 °C. Následně byl aplikován 1% roztok HMP agarózy v 1xPBS, 85 µl na každé podložní sklíčko. Poté byla místa s aplikovanou HMP agarózou ihned překryta krycími sklíčky. V chladničce (při 4 °C) se urychlilo tuhnutí gelu.

3.1.2.2 Inkubace buněk

Buňky byly inkubovány v DMEM (Merck, Germany), v 75 cm^2 lahvích (Greiner Bio-ONE), s testovanými vzorky naředěnými v DMEM s vysokým obsahem glukózy (DMEM s 4500 mg/l glukózy, L-glutamin, pyruvát sodný a hydrogenuhličitan sodný, Sigma-Aldrich) bez fetálního bovinního séra (FBS), aby byly získány vybrané necytotoxické koncentrace (µg/ml) (37 °C, 5 % CO_2). Jako pozitivní kontrola byl na buněčnou kulturu aplikován 0,1% H_20_2 v 1xPBS po dobu 15 minut při 4 °C. Buněčná kultura v kultivačním médiu (DMEM) sloužila jako negativní kontrola.

Po inkubaci byly buňky opláchnuty roztokem 1xPBS (100 µl na jamku) a následně sklizeny trypsinizací, pomocí 50 µl enzymu TrypLE™ Express Enzyme (1x, bez fenolové červeně; Gibco, ThermoFisher Scientific, USA) aplikovaného na dobu ± 10 minut, při 37 °C. Pro zastavení trypsinizace bylo aplikováno 100 µl DMEM + 10 % v/v FBS (VWR, USA).

3.1.2.3 Smíchání buněčné suspenze s LMP agarózou

Sklizená buněčná suspenze byla přemístěna do centrifugační zkumavky a byla provedena centrifugace (3 min, 1500 otáček/min). Následně bylo z buněčného peletu odebráno 25 µl a resuspendováno v 85 µl 1% roztoku agarózy s nízkou teplotou tání (LMP) (Molecular Biology Grade; Qbiogene, MP Biomedicals, USA) temperovaného na 37 °C. Výsledná hustá agarózová suspenze (85 µl) byla napipetována na předem potažená mikroskopická sklíčka a rychle přikryta krycími sklíčky.

3.1.2.4 Lyzace

Po ztuhnutí gelu/agarózy (cca 15 minut) byla krycí sklíčka odstraněna a podložní sklíčka, s buňkami fixovanými v agaróze, byla ponořena do lyzačního pufru s 1% Triton™ X-100 (Merck) při 4 °C na 60 minut, jehož vlivem dochází k odstranění membrány a většiny proteinů z buněk.

Nejprve byl připraven zásobní roztok lyzačního pufru, do kterého byl v den experimentu přidán 1 ml Triton™ X-100 na 100 ml zásobního roztoku. Výsledný roztok byl před použitím vychlazen na 4 °C.

Složení lyzačního pufru: 2,5M NaCl, 100mM EDTA, 10mM Tris a 1% Triton™ X-100, pH 10, 4 °C. Výsledná hodnota pH se upraví pomocí NaOH.

Lyzační pufr – zásobní roztok 1000 ml:

Látka / roztok	**Množství na 1000 ml**	**Příprava, skladovací podmínky**
2,5M NaCl	146,40 g	Látky se doplní deionizovanou vodou na objem 1000 ml. Pomocí NaOH se upraví pH na výslednou hodnotu pH 10. Uchovává se při pokojové teplotě.
100mM EDTA	37,20 g	
10mM Tris	1,21 g	
NaOH	± 8,2 g	

3.1.2.5 Elektroforéza

V den pokusu byl připraven alkalický elektroforetický pufr (2000 ml) ze zásobních roztoků a vychlazen na 4 °C. Po lýze buněk byla sklíčka promyta destilovanou vodou. Dalším krokem bylo umístění sklíček do elektroforetické vany (Sub-Cell Model 192 Cell, 2000 ml) a jejich ponoření do dostatečného objemu chlazeného (4 °C) alkalického elektroforetického roztoku (300mM NaOH a 1mM EDTA, pH > 13) na 40 minut. Následně byla provedena elektroforéza za podmínek 0,8 V/cm a 350 mA, 20 min, při 4 °C.

Elektroforetické zásobní roztoky:

Látka / roztok	Množství	Příprava, skladovací podmínky
10N NaOH	200 g / 500 ml destil. vody	200mM EDTA je možné skladovat max. 2-3 týdny.
200mM EDTA	7,4448 g / 100 ml deioniz. vody	

Elektroforetický roztok (v den experimentu):

Látka / roztok	Množství	Příprava, skladovací podmínky
10N NaOH	60 ml	Roztoky v předepsaném množství doplnit do 2000 ml deioniz. vodou a promíchat. Uchovávat při 4 °C.
200mM EDTA	10 ml	

3.1.2.6 Neutralizace

Po elektroforéze byla sklíčka opatrně přenesena do skleněných kyvet a pečlivě promyta (dvakrát 5 minut) neutralizačním pufrem (0,4M Tris; pH 7,5; 4 °C) a osušena.

Neutralizační pufr (Tris), zásobní roztok 1000 ml:

Látka / roztok	Množství na 1000 ml	Příprava, skladovací podmínky
0,4M Tris	48,5 g	Pomocí HCl se upraví pH roztoku na hodnotu pH 7,5. Uchovává se při pokojové teplotě. Roztok má nestabilní pH.
HCl		
H_2O deionizovaná		

3.1.2.7 Barvení

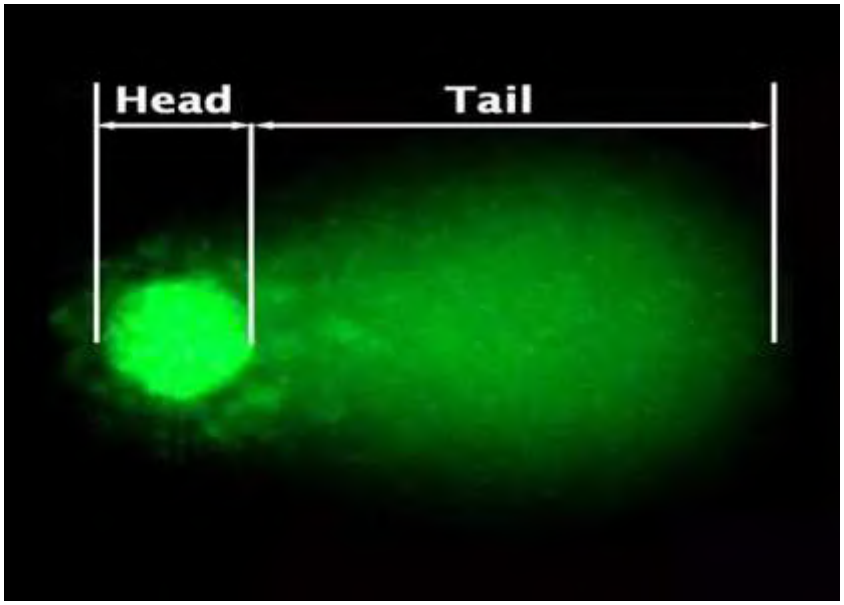
Po neutralizaci byly preparáty obarveny fluorescenčním barvivem SYBR Green I concentrate (10000xDMSO; Merck; ředění dle návodu 1:10 000 v PBS). Na agarózové plochy s buňkami bylo aplikováno 25 µl barviva a následně byly plochy přikryty krycími sklíčky a preparáty byly uloženy na dobu 15 minut do chladničky (4 °C). Při procesu barvení bylo nutné používat rukavice a pracovat v temnu.

3.1.2.8 Mikroskopická analýza, vyhodnocení

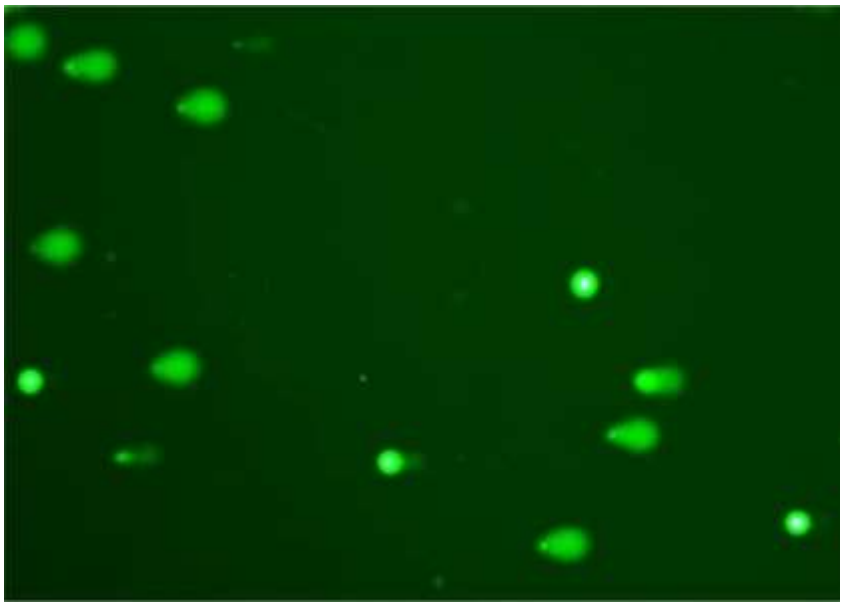
Vzorky byly mikroskopicky analyzovány pomocí fluorescenčního mikroskopu Olympus s připojenou CCD kamerou (Obrázek 14) a vyhodnoceny softwarem CometScore 1.5 (Tritek CometScore Freeware V1.5 Software; TriTek Corp., Sumerduck, VA, USA) (viz Obrázek 15). Experiment byl proveden ve třech cyklech (s použitím triplikátů vzorků).

Z každého testovaného vzorku bylo pro hodnocení náhodně vybráno 100 buněk a střední hodnoty (mediány) z každého měření byly použity pro matematickou a statistickou analýzu. Pro interpretaci výsledků byly použity průměrné hodnoty procentuálního množství DNA v hlavě komety (přímo úměrné intaktní, nefragmentované DNA), nebo množství (%) DNA v ocasu komety (přímo úměrné fragmentované DNA).

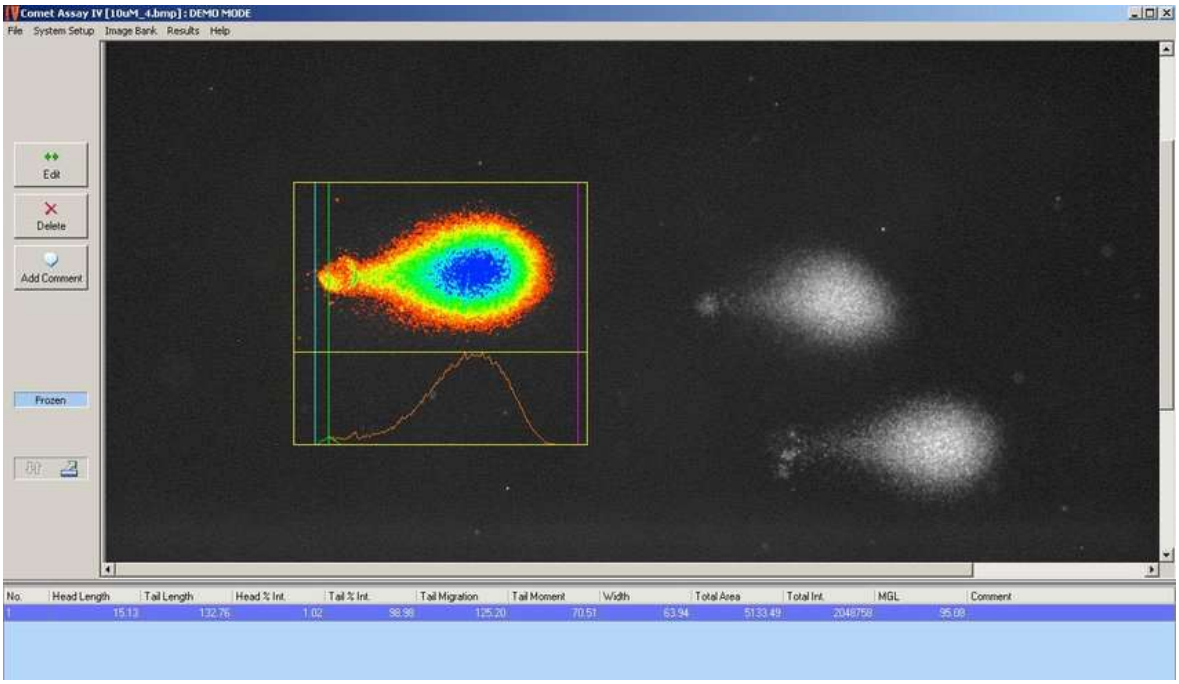
Rozdíly v reakci testovacího systému na zkoumanou látku (v různých koncentracích) byly posuzovány analýzou rozptylu (*ANOVA*) a Dunnettovým *t*-testem. Jako hladina statistické významnosti byla přijata hodnota $p < 0{,}05$. Všechny statistické analýzy byly provedeny pomocí IBM SPSS Statistics for Windows (verze 23.0; IBM Corp., Armonk, NY, USA).



Obr. 13: Stavba komety – dělení na hlavu (Head) a ocas (Tail) (převzato z https://www.slideshare.net/zeeldholakia7/genotoxicity-251354134)



Obr. 14: Obraz testovaného mikroskopického preparátu ve fluorescenčním mikroskopu, tisícinásobné zvětšení, převzato z (https://www.nature.com/articles/s41598-020-75592-7/figures/2



Obr. 15: Příklad měření komet v software Comet Score IV (převzato z https://www.researchgate.net/figure/An-example-of-a-comet-analysed-by-Comet-Assay-IV-The-blue-line-represents-the-start-of_fig6_265190763)

4 Výsledky a diskuze

Tato disertační práce je založena na výsledcích tří publikovaných studií zaměřených na konzervanty a parfémové kompozice (Chrz *et al*., 2020; Chrz *et al*., 2024; Dvořáková *et al*., 2023). Pro testování genotoxických účinků byly využity metody *In vitro* test savčích chromozomových aberací a Alkalický kometový test *in vitro*.

4.1 Parabeny

Parabeny jsou syntetické konzervační látky, široce používané ve spotřebních výrobcích a to nejen v kosmetice, ale i ve farmaceutických výrobcích či potravinách. Jedná se o estery kyseliny p-hydroxybenzoové (PHBA), mezi nejvíce používané patří methylparaben (MP), ethylparaben (EP), propylparaben (PP) a butylparaben (BP) (Güzel Bayülken & Tüylü 2019; Fransway *et al.*, 2019; Matwiejczuk *et al.*, 2020). Jejich rozpustnost a konzervační účinek závisí na délce řetězce, tedy čím kratší uhlovodíkový řetězec, tím lepší rozpustnost ve vodě, avšak menší konzervační účinek. Kvůli zmíněným variabilním vlastnostem se parabeny často aplikují ve směsi (Kapalavavi *et al.* 2014). Existuje řada publikovaných studií zaměřených na potenciální rizika těchto látek. O jejich potenciální genotoxicitě a karcinogenitě pojednává například studie Darbre *et al*. 2004, ve které byly pozorovány nálezy parabenů v biopsiích prsní tkáně a jako zdroj parabenů jsou uvedeny antiperspiranty (Darbre *et al*. 2004). Hodnocení rizik pro člověka se však stalo velmi náročným, protože neprůkazné nebo protichůdné výsledky byly prokázány četnými studiemi *in vivo* a *in vitro* nebo v kombinovaných testovacích bateriích (např. Roszak *et al*., 2017; Barr *et al.,* 2012; Fransway *et al*., 2019; Soni *et al*., 2005; Kang *et al*., 2013b; Darbre & Harvey 2008; Kim *et al*., 2020a; Bilal & Iqbal 2019). Oficiální vědecká stanoviska shrnují, že použití parabenů je pro lidské zdraví bezpečné, protože parabeny se v lidském těle metabolizují na kyselinu benzoovou a vylučují se močí, přičemž kyselina benzoová je v mnoha testech prakticky bez toxických účinků a je nepravděpodobné, že by ovlivňovala s chromozomy (SCF, 1994; SCCP, 2011; SCCP, 2013).

Sedm vzorků esterů kyseliny p-hydroxybenzoové (methylparaben, ethylparaben, propylparaben, isopropylparaben, butylparaben, isobutylparaben, benzylparaben) bylo pro účely naší studie (Chrz *et al*., 2020) dodáno od TOKYO Chemical Industry Co. (Tabulka 1).

Tab. 1: Charakteristika esterů kyseliny p-hydroxybenzoové vybraných pro testování

Substance	IUPAC	CAS	Vzorec	Molekulární hmotnost (g/mol)
Methylparaben	methyl 4-hydroxybenzoate	99-76-3	$C_8H_8O_3$	152,15
Ethylparaben	ethyl 4-hydroxybenzoate	120-47-8	$C_9H_{10}O_3$	166,17
Propylparaben	propyl 4-hydroxybenzoate	94-13-3	$C_{10}H_{12}O_3$	180,21
Isopropylparaben	propan-2-yl 4-hydroxybenzoate	4191-73-5	$C_{10}H_{12}O_3$	180,20
Butylparaben	butyl 4-hydroxybenzoate	94-26-8	$C_{11}H_{14}O_3$	194,23
Isobutylparaben	2-methylpropyl 4-hydroxybenzoate	4247-02-3	$C_{11}H_{14}O_3$	194,23
Benzylparaben	benzyl 4-hydroxybenzoate	94-18-8	$C_{14}H_{12}O_3$	228,25

4.1.1 Výsledky *in vitro* testu savčích chromozomových aberací

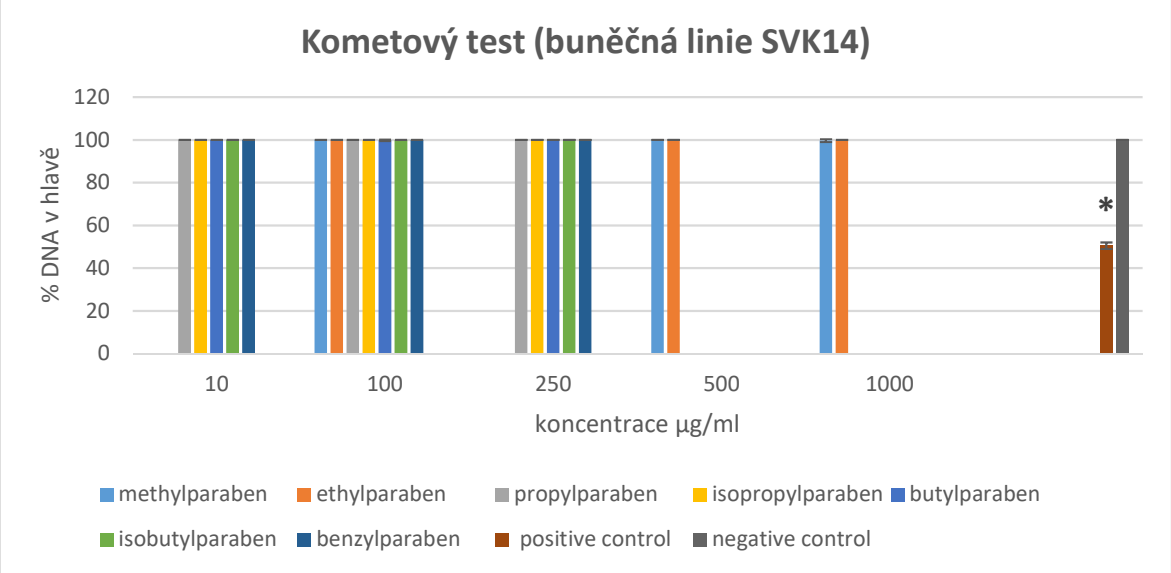
Ve validovaném testu *in vitro* savčích chromozomových aberací nevykazovaly lidské lymfocyty periferní krve zvýšenou míru chromozomálních aberací ve srovnání s negativní kontrolou pro methylparaben, isobutylparaben a benzylparaben. Ethylparaben byl považován za hraniční, ale pro jeho vysokou toxicitu nebylo možné vzorek vyhodnotit ve všech koncentracích. Propylparaben, isopropylparaben a butylparaben vykazovaly vysoké procento aberací a koncentrační závislost u nejvyšších testovaných koncentrací je ve srovnání s negativní kontrolou evidentní (více než dvojnásobná) v případě 26 hodinové inkubace bez MAS (Tabulka 2).

Tab. 2: *In vitro* test savčích chromozomálních aberací. Procento aberantních buněk bylo zaznamenáno po 4 hodinách s/bez MAS a 26 hodinách bez MAS.

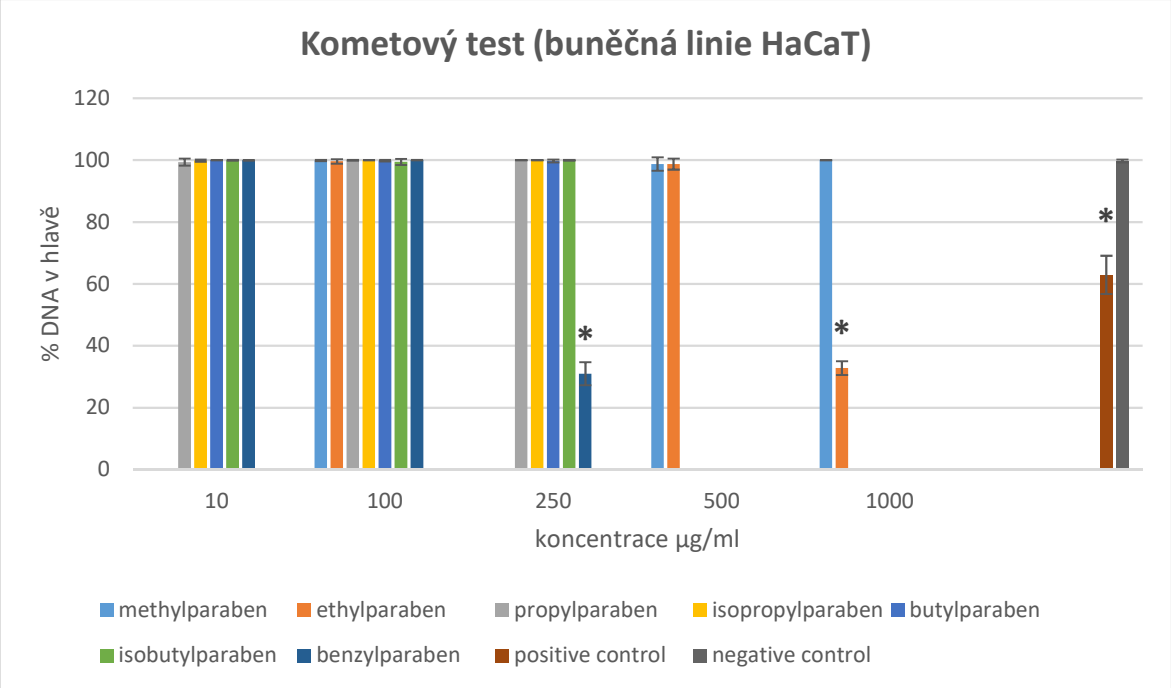
Vzorky / Kontroly	**Koncentrace µg/ml**	**% aberantních buněk**			**Hodnocení**
		4 h s MAS	**4 h bez MAS**	**26 h bez MAS**	
Methylparaben	10	3	4	4,5	**negativní**
	25	4	3	tox	**neprůkazný výsledek kvůli cytotoxicitě**
	50	tox	tox	tox	
	100	tox	tox	tox	
Ethylparaben	10	3	3	3	**hraniční**
	25	5,5	4	tox	**neprůkazný výsledek kvůli cytotoxicitě**
	50	tox	tox	tox	
	100	tox	tox	tox	
Propylparaben	5	4	3,5	2,5	**pozitivní**
	10	2	3	2	
	25	5	6	9	
	50	tox	tox	tox	
Isopropylparaben	5	3	3	2	**pozitivní**
	10	2,5	3	3,5	
	25	4	4,5	7,5	
	50	tox	tox	tox	
Butylparaben	5	5	5	3	**pozitivní**
	10	6	5,5	9	
	25	tox	tox	tox	
	50	tox	tox	tox	
Isobutylparaben	5	3	3	2,5	**negativní**
	10	5	4	4,5	
	25	tox	tox	tox	
	50	tox	tox	tox	
Benzylparaben	1	4	3	2,5	**negativní**
	2,5	5	3	0,5	
	5	2,5	4	1,5	
	10	3,5	3	5	
Negativní kontrola (kultivační medium)			3	2	negativní
Pozitivní kontrola (thio-TEPA 10^{-6}M)			10	15	pozitivní
MAS Negativní kontrola (neexponované buňky s MAS)		5,5			negativní
Pozitivní kontrola (cyklofosfamid 10^{-4}M)		11			pozitivní

4.1.2 Výsledky kometového testu *in vitro* na buněčných liniích SVK14 a HaCaT

Pozorovaná intenzita poškození DNA u buněčné linie SVK14 vystavené některému z parabenů (methyl-, ethyl-, propyl-, isopropyl-, butyl-, isobutyl- nebo benzylparaben) nepřekročila hodnoty negativní kontroly ani při vysokých koncentracích, které byly v MTT testu posouzeny jako cytotoxické (Graf 1). V buněčné linii HaCaT vykazovaly benzylparaben a ethylparaben zvýšenou fragmentaci DNA (benzylparaben, průměrná hodnota 30,9 % DNA v hlavě při 250 µg/ml; ethylparaben, průměrná hodnota 32,8 % DNA v hlavě při 1000 µg/ml), což představovalo hodnoty významně vzdálené od negativní kontroly (99,7 % DNA v hlavě), přičemž fragmentace DNA přesáhla i hodnoty pozitivní kontroly (0,1 % H_2O_2 v 1x PBS, 15 min, 4 °C, střední hodnota 62,9 % DNA v hlavě), jak je uvedeno na Graf 2. Proto byly benzylparaben a ethylparaben v kometovém testu *in vitro* vyhodnoceny jako podezřelé látky z hlediska genotoxicity, protože při vyšších koncentracích mohou vykazovat potenciálně genotoxické účinky. Tento výsledek byl použit jako indikace pro další testování s cílem potvrdit výsledky.



Graf 1: Alkalický kometový test *in vitro* provedený na buněčné linii SVK14. Data (% DNA v hlavě) jsou prezentována jako střední hodnoty ± SD ze tří nezávislých měření. Výsledky byly považovány za statisticky významné, pokud byla hodnota $p < 0,05$ (*). DNA v hlavě je úměrná poškození DNA (čím vyšší hodnota, tím nižší poškození DNA).



Graf 2: Alkalický kometový test *in vitro* provedený na buněčné linii HaCaT. Data (% DNA v hlavě) jsou prezentována jako střední hodnoty ± SD ze tří nezávislých měření. Výsledky byly považovány za statisticky významné, pokud byla hodnota $p < 0,05$ (*). DNA v hlavě je úměrná poškození DNA (čím vyšší hodnota, tím nižší poškození DNA).

4.1.3 Diskuze

Cílem naší studie bylo získat data pro testovanou skupinu chemických látek – parabenů, používaných jako konzervanty v kosmetickém průmyslu. Použili jsme kombinaci dvou *in vitro* toxikologických metod s využitím třech lidských buňkám relevantních nemaligních buněčných systémů. Provedli jsme alkalický kometový test *in vitro* (CMT) s využitím buněčných linií HaCaT a SVK14 a test CA, neboli *in vitro* test savčích chromozomových aberací, s použitím lidských lymfocytů periferní krve. Naše studie prokázala vysokou variabilitu výsledků mezi dvěma metodami *in vitro* a překvapivě také mezi dvěma buněčnými liniemi použitými v kometovém testu. Pozorovaná intenzita poškození DNA v buněčné linii SVK14 nepřesáhla hodnoty negativní kontroly a všechny vzorky byly klasifikovány jako negativní, což naznačuje, že látky, u nichž se významně projevila cytotoxicita, se významně neakumulovaly v DNA ani nenapadaly strukturu DNA, a proto mohou být cytotoxické účinky přisuzovány jiným mechanismům než např. přímému oxidativnímu poškození DNA. Při použití HaCaT buněčné linie byla většina

vzorků také klasifikována jako negativní, nicméně benzylparaben a etylparaben vykazovaly zvýšenou fragmentaci DNA při jedné testované koncentraci. Výsledky z kometového testu jsme považovali za orientační pro další testování a provedli jsme validovaný test chromozomových aberací (CA) s použitím lidských lymfocytů periferní krve. V CA testu byl methylparaben klasifikován jako negativní a ethylparaben jako hraniční, oba však vykazovaly významnou cytotoxicitu. Propylparaben, isopropylparaben, butylparaben byly klasifikovány jako pozitivní a isobutylparaben, benzylparaben jako negativní. Alkalický kometový test byl opakovaně popsán v jiných studiích, kde poskytoval pozitivní výsledky pouze při významně vyšších koncentracích než v testu chromozomových aberací nebo v testu indukce vzniku mikrojader (Giannotti *et al.*, 2002; Kimura *et al.*, 2013; Kawaguchi *et al.*, 2010). Vzhledem k tomu, že kometový test nemusí poskytovat specifickou odpověď s uspokojivou citlivostí při nižších koncentracích, postrádá metabolickou aktivaci a může být ohrožen vyšší cytotoxicitou, nezdá se, že by tyto dva testy mohly v kombinaci slibně korelovat. Za předpokladu, že negativní výsledek *in vitro* by měl být podpořen údaji z alespoň dvou validovaných testů *in vitro*, a s ohledem na to, že v naší laboratoři byl validován pouze jeden biologický test *in vitro*, je třeba výsledky posuzovat s opatrností. V případě neprůkazných výsledků z testování *in vitro* se doporučuje test opakovat nebo použít jiný test *in vitro* (EFSA 2011). Bohužel, zvýšení počtu *in vitro* testů může dále snížit specificitu a zvýšit falešnou pozitivitu. Neprůkazné výsledky mohou být získány i díky zahrnutí metabolického aktivačního systému, rozdílným experimentálním podmínkám, specifičnosti testovacího systému, použitého buněčného typu atd. (Nesslany, 2017). Různé typy savčích buněk mohou vykazovat variabilitu v expresi genů zapojených do opravy DNA, buněčných obranných systémů, řízení buněčného cyklu a apoptózy (např. p53), což má za následek variabilitu v pozorované odpovědi. U periferních lymfocytů byl zjištěn vysoce stabilní karyotyp, avšak při rutinní diagnostice je třeba zvážit i možnou variabilitu mezi dárci (Tweats *et al.*, 2007). Abychom hlouběji zvážili další možné zdroje variability, zaměřili jsme se také na rozpustnost parabenů ve vodě, určující jejich biologickou dostupnost na buněčné úrovni. Provedli jsme také doplňkový test cytotoxicity pomocí 3T3 Balb/c fibroblastů a test Neutral Red Uptake (3T3 NRU Cytotoxicity Assay), který byl proveden podle ECVAM DB-ALM Protocol no. 46. Během mikroskopické kontroly jsme v bezsérových médiích pozorovali různé formace u testovaných vzorků parabenů, jako jsou shluky nebo krystaly,

které nebyly pozorovány při rutinní vizuální kontrole. To naznačuje, že při testování je třeba pečlivě sledovat rozpustnost testovaných látek. Výsledky testu cytotoxicity 3T3 NRU ukázaly další variabilitu. Na základě těchto výsledků jsme navrhli, aby byla v případě parabenů důkladně zkontrolována rozpustnost ve vodních roztocích (tj. kultivačním médiu), protože špatná rozpustnost ve vodě nebo krystalizace může významně ovlivnit biologickou dostupnost parabenů v testovacích systémech *in vitro* i *in vivo* a ohrozit výsledky testování genotoxicity. Provedli jsme stručné vyhledávání údajů o rozpustnosti parabenů ve vodě a našli jsme podobné závěry pro jednotlivé parabeny nebo jejich směsi. Obecně jsou parabeny stabilní na vzduchu a jsou odolné vůči hydrolýze v horké nebo studené vodě a v kyselých roztocích. Antibakteriální potenciál parabenů rostl s délkou esterifikovaného alkoholového řetězce, avšak rozpustnost ve vodě klesala (Soni *et al.*, 2001). Variabilní antimikrobiální potenciál a rozpustnost ve vodě představuje jeden z důvodů aplikace parabenů ve směsi, aby byla zajištěna optimální antimikrobiální aktivita a přijatelná rozpustnost (Güzel Bayülken & Tüylü 2019; Soni *et al.*, 2005; Kapalavavi *et al.*, 2014; Giordano *et al.*, 1999; Perlovich *et al.*, 2005).

4.2 Triclosan a Triclocarban

Triclosan (TCS) a Triclocarban (TCC) se od počátku 40. let 20. století používají jako fungicidy a konzervační látky v mnoha produktech, včetně oděvů, látek, hraček, obalů potravin, podlah v potravinářském průmyslu, stavebních materiálů, zdravotnických potřeb a zejména v produktech pro domácnost a osobní péči, jako jsou mýdla, antibakteriální tuhá/tekutá mýdla, tělová mléka, deodoranty, detergenty, lékařské dezinfekční prostředky, mýdla po holení, dezinfekční prostředky na ruce, zubní pasty, ústní a čisticí vody, kousátka a ubrousky pro děti, atd. (Iacopetta *et al.*, 2021). Tyto dvě chemikálie se liší svou strukturou, ale obě jsou polychlorované aromatické antimikrobiální látky, ve kterých jsou tři atomy vodíku na aromatických kruzích nahrazeny chlorem. Jsou účinné proti mnoha různým bakteriím a také některým houbám a prvokům, protože inhibují syntézu mastných kyselin a vyvolávají narušení integrity membrány (SCCS, 2022, Shrestha *et al.*, 2020).

Dermální aplikace produktů osobní péče je považována za hlavní cestu lidské expozice, ačkoli se mohou do lidského těla dostat také orálně prostřednictvím zubní pasty, ústních vod a zubních ošetření. V důsledku toho byly TCS a TCC detekovány v lidských

vzorcích, jako je krev, mateřské mléko, moč, vlasy a nehty (Zhang *et al.*, 2022; Pycke *et al.*, 2014; Wei *et al.*, 2017; Toms *et al.*, 2011; Kim *et al.*, 2020b; Asimakopoulos *et al.*, 2014; Xue *et al.*, 2015; Iyer *et al.*, 2018; Li *et al.*, 2018; Tian *et al.*, 2023; Yin *et al.*, 2016). Navíc TCS a TCC představují zátěž pro vodní prostředí a riziko kontaminované pitné vody, protože současné čistírny odpadních vod nejsou typicky navrženy pro čištění takových mikropolutantů (Armstrong *et al.*, 2018; Chen *et al.*, 2019). Dlouhodobá expozice vodních organismů TCS a TCC spolu s jejich bioakumulačním potenciálem vedla k jejich detekovatelným hladinám ve vodních potravních řetězcích u druhů, jako jsou řasy, korýši, ryby a mořští savci (Meador *et al.*, 2016; Gomes *et al.*, 2021). Environmentální TCS a TCC by mohly být účinně přijímány potravinářskými plodinami, což vede k další potenciální expozici člověka prostřednictvím konzumace potravin (Lozano *et al.*, 2018; Yang *et al.*, 2020; Vimalkumar *et al.*, 2019). Jejich nepříznivé biologické účinky u lidí včetně endokrinního narušení, vývojových a reprodukčních dopadů (Wei *et al.*, 2017; Wu *et al.*, 2016; Rochester *et al.*, 2017; Aker *et al.*, 2019; Cao *et al.*, 2020; Costa *et al.*, 2020), narušení střevní mikroflóry (Yang *et al.*, 2020; Xie *et al.*, 2020; Sanidad *et al.*, 2022; Zhang *et al.*, 2023b) a zvýšení rezistence na antibiotika (Giuliano *et al.*, 2015; Hartmann *et al.*, 2016; Westfall *et al.*, 2019; Zhang *et al.*, 2023a) vedlo k přísné regulaci používání obou konzervantů.

V EU je podle aktuálního Nařízení (ES) č. 1223/2009 o kosmetických přípravcích povoleno použití TCS jako konzervantu v kosmetických přípravcích s maximální koncentrací 0,3 % v zubních pastách, mýdlech na ruce, tělových mýdlech/sprchových gelech, deodorantech (nikoli sprejích), pudrech na obličej, korektorech skvrn a přípravcích na čištění nehtů před aplikací umělých nehtů, a s maximální koncentrací 0,2 % v ústních vodách (položka 25, příloha V). Použití TCC jako konzervantu je regulováno na maximální koncentraci 0,2 % v konečném produktu (položka 23, příloha V) a v oplachovacích přípravcích na maximální koncentraci 1,5 % s vyloučením použití jako inhibitoru mikroorganismů (položka 100, příloha III). S ohledem na potenciální vlastnosti těchto dvou konzervačních látek narušujících endokrinní systém vydal Vědecký výbor pro bezpečnost spotřebitelů doporučení k aktuálně projednávané novele nařízení o kosmetice, že TCS by se neměl používat v ústních vodách nebo zubních pastách pro děti do 3 let a TCC by neměl být povolen pro použití v ústní vodě a zubní pastě pro děti do 6 let.

Měly by být rovněž zavedeny požadavky na označování s cílem zvýšit ochranu spotřebitelů (SCCS, 2022).

V roce 2019 Evropská komise vyzvala zúčastněné strany, včetně akademických a jiných výzkumných ústavů, orgánů zemí EU, výrobců kosmetických přípravků, výrobců látek vzbuzujících obavy a sdružení spotřebitelů, aby předložily veškeré vědecké informace týkající se hodnocení bezpečnosti 14 látek, včetně TCS a TCC (SCCS, 2022). Předložení jakýchkoli relevantních vědeckých informací bylo požadováno v rámci nařízení o kosmetice a změn jeho příloh uvádějících zakázané, schválené nebo povolené přísady pouze pro omezené použití.

V naší studii zaměřené na genotoxicitu a endokrinní disrupci těchto látek (Chrz *et al.*, 2024) byly použity následující vzorky: Triclocarban (TCC, číslo CAS: 101-20-2, číslo EC: 202-924-1, název IUPAC: N-(4-Chlorfenyl)-N'-(3,4-dichlorfenyl)močovina), a Triclosan (TCS, číslo CAS: 3380-34-5, číslo EC: 222-182-2), název IUPAC: 5-chlor-2-(2,4-dichlorfenoxy)fenol), dodavatel Sigma-Aldrich, USA.

4.2.1 Výsledky *in vitro* testu savčích chromozomových aberací

V *in vitro* testu chromozomových aberací byly obě chemické látky TCS a TCC jasně pozitivní při nejvyšších testovaných necytotoxických koncentracích (10 µg/ml), inkubace po dobu 4 hodin (Tabulka 3). Prodloužená expozice (26 h, bez MAS) při vyšších koncentracích (5, 10 µg/ml) vedla k vyšší cytotoxicitě, proto data nemohla být vyhodnocena jako pozitivní (NE = nehodnoceno).

Tab. 3: *In vitro* test savčích chromozomových aberací. Procento aberantních buněk bylo zaznamenáno po 4 hodinách s/bez MAS a 26 hodinách bez MAS. NE - nehodnoceno, MAS - metabolický aktivační systém.

Vzorky / Kontroly	**Koncentrace µg/ml**	**% aberantních buněk**			**Hodnocení**
		4 h s MAS	**4 h bez MAS**	**26 h bez MAS**	
Triclosan	2,5	5	6	4	**negativní**
	5	4	5	NE	**negativní**
	10	21	10	NE	**pozitivní**
Triclocarban	2,5	4	3	3	**negativní**
	5	7	7	NE	**hraniční**
	10	12	8	NE	**pozitivní**
Negativní kontrola (kultivační medium)		4	3	4	negativní
Pozitivní kontrola (thio-TEPA 10^{-6}M)			9	10	pozitivní
MAS Negativní kontrola (neexponované buňky)		3			negativní
Pozitivní kontrola (cyklofosfamid 10^{-4}M)		10			pozitivní

4.2.2 Výsledky kometového testu *in vitro* na buněčné linii HaCaT

V kometovém testu se procento DNA v ocasu (DNA in Tail) významně zvýšilo při nejvyšších testovaných necytotoxických koncentracích obou testovaných konzervačních látek TCS a TCC a byla pozorována jasná pozitivní reakce závislá na koncentraci (Tabulka 4). Data (% DNA v ocasu) jsou prezentována jako průměrné ± SD hodnoty ze tří nezávislých měření. Výsledky byly považovány za statisticky významné, když byla hodnota $p < 0,05$ (*). DNA v ocasu je přímo úměrná poškození DNA (čím vyšší hodnota, tím větší poškození DNA).

Tab. 4: Kometový test *in vitro*, HaCaT buněčná linie. Data (% DNA v ocasu) jsou prezentována jako střední hodnoty ± SD ze tří nezávislých měření. (*) statisticky významný výsledek s $p < 0,05$.

Vzorky / Kontroly	**Koncentrace µg/ml**	**% DNA in Tail Průměr ± SD**	***p*-value**
Triclosan	2,5	3,75 ± 0,34	**0,060**
	5	13,87 ± 1,51*	**< 0,0001**
	10	15,99 ± 1,64*	**< 0,0001**
Triclocarban	2,5	6,08 ± 0,20*	**0,006**
	5	7,86 ± 0,98*	**0,001**
	10	11,92 ± 2,28*	**0,0005**
Negativní kontrola (kultivační medium)		0,63 ± 0,07	—
Pozitivní kontrola (0,1% H_2O_2)		91,48 ± 0,75	—

4.2.3 Diskuze

V naší studii v testu savčích chromozomových aberací (CA) byly TCS i TCC pozitivní při nejvyšší testované necytotoxické koncentraci (Tabulka 3). Prodloužená expozice (26 h, bez MAS) vedla k vyšší cytotoxicitě, proto data nemohla být použita pro hodnocení. Ačkoli většina vzácně dostupných výsledků v registračních dokumentacích ECHA klasifikovala obě látky jako negenotoxické, nárůst počtu chromozomálních aberací v buňkách čínského křečka V79 vystavených nejvyšší koncentraci TCS (3,0 µg/ml) byl pozorován při 18hodinových a 28hodinových fixačních intervalech bez S9 (Heidemann, 1990), což je v souladu s našimi pozitivními výsledky generovanými na relevantním testovacím systému savců (lidské periferní lymfocyty). Bohužel literární výzkum odhalil, že jsou k dispozici omezené a neprůkazné údaje o chromozomových aberacích indukovaných TCS. TCS byl testován negativně v testech s buňkami CHO nebo v testu mikrojader na myších (kostní dřeň) (SCCP, 2009; SCCS, 2011). Signifikantní ($p \leq 0,05$) koncentračně závislý nárůst aberantních buněk byl pozorován po 96 hodinách expozice TCS u vylíhlých mláďat *Labeo rohita* (podporovaný koncentračně závislým zvýšením nekrotických, apoptotických a mikronukleárních buněk) (Sharma *et al*., 2022). Bylo zjištěno, že neaktivovaný TCS indukuje na dávce závislé zvýšení výtěžku buněk s abnormální morfologií chromozomů v testu *in vitro* savčích chromozomových aberací s

hladinami dávek v rozmezí od 1 do 3 μg/ml (18hodinový sběr) a při 3 μg/ ml (28hodinový sběr). Nejčastěji pozorovaným typem poškození chromozomů byly chromozomové výměny. V *in vivo* testu chromozomových aberací kostní dřeně však nebyly pozorovány žádné známky strukturálních chromozomových aberací (US EPA, 2008).

V případě TCC jsou údaje o indukovaných chromozomových aberacích ještě vzácnější. Jak bylo publikováno v „Screeningovém hodnocení močoviny, N-(4-chlorfenyl)-N'-(3,4-dichlorfenyl) (triklokarbanu)“ zveřejněném kanadskou vládou v březnu 2023 (Government of Canada, 2023), TCC byl negativní v *in vitro* testu chromozomových aberací v buňkách vaječníků čínského křečka, s metabolickou aktivací i bez ní, v koncentracích až 2000 μg/ml (Soap and Detergent Association, 2002). V testech Tox21 byl TCC identifikován jako genotoxický v buněčných liniích s deficitem v drahách opravy DNA (Kim *et al*., 2019). Dostupné omezené údaje byly vyhodnoceny Vědeckým výborem pro bezpečnost spotřebitelů se závěrem, že TCC nebyl shledán jako klastogenní v testu chromozomálních aberací s metabolickou aktivací a bez ní (SCCS, 2022).

V kometovém testu (CMT) provedeném na buněčné linii HaCaT bylo procento DNA v ocasu (DNA in Tail) statisticky významně zvýšeno při dvou nejvyšších testovaných koncentracích po expozici TCS (5, 10 μl/ml) a při všech třech testovaných koncentracích po expozici TCC (2,5, 5, 10 μl/ml). Takový výsledek je podporován např. studií využívající larvy vodního hmyzu *Chironomus riparius*, kde bylo zjištěno, že TCS má genotoxickou aktivitu, protože významně zvyšuje všechny parametry komety (% DNA v ocasu, délka ocasu, Tail moment, Olive moment) ve všech testovaných koncentracích (Martínez-Paz *et al.*, 2013), nebo ve studii binární kombinace TCS a carbendazimu testované na *Daphnia magna* (Silva *et al.*, 2015). Jak TCS, tak TCC způsobily na koncentraci závislé poškození DNA prvoka *Tetrahymena thermophila* v alkalickém kometovém testu, přičemž TCC indukoval závažnější poškození DNA než TCS (Gao *et al.*, 2015). Jiná studie provedená na buňkách HaCaT a L02, ke kterým byl aplikován TCC, však přinesla negativní výsledky (Sun *et al.*, 2019).

Jak je uvedeno v OECD TG 489, pozitivní nálezy v CMT testu nemusí být spojeny pouze s genotoxicitou. Toxicita pro cílovou tkáň může také vést ke zvýšené migraci DNA. Často je pozorována nízká nebo střední cytotoxicita, což ukazuje, že není možné rozlišit migraci DNA indukovanou genotoxicitou od migrace DNA indukovanou cytotoxicitou v

samotném CMT testu. Pokud je však pozorováno zvýšení migrace DNA, doporučuje se provést vyšetření jednoho nebo více indikátorů cytotoxicity, protože to může pomoci při interpretaci nálezů. Zvýšení migrace DNA za přítomnosti jasných důkazů cytotoxicity je třeba interpretovat s velkou opatrností (Burlinson *et al.* 2007; OECD 2014; OECD 2016d). Ve veřejně dostupných registračních dokumentacích (ECHA/12675; ECHA/12075), stanoviscích Vědeckého výboru pro spotřební výrobky (SCCP, 2009) a v nejnovější vědecké literatuře (Sun *et al.*, 2020) byly TCS i TCC opakovaně shledány negativními v Amesově testu provedenému podle OECD TG 471 (OECD, 2020). Antimikrobiální aktivita TCS byla s největší pravděpodobností inhibována enzymy obsaženými v jaterním homogenátu (frakce S9), nebo byl TCS přeměněn na metabolity s nižší cytotoxicitou. To potvrzuje oprávněnost zařazení systému metabolické aktivace do toxikologických metod *in vitro* s cílem zajistit vyšší relevanci pro člověka s ohledem na metabolickou přeměnu xenobiotik v játrech a toxikokinetiku organismu obecně s ohledem na absorpci, distribuci, metabolismus a vylučování *in vivo*. Doporučujeme pečlivě zvážit údaje o cytotoxicitě v jakémkoli *in vitro* testu, protože mohou být získány falešně pozitivní/negativní výsledky, zvláště pokud jsou testována vysoce cytotoxická antimikrobiální činidla. Rostoucí důkazy o genotoxických účincích TCS a TCC na vodní organismy a ryby (Paul *et al.*, 2019; Lee *et al.*, 2023a; Wang *et al.*, 2018; Xu *et al.*, 2015) podporují varování před TCS a TCC a zvyšují úsilí o podrobnější popis mechanismů, kterými se účinky těchto polychlorovaných konzervačních látek projevují. Například oxidační poškození ve specifických buňkách a tkáních (Ma *et al.*, 2022; Zhong *et al.*, 2022; Adhikari *et al.*, 2023; Cui *et al.*, 2023; Alfhili & Lee, 2019), potenciální synergické účinky s jinými látkami přítomnými ve finálních kosmetických přípravcích nebo v životním prostředí (Silva *et al.*, 2015; Lee *et al.*, 2023b; Pashaei *et al.*, 2023; Qu *et al.*, 2021), a hodnocení bezpečnosti jejich aktivních metabolitů, produktů biodegradace nebo fotolýzy, s ohledem na jejich široké použití ve spotřebních výrobcích a jejich přítomnost v životním prostředí (Iacopetta *et al.*, 2021; Zhang *et al.*, 2023a). Nedávné obavy veřejnosti vznesené v souvislosti s potenciálními toxikologickými účinky a akumulací TCS a TCC v životním prostředí vyústily v pokusy najít účinnou náhradu těchto antimikrobiálních látek (Iacopetta *et al.*, 2021), např. analogy TCC (Pujol *et al.*, 2018) obsahující pentafluorsulfanyl nebo benzalkoniumchlorid, benzethoniumchlorid a chloroxylenol.

Tyto látky však dosud nebyly testovány tak intenzivně jako TCS a TCC, a proto mohou představovat větší riziko pro člověka nebo životní prostředí (Sreevidya *et al.*, 2018). Než bude přesvědčivě odůvodněno jejich masivní použití ve spotřebitelských výrobcích, je zapotřebí dalšího zkoumání z hlediska jejich mechanismů účinku a ekologického významu.

4.3 Parfémy

Kosmetické parfémy obsahují směsi vonných látek, např. aromatické nebo fenolické sloučeniny, syntetické vůně, éterické oleje přírodního původu, rozpouštědla a etanol. V závislosti na procentuální koncentraci vonných látek ve finální formulaci parfémového produktu se rozlišuje: parfém (P) 15-40 % vonných složek, parfémovaná voda (EdP) 8-15 %, toaletní voda (EdT) 4-8 % a kolínská voda (EdC) 3–5 %. Kvalita a bezpečné koncentrace jednotlivých složek jsou předpokladem pro bezpečný finální kosmetický produkt (Steineman *et al.*, 2011).

Literární údaje (Wieck *et al*., 2018; Basketter *et al*., 2019; Tai *et al*., 2023) naznačují, že vonné složky mohou vykazovat četné nepříznivé biologické účinky, např. cytotoxicitu, fototoxicitu, kožní senzibilizaci, (foto)genotoxicitu, mutagenitu, reprotoxicitu a endokrinní disruptivitu. Proto byla provedena pilotní studie s vybranými vzorky produktů na bázi vůně, jako jsou deodoranty, toaletní vody a parfémy, s cílem integrovat výsledky z řady alternativních metod bez použití zvířat vhodných pro detekci následujících toxikologických koncových bodů: cytotoxicita (s 3T3 Balb/c fibroblasty); potenciál senzibilizace kůže (metoda *in chemico*, Direct Peptide Reactivity Assay (DPRA), a metoda LuSens *in vitro*, založená na lidských keratinocytech)

Potenciál genotoxicity (*in vitro* alkalický kometový test s buňkami NIH/3T3 a metoda savčích chromozomových aberací *in vitro* u vybraných vzorků pozitivních v kometovém testu); a endokrinní disrupce (*in vitro* test YES/YAS na geneticky upravených kvasinkách). Přítomnost dvaceti čtyř specifických známých alergenů v testovaných vzorcích byla stanovena pomocí GC-MS/MS. Závěrem byl proveden odhad NOAEL (No Observed Adverse Effect Level) směsí alergenů, které byly identifikovány v jednotlivých vzorcích testovaných v této studii (Dvořáková *et al*., 2023).

Pro účely naší studie bylo zakoupeno na území EU 10 produktů, které se používají jako kosmetické parfémy (EdP, 1 deodorant, P, EdT a EdC). V běžné maloobchodní síti byly zakoupeny vzorky 1 – 6; vzorky s označením 7, 8 byly zakoupeny v internetovém prodeji neznačkových produktů; vzorek 9 pocházel z přímého katalogového prodeje; vzorek 10 ze značkové řady produktů byl zakoupen online (viz Tabulka 5).

Tab. 5: Charakterizace vzorků použitých pro studii.

Vzorek	Typ produktu	Země původu
1	EdP	EU
2	EdP	UAE
3	EdP	CHINA
4	EdP	EU
5	Deodorant	EU
6	EdP	KSA
7	P	EU
8	P	EU
9	EdT	EU
10	EdC	EU

4.3.1 Výsledky *in vitro* testu savčích chromozomových aberací

In vitro test savčích chromozomových aberací byl proveden se vzorky 5, 6, 8, 9 a 10 podle OECD TG 473 (OECD, 2016a), aby bylo možné ověřit předpokládaný genotoxický potenciál stanovený v alkalickém kometovém testu *in vitro*. Žádný ze vzorků neindukoval požadované minimální dvojnásobná zvýšení procenta aberantních buněk ve srovnání s negativní kontrolou (viz Tabulka 6). Pozitivní kontrola použitá v testu bez MAS byla správně klasifikována; v testovací verzi s MAS však byla hodnota negativní kontroly relativně vyšší ve srovnání s historickými daty. Test byl přijat jako dodatečné potvrzení negativního genotoxického potenciálu, zatímco pozorované náznaky velmi slabých, koncentračně závislých odpovědí v kometovém testu *in vitro* byly z hlediska genotoxicity hodnoceny jako neprůkazné a spíše jako projev oxidačního stresu. Vzorky byly testovány v triplikátech.

Výsledky uvedené v Tabulce 6 ukazují procento aberantních buněk. Pozitivní výsledek je charakterizován alespoň dvojnásobným zvýšením procenta aberantních buněk ve srovnání s negativní kontrolou).

Tab. 6: *In vitro* test savčích chromozomových aberací. Procento aberantních buněk bylo zaznamenáno po 4 hodinách s/bez MAS a 26 hodinách bez MAS. MAS - metabolický aktivační systém.

Vzorky / Kontroly	**Koncentrace µg/ml**	**% aberantních buněk**			**Hodnocení**
		4 h s MAS	**4 h bez MAS**	**26 h bez MAS**	
5	0,05	2	5	1	**negativní**
	0,1	1	4	0	
	0,15	1	1	2	
6	0,25	4	3	3	**negativní**
	0,5	3	5	2	
	0,75	5	2	5	
8	0,25	3	2	2	**negativní**
	0,5	3	4	4	
	0,75	1	2	5	
9	0,5	1	5	1	**negativní**
	1	3	3	7	
	1,5	6	4	6	
10	0,5	2	4	3	**negativní**
	1	4	5	5	
	1,5	2	3	7	
Negativní kontrola (kultivační medium)			4	5	negativní
Pozitivní kontrola (thio-TEPA 10^{-6}M)			8	15	pozitivní
MAS Negativní kontrola (neexponované buňky s MAS)		6			negativní
Pozitivní kontrola (cyklofosfamid 10^{-4}M)		9			pozitivní

4.3.2 Výsledky kometového testu *in vitro* na buněčné linii NIH/3T3

Alkalický kometový test byl proveden za pomoci buněčné linie fibroblastů NIH/3T3, na kterou byly aplikovány testované vzorky v koncentracích uvedených v Tabulce 7. Záznamy ukazují procento DNA v ocasu (DNA in Tail) při daných koncentracích testovaného vzorku. Tučná čísla ukazují statisticky významnou fragmentaci DNA, závislou na koncentraci, ve srovnání s kontrolním vehikulem. „—“ znamená, že tyto koncentrace nebyly testovány (viz Tabulka 7). DNA v ocasu je přímo úměrná poškození DNA (čím vyšší hodnota, tím větší poškození DNA).

Tab. 7: Kometový test *in vitro*, buněčná linie NIH/3T3. Data (% DNA v ocasu) jsou prezentována jako střední hodnoty ± SD ze tří nezávislých měření. (*) statisticky významný výsledek s $p < 0,05$.

% DNA in Tail									
Vzorky / Koncentrace	**50 µg/ml**	**100 µg/ml**	**150 µg/ml**	**250 µg/ml**	**350 µg/ml**	**500 µg/ml**	**750 µg/ml**	**1000 µg/ml**	**1500 µg/ml**
1	—	—	1,344	0,299	2,329	—	—	—	—
2	—	—	0,002	0,525	0,354	—	—	—	—
3	—	—	0,118	2,006	0,939	—	—	—	—
4	—	—	0,037	1,540	1,210	—	—	—	—
5	0,066	2,823	5,842	—	—	—	—	—	—
6	—	—	—	**0,073**	—	**0,981**	**11,658**	—	—
7	—	—	—	0,008	—	0,969	7,087	—	—
8	—	—	—	1,376	—	**1,129**	**5,055**	—	—
9	—	—	—	—	—	**1,394**	—	**3,043**	**24,002**
10	—	—	—	—	—	**1,557**	—	**2,690**	**14,647**

4.3.3 Diskuze

Kometový test s NIH/3T3 fibroblasty nezjistil žádný významný genotoxický potenciál ve vybraných necytotoxických koncentracích finálních kosmetických přípravků (tj. > 10% zvýšení % DNA v ocasu při aplikaci dvou po sobě jdoucích necytotoxických koncentrací). Nicméně fragmentace DNA závislá na koncentraci, která byla považována za statisticky významnou, byla detekována u vzorků 6, 8, 9 a 10 (Tabulka 7). Proto byla provedena další analýza těchto konkrétních vzorků s použitím validovaného *in vitro* testu savčích chromozomových aberací v souladu s OECD TG 473 (OECD, 2016a) pro potvrzení suspektních výsledků. V tomto druhém testu však u žádného ze vzorků nebyl pozorován genotoxický potenciál (Tabulka 6).

Bohužel, i nadále je obtížné konkurovat konvenčnímu toxikologickému testování *in vivo* založenému na použití laboratorních zvířat, které některé regulační orgány vytrvale podporují, a to navzdory zákazu EU testovat kosmetiku na zvířatech. Primárním cílem naší pilotní studie bylo prokázat užitečnost metod *in vitro* pro predikci rizik a hodnocení rizik finálních (parfémových) kosmetických přípravků. Došli jsme k závěru, že náš navrhovaný

přístup by měl být dále rozvíjen a optimalizován, aby bylo možné přesněji předpovídat potenciální nebezpečí při hodnocení bezpečnosti kosmetických přípravků. Specificky vybrané biologické metody *in vitro* by měly být zahrnuty do souboru větší baterie testů, založené na známých drahách nepříznivých účinků (tzv. „Adverse Outcome Pathways“, AOP) a molekulárních mechanismech vedoucích k těmto nepříznivým účinkům. Takový přístup založený na AOP by mohl odhalit nové toxikologické mechanismy a poskytnout cenná data, která mohou přispět k objasnění specifických interakcí mezi jednotlivými složkami ve finálním kosmetickém přípravku. Interakce mezi složkami mohou mít za následek celkovou nepříznivou biologickou odpověď, i když jsou koncentrace jednotlivých složek považovány za bezpečné.

V naší studii bylo prokázáno, že baterie vybraných biologických testů *in vitro* je prospěšná při screeningu bezpečnosti kosmetických přípravků a jejich složek a může být potenciálně využita jako základ pro další rozvoj kombinovaných toxikologických přístupů v oblasti veřejného zdraví a ochrany spotřebitele.

5 Závěr

Tato disertační práce představuje komplexní pohled na využití alternativních toxikologických metod *in vitro* bez použití laboratorních zvířat, které byly v nedávné době uvedeny jako perspektivní pro testování genotoxicity chemických látek a spotřebních výrobků. S ohledem na zákaz testování kosmetických přípravků a jejich ingrediencí metodami s využitím laboratorních zvířat, tato práce přispívá k pochopení, zdokonalení a rozvoji jediných eticky přijatelných přístupů testování v oblasti kosmetického průmyslu.

Systematickým ověřováním, optimalizací a novým zaváděním *in vitro* toxikologických metod do praxe byly během výzkumu identifikovány silné a slabé stránky jednotlivých metodik, které byly průběžně optimalizovány a kombinovány při aplikaci na skupinu chemických látek využívaných jako konzervanty kosmetických přípravků a parfémy. Aplikace specifických metod poskytla cenné výsledky, které nejenže odpovídají bezpečnostním standardům z hlediska využití validovaných toxikologických metod *in vitro*, ale také poskytují platformu pro další optimalizaci a inovace v oblasti metod, jejichž vývoj je na úrovni výzkumu a procesu standardizace.

V rámci grantového projektu byly posouzeny vybrané chemické látky a kosmetické přípravky metodami vhodnými pro detekci různých koncových bodů genotoxicity. Díky tomu bylo možné zhodnotit, zda výsledky testů odpovídají bezpečnostním standardům pro daný typ výrobku. Současně byly ověřeny strategie pro *in vitro* hodnocení genotoxicity, které jsou široce aplikovány ve výzkumu a praxi.

Disertační práce splnila stanovené cíle a přispěla do souboru poznatků o studiu genotoxicity u spotřebních výrobků a jejich ingrediencí. Výsledky této disertační práce mají přínos pro oblast ochrany veřejného zdraví a bezpečnosti spotřebitelů. Dosažené poznatky mohou sloužit jako základ pro další vývoj a zdokonalení *in vitro* toxikologických metod. Diskuse o regulaci genotoxických testů a standardizaci postupů v oblasti spotřebních výrobků by měla být podnětem pro další kroky v rámci ochrany veřejného zdraví.

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7 Publikační činnost autora

7.1 Práce související s disertační prací

Původní vědecké publikace in extenso v daném oboru uveřejněné v časopisech s IF

1. **Chrz, J.**, Hošíková, B., Svobodová, L., Očadlíková, D., Kolářová, H., Dvořáková, M., Kejlová, K., Malina, L., Jírová, G., Vlková, A., Mannerström, M. (2020) Comparison of methods used for evaluation of mutagenicity/genotoxicity of model chemicals - parabens. Physiological Research, Volume 69, 661-679. https://doi.org/10.33549/physiolres.934615 **IF 2.103**
2. **Chrz, J.**, Dvořáková, M., Kejlová, K., Očadlíková, D., Svobodová, L., Malina, L., Hošíková, B., Jírová, D., Bendová, H., Kolářová, H. (2024) The potential for genotoxicity, mutagenicity and endocrine disruption in Triclosan and Triclocarban assessed through a combination of *in vitro* methods. Journal of Xenobiotics, Volume 14, 15-30. https://doi.org/10.3390/jox14010002 **IF 6.0**
3. Dvořáková, M., Svobodová, L., Rucki, M., Ševčík, V., Hošíková, B., **Chrz, J.**, Bendová, H., Kejlová, K., Očadlíková, D., Malý, M., Kolářová, H., Mannerström, M., Kanďárová, H., Jírová, D. (2023) The Safety Assessment of Cosmetic Perfumes by Using In Chemico and *In vitro* Methods in Combination with GC-MS/MS Analysis. Alternatives to Laboratory Animals, Volume 51, 224-248. https://doi.org/10.1177/02611929231184635 **IF 2.7**

Publikovaná abstrakta – hlavní autor

1. **Chrz, J.**, Vlková, A., Jírová, G., Wittlerová, M., Wittlingerová, Z., Zimová, M., Kolářová, H. (2017) Wastewater from health care facilities – Toxicity for human health and the environment. Abstracts of the 53rd Congress of the European Societies of Toxicology (EUROTOX), September 10–13, 2017, Bratislava, Slovakia, Toxicology Letters, Volume 280, Supplementum 1, p. 203-204. https://doi.org/10.1016/j.toxlet.2017.07.557
2. **Chrz, J.**, Hošíková, B., Svobodová, L., Očadlíková, D., Kolářová, H., Dvořáková, M., Kejlová, K., Vlková, A., Jírová, G., Mannerström, M. (2020) *In vitro* methods used for evaluation of mutagenicity/genotoxicity of selected parabens. Programe & Abstracts: TOXCON 2020. 25th Interdisciplinary Toxicological Conference,

September 3 – 5, 2020, Prague, Interdisciplinary Toxicology, Volume 13, Supplementum 1, p. 23.

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7.2 Ostatní publikace

Publikace článků v odborných časopisech a sbornících s IF

1. Kejlová, K., Bendová, H., **Chrz, J.**, Dvořáková, M., Svobodová, L., Vlková, A., Kubáč, L., Kořínková, R., Černý, J., Očadlíková, D., Rucki, M., Heinonen, T., Jírová, D., Letašiová, S., Kandarova, H., Kolářová, H. (2020) Toxicological testing of a photoactive phthalocyanine-based antimicrobial substance. Regulatory Toxicology and Pharmacology, Volume 115, 104685. https://doi.org/10.1016/j.yrtph.2020.104685 **IF 3.4**
2. Svobodova, L., Kejlova, K., Rucki, M., **Chrz, J.**, Kubincova, P., Dvorakova, M., Kolarova, H., Jirova, D. (2023) Health safety of parabens evaluated by selected *in vitro* methods. Regulatory Toxicology and Pharmacology, Volume 137, 105307. https://doi.org/10.1016/j.yrtph.2022.105307 **IF 3.4**
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1. Kejlová, K., Bendová, H., Sosnovcová, J., **Chrz, J.**, Dvořáková, M. (2017) Hazard identification in newly developed antimicrobials. Proceedings of 8th World Congress on Toxicology and Pharmacology, April 13-15, 2017, Dubai, UAE. Toxicol Open Access, Vol.3, Issue 1 (Suppl), page 74. ISSN 2476-2067 TYOA.

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3. Jírová, G., Vlková, A., Wittlerová, M., Dvořáková, M., Kašparová, L., **Chrz, J.**, Kejlová, K., Wittlingerová, Z., Zimová, M. (2018) Toxicity of hospital wastewater assessed by different bioassays. In: Interdisciplinary Toxicology vol 11, No.1, 2018. TOXCON 2018 Programme & Abstracts, 23. Interdisciplinary Toxicological Conference, June 20-22, 2018, Stará Lesná, Slovakia, p.71. ISSN: 1337-6853 (print version)/13379569 (electronic version).
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5. Jírová, D., Kejlová, K., Dvořáková, M., Bělastová, L., **Chrz, J.**, Rucki, M. (2019) 3RS centre at the National Institute of Public Health in the Czech republic. Abstracts: 24th Interdisciplinary Toxicology 2019, 26-28. 6. 2019, Vyhne, Slovakia, Vol. 12, Supplementum 1, p. 17.
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10. Marketa Dvorakova, Kristina Kejlova, Hana Bendova, Lada Svobodova, Marian Rucki,Vaclav Sevcik, Barbora Hosikova, **Jan Chrz**, Danuse Ocadlikova, Hana Kolarova,Marek Maly, Dagmar Jirova, Helena Kanďárová (2022) Safety of cosmetic perfumes screened in chemico and in vitro in combination with targeted chemical analysis. Abstract Book ESTIV, p.97-98. ESTIV 21.st International Congress, 21.-25. 11. 2022, Barcelona, Spain.
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2. Kejlová, K., Bendová, H., Sosnovcová, J., **Chrz, J.**, Dvořáková, M., Krsek, D., Kolářová, H., Vlková, A., Očadlíková, D. (2017) Hazard identification in newly developed antimicrobials. In: Proceedings of the 20. Conference on Laboratory Animal Science, Jihlava, Czech Republic, 17.-19.5.2017, Physiological Research 2017, Vol.66, Issue 5, pp.1P-2P.
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Příloha č. 1

Comparison of methods used for evaluation of mutagenicity/genotoxicity of model chemicals – parabens

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Comparison of Methods Used for Evaluation of Mutagenicity/Genotoxicity of Model Chemicals - Parabens

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Summary

Growing worldwide efforts to replace (reduce) animal testing and to improve alternative *in vitro* tests which may be more efficient in terms of both time, cost and scientific validity include also genotoxicity/mutagenicity endpoints. The aim of the review article was to summarize currently available *in vitro* testing approaches in this field, their regulatory acceptance and recommended combinations for classification of chemicals. A study using the combination of Comet Assay performed on two cell lines and the Chromosomal Aberration test on human peripheral lymphocytes was performed with the aim to predict the genotoxic potential of selected paraben esters, serving as a model chemical group. Parabens are widely used in consumer products as preservatives and have been reported to exhibit inconclusive results in numerous genotoxicity studies. The Comet Assay identified Ethylparaben and Benzylparaben as potentially genotoxic. The Chromosomal Aberration test revealed weak genotoxic potential in case of Ethylparaben and positive genotoxicity in case of Butylparaben, Propylparaben and Isopropylparaben. The main reasons for variability seem to be limited water solubility of parabens, determining their bioavailability at the cellular level, and absence of metabolic activation in the Comet Assay. The results confirmed that the Comet Assay should serve as a screening test and should not be used as a stand-alone method for classification of genotoxicity. The weight of evidence approach in risk assessment should be supported with data generated with the use of human relevant *in vitro* methods based on cells / tissues of human origin.

Key words

Genotoxicity • Mutagenicity • Alternative Toxicological Testing • *in vitro* Methods • Test Batteries • Parabens

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Introduction

Genotoxicity is one of the most critical toxicological endpoints, highly relevant for public health and environmental protection, including the safety of consumers. The principles of the 3Rs, defined as Replacement, Reduction and Refinement of animal testing by Russell and Burch (1959), represent the ethical basis for the use of laboratory animals in research projects. The Council Directive 2010/63/EU on the protection of animals strongly supports efforts to find alternative methods to animal testing (Beken *et al.* 2016, Directive 2010/63). Numerous *in vitro* testing approaches have been developed with the use of preferentially human relevant biological systems, which often consist of human derived tissue cultures or cell lines (OECD 2017). Unfortunately, the acceptance of *in vitro* toxicological methods alternative to animal testing for genotoxicity endpoints lags behind the progress in technological

development and animal tests remain to be required (Kerecman *et al.* 2017, Coleman *et al.* 2012). Despite of numerous guidelines and publications, considering the benefits of the 3Rs for both animals and science, and substantial advances in the development, validation, and employment of alternative non-animal methods, scientists traditionally tend to prefer the use of animal-based approaches (Combes and Balls 2014). Difficulties in evaluation of *in vitro* genotoxicity endpoints, particularly the physiological background of induction, manifestation and persistence of the genotoxic effects, and also difficulties in transposing and linking the *in vitro* results to *in vivo* conditions may be suggested as the reasons of low acceptance of *in vitro* data. However, a few *in vitro* tests have been already accepted for regulatory purposes, i.e., *in vitro* mammalian chromosomal aberration test (OECD TG 473), *In vitro* mammalian cell micronucleus test (TG 487), and Bacterial reverse mutation test in *Salmonella typhimurium* and *Escherichia coli* (Ames test) (TG 471). Furthermore, *in vitro* systems have been included into OECD TGs designed originally for *in vivo* conditions, *e.g.* the *in vivo* Mammalian Alkaline Comet Assay considers *in vitro* systems to be useful for further investigation of genotoxicity (OECD 2016d). In order to classify a substance as non-genotoxic, a combination of appropriate *in vitro* technologies should be employed (Kerecman *et al.* 2017). Individual *in vitro* methods may exhibit specific advantages and limitations, while their combination may increase sensitivity of safety evaluation (Bhagat 2018). Nevertheless, so far no single *in vitro* test allows detection of the wide range of specific changes in the DNA structure manifested as adverse effects associated either with genotoxicity or mutagenicity (Nesslany 2017). Therefore, combinations of 2 or 3 validated *in vitro* tests of sufficient sensitivity and specificity are currently recommended to be used. Chemical substances may be evaluated as showing no genotoxic potential if all *in vitro* endpoints used are clearly negative while at least two *in vitro* endpoints showing positive results may predict genotoxic potential (EFSA 2011). International working groups and experts from OECD, EURL ECVAM, EPAA, ICCVAM, etc., have been involved in promotion and validation of alternative toxicological methods for regulatory acceptance to replace, reduce or refine (3Rs) *in vivo* testing. Additional data are needed, particularly related to toxicological mechanisms and absorption, distribution, metabolism and excretion (ADME) properties. The development of new approach methodologies (e.g. the development of the OECD QSAR Toolbox, supported by the European Chemical Agency ECHA) should bring high throughput assessment methods, supporting current alternative approaches with human relevant information. Scientific recommendations of testing approaches, and combinations of *in vitro* and *in vivo* methods, have resulted in binding legislation or non-binding recommendations, e.g.: the constantly updated Council regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 (REACH) (EC 2008), OECD Overview on Genetic Toxicology TGs (OECD 2017), ECHA Report on Non-animal Approaches - Current Status of Regulatory Applicability under the REACH, CLP and Biocidal Products Regulations (ECHA 2017), EFSA Statement on Genotoxicity Assessment of Chemical Mixtures (EFSA 2018) and EFSA Scientific Opinion on Genotoxicity Testing Strategies Applicable to Food and Feed Safety Assessment (EFSA 2012).

In vitro approaches available for genotoxicity testing

In vitro endpoints which may be manifested as adverse effects *in vivo* include changes in DNA structure, i.e. numerical chromosomal aberrations (aneuploidy), structural changes (clastogenicity), (double) strand breaks, translocations, deletions, point mutations, gaps, etc. (Turkez *et al.* 2017). A test battery designed for initial screening of genotoxic potential should allow detection of three important genotoxic endpoints, i.e., gene mutations, structural chromosomal aberrations i.e., clastogenicity, and numerical chromosomal aberrations (aneuploidy), in order to understand the genotoxic mode of action (genotoxic endpoint) of the tested substance (EFSA 2011). *in vitro* cell systems mostly included in the OECD TGs, such as mouse lymphoma cells L5178Y, CHO-AS52 and V79 cell lines have been derived from laboratory animals. However, human relevant cell lines derived from malignant cells, such as the e.g. TK6 human lymphoblastoid cells, have been recommended to be used for *in vitro* testing OECD TG 473, 476, 490 (EFSA 2011, OECD 2016b, Ranganatha *et al.* 2016, OECD 2015).

In vitro mammalian cell gene mutation test using the thymidine kinase gene has been recently included into the OECD TG 490 database (OECD 2016e) to identify substances potentially causing gene mutations at the thymidine kinase (*tk*) reporter locus (OECD 2015). The use of *in vitro* systems has indeed been seriously considered useful and included in updated OECD Test Guidelines, such as the OECD TG 489 (*In vivo* Mammalian Alkaline Comet Assay).

The most commonly used methods to assess the genotoxic potential of substances *in vivo*, with possible inclusion of *in vitro* test systems are listed below, on the basis of their principal endpoint of genotoxicity:

Gene mutations:
- Transgenic rodent somatic and germ cell gene mutation assays (OECD TG 488).

Chromosome damage:
- Mammalian erythrocyte micronucleus test (OECD TG 474),
- Mammalian bone marrow chromosome aberration test (OECD TG 475).

Primary DNA damage:
- *in vivo* mammalian alkaline Comet assay (OECD TG 489)

Toxicological test methods designed exclusively as *in vitro* approaches have been developed to be used for detection of the relevant genotoxic endpoints:

Mutations:
- Bacterial reverse mutation test in *Salmonella typhimurium* and *Escherichia coli* (Ames test) (OECD TG 471),
- *in vitro* mammalian cell gene mutation test (OECD TG 476),
- *in vitro* gene mutation assays using the *tk* locus (OECD TG 490).

Chromosome aberrations:
- *in vitro* mammalian chromosomal aberration test (OECD TG 473),
- *in vitro* mammalian cell micronucleus test (OECD TG 487) *(clastogenicity),*
- *in vitro* mammalian alkaline Comet assay (performed with the use of *in vitro* test system, in compliance with OECD TG 489) *(strand breaks).*

The most widely used tests include Bacterial reverse mutation test (OECD TG 471), *in vitro* mammalian cell micronucleus test (OECD TG 487) and *in vitro* mammalian chromosome aberration test (OECD 473).

The Bacterial reverse mutation test (Ames test) has been designed to evaluate the mutagenic potential of test chemicals or mixtures. Ames test is generally used as the first screening method to assess genotoxicity and detection of gene mutations (Bhagat 2018). The assay is based on specific strains *Salmonella typhimurium* and *Escherichia coli* containing identified mutations in amino acid biosynthesis genes at the reporter locus (i.e., histidine [His] or tryptophan [Tryp], respectively) (OECD 1997, OECD 2015). The test detects mutations which revert the identified mutations in the test strains. The revertants are identified by their restored functional capability to synthesize a specific amino acid. The limitation of this method is the use of prokaryotic cells which differ from mammalian cells in factors such as uptake, metabolism, chromosome structure and DNA repair processes. There have been developments to use it in high throughput screening (Flückiger-Isler *et al.* 2004) but the methods have not been developed to a point where they can be routinely used. Substances not directly interacting with DNA may not be detected as mutagenic, which may be relevant especially for substances that have been shown to decrease genomic stability by indirect mechanisms, such as DNA repair systems, cell cycle control and apoptosis (EFSA 2011).

The *in vitro* mammalian cell micronucleus test (MN) has been used for evaluation of cytogenetic damage for decades (Morita *et al.* 1997) in numerous types of test systems, including rodent bone marrow or peripheral blood lymphocytes (Benigni *et al.* 2012, Morita *et al.* 2016, Soeteman-Hernández *et al.* 2016) to identify chemical substances that cause primarily structural chromosomal damage (Corvi *et al.* 2008). The assay detects micronuclei in the cytoplasm of interphase cells and typically employs human or rodent cells lines or primary cell cultures (OECD 2016c). The MN test is almost as sensitive as the chromosomal aberration test (CA) for the detection of clastogens and has the additional advantage of detecting aneugenic substances. The *in vitro* MN test detects the fragments of chromosomes formed into micronuclei, whereas the chromosomal aberration test records visible numerical changes (aneuploidy), translocations and other complex chromosomal rearrangements, that may provide additional mechanistic information (Corvi *et al.* 2008, Kimura *et al.* 2013). Automated systems used for measuring the frequency of micronucleated cells include approaches of flow cytometry (De Boeck *et al.* 2005, Dertinger *et al.* 2011), fluorescent microscopy and image analysis (Parton *et al.* 1996), or laser scanning cytometry (Styles *et al.* 2001). The *in vitro* mammalian cell micronucleus test (OECD TG 487) can be conducted in

the presence or in the absence of cytochalasin B (cytoB), which is used to block cell division and generate binucleated cells and may be combined e.g. with kinetochore staining or fluorescence in situ hybridisation (FISH) (EFSA 2011).

The *in vitro* mammalian chromosome aberration test, based on a proliferating cell population of established cell lines or primary cell cultures in metaphase (Bhagat 2018, OECD 2015), has been used for decades as a biomarker of the early effects of genotoxic carcinogens (Norppa *et al.* 2006, Güzel Bayülken *et al.* 2019). The assay primarily allows the detection of structural aberrations of chromosomes, resulting in specific numerical chromosome aberrations (aneuploidy) in cultured mammalian cells. Additional mechanistic information can be provided using FISH or advanced chromosome staining techniques.

Increasing number of scientific studies have criticized the human relevance of using laboratory animals (e.g. rodents, as the preferred animal model) for assessing hazard and risk of chemicals in humans, as it has become scientifically debatable after consideration of data generated with the use of molecular biology approaches (Phalen *et al.* 2008, Creton *et al.* 2010, Chamanza and Wright 2015, Mowat *et al.* 2017).

The following laboratory animals have been conventionally recommended to be used for genotoxicity testing or as a primary source of *in vitro* cell systems:

- Chinese hamster (*Mesocricetus auratus*) in the Syrian Hamster Embryo (SHE) cell transformation test, Chinese hamster ovary (CHO) and Chinese hamster lung (CHL) cells
- Deer mouse *(Peromyscus leucopus, Peromyscus maniculatus*) (He and Toth 2017)
- Laboratory mouse (*Mus musculus* var. *alba)*
- Rat (*Rattus rattus*), (*Rattus norvegicus* var. *alba*)

Rodent cell lines currently used in genotoxicity tests may exhibit specific deficiencies. CHO cells have been reported to carry a mutant p53 sequence and lack of G1 checkpoint; CHL cells have been reported to exhibit altered regulation of p53 expression; V79 and L5178Y mouse lymphoma cells were found to synthesize a dysfunctional p53 protein (Chaung *et al.* 1997, Storer *et al.* 1997). These issues may increase the test sensitivity, but, in return, induce false positive results (Kirkland *et al.* 2011). The specific cell type used in the bioassay may strongly affect the cellular response and *in vitro* predictivity (Fowler *et al.* 2012). The deficiencies and limits of rodent cell lines have led to efforts to develop and prefer *in vitro* systems based on cells of human origin, which are efficient for DNA repair and for p53 (Kirkland *et al.* 2005, Kirkland *et al.* 2007, Nesslany 2017). An increasing number of studies based on human relevant *in vitro* systems, have demonstrated promising results, indicating significant sensitivity and specificity of the novel approaches. Computational modeling approaches have been suggested for predicting the genotoxic potential and carcinogenicity of chemicals, and have been included in testing strategies in pharmacology (Wu and Wang 2018). Human derived reconstructed 3D epithelium tissue models (RhE) consisting of reconstructed epidermis and functional stratum corneum have been used to establish novel *in vitro* human reconstructed skin micronucleus (MN) and Comet (CMT) assays designed to measure genotoxicity endpoints induced in dividing human keratinocytes after dermal exposure (Kirsch-Volders *et al.* 2011, Reisinger *et al.* 2018, Roy *et al.* 2016, Pfuhler *et al.* 2020a, Pfuhler *et al.* 2020b). Examples of three-dimensional organ models, multicellular spheroids of liver cells (Xu *et al.* 2003) and bone marrow cell cultures (Dal Negro *et al.* 2006) as well as novel technologies using human organ models on-a-chip have been recently introduced (Andersen *et al.* 2014, Alépée *et al.* 2014, Marx *et al.* 2016) with the aim to maximally mimick physiological conditions (Rossini and Hartung 2012). Experimental studies have used e.g. exfoliated oral epithelium cells from buccal smears of healthy donors in the micronucleus test. Buccal smears have been reported as a valuable source of primary cells, however, of limited standardization (Bortoluzzi *et al.* 2014, Jyoti *et al.* 2012, Jyoti *et al.* 2015). For routine *in vitro* toxicological testing, standardized (non-malignant) cell lines may provide more relevant response in standardized conditions. Human bronchial epithelial cell line BEAS-2B was used for detection of genotoxicity of engine emissions in *in vitro* MN assay (Cervena *et al.* 2017). Human derived cell lines that have been used in MN assay include primary human lymphocytes, lymphoblastoid cell line TK6, HepaRG cell line, HT29 (Human colon cancer cell line), Caco-2 cell line (Human heterogenous epithelial colorectal adenocarcinoma), HepG2 (hepatocellular carcinoma) (Allemang *et al.* 2018, Wieczerzak *et al.* 2019, Puerto *et al.* 2018, McCarrick *et al.* 2019). Primary human peripheral lymphocytes have been found to potentially generate fewer false positive results than e.g. V79, L5178Y and TK6 cells (Kirkland *et al.* 2007, OECD 2015). However, not all the systems

have been extensively validated so far, and their use is recommended only if their performance meets the acceptance criteria. Conflicting data have been often obtained in genotoxicity studies, depending on the substances analyzed and methods used, indicating low analytical sensitivity to fully cover the complex causation of genotoxicity, mutagenicity and carcinogenicity. Comparison of the results may be compromised by variability in practical procedure steps or test systems used. Therefore, combinations of toxicological methods (*in vitro, in vivo*) have been proposed in order to increase the sensitivity of genotoxicity testing.

Combinations of methods recommended for genotoxicity/mutagenicity/carcinogenicity testing

Combinations of tests for the screening of carcinogenicity were proposed already in 1974 (Stoltz *et al.* 1974). Most recently, the combination of Ames test, *in vivo* MN, *in vivo* CA and CMT assay has been reported to increase the sensitivity of a testing battery proposed for detection of carcinogens (Kang *et al.* 2013a, Kim and Margolin 1999, Morita *et al.* 2016), alternatively the combination of Ames test and *in vivo* MN has been also reported to show promising sensitivity (Shelby and Zeiger 1990) or combination of MN and CMT assays (Recio *et al.* 2010, Vasquez 2010, Kang *et al.* 2013a). Significant sensitivity was suggested for combination of *in vivo* CMT (90 %) and *in vitro* CMT (86.9 %), *in vivo* MN + *in vivo* CA (88.6 %), *in vivo* MN + *in vivo* CMT (92.5 %), *in vitro* CMT + *in vivo* MN (95.6 %) (Bhagat 2018, Kimura *et al.* 2013). The highest sensitivity has been observed in combination of *in vivo* CMT + *in vivo* CA (96.7 %) (Bhagat 2018, Madrigal-Bujaidar *et al.* 2008, Pfuhler *et al.* 2009, Bowen *et al.* 2011). However, the general development of toxicological testing, performed according to the 3R principles, allows the inclusion of exclusively *in vitro* endpoints in suitable combinations into testing batteries without the use of animals. Assuming that a stand-alone single *in vitro* test cannot be expected to predict all key events potentially resulting in persistent genotoxicity and/or mutagenicity, the combination of *in vitro* assays should include tests with sufficient sensitivity (in order to avoid false negative results) and specificity (in order to avoid false positive results). A standard battery of *in vitro* genotoxicity tests, recommended to be suitable for regulatory purposes, should include 2 or 3 validated tests with at least one test on bacteria and one test on cell cultures (Nesslany 2017). The complex aspects of the induction and causation of the adverse effects, representing potential connections between genotoxicity, mutagenicity and carcinogenicity have to be deeply considered. Genotoxicity has been referred to as an induced damage to DNA, including alterations of the DNA structure, information content and segregation, without direct connection to mutagenicity. Therefore, *in vitro* genotoxicity testing was recommended to include tests detecting DNA strand breaks, DNA adduct formation or mitotic recombination, in addition to specific tests for mutagenicity (Dearfield *et al.* 2011, EFSA 2011, UK Committee on Mutagenicity COM 2011).

Exclusive *in vitro* approaches are strongly recommended in case of dermal exposure and are the only available testing approaches in case of cosmetics and cosmetic ingredients. In case of cosmetic ingredients, the evaluation of the potential for mutagenicity to be annexed in the Regulation (EC) No 1223/2009 should include tests to provide information on three genotoxic endpoints, i.e.: mutagenicity at the gene level, chromosome breakage and/or rearrangements (clastogenicity) and numerical chromosome aberrations (aneuploidy).

The European Scientific Committee on Consumer Safety recommended two tests for the base level testing of cosmetic ingredients, represented by the following test systems:

- Bacterial Reverse Mutation Test (OECD 471) as a test covering gene mutations,
- *In vitro* Micronucleus Test (OECD 487) as a test for both structural (clastogenicity) and numerical (aneugenicity) chromosome aberrations.

Confirmative or supportive evidence may be obtained with the use of tests detecting primary DNA damage (Kirkland *et al.* 2011).

Metabolic activation

Certain chemicals may exhibit genotoxicity only after metabolic activation (indirect genotoxins), therefore it is recommended to include metabolic activation system (MAS) to mimic the situation *in vivo* (Tice *et al.* 2000, Nesslany 2017). Cultivation *in vitro* without MAS may lead to downregulation of enzyme genes and low function of detoxification systems involved in reduction of reactive oxygen species (Oesch *et al.* 2019). The most common system used for metabolic activation is the S9 fraction, prepared traditionally from the liver of rodents (usually rats), but nowadays also human S9 fractions are

commercially available (EFSA 2011, Hong *et al.* 2018). S9 fraction has, however, been reported to compromise the *in vitro* results, as the efficacy of metabolic activation may be specific (depending on e.g. age or sex) and cytotoxicity or variable mutagenic response may be observed (Nishimuta *et al.* 2013, Cox *et al.* 2016, Tweats *et al.* 2007a). Moreover, S9 fractions lack cellular compartmentalization and transporter functions. Human HepaRG cell line, instead, exhibit major CYP activities and other liver specific functions comparable to primary hepatocytes, and has unique potential for competent metabolic activation and may be used as the source of MAS (Josse *et al.* 2012).

Aim of the study

More data from the use of *in vitro* bioassays and test systems are necessary to be generated in order to evaluate the predictivity, sensitivity and specificity of alternative methods suitable for genotoxicity assessment, as still a limited amount of data has been available. Therefore, we carried out a study using the combination of CMT (Comet) assay performed using two non-malignant keratinocyte cell lines HaCaT and SVK14, and the CA (Chromosomal aberration) test using human peripheral lymphocytes, with the aim to predict the genotoxic potential of selected paraben esters, used as a model chemical group. Parabens are widely used in consumer products as preservatives and have been reported to exhibit inconclusive results in numerous genotoxicity studies. We describe the advantages and limits of the combined *in vitro* approaches and also introduce practical issues and details which may help to develop a standardized approach.

Materials and Methods

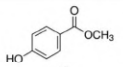
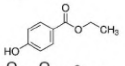
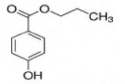
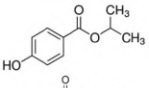
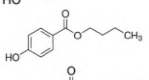
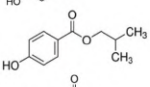
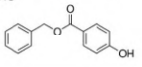
Tested chemicals

Paraben esters (methylparaben, ethylparaben, propylparaben, isopropylparaben, butylparaben, isobutylparaben, benzylparaben) were supplied from (TCI) TOKYO Chemical industry CO., LTD and used as test substances (Table 1).

Cell lines and culture conditions

Commercially available non-tumor cell lines derived from human keratinocytes, i.e. HaCaT cell line and SVK14 cell line (both from ATCC, USA) were used in the *in vitro* CMT assay. For the CA assay, human peripheral blood was obtained by venepuncture from a healthy female human volunteer (non-smoker, non-drinker who was not exposed to radiation, infections or medication for less than one month before the date of collection). The study, performed in compliance

Table 1. Tested chemicals and their formulas.

Substance	IUPAC Name	CAS	Formula	Structural Formula	Molecular weight
Methylparaben	methyl 4-hydroxybenzoate	99-76-3	$C_8H_8O_3$		152.15
Ethylparaben	ethyl 4-hydroxybenzoate	120-47-8	$C_9H_{10}O_3$		166.17
Propylparaben	propyl 4-hydroxybenzoate	94-13-3	$C_{10}H_{12}O_3$		180.21
Isopropylparaben	propan-2-yl 4-hydroxybenzoate	4191-73-5	$C_{10}H_{12}O_3$		180.20
Butylparaben	butyl 4-hydroxybenzoate	94-26-8	$C_{11}H_{14}O_3$		194.23
Isobutylparaben	2-methylpropyl 4-hydroxybenzoate	4247-02-3	$C_{11}H_{14}O_3$		194.23
Benzylparaben	benzyl 4-hydroxybenzoate	94-18-8	$C_{14}H_{12}O_3$		228.25

with the WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects (1964, amended 2013) and the International Ethical Guidelines for Health-related Research Involving Humans (CIOMS 2016), was approved by the Ethical Review Committee of the National Institute of Public Health.

MTT assay

The *in vitro* cytototoxic effect of the parabens used on the two cell lines SVK14 and HaCaT (both human keratinocytes; ATCC, USA) was measured by MTT assay (Mosmann, 1983). Each paraben (diluted in DMEM) was applied to cells at selected concentrations and incubated for 24 hours. After incubation, DMEM (Sigma Aldrich, USA) was replaced by a solution containing 50 µl of 0.5 mg ml^{-1} MTT (Sigma Aldrich, USA) dissolved in 1x PBS. Afterwards, the cell lines were incubated with MTT for 4 hours at 5 % CO_2 and 37 °C. The MTT solution was then replaced with 100 µl of DMSO to ensure the formazan crystals to be properly dissolved. The absorbance level was measured by a Tecan Infinite Pro200 reader (Tecan, Switzerland).

In vitro Comet assay (CMT)

The method was used based on OECD TG 489 with modifications, i.e. using human relevant non-tumor cell lines derived from human keratinocytes (Jiravova *et al.* 2016). Test substances at pre-tested selected concentrations in DMEM were applied to the cell culture for 24 hours. As a positive control, 1 % H_2O_2 in PBS was applied to the cell culture for 15 minutes at 4 °C. Culture medium (DMEM) served as negative control. After incubation, cells were harvested using TrypLE (Gibco, 12604-021) for +/- 10 min. DMEM with fetal bovine serum was applied to stop the trypsinization. Harvested cell suspension was centrifuged and the cell pellet was collected. A 1 % low melting point (LMP) agarose solution, tempered at 37 °C, was mixed with the cell pellet. The resulting dense agarose suspension was pipetted onto pre-coated microscopic slides and quickly covered with coverslips. The agarose was solidified at 4 °C in the refrigerator and then the coverslips were carefully removed, preventing the damage of the sample. Slides were immersed in lysis buffer with 1 % Triton X-100 and incubated in glass Coplin Jars for 60 minutes at 4 °C in order to ensure sufficient cell lysis. In the next step, slides were placed in an electrophoretic tank (Sub-Cell Model 192 Cell, 2,000 ml) and embedded in sufficient volume (slightly covering the slides) of chilled (4 °C) alkaline electrophoretic solution for 40 minutes. Electrophoresis was performed under the following conditions: 350 mA, 0.8 V cm^{-1}, for 20 minutes. After electrophoresis, slides were carefully transferred and rinsed twice for 10 minutes with neutralization buffer (pH 7.5, 4 °C). The slides were stained with SYBR Green I and evaluated microscopically using a fluorescence microscope with a CCD camera (Olympus). The experiment was repeated in three runs in triplicates. From each sample, 100 cells were selected for evaluation using the CometScore 1.5 software. The median values from each measurement were used for mathematical and statistical analysis, and mean values of the amount of DNA in the head of the comet (directly proportional to the intact, non-fragmented DNA), the Olive momentum and DNA in the tail were evaluated. The values of the DNA in the head of the comet were used for interpretation of results. Analysis of variance (*ANOVA*) and Dunnett *t*-tests were used for statistical analysis. A p value < 0.05 was used as the level of statistical significance. All statistical analyzes were performed using IBM SPSS Statistics for Windows, version 23.0. Armonk, NY: IBM Corp.

In vitro mammalian chromosome aberration test (CA)

The test was performed in accordance with OECD TG 473 (OECD 2016a). Peripheral blood lymphocytes were collected from a healthy donor and cultured in RPMI-1640 medium with L-glutamine and $NaHCO_3$ (SIGMA) supplemented with 10 % heat-inactivated bovine serum (Bioveta, Czech Republic). Exogenous source of metabolic activation system (MAS), i.e. a cofactor-supplemented post-mitochondrial fraction prepared from rodent livers (Wistar rats, males, 7-8 weeks old) treated with an enzyme-inducing agent (polychlorinated biphenyl Delor), was used to model mammalian metabolic activation. Cells were exposed to the test substances with or without MAS. 48 hours after the start of the culture, the cells were treated for 4 hours with the test substance and positive control chemicals (Thio-TEPA 10^{-6}M without MAS and cyclophosphamide 10^{-4}M with MAS) and cultured for 24 hours. Culture medium was used as negative control. Then, the cell cultures were exposed for 2 hours to a metaphase arresting agent (colchicine), harvested and subjected to hypotonic cell treatment, fixation, and staining (5 % Giemsa). Metaphase cells (at least 200 well-spaced metaphases with 46 ± 2 centromeres) were analyzed

microscopically for the presence of chromosomal aberrations. Gaps were recorded separately and were not included in the total frequency of aberrations. The percentage of cells with structural chromosomal aberrations was evaluated. At least a 2-fold increase in aberration cells compared to the negative control was a criterion for determining a positive result including a concentration-related increase in the number of cells with chromosomal aberrations.

Results

MTT assay

The results of MTT assay have shown concentration-dependent responses of all samples (Fig. 1, 2). Variability was observed in the used two *in vitro* test systems. Methylparaben was shown to exhibit the weakest cytotoxicity, as the detected cytotoxic concentration (below IC50 value) was 750 µg/ml in the HaCaT cell line and 500 µg/ml in the SVK14 cell line. Ethylparaben and Benzylparaben exhibited cytotoxicity at 500 µg/ml in both *in vitro* cell systems. Butylparaben exhibited cytotoxicity at 100 µg/ml in the HaCaT cell line and 500 µg/ml in the SVK14 cell line. Isobutylparaben and Propylparaben have shown similar results, as the detected cytotoxic concentration (below IC50 value) was 50 µg/ml for both Isobutylparaben and Propylparaben in the HaCaT cell line and 100 µg/ml in the SVK14 cell line. Isopropylparaben exhibited lower cytotoxicity in the SVK14 cell line, as the detected cytotoxic concentration (below IC50) was 500 µg/ml, however, in the HaCaT cell line, the first cytotoxic concentration (below IC50) was 125 µg/ml (data not shown).

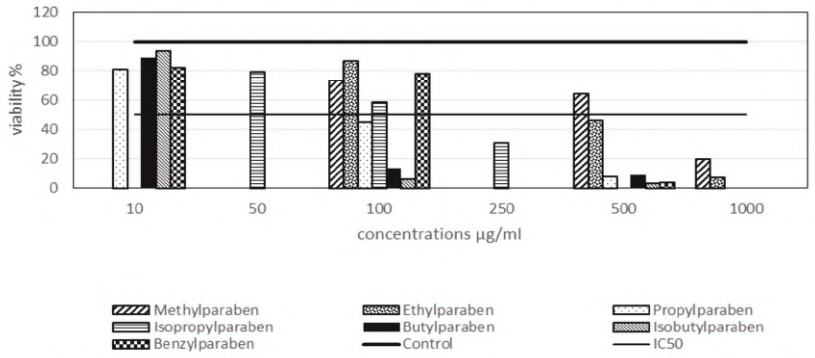


Fig. 1. MTT assay, HaCaT cell line. Bold full line represents the value of solvent control (100 %). Full line represents the cut-off value in comparison with solvent control (IC50).

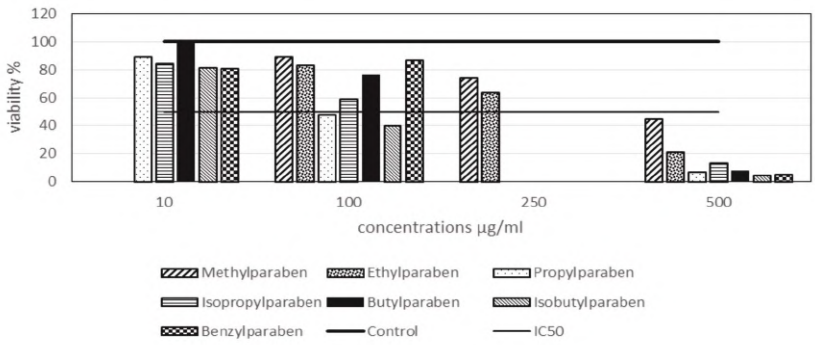


Fig. 2. MTT assay, SVK14 cell line. Bold full line represents the value of solvent control (100 %). Full line represents the cut-off value in comparison with solvent control (IC50).

Comet assay in vitro in SVK14 cell line and HaCaT cell line

The observed intensity of DNA damage in the SVK14 cell line exposed to any of the parabens (Methyl-, Ethyl-, Propyl-, Isopropyl-, Butyl-, Isobutyl- or Benzylparaben) did not exceed the values of negative control even at high concentrations which were observed as cytotoxic in the MTT assay (Fig. 3). In the HaCaT cell line, Benzylparaben and Ethylparaben exhibited increased fragmentation of DNA (Benzylparaben, mean value 30.9 % DNA in head at 250 µg/ml; Ethylparaben, mean value 32.8 % DNA in head at 1000 µg/ml), which represented values significantly distant from the negative control (99.7 % DNA in head), whereas the fragmentation of DNA exceeded even the values of positive control (0.1 % H_2O_2 in 1x PBS, 15 min, 4 °C, mean value 62.9 % DNA in head), as reported in Figure 4. Therefore, Benzylparaben and Ethylparaben were evaluated as suspected genotoxic substances in the *in vitro* Comet assay, as they may potentially exhibit genotoxic effects at higher concentrations. This result was used as an indication for further testing with the aim to confirm the results.

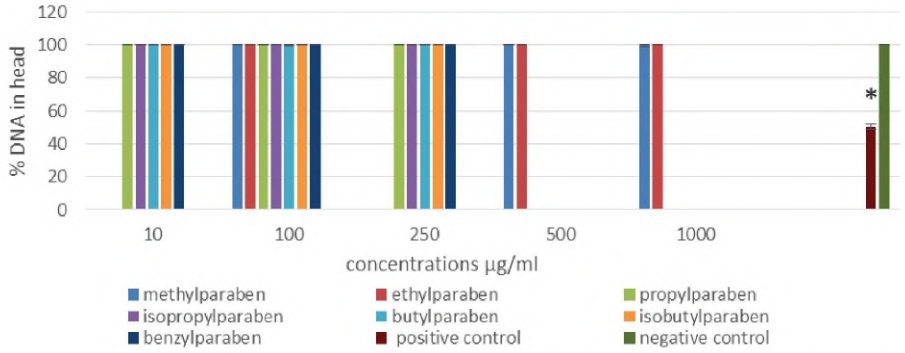


Fig. 3. Comet assay *in vitro*, SVK14 cell line. Data (% DNA in head) are presented as median ± SD values from three independent measurements. The results were considered statistically significant when $p < 0.05$ (*). The DNA in head is proportional to the DNA damage (the higher value, the lower DNA damage).

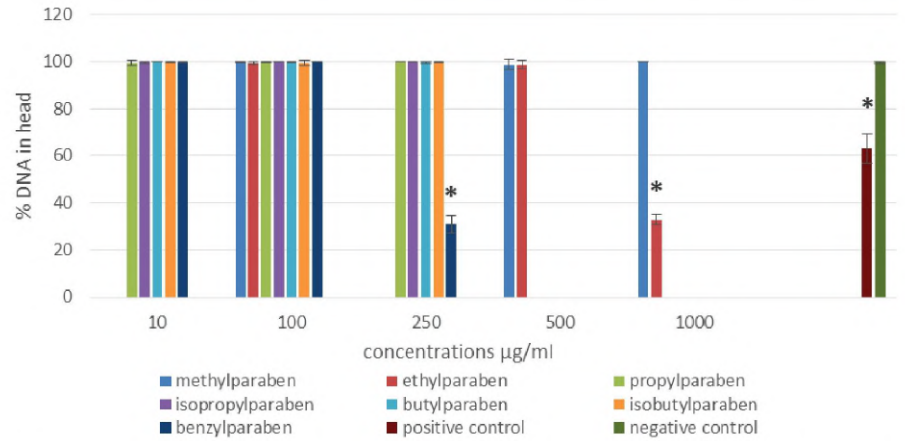


Fig. 4. Comet assay *in vitro*, HaCaT cell line. Data (% DNA in head) are presented as median ± SD values from three independent measurements. The results were considered statistically significant when $p < 0.05$ (*). The DNA in head is proportional to the DNA damage (the higher value, the lower DNA damage).

Table 2. *In vitro* mammalian chromosome aberration test.

Samples / Controls	% abberant cells				Evaluation
	Concentration µg/ml	4 h with MAS	4 h without MAS	26 h without MAS	
Methylparaben	10	3	4	4.5	negative
	25	4	3	tox	inconclusive result due to cytotoxicity
	50	tox	tox	tox	
	100	tox	tox	tox	
Ethylparaben	10	3	3	3	borderline
	25	5.5	4	tox	inconclusive result due to cytotoxicity
	50	tox	tox	tox	
	100	tox	tox	tox	
Propylparaben	5	4	3.5	2.5	positive
	10	2	3	2	
	25	5	6	9	
	50	tox	tox	tox	
Isopropylparaben	5	3	3	2	positive
	10	2.5	3	3.5	
	25	4	4.5	7.5	
	50	tox	tox	tox	
Butylparaben	5	5	5	3	positive
	10	6	5.5	9	
	25	tox	tox	tox	
	50	tox	tox	tox	
Isobutylparaben	5	3	3	2.5	negative
	10	5	4	4.5	
	25	tox	tox	tox	
	50	tox	tox	tox	
Benzylparaben	1	4	3	2.5	negative
	2.5	5	3	0.5	
	5	2.5	4	1.5	
	10	3.5	3	5	
Negative control (non-treated cells)			3	2	
Positive control (thio-TEPA)			10	15	
MAS Negative control (non-treated cells with MAS)		5.5			
Positive control (cyclophosphamide)		11			

The percentage of aberrant cells is recorded after 4h with/without MAS and 26h without MAS (MAS - metabolic activation system). Due to the toxicity of the test substance at a given concentration, mitoses were not evaluable (designated tox).

In vitro mammalian chromosome aberration assay

In the validated *in vitro* mammalian chromosome aberration assay, human peripheral blood lymphocytes did not show an increased rate of chromosomal aberrations compared to the negative control for Methylparaben, Isobutylparaben and

Benzylparaben. Ethylparaben was considered borderline, but due to its high toxicity it was not possible to evaluate the sample in all concentrations. Propylparaben, Isopropylparaben and Butylparaben had a high percentage of aberrations and the concentration dependence at the highest tested concentrations is evident compared to the negative control (more than 2-fold) in the case of the 26-hour incubation without MAS (Table 2).

Discussion

The aim of our study was to generate data with the use of two *in vitro* toxicological methods in combination, performed in three human relevant non-malignant cell systems. We performed the *in vitro* Comet assay using the HaCaT and SVK14 cell line and the CA assay using human peripheral blood lymphocytes. Our study demonstrated high variability of results between the two *in vitro* methods, and surprisingly also between the two cell lines used in the Comet assay. The observed intensity of DNA damage in the SVK14 cell line did not exceed the values of negative control and all the samples were classified as negative, indicating that the substances neither significantly accumulated in the DNA nor attacked the DNA structure, thus, their cytotoxicity may be attributed to other mechanisms than direct DNA damage. With the use of the HaCaT cell line, most samples were also classified as negative, however, Benzylparaben and Ethylparaben exhibited an increased fragmentation of DNA at one tested concentration. We considered the results from the Comet assay as indicative for further testing and performed a validated CA assay using human peripheral blood lymphocytes. In the CA assay, Methylparaben was classified as negative and Ethylparaben as borderline, however, both exhibited significant cytotoxicity. Propylparaben, Isopropylparaben, Butylparaben were classified as positive, and Isobutylparaben, Benzylparaben as negative. Comet assay has been repeatedly described in other studies to give positive results only at significantly higher concentrations than in the CA or MN test (Giannotti *et al.* 2002, Kimura *et al.* 2013, Kawaguchi *et al.* 2010). As the Comet assay may not give a specific response with satisfactory sensitivity at lower concentrations and lacks metabolic activation, or the CA test may be compromised by higher cytotoxicity, the two tests do not seem to correlate promisingly. Assuming that a negative *in vitro* result should be supported by data from at least two validated *in vitro* tests, and with regard that only one *In vitro* bioassay has been validated in our laboratory, the result has to be considered with caution. In case of inconclusive results from *in vitro* testing, repeating the test or applying another *in vitro* test is recommended (EFSA 2011). Unfortunately, increasing the number of *In vitro* assays may further reduce specificity and increase false positivity. Inconclusive results may be obtained due to inclusion of metabolic activation system, experimental conditions, specificities of the test system, cell type used, etc. (Nesslany 2017). Various mammalian cell types may exhibit variability in expression of genes involved in DNA repair, cellular defence systems, cell cycle control and apoptosis (e.g. p53), resulting in different response to chemicals. Lymphocytes have been reported to be karyotypically stable, however, when used in the CA test, mitogens have been observed to induce the resting phase. Characterization of *in vitro* test systems should be performed and donor-to-donor variation avoided (Tweats *et al.* 2007b). In order to deeply consider other possible sources of variability, we focused also on water solubility of parabens, determining their bioavailability at the cellular level. We also performed additional cytotoxicity test using 3T3 Balb/c fibroblasts and Neutral Red Uptake assay (3T3 NRU Cytotoxicity Assay), which was performed according to ECVAM DB-ALM Protocol no. 46 (data not published). Interestingly, during microscopic inspection, we observed various paraben-borne formations in the serum-free media, such as clusters or crystals, which were not observed during routine visual inspection. Moreover, the results of 3T3 NRU Cytotoxicity Assay have shown further variability. Therefore, we suggest that solubility in water solutions (i.e., culture media) should be thoroughly inspected in case of parabens, as poor water solubility or crystallization may significantly affect the bioavailability of parabens in both *in vitro* and *in vivo* test systems and compromise the results of genotoxicity testing. We performed a brief data search upon water solubility of parabens and found similar conclusions for individual parabens or their mixtures. Generally, parabens are stable in air and are resistant to hydrolysis in hot or cold water and in acidic solutions. The antibacterial potential of parabens increased with the length of esterified alcohol chain, however, the water solubility decreased (Soni *et al.* 2001). Variable antimicrobial potential and water solubility represents one of the reasons for application of parabens in mixture, to ensure optimal antimicrobial

activities and acceptable solubility (Charnock and Finsrud 2007, Güzel Bayülken and Tüylü 2019, Soni *et al.* 2005, Kapalavavi *et al.* 2014, Giordano *et al.* 1999, Perlovich *et al.* 2005).

The results of our study are in agreement with numerous toxicological studies on parabens mutagenicity, genotoxicity and carcinogenicity, which often present ambiguous or warning conclusions. Potential genotoxicity and carcinogenicity has frequently been attributed to parabens, e.g. paraben esters have been reported to be detected in breast tissue biopsies, and antiperspirants have been suggested to be the source (Darbre *et al.* 2004). However, the risk assessment for humans has become highly challenging, as inconclusive or contradictory results have been demonstrated by numerous studies *in vivo* and *in vitro* or in combined test batteries (e.g., Tayama *et al.* 2008, Roszak *et al.* 2017, Barr *et al.* 2012, Fransway *et al.* 2019, Soni *et al.* 2005, Kang *et al.* 2013b, Darbre and Harvey 2008, Kim *et al.* 2020, Bilal and Iqbal 2019). Official scientific opinions summarize in their studies that the use of parabens is safe for human health as parabens are metabolised to benzoic acid in the human body and excreted in the urine, with benzoic acid being practically free of toxic effects in numerous tests and unlikely to interfere with chromosomes (SCF 1994, SCCP 2011, SCCP 2013). The results of our study indicate that genotoxicity testing should be strictly interpreted exclusively for the specific *in vitro* test system used in the bioassay, and standard bioavailability of the substance has to be thoroughly ensured. Moreover, as the genotoxicity of parabens is metabolism-dependent, a most relevant MAS should be included. With regard to the above limitations and observations of *in vitro* bioassays, our aim is to implement more *in vitro* methods based on human relevant cells and tissues into our laboratory, to achieve a sufficient battery of non-animal toxicological methods.

Conflict of Interest

There is no conflict of interest.

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Příloha č. 2

The Potential for Genotoxicity, Mutagenicity and Endocrine Disruption in Triclosan and Triclocarban Assessed through a Combination of *In vitro* Methods

Chrz, J., Dvořáková, M., Kejlová, K., Očadlíková, D., Svobodová, L., Malina, L., Hošíková, B., Jírová, D., Bendová, H., Kolářová, H.

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The Potential for Genotoxicity, Mutagenicity and Endocrine Disruption in Triclosan and Triclocarban Assessed through a Combination of In Vitro Methods

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Abstract: Triclosan and Triclocarban, preservatives widely used in cosmetics and other consumer products, underwent evaluation using a battery of new-approach methodologies in vitro (NAMs). Specifically, the Microplate Ames Test (MPF™ Test, Xenometrix, Allschwil, Switzerland) was employed to assess mutagenicity, the Comet assay in vitro on the HaCat cell line and the Mammalian Chromosome Aberration Test were utilized to evaluate genotoxicity, and the XenoScreen® YES/YAS assay was applied to investigate endocrine disruption. The chemicals did not exhibit any positive responses for mutagenicity. However, the mammalian chromosome aberration test identified both chemicals as being positive for genotoxicity at 10 µg/mL. In the Comet assay, the percentage of DNA in the tail significantly increased in a concentration-dependent manner (at 5 and 10 µg/mL for Triclosan, at 2.5, 5, and 10 µg/mL for Triclocarban). The positive response depended on the increasing concentration and the duration of exposure. Triclosan, but not Triclocarban in any of the endocrine assays performed, indicated a potential for endocrine activity in the anti-estrogenic and anti-androgenic assays. The positive in vitro results detected were obtained for concentrations relevant to final products. The alarming findings obtained with the use of new-approach methodologies (NAMs) justify the current precautionary regulatory approach, limiting the use of these preservatives.

Keywords: genotoxicity/mutagenicity; preservatives; chromosome aberrations; Ames test; Comet assay; endocrine disruption

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1. Introduction

Triclosan (TCS) and Triclocarban (TCC) have been used for decades, since the early 1940s, as fungicides and preservatives in various products, including clothing, fabric and leather finishing agents, toys, food packaging, floors in the food industry, construction materials, medical supplies and particularly in household and personal care products, such as soaps, antibacterial bar/liquid soaps, body lotions, deodorants, detergents, medical disinfectants, aftershave soaps, hand sanitizers, toothpastes, handwashes and mouthwashes, body washes, cleansing lotions, baby teethers and wipes, detergents, etc. [1]. The two chemicals differ in their structures, but both are polychlorinated aromatic antimicrobials, in which three hydrogen atoms on aromatic rings are substituted with chlorine. They are effective against many different bacteria, as well as some fungi and protozoa, as they inhibit fatty acid synthesis and induce the disruption of membrane integrity [2,3].

Dermal application of personal care products is believed to be the main route of human exposure, although they may also enter the human body orally through toothpaste, mouthwashes and dental treatments. As a result, TCS and TCC have been detected in human samples such as blood [4–6], breast milk [7,8], urine [9–12], hair and nails [13,14].

Moreover, TCS and TCC represent a burden for the aquatic environment and pose a risk of contaminating drinking water, as current wastewater treatment plants are not typically designed for the treatment of such micropollutants [15,16]. The long-term exposure of aquatic organisms to TCS and TCC, coupled with their bioaccumulation potential, has led to detectable levels of them throughout aquatic food chains in species such as algae, crustaceans, fish and marine mammals [17,18]. Environmental TCS and TCC could be efficiently taken up by food crops, leading to additional potential human exposure through food consumption [19–21]. Their adverse biological effects in humans, including endocrine disruption, developmental and reproductive impacts [6,22–26], gut microbiota disturbance [21,27,28] and the elevation of antibiotic resistance [29–32], have led to strict regulations on the usage of both preservatives.

In the EU, according to the current regulation, (EC) No. 1223/2009 [33], on cosmetic products (Cosmetics Regulation), TCS is allowed to be used as a preservative in cosmetic products with a maximum concentration of 0.3% in toothpaste, hand soaps, body soaps/shower gels, deodorants (not sprays), face powders, blemish concealers and nail cleansing products prior to the application of artificial nails. Additionally, it is allowed in mouthwashes with a maximum concentration of 0.2% (entry 25 Annex V). The use of TCC as a preservative is regulated to a maximum concentration of 0.2% in the final product (entry 23 Annex V), and to a maximum concentration of 1.5% in rinse-off products, excluding its use as an inhibitor of microorganisms (entry 100, Annex III). Concerning the potential endocrine disrupting properties of these two preservatives, the Scientific Committee on Consumer Safety has issued recommendations for the currently discussed amendment to the Cosmetic Regulation. It suggests that TCS should not be used in mouthwash or toothpaste for children under 3 years of age, and TCC should not be allowed for use in mouthwash and toothpaste for children under 6 years of age. Labelling requirements should also be introduced with the aim of enhancing the protection of consumers [2].

In 2016, the U.S. Food and Drug Administration (FDA) banned the use of 19 antimicrobial agents, including TCC and TCS, in personal care products, due to their disputable contribution to preventing bacterial diseases, their potential role in the development of resistance in microorganisms, their ecotoxicity and the potential health risks for humans. The FDA concluded that the total available data regarding the safety profile of TCS and TCC do not contain sufficient information to determine whether these preservatives are generally recognized as safe for use in consumer antiseptic wash products [34].

In 2019, the European Commission invited interested parties, including academic and other research institutes, EU countries' authorities, manufacturers of cosmetic products, producers of substances of concern and consumers associations, to submit any scientific information relevant to the safety assessment of 14 substances, including TCS and TCC [2]. The submission of any relevant scientific information, including the endocrine-disrupting potential of ingredients used in cosmetic products, was required in the framework of the Cosmetics Regulation and the amendment of its annexes listing ingredients that are banned, approved or only authorized for limited use.

Animal testing is prohibited for cosmetic ingredients and final products in the EU by the Cosmetics Regulation. Testing chemical substances of toxicological concern in human volunteers for toxicity endpoints, such as genotoxicity, mutagenicity, carcinogenicity and endocrine disruption, is ethically unacceptable. The use of in vitro toxicological methods remains the only available approach to assess the hazard and risks associated with the use of chemical preservatives present in cosmetic products. Suitable combinations of new-approach methodologies (NAMs) and test systems of human origin (mainly with regard to human genes, receptors, proteins, cells and tissues) providing mechanistic data of the highest human relevance, have recently been suggested as a scientifically valid approach and are currently utilized as efficient non-animal tools for the assessment of genotoxicity and endocrine disruption (OECD Test Guidelines, http://www.oecd-ilibrary.org/ accessed on 15 December 2023). Bacterial strains have been broadly used to predict the potential of mutagenicity. In the Ames test, the assay is usually performed with bacterial strains

of *Salmonella typhimurium* or *Escherichia coli*, both in the absence or presence of an S9 fraction from rat liver used to mimic the metabolic function of mammalian systems in vivo. This may eventually disqualify this in vitro method from being a completely non-animal procedure [35]. However, the use of in vitro methods (NAMs) that include metabolic activation still significantly reduces the number of laboratory animals needed and provides testing with higher human relevance.

With regard to the European Commission's call for data submission, we have selected a battery of in vitro toxicological methods, namely the Ames Test (MPF™ Test, Xenometrix, OECD TG 471) with *S. typhimurium* strains TA98, TA100, TA1535 and TA1537; the Comet assay in vitro performed on the HaCat cell line (non-tumor human keratinocytes); the Mammalian Chromosome Aberration Test on human peripheral lymphocytes (OECD TG 473); and XenoScreen YES/YAS assay (Xenometrix, Allschwil, Switzerland). We used these assays (NAMs) in combination to screen TCS and TCC for genotoxic and mutagenic properties, and their potential for endocrine disruption, applying the most feasible non-animal approach.

2. Materials and Methods

2.1. Chemicals

Triclocarban, (CAS No.: 101-20-2, EC No.: 202-924-1, IUPAC name: N-(4-Chlorophenyl)-N′-(3,4-dichlorophenyl)urea), (TCC), and Triclosan, (CAS No.: 3380-34-5, EC No.: 222-182-2, IUPAC Name: 5-chloro-2-(2,4-dichlorophenoxy)phenol), (TCS), were supplied by Sigma-Aldrich Co., St. Louis, MI, USA, and used as test substances. Their chemical structures are depicted in Figures 1 and 2.

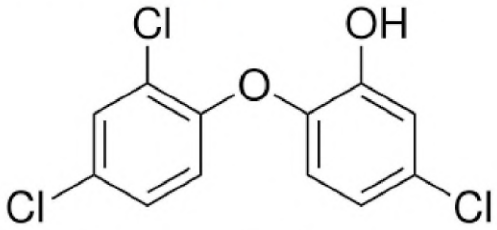

Figure 1. Chemical structure of Triclosan.

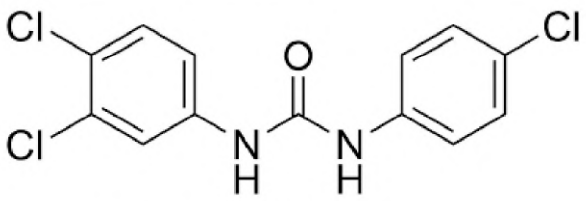

Figure 2. Chemical structure of Triclocarban.

2.2. Metabolic Activation

An exogenous source of the metabolic activation system (MAS), i.e., a cofactor-supplemented post-mitochondrial fraction prepared from rodent livers (Wistar rats, males, 7–8 weeks old) treated with an enzyme-inducing agent (Aroclor 1254 or Phenobarbital/β-Naphtoflavone), was used to model mammalian metabolic activation. This production was conducted at the National Institute of Public Health, Prague, Czech Republic, under project No. MZDR 37519/2019-4/OVZ, and approved by the Czech Ministry of Health.

2.3. Methods

2.3.1. Bacterial Reverse Mutation Test (Ames MPF™ Test)

Ames MPF™ Test (Xenometrix AG, Allschwil, Switzerland) is a commercially available bacterial-modified bacterial plate method, based on the procedure described in OECD TG 471 [36], used for identifying compounds with genotoxic activity. The assay was conducted following the provided standard operating procedure (SOP) and utilized standardized materials and chemicals [37]. Four *Salmonella typhimurium* strains (TA98, TA100, TA1535, TA1537) were employed. All positive control chemicals (2-nitrofluorene (2-NF), 4-nitroquinoline N-oxide (4-NQO), N4-aminocytidine (N4-ACT) and 9-aminoacridine (9-AAc)) for tests with the *S. typhimurium* strains without S9 (S9−) and 2-aminoanthracene (2-AA) for tests in the presence of S9 (S9+) were supplied with the kit. The rat liver S9 fraction, including S9 100/1537 Booster solution, was also provided and was specific for each *S. typhimurium* strain with or without metabolic activation (S9+/S9−), as specified in the supplied SOP. Growth medium was used as the negative control. Briefly, 10 mL growth medium was mixed with 25 µL of the frozen stock culture of the tested strains (TA98 and TA100) in the presence of 10 µL ampicillin (Xenometrix). The strains were incubated at 37 °C in a shaking incubator (NB-205, N-BIOTEK) at 250 rpm for 14–16 h, overnight. The culture density was verified by measuring OD at 600 nm (BioTek Eon High Performance Microplate Spectrophotometer, Agilent Technologies, Santa Clara, CA, USA). The required OD values for the cultures to be suitable for testing should be at least 2.0, and for the negative control (growth medium without culture), should be <0.05. For testing, 10 µL aliquots of test items were added to plates S9−/S9+ with a final concentration of S9 fraction of 4.5%. Three pre-screened non-cytotoxic concentrations were evaluated in triplicate. Then, 240 µL/well of the suspension was transferred to 24-well plates, sealed with breathable tapes and incubated at 37 °C for 90 min with shaking at 250 rpm. After 90 min of incubation, a reversion indicator medium (Xenometrix) was dispensed into each well of the 24-well plates. The indicator medium was gently mixed, and 50 µL/well was transferred to a 384-well microtiter plate. Each column of the 24-well plate was transferred into one half of a 384-well microtiter plate, effectively dividing each sample among 48 wells. Three plates were used per strain with/without S9 (S9+/S9−). The 384-well microtiter plates were placed in plastic bags to reduce evaporation and were incubated at 37 °C for approximately 48 h. The average number of triplicate wells containing revertants per culture/dose was calculated. The combined criteria 'Fold increase (FI) over baseline ≥ 2' and 'Binomial B-value ≥ 0.99' were used to score individual doses as positive (software provided by the kit manufacturer Xenometrix: YES/YAS Calculation Workbook in MS Excel, created by M. Kamber, Xenometrix AG, 2012).

2.3.2. In Vitro Mammalian Chromosome Aberration Test

OECD TG 473 [38] was followed as the main methodological guideline. Peripheral blood lymphocytes were used as the test system. A metabolic activation system (S9 liver fraction), i.e., a cofactor-supplemented post-mitochondrial fraction prepared from rodent livers (Wistar rats, males, 7–8 weeks old) treated with an enzyme-inducing agent (Aroclor 1254 or Phenobarbital/β-Naphtoflavone), was employed to model mammalian metabolic activation. After 48 h of cultivation, the test system was exposed to the test chemicals at previously selected non-cytotoxic concentrations: with or without metabolic activation (+/− S9), positive controls (Thio-TEPA 10^{-6} M without S9, Sigma Aldrich, Germany; Cyclophosphamide 10^{-4} M with S9, Bristol Myers Squibb, USA) and negative control (culture medium). The exposure period was 4 h with or without S9 (+/− S9) and 26 h without S9 (−S9). Following the exposure period, the test system was exposed to Colchicine for 2 h (Sigma Aldrich, Darmstadt, Germany) to arrest the cell cycle in metaphase. The cell cultures were harvested and subjected to hypotonic treatment and fixation on glass slides. Samples on glass slides were stained with 5% Giemsa (Penta, Slovakia, Czech Republic). At least 200 metaphase cells with well-spaced 46 +/−2 centromeres were subjected to microscopic analysis and evaluated for the frequency of chromosome aberrations (i.e., the

percentage of metaphase cells with chromosome aberrations). The criterion for evaluating a result as positive was at least a 2-fold increase in the number of cells with chromosome aberrations compared to the number observed for the negative control. The test was performed at least in duplicate.

2.3.3. In Vitro Comet Assay

OECD TG 489 [39] was followed as the main methodological guideline with modifications as follows: a human relevant cell line derived from human keratinocytes (HaCaT cell line, ATCC, USA) was used as the test system as described previously [40]. Test chemicals dissolved in culture medium (DMEM, Sigma Aldrich, Saint Louis, MO, USA) were applied to the 80% confluent cell culture monolayer at selected non-cytotoxic concentrations (24 h exposure period, 37 °C). Additionally, 1% H_2O_2 in PBS (15 min exposure period, 4 °C) was used as the positive control, and culture medium (DMEM, Sigma Aldrich, USA) was used as the negative control. After treatment, the morphology of the cells was microscopically inspected, and doses of test compounds with observed signs of cytotoxicity were excluded from further steps. TrypLE (Gibco, ThermoFisher Scientific, Waltham, MA, USA) was applied to the cell monolayer (for +/− 10 min) to gently harvest the cells. The cell suspension was centrifuged (6 min, 1000× *g*) and cell suspension samples were prepared by mixing the cell pellets with 85 µL of 1% low melting point (LMP) agarose (ThermoFisher, USA, 37 °C), dropping 85 µL of the LMP agarose cell suspension onto glass slides pre-coated with agarose gel (1% high melting point (HMP) agarose, ThermoFisher, USA) and covering with coverslips. Samples on glass slides with coverslips were solidified at 4 °C, and after removing the coverslips, the samples were lysed in a lysis buffer (1% Triton X-100, Serva, Heidelberg, Germany, 1 h, 4 °C) in glass Coplin Jars. After lysis, the agarose samples were subjected to pre-incubation in alkaline electrophoretic solution (4 °C, 40 min) in the electrophoretic tank (Sub-Cell Model 192 Cell, 2.000 mL). After pre-incubation, alkaline electrophoresis was performed (380 mA, 0.8 V cm^{-1}, 20 min). After electrophoresis, the agarose samples were rinsed twice in a neutralization buffer for 10 min (0.4 M Tris, pH 7.5, 4 °C), stained (SYBR Green I, Invitrogen, Waltham, MA, USA) and subjected to microscopic evaluation (fluorescence microscope with a CCD camera, Olympus, Japan). The test was performed in triplicate, and the mean +/− standard deviation was calculated. At least 100 cells from each stained agarose sample were evaluated using automated software (CometScore 1.5, Sumerduck, VA, USA). The mean values of the amount of DNA in the head, the Olive momentum, and the amount of DNA in the tail of the comets were assessed (ANOVA, Dunnett *t*-tests, IBM SPSS Statistics for Windows, version 23.0 Armonk, NY, USA: IBM Corp.). The mean values of the DNA in the tail of the comets were used for interpretation. The criterion of a p-value < 0.05 was used as the threshold for statistically significant results. Provided that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if: (a) at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control, (b) the increase is dose-related when evaluated with an appropriate trend test, (c) any of the results are outside the distribution of the historical negative control data for a given species, vehicle, route, tissue and number of administrations. When all of these criteria are met, the test chemical is then considered able to induce DNA strand breakage in the tissues studied in this test system. If only one or two of these criteria are satisfied (i.e., in the case the response is neither clearly negative nor clearly positive, and not all the criteria listed above are met), and in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations should be conducted, if scientifically justified [39].

2.3.4. XenoScreen® YES/YAS Assay

Yeast Estrogen Screen/Yeast Androgen Screen (Xenometrix AG, Allschwil, Switzerland) is a yeast-based reporter gene assay designed for screening chemical compounds for estrogenic and androgenic agonistic/antagonistic activities, indicating endocrine disruption

potential. The assay was performed in duplicate according to the manufacturer's standard operating procedure [41], using supplied standardized chemicals. The positive controls used were: 17β-estradiol (10^{-8} M) in the agonistic (estrogenic) assay; 4-hydroxytamoxifen (10^{-6} M) in the antagonistic (anti-estrogenic) assay; 5α-dihydrotestosterone (10^{-6} M) in the agonistic (androgenic) assay; and flutamide (10^{-8} M) in the antagonistic (antiandrogenic) assay. Pre-cultured cell suspensions of two recombinant *Saccharomyces cerevisiae* strains expressing human estrogen (hERα) and androgen (hAR) receptors were prepared according to the manufacturer's instructions, and were exposed to the test samples for 48 h on an orbital shaker (at 31 °C). The optical density (OD) of the red product resulting from the conversion of the yellow substrate after secretion of β-galactosidase was measured at 570 nm on BioTek Eon High Performance Microplate Spectrophotometer (BioTek, Agilent Technologies, Santa Clara, CA, USA). The OD570 of the end product, compared with the controls, directly correlates with the endocrine activity of the test samples. An increase of ≥10% in the absorbance value of the sample compared to that of the vehicle control (i.e., non-treated growth culture medium) was considered a potentially positive response in the agonistic assays (this was a precautionary and conservative evaluation). A decrease of ≥20% in the absorbance value compared to that of the negative control was considered a potentially positive response in the antagonistic assays, assuming a general biological variability of 20%.

3. Results

3.1. Bacterial Reverse Mutation Test (Ames MPF™ Test)

The Ames MPF™ Test was performed on four *S. typhimurium* strains, TA98, TA100, TA1535, TA1537, with and without metabolic activation (S9+/S9−) at three concentrations pre-screened for cytotoxicity and presumed to be non-toxic for the test system. The viable growing culture was visually inspected for turbidity/bacterial colonies in the wells, as recommended in the supplied SOP. However, TCS showed high cytotoxicity in all *S. typhimurium* strains without metabolic activation (S9−). Weak spontaneous mutations under 1% (SM < 1%) in strains TA98 and TA1537 with metabolic activation (S9+) were observed for TCS. Neither TCS nor TCC exhibited a clear concentration-dependent response in the mean number of revertant wells, nor did they show multiple positive responses with a fold increase (FI, fold inductions in revertant numbers over the baseline) ≥ 2 compared to the value of the baseline. At a concentration of 0.5 µg/mL, TCS tested with the *S. typhimurium* strain TA 1537 S9+ (*) showed an outlier value of FI ≥ 2, and this result has been concluded as unclear after being subjected to careful inspection and further evaluation. Fold inductions in revertant numbers over the baseline (FI) are generally not considered positive if they are less than 2.0. Below this FI value, the data are considered unreliable with respect to determining mutagenicity. A compound that shows a clear dose response and/or yields multiple FIs greater than 2.0 is classified as a mutagen. The result was not confirmed by further evaluation, as another observed FI value was ≤2 (0.5). The positive and negative control values and the values of the baseline were within the acceptable limits for an experiment to be considered valid. The mean value of the positive control for the *S. typhimurium* strain TA98 without metabolic activation (S9−) should be 40 at minimum. Thus, the value of 39.67 (**) observed in the TA98 strain was carefully inspected. As two out of three individual replicate values were ≥40, one replicate was considered as a borderline outlier (37), and the response of the positive control was accepted as being valid after an expert judgement and repeated testing within acceptable limits. None of the tested compounds were evaluated as being mutagenic in the Ames MPF™ Test (Table 1).

3.2. In Vitro Mammalian Chromosome Aberration Test (CA)

In the in vitro mammalian chromosome aberration test, both chemicals, TCS and TCC, were clearly positive at the highest tested pre-screened non-cytotoxic concentration (10 µg/mL) after treatment for 4 h (see Table 2). Prolonged exposure (26 h, without S9)

at higher concentrations (5, 10 µg/mL) resulted in higher cytotoxicity, therefore, the data could not be evaluated as positive (NE = not evaluated).

Table 1. Results of the Ames MPF™ Test (Xenometrix, Switzerland). *S. typhimurium* strains TA98, TA100, TA1535, TA1537 were tested with and without metabolic activation (S9+/S9−). Mean numbers of revertant wells from triplicates are presented for the tested concentrations of TCS (0.5–1.0–2 µg/mL) and TCC (2.5–5.0–10 µg/mL), negative control (NC, culture growth media) and positive control chemicals (PC) specific for each *S. typhimurium* strain with or without S9. A fold increase (FI, inductions in the number of revertant wells) greater than 2.0 at multiple concentrations is required for a result to be evaluated as being positive for mutagenicity. The mean numbers of revertant colonies of the positive control chemicals (PC) are proposed in the supplied SOP to have a threshold of >3-fold as the baseline for TA98, TA1535 and TA1537, and >2-fold as the baseline for TA100. The presented data were generated in two separate runs, in triplicate.

Triclosan								
***S. typhimurium* strain**	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**	
S9 −/+	**S9−**	**S9+**	**S9−**	**S9+**	**S9−**	**S9+**	**S9−**	**S9+**
0.5 µg/mL	0.00	4.33	0.00	3.33	0.00	0.33	0.00	2.00
FI	0.00	0.84	0.00	0.48	0.00	0.33	0.00	2.00 *
1.0 µg/mL	0.00	6.00	0.00	2.00	0.00	0.00	0.00	1.33
FI	0.00	1.16	0.00	0.29	0.00	0.00	0.00	1.33
2.0 µg/mL	0.00	6.67	0.00	0.00	0.00	0.67	0.00	0.33
FI	0.00	1.29	0.00	0.00	0.00	0.67	0.00	0.33
NC	2.67	2.67	4.00	4.00	0.33	0.33	1.33	0.33
Baseline	5.18	5.18	7.00	7.00	1.00	1.00	2.86	1.00
PC	39.67 **	48.00	48.00	48.00	48.00	48.00	48.00	44.00
FI	7.65	9.26	6.86	6.86	48.00	48.00	16.78	44.00
Triclocarban								
***S. typhimurium* strain**	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**	
S9 −/+	S9−	S9+	S9−	S9+	S9−	S9+	S9−	S9+
2.5 µg/mL	0.00	4.00	1.67	5.33	0.00	0.67	2.67	2.33
FI	0.00	0.64	0.19	0.91	0.00	0.14	0.89	1.17
5.0 µg/mL	2.33	4.33	3.33	4.67	0.67	1.00	2.33	1.33
FI	0.39	0.69	0.37	0.80	0.33	0.21	0.78	0.67
10.0 µg/mL	3.33	4.33	2	5.67	0.00	0.67	1.00	1.67
FI	0.56	0.69	0.22	0.97	0.00	0.14	0.33	0.83
NC	3.67	5.67	9.00	3.33	1.00	3.00	3.00	2.00
Baseline	5.98	6.24	9.00	5.85	2.00	4.73	3.00	2.00
PC	41.00	40.67	47.33	42.67	44.33	39.67	45.67	37.67
FI	6.86	6.51	5.26	7.29	22.17	8.38	15.22	18.83

3.3. In Vitro Comet Assay

In the in vitro Comet assay, the percentage of DNA in the tail (mean % of DNA in the tail ± SD) significantly increased at the highest tested non-cytotoxic concentrations of both tested preservatives, TCS and TCC, and a clear concentration-dependent positive response has been observed (see Table 3). The results (from three independent measurements) were considered statistically significant when the p-value was <0.05 (*).

Table 2. In vitro mammalian chromosome aberration test (% aberrant cells, 4 h +/− S9, 26 h −S9, NE—non-evaluated).

Samples/ Controls	Concentration µg/mL	% Abberant Cells			Evaluation
		4 h + S9	4 h − S9	26 h − S9	
Triclosan	2.5	5	6	4	negative
	5	4	5	NE	negative
	10	21	10	NE	positive
Triclocarban	2.5	4	3	3	negative
	5	7	7	NE	borderline
	10	12	8	NE	positive
Negative control 1 (culture medium)		4	3	4	negative
Positive control 1 (Thio-TEPA, 10^{-6} M)			9	10	positive
Negative control 2 (+S9)		3			negative
Positive control 2 (cyclophosphamide 10^{-4} M)		10			positive

Table 3. In vitro Comet assay, HaCaT cell line. % DNA in tail (mean ± SD values). (*) statistically significant result with $p < 0.05$.

Samples/ Controls	Concentration µg/mL	% DNA in Tail Mean ± SD	*p*-Value
Triclosan	2.5	3.75 ± 0.34	**0.060**
	5	13.87 ± 1.51 *	**<0.0001**
	10	15.99 ± 1.64 *	**<0.0001**
Triclocarban	2.5	6.08 ± 0.20 *	**0.006**
	5	7.86 ± 0.98 *	**0.001**
	10	11.92 ± 2.28 *	**0.0005**
Negative control (culture medium)		0.63 ± 0.07	-
Positive control (1% H_2O_2)		91.48 ± 0.75	-

3.4. *XenoScreen® YES/YAS Assay*

TCC has not been assessed as being positive for any endocrine activity in any of the XenoScreen® assays included in the study and was not highly cytotoxic. As depicted in Figures 3 and 4, TCS exhibited a strong concentration-dependent positive response in both the anti-estrogenic assay and the anti-androgenic assay (where it demonstrated even greater potency than the positive control, Flutamide). Fungicidal activity against *S. cerevisiae* was carefully monitored during the study through repeated turbidity measurements of the culture at OD690. TCS has demonstrated significant cytotoxic (fungicidal) effects compared to the positive control and the negative (vehicle) control, as well as TCC (which showed only mild cytotoxicity in *S. cerevisiae* strain hERα within acceptable limits). Only five non-cytotoxic concentrations of TCS were deemed to be acceptable for evaluating endocrine-disrupting activity in the transfected *S. cerevisiae* strain hAR with the incorporated human androgen receptor (hAR) (i.e., 3.16×10^{-8}–3.16×10^{-6} M). Additionally, only six non-cytotoxic concentrations of TCS were evaluated in the transfected *S. cerevisiae* strain with the incorporated human estrogen receptor α (hERα) (3.16×10^{-8}–1.06×10^{-5} M).

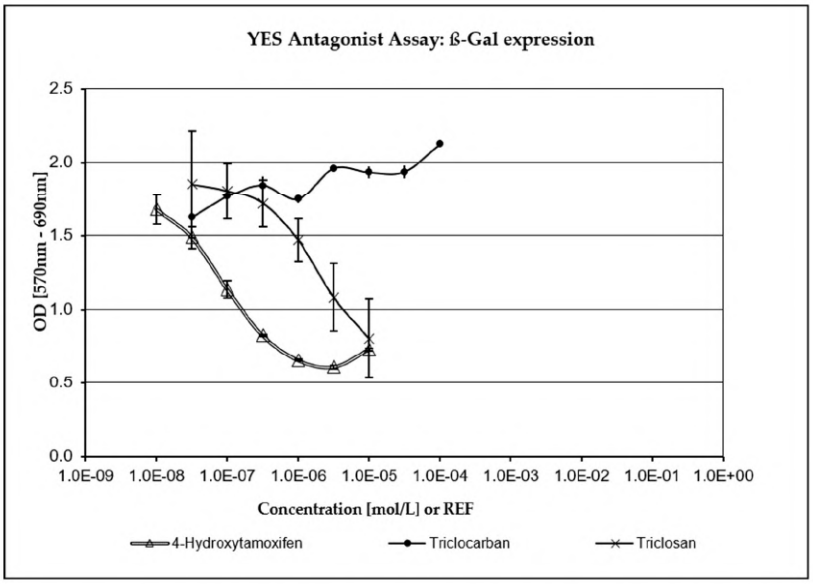

Figure 3. Anti-estrogenic activity. TCS exhibited a strong concentration-dependent response compared to the positive control (4-Hydroxytamoxifen).

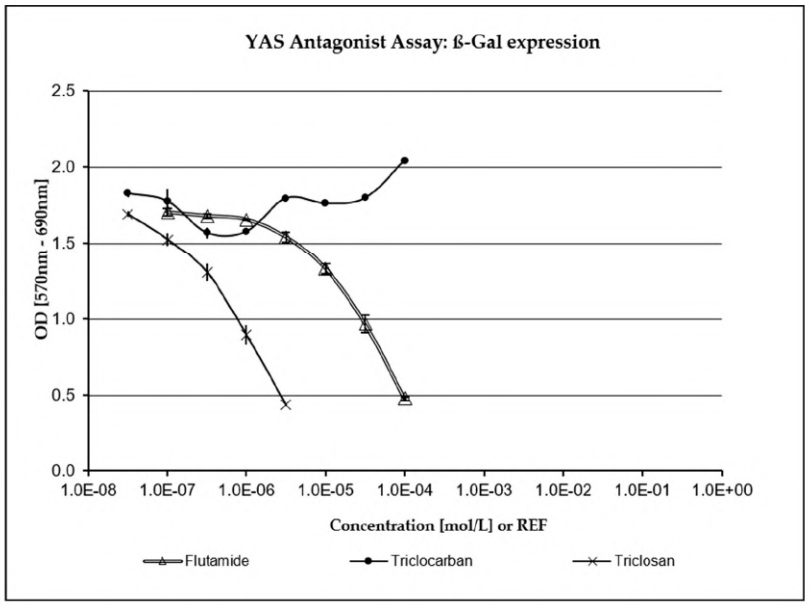

Figure 4. Anti-androgenic activity. TCS exhibited a strong concentration-dependent response compared to the positive control (Flutamide).

4. Discussion

In publicly available registration dossiers [42,43], in the opinions of the Scientific Committee for Consumer Products [44] and in recent scientific literature [45], both TCS and TCC have consistently produced negative results in the Ames test performed according to OECD TG 471 [36]. These findings align with the results of our study, utilizing the modified microplate Ames MPF™ test. In our study, a higher cytotoxicity was observed in all *S. typhimurium* strains tested without metabolic activation (S9−) for TCS compared to TCC. It is likely that the antimicrobial activity of TCS was inhibited by enzymes contained in the liver homogenate (S9 fraction), or that TCS was converted into metabolites with lower cytotoxicity. This underscores the justification of including S9 in toxicological methods in vitro to ensure higher human relevance regarding the metabolic conversion of xenobiotics in the liver and the toxicokinetics of the organism, considering its absorption, distribution, metabolism and excretion in vivo. We suggest carefully considering cytotoxicity data in any in vitro assay, as false positive/negative results may be obtained, especially when testing highly cytotoxic antimicrobials. We argue that testing antimicrobials using the Ames test as a stand-alone in vitro assay cannot provide reliable information on the mutagenic potency of the test substance in mammals (eukaryotes). Therefore, the obtained results should be confirmed in other biological testing more relevant to humans and less sensitive to antimicrobial activity. However, neither TCS nor TCC showed any potential for mutagenicity in the modified microplate Ames MPF™ test. This aligns with previous results from recent studies on the classical plate-format Ames test, where treatment with 10 µg/plate TCS also produced inhibitory effects, while 1 and 0.1 µg/plate TCS did not markedly affect the number of colonies or frequency of mutants in *S. typhimurium* strains [46]. Historical data from the classical Ames test, which used *S. typhimurium* and the yeast *S. cerevisiae*, are included in the registration dossier for TCS in the ECHA database, and they consistently generated negative results [42].

For TCC, the data on genotoxicity are scarce, however, recent studies have consistently produced negative results, and TCC was evaluated as being non-genotoxic, as supported by data available in the registration dossier of the ECHA database [2,43,45].

In the mammalian chromosome aberration test, both TCS and TCC tested positive at the highest non-cytotoxic concentration in our study (Table 2). Prolonged exposure (26 h, without S9) resulted in higher cytotoxicity, therefore, the data could not be accepted for evaluation. Although the majority of the scarce results available in the ECHA registration dossiers classified both substances as non-genotoxic, a concentration-related increase in chromosomal aberrations in Chinese Hamster V79 cells exposed to the highest concentration of TCS (3.0 µg/mL) at the 18 h and 28 h fixation intervals without S9 was observed [47]. This observation aligns with our positive results generated on a mammalian-relevant test system (human peripheral lymphocytes). The relatively limited number of studies available in the ECHA database or in the SCCS reports provided rather sparse data on chromosome aberrations induced by TCS, which tested negative in assays with CHO cells or in the micronucleus test using mice (bone marrow) [44,48]. A significant ($p \leq 0.05$) concentration-dependent increase in aberrant cells was observed after *Labeo rohita* hatchlings were exposed to TCS for 96 h (supported by a concentration-dependent increase in necrotic, apoptotic and micronucleated cells) [49]. Nonactivated TCS was found to induce a dose-related increase in the yield of cells with abnormal chromosome morphology in the in vitro mammalian chromosomal aberration test with dose levels ranging from 1 to 3 µg/mL (18 h harvest) and at 3 µg/mL (28 h harvest). The most frequently observed type of chromosome damage was exchange figures. However, no signs of structural chromosomal aberrations were observed in the in vivo bone marrow chromosomal aberration test [50].

In the case of TCC, data on induced chromosomal aberrations are even more scarce. As concluded in the "Screening Assessment of Urea, N-(4-chlorophenyl)-N′-(3,4-dichlorophenyl) (Triclocarban)" published by the Government of Canada in March 2023 [51], TCC tested negative in an in vitro chromosome aberration test in Chinese hamster ovary cells, both with and without metabolic activation, at concentrations up to 2000 µg/mL [52]. In Tox21

assays, TCC was identified as genotoxic in cell lines deficient in DNA repair pathways [53]. The limited data available have been evaluated by the Scientific Committee on Consumer Safety, concluding that TCC was not found to be clastogenic in the chromosomal aberration test with and without metabolic activation [2].

In the Comet assay performed on the HaCaT cell line, the percentage of DNA in the tail was statistically significantly increased at the two highest tested concentrations upon exposure to TCS (5, 10 µg/mL) and at all three tested concentrations upon exposure to TCC (2.5, 5, 10 µg/mL). Such results are supported, for example, by a study using aquatic larvae of the insect *Chironomus riparius*, where TCS was found to have genotoxic activity as it significantly increased all the comet parameters (% DNA in tail, tail length, tail moment, Olive tail moment) at all tested concentrations [54], or in a study of the binary combination of TCS and carbendazim tested on *Daphnia magna* [55]. Both TCS and TCC caused concentration-dependent DNA damage to the protozoan *Tetrahymena thermophila* in the alkaline Comet assay, with TCC inducing more severe DNA damage than TCS [56]. However, another study performed on HaCaT and L02 cells treated with TCC provided negative results [46].

As stated in the OECD TG 489, positive findings in the Comet assay may not be solely associated with genotoxicity. Target tissue toxicity may also result in increased DNA migration. Low or moderate cytotoxicity is often observed, making it challenging to distinguish DNA migration induced by genotoxicity versus DNA migration induced by cytotoxicity in the Comet assay alone. However, when increases in DNA migration are observed, performing an examination of one or more indicators of cytotoxicity is recommended, as this can aid in interpreting the findings. Increases in DNA migration in the presence of clear evidence of cytotoxicity should be interpreted with caution [39,57,58].

The growing evidence for the genotoxic effects of TCS and TCC on aquatic organisms and fish [59–62] supports warnings about TCS and TCC and raises efforts to clarify the mechanisms by which the effects of these polychlorinated preservatives occur in more detail, including oxidative damage in specific cells and tissues [63–67], potential synergistic effects with other substances present in final cosmetic products or the environment [55,68–70] and evaluations of the safety of their active metabolites, products of biodegradation or photolysis, considering their widespread use in consumer products and their presence in the environment [1,71]. Numerous inconclusive data and variability in test systems support the need to employ test systems highly relevant to humans, i.e., human-derived cell lines or 3D reconstructed human tissue models should be preferably used for safety assessment in vitro.

The potential for endocrine disruption elicited by TCS and TCC was examined in this study using the commercially available in vitro test, XenoScreen YES/YAS (Xenometrix, Switzerland). Severe cytotoxicity and the highly positive response of TCS were observed at five low, non-cytotoxic concentrations in both antagonistic assays conducted (anti-estrogenic assay, anti-androgenic assay). This was compared to the relevant positive controls (4-Hydroxytamoxifen in the anti-estrogenic assay, Flutamide in the anti-androgenic assay) and the vehicle control (culture medium providing a negative response). Cytotoxicity data were considered, and only non-cytotoxic concentrations were selected for the evaluation of endocrine activity. TCC did not show any conclusive positivity or cytotoxicity in the XenoScreen YES/YAS assay. The anti-estrogenic and anti-androgenic behavior of TCS was also confirmed in another recent study utilizing XenoScreen YES/YAS assay [72]. It was reported that TCC exhibited agonist activity on the androgen receptor and estrogen receptor alpha, and antagonist activity on glucocorticoid and the thyroid receptor. TCS showed antagonist effects on the androgen receptor, estrogen receptor alpha, glucocorticoid and the thyroid receptor [73].

Recent public concerns regarding the potential toxicological effects and environmental accumulation of TCS and TCC have led to efforts to find efficient replacements for these antimicrobials [1], such as pentafluorosulfanyl-containing Triclocarban analogs [74] or benzalkonium chloride, benzethonium chloride and chloroxylenol. However, these substances

have not been tested as extensively as TCS and TCC and may, therefore, pose a greater risk to humans or the environment [75]. Further investigation is required to understand their mechanisms of action and ecological significance before their introduction into widespread use in consumer products can be conclusively justified.

5. Conclusions

The potential for mutagenicity, genotoxicity and endocrine disruption in Triclosan and Triclocarban was assessed using NAMs, representing the most suitable non-animal in vitro approach for testing cosmetic ingredients in the EU. The results suggest that both chemicals may exhibit a potential for genotoxicity, depending on the specific in vitro method used, highlighting the advantageous use of NAMs in combination. Triclosan demonstrated some potential for endocrine disruption, specifically anti-estrogenic and anti-androgenic effects. Our study aimed to enhance the toxicological profiles of both preservatives, as there was a lack of in vitro data generated using recently implemented NAMs of higher human relevance, validated or sufficiently standardized for testing cosmetic ingredients. We believe that, in response to the recent European Commission call for data, the provided results could contribute to discussions and the amendment of regulatory measures aimed at enhancing the safety of cosmetic ingredients within consumer products. Our study seeks to illustrate that NAMs based on human-relevant cell lines or transfected microorganisms can yield valuable results when employed in combination. The broader implementation of human-relevant, validated and standardized NAMs should be encouraged, considering their greater acceptance in human risk assessment for regulatory purposes.

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Příloha č. 3

The Safety Assessment of Cosmetic Perfumes by Using *In Chemico* and *In vitro* Methods in Combination with GC-MS/MS Analysis

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The Safety Assessment of Cosmetic Perfumes by Using *In Chemico* and *In Vitro* Methods in Combination with GC-MS/MS Analysis

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Abstract

Animal testing has been prohibited for the safety assessment of cosmetic ingredients or finished products. Thus, alternative non-animal methods, followed by confirmatory clinical studies on human volunteers, should be used as the sole legally acceptable approach within the EU. The safety assessment of cosmetic products requires the involvement of multiple scientific disciplines, including analytical chemistry and biomedicine, as well as *in chemico*, *in vitro* and *in silico* toxicology. Recent data suggest that fragrance components may exert multiple adverse biological effects, e.g. cytotoxicity, skin sensitisation, (photo)genotoxicity, mutagenicity, reprotoxicity and endocrine disruption. Therefore, a pilot study was conducted with selected samples of fragrance-based products, such as deodorant, eau de toilette and eau de parfum, with the aim of integrating results from a number of alternative non-animal methods suitable for the detection of the following toxicological endpoints: cytotoxicity (with 3T3 Balb/c fibroblasts); skin sensitisation potential (*in chemico* method, DPRA); skin sensitisation potential (LuSens *in vitro* method, based on human keratinocytes); genotoxicity potential (*in vitro* Comet assay with 3T3 Balb/c cells); and endocrine disruption (*in vitro* YES/YAS assay). The presence of twenty-four specific known allergens in the products was determined by using GC-MS/MS. The strategies for estimation of the NOAEL of a mixture of allergens, which were proposed by the Scientific Committee on Consumer Products in their 'Opinion on Tea tree oil' document and by the Norwegian Food Safety Authority in their 'Risk Profile of Tea tree oil' report, were used as models for the NOAEL estimation of the mixtures of allergens that were identified in the individual samples tested in this study.

Keywords

3Rs, allergens, alternative methods to animal testing, cosmetics, cytotoxicity, endocrine disruption, genotoxicity, *in vitro* methods, mixtures, perfumes, sensitisers, skin sensitisation, Three Rs

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The key abbreviations used throughout this paper are listed in the *Appendix*.

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Introduction

Animal testing of cosmetic ingredients and finished cosmetic products has been banned in the EU for all toxicological endpoints since 2013. Regulation *EC No 1223/200* on cosmetic products (Cosmetic Regulation) provides a list of ingredients banned for use in cosmetics (Annex II) or approved/authorised only for limited use (Annex III, IV, V, VI).[1] A list of cosmetic ingredients with sufficiently described toxicological properties is not available; thus, in accordance with the Cosmetic Regulation, persons responsible for the marketing of a cosmetic product in the EU are allowed to use a specific ingredient, provided that a safety assessment procedure (Cosmetic Product Safety Report) was completed according to Annex I, and that this can be made available for control by the authorities upon request.

The safety assessments of most regulated ingredients have already been performed on the basis of available toxicological data. With regard to newly considered toxicological properties, data for many ingredients remain incomplete, especially for systemic immunotoxicological or neuroendocrine effects. In general, finished cosmetic products represent mixtures of numerous ingredients. The subject of a safety assessment is the presence and toxicological properties of each individual ingredient. Certain individual cosmetic ingredients, however, may also be composed of numerous individual components — particularly those derived from, for example, natural and biological extracts or biological fluids. The toxicological properties of any combined mixture, such as a finished cosmetic product, depend not only on the toxicological properties of the individual components, but also on their combined interactions — even when individual components are present at what are considered to be low and safe concentrations. Considering these facts, a reasonable approach for predicting the biological activity of a finished cosmetic product by means of sensitive *in chemico* and *in vitro* methods is suggested. Depending on bioavailability, type and frequency of exposure, the biological effects that are predicted *in vitro* may also indicate the potential for biological effects *in vivo*, and the *in vitro* potential to predict relevant adverse reactions *in vivo* should not be underestimated.

Numerous factors — such as the nature of production, biological variability, non-standardised quality, stability or diverse origin of mainly natural components (e.g. natural extracts, plant ingredients, essential oils, raw materials, biological fluids, etc.) — may influence the biological effects of a finished cosmetic product. For example, natural products may show batch-to-batch variability, depending on the season, origin of the plants, etc. The use of indirect distribution channels may unfortunately allow for low-quality products to enter the retail market and reach final consumers, which is often the case for cosmetic perfumes. Low-quality products have been shown to exhibit variable composition, leading to diverse toxicological properties.[2–4]

Cosmetic perfumes represent chemical mixtures containing components of both synthetic and natural origin. Detailed information on many chemical substances, including their No Observed Adverse Effect Level (NOAEL) values, are publicly available in Registration Dossiers on the website of the European Chemicals Agency (ECHA). The list of cosmetic ingredients is given in the cosmetic nomenclature according to the INCI (i.e. International Nomenclature of Cosmetic Ingredients), which can be searched by name or number in the European CosIng database (Cosmetic Ingredient Database, https://ec.europa.eu/growth/sectors/cosmetics/cosing_en).

In the final product, the components are sorted in descending order, according to their percentage distribution in the final volume (e.g. weight/weight or volume/volume, according to the type of solid or liquid cosmetic product, respectively). Quality ingredients at safe concentrations are prerequisites for a safe final cosmetic product. Water and ethanol (EtOH) are the most common polar solvents used in cosmetic perfumes. The toxicological properties of EtOH have been repeatedly characterised, including, for example, its skin irritation potential (*in vivo* skin irritation study in rabbits, Organisation for Economic Co-operation and Development (OECD) Guideline 404: Acute dermal irritation/corrosion[5]) and the NOAEL for repeated dose toxicity (*in vivo* study in rats, oral route, 90-day study[6]).

Most of the toxicological data on chemical substances have been generated historically with *in vivo* models and with studies not fully relevant to humans, especially with regard to toxicokinetics and toxicodynamics, metabolism, systemic toxicity, etc. Certain chemicals remain insufficiently characterised, making the provision of additional information via data-generation with standardised methods highly valuable to the safety assessors.

One very important part of a cosmetic formulation, which may be potentially hazardous from the toxicological point of view, is the fragrance mix (fragrance formulation), which is usually composed of complex aromatic or phenolic-ring compounds, synthetic scents, musks, essential oils, solvents and fixatives.[7] Depending on the percentage concentration of the fragrance in the final formulation of a perfume product, there is a distinction between 'eau de parfum' (EDP; 10–15% fragrance), 'eau de toilette' (EDT; 5–10% fragrance) and 'eau de cologne' (EDC; 3–5% fragrance). Due to the variable composition of cosmetic perfumes, health problems such as skin irritation, skin sensitisation, headaches or asthma attacks have been reported, predominantly in consumers with sensitive skin or with a predisposition to skin or respiratory allergies.[8–11]

Current consumer trends indicate a preference toward the use of naturally derived ingredients (usually labelled as 'bio', 'organic' or 'natural'). These ingredients may,

however, be associated with certain impurities which can lead to potentially significant adverse effects. On the other hand, synthetic fragrances may also include substances of toxicological concern, for which new toxicological properties are gradually identified on the basis of scientific progress, e.g. endocrine activity, cytotoxicity, skin sensitisation or mutagenic potential.[9–12] Since animal testing is prohibited for cosmetic testing, and testing of chemical substances of toxicological concern in human volunteers (particularly for systemic toxicity endpoints) is ethically unacceptable, the use of *in vitro* toxicological methods, ideally based on human relevant cells and tissues, remains a suitable approach to assess the hazard and risks associated with the aromatic compounds present in cosmetic perfumes and other cosmetic products.

A number of *in vitro* methods have been validated in the last decade to reduce and replace animal testing for regulatory purposes. Although primarily validated for the classification and labelling of chemicals or chemically well-defined mixtures, the applicability of an *in vitro* method for the testing of even less defined mixtures should not be explicitly ruled out, unless there is specific evidence that the method is not sufficiently predictive. As there is no other option for testing finished cosmetic products and their ingredients (in general, considered as chemicals), the use of *in vitro* toxicological methods, especially based on cells and tissues of human origin, combined in specific batteries with sufficient robustness and predictive power, has become inevitable for the prediction of toxicological endpoints and potential adverse biological effects, prior to confirmatory assessment of their absence in human volunteers.

The current paper describes a pilot study, which was conducted with selected fragranced-based cosmetic products, with the aim of demonstrating that the integration of results from specific advanced *in vitro* toxicological methods could be used in the safety assessment of finished cosmetic products. This combined approach could potentially become a powerful tool for use in advanced human-relevant toxicological assessment. The following toxicological endpoints and methods were used: cytotoxicity (with 3T3 Balb/c fibroblasts); skin sensitisation potential (*in chemico* method, DPRA); skin sensitisation potential (LuSens *in vitro* method, based on human keratinocytes); genotoxicity potential (*in vitro* Comet assay, with 3T3 Balb/c cells); and endocrine disruption (*in vitro* YES/YAS assay). The presence of twenty-four specific known allergens in the products was determined by using gas chromatography-tandem mass spectrometry (GC-MS/MS).

Materials and methods

Samples

Test samples were taken from ten products representing different types of cosmetic perfume, which were purchased in the EU market before 2018 (Table 1). These comprised: EDP (five samples); parfum (two samples); EDT (one sample); parfum deodorant (one sample); and EDC (one sample). Samples 1–6 were purchased through the regular retail network; Samples 7 and 8 were purchased through online sales of non-branded products; Sample 9 was purchased through direct catalogue sales; and Sample 10 was purchased through online sale of an original brand line distributed also by wholesale channels. The countries of product origin were both EU and non-EU based (see Table 1).

The 3T3 NRU cytotoxicity assay

The assessment of cytotoxicity was performed according to DB-ALM Protocol No. 3: The FRAME Modified Neutral Red Uptake Cytotoxicity Test.[13] In brief, Balb/c 3T3 cells (L1, ECACC No. 86052701) in suspension at a concentration of 10^5 cells/ml were seeded in 96-well plates (100 µl/well) (TPP, Switzerland) and pre-incubated for 24 hours (37°C, 5% v/v CO_2) until not more than 90% confluent. The

Table 1. The list of samples tested in this study.

Sample	Type of product	Seller	Country/region of origin
1	EDP	Retail	EU
2	EDP	Retail	UAE
3	EDP	Retail	China
4	EDP	Retail	EU
5	Parfum deodorant	Retail	EU
6	EDP	Retail	Saudi Arabia
7	Parfum	Online/direct sale	EU
8	Parfum	Online/direct sale	EU
9	EDT	Online/direct sale	EU
10	EDC	Online/wholesale	EU

EDP = 10–15% fragrance eau de parfum; EDT = 5–10% fragrance eau de toilette; EDC = 3–5% fragrance eau de cologne.
Note: All products were intended for topical exposure.

cells were treated with the test samples (200 µl of sample per well), which had been diluted in culture medium without Newborn Calf Serum (NCS; Gibco, ThermoFisher, Cat. No. 16010159), in four replicates, at five test concentrations (100, 250, 500, 1000 and 2500 µg/ml). After 24 hours of exposure, the viability of the cells was evaluated microscopically. Afterwards, the samples were aspirated and Neutral Red dye (NR; Sigma-Aldrich, St Louis, MO, USA, Cat. No. 861251) was dissolved in culture medium without NCS and added to the wells (200 µl/well, 0.05 mg/ml). After a 3-hour incubation at 37°C, the NR solution was removed and the cells were gently washed with PBS (100 µl per well). A fixative solution (EtOH/acetic acid/water) was added (200 µl/well) and the NR was extracted from the lysosomes by 10 minutes of gentle shaking. The extracted NR dye, taken up by the viable cells during incubation, was measured with a fluorescence-luminescence reader (FLX800TBI; BioTek, Winooski, VT, USA) at 530/590 nm. Cytotoxicity (i.e. reduction of viability) was expressed as a concentration-dependent reduction of NR uptake, expressed as relative fluorescence units (RFUs), compared to the vehicle-treated controls (representing 100% viability). Test samples that resulted in RFU values lower than 70% of the value exhibited in the vehicle-treated control were considered to be cytotoxic.

The two test methods used for the detection of skin sensitisation

Direct peptide reactivity assay (DPRA): Reactivity of the test samples with a model cysteine-containing heptapeptide (Ac-RFAACAACOOH)[14] was used to predict their skin sensitising potential, according to OECD Test Guideline (TG) 442C,[15–17] optimised for mixture samples. The peptides were prepared and purified by Centic Biotec (Heidelberg, Germany) to > 98.56% purity.

Two versions of a volumetric approach were used to test each of the samples: In the first version, 50 µl of sample were diluted in 200 µl of acetonitrile, resulting in a final volume of 250 µl (i.e. sample concentration = 20%). Then, a 250 µl aliquot of this 20% sample was mixed with 750 µl of a 0.667 mM peptide stock solution prepared in phosphate buffered saline pH 7.5 (PBS), resulting in further 4-fold dilution in the reaction solution (final total volume = 1 ml). Thus, the final test sample concentration in the reaction was 5% (i.e. 50 µl of the original sample in 1 ml total reaction volume).

In the second version, 250 µl of undiluted sample were directly mixed with 750 µl of a 0.667 mM peptide stock solution prepared in PBS, resulting in a final test sample concentration of 25% in the reaction (i.e. 250 µl of the original sample in 1 ml total reaction volume).

Acetonitrile (ACN) (Hypergrade for LC-MS; Merck, Germany) and EtOH (Gradient Grade for liquid chromatography; Merck) were used as the vehicle controls (VCs). Cinnamic aldehyde (CAS 104-55-2; Sigma-Aldrich) was used as the positive control (PC) at a concentration of 100 mM in ACN. Peptide reactivity was reported as peptide depletion, based on the percentage decrease in the concentration of non-reacted peptide in the sample reaction solution, relative to the average non-reacted peptide concentration measured in the vehicle controls (ACN, EtOH). Briefly, 750 µl of a 0.667 mM peptide stock solution prepared in PBS and 250 µl of sample dissolved in acetonitrile were mixed. The final reaction, containing 0.5 mM of the peptide and 50 or 250 µl of the test sample at final test concentrations of 50 or 250 µl/ml (i.e. 5% and 25%, respectively), was mixed and incubated in the dark for 24 hours at 25°C. Control samples and standards used for defining the calibration curve for each analysis were prepared, and ranged from 0 to 0.534 mM.

According to DB-ALM Protocol No. 154: Direct Peptide Reactivity Assay (DPRA) for Skin Sensitisation Testing,[18] the Limit of Detection (LOD) of both peptides is 0.0167 mM, corresponding to a depletion value of 3.0%. Values lower than 3.0%, and negative values up to –10.0% depletion of the peptide, are considered as 'zero' and should be used as such when calculating the mean. However, if the percentage peptide depletion is lower than –10.0%, it should be considered that this may be due to co-elution, inaccurate peptide addition to the reaction mixture, or just baseline 'noise'. All samples were prepared in triplicate. Following incubation, the peptide was quantified by reverse-phase HPLC with an external standard linear calibration. The UV spectrum was measured in the range from 210 to 400 nm, to permit verification of the peptide peak identity. A percentage peptide depletion of above 13.8% was designated as a positive result.

LuSens skin sensitisation assay: The LuSens assay was performed according to OECD TG 442D[19–21] with optimisation for a gravimetric approach. A genetically modified human keratinocyte cell line, LuSens, was kindly provided by BASF (Germany). Cells were maintained in T75 flasks with 20 ml of growth medium (Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS Superior, 1% penicillin/streptomycin and 0.0005% puromycin dihydrochloride, all from Merck), at 37°C in a humidified atmosphere with 5% CO_2, to a confluence of 80–90%. Cells were propagated twice a week as follows: Cells were trypsinised, seeded at density of 0.7×10^6 cells in T75 culture flasks containing 20 ml of culture medium, and incubated at 37°C in a humidified atmosphere with 5% CO_2. Cell suspensions from passage 4 to 16 were used in the experiments.

The ten selected test samples were weighed and dissolved in dimethylsulphoxide (DMSO) to prepare stock solutions. The stock solutions were further diluted in the culture medium and tested at six final concentrations (3.1, 6.3, 12.5, 25.0, 50.0 and 100 µg/ml). The following controls were included

in each test run: 120 µM ethylene glycol dimethacrylate (EGDMA, positive control); 5000 µM DL-lactic acid (LA, negative control); and 1% DMSO (vehicle control) (Merck). The cells, at 70% confluency, were exposed to the prepared test samples for 48 hours (37°C, 5% CO_2). Steady-Glo® solution (Promega, East Port Life Science, Prague, Czech Republic) was used, according to the manufacturer's instructions, to quantify the induction of reporter gene expression (*luc*). Luminescence was measured with a GloMax® luminometer (Promega). Simultaneously, the MTT viability assay[22] was performed for the assessment of cytotoxicity induced by the samples, by using a BioTek microplate spectrophotometer.

Test samples were considered to show sensitising potential when the fold-change induction of reporter gene expression (in Relative Light Units (RLUs)) was greater than or equal to 1.5, as compared to the vehicle control. In addition, cell viability had to be greater than or equal to 70%, at all sample concentrations evaluated.

The two test methods used for the detection of genotoxic potential

In vitro *Comet assay*: Genotoxicity leading to DNA damage was tested with NIH 3T3 cells (mouse embryonic fibroblasts; ECACC, UK). In brief, NIH 3T3 cells were incubated in DMEM (Merck) in 75 cm^2 flasks (Greiner Bio-ONE), with the test samples diluted in high-glucose DMEM without fetal bovine serum (FBS) (DMEM with 4500 mg/l glucose, L-glutamine, sodium pyruvate and sodium bicarbonate; Sigma-Aldrich, Cat. No. D6429) to give selected non-cytotoxic concentrations (µg/ml), for 24 hours (37°C, 5% CO_2). The cells were harvested by trypsinisation with TrypLE™ Express Enzyme (1×, no phenol red; Gibco, ThermoFisher Scientific, USA, Cat. No. 12604-021), followed by collection in DMEM + 10% v/v FBS (VWR, USA) and centrifugation at 150*g* to obtain a cell pellet.

After aspiration of the supernatant, the cell pellet was resuspended in 1% low melting point (LMP) agarose (Molecular Biology Grade; Qbiogene, MP Biomedicals, USA) heated to 37°C. The cell/LMP agarose suspension was carefully applied onto microscope slides pre-coated with solidified agarose and covered with coverslips. After solidification, the coverslips were removed and the slides immersed in lysis buffer with 1% Triton™ X-100 (Merck) at 4°C for 60 minutes. In the next step, the slides were placed in an electrophoretic tank (Bio-Rad Sub-Cell Model 192 Cell) and immersed for 40 minutes in cold (4°C) alkaline electrophoretic solution (see manufacturer's instructions). Electrophoresis was performed under the following conditions: 350 mA, 0.8 V/cm, 20 minutes. After electrophoresis, the slides were carefully rinsed twice for 10 minutes with a neutralisation buffer (0.4 M Tris-HCl, pH 7.5) at 4°C, stained with SYBR Green I (Merck), and microscopically evaluated by using a fluorescence microscope with CCD camera (Olympus) and CometScore 1.5 software (Tritek Comet Score Freeware V1 5 Software; TriTek Corp., Sumerduck, VA, USA). The experiment was performed in three runs (using triplicates of samples); 100 cells from each sample were selected for evaluation. Median values for the amount of Olive Tail Moment (OTM), DNA in the head and DNA in the tail (which is directly proportional to the damaged DNA), were calculated and evaluated. Differences in response, due to the various concentrations, were analysed with analysis of variance (ANOVA) and Dunnett's *t*-test. The value of $p < 0.05$ was adopted as the level of statistical significance. All statistical analyses were conducted with IBM SPSS Statistics for Windows (Version 23.0; IBM Corp., Armonk, NY, USA).

In vitro *mammalian chromosome aberration test*: This test identifies the agents that cause structural chromosome and chromatid aberrations in cultured mammalian cells. The test was performed in compliance with the procedure published in OECD TG 473.[23] In brief, peripheral blood lymphocytes from a healthy donor were cultured in RPMI-1640 medium with L-glutamine and $NaHCO_3$ (all from Sigma-Aldrich), supplemented with 10% heat-inactivated bovine serum (Bioveta, Czech Republic) at 37°C, 5% CO_2 (in Falcon 12.5 cm^2 tissue culture flasks). Two versions of the assay were performed in parallel (i.e. with or without metabolic activation stimulation (MAS)), according to the test guideline. In the case of MAS, an exogenous source of stimulation, i.e. S9 fraction (Life Technologies, Czech Republic; Cat No. RTS9-PL), was used. Cell cultures were exposed to the test samples both with and without MAS.

Forty-eight hours after culture initiation, the cells were treated for 4 hours with the test substances and the positive control chemicals (without MAS, thio-TEPA (Sigma-Aldrich); with MAS, cyclophosphamide (Cytoxan, Bristol-Myers Squibb, USA)) and cultured for 24 hours at 37°C, 5% CO_2. Colchicine (Sigma-Aldrich) was then added as a metaphase-arresting substance, and the cells were incubated for a further two hours. The cells were then harvested and processed separately, by using hypotonic treatment, fixation and staining with 5% Giemsa.[24] At least 200 well-spread metaphases with 46 ± 2 centromeres were analysed microscopically, and the percentage of cells with structural chromosome aberrations was calculated and evaluated. The criteria for determining a positive result included a concentration-related increase in the number of cells with chromosome aberrations, and/or at least a 2-fold increase in the percentage of aberrant cells, as compared to the negative control.

A yeast-based reporter gene assay for the detection of endocrine disruption potential

Xenoscreen® YES/YAS (Yeast Estrogen Screen/Yeast Androgen Screen; Xenometrix, Allschwil, Switzerland) is

a yeast-based reporter gene assay that is designed for the screening of chemical compounds for oestrogenic and androgenic agonistic/antagonistic activities (i.e. endocrine disruption potential). The assay was performed according to the manufacturer's standard operating procedure, using the supplied standardised chemicals,[25] with optimisation for a gravimetric approach. In brief, the samples were weighed and diluted in DMSO, to a stock concentration of 10 mg/ml. Liquids were pipetted into a glass tube and weighed, and the DMSO was quickly added according to the weight/volume ratio, resulting in a final stock concentration of 10 mg/ml. The stock solutions were diluted in the reaction solution to a final volume of 200 µl at logarithmic non-cytotoxic concentrations, starting at 100 µg/ml as the highest concentration tested. The (solvent only) negative response control (NC) was 1% DMSO, as it was previously verified in-house that this concentration was not cytotoxic to the test system. The positive controls used were: 17β-oestradiol (10^{-8} M) in the agonistic (oestrogenic) assay; 4-hydroxytamoxifen (10^{-6} M) in the antagonistic (anti-oestrogenic) assay; 5α-dihydrotestosterone (10^{-6} M) in the agonistic (androgenic) assay; and flutamide (10^{-8} M) in the antagonistic (anti-androgenic) assay.

The pre-cultured cell suspensions of two recombinant *Saccharomyces cerevisiae* strains expressing human oestrogen (hERα) and androgen (hAR) receptors were prepared according to the manufacturer's instructions and exposed to the test samples, at the selected non-cytotoxic concentrations of 3, 10, 30 and 100 µg/ml, for 48 hours on an orbital shaker (at 31°C). The optical density (OD) of the red product resulting from the conversion of the yellow substrate after secretion of β-galactosidase was measured at 570 nm on a BioTek Eon High Performance Microplate Spectrophotometer. The OD_{570} of the end product, in comparison with the controls, directly correlates with the endocrine activity of the test samples. An increase of ≥ 10% in the absorbance value of the sample compared to that of the NC was considered to be a potentially positive response in the agonistic assays; this is a precautionary and conservative evaluation. A decrease of ≥ 20% in the absorbance value compared to that of the NC was considered to be a potentially positive response in the antagonistic assays, assuming a general biological variability of 20%.

Chemical analysis with gas chromatography-tandem mass spectrometry (GC-MS/MS)

The presence of twenty-four specific known allergens in the products was determined by using GC-MS/MS. Samples were diluted with respect to the standard composition of the matrix (EtOH/water) and wide range of analyte concentrations. The samples for allergen analysis were diluted in acetone to obtain 40:1 and 4000:1 dilutions (w/w). After the addition of specific internal standards for each of the individual analyte/allergen (1/100 of solution weight), diluted samples were directly analysed. Individual stock solutions, at concentrations in the region of 20,000 µg/g, were prepared in acetonitrile and stored at –20°C. Additional dilutions of the stock solutions, for the preparation of the standard solutions used for the calibration curve, were performed in acetone. The calibration curve for allergen analysis ranged from 0.1 to 15 µg/g. Subsequently, internal standards (1/100 of solution weight) to obtain the final concentration of 0.5 µg/g in solution were added to each calibration level. The GC-MS/MS analyses were performed by using a Thermo Trace 1310 gas chromatograph coupled with Thermo Quantum XLS Ultra triple quad mass detector (Thermo Scientific, USA). Separation was performed on a Rxi-17MS column (20 m × 0.18 mm internal diameter, 0.18 µm film thickness; Restek, USA). Helium (99.999%; Linde, Czech Republic) was used as the carrier gas at a constant column flow of 0.6 ml/min. The injector was operated in split mode (split ratio 20:1; 1 µl) and the injector temperature was kept at 250°C. The oven temperature was programmed from 50°C (held for 1 minute), then ramped at 5°C/min to 158°C, 1°C/min to 163°C, 35°C/min to 290°C, and then held at 290°C for 2 minutes. Transfer line and ion source temperatures were set at 300°C and 200°C, respectively. The mass spectrometer detector (MSD) was set to selected reaction monitoring (SRM) mode. Data acquisition and evaluation were performed by using Xcalibur™ and Tracefinder software (Thermo Scientific).

Estimation of NOAEL values for the finished products

The NOAEL is an important toxicological parameter that indicates the concentration, found by experiment or observation, at which a substance causes no detectable adverse alteration of morphology, functional capacity, growth, development or life span of the target organism under defined conditions of exposure.[26] Conventional toxicological methods, by which NOAEL values have been determined for many years, are based on *in vivo* test systems, i.e. laboratory animals such as mice, rats, rabbits, guinea pigs, etc. Advances in science and biomedicine in the past twenty years have led to the establishment of a broad consensus in toxicology that there is a need to ensure greater relevance of testing systems for humans, especially in view of the complex mechanisms that are involved, e.g. developmental, immunological or neuroendocrine pathways. Data demonstrating the efficacy of potential advanced approaches are increasing — for example, Bauch et al.[27] documented the effective use of human-relevant *in vitro* safety testing methods, combined with *in vivo* data evaluation, to provide relevant toxicological information for the assessment of human health risk. The assessment for tea tree oil (TTO)

may be used as a further example of an advanced toxicological approach.[28,29]

The approaches and templates described in the Scientific Committee on Consumer Products' 'Opinion on Tea tree oil',[28] as well as in the Norwegian Food Safety Authority's 'Risk Profile of Tea tree oil — TTO',[29] were used as models for the in-house pilot NOAEL estimation of the mixture of allergens (both including and excluding EtOH in the estimation) in the current study, representing the aim of estimating a NOAEL value of a finished cosmetic product. The approach for TTO was considered to be a relevant model for the toxicological evaluation of mixtures, as TTO consists of more than 100 constituents (mainly mono-terpenes, sesquiterpenes and their alcohols). In accordance with the approach for TTO, the default skin penetration rate of 100%, in order to correlate as much as possible to 100% oral bioavailability, was assumed as the most conservative and precautionary (see the recommendation in the Norwegian Food Safety Authority's 'Risk Profile of Tea tree oil — TTO'[29]). However, the maximum use levels for leave-on products and the impact of chemical volatility on skin absorption after finite dose application had also to be considered. In our estimation, the detected percentage concentrations of individual allergens were further normalised (i.e. divided by 100), in order to represent realistic expected use for leave-on products (1% as the maximum use levels for leave-on products, see the Norwegian Food Safety Authority's 'Risk Profile of Tea tree oil — TTO'[29]).

The direct extrapolation, from a NOAEL for an individual constituent to a NOAEL for a mixture (or a final cosmetic product), might be only acceptable when no other constituent is reported to affect the same target. Because this could be the case for EtOH, particularly for cytotoxicity endpoints, the estimations were performed with and without EtOH. To estimate a NOAEL for a mixture based on available toxicological data, information on the estimated constituent-specific NOAEL, as well as its relative presence in the mixture (in the final cosmetic product), needs to be considered. When available appropriate data from individual constituents are used, a NOAEL may be estimated by using the following published formula[28]:

((% concentration of constituent 1/NOAEL (in mg/kg) of constituent 1) + (% concentration of constituent 2/NOAEL (in mg/kg) of constituent 2) + …etc.) × NOAEL = 100%

Based on the results of the GC-MS/MS chemical analysis, the concentrations of the individual allergens and EtOH were determined in µg/g and converted to percentage concentrations (and, as stated above, further normalised with regard to the expected use level for leave-on products, i.e. divided by 100). The specific NOAEL values (mg/kg) for the individual allergens determined by GC-MS/MS, and for EtOH (oral, rat, 9400 mg/kg[6]), were retrieved from publicly available databases and rounded to whole numbers (considered sufficiently precise for a pilot model estimation). The databases were checked and the individual NOAEL values updated repeatedly (however, further updates in the ECHA database may be expected in the future). Each individual value of the concentration of the individual allergen/100 was further divided by the value of the individual NOAEL (mg/kg), according to the published formula.[28] The resulting values of all individual constituents were summed (with or without EtOH). The final estimated NOAEL value of the allergen mixture, representing the NOAEL value of the finished product, based on the percentage concentration of each allergen present in the sample with an available known NOAEL value (oral, rat, mg/kg), was expressed as the result of the fraction 1/sum of normalised values (in two versions of the calculation, either including or excluding the value for EtOH).

EtOH is used in perfumes as the main ingredient (e.g. up to a concentration of 85%). As it was unclear whether the EtOH concentration would impact upon the final NOAEL values of the finished products, both versions of the estimation (i.e. either with or without EtOH) were performed. It was considered that EtOH is applied in a finite dose — thus, its effects as a chemical enhancer may not be obvious, as it may quickly evaporate from the skin surface, limiting its enhancing effect.[30] On the other hand, the impact of chemical volatility on skin absorption after the finite dose application should be also considered,[31] as EtOH may affect, for example, the penetration of other components and their bioavailability. Due to the high concentrations of EtOH in the finished products, it was necessary to select and take the NOAEL of EtOH[6] into account, in order to compare whether the NOAEL values of the finished products would be affected by the presence of EtOH in their formulations. The NOAEL for EtOH was retrieved from the ECHA database and selected as 9400 mg/kg/bw/day, for oral route, mouse, female (90 day).[6]

Results

The 3T3 NRU cytotoxicity assay

The ten samples were assessed in the 3T3 NRU assay (Figure 1). Sample 5 (the only sample of a 'parfum deodorant') exhibited the highest cytotoxicity, with effects apparent from 250 µg/ml. Samples 1, 2 and 3 (an 'EDP'), exhibited cytotoxicity from 500 µg/ml. Samples 4, 6, 7 and 8 exhibited cytotoxicity from 1000 µg/ml. Samples 9 and 10 were cytotoxic only at the highest concentration tested, i.e. 2500 µg/ml. The horizontal solid black line represents the cut-off value of cytotoxicity (70% of the solvent control value). The ascending order of cytotoxicity of the samples, based on the number of cytotoxic

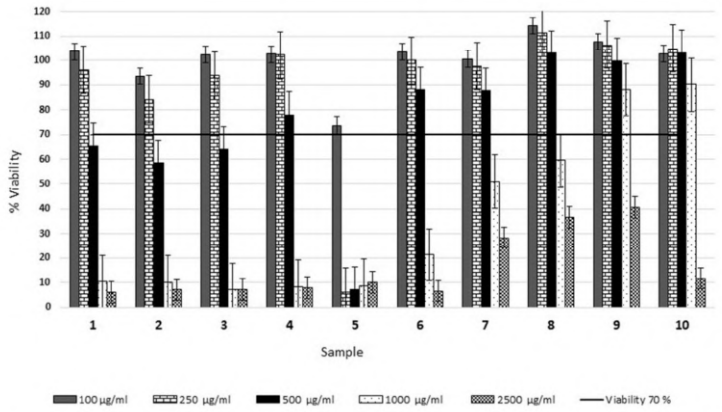

Figure 1. Results obtained for the 10 samples (four replicates) in the 3T3 NRU cytotoxicity assay. The 3T3 NRU cytotoxicity assay was used to assess the cytotoxicity of the samples against BALB/c 3T3 cells. The horizontal solid black line represents the cut-off value of cytotoxicity (70% of the solvent only control value).

concentrations and the highest cytotoxicity observed, was as follows: 9 < 10 < 8 < 7< 6 < 4 < 1 < 3 < 2 < 5 (i.e. Sample 9 exhibited the lowest cytotoxicity and Sample 5 exhibited the highest cytotoxicity).

Direct peptide reactivity assay (DPRA)

The relative depletion of cysteine peptide caused by Samples 1–10, at final concentrations of 50 µl/ml and 250 µl/ml in the reaction mixture, is summarised in Figure 2. The solid horizontal line represents the cut-off value for the positive DPRA prediction (13.89% of peptide depletion). Sample 5 exhibited the highest depletion of cysteine peptide — however, this result was considered likely to be a false positive, due to the zinc content of the sample (metals are reported to interfere with the DPRA, as described in OECD TG 442C[15]). Samples 6, 7 and 8 exhibited a positive response (relative depletion of cysteine peptide higher than 13.89%). ACN and EtOH were the negative (vehicle) controls. Cinnamic aldehyde in acetonitrile (100 mM stock solution), at final concentration of 5 mM in reaction solution, was used as the positive control (PC).

LuSens skin sensitisation assay

The results obtained for nine out of the ten samples are summarised in Figure 3. Due to high cytotoxicity, apparent from a concentration of 25 µg/ml, Sample 2 could be tested only at very low concentrations, as it appeared to be highly cytotoxic to LuSens cells in the MTT assay, performed in parallel. However, Sample 2 was not highly cytotoxic in the later performed NRU assay, which indicates that Sample 2 may affect mitochondrial activity (i.e. the activity of mitochondrial reductases), which is the endpoint of MTT assay as an early marker of toxicity, rather than disturbing the lysosomal membranes, which is the NRU assay endpoint. At the low concentrations tested, it did not show any response *in vitro* that indicated any skin sensitisation potential. For these reasons, Sample 2 is not included in Figure 3; instead, it is shown separately in Figure 4. Among the samples shown in Figure 3, four of them provided positive responses: Sample 6 was predicted as positive at all three non-cytotoxic concentrations (100, 50 and 25 µg/ml); Sample 7 showed a positive response at two concentrations (50 and 100 µg/ml); Sample 8 exhibited a positive response at one concentration (100 µg/ml), whereas at 50 µg/ml it was very close to the 1.5-fold cut-off value (at 1.47); and Sample 9 exhibited a positive response only at one concentration (100 µg/ml).

In vitro *Comet assay*

The genotoxic potential of the fragrance-based product samples was evaluated with the *in vitro* Comet assay, in three independent experimental runs. None of the samples tested induced an increase of more than 10% with respect to DNA in the tail, at two consecutive non-cytotoxic concentrations (Table 2). Based on the statistical methods used, the concentration-dependent responses of Samples 5 and 7 could not be evaluated for statistical significance, due to the high standard deviation between the individual runs. Statistically significant concentration-dependent DNA fragmentation was observed in Samples

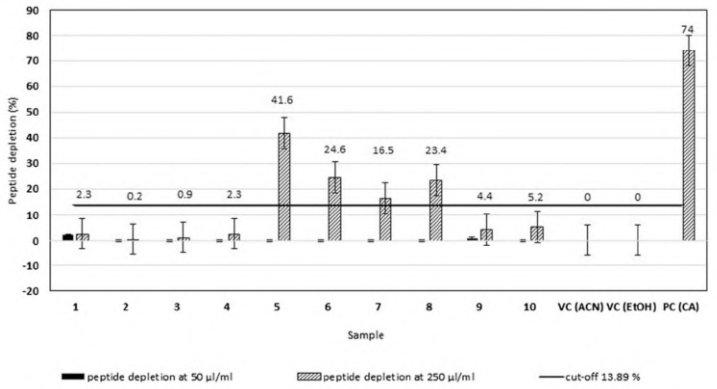

Figure 2. The assessment of skin sensitisation potential with the Direct Peptide Reactivity Assay (DPRA). Reactivity of the test samples with a model cysteine-containing heptapeptide (Ac-RFAACAACOOH) was used to predict their skin sensitising potential. The solid horizontal line represents the cut-off value for the positive DPRA prediction (13.89% of peptide depletion). Samples were tested in triplicate. ACN = acetonitrile; EtOH = ethanol; CA = cinnamic aldehyde; PC = positive control; VC = vehicle control.

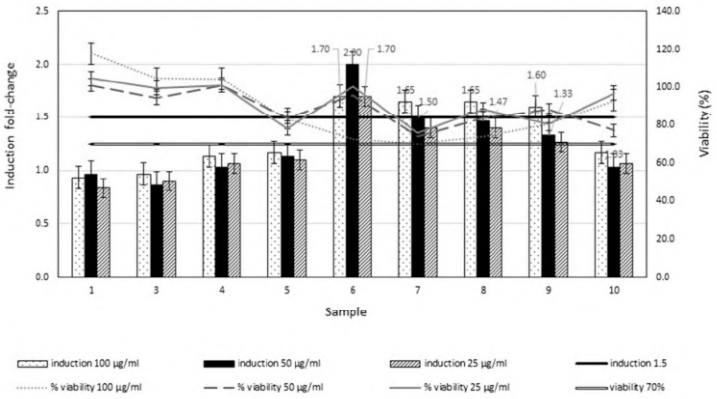

Figure 3. The assessment of skin sensitisation potential with the LuSens assay. The LuSens skin sensitisation assay uses a human keratinocyte ARE reporter cell line, with the induction of reporter gene expression used as an indicator of sensitisation potential. Sample 2 is not included in this figure, due to high its cytotoxicity (instead, see Figure 4). The solid black line represents the cut-off value for positive induction (fold-change of 1.5× the vehicle control value). The double line represents the cut-off value for cytotoxicity (70% viability, as compared to the vehicle control). The grey dotted line represents the viability of samples at 100 µg/ml; the grey dashed line represents the viability of samples at 50 µg/ml; and the grey solid line represents the viability of samples at 25 µg/ml. Samples were tested in triplicate.

6, 8, 9 and 10 (Table 2). These samples were evaluated for suspected genotoxicity and due to the suspected genotoxic potential, they were further evaluated with the *in vitro* mammalian chromosome aberration test (see Table 3). Sample 5 was also included in this analysis to evaluate its genotoxic potential given the observed concentration-dependent response at relatively low concentrations. Samples were tested in triplicate in each experimental run.

In vitro *mammalian chromosome aberration test*

The *in vitro* chromosome aberration test was performed with Samples 5, 6, 8, 9 and 10, according to OECD TG

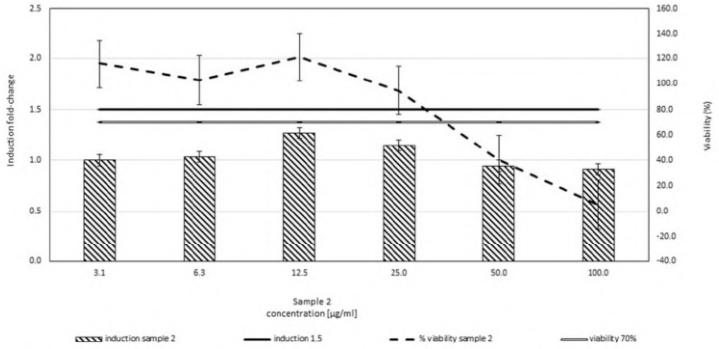

Figure 4. Assessment of the skin sensitisation potential of Sample 2 with the LuSens assay. Sample 2 did not reach the cut-off value of fold-change in induction relative to the vehicle control (1.5-fold) at any non-cytotoxic concentration that could be evaluated. The black dashed line represents cell viability, which should be above 70% (the cut-off value for cytotoxicity, represented by the double line). The solid black line represents the cut-off value of induction (fold-change of 1.5× the vehicle control value). Sample 2 was tested in triplicate.

Table 2. Assessment of genotoxic potential *in vitro* by using the Comet assay with 3T3 Balb/c fibroblasts.

	% DNA in the tail								
Sample No./Conc.	50 µg/ml	100 µg/ml	150 µg/ml	250 µg/ml	350 µg/ml	500 µg/ml	750 µg/ml	1000 µg/ml	1500 µg/ml
1	—	—	1.344	0.299	2.329	—	—	—	—
2	—	—	0.002	0.525	0.354	—	—	—	—
3	—	—	0.118	2.006	0.939	—	—	—	—
4	—	—	0.037	1.540	1.210	—	—	—	—
5	0.066	2.823	5.842	—	—	—	—	—	—
6	—	—	—	**0.073**	—	**0.981**	**11.658**	—	—
7	—	—	—	0.008	—	0.969	7.087	—	—
8	—	—	—	1.376	—	**1.129**	**5.055**	—	—
9	—	—	—	—	—	**1.394**	—	**3.043**	**24.002**
10	—	—	—	—	—	**1.557**	—	**2.690**	**14.647**

The Comet assay was performed by treating 3T3 Balb/c fibroblasts with the test samples at the concentrations indicated. The entries show the percentage of DNA in the tail at the given concentrations of test sample. The bold numbers indicate statistically significant concentration-dependent fragmentation of DNA, as compared to the vehicle controls. '—' indicates that these concentrations were not tested.

473[23] to follow-up the suspected genotoxic potential determined in the Comet assay. None of the samples induced the required minimum 2-fold increase in the percentage of aberrant cells in comparison with the negative control (see Table 3). The positive control used in the test without MAS was correctly classified; however, in the test version with MAS, the NC value was relatively higher compared to historical data. The test was accepted as a supplementary confirmation of negative genotoxic potential, whereas the observed indications of very weak dose-dependent responses in the *in vitro* Comet assay were evaluated as inconclusive for genotoxicity and rather as a manifestation of oxidative stress. Samples were tested in triplicate.

Yeast-based reporter gene assay

This assay was performed to detect endocrine disruption potential. The presented data show the responses of samples at the highest non-cytotoxic concentration tested (100 µg/ml) (Figure 5).

Figure 5a shows a potential agonistic activity on the human oestrogen receptor α. The dark grey column represents a response predicted as potentially positive — Sample 6 can be evaluated as exerting weak agonistic activity on the human oestrogen receptor α, as there was a greater than 10% increase (20.2%) compared to the negative (vehicle) control value (1.37 *versus* 1.14, respectively).

Table 3. Assessment of genotoxic potential with the *in vitro* mammalian chromosome aberration test.

		% Aberrant cells		
Sample	Concentration (μg/ml)	Exposure: 4 hours with MAS	Exposure: 4 hours without MAS	Exposure: 26 hours without MAS
5	0.05	2	5	1
	0.1	1	4	0
	0.15	1	1	2
6	0.25	4	3	3
	0.5	3	5	2
	0.75	5	2	5
8	0.25	3	2	2
	0.5	3	4	4
	0.75	1	2	5
9	0.5	1	5	1
	1	3	3	7
	1.5	6	4	6
10	0.5	2	4	3
	1	4	5	5
	1.5	2	3	7
NC with MAS		6	—	—
PC (cyclophosphamide with MAS)		9	—	—
NC without MAS		—	4	5
PC (thio-TEPA without MAS)		—	**8**	**15**

The chromosome aberration test was performed on human peripheral blood lymphocytes by exposing them to the test samples, with or without metabolic activation. The entries show the percentage of aberrant cells. The bold entries indicate a positive result (as characterised by a ≥ 2-fold increase the percentage of aberrant cells, in comparison with the negative control). MAS = metabolic activation stimulation; NC = negative control; PC = positive control.

The data for potential antagonistic activity on the human oestrogen receptor α are shown in Figure 5b. Sample 10 (an EDC) may be considered to exert the highest antagonistic activity (i.e. anti-oestrogenic activity), as the value obtained was more than a 50% decrease compared to the value of the negative (solvent) control (1.07 *versus* 2.85, respectively), and a concentration-dependent response was observed (data not shown). In addition, Sample 2 (an EDP) was also shown to exert antagonistic potential on the human oestrogen receptor α, as there was a greater than 25% decrease, as compared to the negative control (2.12 *versus* 2.85, respectively).

The data showing potential agonistic activity on the human androgen receptor are presented in Figure 5c. All samples exhibited a negative response in the androgenic assay, as none of the values showed the required minimum 10% increase, as compared to the value of the negative (solvent) control (i.e. 1.46). Thus, none of the samples could be predicted as being human androgen receptor agonists.

The data showing potential antagonistic activity on the human androgen receptor are presented in Figure 5d. Sample 2 (an EDP) may be evaluated as exerting antagonistic activity on the human androgen receptor (i.e. anti-androgenic), as there was a more than 35% decrease compared to the negative (solvent) control (1.10 *versus* 1.73, respectively), and a concentration-dependent response was observed (data not shown). Sample 1 (an EDP), Sample 5 (a parfum deodorant), Sample 7 (reported as a Parfum FEMME containing a pheromone), Sample 8 (reported as a Parfum HOMME containing a pheromone) and Sample 9 (an EDT) could all be evaluated as displaying weak antagonistic activity, as they exhibited at least a 20% decrease compared to the negative (solvent) control (1.35, 1.25, 1.33, 1.33 and 1.29 *versus* 1.73, respectively). However, no concentration-dependent responses were observed.

Chemical analysis

The GC-MS/MS analysis of the ten samples was used to determine the presence of twenty-four specific known allergens. They were shown to be present in the samples at various concentrations and in a wide range of combinations (see Table 4). The presence of specific allergens was studied, particularly allergens subjected to regulatory measures, those with lower NOAEL values, or those detected at higher concentrations.

Despite its previous widespread use in consumer products, buthylphenyl methylpropional (NOAEL 25 mg/kg) has been recently classified by the ECHA Risk Assessment Committee (RAC) as reprotoxic 1B; it was detected in the majority of the samples tested. The buthylphenyl methylpropional concentrations in the

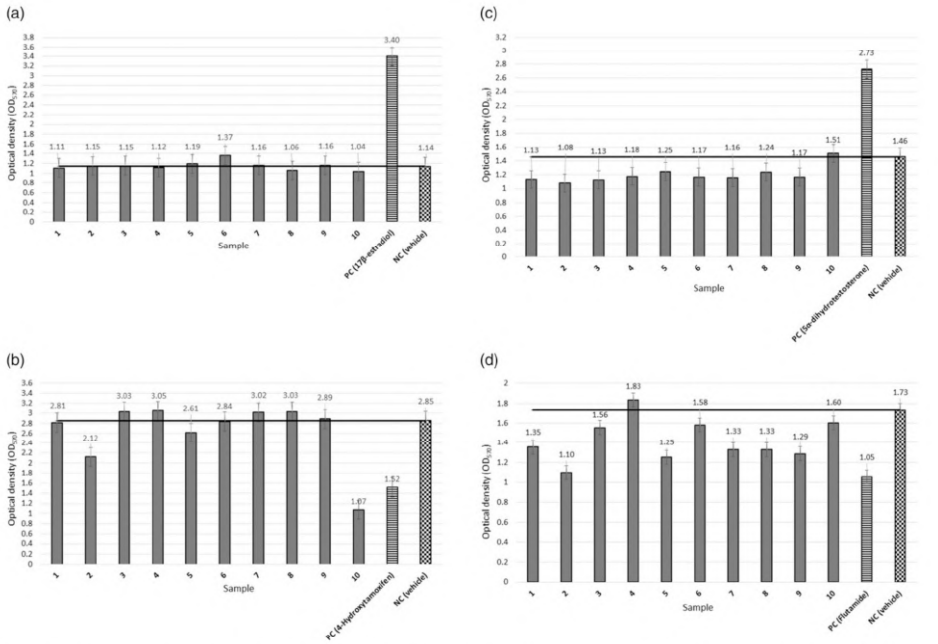

Figure 5. Assessment of endocrine disruption potential with a yeast-based reporter gene assay. (a) Agonistic activity on the human oestrogen receptor α. (b) Antagonistic activity on the human oestrogen receptor α. (c) Agonistic activity on the human androgen receptor. (d) Antagonistic activity on the human androgen receptor. The graphs show the responses of samples at the highest non-cytotoxic concentration tested (100 μg/ml): (a) agonistic activity on the human oestrogen receptor α; (b) antagonistic activity on the human oestrogen receptor α; (c) agonistic activity on the human androgen receptor; and (d) antagonistic activity on the human androgen receptor. The positive controls (PCs) were: in (a) 10^{-8} M 17β-oestradiol; in (b) 10^{-6} M 4-hydroxytamoxifen; in (c) 10^{-6} M 5α-dihydrotestosterone; and in (d) 10^{-8} M flutamide. The dark grey columns in each graph indicate responses that are predicted as potentially positive with respect to the specific agonistic/antagonistic activity. Positive agonistic activity is defined as an increase of ≥ 10% in the absorbance value of the sample as compared to the negative (vehicle) control (NC) value (1% DMSO). Positive antagonistic activity is defined as a decrease of ˃ 20% in the absorbance value of the sample compared to that of the negative (vehicle) control (NC). The NC values are indicated by the black horizontal bars on each graph. Samples were tested in duplicate in every case.

samples can be ranked (from the lowest concentration to the highest) as: 4, 10 (both < LOQ (below the limit of quantification)) < 9 < 6 < 2 < 3 < 1 < 8 < 5 < 7. High (outlier) concentrations of butylphenyl methylpropional were found in Samples 5 and 7, compared to the other samples.

Outlier results in *in vitro* biological effects were also the subject of thorough evaluation. Higher cytotoxicity (i.e. lower activity of mitochondrial reductases in the MTT assay) was observed in Sample 2 — therefore, specific allergens for which the concentrations were markedly different in Sample 2, as compared to the other samples, were considered. The following substances were detected only in Sample 2:

- hydroxyisohexyl 3-cyclohexene carboxaldehyde (270 μg/g, NOAEL 100 mg/kg);
- farnesol (4300 μg/g, NOAEL 266 mg/kg);
- cinnamyl alcohol (34 μg/g, NOAEL 1000 mg/kg); and
- benzyl cinnamate (41 μg/g, NOAEL 600 mg/kg).

In addition, the percentage concentrations of the following substances exhibited outlier values in Sample 2, but not in the other samples:

- benzyl benzoate (17,000 μg/g, NOAEL 781 mg/kg);
- geraniol (3000 μg/g, NOAEL 300 mg/kg); and
- citronellol (3100 μg/g, NOAEL 1000 mg/kg).

Table 4. The content of specific allergens in each sample.

CAS	Analyte / Sample No.	GC-MS identification (μg/g)										LOQ (μg/g)
		1	2	3	4	5	6	7	8	9	10	
127-51-5	Alpha-isomethyl ionone	250	820	< LOQ	< LOQ	4900	260	36	2100	1.2	17	4.6
122-40-7	Amyl cinnamal	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	9.6	4.2
101-85-9	Amylcinnamyl alcchol	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	4.9
105-13-5	Anise alcohol	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	5.7
100-51-6	Benzyl alcohol	68	220	260	5.3	72	< LOQ	540	< LOQ	15	37	3.9
120-51-4	Benzyl benzoate	< LOQ	17000	420	16	< LOQ	290	4.8	< LOQ	< LOQ	140	3.5
103-41-3	Benzyl cinnamate	< LOQ	41	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	3.4
118-58-1	Benzyl salicylate	4400	250	2000	< LOQ	8000	55	2800	1300	7200	11	4.2
80-54-6	Butylphenyl methylpropional	4600	34	1600	< LOQ	9200	21	9700	3800	6.5	< LOQ	4.1
104-55-2	Cinnamal	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	24	< LOQ	< LOQ	< LOQ	48	5.1
104-54-1	Cinnamyl alcohol	< LOQ	34	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	4.7
5392-40-5	Citral	< LOQ	56	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	400	420	208	4.1
106-22-9	Citronellol	740	3100	320	720	400	1000	1900	350	360	140	3.4
91-64-5	Coumarin	< LOQ	86	< LOQ	< LOQ	< LOQ	2100	1200	30	< LOQ	2000	3.2
5989-27-5	D-Limonene	12000	950	1300	2600	1200	1100	3600	17000	16000	5500	3.2
97-53-0	Eugenol	< LOQ	130	24	< LOQ	< LOQ	27	14	200	< LOQ	41	3.8
4602-84-0	Farnesol	< LOQ	4300	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	3.9
106-24-1	Geraniol	260	3000	190	370	23	6.9	2100	750	900	110	3.1
101-86-0	Hexyl cinnamal	1600	140	5600	< LOQ	390	1000	550	< LOQ	1700	< LOQ	3.5
107-75-5	Hydroxycitronellal	< LOQ	170	< LOQ	< LOQ	220	830	3500	100	< LOQ	< LOQ	5.1
31906-04-4	Hydroxyisohexyl 3-cyclohexene carboxaldehyde	< LOQ	270	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	5.9
97-54-1	Isoeugenol	< LOQ	< LOQ	3.7	16	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	3.4
78-70-6	Linalool	46	2000	740	730	8.8	960	14000	19000	4500	1100	3.4
111-12-6	Methyl 2-octynoate	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	3.5
Total content		**23964**	**32601**	**12457.7**	**4457.3**	**24413.8**	**7673.9**	**39944.8**	**45030**	**31102.7**	**9361.6**	

< LOQ = below the limit of quantification.

Further, we focused on the concentration of allergens with lower individual NOAEL values and the possible associations with observed biological effects, such as higher cytotoxicity. For example, the disruption of lysosomal membrane, as detected by the NRU assay, was observed in Sample 5. A significantly higher concentration of alpha-isomethyl ionone (4900 µg/g, NOAEL 4 mg/kg) was detected in Sample 5, as compared to the other samples. Furthermore, higher concentrations of coumarin (NOAEL 138 mg/kg) were detected in Sample 6 (2100 µg/g), Sample 7 (1200 µg/g) and Sample 10 (2000 µg/g), as compared to the other samples.

These observations indicate that attention should be paid to substances present in mixtures at higher concentrations, with lower NOAEL values. In the case of biological effects being observed *in vitro*, such results may indicate the potential for adverse effects *in vivo*, possible combined interactions, and potentiating or synergic effects.

Table 5 shows the total content of allergens and EtOH. The samples can be listed according to the total content of allergens detected, as follows (from the sample with the lowest to the sample with the highest concentration): 4 < 6 < 10 < 3 < 1 < 5 < 9 < 2 < 7 < 8. Likewise, the samples can be listed according to the presence of EtOH, as follows (from the sample with the lowest concentration): 5 < 2 < 3, 4, 7, 9 < 1, 6, 8 < 10.

NOAEL values (mg/kg) of individual allergens

The NOAEL values of the individual allergens (and also EtOH) were retrieved from publicly available databases (see Table 6).

NOAEL values (mg/kg) of finished products

The NOAEL values (mg/kg) were estimated according to the approach described in the Scientific Committee on Consumer Products' 'Opinion on Tea tree oil'[28] and the Norwegian Food Safety Authority's 'Risk Profile of Tea tree oil — TTO',[29] with or without inclusion of the values for EtOH, representing the NOAEL values of the finished products (Table 7). The samples can be sorted according to the NOAEL of the finished product as follows (in descending order, from the highest value): EtOH value excluded: 4 > 10 > 9 > 6 > 3 > 2 > 1 > 7 > 8 > 5; EtOH value included: 4 > 10 > 9 > 6 > 3 > 2 > 1 > 7 > 8 > 5.

Summary of the results

A summary of the data obtained is shown in Table 8. The results of the *in vitro* biological effects (cytotoxicity, skin sensitisation, endocrine disruption, genotoxicity) were collated and roughly interpreted by the assignment of shading intensity to each set of results, followed by a cross-scale comparison across the range of assays. Similarly, the GC-MS/MS results were summarised and were roughly interpreted by using a shading intensity gradient. Finally, the samples were sorted, according to a number of selected criteria (see Table 8), to indicate the predicted product safety (from the safest, based on the chosen criterion).

Discussion

Cytotoxicity

Based on the cytotoxicity results (Table 8), the samples can be sorted in ascending order of cytotoxicity as follows: 9 < 10 < 8 < 7 < 6 < 4 < 1 < 3 < 2 < 5, i.e. Sample 5 (a deodorant) exhibited the highest cytotoxicity and Sample 9 the lowest. The cytotoxicity results seem to be inversely correlated with the content of EtOH — for example, based on GC-MS/MS analysis, Sample 5 contained the lowest concentration of EtOH (70%), but exhibited the highest level of cytotoxicity, Sample 2 contained the second lowest concentration of EtOH (75%) and was the second most cytotoxic — moreover, treatment with this sample also resulted in low viability in the MTT assay. Hence, we propose that the high cytotoxicity observed with Samples 5 and 2 is not attributed to EtOH content, but to other components in the products. Sample 5 was the only deodorant product tested, which probably had a specific composition that contained a higher number of specific (cytotoxic) compounds than the other products tested. The conclusion relating to the importance of the specific composition may be supported by the observation that the highest number of constituents (allergens), i.e. 18 out of 24, was detected in Sample 2, despite this sample not being characterised by the highest total allergen content or a notably high concentration of EtOH (see

Table 5. The total content of allergens and ethanol (EtOH).

	GC/MS identification									
Content / Sample No.	1	2	3	4	5	6	7	8	9	10
EtOH content (%)	85	75	80	80	70	85	80	85	80	90
Total allergen content (µg/g)	23964	32601	12457.7	4457.3	24413.8	7673.9	39941.8	45030	31102.7	9361.6
Total allergen content (%)	2.40	3.26	1.25	0.45	2.44	0.77	3.99	4.50	3.11	0.94

Table 6. The NOAEL values of the individual allergens.

CAS	Substance	Estimated NOAEL (study description)	Reference
127-51-5	Alpha-isomethyl ionone	4 mg/kg bw/d (OECD Guideline 408)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/18602/7/6/2
122-40-7	Amyl cinnamal	36 mg/kg/bw/day	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/20550/7/6/2/?documentUUID=3e3bf44b-2c32-43dd-abb7-6515933f1c49
101-85-9	Amylcinnamyl alcohol	200 mg/kg/bw/day (NTP, 2003)	http://fragrancematerialsafetyresource.elsevier.com/sites/default/files/GS7-cinnamic_acid-related/esters.pdf
105-13-5	Anise alcohol	400 mg/kg bw/d (OECD Guideline 422)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/16802/7/6/2
100-51-6	Benzyl alcohol	400 mg/kg (study equivalent to OECD Guideline 451, supervised by NTP, NTP TR 343, 1989)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/14748/7/6/2
120-51-4	Benzyl benzoate	781 mg/kg bw/day	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/13634/7/1
103-41-3	Benzyl cinnamate	600 mg/kg bw/day (OECD Guideline 422)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/13634/7/1
118-58-1	Benzyl salicylate	360 mg/kg bw/day (OECD Guideline 408)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/16100/7/6/2
80-54-6	Butylphenyl methylpropional	25 mg/kg bw/day (OECD Guideline 408)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/13572/7/6/2
104-55-2	Cinnamal	656 mg/kg/day	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/14462/7/6/2
104-54-1	Cinnamyl alcohol	1000 mg/kg bw (OECD Guideline 407)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/12023/7/6/2
5392-40-5	Citral	Repeated dose toxicity (rat, microcapsule): NOAEL 570 mg/kg	https://hpvchemicals.oecd.org/UI/handler.axd?id=0ea83202-3f4f-4355-be4f-27ff02e19cb9
106-22-9	Citronellol	1000 mg/kg bw/d (corresponding to 290 mg/kg bw/d citronellyl acetate)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/14242/7/6/2
91-64-5	Coumarin	138 mg/kg/bw/day	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/11472/7/6/2
5989-27-5	D-Limonene	1650 mg/kg bw/day (OECD Guideline 408)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/15256/7/6/2/?documentUUID=53fd778e-721f-4cb8-af51-fa22ec86a02f
97-53-0	Eugenol	1250 mg/kg/bwt/d (US FDA, 1993)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/13694/7/6/2
4602-84-0	Farnesol	266 mg/kg/day (OECD 422)	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/23466/7/6/2
106-24-1	Geraniol	300 mg/kg bw/day	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/14184/7/6/4
101-86-0	Hexyl cinnamal	125 mg/kg bw/day	https://www.tga.gov.au/book-page/27-amyl-hexyl-cinnamaldehyde
107-75-5	Hydroxycitronellal	492 mg/kg bw/day	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/12695/7/6/2
31906-04-4	Hydroxyisohexyl 3-cyclohexene carboxaldehyde	100 mg/kg bw/day	https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_074.pdf
97-54-1	Isoeugenol	LOAEL 250 mg/kg/day	https://www.heraproject.com/files/19-F-05-HERA/Isoeugenol/(corrections/May/2005).pdf
78-70-6	Linalool	532 mg/kg bw/day	https://echa.europa.eu/cs/registration-dossier/-/registered-dossier/14501/7/6/2
111-12-6	Methyl 2-octynoate	No data available in the ECHA Database for repeated dose toxicity	—

Table 7. The estimated NOAEL values for the finished products.

Sample No.	1	2	3	4	5	6	7	8	9	10
Type of product	EDP	EDP	EDP	EDP	Parfum deodorant	EDP	Parfum	Parfum	EDT	EDC
NOAEL of finished product mg/kg bw/day, EtOH excluded	3563.1	3730.4	8423.9	200039.7	617.2	10546.2	2156.3	1368.4	17714.8	39284.1
NOAEL of finished product mg/kg bw/day, EtOH included	2694.8	2874.8	4906.4	11098.1	590.1	5398.2	1822.0	1217.8	7064.3	8250.8

The samples were ranked according to their NOAEL values. The grey-shading indicates their potential toxicity, from the highest NOAEL values (lower toxicity, lighter shading) to the lowest NOAEL values (higher toxicity, darker shading). EDP = 10–15% fragrance eau de parfum; EDT = 5–10% fragrance eau de toilette; EDC = 3–5% fragrance eau de cologne.

Table 8). Sample 2 was cytotoxic, not only to 3T3 Balb/c fibroblasts, but also to human keratinocytes, exhibiting the highest level of cytotoxicity in the LuSens assay.

It is notable that the results of the GC-MS/MS analysis and the declared composition on the packaging of Sample 2 showed discrepancies. The declared list of ingredients on the packaging of Sample 2 stated: "Fragrance (perfume), alcohol, demineralized water, contains: amyl cinnamal, benzyl salicylate, citral, citronellol, coumarin, eugenol, geraniol, hexylcinnamal, isoeugenol, butyl phenyl methyl propional, D-limonene, linalool". According to our GC-MS/MS results, benzyl alcohol, citral, hydroxycitronellal, alpha-isomethyl ionone, cinnamyl alcohol, farnesol, benzyl benzoate, hydroxyisohexyl 3-cyclohexene carboxaldehyde and benzyl cinnamate were present in the sample, but these compounds were not declared on the packaging. For Sample 5, all of the allergens detected by GC-MS/MS correlated with the declared list of ingredients on the packaging, which stated: "Alcohol denat., aqua, perfume, PEG-40 hydrogenated castor oil, zinc ricinoleate, tetrahydroxypropyl ethylendiamine, laureth-3, propylene glycol, citric acid, ethanolamine, benzyl salicylate, citronellol, geraniol, hexyl cinnamal, hydroxycitronellal, limonene, butylphenyl methylpropional, alpha-isomethyl ionone".

In general, the mechanisms leading to cytotoxicity of a product ingredient toward isolated cells can be related to, for example, the chemical's ability to cause structural changes within cell membrane proteins. Such mechanisms might have been involved in the cytotoxicity of Sample 5, as it appeared to exhibit high protein reactivity — Sample 5 had the highest level of observed peptide depletion in the DPRA. Other mechanisms associated with cytotoxicity can include: the induction of reactive oxygen species (ROS); the promotion of apoptosis; the inhibition of cell division; and cytostatic effects. Moreover, in the Comet assay, which detects, in particular, oxidising DNA damage (besides other mechanisms leading to DNA fragmentation), Sample 5 exhibited a concentration-dependent increase in the level of DNA in the tail at relatively low concentrations. This result was not interpreted as positive, because it was below the cut-off value of an increase of at least 10% DNA in the tail. However, it could be indicative of possible oxidative stress. Interestingly, the highest cytotoxicity was in correlation with the lowest NOAEL value of the final product estimated for Sample 5, which was not the case for Sample 2.

Skin sensitisation

Based on the total content of all twenty-four allergens, the samples can be sorted in ascending order as follows: 4 < 6 < 10 < 3 < 1 < 5 < 9 < 2 < 7 < 8. The total content of allergens, as detected by GC-MS/MS analysis, correlated, to some extent, with the skin sensitisation results — at least one of the tested concentrations of Samples 8, 7 and 9 exhibited activity in both assays used for screening skin sensitisation potential (i.e. DPRA and LuSens assay). Sample 2 could not be assessed reliably in the LuSens assay, due to very high cytotoxicity toward human keratinocytes (the LuSens cell line). One would hypothesise that such an outlier result could be attributed to specific ingredients exclusively present in Sample 2 (see Table 4) — for example, farnesol (4300 µg/g), hydroxyisohexyl 3-cyclohexene carboxaldehyde (lyral, 270 µg/g), cinnamyl alcohol (34 µg/g) and benzyl cinnamate (41 µg/g). Alternatively, the outlier result could be due to certain ingredients being present at higher concentrations relative to the other samples (e.g. eugenol, citronellol, geraniol, benzyl benzoate), or due to the presence of chemicals not listed on the packaging.

As stated above, our GC-MS/MS analysis and the declared composition on the packaging of Sample 2 showed discrepancies. The declared list of ingredients on the packaging of Sample 2 stated: "Fragrance (perfume), alcohol, demineralized water, contains: amyl cinnamal, benzyl salicylate, citral, citronellol, coumarin, eugenol, geraniol, hexylcinnamal, isoeugenol, butyl phenyl methyl propional, D-limonene, linalool". According to our GC-MS/MS results, benzyl alcohol, citral, hydroxycitronellal, alpha-isomethyl ionone, cinnamyl alcohol, farnesol, benzyl benzoate, hydroxyisohexyl 3-cyclohexene

Table 8. Summary of the results.

Sample No.	1	2	3	4	5	6	7	8	9	10
Type of Product	**EDP**	**EDP**	**EDP**	**EDP**	**Parfum deodorant**	**EDP**	**Parfum**	**Parfum**	**EDT**	**EDC**
Biological effects										
Cytotoxicity (number of cytotoxic concentrations)	+++	+++	+++	++	++++	++	++	++	+	+
Skin sensitisation *in chemico* (DPRA)	N	N	N	N	FP	P	P	P	N	N
Skin sensitisation *in vitro* LuSens	N	N	N	N	N	+++	++	++	+	N
Agonistic activity on hERα (oestrogenic)	N	N	N	N	N	P	N	N	N	N
Antagonistic activity on hERα (anti-oestrogenic)	N	P	N	N	N	N	N	N	N	P
Agonistic activity on hAR (androgenic)	N	N	N	N	N	N	N	N	N	N
Antagonistic activity on hAR (anti-androgenic)	P	P	N	N	P	N	P	P	P	N
Genotoxic potential (Comet assay)	N	N	N	N	susp.	susp.	N	susp.	susp.	susp.
Genotoxicity (Chromosomal abberation test)	n.t.	n.t	n.t	n.t	N	N	n.t.	N	N	N
Frequency of positive biological effects	2	3	1	1	3	4	4	4	3	2
GC-MS/MS analysis										
Number of allergens detected	9	18	11	7	10	13	13	11	10	13
Total content of allergens (%)	2.4	3.3	1.2	0.4	2.4	0.8	4.0	4.5	3.1	0.9
EtOH content (%)	85	75	80	80	70	85	80	85	80	90
NOAEL values										
NOAEL mg/kg bw/day, EtOH excluded	3563.1	3730.4	8423.9	200039.7	617.2	10546.2	2156.3	1368.4	17714.8	39284.1
NOAEL mg/kg bw/day, EtOH included	2694.8	2874.8	4906.4	11098.1	590.1	5398.2	1822.0	1217.8	7064.3	8250.8
Criteria of safety										
Samples sorted according to cytotoxicity (ascending order, from the lowest number of cytotoxic concentrations)	9 < 10 < 8 < 7 < 6 < 4 < 1 < 3 < 2 < 5									
Samples sorted according to the total content of EtOH (ascending order, from the lowest value)	5 < 2 < 3, 4, 7, 9 < 1, 6, 8 < 10									
Samples sorted according to the content of alpha-isomethyl ionone	4, 3 < 9 < 10 < 7 < 1 < 6 < 2 < 8 < 5									
Samples sorted according to the content of butylphenyl methylpropional	4, 10 < 9 < 6 < 2 < 3 < 1 < 8 < 5 < 7									
Samples sorted according NOAEL, EtOH excluded (descending order, from the highest value)	4 > 10 > 9 > 6 > 3 > 2 > 1 > 7 > 8 > 5									
Samples sorted according NOAEL, EtOH included (descending order, from the highest value)	4 > 10 > 9 > 6 > 3 > 2 > 1 > 7 > 8 > 5									
Samples sorted according to the total content of allergens (ascending order, from the lowest value)	4 < 6 < 10 < 3 < 1 < 5 < 9 < 2 < 7 < 8									
Samples sorted according to the number of individual allergens (ascending order, from the lowest number)	4 < 1 < 3, 5, 8, 9 < 6, 7, 10 < 2									
Samples sorted according to the frequency of biological effects (ascending order, from the lowest frequency)	4, 3 < 1, 10 < 2, 9, 5 < 6, 7, 8									

The grey shading reflects the potential toxicity predicted in the *in vitro* biological tests, the predicted NOAEL values, and the detected allergen content, number of allergens and EtOH content. The lower the toxicity, the lighter the shading, and the higher the toxicity, the darker the shading. N = negative; P = positive; FP = false positive; n.t. = not tested; susp. = suspected.

carboxaldehyde and benzyl cinnamate were present in the sample, but these compounds were not declared.

The high cytotoxic activity of farnesol toward specific cell lines has been confirmed previously. However, keratinocytes were reported to be the least sensitive.[32,33] In addition to the substances mentioned above, the highest content of benzyl benzoate (17,000 µg/g) and a relatively high content of benzyl alcohol (200 µg/g) were detected in Sample 2 by GC-MS/MS, as compared to the other samples. Benzyl alcohol was reported to induce genotoxicity in the *in vitro* Comet

assay at high concentrations (25 and 50 M).[34] It would be interesting to verify the cytotoxicity exhibited by Sample 2 in the LuSens cell line in a separate study, using the MTT assay to determine the effects of these specific chemicals, or their mixtures, on other cell lines tested in parallel with the standard LuSens cell line. Benzyl benzoate, benzyl cinnamate and benzyl salicylate (all detected in Sample 2) were previously reported to possibly bind with high affinities to HLA (human leukocyte antigen) molecules and to be non-reactive toward cysteine.[35] This would be consistent with the (potentially false) negative results obtained with Sample 2 in the DPRA (only a cysteine-containing peptide was used) and potentially also in assays (including the LuSens assay) that are able to detect substances that can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1) by, for example, covalent modification of its cysteine residue, through the Keap1-Nrf2-ARE pathway (a regulatory pathway of cytoprotective responses to electrophile and oxidative stress). Further studies based on selected *in vitro* assays could clarify which of the mechanisms suggested above, including degradation and (auto)oxidation, could be involved in the higher levels of cytotoxicity (i.e. lower activity of mitochondrial reductases) observed in the LuSens assay, or the (potentially false) negative results in the DPRA. Substances non-reactive to cysteine might not be efficiently detected in the DPRA (with a cysteine-containing peptide only), or in the assay based on the mechanism of covalent modification of the cysteine residue of the sensor protein, Keap1. Indeed, among all of the samples, the highest benzyl benzoate concentration was detected in Sample 2 (17,000 μg/g).

It is important also to critically consider the growing extent of information on the limitations of the assays used, in order to better understand all of the mechanisms involved. The DPRA is not applicable for the testing of metal compounds (known to react with proteins through mechanisms other than covalent binding), or for complex mixtures or substances of unknown or variable composition, complex reaction products, or biological materials (as described in the OECD TG 442C[15]). The DPRA lacks metabolic capacity, which is also very limited in cell-based assays (including the LuSens assay). Thus, pro-haptens requiring enzymatic activation to exert their sensitisation activity cannot be detected with these methods directly, without prior enzymatic activation. Pre-haptens activated by auto-oxidation may provide (false) negative results. Moreover, chemicals with preferential reactivity toward amino acids other than cysteine or lysine (e.g. nucleophilic sites in histidine) may also lead to false negative results, especially in the DPRA (specific to cysteine or lysine peptides). Cysteine and lysine peptides represent different types of nucleophiles which cover different reaction mechanisms. Therefore, both test systems (cysteine and lysine peptides) should be used to cover a broader spectrum of reactions in the DPRA. If borderline results are obtained (i.e. mean percentage depletion falls in the range of 3% to 10% for the cysteine 1:10/lysine 1:50 prediction model, or cysteine percentage depletion falls in the range of 9% to 17% for the cysteine 1:10 prediction model), then additional testing is recommended. In particular, if negative results are obtained in these ranges (i.e. 3% to 6.38% for the cysteine 1:10/lysine 1:50 prediction model, or 9% to 13.89% for the cysteine 1:10 prediction model), a second run should be conducted, as well as a third one if there are discordant results between the first two runs.

False positive predictions can be obtained in the DPRA for substances that do not covalently bind to the peptide, but are able to promote its oxidation (i.e. cysteine dimerisation). In the cell-based assays (including the LuSens assay), substances with an exclusive reactivity toward nucleophiles other than cysteine's sulfhydryl group (e.g. lysine residues) can be detected as false negatives. Also, due to the assay's limited metabolic capacity, false negative results of pro-haptens may be presented. Regarding the specific constituents, the sensitisation potential of benzyl benzoate, benzyl cinnamate, or benzyl salicylate may rely on other than biotic or abiotic activation,[36] as pre-haptens are activated via auto-oxidation. On the contrary, chemicals that act as chemical stressors may lead to false positive results. Highly cytotoxic chemicals cannot always be reliably assessed, as cell viability needs to be > 70%. Substances that interfere with the luciferase enzyme can affect the luciferase activity either by increasing (e.g. phytoestrogens) or inhibiting the luminescence. Therefore, the European Chemicals Agency has published guidance for use of the above described assays. For the LuSens assay and the DPRA, the information obtained should be used in combination with other information within a weight-of-evidence approach, and not as a standalone test method for fulfilling REACH information requirements. The DPRA, however, can be used for potency categorisation of a skin sensitising substance within ITS DA.[37]

The current adverse outcome pathway (AOP) for skin sensitisation describes four key events: i) covalent modification of epidermal proteins; ii) keratinocyte activation; iii) dendritic cell activation; and iv) T-cell response. The hapten (and pro-hapten) model has been described and accepted for the molecular explanation of skin sensitisation induced by chemicals. Chemicals may form hapten–carrier complexes *in vivo* by direct chemical covalent interaction with a soluble or cell-bound protein, by biotransformation to form a reactive metabolite able to bind to a carrier protein, or by degradative changes to the parent molecule forming reactive groupings. Further advanced investigations, however, complement the (pro)-hapten model and suggest more mechanisms to be included, as the (pro)-hapten interactions may not be sufficient as the sole molecular explanation for chemically induced hypersensitivity. Modified proteins have been already described to be immunogenic not only for T-cells, but also for B-cells, inducing a humoral response, potentially even leading to

autoimmune responses. Notably, growing evidence has suggested that certain chemicals may elicit new mechanisms leading to immune system induction, such as the p-i concept, describing the production of novel antigens presented to T-cells via the (allele specific) HLA system and the binding of non-reactive chemicals to immune receptors, particularly T-cell receptors (TCR). Hapten-independent mechanisms have been investigated and described, including: noncovalent MHC interactions; alternative or newly identified signalling pathways; newly described molecular mechanisms underlying hapten recognition by T-cells; conversion of pro-haptens into haptens in various extrahepatic tissues with metabolic and immunological capacity; interactions with HLA class I molecules in a specific noncovalent manner, leading to the presentation of altered peptides; and mechanisms of autoimmunity induced by xenobiotics.[38–49] If hapten-independent mechanisms are broadly confirmed by reproducible data, additional *in vitro* assays may have to be developed to cover the induction of sensitisation through, for example, interaction with HLAs and selected hapten-independent mechanisms.[35,36]

To summarise the results of skin sensitisation obtained in our study, the *in chemico* DPRA predicted skin sensitisation potential for Samples 5, 6, 7 and 8 (Figure 2). At a concentration of 250 µl/ml, Samples 5, 6, 7, 8 showed potential positive reactivity with the cysteine peptide. Of these, Sample 5 showed the highest reactivity, Sample 7 showed lower reactivity, and the results for Samples 6 and 8 were both lower but similar. Thus, the highest level for cysteine peptide depletion was obtained with Sample 5. Such a result could be appropriate justification for assigning the product to a higher reactivity class — however, the composition of the finished cosmetic product must also be further carefully considered. According to the composition of the perfume, one of the constituents is a zinc compound (zinc ricinoleate). As mentioned above, and also according to the Test Guideline OECD 442C,[15] the DPRA is not applicable for the testing of metal compounds, since they are known to react with proteins through mechanisms other than covalent binding. Thus, based on the zinc content in Sample 5, the DPRA result was evaluated as a false positive — indeed, this positive result was not confirmed by further testing in the LuSens assay. Therefore, in the DPRA, Samples 6, 7 and 8 were finally predicted as positive, as they exhibited a percentage peptide depletion higher than the cut-off value (13.8%). The rest of the samples at 250 µl/ml, and all the samples at 50 µl/ml, showed very low (< 13.8%) or no reactivity. Thus, they could be predicted as negative (non-sensitisers) by the DPRA. However, these results should be further verified, as the DPRA should not be used as a stand-alone assay.

In our study, the predictions obtained with the DPRA were further verified by using the LuSens *in vitro* assay. Both assays predicted Samples 6, 7 and 8 as being positive, showing a positive and/or borderline result for at least two concentrations evaluated in the LuSens assay and for one concentration evaluated in the DPRA. Sample 9 might be evaluated as a very weak or suspected sensitiser, as it was negative in the DPRA and positive in LuSens at one concentration only. Skin sensitisation potential was detected with the LuSens assay at three concentrations (at 12.5 µg/ml for Sample 6, at 50 and 100 µg/ml for Sample 7, and at 100 µg/ml for Samples 8 and 9; Figure 3). Sample 2 was considered negative in the DPRA and, due to its high cytotoxicity, only very low concentrations (< 25 µg/ml) could be used for its evaluation in the LuSens assay, which generated negative results (Figure 4). As mentioned above, the total allergen content was the highest in Samples 7 and 8, which were predicted as skin sensitisers with low reactivity in both assays (DPRA and LuSens). However, while the allergen content of Sample 6 was lower than the average and median values (2.31% and 2.44%, respectively), the product exhibited moderate reactivity with the cysteine peptide and also gave the highest response in the LuSens assay. Apparently, the content of specific substances with a particular affinity and reactivity for proteins may become another indicator for suspected skin sensitisation potential, whereas new mechanisms and interactions should be considered in line with the recent scientific investigation (for example, binding to specific molecules such as HLAs, etc.). Thus, in order to screen the safety of cosmetic products with regard to skin sensitisation, a sophisticated combined approach seems to be the most appropriate — perhaps making good use of more recently developed *in vitro, in chemico and in silico* methods (e.g. ADRA, H-Clat, U-Sens, GARD, SENS-IS, mMUSST, OECD QSAR Toolbox, EpiSensA, etc.) with a special focus on the sensitising potential of individual allergens, as well as on the activity of defined combinations, as described in OECD TG 442E[50] and in other relevant OECD guidelines.[16,51]

Regarding the total content of all allergens, the lowest total concentration (0.44%) was found in Sample 4 (see Table 5). Sample 4 exerted a biological effect only in one case (medium cytotoxicity). Samples 7 and 8 had the highest total content of allergens (4.0% and 4.5%, respectively), and were also found to have the lowest NOAEL values (Table 7). Despite this promising general association between the total allergen content of Samples 7 and 8 and the high frequency of adverse biological effects exerted by each (i.e. four out of eight possible effects tested), it was decided that a more targeted approach was needed. This approach focused on the specific reported toxicological properties of the individual allergens present in the samples, with the aim of obtaining more precise information on any observed effects. Any outlier concentrations of these individual allergens, as compared to their concentrations in the other samples, were also carefully considered.

The substance, 2-(4-tert-butylbenzyl)propionaldehyde (BMHCA, lysmeral/lilial; CAS No. 80-54-6, with

the INCI name of butylphenyl methylpropional), has been recently reassessed, on the basis of some newly-identified toxicological properties. This substance is a fragrance ingredient historically used in many cosmetics (e.g. deodorants, make-up products, face and hand creams, body lotions, hair styling products, soaps and shower gels, shampoos, fragrances), as well as in non-cosmetic products (e.g. household cleaners and detergents, biocidal products, coatings and paints, inks, polishes, and scented articles such as clothes, toys and paper items). Despite its previous widespread use in consumer products, the substance has been recently classified by the ECHA Risk Assessment Committee (RAC) as reprotoxic 1B (listed in Annex VI as a reprotoxic category 1B substance).[1]

The Scientific Committee on Consumer Safety has previously concluded that butylphenyl methylpropional is not considered safe for use in cosmetics,[52] and that any exception for its use was unjustified. In addition to being recently classified as toxic to reproduction, a majority of data submitters agree that the substance is skin sensitising. Moreover, the substance has been under assessment as a potential endocrine disrupter in the ECHA database.[53] Cosmetic products and their ingredients represent poorly defined mixtures. Within these mixtures, various combinations of ingredients can lead to a range of adverse biological effects arising from the use of the finished product. Thus, it is unfortunately difficult to identify the individual ingredient that is responsible for any such effect in this scenario. Therefore, we support the recommendations that the potential to cause adverse health effects through sensitisation should be evaluated by a combination of biological *in vitro* methods,[27] in order to obtain relevant mechanistic data. In the case of a positive result, further human relevant non-animal toxicological evaluation may be performed, considering the composition of the finished product, the toxicological evaluation of the individual ingredients, and the bioavailability related to the intended use and exposure, etc.

Butylphenyl methylpropional was detected in some of the samples, which can be listed as follows (in ascending order of compound concentration): 4, 10 (both below the LOQ) < 9 < **6** < 2 < 3 < 1 < **8** < 5 < **7**. Three of the samples (in bold font), were positive for skin sensitisation potential. Eight out of the ten samples (i.e. not Sample 3 or Sample 4) were positive in at least one endocrine assay.

Hydroxyisohexyl 3-cyclohexene carboxaldehyde was detected only in Sample 2. This sample exhibited high cytotoxic toward the LuSens cell line, was positive in the anti-oestrogenic assay and contained the highest number of individual allergens. Moreover, Sample 2 also had the highest concentrations of citronellol, geraniol, farnesol and benzyl benzoate. These results support our proposed targeted approach, which focuses on specific ingredients, and demonstrates the importance of such approaches for a more precise hazard prediction resulting from biological interactions with specific ingredients in the finished cosmetic product.

Endocrine disruption

The results of the endocrine disruption assays indicate that the extent of endocrine activity may be attributed either to the total content of active substances, or to the presence of specific substances and their possible synergic/additive or potentiating effects. With regard to endocrine disruption, the results can be briefly summarised as follows: Sample 6 was positive in the oestrogenic assay; Samples 2 and 10 were positive in the anti-oestrogenic assay; and Samples 1, 2, 5, 7, 8 and 9 were positive in the anti-androgenic assay. The anti-androgenic activities of these samples should be noted and further investigated, as they appeared to occur rather extensively. As mentioned above, a relatively high total content of allergens was detected in Samples 1, 2, 5 and 9, with Samples 7 and 8 having the highest total content of allergens. This suggests a significant correlation between anti-androgenic activity and the total allergen content. However, as noted earlier, it is problematic to find an association between endocrine disrupting activity and the content of a specific individual ingredient. For instance, in Samples 6, 7 and 10, a high content of coumarin (2100, 1200 and 2000 μg/g, respectively) was detected, as compared to the other samples. However, it would be premature to make any direct association between this substance and the endocrine disrupting activity of these three samples because, for example, Sample 7 also had the highest concentration of butylphenyl methylpropional (9700 μg/g) and hydroxycitronellal (3500 μg/g).

In Samples 1, 5 and 9, a higher content of benzyl salicylate (4400, 8000 and 7200 μg/g, respectively) was detected, whereas Samples 1, 5, 7 and 8 contained two orders of magnitude higher concentration of butylphenyl methylpropional (4600, 9200, 9700, 3800 μg/g, respectively), as compared to the other samples.

Sample 2 was the only sample containing hydroxyisohexyl 3-cyclohexene carboxaldehyde (270 μg/g), farnesol (4300 μg/g), cinnamyl alcohol (34 μg/g) and benzyl cinnamate (41 μg/g), whereas the concentrations of benzyl benzoate (17,000 μg/g), geraniol (3000 μg/g) and citronellol (3100 μg/g) were outlier values in this particular sample. Additionally, Samples 7 and 8 were atypical in terms of their high linalool content (14,000 and 19,000 μg/g, respectively), and Samples 1, 8 and 9 were found to contain the highest concentration of D-limonene (12,000, 17,000 and 16,000 μg/g, respectively).

Thus, these assay results and the GC-MS/MS analyses may indicate a possible specific link between the total allergen content and endocrine disrupting activity. Again, the results emphasise the importance of our proposed targeted approach, which could be a part of further research with respect to the content of specific compounds, their individual endocrine disrupting activity, as well as the activity of their combinations in this respect.

Genotoxicity

Another biological effect that was assessed in our study was genotoxicity. The *in vitro* Comet assay, with 3T3 Balb/c fibroblasts, did not detect any significant genotoxic potential at the selected non-cytotoxic concentrations (i.e. > 10% increase of % DNA in the tail at two consecutive non-cytotoxic concentrations). However, a concentration-dependent fragmentation of DNA, which was considered to be statistically significant, was detected with Samples 6, 8, 9 and 10 (Table 2). Thus, additional *in vitro* analysis of these particular samples was performed by using the validated *in vitro* mammalian chromosome aberration test, in compliance with OECD TG 473,[23] to confirm any genotoxic effects. However, no genotoxic potential was observed for any of these samples in this second assay (Table 3). Future research may focus on testing a wider range of concentrations for DNA fragmentation potential.

NOAEL values

The values of the individual NOAELs, with a preference for data from *in vivo* studies in rats, oral route and 90-day study, were retrieved from ECHA's publicly available database. Where insufficient data were available based on these preferred criteria, alternative information was retrieved preferentially from the same ECHA database, although other reliable sources were used in certain cases (see Table 6 for further details). The lack of data in some cases, alongside the new and ongoing submissions to the database, have to be carefully considered when obtaining the NOAEL values for comparative purposes. Thus, the approach used in this pilot study should be seen as demonstrational, indicating how it is possible, in theory, to work with available (i.e. historical) data and use them for the assessment of mixtures.

Regarding the estimation of NOAEL values for the finished products, two versions of the calculation were performed — i.e. either including or excluding the value for EtOH — and the corresponding values were compared (see Tables 7 and 8) and the samples sorted according to the NOAEL of the finished product: EtOH value excluded (descending order from the highest value): 4 > 10 > 9 > 6 > 3 > 2 > 1 > 7 > 8 > 5; EtOH value included (descending order from the highest value): 4 > 10 > 9 > 6 > 3 > 2 > 1 > 7 > 8 > 5. Obviously, the content of EtOH (or any other solvent) in a finished product should always be considered in order to obtain more precise results, as it may affect the value of the NOAEL. However, in our study, after updating the NOAEL values of individual constituents carefully, EtOH had no or minimal impact on the sorting order of samples with regard to their final NOAELs. Thus, in this study, EtOH did not appear to have a particularly strong effect in this respect.

To summarise the results obtained for the estimated NOAEL values of the ten finished products, Samples 5, 8 and 7 had the lowest final NOAEL values, and Samples 4, 10 and 9 had the highest (see Tables 7 and 8). These findings are in agreement with the other criteria assessed in this study, except for the extent of cytotoxicity and EtOH content. With regard to the content of specific allergens, Sample 5 contained high concentrations of alpha-isomethyl ionone (4900 µg/g), butylphenyl methylpropional (9200 µg/g) and benzyl salicylate (8000 µg/g) as compared to the other samples. This influenced the estimation of the final NOAEL value, as the individual NOAEL values for two of these allergens were the lowest (4 mg/kg for alpha-isomethyl ionone, 25 mg/kg for butylphenyl methylpropional) and also the individual NOAEL value for benzyl salicylate was relatively lower (360 mg/kg).

The authors claim that their approach represents an in-house example of a pilot NOAEL estimation as a part of research work based on available information in the literature, and does not represent any official risk assessment results or verified regulatory approach. Expert approaches verified or recommended directly by regulatory authorities, and guidance on how to derive NOAEL values of final cosmetic products or defined mixtures, as well as further expert guidance in this field for risk assessment of less defined mixtures, will be most appreciated.

Results round-up

The overall results from this study are summarised in Table 8. Sample 4 may be evaluated as the safest, based on its estimated final NOAEL. This conclusion is supported by the lowest frequency of adverse biological effects exerted (only one case, i.e. medium cytotoxicity), and by the results obtained in the GC-MS/MS analysis, which showed that Sample 4 contained the lowest concentration of allergens, the lowest number of individual allergens, and the lowest concentration of butylphenyl methylpropional. The parameters that were evaluated as 'average' for Sample 4, and not the 'safest' or 'lowest', were the concentration of EtOH and the extent of cytotoxicity. Thus, cytotoxicity and EtOH concentration do not seem to be precise indicators of safety, especially if used alone, as our pilot study shows that many more characteristics, parameters and endpoints need to be evaluated.

When evaluating the results obtained for the products that were proposed to be the 'least safe', specific criteria and combinations of indicators should be carefully considered. In the current study, the highest frequencies of adverse biological effects were exerted by Samples 6, 7 and 8 (four adverse effects each) and Sample 2 (three adverse effects each). It is notable that, of these particular samples, Sample 2 contained the highest number of individual allergens (18 allergens), as detected by GC-MS/MS, followed by Samples 6 and 7 and 10 (13 allergens). With regard to the total allergen content, the highest percentage concentrations were detected in Samples 7 and 8 (4.0% and 4.5%, respectively), and higher numbers than the median value (2.42%) or the average value (2.31%) were detected in Samples 1, 2, 5, 7, 8 and 9.

When the samples were sorted in descending order, based on the NOAEL values (from the highest ('safest') value), Samples 1, 2, (3), 5, 7 and 8 were repeatedly placed in the second (i.e. lower, 'less safe') half of the sequence. Thus, attention obviously needs to be paid to the specific allergens detected in these samples, especially to those with low individual NOAEL values. For example, Samples 1, 3, 5, 7 and 8 typically had higher concentrations of butylphenyl methylpropional (with the individual NOAEL value 25 mg/kg), and Samples 1, 2, 5, 6, and 8 had higher concentrations of alpha-isomethyl ionone (with the individual NOAEL value of 4 mg/kg), as compared to the other samples (see Tables 5 and 8). Compared to the other samples, we could conclude that Samples 1, 2, 5, 6, 7 and 8 may be evaluated as being less safe, based on either lower values of the estimated final NOAEL, or the higher frequency of biological effects, or the higher content (or number) of allergens, or the content of allergens with lower individual NOAEL values.

Conclusions

The primary aim of our pilot study was to demonstrate the usefulness of a range of *in chemico* and *in vitro* methods for hazard prediction and risk assessment of final (perfume) cosmetic products. The endpoints included were: cytotoxicity, detected with 3T3 Balb/c fibroblasts (ISO 10993-5); skin sensitisation potential, determined with the *in chemico* DPRA method and with the *in vitro* LuSens assay (based on human keratinocytes); genotoxicity, assessed with the *in vitro* Comet assay in 3T3 Balb/c fibroblasts and the chromosome aberration test; and endocrine disruption, evaluated by means of the commercially available YES/YAS assay. These methods were used in combination with the targeted analysis of selected known allergen content by GC-MS/MS. NOAEL estimation was also included in the study, for the prediction of safety of the cosmetic fragrance-based products tested, which represent chemical mixtures containing a number of active substances at very low concentrations.

With regard to the use of non-animal alternative approaches, one objective of our pilot study was to demonstrate the potential benefits and exploratory power of methods coming from a wide range of toxicological disciplines, for use as alternatives to animal experimentation. Unfortunately, it remains difficult to compete with conventional *in vivo* toxicology testing based on the use of laboratory animals, which is persistently supported by some regulators, despite the EU ban on the animal testing of cosmetics.

Another goal of the current study was to contribute new insight into the safety evaluation of cosmetic products and their ingredients. The NOAEL values of the finished products were estimated, based on the NOAEL values of the detected individual allergens, and calculated with or without inclusion of the value for EtOH. When the products were sorted according to their estimated NOAEL values, with and without EtOH, the rankings were satisfactorily correlated. The estimated NOAEL values also correlated satisfactorily with the frequency of the observed *in vitro* biological adverse effects, the total content of allergens, the number of individual allergens in the formulation, and the presence of specific allergens.

The combination of *in chemico* and *in vitro* methods, targeted GC-MS/MS analysis and estimation of NOAEL values, appears to represent a promising screening tool for hazard prediction and safety assessment of mixtures of allergens in finished cosmetic products. Good correlation was observed between the results obtained from the various methods. The estimated NOAEL values of the finished products seem to be promising indicators of safety, when supported by the results of several other parameters (i.e. the frequency of adverse biological effects, the total allergen content, the number of individual allergens in the finished products, and the content of certain substances with low individual NOAEL values, such as butylphenyl methylpropional or alpha-isomethyl ionone).

We conclude that our suggested approach should be further developed and optimised, for the more accurate prediction of potential hazards and safety assessment of fragrance-based cosmetic products. Specifically chosen biological *in vitro* methods should be included in a test battery, based on known adverse outcome pathways (AOPs) and molecular mechanisms leading to adverse effects. Such an AOP-based approach could uncover new toxicological mechanisms, and provide valuable mechanistic data that may contribute to the clarification of mechanisms and specific interactions between the individual ingredients in the finished cosmetic product. Individual interactions between ingredients may result in an overall adverse biological response, even though the concentrations of the individual components are considered safe.

The use of advanced human-relevant toxicological approaches *in vitro* (i.e. without involving the use of laboratory animals), prior to testing in human volunteers, is not only ethically acceptable, but also in full accordance with the precautionary principle. Here, a battery of selected *in vitro* biological assays was demonstrated to be positively beneficial in the screening of cosmetic products and their ingredients for

toxicological properties. Thus, it could be potentially used as a basis for the further development of combined toxicological approaches in the field of public health and consumer protection.

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Appendix

Table A1. Key abbreviations used in the paper.

ACN	Acetonitrile
AOP	Adverse Outcome Pathway
CA	Cinnamic aldehyde
CLP	Classification, Labelling and Packaging
DPRA	Direct Peptide Reactivity Assay
EC	European Commission
EDC	3–5% fragrance eau de cologne
EDP	10–15% fragrance eau de parfum
EDT	5–10% fragrance eau de toilette
EtOH	Ethanol
GC-MS/MS	Gas Chromatography-Tandem Mass Spectrometry
HPLC	High-performance Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MAS	Metabolic Activation Stimulation
MSD	Mass Spectrometer Detector
N	Negative
NC	Negative control
NOAEL	No Observed Adverse Effect Level
NR	Neutral Red
OECD	Organisation for Economic Co-operation and Development
P	Positive
PC	Positive control
PBS	Phosphate Buffered Saline (pH 7.5)
RAC	Risk Assessment Committee
RFU	Relative Fluorescence Unit
RLU	Relative Light Unit
ROS	Reactive Oxygen Species
SD	Standard Deviation
SRM	Selected Reaction Monitoring
VC	Vehicle control
YES/YAS	Yeast Estrogen Screen/Yeast Androgen Screen