

MESTRADO
CONTROLO DA QUALIDADE

Toxicological Interactions of Food Contaminants in Neurotoxicity *in Vitro*

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M
2023



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**Toxicological Interactions of Food Contaminants in
Neurotoxicity *in Vitro***

Dissertação do 2º Ciclo de Estudos Conducente ao
Grau de Mestre em Controlo da Qualidade

Trabalho realizado sob a orientação de:
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September 2023

DECLARAÇÃO DE REPRODUÇÃO

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Agradecimentos

Esta dissertação não teria sido possível se não fossem as pessoas que me acompanham tanto na vida pessoal como na vida académica.

Em particular, agradeço ao Professor Miguel por todo o apoio e orientação durante este percurso. Agradeço muito a sua disponibilidade e abertura para discutir ideias que me fez crescer e abraçar o meu espírito crítico e curiosidade.

Agradeço ainda à Professora Isabel pela sua orientação e oportunidade de trabalhar e fazer parte da sua equipa.

Um agradecimento especial para a Mafalda, a Marta e a Margarida, cujo otimismo e boa disposição são um essencial para trabalhar no laboratório. Muito obrigada por me terem acolhido tão bem e por todos os vossos conselhos e apoio.

No meu círculo interno, deixo o meu maior obrigada aos meus amigos por estarem ao meu lado, em particular à Manuela por ser tão paciente comigo.

Ao Rui, o meu maior pilar durante estes dois anos. Obrigada por aturares a minha insanidade mental, te rires comigo das minhas desgraças, mas especialmente por sempre teres acreditado em mim e garantido que não desistia mesmo quando a vida se complica.

À minha querida mãe, obrigada pelo carinho e por estares sempre do meu lado. Dedico este documento ao pai.

Resumo

A alimentação e fatores ambientais têm sido apontados como fatores de risco das doenças neurodegenerativas (DNs). A dieta é uma frequente fonte de contaminantes de diferentes classes que exibem neurotoxicidade. Embora, estes contaminantes existam em concomitância na alimentação humana, estudos científicos relativamente aos efeitos combinados de contaminantes são escassos. O objetivo deste trabalho refere a avaliar quantitativamente as interações entre contaminantes alimentares de diferentes classes em células de neuroblastoma humanas (SH-SY5Y). A citotoxicidade individual da acrilamida (ACR), aflatoxina B1 (AFB1), benzo[a]pireno (BaP), cádmio (Cd) e cipermetrina (CYP) foi determinada após 72 horas por ensaio de MTT. As combinações ACR/AFB1, ACR/Cd, AFB1/Cd, ACR/AFB1/Cd em rácios equipotentes e CYP/ACR, CYP/AFB1, CYP/Cd em rácios constantes não equipotentes foram avaliadas através dos respetivos índices de combinações (IC) calculados por duas abordagens: o modelo de Chou-Talalay (CT) e de CISNE. Citotoxicidade de uma mistura de contaminantes realística simulada a partir de uma dieta e contaminantes metabolizados com fração humana S9 foi também alvo de estudo, bem como a expressão genética de BAX, BCL2, CASP3, NF- κ B, p and MAPT. Toxicidade individual dos compostos quando em contacto com SH-SY5Y podem ser ordenados da seguinte forma Cd > AFB1 > CYP > ACR > BaP. As concentrações pertinentes para a exposição alimentar (IC5), a maioria das combinações binárias exibe um comportamento antagónico (IC>1), com exceção da combinação ACR/AFB1 que é considerada aditiva/moderadamente sinérgica pelos modelos CT e CISNE, respetivamente. Globalmente, para as restantes combinações binárias, o modelo de CISNE calcula valores de CI mais conservadores do que CT para efeitos extremos. Em doses baixas cujo efeito é próximo de zero, os modelos apresentam um veredicto substancialmente diferencial sobre a mistura AFB1/Cd: CT considera a interação sinérgica (IC de 0,51), enquanto CISNE estima antagonismo moderado (IC de 1,38). Adicionalmente, uma mistura complexa de contaminantes simulada a partir uma dieta (MIX) causou uma inibição

da viabilidade celular em 10% para o percentil 95 (P95). Os resultados da citotoxicidade dos contaminantes metabolizados, revelam que os metabolitos podem exibir efeitos neurotóxicos consideráveis. Por último, a expressão genética de BAX, BCL2, CASP3, NF- κ B e MAPT foi avaliada para contaminantes individuais e misturas selecionadas, observando-se alterações em relação aos controlos experimentais. Notavelmente, a MIX P95 aumenta a expressão de NF- κ B, resultado concordante com o estado crónico de inflamação induzido por diferentes contaminantes e relacionado com múltiplas doenças crónica. De modo geral, os resultados obtidos destacam a necessidade de investigação em relação à exposição de contaminantes alimentares em contexto de neurotoxicidade e a necessidade de um guia para a avaliação das interações toxicológicas de substâncias consideradas de risco para a saúde humana.

Palavras-Chave: neurotoxicidade, células SH-SY5Y, contaminantes alimentares, interações, método Chou-Talalay, método CISNE, avaliação de risco

Abstract

Diet and environmental factors are seen as potential risk factors for neurodegenerative diseases (NDs). Diet is a frequent source of exposure to contaminants from different classes that exhibit neurotoxicity. Although, these contaminants co-occur in human diet, there is a lack of scientific studies portraying the neurotoxic effects of combinations of contaminants. The main goal of this work is to quantitatively evaluate interactions between contaminants of different classes in SH-SY5Y neuroblastoma human cells. Individual toxicity of acrylamide (ACR), aflatoxin B1 (AFB1), benzo[a]pyrene (BaP), cadmium (Cd) and cypermethrin (CYP) was performed through MTT assay at 72 hours. Combinations ACR/AFB1, ACR/Cd, AFB1/Cd, ACR/AFB1/Cd at equipotent constant ratios, and CYP/ACR, CYP/AFB1, CYP/Cd at non-equipotent constant ratios were assessed through calculations of combination indexes (CI) using two approaches the Chou-Talalay model (CT) and CISNE. A complex realistic mixture of contaminants simulated from a diet and S9 fraction metabolized contaminants cytotoxicity was also assessed. Gene expression of BAX, BCL2, CASP3, NF- κ B, and MAPT was evaluated for individual contaminants and selected mixtures. Individual contaminant toxicity rank in SH-SY5Y was Cd > AFB1 > CYP > ACR > BaP. At pertinent levels for food exposure (IC₅₀), most binary combinations are antagonistic (CI>1), with exception of ACR/AFB1 combination which is considered additive/moderate synergistic via CT and CISNE model, respectively. Globally for the other combination pairs, CISNE computes more conservative CI values than CT at extreme effects. Additionally, a complex realistic mixture of contaminants simulated from a diet caused a 10% inhibition of cell viability at the 95th percentile (P95). The results of in vitro metabolism of food contaminants reveal that metabolites can exhibit considerable neurotoxic effects. Finally, the gene expression of BAX, BCL2, CASP3, NF- κ B, and MAPT was evaluated for individual contaminants and selected mixtures, observing alterations in relation to controls. Notably, MIX P95 increases the expression of NF- κ B, which is concordant with the chronic state of inflammation induced by different contaminants

and associated with multiple non-communicable chronic diseases. Overall, these findings underscore the need for further research to comprehensively understand the potential neurotoxicity risks associated with exposure to multiple contaminants, with implications for proper risk assessment, and the need for guidance documents on evaluation of interactions of substances with toxic potential to human health.

Keywords: neurotoxicity, SH-SY5Y cells, food contaminants, interactions, Chou-Talalay method, CISNE model, risk assessment

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List of Abbreviations

Aβ	β -amyloid
AAMA	<i>N</i> -acetyl- <i>S</i> -(2-carbamoylethyl)cystein
ACR	Acrylamide
AD	Alzheimer's disease
AFB1	Aflatoxin B1
AFBO	Aflatoxin B1-8,9-epoxide
AFM1	Aflatoxin M1
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
ATSDR	Agency of Toxic Substances and Disease Registry
BBB	Blood brain barrier
BaA	Benz[<i>a</i>]anthracene
BaP	Benzo[<i>a</i>]pyrene
BbF	Benzo[<i>b</i>]fluoranthene
CA	Concentration addition
CASP3	Caspase-3
CI	Combination index
CISNE	Code for the identification of synergism numerically efficient
CM	Complete cell culture medium
CNS	Central nervous system
CONTAM	Panel on Contaminants in the Food Chain
CT	Chou-Talalay
CYP	Cypermethrin
Chr	Chrysene

dbcAMP	Dibutyryl cyclic adenosine monophosphate
DNA	Desoxyribonucleic Acid
DNT	Developmental neurotoxicity
DON	Deoxynivalenol
DRI	Dose-reduction index
EC	European Commission
EFSA	European Food Safety Authority
EPA	U.S. Environmental Protection Agency
FB1	Fumonisin b1
GA	Glycidamide
GAMA	<i>N</i> -acetyl-S-(2-hydroxy-2-carbamoylethyl)cystein
GST	Glutathione-s-transferases
HD	Huntington's disease
Hb	Hemoglobin
IA	Independent action
IARC	International Agency for Research on Cancer
IC	Inhibitory concentration
isoGAMA	<i>N</i> -acetyl-S-(1-carbamoyl-2-hydroxyethyl)cystein
LMIC	Low and middle-income countries
MAPT	Microtubule associated protein tau
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NAMs	New approach methodologies
NCDs	Non-communicable diseases
NDs	Neurodegenerative diseases
NFTs	Neurofibrillary tangles
OECD	Organization for Economic Cooperation and Development
OTA	Ochratoxin A
PAHs	Polycyclic aromatic hydrocarbons
PD	Parkinson's disease
qPCR	Quantitative polymerase chain reaction
RASFF	Rapid alert system for food and feed
ROS	Reactive oxygen species

SEM	Standard error mean
T2	T-2 toxin
TAE	Tris-Acetate-EDTA
TPA	12-o-tetradecanoyl-phorbol-13-acetate
ZEN	Zearalenone

Chapter 1

Background

Dementia is currently the seventh leading cause of death among all diseases and one of the major causes of disability and dependency among older people globally (1). In 2015, almost 50 million people worldwide grappled with the debilitating consequences of dementia, and the number is expected to surpass 130 million people by 2050 (1).

While age is the most decisive known risk factor for cognitive decline, dementia is not an inevitable consequence of aging. The Lancet Commission on dementia prevention, intervention, and care states that modifiable risk factors account for almost 40% of the risk of developing dementia (2). Consider Alzheimer's disease (AD) as an illustrative case: accounting for over 70% of dementia cases, the majority of these are so-called sporadic, with a minimal genetic contribution. (2, 3). Potentially modifiable risk factors include physical inactivity, consequences of traumatic brain injuries and several non-communicable diseases (NCDs) like depression, hypertension, diabetes, hearing issues, mid-life high cholesterol levels and obesity, which are connected to the risk of cognitive impairment and dementia (2). Furthermore, environmental influences such as outdoor and indoor air pollution exposure and lifestyle factors like tobacco and alcohol use and unhealthy diets are gaining more recognition for their role (2).

Despite the multifactorial etiology of neurodegenerative diseases (NDs), there is evidence that dietary and environmental factors, namely contaminants, play a relevant role in their pathogenesis and progression. Commonly widespread contaminants from environmental and dietary sources like acrylamide, heavy metals, dioxins, pesticides, mycotoxins and polycyclic aromatic hydrocarbons, among others, have been associated with neurological disorders, both indirectly or directly, as referenced in (4–8). Heavy metals and pesticides

have been the center of attention concerning the relation between NDs and contaminants (7). Both connect to a higher risk of developing NDs from occupational exposure. Moreover, mycotoxins are naturally occurring contaminants produced by fungal species that contaminate mainly cereal crops and exposure has been associated with cognitive alterations (9–11). Fast-paced society favors processed and ultra-processed food consumption that has a detrimental impact on health, being associated with non-communicable diseases like cardiovascular diseases, obesity and diabetes, underlying cause of NDs (12). Moreover, food processing often leads to high concentrations of harmful substances, such as acrylamide in carbohydrate rich foods, or heterocyclic aromatic amines and polycyclic aromatic hydrocarbons during meat processing. These substances cause oxidative stress and inflammation, pathways connected to dementia risk and cognitive decline (13).

Furthermore, it is essential to consider that exposure to a combination of contaminants, a scenario frequently encountered in food, may elicit a higher degree of toxicity compared to when these components are individually examined (14). Permissible contaminant levels structured by regulatory authorities do not account for potential combined exposure synergisms, and co-exposure to contaminants has not been properly and comprehensively addressed regarding NDs. Thus, these molecules toxicity is usually undermined. Many different approaches have been proposed to evaluate and predict mixture effects. From a regulatory perspective, several guidance documents have been recently published and provide a strategy for mixture risk assessment based on frameworks to investigate potential synergistic effects (15). However, some methodological aspects remain insufficiently defined, with a multitude of mathematical models to choose from and a lack of standardized definitions for antagonism and synergism, making it difficult to come to harmonized regulatory conclusions on antagonistic/synergistic claims (14, 16).

1.1 Aim and objectives

Overall, this dissertation seeks to provide empirical data and insights into the complexities of contaminant interactions, ultimately contributing to more accurate and comprehensive risk assessments in the field of neurotoxicity induced by exposure to ubiquitous diet-related contaminants.

Therefore the aim is to assess the neurotoxic effects of acrylamide (ACR), aflatoxin B1

(AFB1), benzo[a]pyrene (BAP), cadmium (Cd) and cypermethrin (CYP) in human neuroblastoma cells both individually and in combination. More specifically:

- To assess the **isolated effect** of the contaminants in SH-SY5Y cells and its effect after **metabolization** with human liver S9 fraction.
- To evaluate and **quantify interactions** between contaminants through application of the combination index method: CISNE model and Chou-Talalay (CT) model.
- To assess the **effect of a low-dose** real proportion mixture in neuronal viability.
- To evaluate the effect of multiple scenarios of exposures to contaminants in terms of **genes expression** regarding apoptosis pathways and neuronal markers.

Chapter 2

Introduction

This chapter will begin by addressing neurodegeneration and the underlying cellular mechanisms that establish a nexus between contaminants and the processes leading to neurodegeneration. Cell-based models are also introduced as invaluable tools for dissecting neurodegenerative processes. The following sections will address several prominent classes of food contaminants (process-induced contaminants, mycotoxins, polycyclic aromatic hydrocarbons, metals and pesticides), focusing on a representative compound relevant to this dissertation. These sections will also detail occurrence in food, human exposure, metabolism and known neurotoxic effects. The final section infers on combination studies framework since investigating the synergistic, additive or antagonistic interactions between multiple food contaminants will provide a more nuanced understanding of the risks posed by complex mixtures.

2.1 Neurodegeneration and neurotoxicity

Dementia is an umbrella term for several diseases that affect memory, other cognitive abilities and behaviour that interfere significantly with a person's ability to maintain their activities of daily living. Neurodegenerative diseases (NDs) refer to a group of neurological conditions with diversified clinical and pathological manifestations affecting a specific subset of neurons and synapses in distinct brain regions (17). Within this group, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) are the most studied (17).

Neurodegeneration covers the progressive loss of structure or function of neurons, which

leads to the cumulative decline of cognitive and motor function (18). By the time NDs are medically acknowledged, the condition is already well-established in the brain, underpinning degeneration of neuronal circuits and protein accumulation as the significant histopathologic changes in a neurodegenerative brain (17).

There is a growing concern regarding the increasing prevalence of dementia, particularly in low and middle-income countries (LMIC), as these regions witness population ageing at a faster rate than high-income countries and higher frequency of potentially modifiable risk factors (1, 2). These are also the countries in which the so-called exposome have a greater impact, meaning that social inequalities and psycho-social influences, in addition to chemical, biological and physical exposures impact the pathogenesis and progression of NDs. The exposome investigates the role played by all exposures that individuals undergo in their lives in the development of non-communicable diseases (NCDs) (19). For example, it is now widely recognized that systemic chronic inflammation triggered by social and environmental factors is a critical etiological element of many NCDs associated with older age, including NDs (19, 20).

Since neurons do not divide, the nervous system is particularly vulnerable to a lifetime of toxicants exposure, showing indistinct signs of disease later in life (4, 21). As age goes by the sensitivity to contaminants increases, due to impairments in cellular mechanism, and the burden on the detoxifying metabolism system relating to the concomitant exposure to toxicants can lead to a reduced cell capacity to neutralize toxic xenobiotics (22). Thus, the elderly may be more susceptible than are young adults to injury from lipid-soluble chemicals at the same exposure levels. It has been established that persistent organic pollutants are capable of bioaccumulation in adipose tissue, staying deposited for years, suffering a slow process of metabolism and elimination (22). Other lipophilic molecules, like phthalates and PAH and lipophilic mycotoxins, are eliminated from the body more rapidly, but their levels are maintained relatively constant due to continuous exposure through environmental sources or through diet that replaces the quantities lost during metabolism and excretion. A sequential toxicity phenomenon might also be at play: lipid-soluble xenobiotics present in serum help mediate permeation hydrophilic chemicals into the central nervous system (CNS) (6). This mechanism explains how low levels of a variety of environmental contaminants can induce ND (6). Simultaneously, early age exposure to contaminants, namely in the pre- and post-natal life period, can have severe consequences due to the increased susceptibility of the

developing system to alterations, predisposing to the onset of neurodegenerative diseases in later life (5, 23).

Within this intricate landscape between the exposome and NDs, the interplay between neurodegeneration and neurotoxicity emerges as a central focus of investigation. Neurotoxicity studies the adverse impacts of chemical or physical agents on the nervous system, resulting in impaired cognitive function, sensory disturbances, and behavioural abnormalities (4, 24).

Despite having different clinical features and histopathologic anomalies, NDs share basic cellular malfunctions that eventually cause neuronal toxicity and death. These components and processes are potential targets of contaminant neurotoxicity, initiating or perpetuating neurodegeneration through these pathways. Few epidemiological associations between contaminant exposures and neurological disorders have been outlined (9, 25), and growing evidence in cell-based and animal models has identified toxic effects of contaminants in cellular pathways and mechanisms associated with NDs that warrant further investigation (26–28).

One of the common features is the aggregation of misfolded proteins in specific regions of the brain. These protein aggregates include β -amyloid ($A\beta$) plaques in AD, hyperphosphorylated tau in AD and other tauopathies, α -synuclein (α -syn) in PD and other synucleinopathies, TAR DNA-binding protein (TDP-43) in ALS and frontotemporal dementia, and polyglutamine protein aggregates in HD. Neurotoxicants might elicit neurodegeneration by inducing abnormal protein aggregation, contributing to the progressive nature of NDs (4, 24). For example, metals such as lead, mercury, aluminium, cadmium and arsenic, as well as some pesticides and metal-based nanoparticles are associated with enhanced $A\beta$ peptide formation and protein tau hyperphosphorylation, causing neuronal death (5, 23).

Beyond the specific protein accumulation and anatomic vulnerability that impair neuronal function, the neurodegenerative brain also deals with oxidative stress, mitochondrial impairment, abnormalities in the ubiquitin–proteasomal and autophagosomal/lysosomal systems, neuroinflammation and synaptic disruption (17, 18). Multiple neurotoxicants, such as acrylamide, heavy metals, pesticides generate oxidative stress by impairing antioxidant function and increasing reactive oxygen species (ROS) levels. The persistent alteration in the redox status of the cells impacts calcium signalling, proteostasis and mitochondrial function of neurons. Pesticides such as organophosphates, carbamates, and organochlorines, known

as anticholinesterases, induce neurotoxicity by disrupting cholinergic functions (24). Moreover, the presence of these aggressors and other molecules produced in response to their presence by neurons, can lead to microglial activation. This glial-mediated inflammation is known as a "double-edged sword" in NDs, as the chronic production of pro-inflammatory cytokines, that is mediated by neurotoxins, is associated with impaired immune functions and neuroinflammation, a causative factor of NDs (29).

2.2 Neuronal cell-based models

Current chemical testing heavily relies on small *in vivo* animal models, particularly rodents (30). Rodents are used in regulatory assessments of life stage-dependent neurotoxicity, such as developmental neurotoxicity (OECD 426) and adult neurotoxicity (OECD 424) (31). *In vivo* models are regarded as the gold standard in the appraisal of drug toxicity, as these systems incorporate all drug pharmacokinetic aspects, including absorption, metabolism, distribution and excretion (30). However, animal-based testing is constrained by ethical, experimental and financial considerations. These, along with the increasing exposure of the population to potentially neurotoxic compounds through various sources like food, air, water, and occupational settings, pressures the shift from traditional high-dose animal testing approaches towards a more mechanistic approach using *in silico* and *in vitro* model systems (20). This approach not only aligns with the growing concerns about animal welfare, but also promises faster, cost-effective, and more comprehensive evaluations of neurotoxic compounds. The use of these models is aligned with new approach methodologies (NAMs) that overview the mapping of toxicity pathways in human tissues through the compilation of mechanisms of action, and identification of critical pathway perturbations responsible for adverse effects (32). Once this goal is achieved, regulatory decisions will be based on preventing significant disruptions in key toxicity pathways *in vitro*, rather than relying on high-dose responses in animal studies and using correction factors to predict low-dose risks in humans.

Despite their inability to fully replicate the intricate complexity of the *in vivo* state, cell-based models prove invaluable in neurotoxicology (33). They assume a crucial role as primary screening tools, prioritizing compounds for regulatory assessment based on their capacity to induce damage to the nervous system. Moreover, cell-based models excel at

elucidating molecular toxicity mechanisms and identifying specific substance targets. This mechanistic understanding is crucial for unraveling the intricate relationship between neurotoxicants and the development of NDs. Currently, there is a wide variety of *in vitro* models for neurotoxicity and developmental neurotoxicity (DNT) based on primary cell cultures, immortalized cell lines and induced or embryonic stem cells. All these systems have advantages and disadvantages; the choice of one or the other must consider them and what endpoints want to be assessed.

Primary cell culture are primarily from rodent origin, and chemicals effects may be influence by interspecies differences. Among the human options, cell lines have a reduced capability to mimic early development and cytoarchitecture of the nervous system compared with stem cells derived cultures. Still, they have the advantage of having shorter and easier culture protocols and less variability between cultures. Human commercial cell lines are mostly immortalized cell lines derived from tumorigenic cells, that have the advantage over primary cell cultures of allowing unlimited and relatively easy culture, not subjected to the ethical concerns of human primary cell cultures (31, 34). The SH-SY5Y cell line is commonly used to study neurodegeneration, particularly in Parkinson's research, and to evaluate the impact of chemical pollutants on the nervous system.

The SH-SY5Y cell line is a thrice-cloned subline of the neuroblastoma cell line SK-N-SH, established in 1970 from a metastatic bone tumor from a 4-year-old cancer patient (35). In culture, SH-SY5Y cells are a mixture of both adherent and floating cells. The cells grow as clusters of neuroblastic cells with multiple, short, fine cell processes (neurites) (Figure 2.1). Cells will aggregate, form clumps and float. While most studies utilize adherent populations and discard the floating cells during medium changes, the biological significance of the adherent versus floating phenotypes still needs to be explored (34). Another important characteristic of SH-SY5Y cells is the presence of two morphologically distinct phenotypes: neuroblast-like cells and epithelial-like cells. These two phenotypes were initially identified by Biedler's group (35, 36) and were later referred to as "N" and "S" types by Encinas *et al.* (37).

Cultured SH-SY5Y cells can continuously proliferate, mainly as adherent neuroblast-like cells although the cell population contains a low proportion of epithelial-like cells (31, 34). SH-SY5Y neuroblast-like cells present polygonal cell bodies with short processes resembling immature catecholaminergic neurons and can express markers such as tyrosine hy-

droxylase (TH) and dopamine- β -hydroxylase, but more predominantly immature neuronal markers such as nestin and transcriptional regulators.

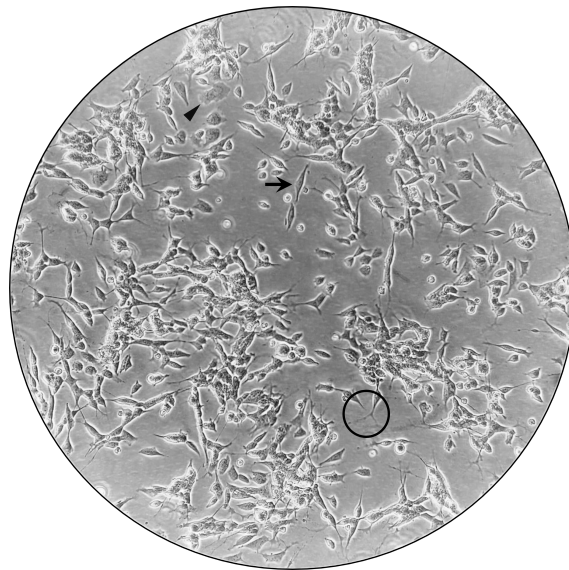


Figure 2.1: Phase-contrast microscopy of undifferentiated SH-SY5Y cells. Cells tend to grow in clusters and at edges of the cluster, cells begin to extend short neurites (circle). SH-SY5Y cells are mainly neuroblastic (N-type, arrows), but occasionally some epithelial-type cells appear (S-type, arrowheads).

Lastly, SH-SY5Y cells can differentiate into a more mature neuronal phenotype. Differentiation of SH-SY5Y cells decreases the proliferation rate, prompts a transition towards a neuron-like morphology (small polarized cell bodies with long neurites) and increased electrical excitability of the plasma membrane, and the expression of mature neuronal markers, such as neurofilament proteins. The specific phenotype adopted by these cells depends on the differentiation method employed; for example, the most commonly used inducing agent, retinoic acid, seems to favour a more cholinergic phenotype while 12-O-tetradecanoylphorbol-13-acetate (TPA) or dibutyryl cyclic adenosine monophosphate (dbcAMP) lead to dopaminergic-adrenergic or adrenergic phenotypes, respectively (31, 34).

Notwithstanding, there remains a notable absence of consensus and standardization of differentiation protocols, and current approaches are unclear on the resulting phenotype (38). Despite differentiated SH-SY5Y possessing a more similar phenotype to dopaminergic neurons, the majority of studies related to Parkinson's research that use SH-SY5Y cells are performed in undifferentiated cells (38). The preference of undifferentiated SH-SY5Y holds true for studying the neurotoxic effects of various chemical pollutants (31).

As a well-established and widely used cell line, SH-SY5Y cells provide a consistent plat-

form for studying the neurotoxic effects of contaminants. However, it is important to note that using SH-SY5Y cells as a model for chemical testing also has limitations. These cells are derived from a cancerous cell line, possibly limiting their relevance to normal neuronal function and their ability to represent the effects of chemical exposure on healthy neurons accurately (20). Furthermore, the concentrations of chemicals used in *in vitro* studies may not reflect realistic exposure levels in humans, and the effects observed in these cell models may not necessarily translate to *in vivo* situations. Additionally, the use of a single cell line only partially captures the complexity of the human nervous system. Different cell types within the nervous system, such as astrocytes and microglia, play important roles in neurotoxicity and influence the responses observed in neuronal cells.

2.3 Diet-related contaminants

The Codex Alimentarius defines contaminant as "*any substance not intentionally added to food or feed for food producing animals, which is present in such food or feed as a result of the production, manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food or feed, or as a result of environmental contamination*" (39).

Diet-related contaminants have various origins, and include agrochemicals (pesticides, veterinary drug residues), natural toxins (mycotoxins), food processing contaminants like polycyclic aromatic hydrocarbons (PAHs), heterocyclic aromatic amines and acrylamide (ACR), environmental sources (PAH, heavy metals) and packaging-derived chemicals. They typically pose a health concern and have a negative impact on the quality of the food or feed, resulting in strict regulations of their levels by national governments and internationally (39).

Over the years, the Panel on Contaminants in the Food Chain (CONTAM) from European Food Safety Authority (EFSA) has published several scientific opinions on the risk assessment of several contaminants in food or feed (40–43). These reports include a review of the occurrence and exposure of humans and animals, toxicokinetic data and toxicological studies to characterize risks for human health accurately. Human dietary exposure assessment play a critical role in ensuring food safety as they serve as the basis for establishing maximum levels of toxicants permitted in specific food products, a responsibility carried out by the European Commission (EC) (44, 45). Certain population groups are prioritized by regulatory agencies as they are more sensitive to contaminants action, such as pregnant

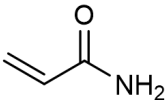
individuals, elderly people, and children (46, 47). Given their lower body weight, children are more susceptible to food contaminants exposure and toxic effects due to the higher absorption rate, poor detoxification elimination capacity, rapid cell proliferation, and immature repairing mechanisms (48). The elderly also show a compromised ability to deal with the adverse affects of toxicants (46).

The following sections overview several prominent classes of food contaminants, focusing on a representative compound relevant to this dissertation: process-induced contaminant acrylamide, mycotoxins (aflatoxin B1), PAHs (benzo[a]pyrene), metals (cadmium), and pesticides (cypermethrin). Details on their occurrence in food, bioavailability, metabolic pathways, and evidence on human neurotoxicity will be addressed.

2.3.1 Acrylamide

Acrylamide (ACR) is a small hydrosoluble compound widely used in producing polymers in wastewater treatments, the pulp and paper industry and the textile industry (49). Identified as a group 2A carcinogen (“possibly carcinogenic to humans”) by the International Agency for Research on Cancer (IARC) in 1994 (42), the presence of ACR in food was only revealed in 2002 after research concluded its formation during Maillard reactions (50, 51). Table 2.1 overviews acrylamide structure, chemical properties and common contaminated foods.

Table 2.1: Overview of acrylamide structure, and chemical properties (Sources: Pubchem, (42)).

ID	Name	Chemical Structure	MW (g/mol)	LogP	CAS
ACR	Acrylamide		71.08	-0.67	79-06-1

2.3.1.1 Occurrence in food and dietary exposure

Foodborne ACR is a product of Maillard reactions between sugars and asparagine during thermal processing (51). Consequently, foods subjected to high-temperature cooking methods like frying, roasting, baking, and toasting tend to contain elevated acrylamide levels (50, 52). Various factors impact acrylamide formation including cooking time, cooking temperatures over 120°C, high asparagine and reducing sugars content and low moisture (51, 52).

ACR is found in processed carbohydrate-rich foods like french fries, bread, biscuits and cakes, and also products like coffee due to the Maillard reactions during processing. These food sources constitute around 40% of caloric intake, thus diet is assumed to be the major source of ACR exposure for the general nonsmoking population (53). These findings indicate that ACR is present in various foods of western diet, including beverage products, staple foods and snack products (54).

The Joint Expert Committee on Food Additives reports that the major foods contributing to the ACR total intake are potato crisps and chips, coffee, pastry and sweet biscuits, and bread (53). Fried and baked food products, such as potato chips, crispbreads and biscuits, contain high levels of ACR, particularly potato chips, in which the ACR content may be up to 8440 $\mu\text{g/kg}$ (54). Although the concentration of ACR in coffee is relatively low, it is a major source of ACR exposure in adults due to the high consumption of coffee (averaging from 522 $\mu\text{g/kg}$ in dried coffee to 1499 $\mu\text{g/kg}$ in dry coffee substitutes) (42, 54).

In 2015, EFSA stated that infants and children were the population group most exposed to acrylamide (average acrylamide intake 0.5–1.9 $\mu\text{g/kg bw/day}$), and infant formulas have a significant share in this exposure (42), being reported levels up to 1821 $\mu\text{g/kg}$ (55). Fernandez *et al.* estimated ACR exposure based on its urinary metabolites in Spanish lactating mothers, and found that the mean value is from 1.2 to 1.9 $\mu\text{g/kg bw/day}$ (56). For the European population, EFSA reports a chronic dietary exposure that on average ranged from 0.4 and 0.9 $\mu\text{g/kg b.w. per day}$ and on the 95th percentile was between 0.6 and 2.0 $\mu\text{g/kg b.w. per day}$ depending on the survey and age group (42). Other reports on specific populations are also in accordance: 0.43 $\mu\text{g/kg bw/day}$ in Poland (57) and 0.38 $\mu\text{g/kg bw/day}$ in Portugal (58).

ACR does not have a clearly established threshold for its effects, meaning that exposure to low doses of ACR may not result in immediate clinically apparent symptoms, but morphological and/or biochemical alterations may be present (53). The tolerable daily intake for neurotoxicity was estimated at 40 $\mu\text{g/kg bw/day}$ for ACR and 70 $\mu\text{g/kg bw/day}$ for glycidamide (GA) (ACR reactive metabolite) while for cancer was estimated to be 2.6 and 16 $\mu\text{g/kg/day}$ for ACR and GA, respectively (59).

2.3.1.2 Bioavailability and metabolism

ACR is easily absorbed in the gastrointestinal tract and is found in circulation and peripheral tissues after ingestion (26, 53). As depicted in Figure 2.2, phase I metabolism of ACR forms glycidamide (GA), after epoxidation reaction by CYP2E1 P450 isoform. Both GA and ACR may form glutathione related metabolites (GAMA, isoGAMA, AAMA and AAMA sulfoxide) by glutathione S-transferases. These metabolites are further degraded to mercapturic acid metabolites, which are eliminated in the urine (26).

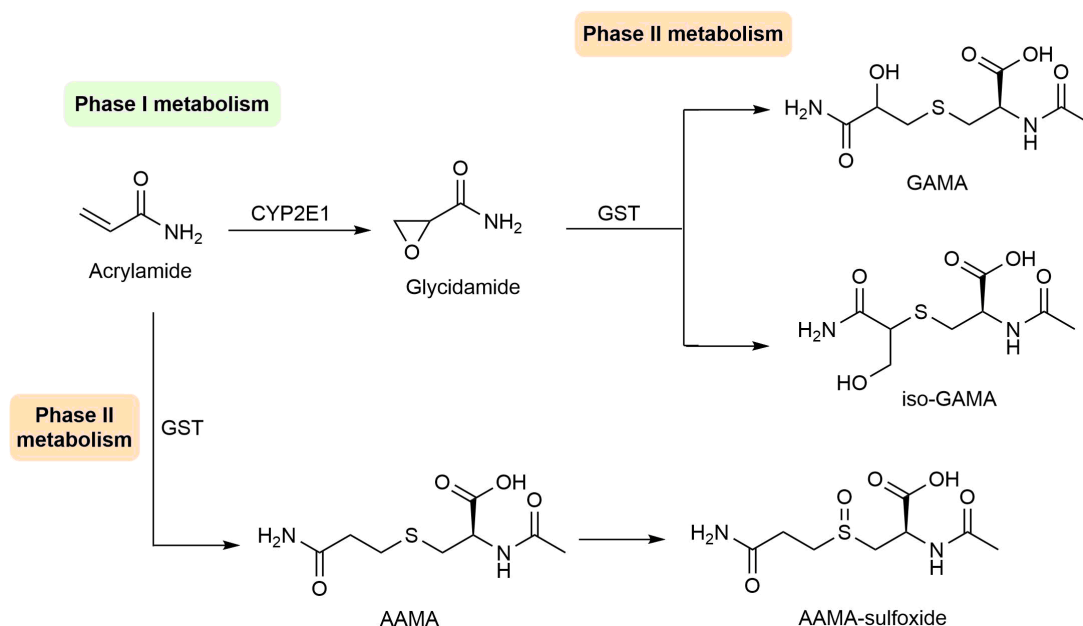


Figure 2.2: Metabolism of acrylamide (26, 42). Both phase I metabolite GA and ACR may form GSH related metabolites (*N*-acetyl-S-(2-hydroxy-2-carbamoyl-ethyl)cystein - GAMA, *N*-acetyl-S-(1-carbamoyl-2-hydroxyethyl)cystein - isoGAMA, *N*-acetyl-S-(2-carbamoyl-ethyl)cystein - AAMA, *N*-acetyl-S-(2-carbamoyl-ethyl)cystein sulfoxide - AAMA sulfoxide) by glutathione-S-transferases (GST). These metabolites are further degraded to mercapturic acid metabolites.

GA corresponds to the major active ACR metabolite, and plays a pivotal role in the genotoxicity and carcinogenicity associated with ACR exposure (42). GA is able to generate covalent DNA adducts, with the N7-guanine adduct (N7-GA-Gua) being the most abundant following ACR exposure (42). While GA-DNA adducts are found in various tissues, covalent ACR-DNA adducts have not been reported in animals or humans (42). Although both ACR and GA can react with proteins, such as hemoglobin (Hb), forming covalent adducts that serve as important biomarkers of exposure (42).

2.3.1.3 Acrylamide-induced neurotoxicity

Since ACR was classified as a Group 2A carcinogen back in 1994, further research identified four critical endpoints for ACR toxicity: carcinogenicity, effects on male reproduction, developmental toxicity, and neurotoxicity (42). Due to its physical and chemical properties, ACR can disperse throughout the body, increasing the risk of tumors in various organs. ACR and its reactive metabolite, glycidamide (GA), can cross the blood-brain barrier and the placenta, potentially causing direct harm to neurons or disrupting vital brain signalling pathways in different lifestages.

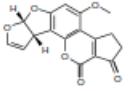
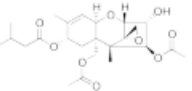
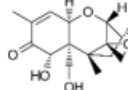
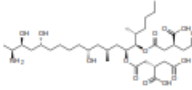
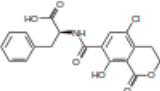
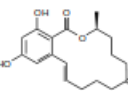
ACR was initially recognized as a neurotoxin due to its ability to cause ataxia, muscle weakness, and peripheral neuropathy in both rodents and humans after occupational exposure (42, 53). However, only a few studies address neurological-related issues related to foodborne ACR exposure. In a cohort study of non-smoking Chinese elderly, dietary exposure to low levels of ACR (0.27 and 0.24 $\mu\text{g/day}$ for men and women, respectively) was associated with a mild decline in cognition or increased risk of poor cognition over 4 years in men but not in women (60). Both chronic exposure and low dosage of ACR have been found to cause changes in brain structure and function in animal models, namely neuronal loss, altered neurotransmitter systems, and behavioural deficits. In rats, chronic low-dose ACR ingestion (up to 10 mg/kg/day) throughout a lifetime including the prenatal period, has been found to cause decrements in food-motivated behaviour in rats and deficits in the development and motor coordination of rat pups (42, 53).

2.3.2 Mycotoxins

Fungi species produce toxic secondary metabolites known as mycotoxins, a hidden danger in food and feed. The Food and Agriculture Organization of the United Nations estimates mycotoxins contaminate 25% of agricultural goods annually around the world (61, 62). So far, more than 300 mycotoxins were identified in over 350 fungi species. Table 2.2 describes some of the most studied prevalent foodborne mycotoxins: aflatoxin B1 (AFB1), trichothecenes (deoxynivalenol (DON), T-2 toxin (T2)), fumonisin B1 (FB1), ochratoxin A (OTA), patulin and zearalenone (ZEN) (61, 62). These mycotoxins are a significant threat to human and animal health, with the potential to cause a range of diseases such as cancer, kidney damage, gastrointestinal issues, reproductive disorders, and immune suppression

(61–63).

Table 2.2: Mycotoxins chemical structure, molecular weight, and partition coefficient and most common food commodities contaminated. From PubChem and (61).

ID	Common Name	Chemical Structure	MW (g/mol)	LogP	Food Commodities
AFB1	Aflatoxin B1		312.28	1.6	Wheat, walnut, maize, cotton, peanuts, eggs, milk, meat
T-2	T-2 Toxin		466.52	0.9	Barley, oats, wheat
DON	Deoxynivalenol		296.30	-0.7	Wheat, maize
FB1	Fumonisin B1		721.83	-0.5	Corn and wheat
OTA	Ochratoxin A		403.81	4.7	Coffee beans, oats, wheat, maize, wine, dried fruits, spices, eggs, meat
PAT	Patulin		154.12	-1	Apples, grapes, plums, peaches, pears, tomatoes
ZEN	Zeranolone		318.36	3.6	Maize, barley, oats, sorghum and wheat

The main mycotoxin-producing genera are *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria* (61, 62). Various factors, such as stress in plants and specific climatic conditions, namely temperature, water activity, pH, oxygen and substrate composition, are required for fungi to produce these toxins and are frequently different from optimal growth conditions (61, 62).

Mycotoxin contamination occurs in the field during crop growth and at various stages of the food chain, including storage, processing, and transportation (61, 64). Crops such as cereals (maize, wheat, barley), consumed widely as staple foods, are particularly vulnerable to mycotoxin contamination (61, 64). Besides cereal grains, spices, soy, peanuts and some vegetables and fruits also deal with mycotoxins (61, 64). Indirect exposure is also a risk for human and animal health since contaminated feed causes harm to livestock and accumulation of mycotoxins produces contaminated milk, eggs and meat (63, 64). The lack of visible mould in food and feed does not necessarily imply the absence of mycotoxins (61). In addition, their resistance to high temperature, chemical or physical processing requires strict crop control and regulations (62). Climate change can further worsen the problem by

altering fungal growth patterns and mycotoxin production (62).

Mycotoxin contamination has significant economic and social impacts, often surpassing established regulation limits (62). In 2022, aflatoxins, were the one of the most recurring hazards notified in the Rapid Alert System for Food and Feed (RASFF) of the European Union (65). In light of this, the present dissertation will center its attention on aflatoxin B1, given its prevalence and known toxicity that poses a significant threat to human health.

2.3.2.1 Aflatoxin B1

Aflatoxins are primarily produced by *Aspergillus* species, including *A. flavus* and *A. parasiticus*, with aflatoxin B1, B2, G1 and G2 being the most found (45). Notably, among these, aflatoxin B1 (AFB1) stands out as the most toxic dietary contaminant. AFB1 is a potent carcinogen and mutagen, earning its classification by the International Agency for Research on Cancer (IARC) as a Group 1 carcinogen (66).

2.3.2.2 Occurrence in food and dietary exposure

AFB1 is found in a wide range of food commodities like cereals, oil seeds, spices, tree nuts, and dried fruits but also animal derived products, namely eggs and meat products (offal) (63). Additional, AFB1 metabolite, known as aflatoxin M1, appears in animal milk after cattle exposure to AFB1 contaminated feed. Both, AFB1 and AFM1 have strong thermal stability (>100°C), thus not being eliminated during pasteurization and sterilization of milk and dairy products (63).

EFSA risk assessment of aflatoxins states that grains and grain-based products are the largest contributors to chronic AFB1 dietary exposure across population groups (43). Greatest variation of AFB1 contamination levels is found in maize, with an average minimum of 2.3 µg/kg and 555.0 µg/kg as maximum average for 46.1% of analysed samples (67). Spices are the food commodity with the highest percentage of positive samples (64.4%), with minimum and maximum AFB1 levels at 0.2 and 25.4 µg/kg, respectively (67).

The exposure levels to AFB1 vary across different age groups (43). Younger population groups are more exposed to AFB1, with the average daily exposure ranging from 0.08 up to 6.95 ng/kg bw. High risk exposure (95th percentile) spans from 0.35 up to 14.01 ng/kg bw per day (43). For adults, the mean exposure ranges from 0.22-0.49 ng/kg bw per day up

to 1.35-3.25 ng/kg bw per day (43). When considering the 95th percentile (P95) exposure values go up to 6.78 ng/kg bw per day (43).

2.3.2.3 Bioavailability and metabolism

Regarding bioavailability, AFB1 shows high capacity to passively cross the gastrointestinal barrier (61, 63). AFB1 has a half-life of 64.4 h in humans, and due to its stability with lysine in human serum AFB1-lys can be seen as biomarker of exposure with a half-life of 2–3 months (68).

Figure 2.3 describes AFB1 metabolism. Phase I reactions are carried out by liver P450 enzyme system (CYP3A4 and CYP1A2), converting this mycotoxin is converted to a more toxic, highly reactive aflatoxin B1-8,9-epoxide (AFBO) (43, 67). Both isomers (endo-8,9-epoxide and exo-8,9-epoxide) possess a highly electrophilic nature that allows them to react spontaneously amine groups in proteins and nucleic acids. Notwithstanding, the exo isomer is considered the most carcinogenic given its higher affinity to form DNA adducts. AFBO can also be further hydrolysed to AFB1-8,9-dihydroxydiol that is in equilibrium with the dialdehyde form that can bind spontaneously to proteins at lysine residues. AFBO detoxification occurs via glutathione conjugation (AFB1-GSH) which is further metabolized into mercapturic acid derivatives to be excreted in urine (43, 67).

Besides AFB1 epoxidation into AFBO, CYP450 enzymes are also responsible by AFB1 hydroxylation that produces less toxic metabolites like aflatoxin M1 (AFM1), aflatoxin Q1, and aflatoxin P1 (43, 67). From this metabolite, AFM1 is the main AFB1 hydroxylated metabolite, being found in mammals and human milk (63). Though less toxic than the parent mycotoxin, carcinogenic and immunosuppressive effects have been described, justifying the Group 2B carcinogen classification and establishment of maximum residue limits in milk (63).

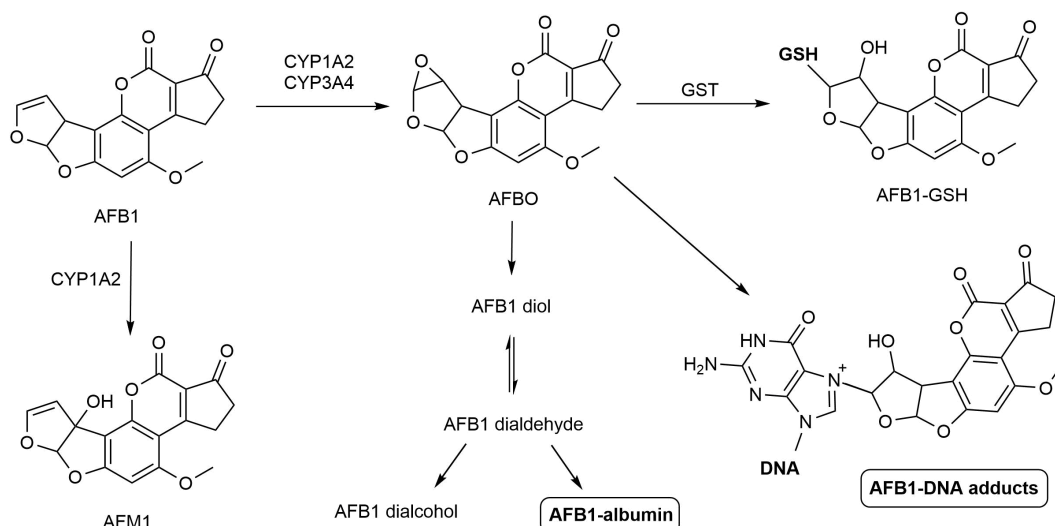


Figure 2.3: Metabolism of aflatoxin B1.

2.3.2.4 Aflatoxin B1-induced neurotoxicity

Chronic AFB1 exposure in children was previously associated with stunting and reduced cognitive ability in low and middle income countries (9), although a systematic review is required to evaluate such conclusions. Ingestion of AFB1 (15.75 $\mu\text{g/kg}$ for eight weeks) by Wistar rats resulted in astrocyte loss in the frontal cortex and neurons in the hippocampus (69). These histological changes were potentially reversible upon AFB1 withdrawal (69). Alsayyah, *et al.*, (70) reports neurodegeneration after 30-day dose AFB1 accumulation, being more severe after 90-days (70). Beyond oxidative stress, AFB1 leads to vasodilation and blood brain barrier (BBB) impairment through induction of endothelial relaxation (69, 70). Rats treated with oral 80 $\mu\text{g/kg}$ AFB1 for 28 days (71) had diminished dopamine levels in cortex and hippocampus region, and the levels of inflammatory and apoptotic markers were elevated. Similar experimental study conditions resulted in anxiogenic behaviour of treated rats (72). After 30 days of dietary exposure to AFB1 (300 $\mu\text{g/kg}$), mice brain integrity was affected, with detection of red-cell infiltration (73). Levels of AFB1 in brain tissue were 60 $\mu\text{g/kg}$ body weight (bw), and metabolite AFBO was not detected, suggesting that AFB1 brain toxicity does not require metabolic activation (73). Detectable behavioural changes and other biochemical parameters, namely the reduction of antioxidant non-enzymatic defenses, were described in rats after acute treatment with AFB1 (250 $\mu\text{g/kg}$) (74). An acute and chronic dosage of AFB1 (0.75 mg/kg) was orally administered in every 3 days for 45 days (75), and resulted in a memory impairment behaviour. The subjects presented a low cognition, high

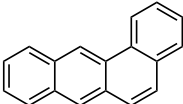
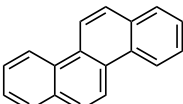
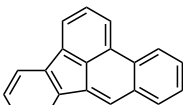
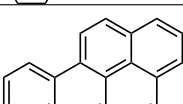
anxiety and lethargy.

2.3.3 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are compounds that exhibit two or more fused aromatic rings and derive from the incomplete combustion or pyrolysis of organic material (41, 76). The first focus on PAH toxicity arises from occupational exposure to these pollutants among chimney sweeps (76). Environmental PAHs can originate from natural sources, such as forest fires and volcanic emissions, and anthropogenic sources, such as coal burning, vehicle exhaust emissions, engine lubricating oils, and cigarette smoke (76, 77). Food consumption is the primary source of non-occupational human exposure for non-smoking individuals (41, 77).

The CONTAM panel has identified four major PAHs (PAH4) in foods presented in Table 2.3: benz[a]anthracene (BaA), chrysene (Chr), benzo[b]fluoranthene (BbF), and benzo[a]pyrene (BaP) (41). BaP has been classified as a Group 1 carcinogen by the IARC and causes teratogenicity, neurotoxicity and immunotoxicity (41). On the other hand, BaA, BbF, and Chr are classified as Group 2B agents (41). The following sections will focus more on BaP, as it serves as the representative molecule for PAHs class.

Table 2.3: Chemical properties of PAH4 (Source: PubChem).

ID	Name	Chemical Structure	MW (g/mol)	LogP	CAS
BaA	benz[a]anthracene		228.29	5.76	56-55-3
Chr	chrysene		228.28	5.81	218-01-9
BbF	benzo[b]fluoranthene		252.31	5.78	205-99-2
BaP	benzo[a]pyrene		253.31	6.13	50-32-8

2.3.3.1 Occurrence in food and dietary exposure

Diet related exposure to PAHs may occur via transference from environmental sources or via food processing. Oil spills and industrial discharges aid PAHs entrance in the food chain via fish and marine invertebrates. Filter-feeding invertebrates like bivalves that accumulate high levels of PAH, are of more concern to human exposure, since fish have a greater ability to metabolize PAHs than bivalves, so the compounds tend to persist more in the latter (78). Airborne and soil PAHs can also contaminate crops. In terms of food processing, PAHs are concentrated in food commodities like vegetable oils and dried grains due to the drying processes (77). In addition certain processing conditions of meat, with direct contact of fat with a flame or incomplete combustion of charcoal, lead to PAH formation. Also, traditional commercial smoking techniques, in which smoke from incomplete wood burning comes into direct contact with the product, can lead to significant contamination by PAHs, if the process is not adequately monitored (79). Temperature, time, humidity, type of smoke used are crucial parameters in controlling PAH formation (41, 79). Thus, PAH have been found in grilled, fried and barbecued meat, as well as, in dried and smoked fish. Nevertheless, analysis of PAHs exposure through diet on the EFSA report concluded that cereal products constitute the major source to these contaminants, next to seafood products followed by oils and fats, vegetables, fruits and processed animal related products (41), ranking determined by consumption patterns or level of contaminants of food groups. However, contribution of food groups to PAHs intake is greatly dependent on home-cooking procedures and dietary habits: smoked fish and meats, grilled and barbecued foods significantly increase the population exposure to PAHs (41, 78). For example in Spain, meat and meat products were found to be the second food group with the highest concentration (total PAHs: 13.4 $\mu\text{g/kg}$) and contribution to PAH dietary intake was only surpassed by cereals (total PAHs: 14.5 $\mu\text{g/kg}$) (80).

The average dietary exposure across European countries for which data are available ranged from 235 ng/day (3.9 ng/kg b.w. per day) for an average consumer and 389 ng/day (6.5 ng/kg b.w. per day) for high dietary consumers in the case of BaP alone, and 1168 ng/day (19.5 ng/kg b.w. per day) and 2068 ng/day (34.5 ng/kg b.w. per day) respectively for PAH4 (41).

2.3.3.2 Bioavailability and metabolism

PAHs enter the human body through the respiratory tract, digestive tract, or the skin. BaP and BbF exhibit reduced bioavailability using *in vitro* intestinal transport model (only 40% and 20% transport efficiency) (81). After absorption and distribution, highest levels are found in the adipose tissue (82). Metabolic activation of BaP can occur in the intestine, but mainly in the liver. Figure 2.4 shows the main steps of BaP metabolism (83). BaP is converted to BaP-7,8-epoxide by CYP1A1/B1, which is further oxidized in a two-step reaction, first by epoxidase hydrolase forming BaP-7,8-dihydrodiol, and then by CYP1A1/B1, resulting in the reactive metabolite BaP-7,8-diol-9,10 epoxide (BPDE). This metabolite can form stable DNA adducts. On the other hand, 7,8-dihydrodiol BaP can also undergo further oxidation, forming catechol and BaP-7,8-dione via aldo-keto reductases. BaP can also be transformed to the reactive radical BaP cation by a one-electron oxidation process catalysed by CYP-peroxidase that can result in chemically unstable alkylation of DNA, which leads to depurination, a potentially mutagenic event (76). Conjugation with endogenous molecules, such as sulphates, glucuronic acid, and GSH, allow PAH to be excreted through the bile or urine.

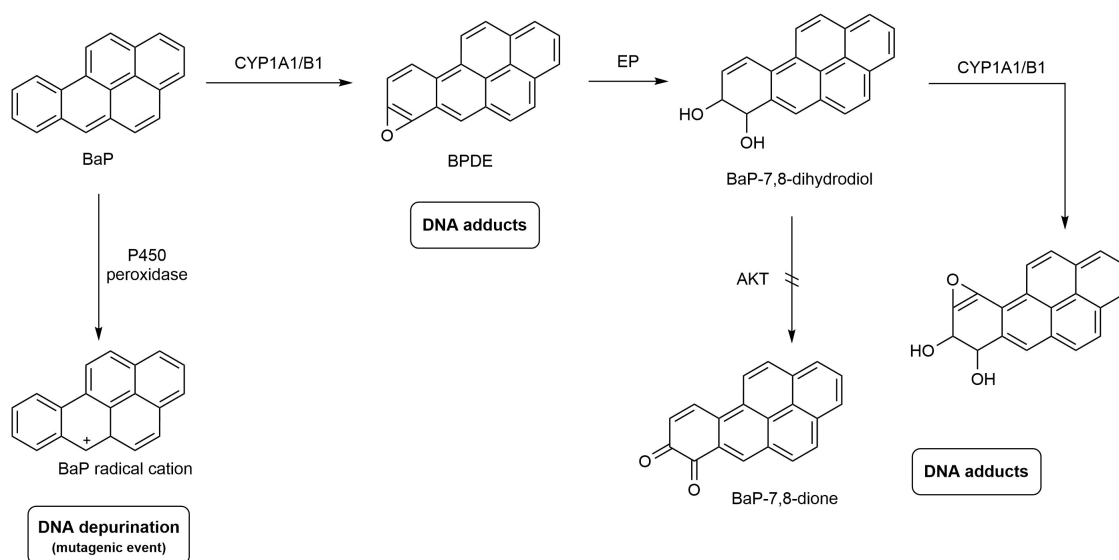


Figure 2.4: First phase metabolism of BaP.

2.3.3.3 Benzo[a]pyrene-induced neurotoxicity

Although BaP neurotoxicity has received considerably less attention than its carcinogenicity, this contaminant can easily cross the BBB given its lipophilicity (27).

BaP elimination from the brain proceeds with a lag phase that likely occurs because lev-

els of the CYP1A1 enzyme are very low in the brain (27). Notwithstanding, circulating BaP metabolites could mediate neurotoxicity (27, 84). The pattern of BaP metabolite concentrations in the plasma and brain of Fisher-344 rats exposed to BaP by oral gavage provokes behavioural alterations (84).

The neurotoxic effects of PAHs during early life have been discussed as a means for NDs later in life (85, 86). Epigenomic alterations might be behind the BaP neurotoxicity in adult zebrafish, given the lasting consequences of altered DNA methylation patterns, as seen in this study in several neurological-related promoters and expression of DNA methyltransferases (86). Interestingly, a consistent chronic low exposure to BaP of zebrafish throughout their life led to similar results (87). Dietary exposure to BaP of mice expressing human α -syn during the prenatal period was found to exacerbate α -syn pathology in adult mice brain, presenting reduced protein clearance capacity and lacking dopamine neurons (88).

In rodent studies, oral exposure to BaP globally caused behavioural alterations (89–91), potentially linked to oxidative stress (90, 92, 93) and modulated by metabolism (84, 91). As previously mentioned, BaP alters NDMA receptor expression in animal models (85, 94, 95), being a more potential serious consequence than this contaminant genotoxicity in the brain (94). BaP chronic exposure induced progressive decline in spatial learning/memory and exploratory behaviours in APP/PS1 mice (an early-onset model for AD) and WT mice, with the ND model exhibiting severer behavioural deficits (96). AD-like pathological changes were found augmented in APP/PS1 mice, namely neuronal loss, A β burden and A β plaque formation and neuroinflammation, with consistent microglia activation. This study suggests that BaP can aggravate neurodegeneration, inducing a fast decline in neuron function in a susceptible model (96).

2.3.4 Heavy metals

Heavy metals are metallic elements known for their high atomic weight, density, and potential toxicity, especially in low concentrations (97). Certain elements, such as iron, copper, and zinc, are vital for various biological processes and enzymatic reactions in trace amounts, yet they can become toxic when present in higher concentrations (97). On the other hand, several heavy metals, including arsenic, cadmium, chromium, lead, mercury, are toxic and have no known beneficial properties for the human body (97). These toxic metals can enter

the environment through human activities, such as industrial processes, mining, and agriculture, persisting in the environment for extended periods. Consequently, this persistent contamination has been associated with severe health consequences (97, 98). The Agency of Toxic Substances and Disease Registry (ATSDR) has identified arsenic, cadmium, and mercury as some of the most hazardous contaminants regarding exposure frequency (97, 98).

Considering these factors, this dissertation will focus on cadmium due to its widespread presence in food and well-documented toxicity, which presents a substantial risk to human health.

2.3.4.1 Cadmium

Cadmium (Cd) is a widespread environmental contaminant originating from sources like fossil fuels, agricultural runoff, mining and industrial processes (98). Human exposure to cadmium primarily occurs through inhalation of cigarette smoke, ingestion of contaminated foods and water consumption (40, 98).

2.3.4.2 Occurrence in food and dietary exposure

Cd exposure occurs primarily through food in the non-smoking population (40). High levels of cadmium were found in algal formulations, cocoa-based products, crustaceans, edible offal, fungi, oilseeds, seaweeds and water molluscs. Based on consumption prevalence, potatoes (13.2%), bread and rolls (11.7%), fine bakery wares (5.1%), chocolate products (4.3%), leafy vegetables (3.9%) and water molluscs (3.2%) contributed the most to cadmium dietary exposure across age groups. EFSA CONTAM Panel has set a tolerable weekly intake of 2.5 $\mu\text{g/kg}$ bw to ensure sufficient protection of all consumers (40). However calculations of lifetime cadmium dietary exposure are close to or exceed these health-based guidance values, depending on the exposure degree (40).

2.3.4.3 Bioavailability

Cd accumulates in the human body due to its slow excretion and a very long biological half-life (10–30 years). The majority accumulates in liver and kidneys, and the older population is more sensitive to Cd and other metals because renal function declines with age (99). Cd was also found in locus ceruleus neurons from human autopsies of individuals with

diverse clinicopathological conditions and causes of death (100).

2.3.4.4 Cadmium-induced neurotoxicity

One of the major concerns associated with heavy metal exposure is their potential to cause neurotoxicity. Studies have suggested that overall heavy metal exposure may contribute to the development or progression of NDs through several potential mechanisms such as oxidative stress, calcium imbalance, neuroinflammation (24). Some studies have suggested that metals may interact with p-tau and promote aggregation of A β , potentially playing a role in the formation of A β plaques characteristic of the disease. Chronic and prolonged cadmium exposure can lead to disruption of BBB tight junctions, ultimately increasing its permeability (101). Once in the brain, this metal accumulates in the choroid plexus at concentrations much greater than those found in the cerebrospinal fluid and in other brain areas (101). Exposure to cadmium is also seen as an etiological factor for sporadic PD. PD patients exhibit elevated blood Cd concentration and proinflammatory lipid metabolites in the brain (102). Accumulation of Cd in the brain leads oxidative stress, inflammation and neuronal death which contribute to cognitive dysfunction (103).

2.3.5 Pesticides

A pesticide is a chemical substance or mixture specifically designed to control, repel, or eliminate various types of pests (104, 105). They include insecticides, antiseptics, disinfectants, anti-bacterial, fungicides, algicides, rodenticides, and herbicides (106). From a chemical perspective, organic pesticides, the majority of which are synthetic, can be grouped divided in organophosphates, organochlorines, carbamates, and pyrethroids (106).

Pesticides play a significant role in modern agriculture and pest management (104). The development and use of pesticides have revolutionized food production, allowing for increased yields and improved food security (104).

However, the use of pesticides also raises important environmental and health concerns (105). Pesticides can have unintended consequences, such as harming non-target organisms, contaminating water sources, and contributing to the development of pesticide-resistant pests. Additionally, there are concerns about the potential impact of pesticide residues on human health, particularly when they accumulate in food products. Pesticide

residues are found in everyday foods and beverages, including, water, fruit juices, and vegetables (105). Along with the chronic consumption of foodstuff grown in pesticide-contaminated soil and water, pesticide home use for pest control can lead to the accumulation of pesticides inside the body (106). This accumulation potentially results in chronic diseases such as neurotoxicity, cancer, necrosis, asthma, reproductive disorders, cardiac diseases, and diabetes (106).

Recent advancements in neurotoxicity research have resulted in a significant milestone – the ban on chlorpyrifos, a widely used organophosphate insecticide. Exposure to this pesticide during critical periods of brain development has been associated with cognitive and behavioural deficits, developmental delays, and other neurological disorders. After decades of research studies raising alarms about the neurotoxic effects of chlorpyrifos (107) the EU banned the use of chlorpyrifos in agriculture in 2020, citing risks to human health, particularly for children. The U.S. Environmental Protection Agency (EPA) established regulatory actions in December 2022, banning chlorpyrifos on foodcrops (108). The controversy surrounding chlorpyrifos illustrates the delicate balance between the benefits of effective pest control and the potential risks posed by certain pesticides (109). It also underscores the importance of ongoing research, risk assessments, and evidence-based decision-making to ensure that pesticide use aligns with human health and environmental safety standards.

A group of pesticides that has gained the interest of researchers due to their neurotoxic effect at different life-stages are pyrethroids.

2.3.5.1 Pyrethroids

Pyrethroids are used to control a wide range of pests, including insects and other arthropods in agriculture, as well as in residential, commercial, and public settings. This class of pesticides is structural similar to natural pyrethrins extracted from plants (110). Chemical structure divides pyrethroids in type I, which includes permethrin and bifenthrin that contain either a primary or secondary alcohol, and type II like cypermethrin and deltamethrin are primary alcohols with a cyano group on the α -carbon of the alcohol (111, 112). Pyrethroids share a common main mechanism of action as activators of voltage-gated sodium channels, thus causing neurotoxicity, cardiotoxicity (111, 112). From the pyrethroids class, we will focus our attention on cypermethrin (CYP).

2.3.5.2 Cypermethrin

Cypermethrin (CYP) (Figure 2.5, MW 416.3 g/mol, logP 6) is comprised of eight enantiomers and typically contains 20-40% α -cypermethrin, which consist of the two most toxicologically active isomers of cypermethrin (110).

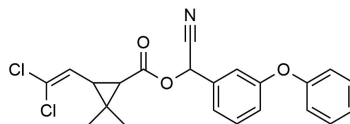


Figure 2.5: Structure of cypermethrin (CYP).

2.3.5.3 Environmental and dietary exposure

CYP has been extensively used as an insecticide for agricultural purposes (111). CYP distribution along food groups varies widely, with greater prevalence in fruits, peanuts, legumes and vegetables (113). A biological monitoring study in Canada of a metropolitan populace exposed to pyrethroids in the diet found that the study population was mainly exposed to permethrin and CYP. CYP is also used in livestock production and is recommended for in-home insect control (114, 115).

2.3.5.4 Bioavailability and metabolism

Due to its lipophilic properties, CYP is found at higher concentrations in adipose tissue, skin, ovaries, kidneys, adrenal glands, and in the liver. CYP is metabolized by cleavage of the ester linkage, followed by conversion to 3-phenoxybenzoic acid (3-PBA), cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (cis-DCCA), and trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (trans-DCCA), and is then excreted into the urine (116).

2.3.5.5 Cypermethrin-induced neurotoxicity

CYP is able to cross the BBB and enter the central nervous system where it acts as voltage-gated sodium channel activator (117). By delaying the closure of the sodium channel, CYP provokes excitotoxicity of neuronal cells.

A meta-analysis reveals there is a positive association between pesticide exposure and AD, confirming the hypothesis that pesticide exposure is a risk factor for AD (118). Pro-

longed exposure to CYP induced AD-like pathology, with increase in levels of A β 42 and hyperphosphorylated tau in the hippocampal and cortical regions (119). Potential mechanisms for this observations could be related to elevated ROS production, mitochondria dysfunction (120, 121).

2.4 Evaluation of toxicological interactions

Toxicology has been directed at studying the mechanisms of adverse effects of isolated compounds on living organisms at high levels of exposure, forming the basis for risk and safety assessment (122). Chemical mixtures in the food or the environment constitute a major health challenge deserving due attention. By studying the toxicity of chemical mixtures instead of their individual constituents, it may be possible to clarify their potential role in the development of chronic diseases, particularly those with long latency periods (123).

The synergism concept has been a controversy in toxicology for over than 100 years, with over 20 arbitrary, non-quantitative synergy definitions are still applied and reported (16). With no clear exact guidelines of synergy definition, governmental regulatory agencies, funding organizations and academia research have no basis to judge or to regulate synergy claims (15, 16). The definition of each interaction effect is based on a comparison of observed effects with the expected effects of combination. If the observed effect is greater than expected, it is defined as a synergism, and if the opposite is true it is defined as an antagonism. The third case, when the expected value is equal to the observed one is called an additive effect (124).

The assessment of the combination effects in terms of synergy, antagonism and additivity, relies on the accurate prediction of the expected effects of a mixture. Many different approaches have been proposed to evaluate and predict mixture effects. Among the several available mathematical models, the remaining of this chapter will introduce some of the historical combinatorial theories and models, namely the arithmetic definition of additivity (14), Bliss independence and Loewe's additivity law and other quantitative approaches like Chou-Talalay's median effect and CISNE (code for the identification of synergism numerically efficient).

2.4.1 Arithmetic definition of additivity

The arithmetic definition of additivity identifies the effect of a combination by simple addition (14):

$$E_{A1+B2} = E_{A1} + E_{B2} \quad (2.1)$$

This means that the expected effect of a combination between contaminant A at a given dose (D1) and contaminant B at a given dose (D2), E_{A+B} corresponds to the sum of individual effects of both contaminants at the given doses (E_{A1} and E_{B2}). Mathematically, since the dose-effect curve is not linear, it leads to inaccurate expectations of mixtures effects (124). In simpler terms, if A and B each inhibits 30%, the additive effect cannot be 60% because if A and B each inhibits 60%, the combined additive effect of 120% is biologically impossible if the maximally inducible effect can only be 100% (124, 125). Despite its inaccuracies, this model was applied to assess mycotoxin interactions in 52 out of 83 studies reviewed in (126).

Nevertheless, other approaches are required to provide more reliable calculations of mixture effects, such that a reference point for the assessment of combination effects in terms of synergisms, additivity, and antagonism can be defined (125).

2.4.2 Reference additivity models

A reference model, often referred to as the null hypothesis of non-interaction, serve as a baseline against which we can measure how drug interactions should ideally occur based on the individual effects of the drugs (127). Any deviation from these reference models is what we use to identify synergy or antagonism, depending on the direction of this deviation (127). Examples of globally accepted reference additivity models are the independent action model and the concentration addition model.

2.4.2.1 Independent action model

Developed in 1939, the independent action (IA) also known as Bliss independence model rests on the assumption that the effects of the individual mixture constituents are independent from one another (128). In mathematical terms:

$$F_{ac} = f_{aA} + f_{aB} - f_{aA} \times f_{aB} \quad (2.2)$$

Where F_{ac} is the fraction affected by combination of drugs A and B, at a given effect, f_{aA} is the fraction affected for drug A and f_{aB} is the fraction affected for drug B.

This implies that the action of substance A is independent and not influenced by substance B, and vice-versa. Bliss independence is most suitable for situations where drugs are expected to act independently, such as when they have different mechanisms of action or affect different pathways. This also means that chemicals that are present below ‘zero effect’ levels are not expected to contribute to the total mixture effect.

Although still commonly used as a basic reference model, there has been much criticism over the validity of the Bliss Independence model (127). Some limitations are related to the model’s assumptions; it assumes a linear relationship between the effects of the drugs (but in reality, drug interactions can be nonlinear) and it assumes that drugs act independently, which is untrue for a large proportion of drug interactions (127). Another missing point often brought up by advocates of the Loewe additivity reference model is that Bliss fails when applied to the “sham mixture” scenario, explained below (127).

2.4.2.2 Concentration addition model

The concentration addition (CA) model or Loewe additivity model relies on the fairly intuitive scenario of a “sham mixture”, where a single drug is mixed with itself. The idea is that a compound combined with itself should yield no interaction effect (127).

In simpler terms, Loewe’s additivity law assumes that the compounds act within the same compartment on the same biological size by the same mechanism. The only difference is in their potency. Considering a two-drug combination, the additive effect can be defined as

$$\frac{D_A}{D_{A_{alone}}} + \frac{D_B}{D_{B_{alone}}} = 1 \quad (2.3)$$

where D_A and D_B is the doses of drug A and B applied in combination for achieve a certain response level (x), and $D_{A_{alone}}$ and $D_{B_{alone}}$ correspond to the doses required to achieve the same effect when applied alone.

To formulate a Loewe additivity model, the dose-response relationship of individual drugs needs to be modeled first. If two agents share the same shape and slope for their concentration-response curves and differ only in potency, then it can be said that they act as a dilution of each other if combined together. Consequently, each compound in the mixture can be re-

placed by an equi-effective concentration of another, without diminishing the overall mixture effect. By implication, this means that every mixture component contributes to the overall combination effect in proportion to its concentration, even below zero effect levels. However, finding compounds that fulfill these requirements is a hard task, which leads to the practically inapplicability of CA model as exemplified in (14).

2.4.3 Quantification of interactions

2.4.3.1 The Chou-Talalay Method

The Chou-Talalay method is one of the most widely used methods for detecting and quantifying synergistic interactions between two or more drugs (16, 124). The basis of this method is the median-effect equation (2.4) of the mass-action law, which unifies the Michaelis-Menten equation for enzyme substrate saturation, the Hill equation for ligand occupancy at high order, the Henderson-Hasselbalch equation for pH ionization, and the Scatchard equation for the receptor binding (124).

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m} \right)^m \quad (2.4)$$

where f_a is the fraction of cells affected (i.e., killed), f_u is the fraction of cells unaffected (i.e., living) and corresponds to $1 - f_a$, D_m is the dose that produces 50% of the maximum effect (i.e., the IC_{50}), and m is a parameter that describes the shape of the dose-response.

The median-effect plot which is a plot of $\log(D)$ vs $\log(\frac{f_a}{f_u})$ yields a straight line with slope (m) and the x-intercept of $\log(D_m)$ (2.5):

$$\log\left(\frac{f_a}{f_u}\right) = m \times \log(D) - m \times \log(D_m) \quad (2.5)$$

The combination index (CI) concept was introduced by Chou T.C. and Talalay P. in 1984 (124). The derived combination index equation for two drugs is:

$$CI = \left(\frac{D_1}{D_{x1}} \right) + \left(\frac{D_2}{D_{x2}} \right) \quad (2.6)$$

where $(D_x)_1$ and $(D_x)_2$ are the individual doses of contaminants to produce a certain effect ($x\%$), while D_1 and D_2 are doses of each contaminant in the binary combination that were

required to achieve the same effect. In this equation, a CI value of 1 indicates additivity, $CI < 1$ indicates synergy, and $CI > 1$ indicates antagonism between the two drugs.

The Dose-Reduction Index (DRI) is another concept that facilitates the comprehension of interaction between two or more contaminants (124). According to Chou *et al.*, the DRI is a measure of how many -fold the dose of each drug in a combination may be reduced at a given effect level compared with the doses of each drug alone. For a two-drug combination, the DRI value for each corresponding drug is given as

$$DRI_1 = \frac{(D_x)_1}{(D)_1}, DRI_2 = \frac{(D_x)_2}{(D)_2} \quad (2.7)$$

where $(D_x)_1$ and $(D_x)_2$ are the individual doses of contaminants to produce a certain effect ($x\%$), while D_1 and D_2 are doses of each contaminant in the binary combination that were required to achieve the same effect. This means that a DRI equal to 1 means that the dose in the combination treatment is the same as the dose when the drug is administered alone, while a DRI greater than 1 indicates that the combination treatment reduces the drug dose while maintaining the desired effect.

2.4.3.2 CISNE (Code for the Identification of Synergism Numerically Efficient)

While the Chou-Talalay method is widely recognized, it does have certain technical challenges in terms of model fitting, which can introduce biases into the estimation of parameters (129, 130). According to Chou's linearization of the median-effect equation (Equation 2.5), by estimating the slope and intercept by least squares fit, Dm is calculated as

$$Dm = e^{\frac{m \times \log(D_m)}{m}} \quad (2.8)$$

This calculation yields sensitive and biased estimation of dose-effects at extremes responses (close to 0% or 100%), and excludes of data points with effects smaller than 0% or larger than 100% since they can not be used in the logarithm on the left side of Equation 2.5. These biases can lead to significant false positive or false negative errors, depending on the slope of the dose-response curve (129, 130). Fitting the same equation as a non-linear regression model estimates model parameters better and reduces the rate of false positives or negatives, especially when the slope (m) deviates from 1. This non-linear regression can

be easily applied using the free CISNE software.

2.4.3.3 Considerations on experimental design

To accurately assess the effects of a complex mixture, it is required to have the individual response profiles of each component as well as the precise composition of the tested mixture (124). *In vitro* studies are helpful to achieve this goal, as they allow for a more controlled environment than animal or human studies. Moreover, cell-based models produce large amounts of highly reliable data in a relatively short period. Recognisably, to ensure greater precision of these predictions, high reproducibility in the assay of choice is demanded. Cell viability assays are commonly employed to study mixtures effects, as an ideal cell viability assay should be safe, rapid, reliable, efficient, time- and cost-effective, and not interfere with the compounds of interest (131). In addition to assay selection, other experimental design parameters require attention like dose density and range applied to the model system, as well as, combination designs.

Fixed-ratio combination design

Figure 2.6 describes several sample design approaches.

A fixed-ratio combination stipulates a fixed ratio of the individual contaminants, rather than varying the dose of each contaminant independently. This approach minimizes the number of combination data points in comparison with matrix design while still providing valuable information on drug interactions (124, 132).

Equipotent ratio mixtures are a key concept in the fixed-ratio combination approach (radial design) (124, 127). In these mixtures, each component is present in the combination at a concentration that yields an equal and predetermined effect level. For instance, if drug A has an IC_{50} of 2 μ M and drug B has an IC_{50} of 4 μ M, an equipotent mixture can be established at a 1:2 ratio, ensuring that both drugs contribute equally to the overall effect. Equipotent ratio mixtures are particularly valuable when studying interactions between well-characterized contaminants and when precise concentration-response data are available for each component (124). Non-equipotent fixed ratio approaches may also be used. This approach is often chosen based on practical considerations, such as safety thresholds or the desire to maintain the concentrations within a reasonable range (124).

Approaches may include edge design in which a contaminant levels remains unaltered.

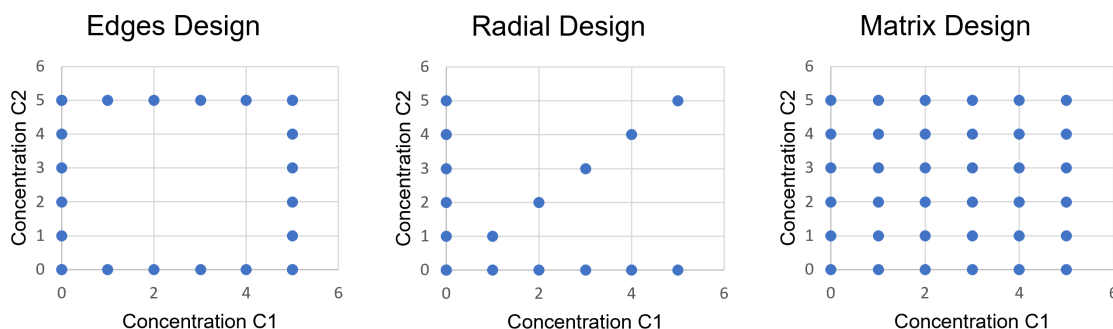


Figure 2.6: Possible configurations of sample design in combination studies.

Low-dose Mixtures

Little is known about the potential adverse effects from long-term exposure to complex mixtures at low doses, close to health-based reference values (122, 123). Evaluation of chemical mixture effects requires an integrated and systematic approach and close collaboration across different scientific fields, particularly toxicology, epidemiology, exposure science, risk assessment and statistics, to properly integrate data from all these disciplines.

To achieve a comprehensive assessment, it is essential to accurately measure human exposure to these contaminants through various methods and tools, including biomonitoring, environmental monitoring, and dietary surveys. Scientific literature provides a great backbone to this approach by providing valuable data concerning analyses and quantification of food contaminants in different foods. Manual summarising of the vast amount of information available in scientific literature is not feasible, so recent novel alternatives like literature mining and machine learning techniques can be employed. Martins *et al.* employed literature mining and machine learning techniques to explore the presence of chemical contaminants in various food groups (113). The study collected data from 254 papers published over the past two decades, resulting in 11723 data points. The distribution of contaminants varied across food groups, with metals being the most studied, followed by PAHs, mycotoxins, pesticides, and HAAs. Metals were found in all food groups, while PAHs were present in seven out of 12 groups. Mycotoxins were found in six groups, and pesticides were found in almost all groups except for meat, eggs, and vegetable oils. This work synthesises diverse sources of information on contaminant presence across various food groups, formulating a realistic

mixture of food contaminants according to their contribution to daily diet consumption.

The intersection of data mining, food contaminants monitorization, and the definition of realistic contaminant mixtures delivers a comprehensive and contextually rich framework for assessing toxicological properties of compounds in complex mixtures, with implications for the development of new food quality control strategies.

Chapter 3

Materials and methods

3.1 Chemicals and materials

Benzo[a]pyrene (CAS 50-32-8, $\geq 96\%$), acrylamide (CAS 79-06-1, $>99\%$), aflatoxin B1 (CAS 1162-65-8, $>98.0\%$), cypermethrin (CAS 52315-07-8, PESTANAL®, analytical standard), were purchased from Sigma Aldrich (St Louis, MO, USA). Cadmium chloride ($\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$, 98%) was kindly provided by the Laboratory of Organic and Pharmaceutical Chemistry.

For cell culture maintenance, high glucose Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and sodium pyruvate, fetal bovine serum (FBS), 0.25% Trypsin/ 0.02% EDTA solution and Penicillin/Streptomycin 100x solution (Pen/Strep) were all purchased from Biowest (Nuaille, France). Phosphate buffer saline tablets (PBS) were from Sigma Aldrich (St Louis, MO, USA). The T-75 cm^2 , T-25 cm^2 flasks, serological pipettes, screw-cap criovials and 96-well and 12-well plates for cell assays were obtained from Sarstedt (Nümbrecht, Germany). The 0.22 μm PES syringe filters were purchased from FilterBio Membrane (Jiangsu, P.R China), while the 2 mL and 5 mL syringes were from BD Emerald (Fraga, Spain). All materials were acquired sterile or underwent prior sterilization before use in cell culture, and all procedures were carried out in a safety hood cabinet.

Dimethyl sulfoxide (DMSO) (CAS 67-68-5, $>99.9\%$) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (CAS 298-93-1, $>98.0\%$) was purchased from Duchefa Biochemie B.V. (Haarlem, The Netherlands). Human liver S9 (pooled, 20 mg/mL) was purchased from Sigma-Aldrich. NADPH tetrasodium salt (CAS 2646-71-1, $>95\%$) and glutathione reduced (GSH) (CAS 70-18-8, $>97\%$) were from Panreac (Darmstadt, Germany).

For gene expression analysis, PureZOL™ RNA Isolation Reagent, iScript cDNA synthe-

sis kit and iTaq Universal SYBR Green Supermix were obtained from BioRad Laboratories (California, USA). Agarose and TAE buffer 10x were obtained from GRiSP (Porto, Portugal). Bioperformance certified water was from Sigma Aldrich (St Louis, MO, USA), while the solvents ethanol absolute and 2-propanol were obtained from Honeywell, Riedel-de-Haën (Seetze, Germany). For materials, 0.2 ml 8-tube PCR strips and caps were from BioRad Laboratories (California, USA), 1.5 mL PCR clean safe-lock tubes were from Eppendorf (Hamburg, Germany), and filtered pipette tips were from Sarstedt (Nümbrecht, Germany). All materials were RNase-free, and procedures were conducted in a PCR workstation.

3.2 Methods

3.2.1 Cell culture

The human neuroblastoma SH-SY5Y cell line was used to assess the neurotoxicity of the diet-related contaminants. SH-SY5Y cells were acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, ACC 209, LOT 17) and maintained at passage numbers below 15 to ensure the preservation of their neuronal profile, as indicated by (133). Cells were maintained in 75 cm² culture flasks in complete cell culture medium (CM) composed of DMEM, supplemented with 15% FBS (v/v), 1% Pen/Strep (v/v) in a humidified incubator at 37°C and 5% CO₂. Subculture procedures were performed once a week at 80-90% cell confluence. Briefly, after CM removal, adherent cells were rinsed with warm sterile PBS and detached using 1 mL 0.25% trypsin-EDTA solution (2 minutes at 37°C and 5% CO₂). Trypsin was neutralized with CM (1:4) and cell density was assessed using the improved Neubauer chamber (Marienfeld Superior, Germany) by adding trypan blue solution to an aliquot of cell suspension (1:2). Cells were passaged at the initial density of at least 50 000 cells/mL. SH-SY5Y cells stock were preserved in 90% FBS and 10% DMSO in screw-cap vials at -80°C. Regular microscopic examination was conducted to detect any anomalies or contaminants and to assess the ratio between floating and adherent cells. Furthermore, mycoplasma testing through qPCR was performed every 3 months.

3.2.2 Cell viability assay

The MTT assay is a widely used colorimetric assay in cell biology and toxicology to assess cell viability and proliferation (131). This assay relies on the ability of viable cells with active NAD(P)H-dependent oxidoreductase enzymes to reduce the yellow water-soluble MTT reagent to a purple formazan product, which is insoluble in aqueous solutions (Figure 3.1). The intensity of the formazan product is proportional to the number of viable cells.

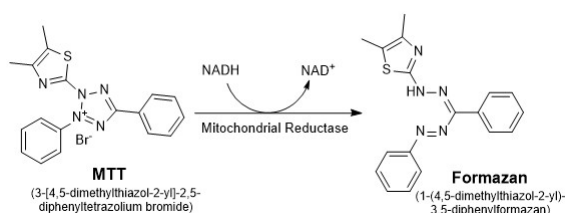


Figure 3.1: Reduction of MTT to formazan crystals by mitochondrial reductases of SH-SY5Y cells (131).

SH-SY5Y cells were seeded in 96-well plates at a density of 50 000 cells/mL. After a 24 h period for cell adhesion at 37 °C, 5% CO₂, cells were exposed to compounds for 72 h, both alone or in mixture, following the experimental design. After 72 h of exposure, the medium was removed and 100 μ L of MTT solution (0.5 mg/mL) was added to each well and plate were incubated for 3 hours at 37 °C, 5% CO₂. The solution was removed, and the purple formazan crystals were dissolved in DMSO (100 μ L). Absorbance was read at 570 nm on the SPECTROstar Nano Microplate reader (Ortenberg, Germany). Cells treated with CM alone and with DMSO (0.5%) were used as controls to ensure the viability of cells during the experiments. All measurements were done in 6 replicates in 3 independent experiments. Results are expressed as percentage of cell viability in relation to adequate solvent control.

3.2.3 S9 fraction incubation

Prior to the incubations, the protein density of the S9 fraction was adjusted to contain 1 mg/mL in 100 mM phosphate buffer, pH 7.4 and stored in aliquots at -80°C. Reaction mixture was prepared in PBS (100 mM, pH 7.4) was consisted of human liver S9 fraction (1 mg/mL final protein concentration), parental compound, prepared in a total volume of 0.5 mL and preincubated in a shaking water bath at 37 °C. After acclimatization, the reaction was initiated by addition of 1 mM NADPH and 2 mM GSH, as previously reported (134). Blank

samples were prepared as described above, but with 0.1% DMSO instead of substrate (S9 vehicle matrix). The reaction was quenched after 3 hours by addition of methanol to the samples (ratio of 1:4 methanol) to precipitate the proteins. Samples were centrifuged at 16,000 g, 10 min, 4 °C, and the supernatant was dried under nitrogen flux. The residues were resuspended in CM for cell viability assays and gene expression analysis.

3.2.4 Gene expression analysis

3.2.4.1 Cell exposure and RNA extraction

SH-SY5Y cells were seeded in 12 well plates (150000 cells/well) and exposed to contaminants for 72 hours. Treatment conditions depicted in 3.2.5 were performed in triplicate and in 3 independent experiments.

Total RNA of SH-SY5Y was extracted with PureZOL™ RNA Isolation Reagent as illustrated in 3.2. PureZOL™ is a phenol and guanidine isothiocyanate-based reagent that effectively denatures proteins, disrupts cells, and separates RNA from other cellular components. Following treatment removal, 500 µL of PureZOL™ was added to each well, and the solution was vigorously mixed for 5 minutes. Technical replicates were combined in 1.5 mL tubes and stored at -80°C to prevent RNA degradation until RNA isolation. Chloroform was added to allow for phase separation, and subsequent centrifugation created a biphasic solution where the lower organic phase contained proteins and DNA while the upper aqueous phase contained RNA. Without interfering with the interface, the aqueous phase was transferred to new 1.5 mL tubes and ice-cold isopropyl alcohol was added to precipitate RNA, given its low solubility in alcohol. After further centrifugation, the RNA pellet was washed with ice-cold 75% ethanol. In the follow-up centrifugation, the supernatant was discarded, and the pellet dried before resuspension in nuclease-free water.

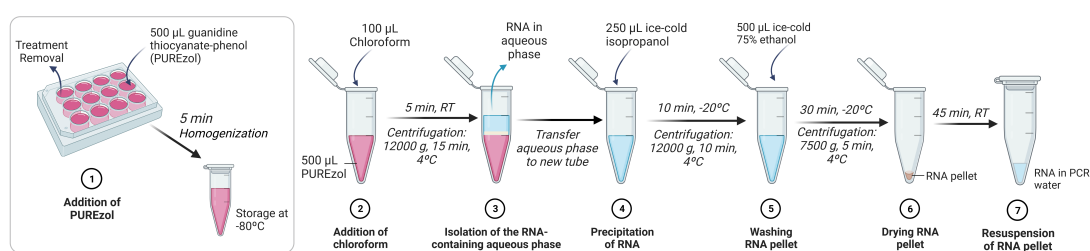


Figure 3.2: Steps in RNA extraction based on Instruction Manual PureZOL™ RNA Isolation Reagent. Created with Biorender.

3.2.4.2 RNA quality and quantification

RNA quantification was performed using a UV-Vis spectrophotometer (Take3 adapter, BioTek plate reader, USA). Absorbance measurements were conducted at 260 nm in duplicate with 2 μL of sample and RNA concentration in samples was calculated based on Lambert-Beer law. Furthermore, the purity of the RNA samples was assessed by determining the A_{260}/A_{280} ratio and (values ≥ 2.0 were considered acceptable). The absorbance at 280 nm indicates protein contamination, which can affect the accuracy of RNA concentration measurements. To standardize the RNA concentrations, all samples were adjusted to a uniform concentration of 100 ng/ μL in nuclease-free water. The integrity of the RNA samples was evaluated through electrophoresis on a denaturing agarose gel in Tris-Acetate-EDTA (TAE) 1x buffer, which was stained with GelRed (Biotium, Fremont, USA) and visualized using a UV light tray (Gel Doc EZ System, Bio-Rad).

3.2.4.3 Reverse transcription quantitative polymerase chain reaction

In a two-step reverse transcription quantitative polymerase chain reaction (RT-qPCR) workflow, the process of RNA quantification is divided into two separate stages. First, the sample's RNA is converted into complementary DNA (cDNA) through the reverse transcription step. Then, this initial cDNA strand is amplified and quantified using qPCR. One significant benefit of this two-step method is its flexibility in configuring reactions and its ability to analyze multiple targets within a single RNA sample. Thus, cDNA synthesis was performed using iScript™ cDNA Synthesis Kit, through two-step PCR (RT-qPCR), in BioRad MJ Mini Thermal Cycler as reported in Table 3.1. The synthesis process involved the transcription of 500 ng of RNA in a 10 μL reactions. cDNA was preserved at -25 °C until analysis.

Table 3.1: Reagents and reaction protocol for cDNA synthesis.

Component	Volume per reaction (μL)	Reaction Protocol	
5x iScript Reaction Mix	2	Priming	5 min at 25°C
iScript Reverse Transcriptase	0.5	Reverse transcription	20 min at 46°C
RNA template	5	RT inactivation	1 min at 95°C
Nuclease-free water	2.5	Hold	10 min at 4°C

The expression levels of genes related to apoptosis and neuronal markers were assessed through quantitative polymerase chain reaction (qPCR). Amplification was performed in CFX

Connect Real-Time PCR System equipment (BIO-RAD) with the following conditions: one denaturation step at 95 °C for 40 s followed by 40 cycles at 95 °C for 15 s and annealing at 60 °C for 60 s. The qPCR reaction mixture was prepared in triplicate and each replicate consisted of cDNA diluted 50 times with nuclease free water (2 μ L), iTaq Universal SYBR Green Supermix (5 μ L), RNase-free water (2.2 μ L) and primer sets at 10 μ M (2 $\times$ 0.4 μ L). Amplification specificity was confirmed by melting curve analysis (65 to 95°C, increment 0.5°C) (135). The expression level of BAX, BCL2, CASP3, NF- κ B and MAPT was calculated by the $2^{-\Delta\Delta C_q}$ method (136). The list of primer sequences is shown in Table 3.2. Primer amplification efficiencies ranged from 90 to 105% and were determined using five data points of a standard curve from 25 to 15625x dilution of cDNA. Treated samples were expressed against reference (CM or vehicle) and normalized to the housekeeping genes GAPDH and β -Actin expression.

Table 3.2: Primers sequences for qPCR.

Target	Forward Sequence (5'- 3')	Reverse Sequence (5'- 3')	References
GADPH	CAACGGATTTGGTCGTATTGG	GCAACAATATCCACTTTACCAGAGTTAA	(137)
β -Actin	CACTCTTCCAGCCTTCCTTCC	GCACTGTGTTGGCGTACAGG	(138)
BAX	CCCCCGAGAGGTCTTTTCC	CCTTGAGCACCAGTTTGCTG	(139)
BCL2	CTTCTTTGAGTTCGGTGGGG	AAATCAAACAGAGGCCGCAT	(139)
CASP3	GTGGAGGCCGACTTCTTGTA	TTTCAGCATGGCACAAAGCG	(139)
NF- κ B	ATGTGGAGATCATTGAGCAGC	CCTGGTCCTGTGTAGCCATT	(140)
MAPT	GATTGGGTCCCTGGACAATA	GTGGTCTGTCTTGGCTTTGG	(28)

3.2.5 Experimental design

3.2.5.1 Preparation of stock solutions

Stock solutions of ACR (10 M) and Cd (1 M) were prepared in ultrapure water. Stock solutions at solubility maximum of AFB1 (35 mM), BaP (100 mM) and CYP (500 mM) in DMSO. Stock solutions were stored at -20°C and working solutions were prepared in CM right before experiments and maximum vehicle solvent percentage was set at 0.5%.

3.2.5.2 Isolated effects of contaminants on SH-SY5Y cells

The neurotoxic effects of the individual contaminants ACR, AFB1, BaP, Cd and CYP were assessed after incubation for 72 hours using the MTT assay (described in section 3.2.2). The range of doses surveyed is described in Table 3.3. The results from isolated contaminants experiments were used to fit a dose-response effect curve. Concentrations were selected to enable a full dose response curve from no effect to the highest viability inhibition as possible. Experiments were conducted in three independent occasions, with 6 replicates per measurement.

Table 3.3: Range of concentrations of contaminants tested isolated in SH-SY5Y cells.

	Levels of concentrations (μM)							
ACR	10000.00	5000.00	2500.00	1250.00	625.00	312.50	156.25	78.13
AFB1	100.00	50.00	25.00	12.50	6.25	3.13	1.56	0.78
BaP	200.00	100.00	50.00	25.00	12.50	6.25	3.13	1.56
Cd	100.00	50.00	25.00	12.50	6.25	3.13	1.56	0.78
CYP	250.00	125.00	62.50	31.25	15.63	7.81	3.91	1.95

3.2.5.3 Metabolic activation and its impact on the neurotoxicity of isolated contaminants

ACR, AFB1, BaP and CYP were incubated with human S9 fraction in duplicate in two different occasions. From contaminants stock solutions, working solution were prepared in PBS right before experiments, with a maximum DMSO percentage set at 0.1%. Incubation with human S9 fraction (section 3.2.3) was done with standard 100 μM of parental compound, as conducted in (134, 141), ACR and CYP. BaP was incubated at 50 μM due to solubility problems with PBS at 100 μM . To avoid DMSO influence on S9 fraction, AFB1 was also incubated at 50 μM .

To compare the neurotoxic effects of non-metabolized and metabolically activated contaminants in SH-SY5Y cells, residues resulting from human S9 fraction incubation were re-constituted in CM at the same volume as S9 reaction (0.5 mL). The aim was to achieve concentrations of these residues that would match the concentration of the parent compound if no metabolism had occurred, equivalent to initial contaminant concentration (50 μM for BaP and 100 μM for ACR and CYP). An additional dilution was done for AFB1 so target concentrations was 10 μM for AFB1 (dilution factor of 5) to be similar to the IC_{50} found in SH-SY5Y cells.

The same concentrations were used for the non-metabolized counterparts. Subsequently, SH-SY5Y cells were exposed for 72 hours to two sets of treatments: non-metabolized and S9 metabolized ACR, AFB1, BaP, and CYP. Control assays included CM, 0.1% DMSO and a S9 vehicle matrix (S9 without any compound with 0.1% DMSO). These experiments were conducted on two separate occasions, with three technical replicates.

3.2.5.4 Combined effects of contaminants in SH-SY5Y cells

To evaluate the combined effects of contaminants, mixtures were designed at constant ratio. This radial approach minimizes the number of combination data points while still providing valuable information on contaminants interactions (Figure 2.6). All combinations effects in SH-SY5Y cells were evaluated through MTT assay after 72 hours of exposure.

BaP was excluded from combination studies due to its inability to achieve a 50% inhibition of cell viability and practical challenges with solubility hindered the evaluation of BaP at higher concentrations.

The neurotoxicity of ACR/AFB1, ACR/Cd, AFB1/Cd and ACR/AFB1/Cd was thus studied at different levels of concentration at equipotent ratios, following the methodology proposed by Chou for better accuracy(124) , so that the effect of each contaminant would be similar. (Table 3.4). Experiments were conducted in three independent occasions, with 6 replicates per measurement.

Table 3.4: Concentrations of binary mixtures of ACR, AFB1 and Cd tested in SH-SY5Y cells.

(A) ACR/AFB1			(B) ACR/Cd			(C) AFB1/Cd			(D) ACR/AFB1/Cd			
Conc ACR (μM)	Conc AFB1 (μM)	IC ₅₀ ratio	Conc ACR (μM)	Conc Cd (μM)	IC ₅₀ ratio	Conc AFB1 (μM)	Conc Cd (μM)	IC ₅₀ ratio	Conc ACR (μM)	Conc AFB1 (μM)	Conc Cd (μM)	IC ₅₀ ratio
3200	40	80:1	3200	20	160:1	40	20	2:1	3200	40	20	160:2:1
1600	20		1600	10		20	10		1600	20	10	
800	10		800	5		10	5		800	10	5	
400	5		400	2.5		5	2.5		400	5	2.5	
200	2.5		200	1.25		2.5	1.25		200	2.5	1.25	
100	1.25		100	0.625		1.25	0.625		100	1.25	0.625	
50	0.625		50	0.3125		0.625	0.3125		50	0.625	0.3125	

CYP individual experimental data below IC₅₀ was not possible to determine (concentrations higher than 500 μM were not tested due to solubility issues in working CM solutions). A non-equipotent fixed ratio approach was followed for CYP binary mixtures (CYP/ACR, CYP/AFB1, CYP/Cd) to avoid excessively high concentrations irrelevant for food-related CYP exposure and considering also CYP insolubility in CM at higher levels. Experiments

were conducted in two independent occasions, with 6 replicates per measurement.

Table 3.5: Concentrations of CYP-containing binary mixtures tested in SH-SY5Y cells.

(A) CYP/ACR			(B) CYP/AFB1			(C) CYP/Cd		
Conc CYP (μM)	Conc ACR (μM)	IC ₅₀ ratio	Conc CYP (μM)	Conc AFB1 (μM)	IC ₅₀ ratio	Conc CYP (μM)	Conc Cd (μM)	IC ₅₀ ratio
500	3200	1:6.4	500	40	12.5:1	500	100	5:1
250	1600		250	20		250	50	
125	800		125	10		125	25	
62.5	400		62.5	5		62.5	12.5	
31.25	200		31.25	2.5		31.25	6.25	
15.625	100		15.625	1.25		15.625	3.125	
7.8125	50		7.8125	0.625		7.8125	1.5625	
3.90625	25		3.90625	0.3125		3.90625	0.78125	

Traditional toxicity studies often involve testing substances in isolation or in binary mixtures across a wide range of concentrations to assess their individual and combined effects for a comprehensive hazard characterization. This approach is valuable for understanding the fundamental toxicological properties of substances and their potential interactions. However, it is our opinion that an approach of testing mixtures based on realistic combinations, ratios, and concentrations of toxicants that can occur in foods is also essential. This approach is more representative of real-world exposure scenarios and can provide valuable insights into the safety of the foods we consume. Thus, in addition to the described dose-response studies, a realistic mixture (MIX) of the studied compounds ACR, AFB1, Cd and CYP was tested at different concentrations in SH-SY5Y cells. Concentrations covered realistic doses and ratios obtained from previous studies within our group as outlined below. The composition of this mixture was determined by analyzing contaminants food occurrence data gathered by literature mining and machine learning techniques (as discussed in section 2.4.3.3) (113) and other population exposure data (58). Briefly, a standard omnivore diet using common food items was formulated, ensuring it provided the daily caloric intake close to 2500 kcal and an acceptable macronutrient distribution (supplementary material 5.1). Daily exposure to contaminants from this data was calculated, taking into consideration each food's contribution to the diet and the weighted average of contaminant levels in each food. Next, different percentiles of exposure were calculated, and the contaminant ratios at each percentile were determined. These ratios were then averaged to create a comprehensive profile of exposure. This profile was used to formulate a real low-dose mixture, which was subsequently employed to evaluate neurotoxicity in SH-SY5Y cells. Serial dilutions of this mixture were prepared so that the levels of exposure covered the 25th, 75th, 95th percentiles (Table 3.6). Experiments were conducted in three independent occasions, with 6

replicates per measurement.

Table 3.6: Concentrations of realistic mixtures of contaminants tested in SH-SY5Y cells.

	Ratios	Levels of concentrations (μM)							
		S1	S2	S3	S4	S5 (P95)	S6 (P75)	S7	S8 (P25)
ACR	59.26	31.93	15.97	7.98	3.99	2.00	1.00	0.50	0.249
AFB1	3.65	1.97	0.98	0.49	0.25	0.12	0.06	0.03	0.015
Cd	6.65	3.58	1.79	0.90	0.45	0.22	0.11	0.06	0.028
CYP	30.45	16.41	8.20	4.10	2.05	1.03	0.51	0.26	0.128
Total	100.00	53.88	26.94	13.47	6.74	3.37	1.68	0.84	0.42

3.2.5.5 mRNA expression

SH-SY5Y cells were exposed for 72 hours to several treatments for mRNA expression analysis. Interest in this analysis arises with the goal to obtain further information regarding contaminants effect both individually and in selected mixtures beyond cell viability. So for isolated contaminants treatments, doses close to the IC_{50} were applied in the case of ACR (80 μM), AFB1 (1 μM) and Cd (0.5 μM). For BaP and CYP, doses applied were 50 μM and 100 μM , respectively. For AFB1/Cd was chosen a dose close to the IC_{50} (total dose 0.3 μM , ratio 2:1), while SH-SY5Y cells were exposed to 25th and 75th percentiles related to MIX combination 3.6. Given the cytotoxicity of preincubated S9 contaminants, a 50x dilution was applied to S9-AFB1 (1 μM final concentration), S9-BaP (1 μM) as well as to the matrix vehicle control S9. Vehicle controls of 0.1% DMSO and CM were also included. Experiments were conducted in three different occasions and two technical replicates.

3.2.6 Data and statistical analysis

Data were reported as mean $\pm$ standard error mean (SEM) in cell viability graphs using GraphPad Prism version 9.3.1 for Windows (GraphPad Software, La Jolla, California, USA) to represent the dose-effect response curves. Grubbs outliers test was performed on experimental data and outliers were removed. One-way ANOVA followed by Dunnett's multiple comparisons test against controls and Tukey's test to determine the statistical differences between the treatment groups was performed. Combination index (CI) method was calculated through the Chou-Talalay approach using Compusyn software version 1.0 (ComboSyn Inc., Paramus, NJ, USA) and CISNE model using CISNE software version 1.0 (University

of Oviedo). Relevant parameters for contaminants interaction evaluation were discussed in section 2.4.3. mRNA expression graphs are presented in $\log_2$ of 2^{-Cq} values for better visualization of under-expressed values. Statistical analyses were performed using GraphPad Prism version 9.3.1 for Windows (GraphPad Software, La Jolla, California, USA). Before comparison between groups, Shapiro-Wilk and Bartlett tests were performed to assess, respectively, the normal distribution and homogeneity of data variances. Quantitative data were examined using one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) post hoc test for multiple comparisons among the groups. Differences were considered statistically significant at $P < 0.05$. Symbols and correspondent p-values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Chapter 4

Results and discussion

4.1 Cytotoxicity of individual food contaminants

Initial cytotoxicity assessments were conducted to select contaminant classes and identify a single representative contaminant. These findings, along with existing literature data on their neurotoxic properties and prevalence in food sources, guided the choice of the following representative contaminants: ACR for processing-induced contaminants, AFB1 for mycotoxins, BaP for PAHs, Cd for heavy metals, and CYP for pesticides.

The effects of ACR, AFB1, Cd, CYP and BaP on SH-SY5Y cells viability, assessed by the MTT assay after 72 h incubation, are presented in Figure 4.1. ACR, AFB1, Cd and CYP inhibit cell viability in a concentration-dependent manner. However, BaP could not show a full dose-response curve to determine IC₅₀. At the highest concentration tested (100 μ M) cell viability was impacted to a maximum of 72.59 % (Fig 4.1E). Higher concentrations were not tested due to solubility problems in CM working solutions, and for this reason, BaP was excluded from combination studies. Other studies verified identical behavior with BaP, reporting no effect in mouse primary neurons or Neuro2a cells after exposure to 80 μ M for 48 h (142).

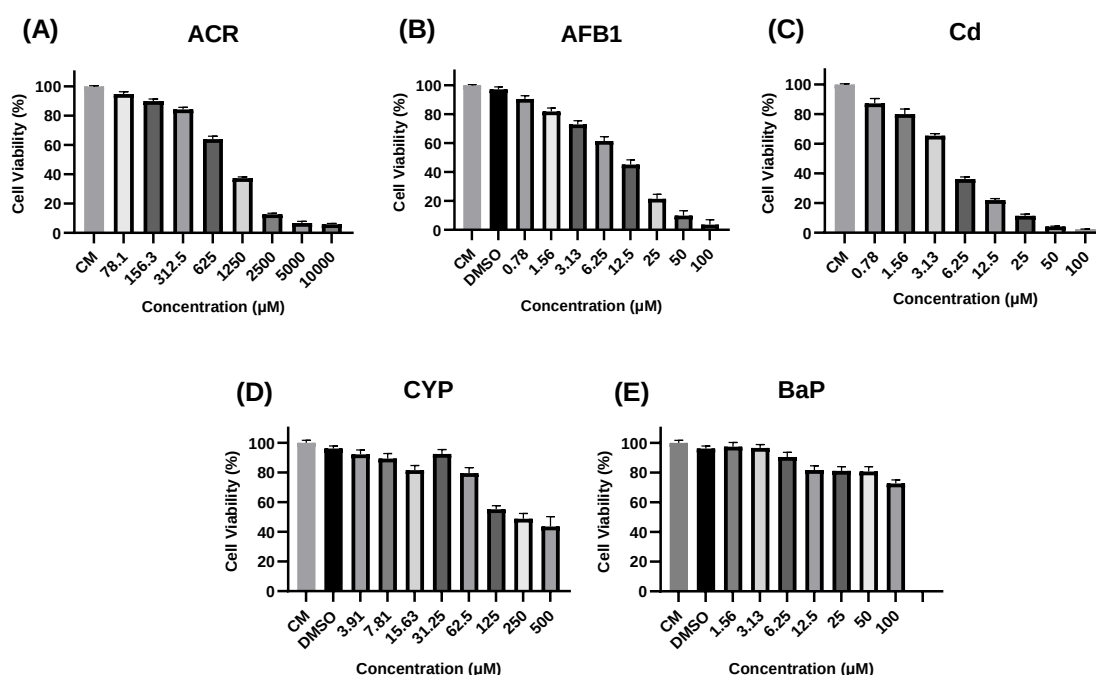


Figure 4.1: Percentage of SH-SY5Y cell viability (%) affected by different doses of contaminants (ACR, CYP, BaP, AFB1 and Cd) after 72 hours of exposure. The results are expressed as a percentage of the respective control mean $\pm$ SEM (CM for ACR and Cd and 0.5% DMSO for AFB1, BaP and CYP).

The models Chou-Talalay (CT) and CISNE were applied to individual dose-response data of contaminants, and calculated parameters are shown in 4.1. Dm (the concentration that produces 50% of effect) and m (slope of the median-effect plot) are calculated from fitting experimental data through linearization of the median-effect equation in the CT model, whereas CISNE takes advantage of modern curve fitting algorithms and obtains the values directly from nonlinear regression. The latter approach allows experimental data points to affect data above 100% or below 0% (from natural experimental fluctuations) not to be ignored and guarantees that data points close to extreme response share the same weight as others. Thus, CISNE reduces the rate of false positive or negative verdicts of synergistic behavior at IC_5 and IC_{90} . Notwithstanding, the CT method is the most prolific approach to quantifying synergy, even with its known limitations (127). Our approach was thus to compare both methods. Interfaces of the CT model (Compusyn software) and CISNE model are very basic. Still, Compusyn provides a more user-friendly manner to obtain and graph data on separate software. In contrast, CISNE only provides Dm and m values and simulated CI values, leaving the user to perform other calculations (e.g., DRI values).

The goodness of fit of the linearization of the median-effect equation (equation 2.5) was evaluated by the linear correlation coefficient (r value) for the CT model (Table 4.1). The standard for *in vitro* studies was met ($r \geq 0.95$) for ACR, AFB1 and Cd (r values of 0.9887, 0.9931 and 0.9955, respectively). CYP did not meet this criterion ($r = 0.9071$) since experimental data below IC_{50} was not possible to determine (concentrations higher than 500 μM were not tested due to solubility issues). All individual contaminants except CYP dose-effect curves present sigmoidal shapes ($m > 1$) for both models.

Table 4.1: Parameters (m , IC_5 , IC_{25} , IC_{50} (Dm), IC_{75} , and IC_{90}) of the isolated dose-effect response curves according to (A) Compusyn software based on CT base-model and (B) CISNE software based on CISNE model. m values signifies the shape of the dose-effect curve ($m = 1$, hyperbolic, $m > 1$ sigmoidal, $m < 1$ flat sigmoidal).

(A) Chou-Talalay									
	m	Dose Range	Units	IC_5	IC_{10}	IC_{25}	IC_{50} (Dm)	IC_{75}	IC_{90}
ACR	1.300	78.1-10000	μM	87.71	155.83	362.77	844.54	1966.07	4577.01
AFB1	1.162	0.08-10	μM	0.78	1.49	3.84	9.88	25.43	65.47
CYP	0.652	3.91-500	μM	2.96	9.32	50.26	270.96	1460.89	7876.42
Cd	1.213	0.8-100	μM	0.39	0.73	1.81	4.47	11.06	27.37
(B) CISNE									
ACR	1.513	78.1-10000	μM	126.57	207.42	428.79	886.43	1832.50	3788.30
AFB1	1.260	0.08-10	μM	0.61	1.19	3.19	8.51	22.74	60.75
CYP	0.804	3.91-500	μM	5.68	14.39	56.44	221.32	867.97	3403.93
Cd	1.118	0.8-100	μM	0.43	0.78	1.86	4.46	10.66	25.50

Comparing CISNE estimated parameters with CT parameters, both models estimated the following rank of toxicity: Cd > AFB1 > CYP > ACR. AFB1 and Cd were determined to be more potent, with a significant difference in the order of magnitude, meaning lower concentration levels are required to induce a determined response (e.g., IC_{50}). ACR was the less potent toxic at the IC_{50} level. Our results show Cd is the most cytotoxic contaminant in SH-SY5Y cells, with an IC_{50} calculated at 4.47 μM for CT and 4.46 μM for CISNE. Previous studies typically report neurotoxicity based on a 24 h incubation period, which cannot address chronic toxicity, especially in the case of substances with a long half-life, like heavy metals. A study in different neuronal cells found SH-SY5Y cells to be the least sensitive to $CdCl_2$ (IC_{50} for cellular ATP level of 67.5 μM) after 24 h, when compared to mouse N1E-115 cell

line and rat PC12 cell line (143). Bovio *et al.* reports a decrease in SH-SY5Y cell viability to 63% after a 24 h exposure with 20 μM CdCl_2 using MTT assay (101). AFB1 also exhibited great neurotoxicity, with an IC_{50} of 9.88 μM for CT and 8.51 μM for CISNE 4.1. In IMR-32 human neuroblast cells, AFB1 IC_{50} toxicity was of 6.18 $\mu\text{g/mL}$ (equivalent to 19.79 μM) after a 24 h (73). Congruent results regarding AFB1 neurotoxicity are reported in other human neuroblastoma models. In SKNMC and SK-N-SH cell lines, the latter being the parental cell line to the SH-SY5Y line, AFB1 is found to have a concentration and time-dependent toxicity, decreasing SK-N-SH cell viability to 58% after incubation of 10 $\mu\text{g/mL}$ (equivalent to 32.02 μM) (144). On the other hand, ACR requires milimolar concentrations to inflict cell death in SH-SY5Y (IC_{50} of 844.54 for CT and 886.43 μM for CT) 4.1. Attoff *et al.* reported similar MTT reduction results in the same cellular model and timepoint (50% loss of viability at 1000 μM) (60). Studies focus on ACR influence on neuronal differentiation (60, 145, 146). In the same study, chronic exposure to picomolar levels of ACR sustained viability and inhibited differentiation of SH-SY5Y cells (60).

CYP leads to significant loss in cell viability starting at 125 μM , decreasing viability to 57.50% (Fig 4.1D). Promthep *et al.* showed that cell viability of SH-SY5Y cells remained unaffected after 24 h exposure to 100 μM to CYP and only decreased cell viability to 80% after 48 h (110). CYP comprises multiple isomers, of which 20-40% consist of racemate α -CYP. α -CYP is more cytotoxic to SH-SY5Y cells with 60 μM lowering viability to 80% just after 24 h of exposure (147). CYP toxicity results should be carefully interpreted, and the isomeric composition should be considered.

4.1.1 Comparison of the cytotoxic effects of non-metabolized and metabolized contaminants in SH-SY5Y cells

The toxicity of several contaminants, including mycotoxins, acrylamide, PAHs, metals, and pesticides, can depend on metabolic activation processes. To assess if differential effects occurred between non-metabolized and metabolized contaminants on neuronal viability, ACR, AFB1, BaP and CYP were previously incubated with human S9 fraction to create metabolically activated samples (section 3.2.3).

Non-metabolized and metabolized pairs of contaminants (e.g., AFB1 and S9-AFB1) were assessed at an equivalent concentration of the parent compound, as outlined in section 3.2.5.

The findings depicted in Figure 4.2 indicate that the S9 vehicle matrix (S9) exhibits toxicity towards SH-SY5Y cells, reducing their viability to 51.1%. The rationale behind this cytotoxicity remains unexplained, despite the application of similar S9 incubation methods prior to *in vitro* studies on other cell lines (134). Kohn and Durham have reported that neurons display greater sensitivity to lower S9 concentrations than non-neuronal cells (148). This observation may provide insight into the matter, even though all proteins were eliminated via precipitation and centrifugation prior to the 72 h exposure to SH-SY5Y cells. Nevertheless, metabolic-activated contaminants differ from S9 vehicle matrix ($p < 0.0001$), and from the respective parental compounds at equivalent concentrations ($p < 0.0001$) with exception of S9-AFB1.

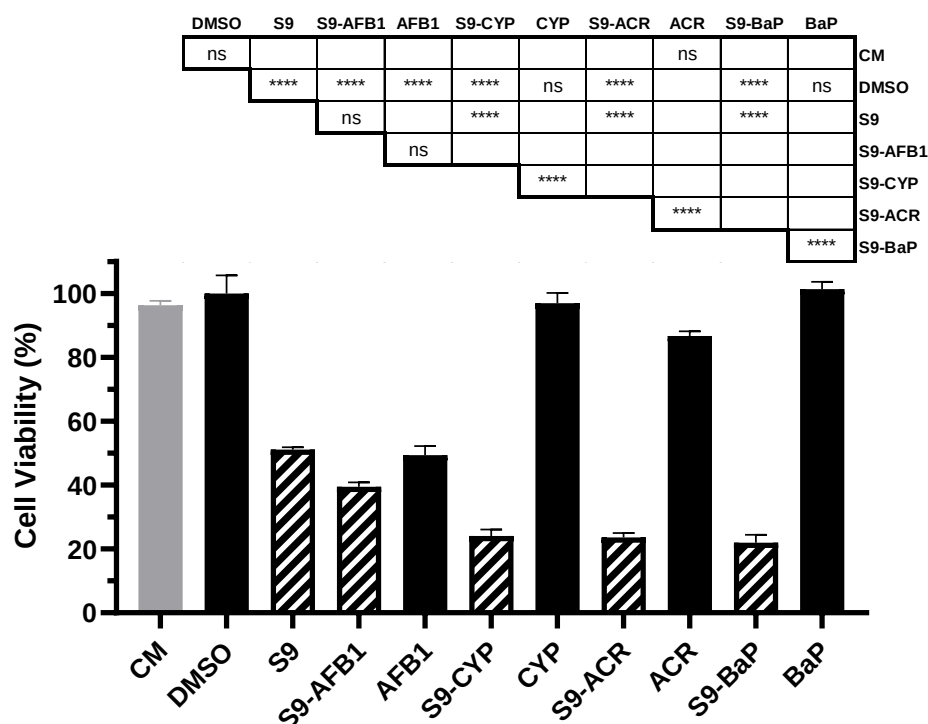


Figure 4.2: Percentage of SH-SY5Y cell viability (%) affected by non-metabolized and metabolized contaminant pairs at comparable total doses after 72 hours of exposure. The results are expressed as a percentage of the respective control mean $\pm$ SEM (CM for ACR, 0.1% DMSO for AFB1, BaP and CYP, S9 vehicle matrix (S9) for S9-AFB1, S9-CYP, S9-ACR, S9-BaP) (Symbols: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

4.2 Cytotoxicity of mixtures of food contaminants

4.2.1 Evaluation of the neurotoxicity of equipotent mixtures of food contaminants

Combined assays of ACR/AFB1, ACR/Cd, AFB1/Cd and ACR/AFB1/Cd were performed at equipotent ratios, so that the contribution to the effect of each contaminant in mixture would be equal (149). Ratios and concentrations assayed are described in 3.2.5. As explained before, combined assays (binary and tertiary mixtures) were done including ACR, AFB1 and Cd due to limitations in dose-response curves for BAP and CYP:

The experimentally determined dose-effect response curves of ACR/AFB1, ACR/Cd, AFB1/Cd and ACR/AFB1/Cd mixtures on SH-SY5Y cells viability are presented in Figure 4.3, in comparison to the dose-effect response curves of the individual mixture constituents.

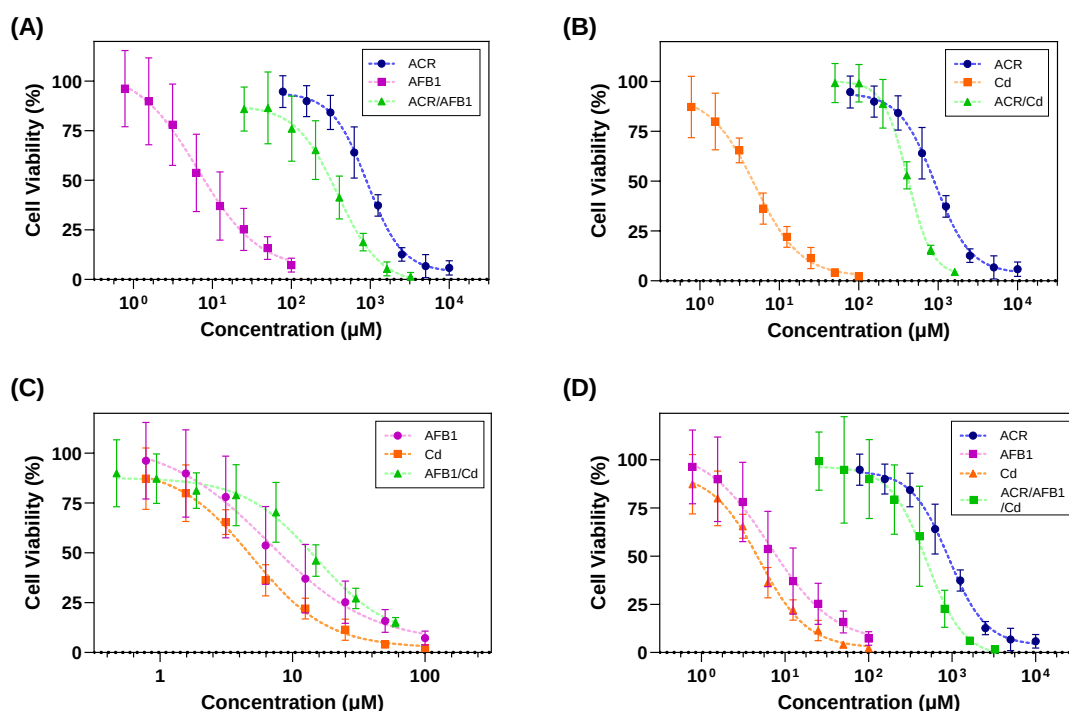


Figure 4.3: Dose-effect response curves of binary mixture ACR/AFB1 (A), ACR/Cd (B), AFB1/Cd (C) and ternary mixture ACR/AFB1/Cd (D) at equipotent ratios.

All combinations inhibited cell viability in a concentration-dependent manner, causing different dose-curve profiles than their constituents. For a comprehensive assessment of combined effects from contaminants interactions, curve parameters and CI should be evaluated. Table 4.2 itemizes combined parameters for ACR/AFB1, ACR/Cd, AFB1/Cd and ACR/AFB1/Cd mixtures. CI values are plotted as a function of effect level (fa) in Figure 4.4 to aid quantitative perception of the type of interactions in a given combination mixture in both CT and CISNE models.

Table 4.2: Parameters (m, IC₅, IC₂₅, IC₅₀ (Dm), IC₇₅, and IC₉₀) and CI values of the combined equipotent dose-effect response curves according to (A) Compusyn software based on Chou-Talalay and (B) CISNE software based on CISNE model. Type of interaction: Strong synergism (++++); Synergism (+++); Moderate synergism (++); Slight synergism (+); Nearly additive (±); Slight antagonism (-); Moderate antagonism (- -); Antagonism (- - -); and, Strong antagonism (- - - -)

(A) Chou-Talalay method

	m	IC5	CI value		IC10	CI value		IC25	CI value		IC50 (Dm)	CI value		IC75	CI value		IC90	CI value	
ACR/AFB1	1.53	38.61	1.05	±	62.86	0.92	±	128.67	0.77	++	263.39	0.64	+++	539.15	0.53	+++	1103.65	0.45	+++
ACR/Cd	2.52	280.23	7.60	----	376.89	5.61	----	582.72	3.60	----	900.96	2.31	---	1392.99	1.49	--	2153.73	0.96	±
AFB1/Cd	0.81	0.30	0.51	+++	0.75	0.68	+++	2.91	1.05	±	11.32	1.62	---	43.93	2.49	---	170.56	3.86	----
ACR/AFB1/Cd	1.77	70.83	3.01	---	107.90	2.48	---	200.38	1.86	---	372.09	1.41	--	690.97	1.06	---	1283.12	0.80	++

(B) CISNE

	m	IC5	CI value		IC10	CI value		IC25	CI value		IC50 (Dm)	CI value		IC75	CI value		IC90	CI value	
ACR/AFB1	1.25	26.76	0.75	++	48.66	0.74	++	117.25	0.72	++	282.52	0.72	++	680.74	0.74	++	1640.26	0.76	++
ACR/Cd	2.67	282.74	6.30	----	373.92	4.77	----	563.96	3.19	---	850.58	2.14	---	1282.87	1.44	---	1934.88	0.98	±
AFB1/Cd	1.03	0.74	1.38	--	1.53	1.51	---	4.46	1.73	---	13.01	1.99	---	37.90	2.30	---	110.42	2.66	---
ACR/AFB1/Cd	1.93	100.55	4.23	----	148.07	3.39	----	261.61	2.47	---	462.19	1.81	---	816.58	1.35	---	1442.69	1.01	±

The foreseen biased behaviour of CT model at extreme fa effects is well visualised when comparing CT and CISNE models (Figure 4.4) (129), despite CT r values above 0.97 for all combinations. In the case of ACR/AFB1 (Figure 4.4A), CISNE prevents an overestimation of synergistic interactions at fa above 0.5, stabilizing ACR/AFB1 combination as moderate synergistic through all range of effect levels. However, at low fa levels IC₅, a relevant synergism is detected by CISNE, while CI denotes a nearly additive effect. These differential effects at relevant doses from food exposure highlight the importance of the models selection and calibration. Fluctuations of CT model on the other hand predict additive affects at IC₅ and IC₁₀ and synergisms for higher inhibitory concentrations. For ACR/Cd combination (Figure 4.4B), strong antagonism is observed IC₅ and IC₁₀ for models. For AFB1/Cd combination (Figure 4.4C), CISNE computes more conservative CI values than CT. In fact, at fa close to 0 (where also doses of contaminants are low) a differential verdict on the type of interaction

from both models: CT calculates a CI value to 0.51, deeming the interaction synergetic while CISNE estimated moderate antagonism (CI value of 1.38). At other fa levels follow an antagonistic trend, towards strong antagonism and moderate antagonism at IC₉₀, for CT and CISNE, respectively. Lastly, ternary mixture is determined as strong antagonistic (CI value of 3.01) and antagonistic (CI value of 4.23) at IC₅ in CISNE and CT, respectively.

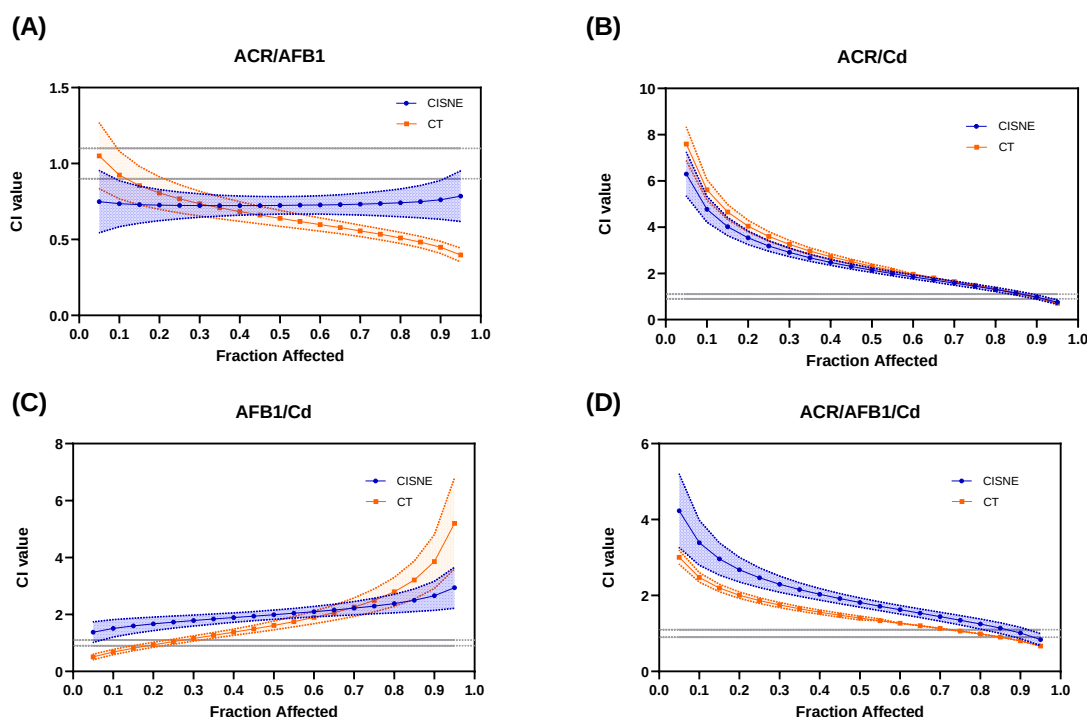


Figure 4.4: Combination index plots for binary combinations and ternary combination using CT model (in orange) and CISNE (in blue). Transparent colored areas that accompany the plots represent the 95% confidence intervals for CI values based on sequential deletion analysis (124). Dashed gray lines indicate the additive zone ($0.9 < CI < 1.1$) that divides synergistic interactions ($CI < 0.9$) from antagonistic interactions ($CI > 1.1$).

CYP binary mixtures were not included in the above determinations since IC₅₀ values were not determined experimentally, as explained before. However, in the next section, CYP binary combinations will be approached at a non-equipotent ratio to collect information concerning CYP interactions. Hence, a global discussion of dose reduction index values (DRI) of synergic combinations (Table 4.4) and polygonograms at five inhibition concentrations, IC₅, IC₂₅, IC₅₀, IC₇₅ and IC₉₀ (Figure 4.7) including all the 4 toxicants will be presented after the following section,

4.2.2 Evaluation of the neurotoxicity of CYP mixtures of food contaminants

A non-equipotent fixed ratio approach was followed for CYP binary mixtures (CYP/ACR, CYP/AFB1, CYP/Cd). Ratios and concentrations assayed are described in 3.5. The dose-effect response curves of CYP/ACR, CYP/AFB1 and CYP/Cd mixtures on SH-SY5Y cells viability are presented in Figure respectively, in comparison to the dose-effect response curves of the individual mixture constituents.

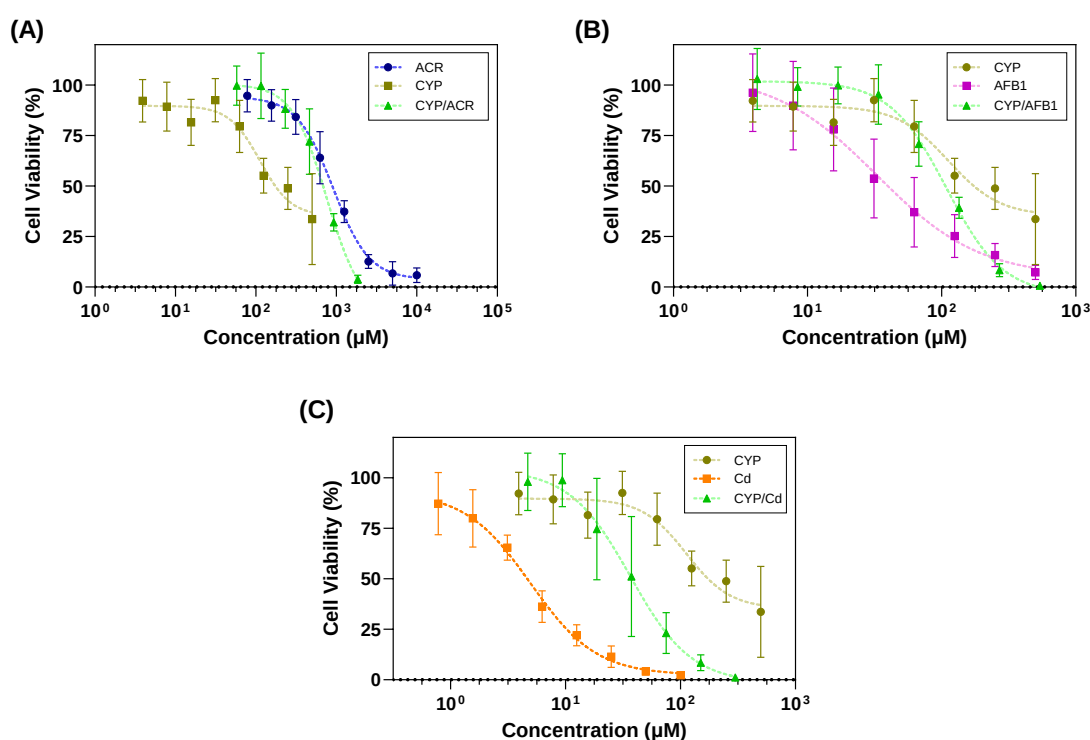


Figure 4.5: Dose-effect response curves of binary mixture (A) CYP/ACR, (B) CYP/AFB1 and (C) CYP/Cd at constant non-equipotent ratios.

All combinations showed a dose-dependent effect, decreasing cell viability at higher concentration ratios. Table 4.2 presents the parameters determined for the binary mixtures CYP/ACR, CYP/AFB1 and CYP/Cd. All r values for CT were above 0.97. As CI values go from 0.23 to 16.53, $\log(CI)$ instead of CI is plotted against F_a to facilitate visualization (Figure 4.6).

Table 4.3: Combined effect parameters (m, IC₅, IC₂₅, IC₅₀ (Dm), IC₇₅, and IC₉₀) of the dose-effect response curves calculated from Compusyn software based on the Chou-Talalay method (A) and from CISNE software based on CISNE model (B). Type of interaction: Strong synergism (++++); Synergism (+++); Moderate synergism (++); Slight synergism (+); Nearly additive (±); Slight antagonism (-); Moderate antagonism (- -); Antagonism (- - -); and, Strong antagonism (- - - -).

(A) Chou-Talalay method

	m	IC5	CI value	IC10	CI value	IC25	CI value	IC50 (Dm)	CI value	IC75	CI value	IC90	CI value
CYP/ACR	2.68	206.84	11.53	273.30	5.49	411.64	2.09	620.03	0.95	933.90	0.50	1406.67	0.29
CYP/AFB1	3.43	41.25	16.86	51.29	7.66	70.67	2.67	97.35	1.07	134.12	0.48	184.78	0.23
CYP/Cd	2.23	10.57	7.47	14.79	4.70	24.22	2.64	39.67	1.60	64.98	1.02	106.42	0.66

(B) CISNE

	m	IC5	CI value	IC10	CI value	IC25	CI value	IC50 (Dm)	CI value	IC75	CI value	IC90	CI value
CYP/ACR	2.45	199.76	6.12	270.98	3.67	424.26	1.87	664.25	1.05	1039.99	0.65	1628.27	0.44
CYP/AFB1	2.29	29.34	8.33	40.64	5.14	65.62	2.60	105.96	1.37	171.10	0.74	276.28	0.41
CYP/Cd	1.82	7.54	4.03	11.38	3.09	20.82	2.17	38.11	1.57	69.74	1.16	127.61	0.87

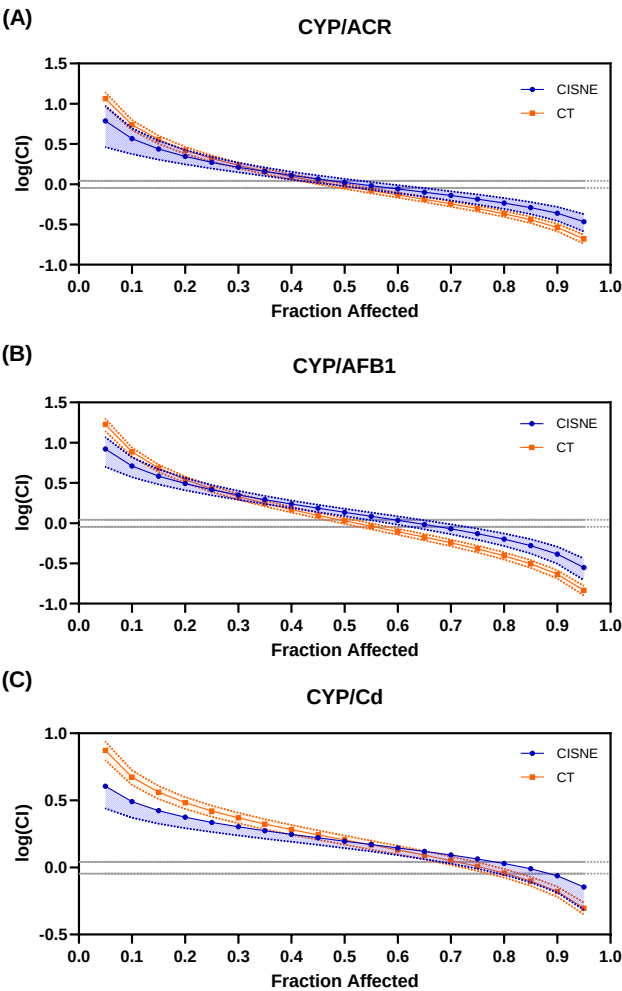


Figure 4.6: Fa-log(CI) plots for binary combinations (A) CYP/ACR, (B) CYP/AFB1 and (C) CYP/Cd using CT model (in orange) and CISNE (in blue). Transparent colored areas that accompany the plots represent the 95% confidence intervals for CI values based on sequential deletion analysis (124). Dashed gray lines indicate the additive zone (0.9<CI>1.1) that divides synergistic interactions (CI<0.9) from antagonistic interactions (CI>1.1).

All CYP binary combinations show antagonistic effects at lower Fa levels, transitioning into additive/synergistic effects at levels superior to Fa 0.5, depending on the model. As observed with previous combinations, the strength of antagonism/synergism is always higher in CT than CISNE: at Fa 0.05 CT determines very strong antagonism ($CI > 10$), while CI evaluates interactions as strong antagonism ($3.3 < CI < 10$), for all CYP binary combinations.

Besides the combined effects parameters and CI plots, used models enable the presentation of interaction effects recurring to more intuitive values as the DRI and polygonogram plots. DRI indicates how many folds the concentration of each compound, in combination, may be reduced to achieve the same level of inhibition, compared with the doses of each compound alone (124). Polygonograms are visual tools designed to help visualize multiple combination interactions. Table 4.4 overviews the DRI values for all combinations tested at equipotent ratios (ACR, AFB1 and Cd) and non-equipotent ratios for CYP combinations. Figure 4.7 describes polygonograms for all binary combinations at five inhibition concentrations, IC₅, IC₂₅, IC₅₀, IC₇₅ and IC₉₀. Both DRI values and polygonograms are based on the CISNE model. CISNE model was chosen to calculate DRI values and conclude on the type of interactions of combinations. This model seems more accurate for low-concentration evaluation of interactions than CT, as stated before. Other comparisons between the two methods were conducted in (150). In this study, the combined effects of platelet antagonists were assessed on ADP-induced platelet activation, and CISNE provided a better fit for experimental data than the CT model.

Table 4.4: Dose reduction index values (DRI) pertaining to CISNE model at inhibition concentration of 5, 10, 25, 50, 75 and 90% for (A) ACR/AFB1, ACR/Cd, AFB1/Cd and ACR/AFB1/Cd mixture and (B) binary combinations CYP/ACR, CYP/AFB1 and CYP/Cd. DRI of synergic combinations is highlighted in a white-orange gradient (from 0 to maximum DRI found, respectively) for easier visualization.

(A)	Ratio	DRI					
		IC5	IC10	IC25	IC50	IC75	IC90
ACR	80:1	4.79	4.32	3.70	3.18	2.73	2.34
AFB1		1.85	1.99	2.20	2.44	2.71	3.00
ACR	160:1	0.45	0.56	0.77	1.05	1.44	1.97
CD		0.25	0.34	0.53	0.84	1.34	2.12
AFB1	2:1	1.24	1.17	1.07	0.98	0.90	0.83
CD		1.75	1.53	1.25	1.03	0.84	0.69
ACR	160:2:1	1.28	1.43	1.67	1.95	2.29	2.68
AFB1		0.50	0.66	0.99	1.50	2.27	3.43
CD		0.70	0.86	1.16	1.57	2.13	2.88

(B)	Ratio	DRI					
		IC5	IC10	IC25	IC50	IC75	IC90
CYP	1:6.4	0.21	0.39	0.98	2.47	6.18	15.47
ACR		0.73	0.89	1.17	1.54	2.04	2.69
CYP	12.5:1	0.21	0.38	0.93	2.26	5.48	13.31
AFB1		0.28	0.40	0.66	1.08	1.79	2.97
CYP	5:1	0.90	1.52	3.25	6.97	14.94	32.01
CD		0.34	0.41	0.54	0.70	0.92	1.20

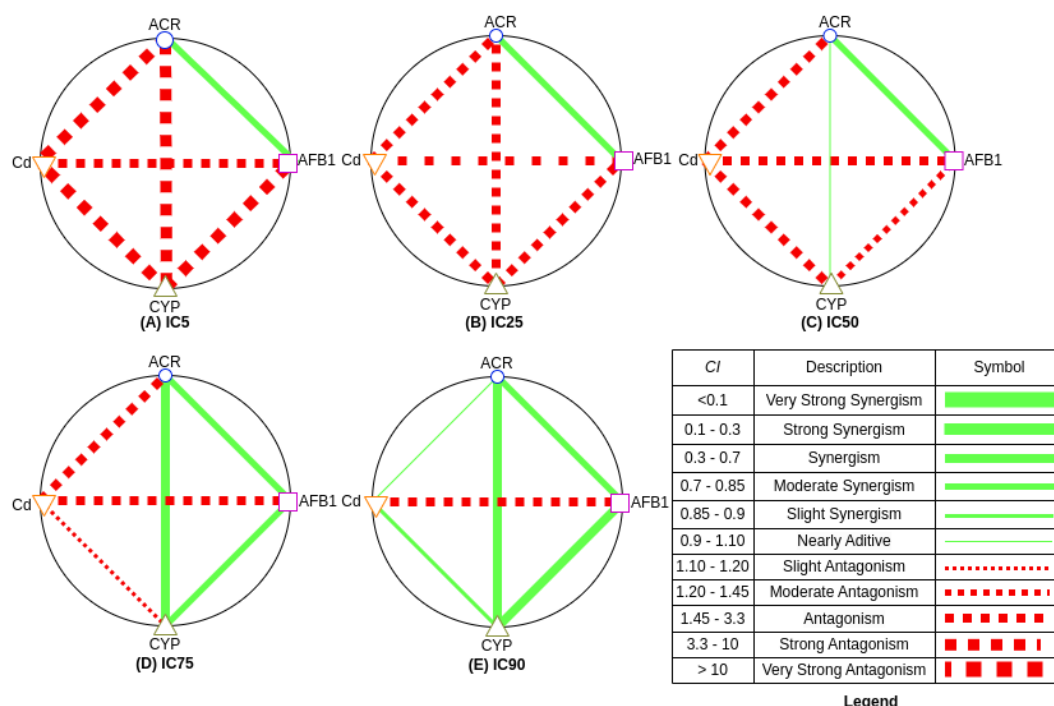


Figure 4.7: Polygonograms of all binary combinations at five inhibition concentrations. Green solid lines indicate synergism, while dashed red lines represent antagonisms. The degree of boldness of the line represents the degrees of synergism or antagonism.

At IC₅ most binary combinations are strongly antagonistic, with exception of ACR/AFB1. In fact ACR/AFB1 combination synergic nature is sustained throughout the full dose-response curve. Greater DRI values at lower doses assume greater importance in assessing human risk because exposure to contaminants in food typically takes place over extended periods at low, continuous levels. So, this combination seems to be of relevance as ACR and AFB1 show a 4.79 and 1.85 concentration times reduction at the IC₅, respectively. AFB1/Cd combination is entirely antagonistic using CISNE model. This however contrasts with CT model that at relevant low doses (IC₅) deems this combination as synergistic. These particular results reiterate the importance of the model choice for combination analysis in toxicity studies. The remaining binary combinations are in majority, antagonistic, except at higher inhibitory concentrations, not so relevant in the context of food exposure.

4.2.3 Evaluation of the neurotoxicity of a realistic low-concentration mixture of food contaminants

Low-concentration mixtures (MIX) of ACR, AFB1, Cd and CYP were used to assess the chronic effect of relevant food exposure levels of contaminants in neuronal cells. Concentra-

tion levels in each dilution are described in section 3.2.5. Results are showed in Figure 4.8 which reveals that MIX at the 95th percentile can inhibit cell viability to 89.84% ($p < 0.05$). This is physiologically relevant since the this level and mixture ratios of contamination can occur in foods presently. Exposure to higher concentrations of the MIX greatly impact SH-SY5Y viability, which inhibition reaches 25% after exposure to MIX4 (which total concentration equals to 6 times more then average mean daily exposure to contaminants through food).

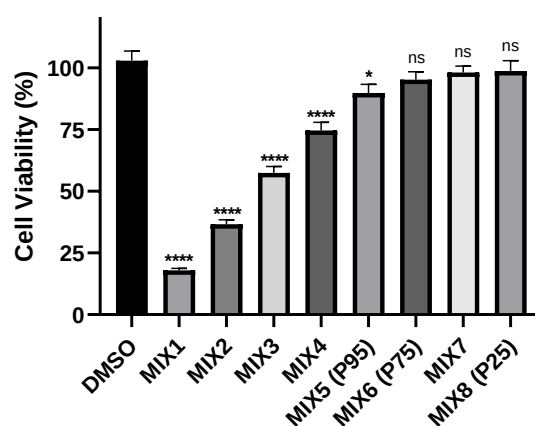


Figure 4.8: Percentage of SH-SY5Y cell viability (%) after 72 h exposure to low-concentration mixtures of ACR, AFB1, Cd and CYP. Serial dilutions of this mixture were prepared so that the levels of exposure covered the 25th, 75th, 95th percentiles.

It is crucial to acknowledge that while prevalence data on contaminants in food offers a valuable starting point, it may not directly correlate with the actual internal concentrations of these contaminants within the body. The relationship between dietary intake and internal exposure is complex and influenced by factors such as the bioaccessibility of contaminants, their absorption in the gastrointestinal tract, and metabolism. Nevertheless, it is worth highlighting that this study represents a pioneering effort as the first instance of employing a complex mixture of contaminants to assess neurotoxicity, adding a unique dimension to our understanding of the potential risks associated with chronic exposure to such mixtures. Moreover, cell viability inhibition effects assayed here are at the very last point of the adverse outcome, which means that many interrelated key events are occurring upstream in the pathway, even at lower doses, whose effects impact neurotoxicity.

4.3 Gene expression analysis

Gene expression of BAX, BCL2, CASP3, NF- κ B and MAPT was explored in the selected model configuring an initial approach to assess toxicity mechanisms imparted by contaminants in the neuronal model.

Bax, a pro-apoptotic member of the Bcl-2 family, is known for its role in promoting cell death by initiating the intrinsic apoptotic pathway. On the contrary, Bcl-2, an anti-apoptotic counterpart within the same family, functions to inhibit apoptosis and promote cell survival. The balance between Bax and Bcl-2 expression levels is a critical determinant of cell fate, and an altered Bax/Bcl-2 ratio can signify a shift towards apoptotic processes or enhanced cell survival (151). Caspase-3, an effector caspase, has long been recognized for its central role in the initiation and execution of apoptosis (152). NF- κ B serves as a critical regulator of neuroinflammatory pathways, influencing the expression of genes associated with immune responses, oxidative stress, and apoptosis (153). Microtubule-associated protein tau (MAPT) is responsible for stabilizing and supporting microtubule, which are essential for neuronal structure and intracellular transport. In tauopathies like AD, tau undergoes abnormal hyperphosphorylation, causing it to detach from microtubules and form neurofibrillary tangles (18).

In this study, SH-SY5Y cells were exposed for 72 hours to isolated contaminants isolated contaminants, metabolized, and mixtures with relevant toxicity determined in the previous sections: ACR, AFB1, BaP, Cd, CYP, metabolic activated AFB1 (S9(AFB1)) and BaP (S9(BaP)), the most synergic binary mixture at low doses (AFB1/Cd) and two concentration levels of MIX at the 25th percentil (P25) and 75th percentil (P75) of exposure, following the experimental design explained in section 3.2.5.5. After exposure, RNA was extracted and quantified and the purity and integrity of the samples was evaluated prior to qPCR experiments (supplementary material 5.2).

4.3.1 Impact of individual and combinations of contaminants in SH-SY5Y cells gene expression

The individual and combined effects of the referred contaminants in the five genes mRNA expression is presented in Figure 4.9.

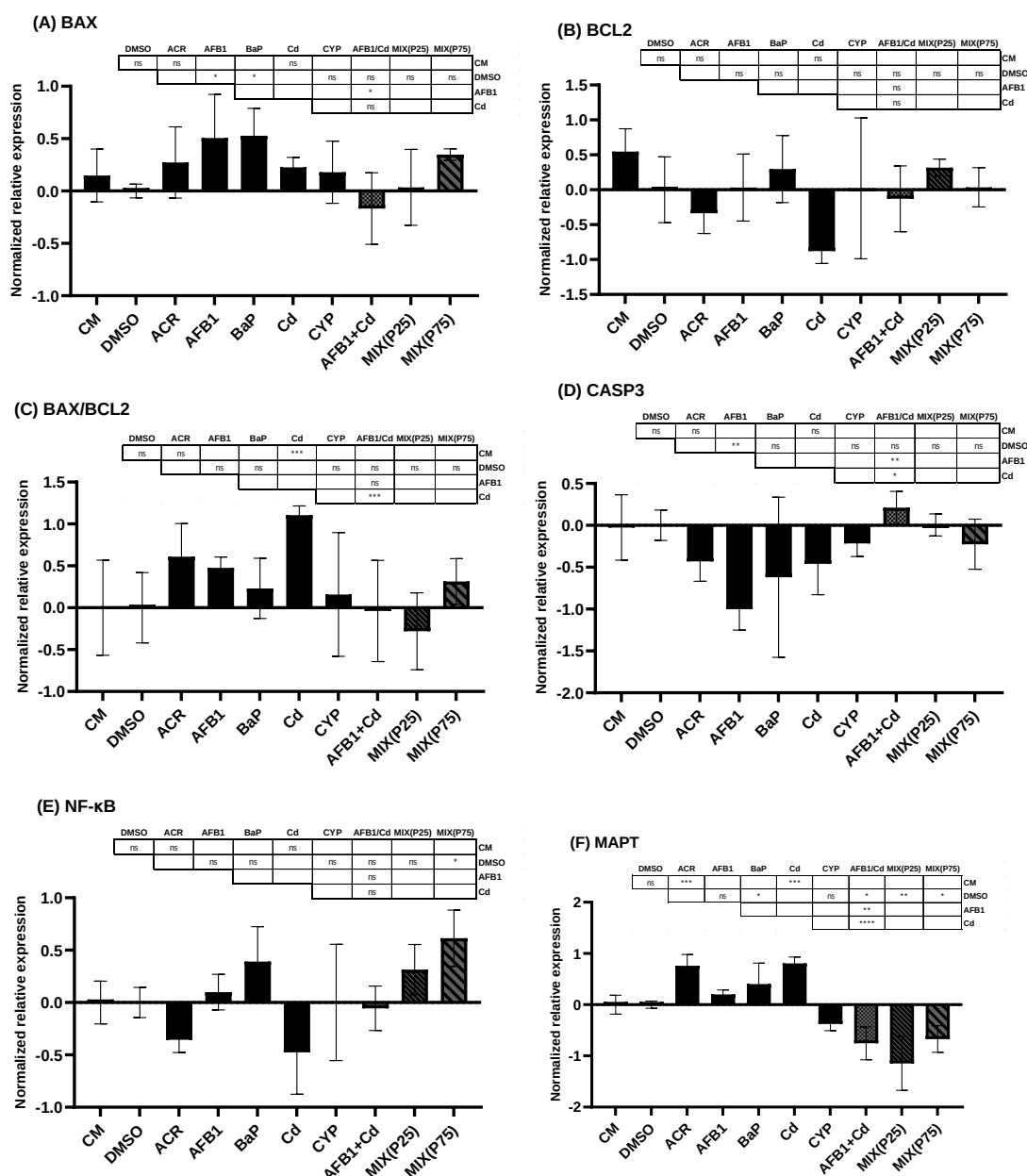


Figure 4.9: Normalized relative expression in log2 scale of (A) BAX, (B) BCL-2, (D) CASP3, (E) NF- κ B and (F) MAPT genes in SH-SY5Y cells. Panel (C) relates to BAX/BCL2 ratio. Cells were exposed for 72 hours to ACR, AFB1, BaP, Cd and CYP, binary mixture AFB1/Cd and MIX at the P25 and P75 concentration levels. Cells treated with DMSO (0.1%) were the negative control. Data represents the mean $\pm$ standard error of three biological replicates (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

BAX mRNA was overexpressed after exposure to AFB1 and BaP (1.46-fold change compared to vehicle control, $p < 0.05$) (Fig 4.9A). No significant differences were observed among other treatments compared to vehicle control, although other isolated contaminants also up-regulate BAX to a less extent (ACR 1.23-fold, Cd 1.17-fold and CYP 1.15-fold). BAX gene upregulation is concentration-dependent in relation to MIX P25 and P75 exposure (1.05-fold and 1.27-fold, respectively). On the opposite, binary mixture AFB1/Cd at the same concentration level than its constituents seems to lead to BAX underexpression (0.91-fold, ns). This intriguing result highlights the potential for complex interactions and divergent outcomes when contaminants are combined.

No significant differences were detected in BCL2 mRNA expression (Fig 4.9B), although a clear trend of downregulated expression is observed for ACR and Cd (0.80-fold and 0.54-fold, respectively), compounds presented cell viability reduction in previous assays. The BAX/BCL2 ratio integrates both BAX and BCL2 mRNA expression results, providing insight into the overall cellular response to contaminant exposure at a global level (Fig 4.9C). Cd shows a BAX/BCL2 ratio of 2.16-fold more than control ($p < 0.001$), predicts that apoptotic pathways are stimulated, as observed in rat cortex brain and hippocampus (151).

Individual exposure to contaminants revealed a downregulation trend in CASP3 expression, with only statistical significance observed for AFB1, resulting in CASP3 underexpression by 0.505-fold ($p < 0.01$) (Fig 4.9D). Our results on CASP3 may seem contradictory to the apoptosis scenario that multiple contaminants induce, according to literature. For example, in IMR-32 cells AFB1 (2 $\mu\text{g/mL}$; equivalent to 6.40 μM) led to mitochondria-dependent apoptosis via BAX and CASP3 mRNA upregulation, while Bcl2 expression remained unaltered (73). However, beyond its canonical function in apoptotic pathways, neuronal caspase 3 has an important role in synaptic plasticity, neurodevelopment, and neurite outgrowth (152). Caspase-3 inhibition dramatically reduces neurite extension and delays differentiation (152). It is still interesting, however, that compounds involved in significant difference in CASP3 expression are AFB1 and its mixture with Cd.

NF- κ B mRNA expression levels did not suffer significant alterations after individual contaminant exposure (Fig 4.9E). Only exposure to MIX(P75) led to a significant upregulation of NF- κ B expression, presenting a 1.55-fold change relative to control ($p < 0.05$); tendency that was already observed at P25 (1.26-fold, ns). This finding aligns with global knowledge of inflammatory potential of exposure to contaminants and makes it interesting to study other

markers involved in inflammation processes.

In what respects MAPT, its upregulation is observed following exposure to ACR (1.71-fold, $p < 0.001$), Cd (1.75-fold, $p < 0.001$) and BaP (1.36-fold, $p < 0.05$) (Fig 4.9F). Interestingly, the opposite was verified for non-individual exposures: MAPT was downregulated by a 0.60-fold for ACR/Cd combination ($p < 0.05$), and expression also decreased for P75 (0.64-fold, $p < 0.05$) and P25 (0.47-fold, $p < 0.01$). It has been shown that in brains of AD patients there is a reduction in MAPT expression, however for PD it seems brain regions express greater levels of MAPT (154). Therefore, both influences of the tested contaminants in MAPT expression are relevant to NDs.

Concerning CYP exposure, no significant differences were found compared to vehicle control (DMSO). Other pyrethroids, like α -CYP induced gene expression alterations that lead to apoptosis, autophagy and necrosis (147, 155).

These results recognize the importance of understanding not only the individual effects of contaminants but also their combined impact. Comparison between AFB1 and Cd to their binary combination at IC_5 level reveals that this combination traces a genetic expression profile independent from its constituents. The impact of the binary mixture on BAX, CASP3 and MAPT expression is significantly different and opposite to the individual effects of AFB1 and Cd (with the exception of AFB1/Cd vs Cd in BAX expression). These insights contribute to our broader understanding of the complex impact of contaminants on neuronal health.

4.3.2 Impact of metabolized contaminants in SH-SY5Y cells gene expression

SH-SY5Y mRNA expression analysis was also conducted to assess differential expression of metabolized and non-metabolized AFB1 and BaP (Fig 4.10).

Comparison between equivalent levels of AFB1 and AFB1(S9) reveals significant differences for BAX (overexpressed by 72.85% when compared to AFB1). CASP3, which was underexpressed after AFB1 exposure, also responded differently to AFB1(S9), eliminating the effect of parental AFB1 (no differences compared to control). In addition, lower levels of metabolized BaP lead to a significantly increased overexpression of BAX and CASP3 when compared to non-metabolized control (77.78% more than BaP). The significant differences observed in genes such as BAX and CASP3 between metabolized and non-metabolized

forms underline the importance of considering metabolic activation when assessing the neurotoxicity and gene expression effects of these contaminants. Other genes did not suffer significant differences.

As previously mentioned in section 3.2.5, a dilution factor of 50 was applied to residues obtained after S9 incubation to counteract S9 matrix effects observed in cell viability assays. However, S9 matrix provokes alterations in the expression compared to the baseline of vehicle control (DMSO) in BAX, CASP3, NF- κ B and MAPT genes (Fig 4.10). Nevertheless, metabolized AFB1 and BaP seem overcome effects of S9 matrix on mRNA expression, with exception of BCL2 which is even more underexpressed in AFB1(S9) and BaP(S9) when compared to S9 fold-change.

While neurons may have limited metabolic capacity, in the context of NDs, a compromised BBB function presents a scenario where even subtle changes in gene expression induced by xenobiotics can have profound effects on neuronal health. The BBB normally serves as a protective barrier, regulating the passage of substances from the bloodstream into the brain. BBB impairment is frequently seen in NDs and leads to an increased permeability, allowing contaminants to enter the brain more readily. This altered dynamics of substance transport can have significant implications for the exposure of neurons to environmental contaminants. Moreover, the metabolism of contaminants within the brain, even if limited in neurons themselves, can occur in surrounding glial cells, such as astrocytes and microglia. These metabolites, when produced, can influence neighbor neurons and contribute to neurotoxicity. The study of other brain or BBB cells expression behaviour when exposed to such contaminants is thus of paramount importance.

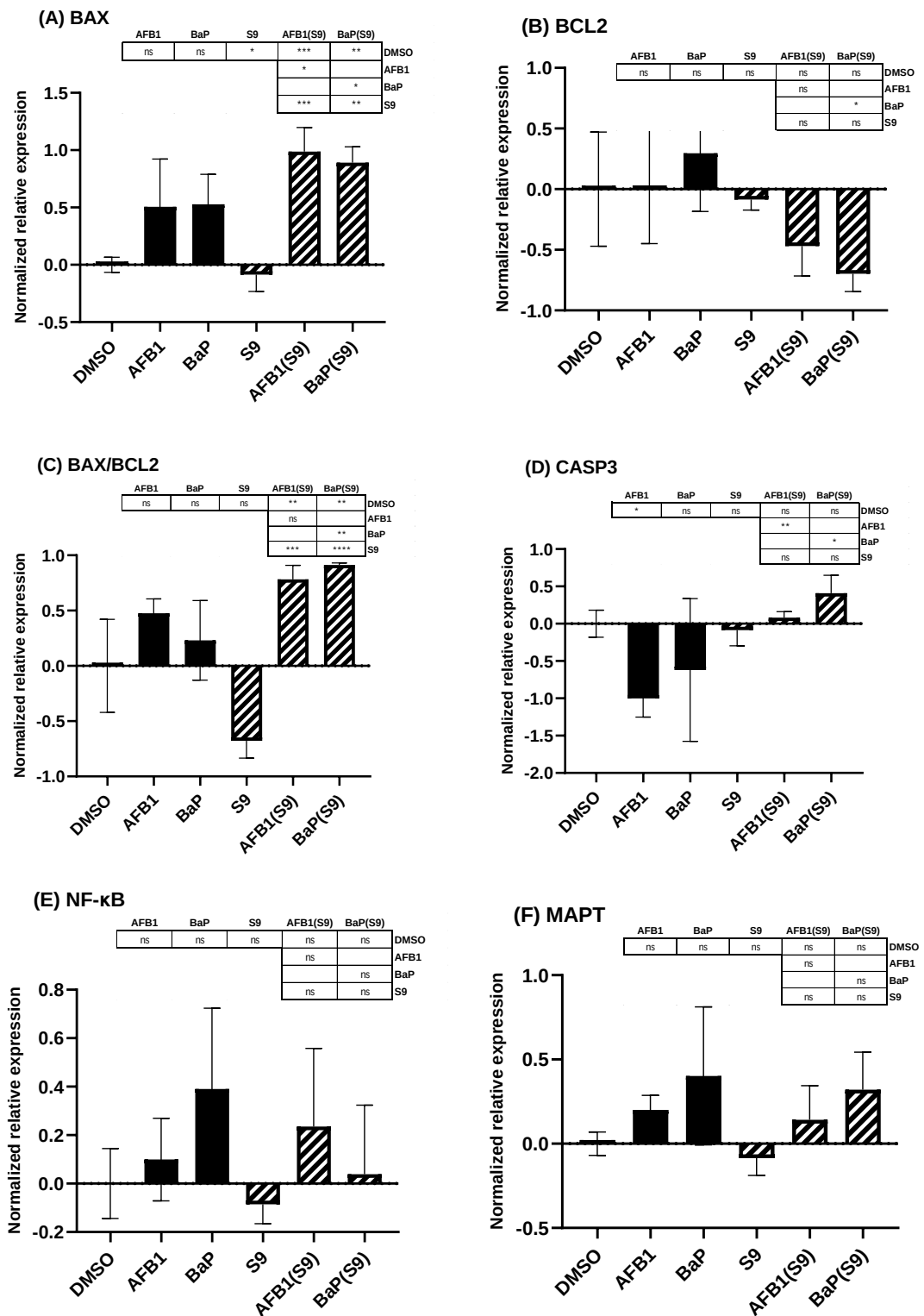


Figure 4.10: Normalized relative expression in log2 scale of (A) BAX, (B) BCL-2, (D) CASP3, (E) NF- κ B and (F) MAPT genes in SH-SY5Y cells. Panel (C) relates to BAX/BCL2 ratio. Cells were exposed for 72 hours to non-metabolized and metabolized AFB1 and BaP. Expression is given relative to DMSO (0.1%) control for AFB1 and BaP, and relate to S9 matrix for AFB1(S9) and BaP(S9). Data represents the mean $\pm$ standard error of three biological replicates (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

Chapter 5

Conclusion

In conclusion, out of the contaminants studied Cd presents the lowest IC_{50} , followed by AFB1, after individual exposure. Metabolization of ACR, AFB1, BaP and CYP with S9 revealed differential results in cell proliferation when compared to non-metabolized contaminants. An unexpected effect of the S9 matrix in SH-SY5Y conditioned some of the results analysis.

CISNE and CT models infer different interactions between contaminants. Contradictory results were obtained when applying CT and CISNE models for AFB1/Cd combination at IC_5 . Cautionary application of CT is advised when studying interactions at low doses due to biased results from linearization issues. Using CISNE model, relevant conclusions pertained to ACR-AFB1, the only synergetic combination at significant levels regarding human risk exposure from foods (IC_5). This interaction is particularly relevant since these contaminants frequently co-occur in cereal foods, highly consumed worldwide.

A realistic mixture of contaminants was successfully constructed pertaining to dietary exposure at different percentiles. A significant difference was observed for 95th percentile MIX (P95) on SH-SY5Y cell viability, decreasing to 90%. Furthermore, different gene profile expression was observed. Notably, MIX P95 enhanced the expression of NF- κ B, that aligns with global knowledge of inflammatory potential of exposure to contaminants.

The assessment of contaminants and their mixtures on the expression of ND-related genes has unveiled intriguing insights, although the level of differential expression was low. It has become evident that these combinations yield a distinctive genetic expression profile, prompting consideration of mixtures of contaminants as distinct entities, occasionally unrelated to the cumulative effects of their individual components.

Overall, these findings underscore the need for further research to comprehensively understand the potential neurotoxicity risks associated with exposure to multiple contaminants, with implications for proper risk assessment, and the need for guidance documents on evaluation of interactions of substances with toxic potential to human health.

The present results open the way for further research that should assess contaminants toxicity of differentiated SH-SY5Y and include the incorporation of advanced *in vitro* models, namely neurons co-culture with microglia. Microglia play a pivotal role in modulating neuroinflammation and can potentiate the effect of contaminants. Therefore, studying the effects of contaminants within the context of neuron-microglia co-cultures can provide a more accurate representation of the complex interplay between contaminants and the brain. This approach will contribute significantly to our understanding of neurodegenerative diseases and the role of food-related and environmental factors in their development, with obvious implications for the improvement of risk assessment and the consequent need to develop new food safety and quality control strategies.

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Supplementary materials

Supplementary material 1

Food groups	Omnivore Diet						
	Food	Energy (kcal)	Quantity (g)	Protein (g)	Fat (g)	Carbohydrates (g)	Fibre (g)
Whole grains	Rice	250.0	200.0	5.0	0.4	56.0	1.6
	Wheat - pasta	204.0	200.0	6.8	1.2	39.8	3.0
	Maize - bread	376.0	200.0	10.6	2.4	74.4	7.4
Tubers	Potato	43.5	50.0	1.2	0.0	9.3	0.8
Vegetables	Cabbage	44.0	200.0	4.4	0.4	0.6	5.8
	Carrot	37.5	150.0	0.9	0.0	1.7	3.9
Fruits	Apple	122.0	200.0	0.4	1.0	15.5	3.8
Dairy food	Cheese	148.2	60.0	18.0	8.4	0.3	0.0
Protein sources	Beef	172.8	90.0	24.2	8.5	0.0	0.0
	Egg	0.0	0.0	0.0	0.0	0.0	0.0
	Salmon	327.6	120.0	24.8	25.3	0.0	0.0
	Beans	41.2	40.0	2.6	0.2	5.8	2.7
	Peanuts	121.0	20.0	5.1	9.9	1.9	1.8
Added fats	Olive oil	450	50	0.1	50.0	0.0	0.0
Total		2338	1580	104	108	205	31

Figure 5.1: Construction of an standard omnivore diet with common foods. Integration of contaminant data on this specific foods according to their quantities in diet provides a low-dose relevant mixture.

Supplementary material 2

Regarding RNA extraction and quantification, all the samples had the ratio 260/280 approximately equal to 2 with exception of P25_C which was disregarded for subsequent analysis.

Table 5.1: RNA concentrations and purity ratio of samples (A260/A280). Denominations A, B and C relate to different biological samples.

Sample Code	Concentration (ng/μl)	A260/A280	Sample Code	Concentration (ng/μl)	A260/A280
AFB1_A	869,1325	2,158	P75_A	149,5015	2,1725
AFB1_B	988,507	2,19	P75_B	113,2915	2,475
AFB1_C	1213,3945	2,174	P75_C	100,224	2,3605
ACR_A	1345,9985	2,203	CM_A	367,541	2,2595
ACR_B	1219,0165	2,1925	CM_B	394,9895	2,2495
ACR_C	993,666	2,157	CM_C	351,042	2,2455
CD_A	1125,8525	2,1915	P25_A	312,245	2,2485
CD_B	633,543	2,144	P25_B	290,999	2,2185
CD_C	1152,4605	2,1655	P25_C	28,9675	1,492
BAP_A	1544,0365	2,2045	S9_BAP_A	275,4815	2,061
BAP_B	875,27	2,165	S9_BAP_B	442,3275	2,212
BAP_C	1426,0545	2,1795	S9_BAP_C	633,379	2,17
DMSO_A	1036,157	2,15	S9_AFB1_A	933,8855	2,278
DMSO_B	998,838	2,1695	S9_AFB1_B	492,462	2,239
DMSO_C	1204,924	2,1495	S9_AFB1_C	533,452	2,235
CYP_A	1018,6615	2,174	S9_CNT_A	606,431	2,182
CYP_B	1285,303	2,1235	S9_CNT_B	448,858	2,214
CYP_C	907,946	2,1635	S9_CNTRL_C	516,58	2,221
AFB1+Cd_A	1120,5115	2,1565			
AFB1+Cd_B	881,5805	2,141			
AFB1+Cd_C	881,2995	2,1335			

RNA integrity was assessed by an agarose gel electrophoresis, as exemplified in Figure 5.2, which allows to accurately separate charged molecules, such as RNA, and examine their conservation status, based on the 18S and 28S subunits. RNA was not degraded and 28S:18S ratio was around 2.0 for all samples (band lines percentages were determined with Image Lab software, BIORAD).

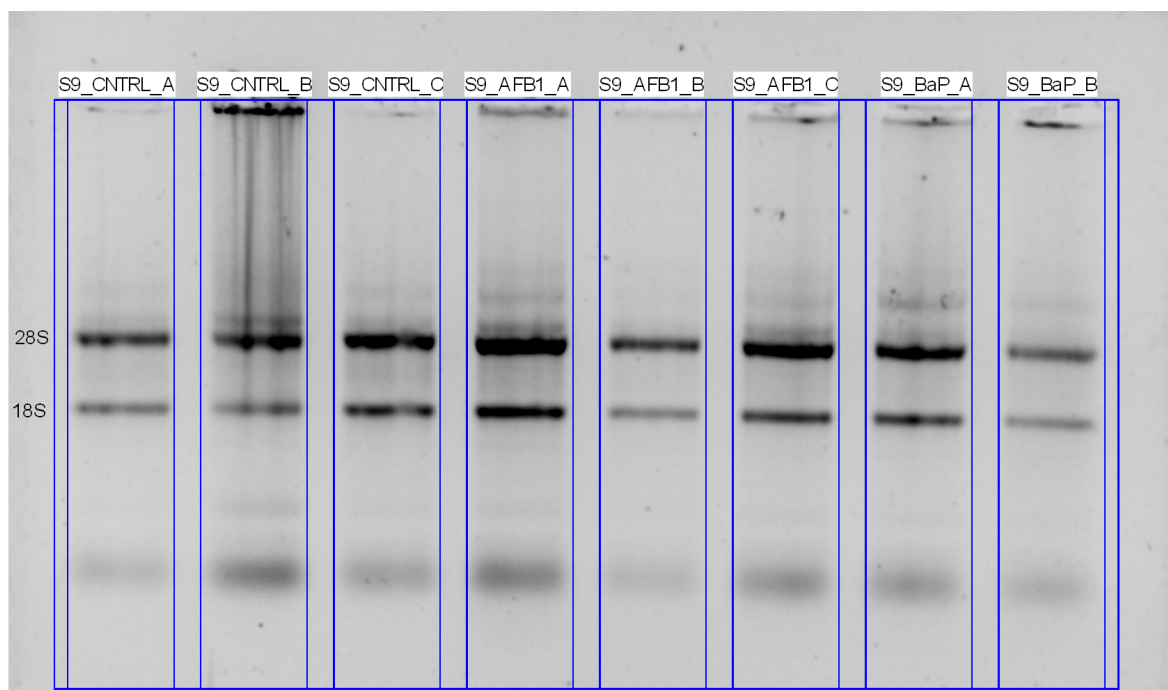


Figure 5.2: Agarose gel electrophoresis for RNA integrity evaluation.