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Environmental Fate Processes and Human Exposure to Rubber-derived Compounds

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“*PhD ist ein bisschen wie klettern. Es kommt manchmal eine schwierige Stelle, und genau da muss man festbeißen und dranbleiben.*“

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Acknowledgements

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i. Abstract

Tire and road wear particles (TRWP) are constantly emitted into the environment due to abrasion at the road surface. Rubber additives and their transformation products (rubber-derived compounds) make up 5% - 10% by weight of tire material, and leach from TRWP into the environment. Thus, rubber-derived compounds are a contaminant class of rapidly emerging concern. Several rubber-derived compounds are toxic to aquatic species, and recent studies report mammalian toxicity as well. Rubber-derived compounds have been detected in human urine, breastmilk, and spinal fluid. However, little is known about the pathways by which humans are exposed to rubber-derived compounds. This PhD thesis investigates exposure to rubber-derived compounds via food and air.

Rubber-derived compounds are expected to reach agricultural environments via recycled wastewater irrigation, biosolids application, and atmospheric deposition, and may then be taken up by edible plants. Five rubber-derived compounds: hexamethoxymethylmelamine (HMMM), benzothiazole (BTZ), N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD), 6PPD-quinone, and 1,3-diphenylguanidine (DPG) were taken up by lettuce plants in a hydroponic system, and translocated to edible leaves. The extent of uptake and translocation varied in relation to compounds' speciation and lipophilicity. The compounds were metabolized by the lettuce plants, and several transformation products showed increased stability in lettuce leaves. When hydroponically-grown lettuce were exposed to tire wear particles, instead of a single spike of rubber-derived compounds, the uptake rates and total concentrations which accumulated in lettuce leaves changed, depending on the leached amounts and leaching kinetics of rubber-derived compounds from tire wear particles.

It is expected that the accumulation of rubber-derived compounds in soil-grown lettuce would be lower than in hydroponically-grown lettuce, since both sorption and transformation can reduce the bioaccessibility of organic compounds in soil. Therefore, plant uptake experiments were conducted with lettuce grown in three soils of varying composition. Tire-derived compounds were taken up by lettuce grown in soil, although the formation of non-extractable residues in soil reduced the bio-accessibility, and thus, uptake of HMMM and 6PPD, especially in soil with a high clay content. DPG and 6PPD-quinone had higher bioaccessibility in all soils. Transformations of rubber-derived compounds were also detected in soil grown lettuce, and their distribution within plants was investigated. Finally, sixteen rubber-derived compounds were analyzed in commercial leafy vegetables. Low concentrations of rubber-derived compounds were found in 71% of samples, confirming that rubber-derived compounds are present in the agricultural environment, and are taken up by edible plants. Based on measured concentrations and dietary survey data, estimated daily intake of rubber-derived compounds via leafy vegetable consumption

are presented. These are slightly lower or similar to estimated daily intake via previously reported exposure routes.

Although most rubber-derived compound research has focused on tires, rock climbing shoes are also made of specialized rubber, and generate particles due to friction with climbing holds in indoor facilities. Elevated concentrations of rubber-derived compounds were measured in rock climbing shoes, and in airborne particulate matter samples from indoor climbing gyms. Based on rubber-derived compound concentrations in airborne particulate matter, the estimated daily exposure via inhalation for climbers and climbing gym employees was calculated, and found to exceed rubber-derived compound exposure from other sources, including for street workers in megacities. The rubber-derived compound profile did not match between climbing shoes and airborne particulate matter, however through a series of control experiments, other sources of rubber-derived compounds to climbing gym air could be excluded. Ozonation experiments confirmed that the rubber-derived compounds in airborne particulate matter were likely transformation products of the rubber-derived compounds in climbing shoes, thereby implicating a consumer rubber product as the source of elevated human exposure to rubber-derived compounds.

Overall, this PhD thesis mechanistically investigates the processes by which rubber-derived compounds reach food and air, and presents the estimated daily intake of rubber-derived compounds via each of these exposure routes.

ii. Kurzfassung

Reifen- und Straßenabriebpartikel (engl. tire and road wear particles, TRWPs) werden durch Reibung an der Straßenoberfläche kontinuierlich in die Umwelt freigesetzt. Gummiadditive und deren Transformationsprodukte (gummiabgeleitete Stoffe) machen 5 bis 10 Gewichtsprozent des Reifenmaterials aus, gelangen aus TRWPs in die Umwelt und sind daher eine Schadstoffklasse von rapide wachsender Bedeutung. Mehrere gummiabgeleitete Stoffe sind toxisch für Wasserlebewesen, und, laut neuerer Studien, auch für Säugetiere. Gummiabgeleitete Stoffe wurden in menschlichem Urin, Muttermilch und Rückenmarksflüssigkeit nachgewiesen, es ist allerdings wenig darüber bekannt, wie Menschen diesen Stoffen ausgesetzt sind. Diese Dissertation untersucht die Exposition gegenüber gummiabgeleiteten Stoffen über Ernährung und Atemluft.

Es wird angenommen, dass gummiabgeleitete Stoffe über die Bewässerung mit recyceltem Abwasser, die Düngung mit Klärschlamm sowie atmosphärische Deposition in landwirtschaftliche Umgebungen gelangen und von essbaren Pflanzen aufgenommen werden. Fünf gummiabgeleitete Stoffe – Hexamethoxymethylmelamin (HMMM), Benzothiazol (BTZ), N-(1,3-Dimethylbutyl)-N'-Phenyl-P-Phenylenediamin (6PPD), 6PPD-Chinon und 1,3-Diphenylguanidin (DPG) – wurden von Salatpflanzen in einem hydroponischen System aufgenommen und in die essbaren Blätter transportiert. Das Ausmaß der Aufnahme und des Transports variierte in Abhängigkeit von der Speziation und Lipophilie der Stoffe. Die Stoffe wurden von den Salatpflanzen metabolisiert, und mehrere Transformationsprodukte zeigten eine erhöhte Stabilität in den Salatblättern. Als der hydroponisch gezogene Salat statt einzelner gummiabgeleiteter Stoffe Reifenabriebpartikeln ausgesetzt wurde, änderten sich die Aufnahmegeschwindigkeiten und die in den Salatblättern angesammelten Gesamtmengen abhängig von ausgelaugten Mengen und den Auslaugkinetiken der gummiabgeleiteten Stoffe aus den Reifenabriebpartikeln.

Es wird angenommen, dass die Akkumulation von gummiabgeleiteten Stoffen in im Boden angebautem Salat geringer ist als in hydroponisch angebautem Salat, da sowohl Sorption als auch Transformation die Bioverfügbarkeit organischer Verbindungen im Boden verringern können. Daher wurden Versuche mit Salat durchgeführt, der in drei Böden unterschiedlicher Zusammensetzung angebaut wurde. Zwar wurden gummiabgeleitete Stoffe von im Boden angebautem Salat aufgenommen, doch die Bildung von nicht extrahierbaren Rückständen im Boden reduzierte die Bioverfügbarkeit und damit die Aufnahme von HMMM und 6PPD, insbesondere in Böden mit hohem Tongehalt. Auch in im Boden angebautem Salat wurden Transformationen von gummiabgeleiteten Stoffen festgestellt, und deren Verteilung in den Pflanzen untersucht. Schließlich wurden sechzehn gummiabgeleitete Stoffe in kommerziell angebauten Blattgemüsen analysiert. In 71 % der Proben wurden niedrige Konzentrationen

gummiabgeleiteter Stoffe gefunden, was das Vorhandensein gummiabgeleiteter Stoffe in landwirtschaftlichen Umgebungen und die Aufnahme durch essbare Pflanzen bestätigt. Basierend auf gemessenen Konzentrationen und Ernährungsumfragedaten werden die geschätzten täglichen Aufnahmemengen von gummiabgeleiteten Stoffen durch den Verzehr von Blattgemüse präsentiert. Diese sind etwas niedriger als die oder ähnlich den geschätzten täglichen Aufnahmemengen über zuvor berichtete Expositionswege.

Während sich die meiste Forschung zu gummiabgeleiteten Stoffen auf Reifen konzentriert, bestehen auch Kletterschuhe aus speziellem Gummi und erzeugen in Indoor-Anlagen durch Reibung mit Klettergriffen Partikel. Sowohl in Kletterschuhen als auch in Schwebstoffproben aus der Luft in Kletterhallen wurden erhöhte Konzentrationen gummiabgeleiteter Stoffe gemessen. Basierend auf den Konzentrationen gummiabgeleiteter Stoffe in den Schwebstoffproben wurde die geschätzte tägliche Exposition durch Inhalation für Kletterer und Angestellte in Kletterhallen berechnet und festgestellt, dass sie die Exposition durch andere Quellen, einschließlich Straßenarbeiter in Megastädten, übersteigt. Die Profile gummiabgeleiteter Stoffe in Kletterschuhen und Schwebstoffproben stimmten nicht überein, durch eine Reihe von Kontrollversuchen konnten jedoch andere Quellen gummiabgeleiteter Stoffe in der Luft der Kletterhalle ausgeschlossen werden. Ozonisierungsexperimente bestätigten, dass die gummiabgeleiteten Stoffe in den Schwebstoffproben wahrscheinlich Transformationsprodukte der gummiabgeleiteten Stoffe in den Kletterschuhen waren und implizierten somit ein Konsumgummiprodukt als Quelle der erhöhten menschlichen Exposition gegenüber gummiabgeleiteten Stoffen.

Insgesamt untersucht diese Dissertation mechanistisch die Prozesse, durch die gummiabgeleitete Stoffe in Lebensmittel und Luft gelangen, und präsentiert die geschätzte jeweilige tägliche Aufnahmemenge von gummiabgeleiteten Stoffen über beide Expositionswege.

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Chapter 5: Anya Sherman†, Thibault Masset†, Lukas Wimmer, Leah K. Maruschka, Lea Ann Dailey, Thorsten Hüffer, Florian Breider, Thilo Hofmann
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CRediT: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – Original Draft, Writing – Review & Editing, Visualization. † Anya Sherman and Thibault Masset contributed equally to this work.

Chapter 1: General Introduction

1.1 Rubber-derived compounds in the environment

In 2021, I moved to Vienna to start my PhD, and Austria introduced the "Klimaticket" – a €3/day all-inclusive national public transportation ticket. In 2024, 13% of Austrian residents hold Klimatickets (I am one of them). However, even in a country with an incredible public transportation offering, the vast majority of Austrians still rely on personal vehicles for transportation: the number of vehicles registered in the country has grown almost every year since 1950, reaching over 730,000 in Vienna alone at the end of 2023[1]. In contrast, only 97,800 Viennese residents hold a Klimaticket[2]. This is even more true in countries with poorer transportation infrastructure - in 2021, 66 million cars were purchased globally, and in the U.S. alone, nearly 5 trillion kilometers were driven[3]. These numbers are predicted to grow over the next decade[4]. The environmental concerns associated with the massive number of automobiles on the road are not limited to exhaust emissions. Non-exhaust emissions from automobile traffic introduce novel entities[5], including tire wear, into the environment.

Tires abrade due to friction at the road surface, forming composite particles of road and tire material (tire and road wear particles – TRWP)[6]. Tire and road wear particles, and their associated chemicals are a pollutant class of increasing concern due to their environmental prevalence and demonstrated ecological harm. This is despite, or perhaps partly because of, the many uncertainties regarding the emissions and fate of TRWP and associated chemicals in the environment. For example, it is generally recognized that TRWP are one of the most abundant types of microplastics emitted into the environment, but the precise fraction remains unclear, with estimates varying between 24 to 94%[7]. Quantification of these particles in the environment is difficult, since the black color of the particle prevents the use of Raman spectroscopy, which is common for quantifying other types of microplastics. Rubber monomers can be measured with pyrolysis-GCMS, and marker additives such as zinc and benzothiazoles can be measured with a variety of methods. Both of these techniques have been employed to quantify TRWP in roadside soils (up to 204 mg/g), air (up to 3.4 μg/m3), surface waters (up to 0.8 mg/mL), and sediments (up to 155 mg/g)[8]. Additionally, several computational approaches have been applied to estimate the flux of TRWP into the environment. One approach uses emission factors (particles generated per distance driven) multiplied by total distances driven per year, while a second approach is based on the annual weight loss of tires, multiplied by the number of tires in use. For example, the German Automobile Club reports an average mass loss of 120 miligrams of tire wear per kilometer driven[9]. Based on multiple national estimates, a global tire wear emission estimate of 0.23 – 4.7 kg/capita/year was proposed[10].

Rubber polymers makes up only 40 – 60% of tire mass, with fillers, oils, and chemical additives composing the rest[6]. These components are present as a mixture, meaning that chemical additives are generally not bound to the polymers. They are thus mobile within TRWP, and can eventually migrate to the particle surface, and be released into the environment[11,12]. Chemicals in plastics are gaining international attention as an environmental and human health concern. In 2023, the United Nations reported that over 13,000 chemicals are associated with plastics, of which 3,200 are chemicals of concern (e.g. carcinogens, endocrine disruptors, ecotoxicants)[13]. In an even more recent report, Wagner et al revised these numbers to 16,000 chemicals in plastics, of which 4,200 are of concern[14]. TRWP are no exception - non-target screenings detected over 200 unique chemical signals in tire extracts, of which more than half were leachable in aqueous systems[12]. In fact, the transportation industry was identified by the United Nations as a priority sector where plastic-associated chemicals of concern are used in a way that results in human or environmental exposure[13].

Many chemical additives are incorporated during the vulcanization process, in which different rubber polymer chains are linked together. Hexamethoxymethylmelamine (HMMM) is commonly used as a rubber cross linker during vulcanization. Other chemicals such as benzothiazoles and phenyl-guanidines are added as vulcanization accelerators, to reduce the temperature and time needed for vulcanization. Additionally, some chemicals are added to impart certain properties to the final rubber product. These include anti-oxidants, such as p-phenylenediamine (PPD) compounds, which form a protective layer on the surface of rubber, preventing oxidative degradation. Many chemical additives are inherently reactive, and form transformation products within rubber, or after release into the environment. Therefore, both intentionally added compounds, and their transformation products (rubber-derived compounds) are of concern as environmental pollutants.

Modeling and laboratory studies suggest that up to 5%[15,16] of TRWP become airborne upon generation. Accordingly, p-phenylenediamines, and their quinone derivatives have been detected in urban airborne particulate matter globally, with typical concentrations of several picograms per cubic meter[17–20], and maximum concentrations of 7.25 ng/m^3 reported for 6PPD-quinone at roadsides in Guangzhou, China[21]. Benzothiazoles, as well as 1,3-diphenylguanidine (DPG), have also been detected in urban air, at concentrations from tens to several hundred picograms per cubic meter[18,22–24]. The non-airborne fraction of TRWP remains on the road surface until being washed off during precipitation events – either to roadside soils or to runoff collection systems. An estimated 66-76% of TRWP accumulate in roadside soils[25], while collected runoff is either discharged directly into surface waters or transported to wastewater treatment plants[6]. HMMM and its transformation products, 6PPD and its transformation products, DPG, benzothiazole and several of its derivatives have all been detected in wastewater effluent[26–28], and surface

waters[28–33]. A majority of TRWP are presumably retained in wastewater treatment plant sludge[25], where benzothiazole has been detected at concentrations up to 50.2 µg/kg[34].

The environmental ubiquity of TRWP and rubber-derived compounds has various ecological consequences. Most studies have focused on toxicity in the aquatic environment, where organisms are usually exposed to a mixture of TRWP and leachable rubber-derived compounds. It is generally accepted that leachable rubber-derived compounds are the main drivers of the toxicity of TRWP[35,36], although the presence of particles enhances toxicity[35]. Traditional analytes, such as zinc and PAHs were first thought to be the rubber-derived compounds responsible for toxicity[37,38]. In 2021, the acute toxicity of 6PPD-quinone to Coho salmon at environmentally relevant concentrations was discovered[39]. Follow up studies revealed that the toxicity of 6PPD-quinone is species-specific, but that multiple aquatic species are sensitive[40–43]. Other rubber-derived compounds also contribute to toxicity - using a concentration addition approach to assess the mixture toxicity of leachate constituents, Dudefoi et al. ascribed 32% of the toxicity observed to rainbow trout cell lines to zinc, 6PPD, DPG, and 2-mercaptobenzothiazole, and hypothesized that the un-accounted for 68% of observed toxicity could be due to the presence of unknown rubber-derived chemicals, including transformation products[35]. This hypothesis was further confirmed with effect- directed analyses which showed toxicity of unknown organic rubber-derived compounds[44].

In recent years, rubber-derived compounds have been detected in more matrices, including drinking water[26], dust[19,45–53], urban air[17–23], and food[54], raising questions about human exposure and toxicity. In fact, benzothiazole, 2-hydroxybenzothiazole, 2-aminobenzothiazole, DPG, 6PPD and 6PPD-quinone have all been detected in human urine[55–58], with enhanced 6PPD and -quinone concentrations in pregnant women[56]. 6PPD was also detected in more than half of 120 breast milk samples collected in Guangzhou, China[59]. 6PPD-quinone was detected in cerebrospinal fluid, with concentrations twice as high in Parkinson's disease patients as in control patients, and 2-hydroxybenzothiazole was detected in adipose tissues[60].

Mammalian toxicity studies of TRWP and rubber-derived compounds remain few and far between. In a study funded by the Tire Industry Project, rats were exposed to TRWP via inhalation, and no toxicity up to 100 µg/m^3 was observed[361]. Based on these data, the same authors proposed a no-observable-adverse-effect-concentration of 55 µg/m^3 for humans[62]. However, an independent study observed sub-acute effects (pulmonary fibrotic injury and early indications of pulmonary fibrosis) in rats exposed to tire wear[63]. In-vitro work with human lung cells has shown DNA damage induced by both tire particles and tire particle extracts, as well as inflammatory responses induced by tire particles[64,65]. One study found that five PPD-quinones contributed 2.9% of the oxidative potential of particulate matter[66], while oral exposure to 6PPD and 6PPD-quinone led to accumulation in mice livers, and induced hepatoxicity and altered hepatic

metabolism[67]. 1,3-diphenylguanidine is listed by ECHA a potential respiratory irritant (as well as harmful if swallowed, and a potential fertility disruptor)[68], and its transformation product, aniline, is an acute and chronic toxicant, due to its effects on the lungs and blood, and is classified by the US EPA as a probably human carcinogen[69,70]. Inhaled benzothiazoles may be of less concern - benzothiazole, 2-aminobenzothiazole, 2-hydroxybenzothiazole, and 2-mercaptobenzothiazole caused cytotoxicity to lung cells only at concentrations over 100 mg/L, and no sub-acute effects (chromosome damage) were observed[71]. However, the same set of benzothiazoles were shown to be endocrine disruptors via a combination of ex-vivo and in-vivo methods[72]. Additionally, a cohort study tentatively linked 2-mercaptobenzothiazole exposure to excess cancer occurrence[73].

Although these findings are fragmented, and insufficient to draw conclusions about the chemical drivers, or mechanisms of rubber-derived compound toxicity via inhalation or ingestion, they do justify further study of human exposure to rubber-derived compounds. It is necessary to identify potential routes of exposure and quantify their relative contributions to overall rubber-derived compound intake. For the exposure routes which dominate human intake of rubber-derived compounds, it is important to study the processes which govern the extent of exposure. Such process-based understanding can inform solutions to mitigate exposure.

1.2 Rubber-derived compounds in the agricultural environment: relevant processes controlling uptake by edible plants

Feeding the growing global population demands increasingly productive agriculture, but a changing climate poses challenges to standard agricultural practices. Alternative irrigation water sources are needed, as global water shortages become more common. Additionally, alternative fertilizers are needed, due to the energy demands of bioavailable nitrogen production, and limited phosphorous resources. Irrigation with recycled wastewater, and application of bio-solids as fertilizer are sustainable strategies to meet the water and nutrient demands of agriculture. However, both have the unintended consequence of introducing wastewater-derived pollutants such as rubber-derived compounds into the agricultural environment.

Rubber-derived compounds enter wastewater treatment plants both associated with TRWP and in dissolved form. TRWP are expected to be predominantly retained in sludge, while dissolved rubber-derived compounds may partition to sludge, transform, or be present in treated effluent. Thus, both irrigation with recycled water and application of sludge as biosolids can introduce rubber-derived compounds to the agricultural environment. It has been estimated that the application of sewage sludge introduces 1400-2800 tons/year of TRWP (2-3% of all non-airborne TRWP) to German agricultural

fields[25]. Additionally, deposition of airborne TRWP onto agricultural fields could occur[10], especially in areas where agriculture and major roadways are co-located. It is expected that TRWP-associated rubber-derived compounds will leach in the upper soil layers, rather than being transported to deeper soil or groundwater[74]. Several rubber-derived compounds have been detected in agricultural runoff, confirming their presence in agricultural soils[26]. Rubber-derived compounds in agricultural soils could be taken up by edible plants, thereby entering into the food chain, as has been well documented for other organic pollutants[75–77].

To be taken up by plants, a compound must be bioaccessible. Bioaccessibility refers to the fraction of compounds which could be taken up by an organism, e.g. dissolved in pore water in the root zone of plants[78,79]. This differs slightly from bioavailability, which also takes into account physiological constraints, such as the potential of a compound to cross cell membranes and enter an organism. Several processes may reduce the bioaccessible fraction of a compound, such as mobility out of the root zone[80], transformation, or sorption[81]. There are many specific mechanisms by which a compound can be transformed or sorbed in soil[77,82–84], and their degree of reversibility determines how likely it is that the compound can become bioaccessible. In the field of pesticide science, these sorptive and transformation processes are grouped into different categories of residue formation[85,86]. Extractable residues are the fraction of compounds which can be extracted with standard aqueous or solvent extraction methods, and are generally either bioaccessible, or can readily become bioaccessible. On the other hand, non-extractable residues can not be extracted from soils using standard extraction methods and will not become bioaccessible unless the soil matrix is substantially altered. Type I non-extractable residues include compounds and their transformation products which are adsorbed or physically entrapped in soil particles; Type II residues include compounds and their transformation products which are covalently bound to soil components. Type III residues are compounds which have been incorporated into biomass or mineralized, and can no longer become bioaccessible under any conditions[87].

Bioaccessible compounds can be taken up by plant roots along with water[77]. A compound may be transported through root cells toward xylem via the symplastic (through cytoplasm, via plasmodesmata – inter-cell channels), transmembrane (through cytoplasm, crossing between cells via diffusion across cell membranes and walls), or apoplastic pathways (through the apoplast – the space between cell membranes and cell walls). Separating the roots tissue and xylem is the Casparian strip – a thick layer of cells where the apoplast is blocked. Thus, only compounds which can cross cell membranes may enter the plants xylem flow, and be transported to aboveground tissue[77,88]. Compounds larger than 400 g/mol can not cross cell membranes, and are thus excluded at the Casparian strip[89]. Due to repulsion at cell membranes, highly hydrophilic compounds have reduced transport in plant roots, as do highly hydrophobic

compounds, due to retention at cell membranes. A logK_{OW} of 1.78 is optimal for translocation of neutral compounds[90]. The transport of ionizable compounds through root cells may also be hindered by electrostatic attraction to negatively charged root tissues, or via ion trapping: a compound which is neutral in the apoplast (pH between 4 and 6.3[91]) diffuses into the cytoplasm (pH between 7.3-8[91]), where it acquires a charge, and is retained electrostatically[92]. Organic compounds may also be taken up passively through membrane channels, such as aquaporins, or ion channels[93–95], or via active transport pathways which involve transporter proteins and consume energy. Uptake via these pathways can exceed uptake via the transpiration stream[93,96].

Plant metabolism can reduce the concentrations of a compound in plant tissues. Plants have relatively non-specific metabolism to detoxify contaminants in three general phases. In Phase I metabolism, the polarity and reactivity of a compound is increased, often via a hydroxylation reaction catalyzed by CYT P450 or similar enzymes[97]. This renders the compound amendable for Phase II conjugation, in which the compound is conjugated with an abundant bio-molecule, most often a sugar or amino acid via the formation of a covalent bond[97]. Conjugates may then be deposited in plant vacuoles or incorporated into cell biomass in Phase III metabolism[98].

Overall, rubber-derived compounds in the agricultural environment may accumulate in edible plants. The accumulation of rubber-derived compounds in edible plants is affected by 1) bioaccessibility of rubber-derived compounds in agricultural soils, which can be reduced via mobility or the formation of non-extractable residues, 2) plant uptake and translocation from root tissues to above ground tissues, and 3) plant metabolism. These processes have not yet been investigated for most rubber-derived compounds, so the accumulation of rubber-derived compounds in edible plants, and the resulting dietary exposure can not yet be assessed.

1.3 Rubber-derived compounds in indoor air: the case of climbing gyms

While exposure to rubber-derived compounds via food has been scarcely investigated, exposure via inhalation has received more attention. Rubber-derived compounds have been detected in airborne particulate matter samples in outdoor urban environments around the world[17–23], with the highest estimated daily exposures for roadside workers[21,24]. In both outdoor and indoor environments, airborne particulate matter eventually settles as dust, and rough correlations have been observed between concentrations of some organic compounds in airborne particulate matter and settled dust[99]. Rubber-derived compounds have been detected in indoor dust[19,45–53], which suggests that they are present in indoor airborne particulate matter, although this has not yet been confirmed with reliable measurements. While outdoor air may introduce rubber-derived compounds to indoor environments, it is also possible

that consumer rubber products other than tires contribute to RDC concentrations in indoor air. For example, rubber-derived compounds were measured in artificial turf[100], and are present in air of indoor turf facilities[101,102]. Rubber-derived compounds have also been detected in a wide range of consumer products, from rubber bands to bottle nipples, although the number of compounds detected, and the measured concentrations were much lower in these consumer products compared to tires and recycled tire products[100]. Lower rubber-derived compound concentrations are likely related to lesser material demands of these consumer products compared to tires.

On the other hand, rock climbing shoes are a consumer rubber product which are highly engineered for specific properties. The rubber soles of rock climbing shoes are specialized for optimal friction between the shoe and the footholds, as well as for varying degrees of flexibility and hardness. Like tires, climbing shoe soles abrade during their intended use, due to friction between shoes and climbing footholds. In the case of indoor climbing gyms, this abrasion occurs in an enclosed environment, where many people are present. Due to physical activity, rock climbers in these facilities may have an elevated respiration rate, thereby increasing their inhalation of airborne rubber particles.

Climbing gyms are an increasingly popular form of recreation, particularly in urban areas - in the US, an estimated 6.36 million people visited indoor climbing halls in 2023[103], and 785 registered climbing gyms were operating in the US and Canada[104], implying that many thousands are employed in these facilities. Therefore, climbing gyms may be a hotspot of rubber additive exposure for climbers and employees.

1.4 Structure and aims of the PhD thesis

This PhD thesis investigates human exposure to rubber-derived compounds via food and air. The first part of the thesis (chapters 2-4) focuses on dietary exposure due to the accumulation of rubber-derived compounds in leafy vegetables. Leafy vegetables were chosen as a representative crop, since they are consumed raw, and due to elevated transpiration in leaves, accumulate higher quantities of organic compounds, as compared to various other fruits and vegetables[75,76]. The second part of the thesis (chapter 5) focuses on inhalation exposure to rubber-derived compounds in indoor air. Climbing gyms were chosen for study since they may represent a previously unstudied hotspot of rubber-derived compound exposure due to the abrasion of highly engineered rubber rock climbing shoe soles. Both parts of the thesis aim to 1) mechanistically investigate the environmental processes which affect the concentrations of rubber-derived compounds in the target medium (leafy vegetables or air), and 2) quantify the concentrations of rubber-derived compounds in real environmental samples, in order to 3) estimate the human exposure to rubber-derived compounds via both routes.

Chapters 2 and 3 focus on mechanistic investigations of the uptake of rubber-derived compounds by leafy vegetables in the agricultural environment. Although there are hundreds of organic rubber-derived compounds[12], a set of five representative compounds was selected, based on their detection frequency in the environment, availability of analytical standards, and wide-ranging physicochemical properties. The compounds selected were hexamethoxymethylmelamine (HMMM), 1,3-diphenylguanidine (DPG), benzothiazole (BTZ), N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD), and 6PPD-quinone. Chapter 2 describes plant uptake experiments which were conducted hydroponically in order to investigate whether rubber-derived compounds can be taken up by lettuce roots, and translocated to lettuce leaves. Plant uptake experiments in soils are described in Chapter 3, which investigates the bioaccessibility of rubber-derived compounds to plants, as well as the influence of several soil properties on bioaccessibility. In both Chapters 2 and 3, high-resolution mass spectrometry was employed to screen lettuce samples for transformation products of target rubber-derived compounds, since it was expected that rubber-derived compounds could be metabolized in plants. Finally, in Chapter 4, we quantified sixteen rubber-derived compounds in commercial leafy vegetable samples. These measurements validated the occurrence of the translocation, bioaccessibility, and metabolism processes investigated in chapters 2 and 3, and allowed us to estimate human exposure to rubber-derived compounds via leafy vegetable consumption.

The fifth chapter of this PhD thesis focuses on a different route of human exposure to rubber-derived compounds: inhalation of rubber particles in indoor rock climbing gyms. We confirmed that rubber-derived compounds are present in rock climbing shoes, and measured rubber-derived compounds in airborne inhalable and respirable particulate matter in five indoor climbing gyms. We sought to implicate rock climbing shoes as the main source of the rubber-derived compounds in climbing gym air, via a series of control experiments to eliminate other sources of rubber-derived compounds, and via ozonation experiments to prove that the rubber-derived compounds measured in climbing gym air could originate from the rubber-derived compounds in rock climbing shoes. Finally, we estimated human exposure to rubber-derived compounds via inhalation for rock climbers and climbing gym employees.

Chapter 2: Uptake, Metabolism, and Accumulation of Tire Wear Particle-Derived Compounds in Lettuce

- Hydroponic Study-

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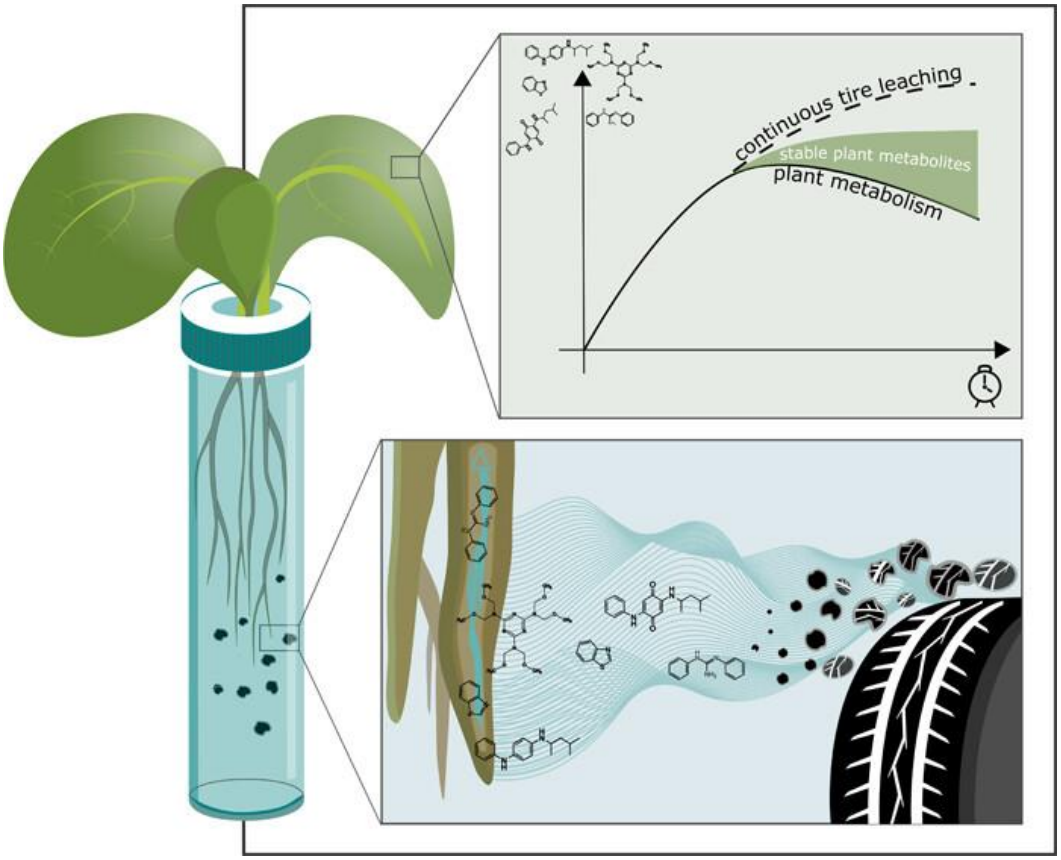

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2.1 Abstract

Tire wear particle (TWP) derived compounds may be of high concern to consumers when released in the root zone of edible plants. We exposed lettuce plants to the TWP-derived compounds diphenylguanidine (DPG), hexamethoxymethylmelamine (HMMM), benzothiazole (BTZ), N-phenyl-N'-(1,3-dimethylbutyl)-p-phenylenediamine (6PPD) and its quinone transformation product (6PPD-q) at concentrations of 1 mg L^{-1} in hydroponic solutions over 14 days to analyze if they are taken up and metabolized by the plants. Assuming that TWP may be a long-term source of TWP-derived compounds to plants, we further investigated the effect of leaching from TWP on the concentration of leachate compounds in lettuce leaves by adding constantly leaching TWP to the hydroponic solutions. Concentrations in leaves, roots and nutrient solution were quantified by triple quadrupole mass spectrometry and metabolites in the leaves were identified by Orbitrap high resolution mass spectrometry. This study demonstrates that TWP-derived compounds are readily taken up by lettuce with measured maximum leaf concentrations between ~ 0.75 µg g^{-1} (6PPD) and 20 µg g^{-1} (HMMM). Although these compounds were metabolized in the plant we identified several transformation products, most of which proved to be more stable in the lettuce leaves than the parent compounds. Furthermore, continuous leaching from TWP led to a resupply and replenishment of the metabolized compounds in the lettuce leaves. The stability of metabolized TWP-derived compounds with largely unknown toxicities is particularly concerning and is an important new aspect for the impact assessment of TWP in the environment.

2.2 Introduction

The extent of continuous tire wear particle (TWP) emissions into the environment remains poorly quantified,[7] but world-wide emissions are estimated to amount to 5.9 million tons per year.[10] TWP are expected to be introduced to farmland soils via several pathways, including atmospheric deposition and road runoff. Additionally, high retention of TWP in wastewater treatment plants (WWTP), with an estimated amount of > 93% of TWP retained, implicates that WWTP sludge is an important source of TWP to farmlands when biosolids are applied as fertilizers.[25] It was estimated that in Germany between 1,400–2,800 t TWP per year are deposited on agricultural land through the application of biosolids.[25]

TWP can introduce a large suite of organic compounds[32,105] to farmland soils with unknown effects on biota. Aside from rubber and fillers, tires contain additives that are, to date, indispensable for their functionality. These include vulcanization accelerators, activators, plasticizers, processing aids, and antioxidants.[106] The vulcanization accelerators 1,3-diphenylguanidine (DPG) and benzothiazole (BTZ) have been detected at concentrations in the µg L^{-1} range in rivers.[11] Although these concentrations are

close to the compounds' predicted no effect concentrations (PNEC) it was shown that at higher concentrations they are toxic to fish embryos.[36,107,108] Similar concentrations have been reported for hexamethoxymethyl melamine (HMMM), a cross-linking agent with over 30 known transformation products.[27,109] TWP have been implicated as the predominant source of these compounds in rivers[11]. Not only the parent compounds, but also their transformation products, many of which are still unknown, may exert harmful effects on biota. For example, the anti-ozonant N-phenyl-N'-(1,3-dimethylbutyl)-p-phenylenediamine (6PPD) is transformed into a significantly more toxic quinone transformation product (6PPD-q), which is responsible for mortalities of coho salmon through the introduction of street runoff into surface waters, where it has been detected in the concentration range of μg L^{-1}.[39,110]

When TWP enter farmland soils, they are expected to release these compounds in the upper soil layers, rather than transporting them to deeper soil horizons.[74] Once released in the root zone, the compounds may be readily available for root uptake by edible plants, as has previously been shown for various pharmaceuticals that were taken up from reclaimed wastewater and detected at concentrations of up to 300 ng g^{-1} in leafy greens,[76] and plastic-derived phthalates that were detected at maximum concentrations of up to 2.4 μg g^{-1} in cultivation experiments spiked at 500 μg kg^{-1}.[111] It was shown that through the uptake of well water up to 80 ng g^{-1} of benzotriazole was present in lettuce plants from an agricultural field in California,[112] but uptake of the broad range of TWP-derived compounds by edible plants has not previously been investigated. Hydrophobic neutral compounds are readily taken up by plant roots, while anionic compounds are mainly repelled by negative charges at cell membranes in the roots.[89,113] Compounds then migrate through the root tissue towards the xylem either via the symplastic pathway (through the cytoplasm via plasmodesmata), or via the apoplastic pathway (through the intercellular space, i.e. cell walls). Along their transport pathways, hydrophobic neutral compounds may partition into root lipids, thereby hindering their translocation to the leaves of plants. For neutral compounds, the mobility within the plant depends on the compound's octanol-water partitioning constant (K_{ow}), with maximum translocation occurring at a log K_{ow} of 1.78.[89,90] For ionic and ionizable compounds, electrostatic interactions with root tissues can also hinder mobility, and K_{ow} alone is an insufficient predictor for their mobility. Weak acids and bases can be ionized in the various root cellular compartments, which have different pH values.[91] Cationic compounds can be retained in the roots due to interactions with negatively charged cell walls, while weak acidic compounds can be subject to ion trapping, in which they diffuse into the cell in their neutral form, and are then trapped as anions in the more alkaline cellular cytoplasm.[91,114] Compounds that are mobile within the plant tissue will passively move upwards with the transpiration stream. However, the transport of organic compounds can exceed transpiration rates due to active transport by transporter proteins, as was shown, e.g., for isothiazolinone, benzotriazole and mercaptobenzothiazole in Arabidopsis plants.[96,115,116] Finally, physical properties of the

compounds can also affect their mobility. The diffusion of compounds > 400 $gmol^{-1}$ through the plant roots is mostly hindered at the Casparian Strip (the suberized center of the root endodermis), preventing them from entering the xylem and hindering them from being translocated into the plant leaves.[89]

The effective accumulation of TWP-derived organic compounds in plants also depends on the plant's metabolism. Within the plant roots and leaves, plant metabolism generally starts with phase I metabolism: an activation of the compound through hydroxylation, hydrolysis or dealkylation[97]. These activation processes can be catalyzed, e.g., by the enzyme cytochrome P450 oxidase mediating the oxidation/hydroxylation of contaminants.[117] These intermediate transformation products are often short-lived and further transformed by phase II metabolism, in which the activated compound forms a covalent bond with sugars or amino acids (transglycosylation or transamination processes). For compounds already containing -OH or $–NH_2$ functional groups, phase I activation may not be necessary because the compound can be directly conjugated at these sites.[89] Conjugates such as glycosylated metabolites are typically more water soluble than the original compound and can thus be transferred back into the roots and excreted into the external substrate, where subsequent deconjugation can occur.[96,118] Furthermore, the conjugated compounds can be deposited in plant vacuoles as a detoxification mechanism.[111,116] The complete metabolization of organic compounds can occur within a few hours.[117]

This study aimed to investigate whether lettuce plants take up TWP-derived compounds, thereby posing a potential risk to human health. We identified transformation products in the plant leaves to understand their fate, and possible human exposure to these compounds. Assuming that TWP may be a long-lasting source of TWP-derived compounds to plants, we further investigated the effect of continuous leaching from TWP on the concentration of TWP-derived compounds and their transformation products in lettuce leaves. We show that the monitoring of parent compounds may lead to an underestimation of human exposure concentrations and emphasize the presence of metabolized TWP-derived compounds with largely unknown toxicities in edible plants.

2.3 Methods

2.3.1 Chemicals and materials

TWP-derived compounds and selected chemical properties are shown in Table S2.1. DPG (Sigma-Aldrich 43029), BTZ (Sigma-Aldrich 61427), 6PPD (Sigma-Aldrich CDS013697), HMMM (TCI Europe T2059), and 6PPD-q (HPC Standards GmbH 687855) were purchased, and stock solutions were prepared and stored in LC-MS grade acetonitrile at 1 gL^{-1}. Ultrapure water was produced by a deionization system (PURELAB Ultra, ELGA LabWater Global Operations, 18.2 MΩ cm). LC-MS grade acetonitrile was purchased from VWR, formic acid from Sigma-Aldrich and sodium chloride (NaCl) from Merck.

Recycled tire granulate with a size distribution of 0.4 – 0.7 mm was provided by an Austrian tire recycling company that produces tire granulate from used truck and car tires.

2.3.2 Exposure experiments

Lettuce seedlings of the type *Valerianella locusta L.* were bought from a local garden supply market, removed from the soil, and thoroughly rinsed with tap water. All plants had well-developed roots and 1.6 ± 0.5 g total fresh weight. Each rosette was fixated in a glass vial containing 30 mL of hydroponic nutrient solution prepared with Blusana® fertilizer and placed in a growth chamber. The light-period was 16 hours day^{-1}, the light intensity 425 µmol $m^{-2}s^{-1}$ (PAR spectrum), 18°C during the day, and 15°C during the night at 75% relative humidity. After 2 days of equilibration with the nutrient solution the vials were spiked with HMMM, DPG and BTZ to a concentration of 1000 µg L^{-1} in the nutrient solution. This concentration was chosen after evaluating three different dosing levels in pre-experiments and was selected to obtain reliable data well above the level of quantification. Due to the degradation in the stock solution, initial concentrations of 6PPD and 6PPD-q were 400 and 200 µg L^{-1}, respectively. In a second experimental set, 3 g of tire granulate were added to each vial, and placed on a magnetic stirring plate at 350 rpm to keep the particles in suspension. Control samples with plants but without compounds/TWP and control samples with compounds/TWP but without lettuce plants were set up and all experiments were run in triplicates. All vials were wrapped in aluminum foil to avoid photodegradation of spiked or leached compounds. The nutrient solution in each vial was replenished every day. Plants were harvested after 0.125, 0.25, 0.5, 1, 2, 4, 7, 10 and 14 days and at each time point the plants and vials were sacrificed. The roots were rinsed with 2 mL of acetonitrile, which was combined with the rest of the nutrient solution of the respective vial. The roots were dried with lint-free tissue and separated from the leaves, and the fresh weight of roots and leaves were recorded. Roots and leaves were immediately frozen at -20 °C, and after the last harvest all samples were freeze-dried in one batch.

2.3.3 Extraction of TWP-derived compounds

After freeze-drying, the plants' dry weight was recorded and root and leaf samples were transferred to centrifuge vials. The plant tissues were extracted using a bead beater (Bead Genie™, Scientific Industries) with stainless steel beads and 2 mL of acetonitrile for 3 min at 4500 rpm. The samples were centrifuged for 5 min at 5,000 g and the supernatant was recovered. This extraction was repeated twice with a cumulative volume of 5.5 mL, which was filtered (0.22 µm nylon syringe filters, VWR 514-0066) into clean glass vials for further analysis. Extraction efficiencies from plant material including loss of analytes to the nylon filters were tested with spiked concentrations of 20 and 100 µg L^{-1} and ranged between 87.6 and 103% (mean 95.3±4.7%, Table S2.2). TWP-derived compounds remaining in the nutrient solution

were extracted by liquid-liquid extraction, adding 2 mL of acetonitrile to 3 mL of nutrient solution, adding 300 mg NaCl for phase separation and recovering the supernatant. This was repeated once and the supernatants were combined and filtered (0.22 µm nylon syringe filters) into clean glass vials for analysis. Extraction efficiencies from nutrient solution including loss of analytes to the nylon filters were tested with spiked concentrations of 50 and 500 µg L^{-1} and ranged between 87.2 and 104 % (mean 93.8±6.0%, Table S2.2).

2.3.4 Liquid chromatography – mass spectrometry measurements

TWP-derived compounds were quantified using ultra performance liquid chromatography coupled to triple quadrupole mass spectrometry (Agilent 6470, hereafter: triple quadrupole-MS) using external standards purchased from Sigma-Aldrich, TCI Europe, and IPC Standards GmbH. Results of the extraction tests imply that the sample matrix did not significantly impact measurements. The limit of quantification (LOQ) was determined as mean concentration of 5 blanks plus 10 times the standard deviation.

To evaluate the uptake and accumulation of the compounds in lettuce roots, the root concentration factor is expressed as:

$$Root\ concentration\ factor = \frac{concentration\ in\ the\ roots\ [\mu g g^{-1}]}{concentration\ in\ nutrient\ solution\ [\mu g L^{-1}]}.$$

As interactions of the compounds with the root tissue can affect their transport into plant leaves, the translocation factor was calculated as:

$$Translocation\ factor = \frac{concentration\ in\ the\ leaves\ [\mu g g^{-1}]}{concentration\ in\ the\ roots\ [\mu g g^{-1}]}.$$

Leaf extracts were additionally measured with an untargeted work flow using ultra performance Orbitrap high resolution mass spectrometry (Thermo Scientific Q Exactive™, hereafter: Orbitrap-HRMS) for transformation product analysis. Measurement parameters for both triple quadrupole-MS and Orbitrap-HRMS measurements are reported in supporting information (Section S2.1, S2.2). To identify transformation products, we first used Compound Discoverer software (Thermo Scientific, Version 3.1.1.12)[119] (Section S2.3, Figure S2.1, Table S2.5) to filter for compounds with an intensity ratio of at least 5 between the spiked lettuce samples and the control lettuce samples. For BTZ, due to contamination of the control samples, this filter was turned off. The transformation products were expected to be structurally similar to their parent compounds, and therefore produce common fragments. Therefore, we used the Molecular Networks tool of Compound Discoverer software to group unknown compounds with

MS1 and MS2 spectral similarity (at least 2 matched centroids, and 50% spectral similarity, FISh scoring) to the metabolized TWP-derived compounds.

We additionally used the Generate Expected Compounds tool of Compound Discoverer software to generate a list of possible transformation products of the TWP-derived compounds, based on plausible activation reactions for the TWP-derived compounds,[27,120] and conjugation reactions previously reported in plants (Table S2.6).[109,116] We then retroactively screened our data for these precursor masses (within 5ppm) with the Find Expected Compounds tool. With this approach, we were able to identify additional compounds of interest which were not present at high enough abundance to trigger MS2 scans in the Orbitrap-HRMS and were thus not identified with our Molecular Networking approach. For these compounds, we generated MS2 spectra by re-measuring samples using an inclusion list. We manually compared these MS2 spectra to the MS2 spectra of the associated parent compounds, and in the case of at least 2 fragment matches we report the compound as a potential transformation product of the original TWP-derived compounds (similar to what the molecular networking tool does automatically).

2.3.5 Sorption to lettuce roots

To evaluate the sorption of the TWP-derived compounds by lettuce root cell walls of the epidermis as well as the apoplast, batch experiments were performed. Clean lettuce roots were freeze-dried, ground to fine powder, suspended in nutrient solution (3.4 gL^{-1}) and equilibrated overnight. Each compound was spiked to a concentration of 50 and 500 $\mu g\ L^{-1}$ in the nutrient solution. The samples were agitated on a reciprocal platform shaker at 125 rpm at room temperature under exclusion of light, followed by centrifugation at 5,000 g and filtration of the supernatant (0.22 µm nylon syringe filter).[89] The amount of analyte remaining in the nutrient solution after sorption by the lettuce roots was quantified by triple quadrupole-MS analysis to calculate distribution coefficients (K_D) between roots and nutrient solution. The amount of analyte sorbed was normalized to dry root biomass to obtain contaminant concentration in $\mu g\ g^{-1}$ of root biomass.

2.3.6 Statistical Analysis

All experiments were run in triplicates, and technical duplicates were analyzed for each sample. All calculations and statistical analyses were done in R, version 4.2.0. To evaluate statistically significant differences in concentrations, ANOVA testing was used. For multi-variate comparisons, Tukey's HSD post-hoc testing was performed and results were visualized with compact letter display (Tables S2.7-S2.11).

2.4 Results and discussion

2.4.1 TWP-derived compounds are taken up by lettuce roots and are translocated into their leaves

The lettuce plants that were exposed to spiked TWP-derived compounds did not show any signs of phytotoxicity as indicated by the recorded biomass and the absence of visible cues of growth inhibition (Table S2.12). Moreover, we observed increased depletion ($p<0.05$) of all TWP-derived compounds from the nutrient solution in the samples containing plants as compared to the control samples (Figure S2.2, Table S2.11). DPG, HMMM and BTZ were stable in the nutrient solution controls over the experimental time span, while the loss of 6PPD and 6PPD-q in the control samples does not account for their depletion when plants were present (Figure S2.2). Accordingly, all five compounds were detected in roots and leaves of the lettuce plants (Figure 2.1a, 2.3).

The concentrations of DPG, HMMM and 6PPD in lettuce leaves peaked after 7 to 10 days at 2.29, 18.0 and 0.78 µg g^{-1}, respectively, followed by a subsequent decline to 1.16, 12.7 and 0.11 µg g^{-1}, respectively. In contrast, the main uptake of BTZ occurred within the first 6 hours (up to 1.24 µg g^{-1}), followed by a rapid decrease in its concentration to 0.17 µg g^{-1} thereafter. Concentration decreases were observed for DPG, BTZ, and 6PPD ($p<0.05$). After 10 days we observed a decreasing trend in HMMM concentration, which might become significant with longer experimental times (> 14 days). Only 6PPD-q showed a continuous increase in concentration over the full 2 weeks of the experiment, with values up to 2.19 µg g^{-1} (Figure 2.1, Table S2.8).

Despite different uptake rates, all five parent compounds could be identified in the lettuce leaves, which can be attributed to their low molecular weight. The diffusion of the studied compounds into the xylem and subsequent translocation into leaves was not hindered at the Casparian strip, as the molecular weights of all studied compounds were below the approximate exclusion threshold of 400 $gmol^{-1}$ (Table S2.1).[89] Further, experimental K_D values for lettuce roots were low for all compounds (0.05-0.7 Lg^{-1}), (Table S2.13) indicating that the translocation was not entirely hindered by sorption of the compounds to the root tissue and that these TWP-derived compounds can passively transit the plasma membranes without the need of secondary active transport mechanisms. Active transport was found to not be a significant contributor to translocation of cyetpyrafen.[121] On the other hand, uptake of benzotriazole and 2-mercaptobenzothiazole into Arabidopsis plants was shown to exceed the transpiration stream by far.[96,116] Thus, the fast uptake of BTZ in lettuce plants (Figure 2.1) may be partly attributed to the contribution of active transporter proteins.

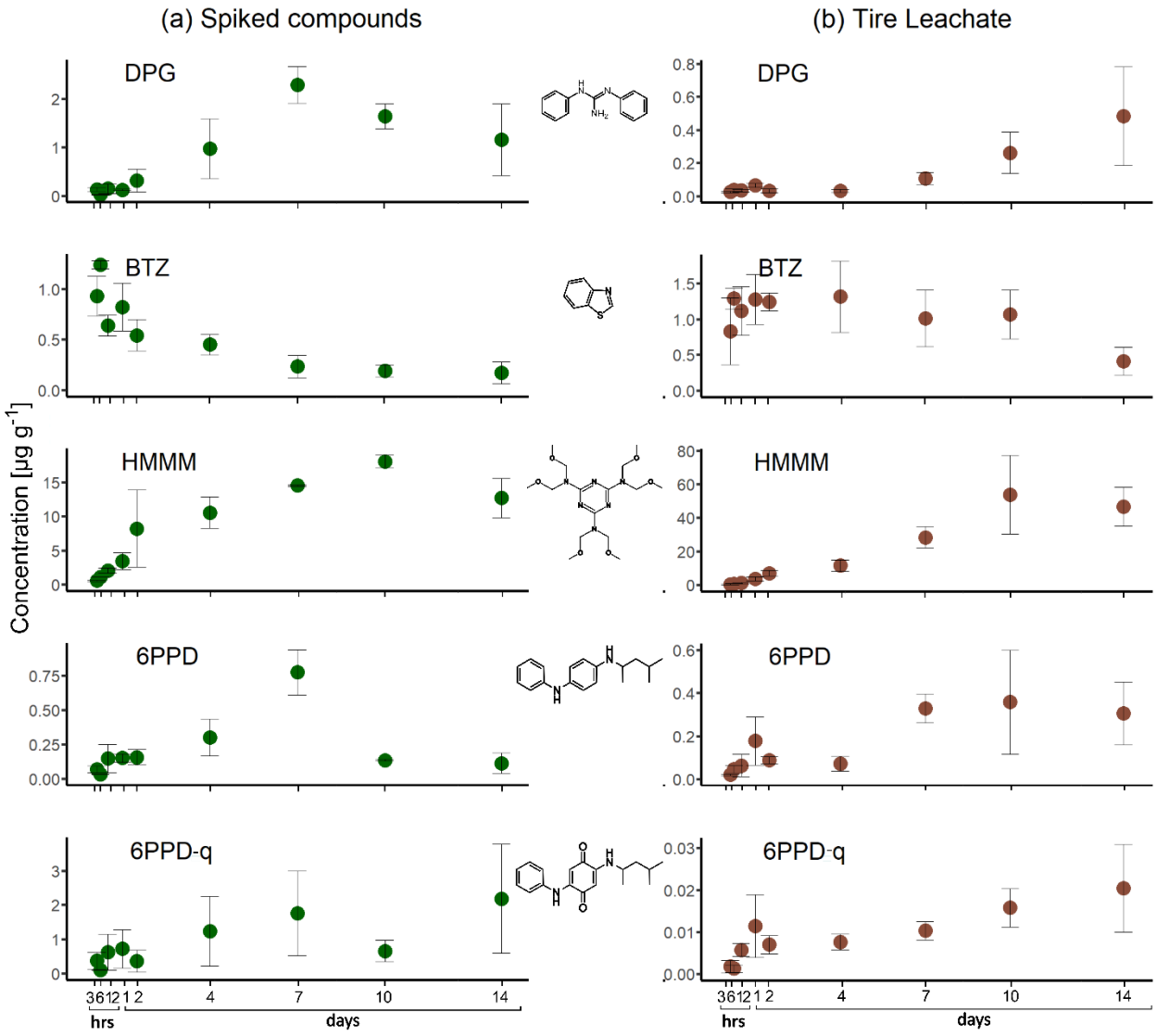


Figure 2.1 Concentration of TWP-derived compounds per unit biomass in lettuce leavesover 14 days after exposure to (a) a single initial spike (green dots) and (b) continuously replenished leaching from TWP in the nutrient solution (brown dots). Error bars represent the standard deviation from triplicate measurements. All five TWP-derived compounds were taken up into lettuce leaves.

However, the peak concentrations and the rate of translocation from roots into leaves differed among the compounds (Figure 2.1**Error! Reference source not found.**a). At the peak concentration, which was reached after 7-10 days, HMMM had accumulated in the leaves at significantly ($p<0.05$) higher concentrations than all other compounds, i.e., at a level of ~20 µg g^{-1}, and showed a higher translocation ($p<0.05$) from roots to leaves (translocation factor>1) (Figure 2.2, Table S2.9). During the first 24 hours

of the experiment, BTZ showed a similarly high translocation factor, and accordingly, HMMM and BTZ were translocated the fastest from lettuce roots to leaves, showing sharp concentration increases in the leaves within the first day of the experiment. In comparison, the translocation factors of DPG, 6PPD and 6PPD-q were approximately 2-3 orders of magnitude lower than that of HMMM and the increase in leaf concentration occurred after a slight delay (Figure 2.1a).

The observed differences in uptake rate and translocation can be explained by differential retention of the compounds in the root tissue. Although the measured K_D values (Table S2.13) were low, there were slight differences in affinities of the compounds to the roots. The measured K_D values followed the order of 6PPD>DPG>HMMM≅BTZ, which corresponds to the observed order of translocation factors of the compounds (Figure 2). The lower translocation factors and higher K_D values for DPG, 6PPD and 6PPD-q not only explain the lower concentrations in the leaves compared to HMMM, but also correspond to their delayed increase in leaf concentration compared to the accumulation of HMMM and BTZ in lettuce leaves within the first day.

The K_D and inversely associated mobilities of TWP-derived compounds in lettuce plants depend on the hydrophobicity and charge of the compounds (Table S2.1).[114] Highly hydrophilic compounds barely pass through lipid membranes in the roots and would require transporter (carrier) proteins in the root membranes. On the other hand, highly hydrophobic compounds can be retained by partitioning into root lipids.[90] The maximum mobility of TWP-derived compounds into and through the roots is therefore expected for neutral compounds with a log K_{ow} of 1.78,[90] which corresponds to that of BTZ (log K_{ow}= 2) and HMMM (log K_{ow}= 1.6) and explains their high translocation factors. Accordingly, BTZ and HMMM barely accumulated in the roots, resulting in low root concentration factors during the entire experiment (Figure 2.3). In comparison, the K_D, and therefore the root concentration factor of DPG are approximately one order of magnitude higher. The higher log K_{ow} of DPG (2.9) indicates that the retention of DPG in the roots is caused by partitioning into root lipids.[113] Furthermore, DPG (pK_a = 10) is present mostly in its cationic form at the physiological pH of both the apoplast (pH 4-6.3) and the cytoplasm (pH 7.3-8), and can therefore be retained by negative charges of the cell walls in the root apoplast.[91] The interaction of polyphenols in root tissues with primary amines may explain the higher root retention of DPG compared to HMMM and BTZ.[89] Although BTZ and HMMM with pK_a values of 7.80 and 7.01, respectively, could be similarly cationic in the apoplast, their pK_a values are closer to the pH values in the cytoplasmic plant compartments, causing them likely not to be fully ionized or to be neutral in this pH range. Due to their mobility in plant tissue, BTZ and HMMM may passively diffuse through the cell membrane, where the more alkaline pH of the cytoplasm is close to the pK_a of both compounds. Thus, BTZ and HMMM in the cytoplasm may be present to a large extent in their neutral form and pass unhindered through the symplastic pathway.

6PPD and 6PPD-q consistently exhibited the lowest translocation factors of all compounds although the differences in translocation factors between the different compounds were generally small (Figure 2.2, Table S2.9). Due to the fast abiotic degradation of 6PPD-q in aqueous solution during the sorption experiments, K_D values could not be reliably determined. Nevertheless, the root concentration factors of 6PPD and 6PPD-q are several orders of magnitude higher than those of the other TWP-derived compounds, indicating stronger sorption of these compounds to root cell walls (Figure 2.3). This higher sorption can be explained by their high log K_{ow} of 5.6, suggesting strong partitioning into root lipids. In addition, 6PPD-q may be able to form H-bonds between its quinone and root polyphenols.[89,122]

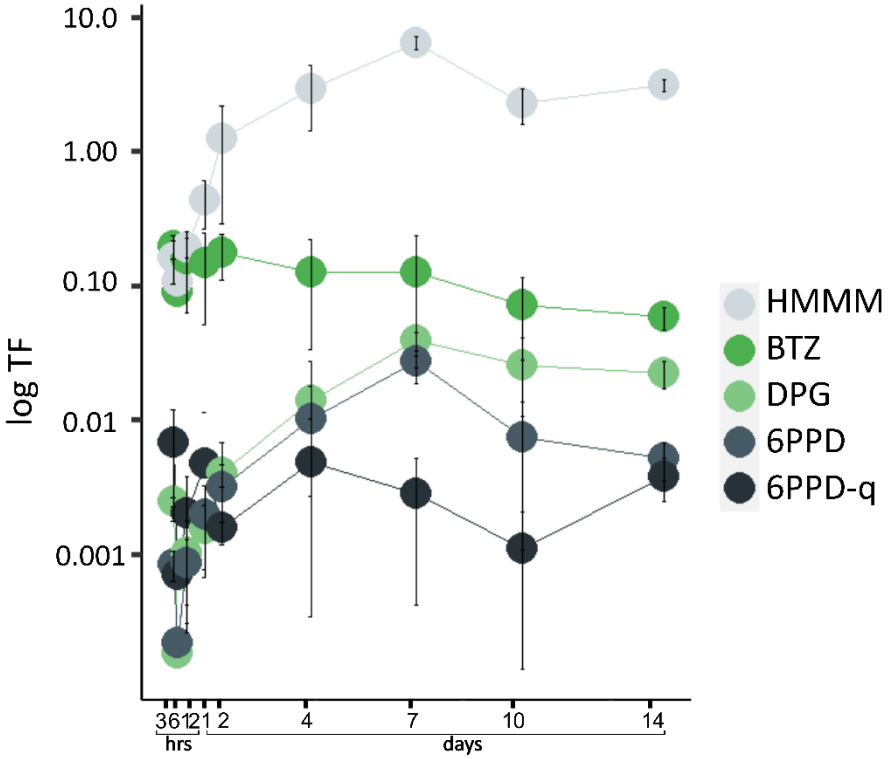


Figure 2.2 Translocation factors (log TF) of TWP-derived compounds from lettuce roots to the leaves after a single initial compound spike. Error bars represent the standard deviation from triplicate measurements.

We cannot rule out that the concentration increase of 6PPD-q was, in part, due to transformation from 6PPD, but we do not expect this to be a very relevant transformation in our experiments. The majority of 6PPD-q is formed via ozonation of 6PPD at tire surfaces[123], which is unlikely to occur in the plants or nutrient solution. Rather, the continuous increase in foliar concentration of 6PPD-q can be explained by the high root concentration factor retaining 6PPD-q and delaying its translocation to the leaves.

Although plant uptake is strongly compound dependent, the data demonstrate that TWP-derived compounds with a variety of structural differences have the potential to be taken up and translocated to edible plant parts. Despite being partially retained in the roots, even hydrophobic and ionic compounds were translocated to lettuce leaves.

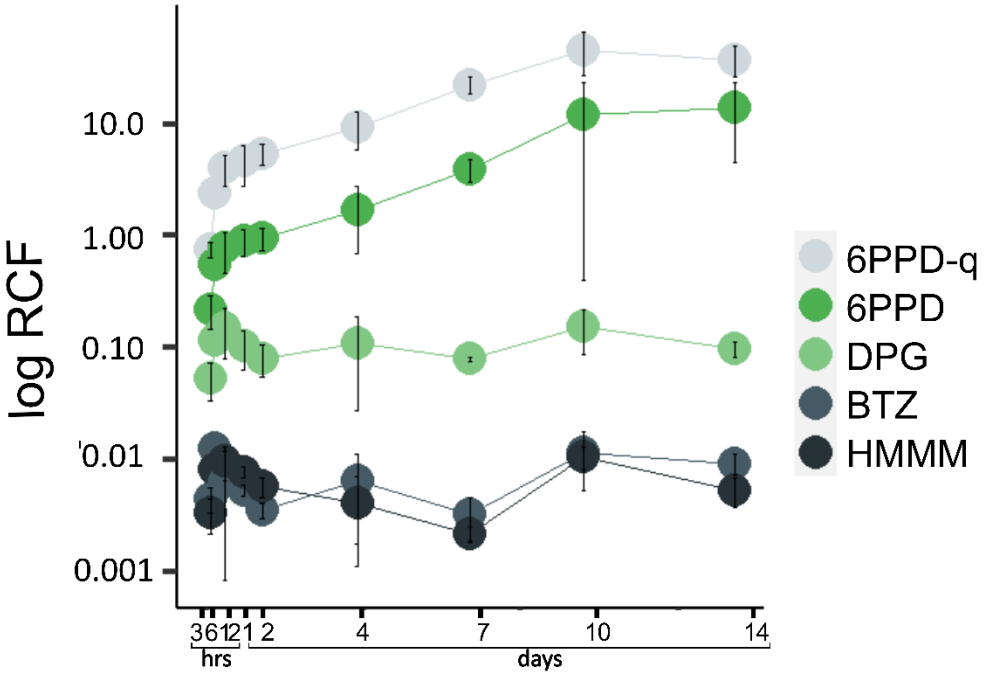


Figure 2.3 . Root concentration factors (RCF) of TWP-derived compounds between nutrient solution and lettuce roots. 6PPD-q (light grey dots), 6PPD (green dots), DPG (light green dots), BTZ (dark grey dots), and HMMM (black dots) after a single initial compound spike. Error bars represent the standard deviation from triplicate measurements.

2.4.2 Lettuce plants metabolize TWP-derived compounds and accumulate their transformation products in the leaves

The concentration increases of 6PPD, DPG, BTZ and HMMM in the lettuce leaves were followed by rapid concentration decreases (Figure 2.1a). Although the upward flow of the xylem is usually higher than the downward flow of the phloem towards to roots, the analytes may have been partly transported back into the roots[124]. To confirm that this decrease in concentration of TWP-derived primary compounds in lettuce was not due to the re-translocation of the compounds to the roots or due to depuration (excretion) into the nutrient solution, we calculated mass balances for all the TWP-derived compounds (Figure S2.3). We found a net mass loss of these compounds in the plant samples compared to the controls, and the decreasing concentration in the leaves suggests that this mass loss can be attributed - at least partly – to

metabolism in the leaves. Metabolites may then undergo various fate processes, such as storage, depuration, or further transformation.

6PPD and DPG reached maximum concentrations at one week of exposure, after which 6PPD declined to concentrations close to the LOQ, while DPG was metabolized to a lesser extent (Figure 2.1a). DPG and 6PPD both contain secondary amines, a functional group which is generally prone to undergo biotransformation reactions,[125] and has been shown to be sites of, e.g., sugar conjugation (transglycosylation) in plants.[116] Thus, DPG and 6PPD may be directly conjugated (i.e., without the need for an activation step),[116,126] which could explain the relatively rapid degradation of DPG and 6PPD in lettuce leaves. HMMM reached a maximum concentration after ~ 10 days, and then decreased, though much more slowly than the other TWP-derived compounds. The slower degradation rate, along with the fact that HMMM has no functional groups directly amenable to conjugation reactions, suggests that HMMM first undergoes a phase I activation reaction, which can be rate-limiting rather than undergoing direct conjugation. [127] 6PPD-q did not appear to reach a maximum concentration within the course of the experiment. BTZ displayed the fastest metabolism, reaching its maximum foliar concentration after 6 hours, and then decreasing to concentrations close to the LOQ for the rest of the experimental duration.

To gain an insight into the fate of the TWP-derived compounds in plants, we measured the leaf extracts with Orbitrap-HRMS, following an untargeted approach. With this approach we detected 19 potential transformation products of the original 5 TWP-derived compounds, identified to varying degrees of confidence.[128] All of these compounds were present at elevated signals in the spiked plant extracts compared to the controls, and were structurally related to the original TWP-derived compounds (Section S2.4, Table S2.14).

6PPD and DPG were readily conjugated. We tentatively identified one of the transformation products of DPG as an acetate conjugate ($TP_{DPG}270$), and the only detected transformation product of 6PPD as a 6PPD-glucose conjugate ($TP_{6PPD}431$).

DPG was more stable in a conjugated form, as the measured intensities of its conjugates increased sharply at around 7 days, and did not decrease substantially thereafter (Figure 2.4, Table S2.10). 6PPD-glucoside, on the other hand, increased and subsequently decreased ($p<0.05$) in concentration, suggesting further metabolism of the 6PPD-glucoside, or alternatively excretion of the conjugated compound. Excretion of glucoside conjugates has been previously demonstrated.[129,130] Conjugation of phytotoxic compounds creates a more water-soluble molecule, which plants can either excrete, store in their vacuoles, or which accumulate through sorption to cell walls, thereby immobilizing the compound and preventing toxic effects to the plant.[120,126] Once in the vacuole, conjugated xenobiotics have been shown

to undergo further degradation reactions.[120,131] In this study, the nutrient solution controls showed that DPG was highly stable in water, while 6PPD degraded very quickly (Figure S2.2). This suggests that the degradation of conjugated compounds in plants may be related to the overall stability of the unconjugated compounds. Since we did not monitor the hydroponic solution for transformation products, we cannot differentiate between excretion and/or further transformation of 6PPD-glucoside. On the other hand, DPG conjugate stability means that DPG could still be present (albeit in its conjugated form) in edible plants at the time of human consumption. This study contributes to the growing body of work demonstrating that monitoring of parent compounds only may lead to an underestimation of exposure risk by ignoring the presence of metabolites, a phenomenon known as "metabolite masking".[116,129,132]

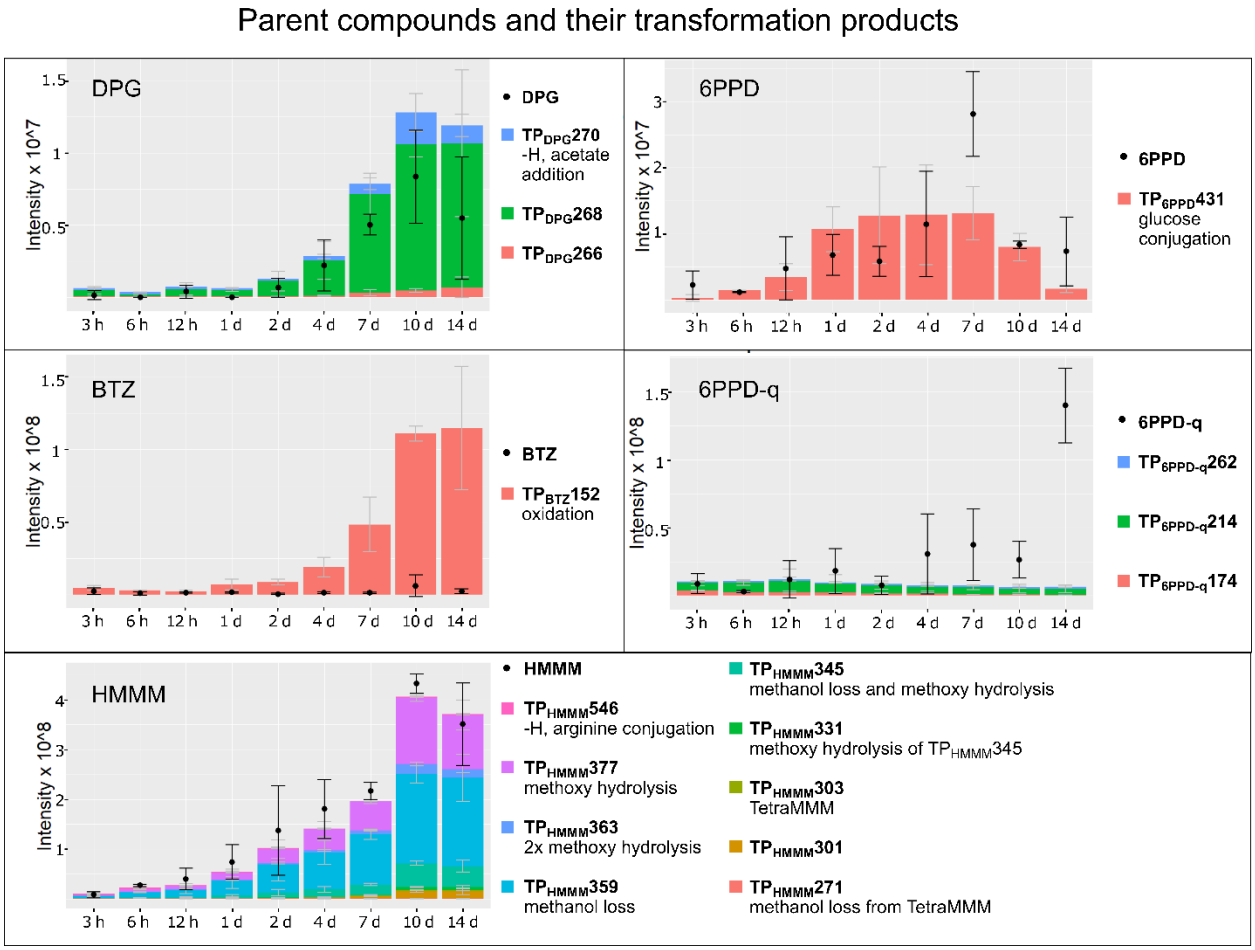


Figure 2.4 Ion intensities of TWP-derived compounds and their transformation products over time as measured by Orbitrap-HRMS. Black dots represent the measured intensity of the parent compounds, while the stacked bar plots represent the cumulative intensities of the transformation products.

6PPD-q is inherently unstable, as we measured rapid degradation in the nutrient solution controls. Interestingly, 6PPD-q appeared to be more stable in the plants, displaying a continuously increasing concentration in the leaves for the entire 2 weeks of the experiment (Figure 2.4). We identified three potential transformation products of 6PPD-q ($TP_{6PPD\text{-}q}214$ with monoisotopic mass 213.20858, $TP_{6PPD\text{-}q}174$ with monoisotopic mass 173.12001, and $TP_{6PPD\text{-}q}262$ with monoisotopic mass 261.13555). To the best of our knowledge, these masses have not previously been reported as transformation products for 6PPD or 6PPD-q. All three transformation products were stable within the plants, with only $TP_{6PPD\text{-}q}174$ displaying a slight decrease ($p<0.05$) in measured intensity over time. The increased stability of 6PPD-q metabolites compared to the 6PPD metabolite may be related to the distinct transformation processes. While glycosylation was a major fate process for 6PPD, leading to potential excretion of the conjugated compound, conjugation was not a major fate process for 6PPD-q.

HMMM was also metabolized by lettuce plants and we could identify 11 potential transformation products of HMMM. After reaching the maximum concentration at 10 days, the transformation products of HMMM did not decrease significantly (Figure 2.4, Table S2.10). In general, HMMM undergoes sequential degradations via only three reaction steps (hydrolysis of a methoxy group, formaldehyde elimination, and N-methylol oxidation to aldehyde).[27,109,133] Although there is growing evidence that the N-methylol oxidation to aldehyde reaction is biologically catalyzed[27,109], we did not observe any transformation products formed via this reaction in the lettuce plants. Rather, the hydrolysis of a methoxy group was the most relevant reaction, producing two of the four major transformation products ($TP_{HMMM}377$ and $TP_{HMMM}363$), both of which have been ubiquitously detected in urban rivers[27,29]. Surprisingly, the transformation product measured at highest abundance in the lettuce leaves ($TP_{HMMM}359$) was previously unreported, with an exact monoisotopic mass of 358.19526 and a proposed molecular formula of $C_{14}H_{26}N_6O_5$. A molecule with this molecular formula cannot be formed via any of the known HMMM transformation reactions, including those which have been shown to be catalyzed by bacteria and fungi[27,109]. This may be due to a reaction pathway catalyzed within plants. Based on the proposed molecular formula of this transformation product, we reason that the reaction might release a methanol from HMMM. We observed the same neutral loss between known transformation product TetraMMM and $TP_{HMMM}271$, implying that $TP_{HMMM}271$ was formed via the same methanol loss pathway from TetraMMM. The transformation product with the third highest intensity is also previously unreported ($TP_{HMMM}345$) and may be a secondary transformation product of $TP_{HMMM}359$ via the known methoxy hydrolysis reaction. Although we identified further transformation products, including a probable arginine conjugate of HMMM ($TP_{HMMM}546$), they were measured at much lower intensities than the aforementioned transformation products. Although measured at lower intensity than other transformation products, the tentative identification of an arginine conjugate of HMMM is worth note.

Amino acid conjugates are not excreted as easily as sugar conjugates, due to the locations of the respective enzymatic reactions,[130,134] which may imply long-term stability of $TP_{HMMM}546$. This paper joins a growing body of evidence showing that amino acid conjugation is a relevant biological transformation for xenobiotics.[96,109,115,129] Some of these novel transformation products of HMMM may be uniquely formed within plants, and we measured four transformation products of HMMM with high intensity, all of which were stable in the leaves over the duration of our experiment.

BTZ was metabolized remarkably quickly in the lettuce leaves. We identified one transformation product of BTZ, $TP_{BTZ}152$ (exact monoisotopic mass of 151.00881, unequivocal molecular formula: C_7H_5NOS) which was measured at very high intensity. The measured intensity of $TP_{BTZ}152$ increased continuously over the course of the experiments, even after BTZ had been depleted (Figure 2.4). The fact that BTZ was stable in nutrient solution (Figure S2.2) but transformed so rapidly to $TP_{BTZ}152$ in the leaves suggests that the reaction was catalyzed by a highly active catabolic enzyme present in the plants. 2-Mercaptobenzothiazole has also been shown to be transformed very rapidly in plants.[116] Because of its structural similarity to purines, BTZ may have entered the purine catabolic pathway, as has been previously shown for benzotriazole in the tryptophan pathway.[96] The exact mass difference between $TP_{BTZ}152$ and BTZ implies the addition of an oxygen atom, which could have been catalyzed by xanthine dehydrogenase, an enzyme which oxidizes the pyrimidine ring of hypoxanthine to xanthine. This reaction would also be feasible for BTZ and would produce the observed compound $TP_{BTZ}152$ (Table S2.14). Purines are catabolized as an energy source in plants[135], and xanthine dehydrogenase is highly active in the leaves of many plant species[136]. Therefore, the presence and gene expression of this enzyme in plants could explain the rapid transformation of BTZ to $TP_{BTZ}152$ in plant leaves. In the following step of purine catabolism in plants xanthine dehydrogenase catalyzes the oxidation of the imidazole ring. However, the stability of $TP_{BTZ}152$ and the fact that we did not detect this next oxidation product, suggest that BTZ is not further metabolized by the purine catabolic pathway. An alternative hypothesis for the rapid formation of $TP_{BTZ}152$ is oxidation of the reduced sulfur in benzothiazole, as has been shown for benzisothiazolinone.[115] The metabolism of BTZ that we demonstrate here may have wider implications on the plants' biochemical processes, as has been recently shown for benzisothiazolinone.[115] Future work should monitor the activities of relevant enzymes, or the up- or down-regulation of endogenous plant compounds. Regardless of the site of oxidation, $TP_{BTZ}152$ was likely a stable product of BTZ metabolism and is expected to accumulate in lettuce leaves during the growth period.

2.4.3 Leaching from tire wear particles (TWP) continuously replenishes metabolized compounds in plants

While some of the transformation products of TWP-derived compounds accumulated in lettuce leaves, the concentration of the parent compounds quickly decreased. However, with TWP as major source of these contaminants, they are likely continuously supplied over the plant's growth period. Therefore, in a second scenario, we exposed lettuce plants to TWP, which continuously released the five compounds into the nutrient solution (Figure S2.2). In spite of substantial compound concentrations leaching from the particles, we did not observe phytotoxic effects of TWP or their leachates on lettuce plants, as confirmed by visual inspection of the experimental plants as well as the recorded biomass (Table S2.12).

Compared to the treatment with a single initial TWP-derived compound spike, the concentrations of all compounds in lettuce leaves were more stable or continuously increased when exposed to TWP, due to the resupply from constant leaching of TWP (Figure 2.1b). The concentration of DPG, which was degraded following a peak in concentration after 7 days in the spike experiment (**Error! Reference s ource not found.**a), continuously increased until the end of the experiment when plants were exposed to TWP (**Error! Reference source not found.**b). The concentrations of HMMM and 6PPD remained stable u ntil the end of the experiment instead of decreasing after 7 days and the concentration of BTZ, which was otherwise metabolized within the first day of the experiment, decreased much later and more slowly when exposing lettuce plants to TWP (**Error! Reference source not found.**b).

These TWP-derived compound concentrations in lettuce leaves resulted from compound uptake, translocation, and metabolism, but also the differential leaching rates of the compounds from TWP. The leaching rate of DPG remained stable over the first 7 days and then decreases only slightly (Figure S2.2), and this continuous resupply of DPG into the nutrient solution led to a constant concentration increase in lettuce leaves. In comparison, the leaching rates of HMMM and BTZ were particularly high in the beginning and therefore accumulated in lettuce leaves to much higher concentrations than DPG. However, the leaching rates of HMMM and BTZ plateaued after approximately four days, which resulted in a concentration decrease in the leaves as soon as the leaching rate and plant uptake rates declined below those of plant metabolism of those compounds. 6PPD and 6PPD-q instantaneously decompose in the nutrient solution once they leach from TWP, but even so they showed a more continuous concentration increase in the leaves compared to the spike experiment.

In the soil environment the leaching behavior of TWP likely differs from the hydroponic experiments, but tire additives are expected to migrate through the TWP and leach out.[6] For example, amine stabilizers such as 6PPD are designed to freely migrate through the tire rubber towards the surface,

allowing them to serve as anti-ozonants,[137] with the side-effect that they continuously leach into the environment. DPG, BTZ and HMMM are used as vulcanization accelerators and cross-linking agents, which requires a good dispersibility in rubber,[138] so that the compounds which are not consumed during vulcanization[139] leach readily into the environment making them available for plant uptake.

2.5 Environmental implications

The uptake and accumulation of five TWP-derived compounds by lettuce plants and the subsequent translocation of the compounds into leaves may be of concern to consumers, particularly because lettuce plants are eaten raw. The fact that even the hydrophobic TWP-derived compounds, 6PPD and 6PPD-q, were readily taken up by lettuce is concerning as it shows that uptake is not restricted to specific molecular characteristics, such as molecular weight and hydrophobicity.

While 6PPD and DPG were likely conjugated by e.g. transglycosylation, HMMM, 6PPD-q, and BTZ were degraded via various pathways. We here for the first time report conjugation of TWP-derived compounds in edible plants, and we further propose novel transformation products of HMMM and 6PPD-q, including an amino acid conjugate of HMMM. With the exception of 6PPD, plant metabolism formed more stable products that accumulated in lettuce leaves.

Conjugation reactions are generally reversible, and deconjugation has been demonstrated in the human digestive system[129]. Especially in the case of DPG, conjugation implies that DPG is preserved in the edible part of lettuce plants, and humans could be exposed to it during consumption following deconjugation. For the other TWP-derived compounds with stable transformation products, humans may be exposed to these transformation products, with unknown toxicities. Further work is needed to determine the toxicity of TWP-derived compounds and their transformation products, and to determine the environmental levels of these compounds in agricultural products.

The uptake of TWP-derived compounds was studied for exposure conditions in hydroponic cultures. Under environmental conditions, the compounds can be sorbed to soil constituents, decreasing the bioavailability of these compounds in soils.[78] Other compounds such as pharmaceuticals were shown to sorb to the soil matrix and to quickly equilibrate with soil pore water. This process is highly dependent on soil properties, as well as on compound properties. The charge state of positively ionizable compounds (HMMM, 6PPD, DPG, and BTZ) is determined by soil pH, and in the case that the compounds are present in cationic form, sorption is controlled by the soil's cation exchange capacity.[140] The availability of neutral compounds is determined by the soil's organic carbon content, and the compound's $\log K_{ow}$.[140] For example, although 6PPD-q displayed a relatively high root concentration factor under hydroponic conditions (Figure 2.3), in soils with a high organic carbon content it is likely that due to the compound's

$\log K_{ow}$ of 5.0-5.5, 6PPD-q is expected to remain bound to soil rather than being available for plant uptake. Biosolids have a high organic carbon content, which may decrease the availability of neutral tire-derived compounds for plant uptake.[141] Additionally, co-contaminants could also affect plant uptake. For example, cellular stress induced by mercury was demonstrated to reduce plant uptake of oxytetracycline.[142] Growth stage of the plant may also impact the accumulation of tire-derived compounds. It has been shown that premature cabbage take up pharmaceuticals and chemicals derived from personal care products at higher amounts than mature cabbage.[75] If plants are exposed to tire-derived compounds during early growth stages, uptake may be even higher than what we have demonstrated here, and stable metabolites may accumulate over the plants' entire life cycle. Under field conditions, further degradation of the compounds may occur over the growth cycle of lamb's lettuce which spans over approximately 1 month.[143] However, the observed stability of the identified transformation products after two weeks suggests that even considering longer growth cycles in the field these transformation products may still be present at harvest.

Organic compounds generally accumulate to a lesser extent in fruit vegetables than in leafy greens or root vegetables,[75,76] because root-shoot transport of organic compounds is assumed to be mostly via xylem, and xylem influx to fruits is limited.[77] Studies comparing uptake of organic compounds by multiple crops have found that for some compounds, leafy greens such as lettuce and spinach are the strongest accumulators, while other compounds show higher accumulation in root vegetables.[75,76] In this study, we also demonstrated that preferential accumulation in roots or leaves is compound-dependent. Compounds such as 6PPD-q, which were found to accumulate in lettuce roots, may be of more concern in root vegetables, such as carrots or radish.

The dosing levels used in the experiments were chosen rather high compared to environmentally relevant doses.[112] A change in dosing level was previously shown to linearly correlate with the uptake of carbamazepine and diclofenac by lettuce,[144] and pre-experiments conducted in the course of this study demonstrated similar trends at different dosing levels. On the other hand, the assimilation of 2-mercaptobenzothiazole was higher at lower exposure concentrations[116] so that a slightly different response at environmentally relevant concentrations may not be excluded. An unresolved, yet critical aspect may be the degradation of these compounds by soil microbial communities before they are taken up by the plants, which should be investigated in the future.

Depending on the input pathway to agricultural fields, some TWP-derived compounds may leach from TWP before they enter the soil. For air-borne TWP, compounds may be lost through volatilization during atmospheric transport, but the majority of the compounds are expected to be released once TWP enter the agricultural soils and come in contact with soil pore water. There, the compounds are released

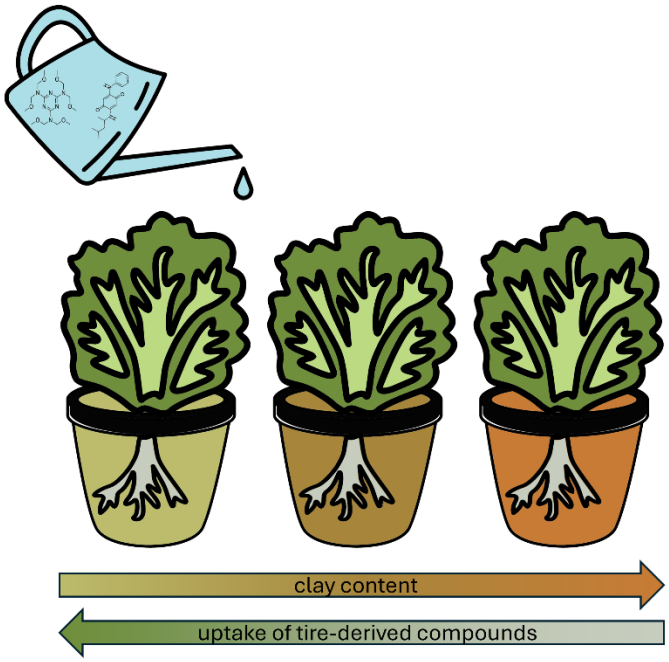
over time and can pose a long-lasting source of toxic TWP-derived compounds to edible plants. In contrast, TWP that are deposited on the road are exposed there to sunlight and rain, before entering a (mixed) sewer system and then a WWTP.[11,145] In this case, TWP may have already released substantial amounts of the compounds, due to prolonged contact with the aqueous phase, and therefore not generate a continuous supply on agricultural fields. However, the compounds may be introduced to agricultural fields if waste-water, which has been shown to carry large amounts of TWP-derived compounds,[11,133] is used for irrigation. In this study, we used TWP from recycled tires. Despite the previous usage and environmental exposure of this recycled tire material, it leached considerable amounts of compounds. The leaching from aged tire material indicates that intraparticle diffusion of DPG, HMMM and BTZ, i.e., their migration to the tire surface, can limit the release of these compounds during their transport in the environment. Thus, upon reaching agricultural fields, TWP may continue leaching tire additives, making them accessible for plants and entering the human food web.

This study showed that TWP may be a continuous source of TWP-derived compounds to edible plants and transformation products accumulate in the leaves of lettuce. This may become critical if regulatory thresholds are only defined according to the concentrations of original TWP-derived compounds, while underestimating the sum of parent compounds and transformation products with up to date largely unknown toxicities.

Chapter 3: Uptake of Tire-wear Derived Compounds by Lettuce Grown in Three Soils

-Soil Study-

Anya Sherman, Luzian Elijah Hämmerle, Evyatar Ben Mordechay, Benny Chefetz, Thilo Hofmann



Manuscript in preparation.

3.1 Abstract

Tire-wear derived compounds have been recently detected in commercial leafy vegetables, but processes governing their uptake and accumulation in agricultural environments under realistic conditions remain unclear. Previous work has investigated the uptake, translocation, and metabolism of tire-wear derived compounds by lettuce plants grown in hydroponic systems, but it is expected that the bioaccessibility of these compounds will change in agricultural soil systems. In this study, lettuce plants grown in three agricultural soils were exposed to five tire-wear derived compounds via regular irrigation with spiked water over their entire growth period. The accumulation of hexamethoxymethylmelamine (HMMM) and N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD) in lettuce plants was limited by the formation of non-extractable residues in all three soils; relatively high bioaccessibility of 1,3-diphenylguanidine (DPG) and 6PPD-quinone enabled greater accumulation. Accumulation of all tire-wear derived compounds was greater in lettuce grown in soils with higher sand, and lower clay content. Potential transformation products of the tire-wear derived compounds were also observed in lettuce plants. The distribution of smaller, more polar transformation products between parts of the plants, and between plants grown in different soils generally matched the distribution of the parent compounds, while there were generally no statistically significant trends in the distribution of larger, less polar transformation products. This study provides a better understanding of the uptake of tire-wear derived compounds by lettuce plants in the agricultural environment.

3.2 Introduction

Tire additives (5% - 10% of tire material by weight)[6] are a contaminant class of emerging concern due to the high entry rate of tire and road wear particles into the environment (0.9 to 2.5 kg/capita/year)[7] and their potential to leach into their surrounding ecosystems. Tire additives include vulcanization accelerators such as benzothiazoles and guanidine derivatives, such as 1,3-diphenylguanidine (DPG), cross-linkers such as hexamothymethylmelamine (HMMM), and antioxidants such as phenylene-diamines (PPDs). Many of these additives are reactive and produce transformation products during tire production (vulcanization agents), use (antioxidants), or once in the environment. For example, 6PPD (a widely used anti-ozonant) reacts with ozone to form a quinone product (6PPD-quinone), which is highly toxic to several fish species at environmentally relevant concentrations[31,43,146,147].

Tire-wear derived compounds (additives and their transformation products) enter the urban water cycle primarily through road runoff and thus are frequently detected in wastewater and in surface waters [17,26–29]. Thus, irrigation with treated wastewater or surface water, especially in arid and semi-arid climates where unconventional water sources are needed for routine irrigation, exposes the agro-environment to tire-wear derived compounds. Treated wastewater already makes up more than 50% of Israel's water

supply in the agricultural sector[148]. In Spain and Italy, a total of 821,920 m^3 and 123,288 m^3 are applied to fields each day, respectively[149]. The usage of treated wastewater for irrigation proposes is expected to increase globally over the next years, to meet increasing demands on agricultural systems in the context of rising temperatures and prolonged droughts. Within Europe, recent legislation will also increase the use of treated wastewater for irrigation[150]. Additional sources of tire-wear derived compounds to agricultural fields may be via aerial transport of tire particles from nearby roads[151] and the application of treated biosolids, which is estimated to deposit 1400 – 2800 tons per year of tire wear particles on agricultural fields in Germany[25].

Tire-wear derived compounds, such as HMMM, 6PPD-quinone, benzothiazole and others have been reported in commercial crops, reaching up to 665 ng/g dry weight[152]. These findings confirm the presence of tire-wear derived compounds in the agricultural environment and demonstrate their bioaccessibility, leading to uptake by commercial crops. However, so far, no correlations between tire-wear derived compound uptake and known growth conditions, such as soil properties or irrigation system have been observed. In previous hydroponic experiments, we mechanistically evaluated the uptake of five tire-wear derived compounds by lettuce plants using hydroponic experiments[153]. HMMM exhibited the highest plant uptake and translocation of all studied compounds, reaching a maximum concentration of 18.0 μg/g in lettuce leaves. DPG reached a maximum concentration of 2.3 μg/g in lettuce leaves. 6PPD and 6PPD-quinone were mostly retained in lettuce roots due to their lipophilicity, reaching maximum concentrations in the leaves of 0.8 μg/g and 2.2 μg/g respectively[153]. Benzothiazole reached maximum concentrations of 1.2 μg/g in the lettuce leaves within 6 hours, after which it was metabolized, as has been reported for several benzothiazole, benzotriazole, and benzimidazole derivatives[96,112,115,154,155]. In hydroponic experiments compounds are fully bioaccessible for plant uptake, which facilitates the study of plant uptake and metabolic processes, but differs from standard conditions in the agricultural environment, where only the fraction of compounds dissolved in soil solution is assumed to be bioaccessible[78,79]. In the soil matrix, organic compounds such as tire-wear derived compounds can undergo many fate-determining processes that would reduce the total bioaccessible amount in soil solution. Hydrophilic, mobile compounds may be transported out of the root zone of plants[80], and sorption or degradation can reduce bioaccessible concentrations of non-mobile compounds which do remain in soil solution in the plants' root zone.[81]. The mechanisms by which organic compounds can interact with soil components have been extensively reviewed in the literature and are difficult to disentangle experimentally[82,84]. For this reason, it is common in research and regulatory assessments to group these fate processes into pools of "extractable residues" and "non-extractable residues"[85,86]. Extractable residues can be extracted via common laboratory methods, such as water or solvent extractions, that do not substantially alter the soil matrix and

are either bioaccessible, or can easily become bioaccessible to plants due to desorption processes. Non-extractable residues are classified as organic compounds and their transformation products that are adsorbed or physically entrapped within the soil particles (type I non-extractable residues), covalently bound to soil particles (i.e., chemical sorption; type II non-extractable residues), or assimilated into soil biomass (type III non-extractable residues)[87]. Types I and II non-extractable residues are expected to be released to soil solution only if the soil matrix is substantially altered, while Type III non-extractable residues can no longer become bioaccessible[85]. Previous work on soil sorption of tire-wear derived compounds suggests that HMMM and DPG do form large fractions of non-extractable residues in soils[156], and up to 95% immobilization of 6PPD-quinone was observed in a study with bioretention cells[157], so we hypothesized that bioaccessibility, and therefore plant uptake of these compounds in the agricultural environment might be much lower than suggested by hydroponic experiments.

Under hydroponic conditions, transformation products of HMMM, DPG, 6PPD, 6PPD-quinone, as well as several benzothiazole and benzotriazole derivatives have been reported in plants and fungi[96,109,116,153], and the same benzotriazole transformation products were also found in plants grown in soil[112]. However, transformation of most tire-wear derived compounds has not yet been confirmed in plants grown under realistic conditions.

In this study we investigated the uptake of HMMM, DPG, Benzothiazole, 6PPD and 6PPD-quinone (deuterated form) by lettuce plants grown in three soils. These model compounds represent a variety of tire-wear derived compound classes, and have different physico-chemical behavior with p*K*a ranging from 10 to -4, and a log *D* ranging from 0.3 to 4.3. All compounds were continuously introduced to the system via the irrigation water, since this represents a likely exposure scenario in farming. It is expected that tire-wear derived compounds are primarily translocated passively, so lettuce plants were separated into inner and outer leaves, to confirm that accumulation is higher in outer leaves where there is increased transpiration. The three soils varied in composition, as we expected that soil properties including cation exchange capacity and organic carbon content would influence bioaccessibility of the compounds. We hypothesized that the formation of non-extractable residues rather than mobility would reduce bioaccessility of tire-wear derived compounds, and we confirmed this using recovery experiments and depth-dependent soil sampling. We additionally used non-target mass spectrometry to screen for transformation products of tire-wear derived compounds in the lettuce leaves, and to evaluate their distribution within plants, and between plants grown in different soils.

3.3 Materials and Methods

3.3.1 Experimental Design

Plant uptake experiments were carried out in a greenhouse (Faculty of Agriculture, Food, and Environment, the Hebrew University of Jerusalem), between August and September 2023. Lettuce plants (*Lactuca sativa*) seedlings were obtained from a nursery (Hishtil, Israel) and planted in 3 L pots containing soil collected from lysimeter stations in the Negev region of Israel, which have been irrigated with freshwater since 2001. Three different soils collected from Nir Oz, Ein Hashlosha, and Sa'ad areas in Israel were selected for this study, all of which have been characterized and described previously[158–161]. Soil properties are presented in Table S3.1. Three replicates (individual pots, with one lettuce each) were included for each soil type. Pots with lettuce plants, which were not exposed to tire-wear derived compounds were also included in triplicate for each soil type, as negative controls.

Initially, three seedlings were planted in each pot. Plants were irrigated using drip irrigation (0.5 L/h) three times a day, with each irrigation cycle delivering 150 – 200 mL of water. After a 12-day acclimatization period, the seedlings were thinned to one per pot, and the exposure period began. During the exposure period (26 days), automatic irrigation was skipped every 2-3 days, and plants were manually watered with 100 mL of irrigation solution containing 1 µg/mL of a mixture of five tire-wear derived compounds. In total, each plant was irrigated 10 times with the tire-wear derived compound solution, for a total dose of 1mg over the course of 26 days.

Benzothiazole (BTZ), 1,3-diphenylguanidine (DPG), hexamethoxymethylmelamine (HMMM), N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine (6PPD), and d5-6PPD-quinone were spiked into the irrigation water. Compounds were the same as in our previous hydroponic study and were selected to span different compound classes. Compound properties are provided in Table S3.2. The deuterated form of 6PPD-quinone was used to differentiate between 6PPD-quinone which was spiked into the irrigation water and 6PPD-quinone which was formed via transformation of 6PPD.

After 38 total days of growth, the lettuce had reached a commercial size and were harvested. The outer ten leaves were separated from the inner leaves. All leaves were lyophilized, homogenized using a blender, and mailed to Vienna for extraction and analysis. The upper ten centimeters of soil were separated from the lower part of the soil, and the majority of root biomass was removed manually from the top soil. Upper and lower soil samples were lyophilized, homogenized, and sub-samples were mailed to Vienna for extraction and analysis.

3.3.2 Chemicals

Acetonitrile (LCMS Grade) was purchased from Sigma-Aldrich. Formic acid for HPLC was purchased from Merck. d5-6PPDq was from HPC Standards®. DPG, benzothiazole, and 6PPD were purchased from Sigma-Aldrich. HMMM was purchased from TCI Europe. D4-Benzothiazole was purchased from LGC Standards. Ultrapure water was produced by a deionization system (PURELAB Ultra, ELGA LabWater Global Operations, 18.2 MΩ cm).

3.3.3 Sample Extraction and target analyses (UPLC-Triple quadrupole MS)

All lettuce and soil samples were extracted in Vienna between November and December 2023. A slightly modified version of our previously published plant extraction protocol was used to extract all lettuce samples[153]. Briefly, 1 mg of homogenized biomass was added to a 15 mL centrifuge tube, along with 4 g stainless steel beads, surrogate standard (500 ng d4-benzothiazole) and 4 mL HPLC-grade acetonitrile. Samples were extracted via bead beating at 3000 strokes per minute for three minutes, followed by centrifugation at 5000 rpm for 5 minutes, after which 1 mL of supernatant was extracted to a separate vial. This extraction was repeated twice, with 2 mL of acetonitrile added and collected each time. All extracts were combined for a total extract volume of 5 mL, vortexed, and filtered through a 0.22 µm nylon filter, and stored at 4°C until analysis.

Soil samples were extracted using a QuEChERS extraction protocol adapted from the literature[79]. The addition of a buffer (MOPS pH 8) as well as the addition of a divalent cation (Ca^{2+}, 10 mM) were tested during method optimization: 5 µg of all compounds were spiked into half of the soils bulk volume of acetonitrile, vortexed, and then added to 2 g soil in an Eppendorf tube. Soil was thoroughly saturated, and headspace was minimal, which maximized interaction of the tire-wear derived compounds and the soil. Soil was agitated at 250 rpm for 24 hours in the dark, and then dried at 60° C for 2 hours to remove acetonitrile. Soil was then transferred to a 15 mL centrifuge tube and extracted. Neither the use of buffer, nor a divalent cation enhanced overall extraction recovery (Table S3.3). Thus, the extraction method used for soil samples was as follows: soil samples were ground using a mortar and pestle, then 2.5 g subsamples were weighed into 15 mL polypropylene centrifuge tubes. Surrogate standard (2.6 µg d4-Benzothiazole) and 4 g of stainless steel beads were added, along with 2 mL of 150 mg/L EDTA solution. Samples were agitated via bead beating at 4800 strokes per minute for 3 min. Then 5 mL of ACN was added and bead beating was repeated, followed by the addition of 2 g Na_2SO_4 and 1 g NaCl and a third round of bead beating. Samples were centrifuged at 5000 RPM for 30 min, then 4 mL supernatant was transferred into a clean glass vial, followed by the addition of 2 mL acetonitrile to the soil. Bead beating and centrifugation were repeated, then 2 mL of supernatant was removed and combined with the first

extract. Extracts were mixed and filtered through a 0.22 µm nylon syringe filter and stored at 4°C until analysis.

All lettuce and soil extracts were measured using ultra performance liquid chromatography coupled to triple quadrupole mass spectrometry (Agilent 6470) according to our previously published method[153]. Raw data were processed using Agilent Quantitative Analysis software.

3.3.4 QA/QC

Soil and lettuce samples were extracted in batches of 18 samples. Two method blanks (no matrix) were extracted along with each batch, to monitor any contamination that may have arisen during extraction. Matrix-matched calibrations were prepared to account for matrix effects and recovery: After an initial screening to identify control lettuce and soil samples with no detectable tire-wear derived compounds, these clean samples were spiked with known concentrations of the tire-wear derived compound mixture and extracted as described previously.

No tire-wear derived compounds were detected in any method blanks. Limits of quantification of all tire-wear derived compounds are provided in Table S3.5. Due to high limits of quantification (7.5 ng/mL which correspondes to 18 ng/$g_{dry\ weight}$ in soil, and 10 ng/mL which corresponds to 50 ng/$g_{dry\ weight}$ in leaves), benzothiazole could not be quantified in any soil or leaf samples. HMMM, DPG, d5-6PPD-quinone and 6PPD all had satisfactory limits of quantification in soil and lettuce (0.1 – 1 ng/mL, which corresponds to concentrations in biomass of 0.2 – 5 ng/$g_{dry\ weight}$, Table S3.5). HMMM, d5-6PPD-quinone, and 6PPD were not detected in any control soil samples. DPG was consistently detected at trace levels in the Ein Hashlosha and Sa'ad control soil samples, and in one Nir Oz control soil sample (Table S3.6). Soil from the lysimeter stations has been irrigated with fresh water for 23 years[158], and DPG is frequently detected in fresh waters[26,110], which could explain the trace levels of DPG found in these soils. No tire-wear derived compounds were detected in any control lettuce leaves, except for d5-6PPD-quinone in the outer leaves of a lettuce sample grown in Sa'ad soil at a trace concentration of 0.5 ng/g dry weight.

3.3.5 Sample extraction and non-target analyses (UPLC-Orbitrap MS)

All lettuce samples were re-extracted for non-target analyses, using a protocol which was developed to concentrate transformation products, as well as improve chromatography, and therefore peak-picking of more polar (early eluting) analytes. 1 g of homogenized plant material was extracted as for the target analyses, and then concentrated with N_2 blowdown evaporation to 1 mL. 1.5 mL Ultrapure water was added to the concentrated extract, and vortexed for 30 seconds, to cause chlorophyll precipitation. The sample was immediately filtered through a 0.22 µm nylon syringe filter which effectively removed all chlorophyll precipitates. 300 mg NaCl were added to the filtrate, and vortexed for 30 seconds, which

caused a phase separation between the ultrapure water and acetonitrile. The acetonitrile phase was collected using a Pasteur pipette and stored at 4°C until analysis.

Samples were measured using ultra performance liquid chromatography (Thermo Fisher Scientific – Vanquish Horizon) coupled to Orbitrap mass spectrometry (Thermo Fisher Scientific - Orbitrap Exploris™ 240). In-needle mixing was used to reconstitute samples to 50% acetonitrile before injection, which improved chromatography of early eluting analytes, while avoiding loss of analytes which are unstable in aqueous solution. Detailed method parameters are provided in Section S3.1. MS^2 spectra were acquired using AquireX software, and all samples were measured in full scan mode with a resolution of 240,000.

Data were processed using Compound Discoverer software, using a modified version of our previously published workflow[153,162]. Detailed data processing workflow is provided in Figure S3.1 and relevant parameters are provided in Table S3.4. Differential analysis was used to identify compounds which were up-regulated in exposed plants versus control plants. Statistical parameters (log 2 fold change and p-value) were selected as the highest values at which all parent compounds were up-regulated. From these up-regulated compounds, potential transformation products were identified with three methods: suspect screening, expected compounds analysis, and molecular networking, as has been previously described[153,162]. Candidate transformation products were further filtered to only those containing at least one diagnostic fragment of the associated parent compound in its MS^2 scan. Diagnostic fragments used for each parent compound are provided in Section S3.2. Finally, a manual check of all potential transformation products was performed to remove in-source fragments as well as transformation products for which the fragment matching was based on noisy, or otherwise low-quality MS2 spectra. Details about all potential transformation products, as well as chromatograms and MS2 spectra are provided in Section S3.2.

3.4 Results and Discussion

The accumulation of HMMM, DPG, BTZ, 6PPD, and 6PPD-quinone (deuterated form) in lettuce grown in three soil types were measured to investigate whether these compounds are bioaccessible to lettuce grown in soil, and how various soil properties influence bioaccessibility. HMMM, DPG, 6PPD, and d5-6PPD-quinone were detected in lettuce samples in all three soil types, at maximum concentrations of 66 ng/g, 33 ng/g, 3.7 ng/g and 94 ng/g respectively (Figure 3.1), indicating that these compounds are bioaccessible, and are taken up and accumulate in lettuce plants.

The outer ten lettuce leaves exhibited substantially higher concentrations of all tire-wear derived compounds than the inner leaves (Figure 3.1). This has been observed for carbamazepine, and is likely

due to higher transpiration rates of outer lettuce leaves, which are exposed to the atmosphere, than the protected inner leaves[161]. The outer leaves are also older than the inner leaves, which may also contribute to increased accumulation. The outer leaves had more variable concentrations of all tire-wear derived compounds than the inner leaves (Figure 3.1). The variability was consistent between compounds – that is, the highest concentrations of all tire-wear derived compounds were measured in the same lettuce plant, and the lowest concentrations of all tire-wear derived compounds were measured in the same lettuce plant. This is likely due to variation in transpiration rates between individual lettuce plants, as higher transpiration rates have been shown to increase accumulation of organic compounds[163]. Transpiration-dependent accumulation indicates that tire-wear derived compounds are taken up passively with the transpiration stream, rather than via active pathways, in which case uptake can exceed transpiration rates[115]. Passive uptake via the transpiration stream is generally understood to drive xenobiotic uptake in plants[121,164], although there is emerging evidence that other uptake processes, such as the plasma membrane H+-ATPase pump, aquaporin, and ion channels are also relevant[95,96,115,155]. Measurement of transpiration rates, or experiments with uptake pathway inhibitors would be needed to conclusively determine the dominant uptake pathway of the tire-wear derived compounds.

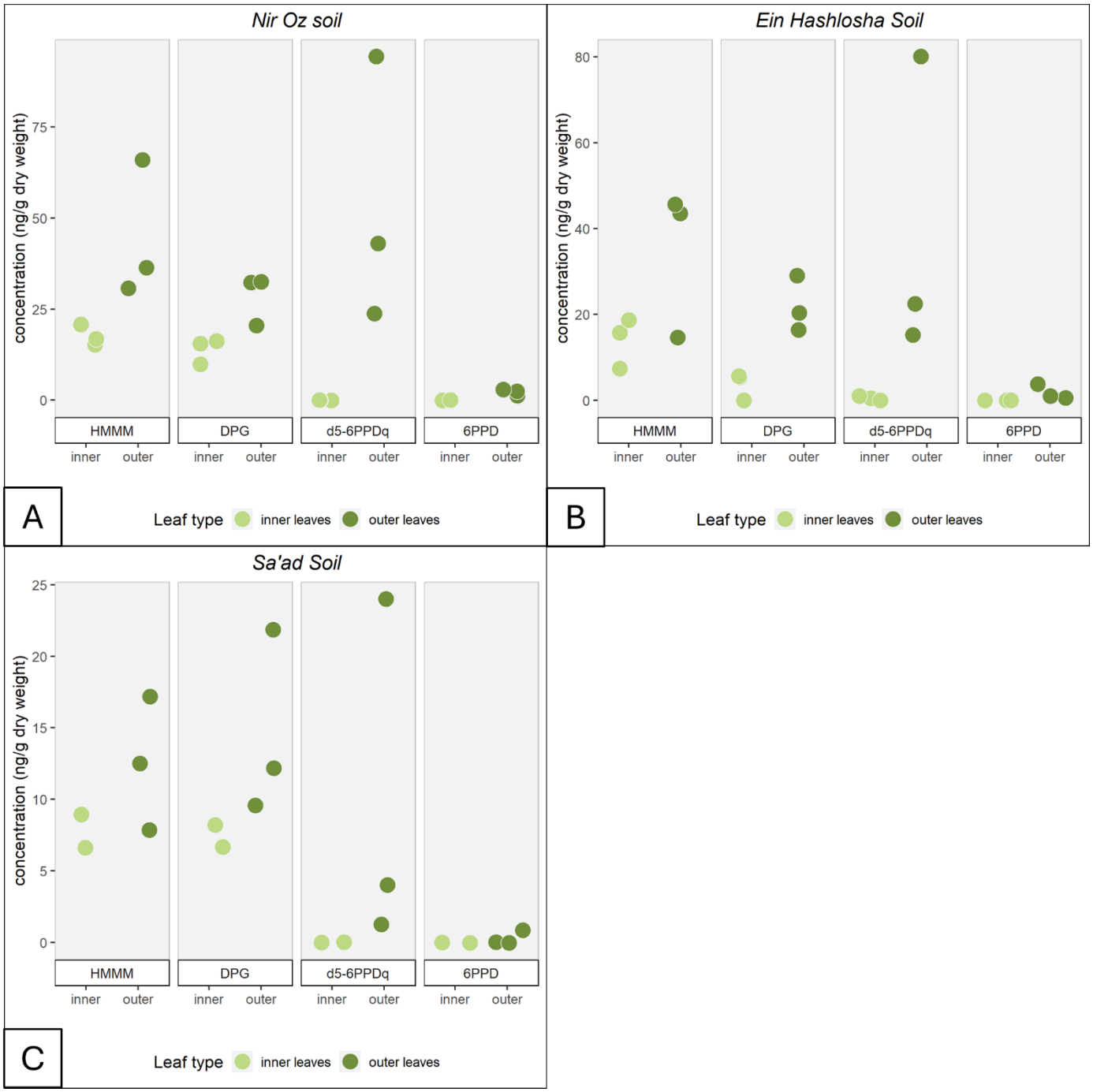


Figure 3.1 Concentrations of tire-wear derived compounds in inner and outer leaves of lettuce grown in A) Nir Oz soil, B) Ein Hashlosha and C) Sa'ad soils

Concentrations of HMMM, DPG, and d5-6PPD-quinone in the outer leaves of plants grown in the same soil type were similar (Nir Oz soil: 23.7 – 94.5 ng/g, Ein Hashlosha soil: 14.7 – 80.1 ng/g, Sa'ad soil: 1.3 – 24.0 ng/g, Figure 3.1). 6PPD concentrations were much lower (Nir Oz soil: 1.1 – 2.9 ng/g, Ein Hashlosha soil: 0.6 – 3.7 ng/g, Sa'ad soil: <LOQ – 0.9 ng/g, Figure 3.1). This is very different than what

we observed in previous hydroponic studies, where HMMM concentrations exceeded DPG concentrations by approximately one order of magnitude, and 6PPD and 6PPD-quinone concentrations by two orders of magnitude[153]. The uptake and translocation of tire-wear derived compounds by lettuce plants is unlikely to differ between the two studies; the differences in results are rather related to the bioaccessibility of the tire-wear derived compounds in a hydroponic compared to a soil system. In a hydroponic system, all of the compounds added to the hydroponic solution are assumed to be bioaccessible, while in a soil system, biotic and abiotic transformations, as well as vertical mobility or formation of non-extractable residues may reduce the bioaccessibility of some tire-wear derived compounds [81].

To investigate the vertical mobility of the tire-wear derived compounds in our pots, we divided the soil into a top fraction (upper 10 centimeters), and a bottom fraction. In the upper ten centimeters, where lettuce root density was highest[165], concentrations of the four tire-wear derived compounds ranged from 0.3 – 27.8 ng/g dry weight in the Nir Oz soil, 0.5 – 44.0 ng/g dry weight in the Ein Hashlosha soil, and 0.5 – 69.1 ng/g dry weight in the Sa'ad soil (Figure 3.2). In the bottom of the Nir Oz soil, all compounds were below limit of quantification while concentrations in the bottom of the Ein Hashlosha and Sa'ad soils were consistently lower than in the top soil (Figure 3.2). Therefore, vertical mobility within our pots, or out of the pot entirely, did not reduce the bioaccessibility of tire-wear derived compounds to lettuce plants.

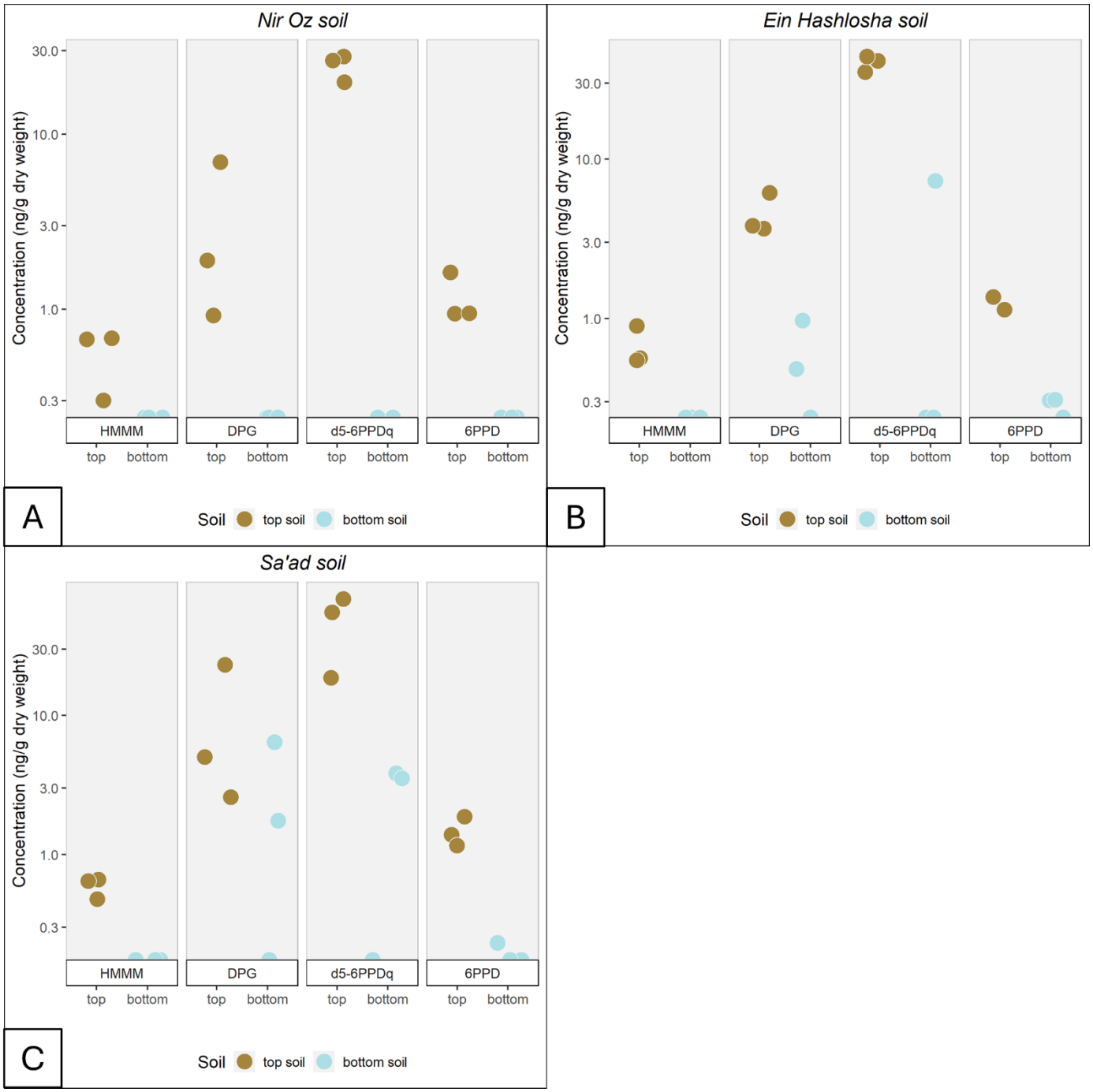


Figure 3.2 Soil concentrations of tire-wear derived compounds in top (upper 10 centimeters) and bottom of A) Nir Oz soil, B) Ein Hashlosha and C) Sa'ad soils

A QuEChERS extraction method was adapted from the literature[79], and optimized to achieve maximum recovery and minimum standard deviation for the five tire-wear derived compounds. Both the addition of a buffer, and a divalent cation (Ca^{2+}) reduced the stability of 6PPD and d5-6PPD-quinone, and

buffer addition generally increased the standard deviation of recoveries of all compounds (Table S3.3). Thus, neither a buffer nor a divalent cation were included in the final extraction method. Control extractions without a soil matrix yielded recoveries between 94% and 99%, with a maximum standard deviation of 9% for DPG, BTZ, HMMM, and d5-6PPD-quinone. The recovery of 6PPD was lower (61%, standard deviation 3%), due to its instability in aqueous solution over the 24 hour equilibration period[123]. In the presence of soil, recovery dropped substantially for BTZ (37% to 56%), HMMM (7% to 20%), and 6PPD (23% to 43%, Table S3.3) due to the formation of non-extractable residues. In the presence of all three soils, recoveries of DPG (99% - 107%) and d5-6PPD-quinone (83% to 96%) remained high, indicating that these compounds were mostly present as extractable residues. Accordingly, in the upper ten centimeters of the Nir Oz soil, extractable concentrations of the four tire-wear derived compounds followed the order of d5-6PPD-quinone (19.8 - 27.8 ng/g dry weight) > DPG (0.9 - 6.9 ng/g dry weight) > 6PPD (0.9 - 1.6 ng/g dry weight) > HMMM (0.3 – 0.7 ng/g dry weight). This order held true in the Ein Hashlosha soil: d5-6PPD-quinone (35.2 – 44.0 ng/g dry weight) > DPG (3.7 – 6.1 ng/g dry weight) > 6PPD (1.1 – 1.4 ng/g dry weight) > HMMM (0.5 – 0.9 ng/g dry weight), and in the Sa'ad soil: d5-6PPD-quinone (23.0 – 69.1 ng/g dry weight) > DPG (1.9 – 23.0 ng/g dry weight) > 6PPD (1.2 – 1.9 ng/g dry weight) > HMMM (0.5 – 0.7 ng/g dry weight). Low extractability of HMMM from four soils has been previously reported, ranging from 8.1 to 66%[156], indicating that the non-extractable residues of HMMM form in many soil types. Tang et al reported substantially lower DPG recoveries between 4.9% and 60% from four soils[156]. The differences could be either due to different interactions of DPG with soils of distinct compositions, or an artifact of different extraction methods[156]. Although non-extractable residues of Type I (adsorbed or entrapped) and Type II (covalently bound) can be released to soil solution eventually[87], including via the disruption of soil structure by plant roots[166], it can be assumed that the fraction of tire-wear derived compounds which were non-extractable at the time of lettuce harvest were not accessible to the lettuce plants during their growth, while the extractable fractions were likely bioaccessible.

Based on this assumption, we calculated bioconcentration factors as the ratio of the compounds measured in the lettuce leaves to the extractable concentration measured in the top soil:

$$BCF = \frac{leaves}{soil_{extractable}}.$$

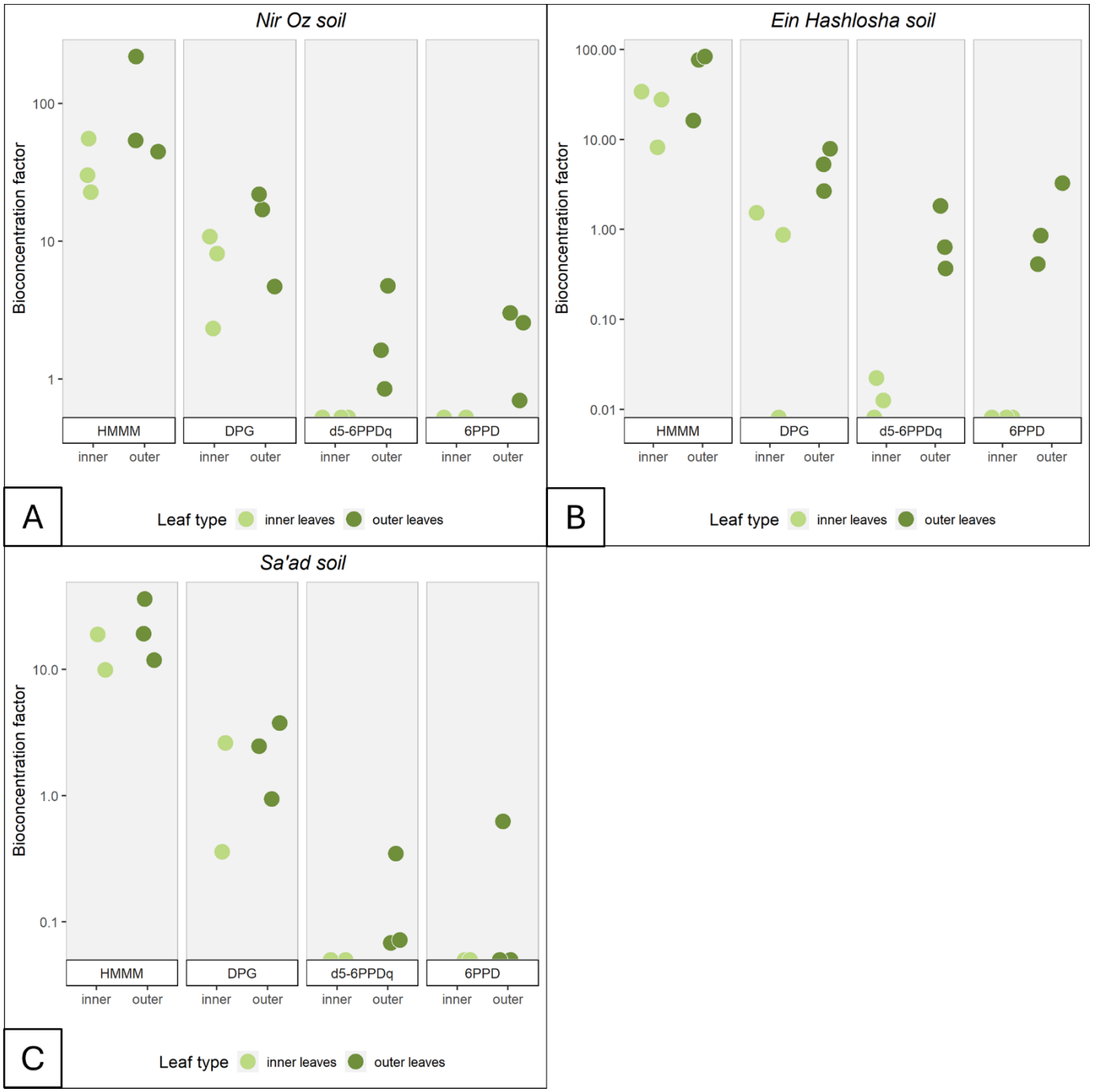


Figure 3.3 Bioconcentration factors of tire-wear derived compounds in inner and outer leaves of lettuce grown in A) Nir Oz soil, B) Ein Hashlosha and C) Sa'ad soils

Bioconcentration factors for both the inner and outer leaves follow the order HMMM > DPG > d5-6PPD-quinone ≈ 6PPD (Figure 3.3). This order matches what we observed in our hydroponic experiments, confirming that lettuce uptake and translocation of the tire-wear derived compounds is

consistent between the two systems. The bioconcentration factors calculated in this study, as well as those from our hydroponic study indicate that HMMM has a very high uptake and translocation potential, due to its partially neutral charge within plant cytoplasm, and its $logK_{ow}$ of 1.6, which is very close to the theoretical optimum value of $logK_{OW}$=1.8[90]. However, in all tested soils, the majority of HMMM formed non-extractable residues and was thus only of limited bioaccessibility to plants (Table S3.3). DPG showed lower bioconcentration factors than HMMM in both studies, due to its higher lipophilicity ($logK_{OW}$=2.9), and its positive charge in the plants' apoplast and cytoplasm. In contrary to HMMM, DPG was generally bioaccessible in our tested soils, although this may not be true in all soils[156]. d5-6PPD-quinone and 6PPD are mostly retained in the roots of plants due to their lipophilicities ($logK_{OW}$ of 4.30 and 5.6 respectively), and thus have the lowest bioconcentration factors of all studied tire-wear derived compounds[153]. Similar to DPG, the high bioaccessibility of d5-6PPD-quinone in soil compensates for its low bioconcentration factor, and concentrations of d5-6PPD-quinone and DPG in plants were in the same range as concentrations of HMMM. 6PPD was present in leaves at much lower concentrations, due to the combination of its low bioaccessibility and low bioconcentration factor.

The distribution of compounds between outer and inner leaves, top and bottom soil, and calculated bioconcentration factors displayed consistent trends in all three soils (Figures 3.1 – 3.3). However, absolute concentrations measured in the lettuce leaves varied from soil to soil (Figure 3.4). The differences are most likely related to the bioaccessibility of the compounds in soils of varying composition. All compounds had the highest concentrations in lettuce plants grown in the Nir Oz soil (Figure 3.4), which was composed of 82.5% sand, and only 5% clay (Table S3.1). Plants grown in the Ein Hashlosha soil, which was composed of 45% sand, and 12.5% clay had the second highest tire-wear derived compound concentrations, while plants grown in the Sa'ad soil, with 24.5% sand, and 27% clay had the lowest tire-wear derived compound concentrations. Sand, which is mainly composed of quartz in the 2 to 0.02 mm size fraction, has a very low sorption potential, and was shown to correlate to lower sorption of seven pharmaceuticals in 13 soils[140]. On the other hand, organic cations can bind strongly to clay, due to its high specific surface area, negative charges, and large cation exchange capacity[82]. The cation exchange capacity of the three soils increased with increasing clay content: 11 meq/100g in the Nir Oz soil, 17 meq/100g in the Ein Hashlosha soil, and 22.5 meq/100g in the Sa'ad soil. Cationic exchange with clay minerals would implicate the formation of Type I non-extractable residues[87], thereby reducing the compounds' bioaccessibility. In one study of four agricultural soils, clay content correlated with sorption of five out of six studied pharmaceuticals[167]. In another study, clay content was found to drive higher sorption of cationic compounds[140]. At the soils pH (7.45 – 7.65 in all soils), DPG is expected to be cationic (basic pKa of 10). 6PPD, with a basic pKa of 6.7[168], is mostly neutral. Assuming a basic pKa of 7.01 for HMMM[27], HMMM is also mostly neutral, although it should be noted that modelled pKa values

for HMMM vary widely[27,156], and we are not aware of any measured values. 6PPD-quinone is exclusively neutral in the environment, with a predicted basic pKa of less than 0[30]. Cationic exchange with clay minerals could thus explain the lower bioaccessibility of DPG in soils with higher clay content, but other mechanisms are likely relevant for the neutral compounds.

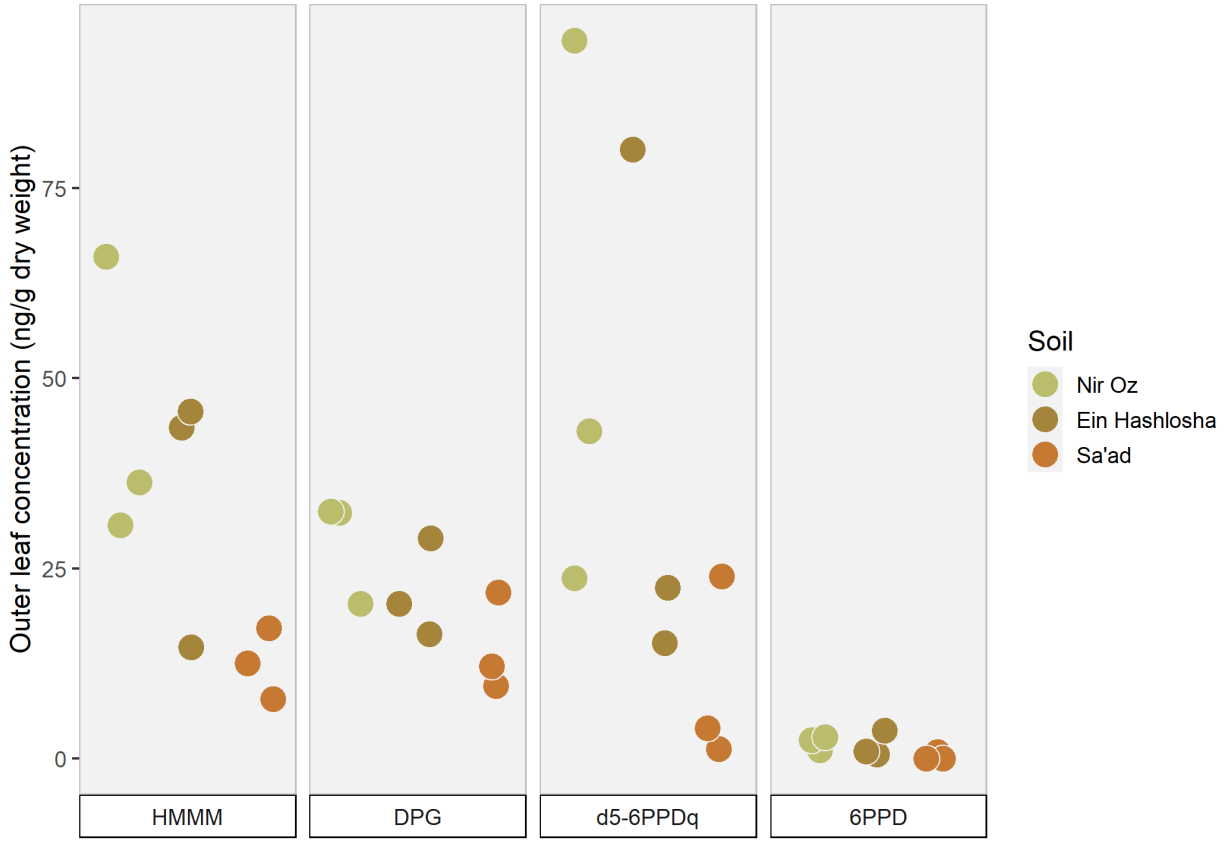


Figure 3.4 Concentrations of tire-wear derived compounds in the outer leaves of plants grown in three soils . Lettuce grown in the Nir Oz soil (yellow) had the highest concentrations of all tire-wear derived compounds, while lettuce grown in the Sa'ad soil (red) had the lowest concentrations of all tire-wear derived compounds.

Organic compounds can also form non-extractable residues via interactions with soil organic matter[87], and organic carbon is a major predictor of sorption - this is especially true for neutral, hydrophobic compounds[140,167,169]. The soils used in these experiments have a very low organic carbon content: 1.04% in Nir Oz soil, 2.65% in Ein Hashlosha soil, and 2.27% in Sa'ad soil (Table S3.1). At such low levels, it is unlikely that organic carbon plays a large role in the partitioning of the tire-wear derived compounds investigated between soil and soil solution. However, the especially low organic carbon content could contribute to higher bioaccessibility of neutral tire-wear derived compounds in the Nir Oz soil.

The studied tire-wear derived compounds are reactive and form transformation products[27,123], including within plants[153]. We therefore screened lettuce leaves for transformation products using a non-target approach. Sixteen compounds were selected as potential transformation products that were up-regulated in exposed plants versus control plants (log_2 fold change ≥ 1, p-value ≤ 0.05), and shared at least one diagnostic fragment with one of the parent compounds (Section S3.2). Two compounds contained diagnostic fragments of multiple parent compounds: TP_205 was annotated as a potential transformation product of d5-6PPD-quinone and 6PPD. TP_210 was annotated as a potential transformation product of DPG and d5-6PPD-quinone. This suggests that the diagnostic fragments used for transformation product annotation may not have been specific enough.

Three potential transformation products of HMMM were identified (Section S3.2), all with a confidence level of 2a according to Schymanski et al[128]. All three transformation products have lower retention times and molecular weights than HMMM (Figure 3.5A), which is typical of bacterial and abiotic transformations, suggesting that these transformation products may have been formed in the irrigation water or soil, and then taken up by the plants. Indeed, all three of these transformation products are known to be formed both biotically and abiotically in aerobic aquatic environments, and are commonly detected in surface waters[27,133]. Alternatively, these transformation products may have been formed via Phase I transformations within the plants, which generally activate an organic compound and increase its polarity[97]. All three transformation products had lower peak areas than HMMM.

Seven compounds were identified as potential transformation products of DPG (Section S3.2). In contrast to HMMM, all potential transformation products of DPG had higher retention times than DPG, and all but one had higher molecular weights (Figure 3.5 B). This implies Phase II conjugation reactions, which almost certainly occur within plants. During Phase II metabolism, sugar or amino acids can be covalently bound to Phase I transformation products of a compound[97]. All transformation products had lower peak areas than DPG.

Five potential transformation products of 6PPD, and three of d5-6PPD-quinone were identified. For both 6PPD and d5-6PPD-quinone, a mix of Phase I and Phase II transformation processes seem to be relevant, as both larger and smaller, and more and less polar transformation products were formed (Figure 3.5 C-D). 6PPD-quinone (non-deuterated form) was detected in several spike lettuce samples but was not annotated by our workflow, likely due to its very low concentrations. TP_d56PPDq_379 had higher peak areas than d5-6PPD-quinone in the plant leaves, while the other two transformation products had lower peak areas. Almost all potential transformation products of 6PPD had higher peak areas than 6PPD. This could imply that in addition to low bioaccessibility and plant uptake, the very low concentrations of 6PPD

in plant leaves could also be due to extensive transformation, as has been reported in a variety of environments[30,123,170].

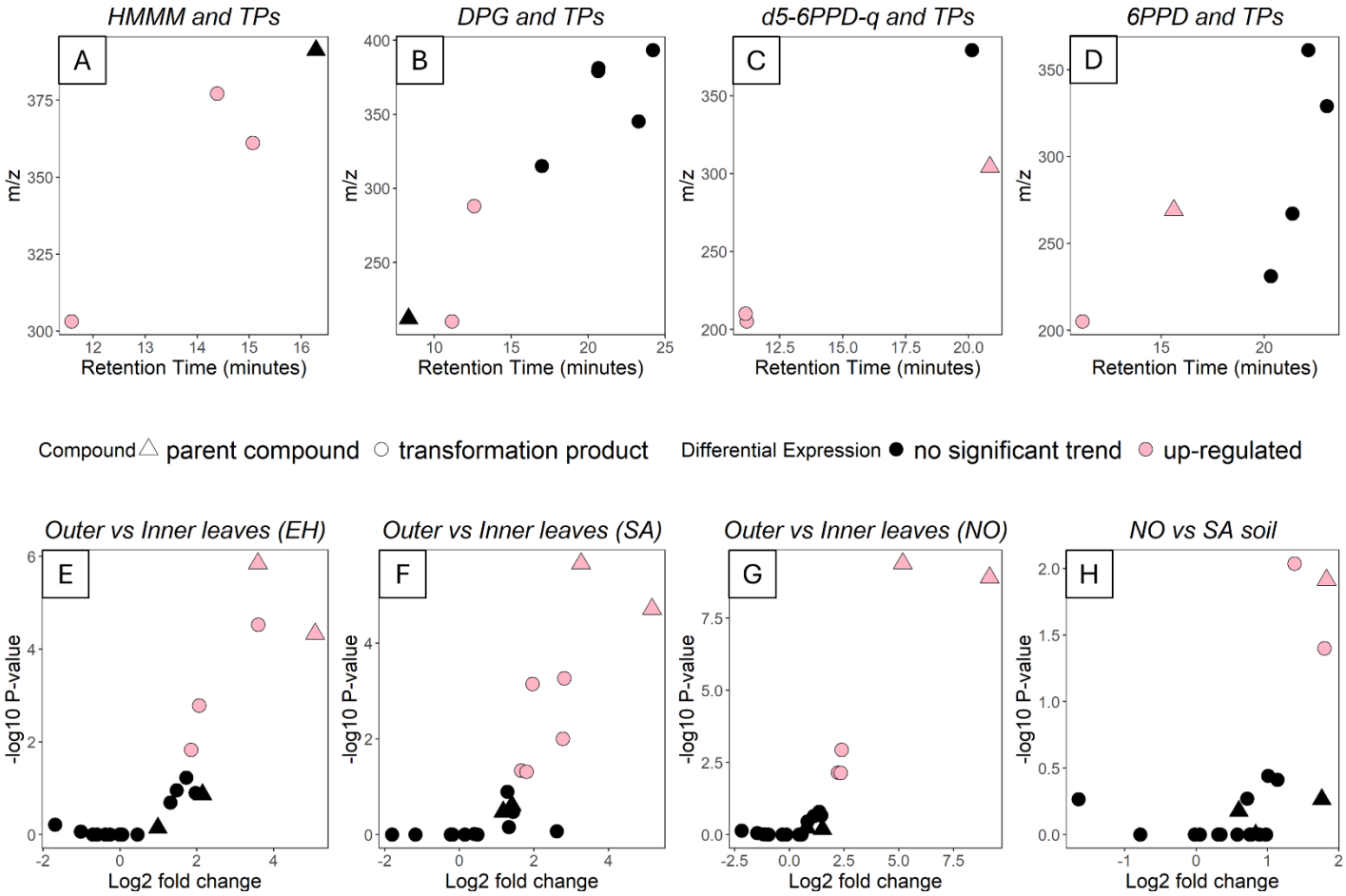


Figure 3.5 Potential transformation products and parents compounds mass to chare ratio (proxy for molecular size) versus retention time as determined by reverse-phase liquid chromatography (proxy for polarity) for HMMM (A), DPG (B), d5-6PPD-quinone (C), and 6PPD (D). Parent compounds are represented by triangles, and potential transformation products by circles. Pink color is used for compounds which were up-regulated in at least one differential analysis, and black color is used for compounds which showed no statistically significant trend in any of the differential analyses. Differential analyses were used to compare the outer leaves and inner leaves of plants grown in Ein Hashlosha soil (E), Sa'ad soil (F), Nir Oz soil (G), and to compare outer leaves of plants grown in the Nir Oz soil to plants grown in the Sa'ad soil (H). Pink color represents a statistically significant up-regulation at $\log_2$ fold change ≥ 1, p-value ≤ 0.05.

Differential analyses were used to visualize whether the transformation products were found in the same plants, and same parts of the plants as the parent compounds. Outer and inner leaves were compared for each given soil (Figure 3.5 E-G), and the outer leaves of plants grown in the Nir Oz soil were compared with the outer leaves of plants grown in the Sa'ad soil (Figure 3.5 H). At a $\log_2$ fold change of 1, and a p-value of 0.05, all transformation products either showed no statistically significant difference between sample types (black color in Figure 3.5), or were up-regulated (pink color in Figure 3.5) in the outer leaves and in the Nir Oz soil plants. The large number of potential transformation products which showed no statistically significant trend could be due to the limited statistical power of these analyses – the experiment was only conducted in triplicate, and many of the compounds were detected only at trace levels.

The compounds which were up-regulated in the outer leaves compared to the inner leaves, and in the Nir Oz soil compared to the Sa'ad soil were generally the smaller, more polar transformation products, which is shown by the concentration of pink points in the lower left corners of Figures 3.5 A-D. If these are indeed Phase I transformation products formed within the plants, it is unsurprising that they are up-regulated in the same samples where the majority of the parent compounds were found (outer leaves and Nir Oz soil plants). On the other hand, if these transformation products were already formed in the soil system, the similar trends in distribution suggest similar bioaccessibility and plant uptake trends to the parent compounds. Specifically, up-regulation in outer leaves suggests that a compound is predominantly taken up passively via the transpiration stream, and thereby accumulates in outer leaves which have a higher transpiration rate. Indeed, passive uptake in plants is greater for smaller compounds, which can more readily cross cell membranes[89]. Additionally, passive uptake is greatest for compounds of intermediate polarity, with a theoretical optimal log K_{ow} of 1.8[90]. DPG, d5-6PPD-quinone, and 6PPD all have log K_{OW} values above the theoretical optimum of 1.8 for translocation[90] (Table S3.2), so it is unsurprising that their more polar transformation products seem to be passively taken up via the transpiration stream. Up-regulation in Nir Oz soil plants as compared to Sa'ad soil plants implies that smaller and more polar transformation products may also be more bioaccessible in soil with a high sand and low clay content.

Larger, less polar transformation products (likely conjugates) generally showed no statistically significant trend in any of the differential analyses (concentration of black points in the top right of Figures 3.5 A-D). Since conjugates were almost certainly formed within the plants, it is surprising that they were not up-regulated in samples where the majority of the parent compounds were found, suggesting that these conjugates may undergo further fate processes within the plants. Conjugates can undergo "Phase III" metabolism, in which they are either deposited in vacuoles, or incorporated into cell

walls, forming bound, or non-extractable residues[98,171]. Phase III metabolites have been shown to represent up to 83-93% of organic compounds in plant leaves[166]. It is thus plausible that the lack of statistically significant trends in the distribution of large apolar transformation products is due to these compounds being transported to other parts of the plants or immobilized via Phase III metabolic processes.

Since Phase III metabolites are likely not extractable with our method, nor with any standard plant extraction methods[98,171], further study is needed to investigate their distribution in lettuce plants. The use of ^{14}C labelled compounds would enable the quantification of Phase III metabolites, as well as Phase I and Phase II transformation products which were not identified with our workflow[166]. ^{14}C labeling would also enable quantification of non-extractable residues in soils, and could provide insights into the type of non-extractable residues formed[85]. In general, mechanistic studies are needed to understand the sorptive or transformation processes which reduce the bioaccessibility of tire-wear derived compounds to plants. Such studies should also include a wider range of soil types, including soils with higher organic carbon contents.

Chapter 4: Uptake of tire-derived compounds in leafy vegetables and implications for human dietary exposure

-Detection Study-

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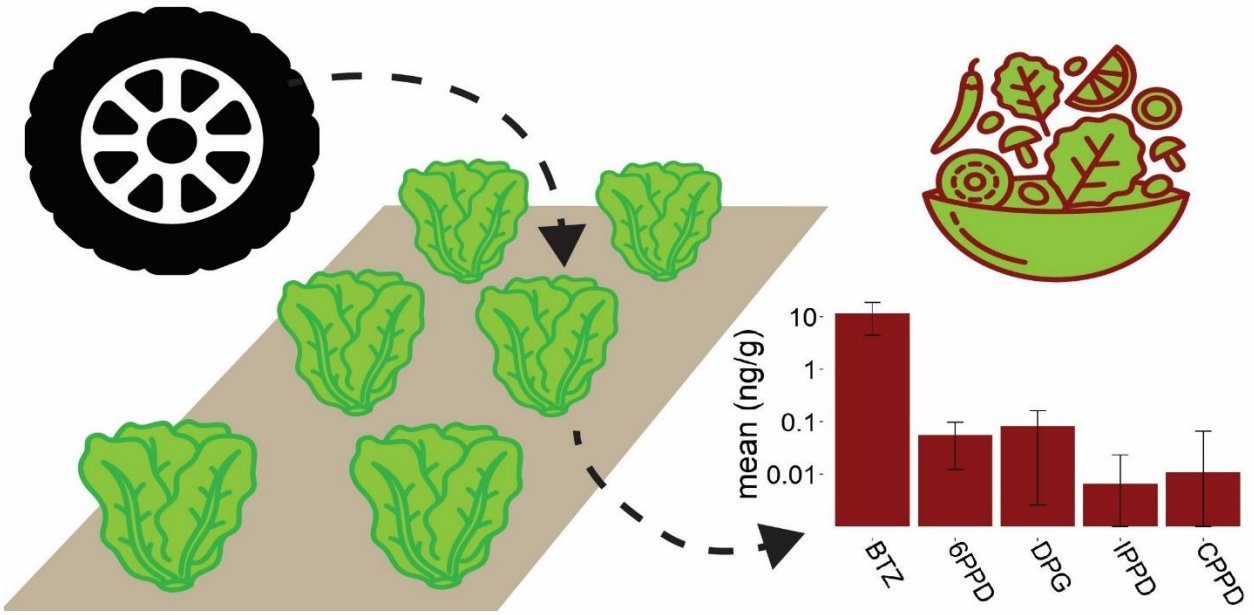

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4.1 Abstract

Introduction: Tire and road wear particles are one of the most abundant types of microplastic entering the environment. The toxicity of tire and road wear particles has been linked to their organic additives and associated transformation products. Tire and road wear particles, and associated tire-derived compounds are introduced to the agricultural environment via atmospheric deposition, irrigation with reclaimed wastewater, and the use of biosolids (treated sewage sludge) as fertilizer. In the agricultural environment, these tire-derived compounds could be taken up by edible plants, leading to human exposure.

Methods: Sixteen tire-derived compounds were measured in twenty-eight commercial leafy vegetable samples from four countries. Based on the results, the estimated daily intake of these tire-derived compounds was calculated due to leafy vegetable consumption based on local diets under a mean and maximum concentration scenario.

Results: In commercial leafy vegetables, six tire-derived compounds were detected: benzothiazole (maximum concentration – 238 ng/g dry weight), 2-hydroxybenzothiazole (maximum concentration – 665 ng/g dry weight), DPG (maximum concentration – 2.1 ng/g dry weight), 6PPD (maximum concentration – 0.4 ng/g dry weight), IPPD (maximum concentration – 0.1 ng/g dry weight), and CPPD (maximum concentration – 0.3 ng/g dry weight). At least one compound was present in 71% of samples analyzed. The estimated daily intake for DPG ranged from 0.05 ng/person/day in the mean scenario to 4.0 ng/person/day in the maximum scenario; benzothiazole ranged from 12 to 1296 ng/person/day; 6PPD ranged from 0.06 to 2.6 ng/person/day; IPPD ranged from 0.04 to 1.1 ng/person/day; CPPD ranged from 0.05 to 2.6 ng/person/day.

Discussion: Statistical analyses did not reveal correlation between known growth conditions and tire-derived compound concentrations in the leafy vegetable samples. The estimated daily intake via leafy vegetable consumption was generally lower than or comparable to the estimated daily intake via other known sources. However, we show that tire-derived compounds are taken up by foodstuff, and exposure might be higher for other produce. Future studies are needed to uncover pathways of tire-derived compounds from road to food, assess the exposure to transformation products, and investigate the biological effects associated with this exposure.

4.2 Introduction

Abrasion of tires at the road surface generates tire and road wear particles, which represent a major flux of microplastics into the environment. Estimates of tire and road wear particle emissions range widely, from 0.9 to 2.5 kg/capita/year, which corresponds to between 24% and 94% of total microplastic emissions [7], emphasizing that tire and road wear particle pollution is highly relevant, but still poorly understood. The amount of tire and road wear particles is expected to increase in future years due to the continuous increase in the number of cars on the road. Upon generation at the road surface, 0.1 – 10% of tire and road wear particles become airborne [172], and can potentially be transported over long distances [10]. The largest fraction of tire and road wear particles (90 - 99.9%) accumulates at the road surface and is flushed during road-wash events into roadside soils, receiving water bodies, or sewer systems. Many cities have combined sewer and runoff systems, in which road-wash is collected along with sewage in wastewater treatment systems. Thus, it is expected that biosolids (treated sewage sludge) are contaminated with tire and road wear particles. Biosolids are used in many countries for fertilization, and an estimated 1400 – 2800 tons per year of tire wear particles are deposited on agricultural fields in Germany via this route [25]. Treated wastewater effluent is also used in many countries for irrigation, with the unintended risk of introducing pollutants to agricultural fields [77,173,174].

One of the main concerns associated with tire and road wear particles is their high chemical additive content. Tire and road wear particles have been estimated to contain 5-10% organic and inorganic additives by weight, added among other functions, as processing aids, antioxidants, and plasticizers [6]. These additives and their transformation products, i.e., tire-derived compounds, are not chemically bound within the rubber matrix, and many leach into the environment. Some of the main chemical additives are vulcanization accelerators including benzothiazoles, and several guanidine derivatives such as 1,3-diphenylguanidine (DPG), which comprise 0.5% of tire mass each [175], corrosion inhibiting benzotriazoles, the crosslinking agent hexa(methoxymethyl)melamine (HMMM), and p-phenylenediamine compounds (PPDs), which are used as antiozonants, and represent 0.8% of tire mass [175]. These tire-derived compounds are ubiquitously detected in the environment. Benzothiazole is detected in surface water up to 2500 µg/L, wastewater up to 49.3 µg/L, sludge up to 50.2 µg/kg, and air up to 32 µg/m^3 [28]. DPG has recently been detected in wastewater effluent at concentrations up to 150 ng/L and surface waters up to 1.9 µg/L [26]. HMMM has been detected in wastewater treatment plants at concentrations up to 60.8 µg/L [27], as well as rivers at concentrations up to 1.6 µg/L [29]. PPDs and PPD-quinones have been detected in air, water, and soils at sum concentrations of 11.0 pg/m^3, 2.3 µg/L, and 776 ng/g, respectively [17]. Both benzothiazoles and DPG have been associated with toxic effects of tire and road wear particles to fish, albeit above environmentally relevant concentrations [36]. Quinone derivatives of PPDs (PPD-quinones)

have been shown to be highly toxic to some aquatic species at environmental concentrations, with the most notable example being 6PPD-quinone [176].

There are multiple pathways by which tire-derived compounds can reach the agricultural environment, but exact pathways and mass fluxes are unknown. The airborne fraction of tire and road wear particles can deposit onto agricultural soils [151], especially where agriculture and major roadways are in close proximity. Reclaimed wastewater containing tire-derived compounds may be applied to agricultural fields for irrigation, mainly in areas of water scarcity. For example, reclaimed wastewater comprises more than 50% of Israel's agricultural water supply [148]. Irrigation with reclaimed wastewater is prohibited in Switzerland [177], but practiced in several other countries in Europe, particularly in the Mediterranean region. According to a 2008 report, Spain uses 821,920 m^3/day and Italy uses 123,288 m^3/day [149] of reclaimed wastewater. The use of reclaimed wastewater for irrigation is expected to increase globally over the next years, especially in Europe due to new EU regulation [150]. Based on the high density of tire and road wear particles (1.5 – 2.2 g/cm^3) relative to water, and reported retention of other types of microplastic particles in wastewater treatment plants, it is assumed that tire and road wear particles are retained in sludge during wastewater treatment [25]. After further treatment, sludge may be used as an alternative fertilizer. Although the presence of contaminants in sludge has led to a ban of its use in agriculture in Switzerland [178], each year 755 kilotons dry mass of sludge is applied to land as biosolids in Spain, and 316 kilotons dry mass in Italy [179]. This practice is expected to increase globally due to both the energy demands associated with bioavailable nitrogen production, and global phosphorous scarcity.

Plant uptake of contaminants of emerging concern is well documented in the literature [75,77,180]. Hydroponic studies have demonstrated plant uptake and translocation from roots to leaves of the tire-derived compounds HMMM (maximum concentration 18.0 µg/g), 6PPD (maximum concentration 0.75 µg/g), 6PPD-quinone (maximum concentration 2.19 µg/g), DPG (maximum concentration 2.29 µg/g), benzothiazole (maximum concentration 1.24 µg/g) [153], as well as 2-mercaptobenzothiazole (maximum concentration 1.12 µg/g) [155]. However these experiments were conducted under controlled conditions and at higher concentrations than are expected to occur in the environment. Uptake by plants grown in soils is more complex, involving chemical, physical and biological processes affecting the availability of a compound to the plant. Strong sorption to soil has been demonstrated for several tire-derived compounds, with HMMM displaying a Freundlich sorption coefficient (log K_f) up to 1.19 and DPG up to 2.88 [156], suggesting that plant uptake of tire-derived compounds may be lower in the soil environment as compared to hydroponic studies. The potential for enhanced solubilization due to the presence of plant root exudates, as well as biotic and abiotic transformations of tire-derived compounds in soil further complicate the extrapolation of results obtained in hydroponic studies to the agricultural environment.

The increasing body of research demonstrating biological effects of tire-derived compounds [31,36,176], as well as their recent detection in human urine [55,57] warrant a thorough human exposure assessment. Human exposure to tire-derived compounds via inhalation, dust ingestion, and drinking water have been demonstrated [17,21,45,48], however, the concentration of tire-derived compounds in commercial produce and the exposure via dietary intake is unknown. Leaves have been demonstrated to accumulate more organic contaminants than other plant organs [75,76,169], so we hypothesized that leafy vegetables may be contaminated with tire-derived compounds and contribute to the overall human exposure. Due to differences in reclaimed wastewater and biosolids use in different regions of the world, we hypothesized that tire-derived compound contamination of leafy vegetables would depend on national agriculture policy. Therefore, in this study we screened sixteen tire-derived compounds in twenty-eight leafy vegetable samples grown in four countries (Israel, Switzerland, Italy, Spain) in order to estimate human exposure via dietary intake.

4.3 Materials and Methods

4.3.1 Sample Collection and Processing

Two sets of leafy vegetable samples, collected from Switzerland and Israel, were analyzed in this study. In total, fifteen leafy vegetables (grown in Switzerland, Italy, or Spain) were purchased in triplicate in grocery stores in Switzerland as part of an investigation by the Swiss consumer magazine K-TIPP between February and April 2023. Information regarding use of reclaimed wastewater or biosolids was unavailable for these samples. The leaves were transported frozen to our laboratory, where they were lyophilized. The dry leaves were then homogenized using a blender to obtain representative samples of the entire biomass. A total of thirteen leafy vegetable samples were collected in Israel during 2017, each representing a different commercial field. All samples from Israel were irrigated with reclaimed wastewater. About 10-15 plants were collected from each field and mixed in the lab to form a composite sample representative of each field. Details regarding the sampling procedure and locations are provided in Ben Mordechay et al 2021 [76]. The samples were lyophilized and stored frozen (-20°C). The dry samples were shipped frozen to Austria (April 2023) for analysis. Details about individual samples, including the wastewater treatment plant where the reclaimed wastewater was produced are provided in Table S4.1.

Samples from both sample sets were extracted between March and May 2023. Three sub-samples of 1.5 g were added to 50-mL polypropylene centrifuge tubes (Nunc). Isotopically labelled internal standards (0.67 µg of benzothiazole-d4 and 6PPD-quinone-d5) were added to each of the sub-samples. To each tube, 4 g stainless steel beads and 30 mL acetonitrile (LCMS grade, Analytics Shop) were added. Samples were homogenized via bead beating for 3 min (3000 strokes per min) and centrifuged (17000 *g*, 20 min,

and 20°C). Twenty mL of supernatant was extracted to a separate glass vial. Then, 4 mL of acetonitrile was re-added to the tube containing the biomass, and extraction was repeated twice following the same procedure, for a total extracted volume of 28 mL per sub-sample. All sub-samples (28 mL) were combined, then filtered (0.2 µm nylon filters) using vacuum filtration. Filtered extract was then pre-concentrated to 5mL using rotary evaporation (226 mBar, 60 °C). Due to precipitation, concentrated samples were re-filtered through 0.2 µm nylon filters before analysis.

4.3.2 Analysis

Samples were analyzed using ultra performance liquid chromatography coupled with triple quadrupole mass spectrometry (Agilent 6470), operated in multiple reaction monitoring (MRM) mode. The following compounds were analyzed: benzothiazole (BTZ), 2-hydroxybenzothiazole (20H-BTZ), 2-aminobenzothiazole (2amino-BTZ), 2-mercaptobenzothiazole (2SH-BTZ), 5-methyl-1H-benzotriazole (5M-1H-BTR), aniline, 1,3-diphenylguanidine (DPG), hexa(methoxymethyl)melamine (HMMM), and the phenylenediamine compounds: 6PPD, IPPD, CPPD, DPPD and their associated quinones: 6PPDq, IPPDq, CPPDq, DPPDq. Molecular structures and physicochemical parameters of all tire-derived compounds are provided in Table S4.2. Chromatographic and MRM parameters are provided in Table S4.3. Raw data were processed using Agilent Quantitative Analysis software, and detailed peak picking settings are provided in Section S4.1.

Samples were additionally analysed using Orbitrap high resolution mass spectrometry (Thermo Scientific Q Exactive). Measurement details are provided in Section S4.2. A suspect screening was applied to search for previously reported tire-derived compounds and transformation products for which we do not have standards [27,30,36,96,109,116,123,133,170,181]. Features with an exact mass match to a suspect of Δppm < 5ppm were considered potential transformation products, and the MS2 spectra were manually checked for fragments matching those reported in the literature, those of the respective parent compound, or a good FISH (in-silico fragmentation) annotation, in the case where a structure is known or has been proposed. In addition to the suspect screening, we attempted to apply two non-target data analysis methods to search for transformation products. The first approach was using the Expected Compounds feature of Compound Discoverer software, which applies known Phase I and Phase II transformations in-silico to a list of compounds. In this case, we applied common Phase I and Phase II transformations which have been previously reported to occur in plants [96,109,116,182] to our sixteen target tire-derived compounds, and then screened acquired data for exact mass matches to these expected compounds. The second approach was to use the Molecular Networking feature of Compound Discoverer, which links compounds based on shared molecular fragments from MS2 spectra. Further details of suspect and non-target analyses are provided in Section S4.3.

4.3.3 Quality assurance and quality control

Matrix-matched calibrations were prepared by extracting reference material (i.e., samples containing no tire-derived compounds) and spiking it to twelve nominal concentrations from 0.11 to 556 ng/g. Analytical blanks were measured after every six samples. Limit of quantification was determined as the lowest concentration within the linear range of the calibration, or the mean plus three times the standard deviation of all analytical blanks. Six method blanks (no plant material) were extracted along with samples. Three compounds (DPG, 2-hydroxybenzothiazole, and benzothiazole) were detected sporadically in method blanks – for these compounds, the mean concentration measured in method blanks was subtracted from the concentrations measured in samples. Recovery tests were performed by spiking dry reference material at nominal concentrations of 1, 10, and 100 ng/g, and extracting as described above. For all tire-derived compounds, limit of quantification (ng/g) and recovery (%) are presented in Table S4.4.

All samples were measured in duplicate, and measurement order was randomized to eliminate bias from instrument carryover. Concentrations in measurement duplicates were averaged. In the case that one duplicate was below the limit of quantification, its value was set to 0. It is important to note that this is a conservative approach to data treatment, and actual concentrations may be slightly higher than what we report. Samples for which one duplicate was below the limit of quantification, or for which variance between measurement duplicates exceeded 30% were annotated (Figure 4.1).

4.3.4 Calculations and Statistical Analyses

All data were analyzed in R (Version 4.3.1). Divisive hierarchical clustering analysis (HCA) was applied to all samples. Compound concentrations were first centred and scaled, with concentrations below limit of quantification set to half of the limit of quantification. Dissimilarities between samples were calculated using the Manhattan algorithm (sum of absolute differences) from the cluster R package, and dendrograms were visualized using the factoextra R package. To evaluate individual relationships between specific variables and tire-derived compound contamination, samples were grouped by country of growth, leafy vegetable type, wastewater treatment plant of irrigation water origin, compost application, and growth season. For the last three variables, information was only available for samples from Israel, thus other samples were excluded from these two analyses. Analysis of Variance (ANOVA) was then performed to check for statistically significant variation in either cumulative tire-derived compound concentration or number of tire-derived compounds detected between the different groups. Exposure was estimated for the adult Israeli population based concentrations in samples from Israel, and consumption data from the 2nd Israeli National Health and Nutrition Survey [183]. Exposure to tire-derived

compounds in leafy vegetables was compared with exposure to pharmaceuticals from the same leafy vegetable samples, which was assessed in Ben Mordechay et al. 2022 [183]. Exposure was estimated for the adult Swiss population based on concentrations in samples purchased in Switzerland. Swiss leafy vegetable consumption data was not readily available, but the EFSA Comprehensive European Food Consumption Database [184] provided consumption data for neighbouring countries Austria, France, and Italy, so Swiss consumption was based on a weighted average of those values. Exposure was calculated with the following equation: $EDI = C \times D$, where C represents the concentration of a given compound in leafy vegetable samples (ng/g wet weight), D represents the mean daily dose of leafy vegetables in each adult population (g consumed/person/day). The mean scenario was calculated using the mean concentration and mean leafy vegetable consumption, while the maximum scenario was calculated using the maximum concentration and 95th percentile leafy vegetable consumption.

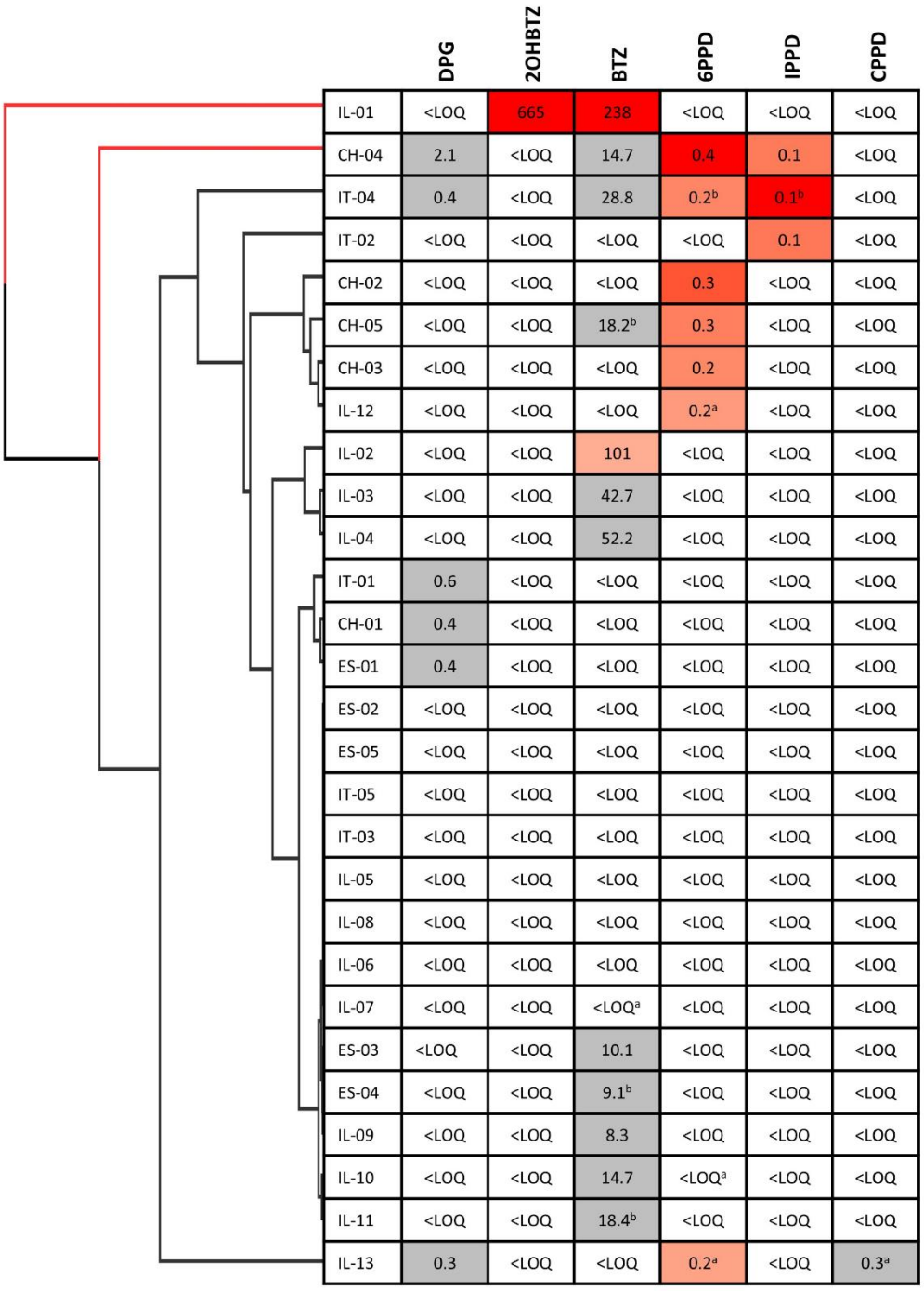
4.4 Results

Out of the sixteen compounds analyzed, six were detected in at least one leafy vegetable sample: benzothiazole (maximum concentration – 238 ng/g), 2-hydroxybenzothiazole (maximum concentration – 665 ng/g), DPG (maximum concentration – 2.1 ng/g), 6PPD (maximum concentration – 0.4 ng/g), IPPD (maximum concentration – 0.1 ng/g), CPPD (maximum concentration – 0.3 ng/g). The number of tire-derived compounds per sample ranged between 0 (*n*=8 samples) and four (*n*=2 samples). At least one tire-derived compound was detected above limit of quantification (LOQ) in 71% of the samples. All measured concentrations are presented in Figure 4.1.

Out of the sixteen tire-derived compounds analyzed, benzothiazole was the most frequently detected (detection frequency of 42.9%). 2-aminobenzothiazole, 2-mercaptobenzothiazole, and 2-hydroxybenzothiazole were not detected (with the exception of 2-hydroxybenzothiazole in CH-04). The only benzotriazole derivative analyzed, 5-methyl-1H-benzotriazole, was not detected. DPG was detected in 21.4% of samples. The detection frequencies of 6PPD, IPPD, and CPPD were 25%, 10.7%, and 3.6%, respectively. DPPD was not detected, nor were the respective quinone derivatives. HMMM and aniline were also not detected.

Divisive hierarchical clustering analysis was performed to identify groups of samples with similar patterns in tire-derived compound concentrations. Most samples clustered together, with the exception of two samples: one lettuce grown in Switzerland (CH-04) contained the highest measured concentrations of DPG (2.1 ng/g dry weight) and 6PPD (0.4 ng/g dry weight), and lettuce grown in Israel (IL-01) contained the highest measured benzothiazole concentration in any sample (238 ng/g dry weight), and the only detection of 2-hydroxybenzothiazole (665 ng/g dry weight), which was also the highest measured

concentration of any compound. Plastic factories were located next to lettuce growth sites or distribution centers for both samples, but our data did not allow us to prove that they were a source of emissions. In addition, sample CH-04 was next to a major highway, potentially explaining the high concentrations (further discussion on these two samples is provided in Section S4.4). For these reasons, the two samples were classified as outliers and excluded from further analyses. Analysis of Variance (ANOVA) did not reveal any statistically significant ($p<0.05$) relationships between tire-derived compound contamination in leafy vegetables and growth country, crop type, recycled wastewater source, compost application, or growth season (Table S4.5).



	DPG	2OHBTZ	BTZ	6PPD	IPPD	CPPD
IL-01	<LOQ	665	238	<LOQ	<LOQ	<LOQ
CH-04	2.1	<LOQ	14.7	0.4	0.1	<LOQ
IT-04	0.4	<LOQ	28.8	0.2[b]	0.1[b]	<LOQ
IT-02	<LOQ	<LOQ	<LOQ	<LOQ	0.1	<LOQ
CH-02	<LOQ	<LOQ	<LOQ	0.3	<LOQ	<LOQ
CH-05	<LOQ	<LOQ	18.2[b]	0.3	<LOQ	<LOQ
CH-03	<LOQ	<LOQ	<LOQ	0.2	<LOQ	<LOQ
IL-12	<LOQ	<LOQ	<LOQ	0.2[a]	<LOQ	<LOQ
IL-02	<LOQ	<LOQ	101	<LOQ	<LOQ	<LOQ
IL-03	<LOQ	<LOQ	42.7	<LOQ	<LOQ	<LOQ
IL-04	<LOQ	<LOQ	52.2	<LOQ	<LOQ	<LOQ
IT-01	0.6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CH-01	0.4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ES-01	0.4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ES-02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
ES-05	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IT-05	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IT-03	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IL-05	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IL-08	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IL-06	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IL-07	<LOQ	<LOQ	<LOQ[a]	<LOQ	<LOQ	<LOQ
ES-03	<LOQ	<LOQ	10.1	<LOQ	<LOQ	<LOQ
ES-04	<LOQ	<LOQ	9.1[b]	<LOQ	<LOQ	<LOQ
IL-09	<LOQ	<LOQ	8.3	<LOQ	<LOQ	<LOQ
IL-10	<LOQ	<LOQ	14.7	<LOQ[a]	<LOQ	<LOQ
IL-11	<LOQ	<LOQ	18.4[b]	<LOQ	<LOQ	<LOQ
IL-13	0.3	<LOQ	<LOQ	0.2[a]	<LOQ	0.3[a]

Figure 4.1 Concentrations of tire-derived compounds in twenty-eight leafy vegetable samples. Samples are grouped by hierarchical clustering analysis, shown with the dendrogram left of the table. All units are ng/g dry weight. Superscript a indicates that one measurement duplicate was below limit of quantification. Superscript b indicates that variance between measurement duplicates was >30%. Gray color indicates trace detections of tire-derived compounds (detections in a concentration range associated with >40% error, Table S4.4)

With a suspect screening, we tentatively identified two tire-derived compound transformation products which have been previously reported. HMMM TP546 was identified in hydroponically grown lettuce plants exposed to HMMM[153], and was proposed to be an amino acid conjugate. Here, HMMM TP546 was detected with a maximum signal in samples CH-05 and ES-02. 6PPD TP194 is a known transformation product of 6PPD which has previously been reported in snow and wastewater treatment plant influent [123], and was detected with a maximum signal in sample IL-12. According to Schymanski et al 2014 [128], both compounds were assigned a confidence level of 3, "tentative candidate", since their exact mass and several MS^2 fragments correspond to proposed compounds, but MS^2 spectral matching was not thorough enough to merit higher confidence (details Section S4.5). We were unable to identify any further transformation products with non-target analyses, so exposure was assessed based on only the sixteen target tire-derived compounds.

Estimated daily intake (EDI) via leafy vegetable consumption of the five detected tire-derived compounds for the Israeli and Swiss adult populations are presented in Table 4.1. Based on mean concentrations measured, and mean leafy vegetable consumption, $EDI_{leafy\ vegetable}$ ranged from 0.04 ng/person/day of IPPD to 52 ng/person/day of benzothiazole. Under the maximum concentration scenario, $EDI_{leafy\ vegetable}$ was between one and two orders of magnitude higher for all compounds, and ranged from 1.1 ng/person/day of IPPD to 1296 ng/person/day of benzothiazole.

Table 4.1 Leafy vegetable consumption (g/person/day) and estimated daily intake (ng/person/day) via consumption of leafy vegetables of six tire-derived compounds for adult national populations. Both the mean scenario and maximum scenario are shown, (mean / maximum).

	Leafy vegetable consumption	DPG	BTZ	6PPD	IPPD	CPPD
Israel	22 / 104	0.05 / 2.9	52 / 1296	0.06 / 1.9	<LOQ	0.05 / 2.6
Swiss	43 / 127	0.3 / 4.0	12 / 313	0.2 / 2.6	0.04/ 1.1	<LOQ

4.5 Discussion

The uptake of pharmaceuticals from reclaimed wastewater into edible crops has been well established with both mechanistic [78,79,89,114,185–187] and monitoring studies [75,76]. Several laboratory studies have demonstrated that other classes of compounds, including industrial compounds [130,188,189], plasticizers [190,191], and even tire-derived compounds [112,153,155] can be taken up by plants. However, the occurrence of tire-derived compounds in consumer produce has not previously been established. Here, we report

concentrations of sixteen tire-derived compounds in commercial leafy vegetable samples grown in four countries (Switzerland, Italy, Spain, and Israel). The samples collected in Israel have been previously analyzed for 65 pharmaceuticals [76], enabling a direct comparison of detection frequency and concentration between tire-derived compounds and pharmaceuticals (Table S4.6). Generally, the samples with the highest pharmaceutical concentrations detected also had the highest tire-derived compound concentrations. Concentrations of individual compounds in the Israeli sample set ranged greatly, both for tire-derived compounds (not detected to 665 ng/g) and pharmaceuticals (not detected to 1470 ng/g).

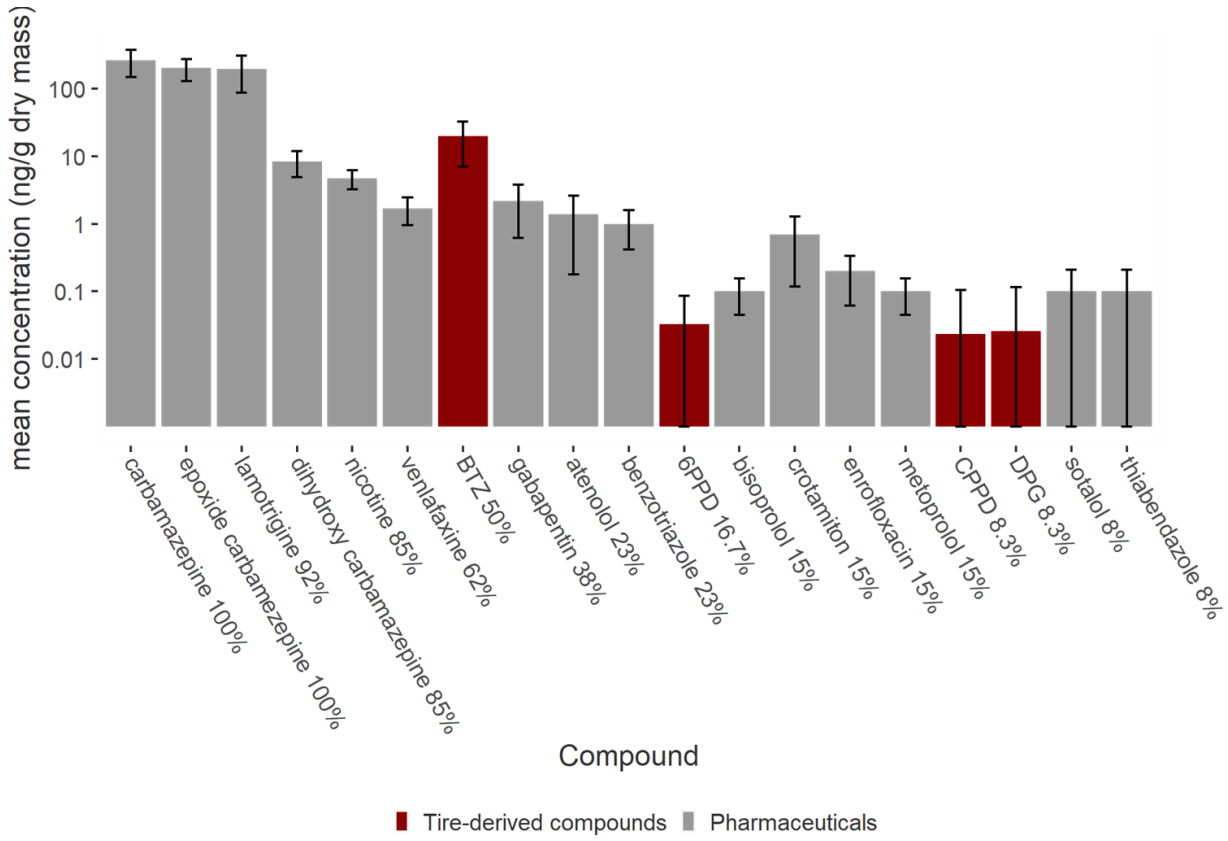


Figure 4.2 Mean concentrations of pharmaceuticals (gray) and tire-derived compounds (red) measured in leafy vegetable samples from Israel. Standard errors are shown with error bars. Detection frequencies are shown along with the compound names.

Detection frequencies also varied greatly among both tire-derived compounds in the sample set from Israel (0% to 38.5%) and pharmaceuticals (0% to 100%). This variability is unsurprising since the detection frequencies of both tire-derived compounds and pharmaceuticals in wastewater and surface water bodies also varies highly from compound to compound, due to differences in emissions and

environmental stability [11,32,133,192–194]. In the agricultural environment, soil sorption, microbial transformation, extent of plant uptake and translocation, and plant metabolism vary greatly for compounds with different chemical structures [77–79,140,154,158,167,187]. Benzothiazole was the tire-derived compound with the highest detection frequency of 50% in the samples from Israel, and 42.9% in all samples (Figure 4.2). Six pharmaceuticals exhibited even greater detection frequencies in leafy vegetables: carbamazepine (100%) and two of its metabolites, epoxide carbamazepine (100%), dihydroxy carbamazepine (85%), lamotrigine (92%), nicotine (85%), and venlafaxine (62%). These compounds were also detected at high frequency in rivers due to their consistent use and emission [194]. While pharmaceuticals are generally used consistently throughout the year, tire-derived compounds enter wastewater mainly during runoff events, and thus exhibit lower detection frequencies in the environment [11,32]. Despite inconsistent detections for individual tire-derived compounds, 69% of the samples from Israel contained at least one tire-derived compound, compared with 100% of the same samples which contained at least one pharmaceutical [76].

Detection frequencies of individual tire-derived compounds could be interpreted based on their physicochemical properties (Table S4.2) and known environmental behavior. It is known that benzothiazole [153], as well as 2-mercaptobenzothiazole [155] can be readily taken up by plants. Unsubstituted benzothiazole was frequently detected (42.9% of samples), while 2-mercaptobenzothiazole, 2-hydroxybenzothaizole, and 2-aminobenzothiazole were not detected. Higher limits of quantification could contribute to the lack of detections for two compounds (75.6 and 55.6 ng/g for 2-mercaptobenzothiazole and 2-hydroxybenzothiazole, respectively), but 2-aminobenzothiazole had a very low limit of quantification (1.1 ng/g). Similarly, 5methyl-1H-benzotriazole was not detected (limit of quantification 2.8 ng/g), although unsubstituted benzotriazole was detected in 23% of the same leafy vegetable samples when they were previously analyzed [76]. In addition to analytical bias, the differences in detection frequencies could be related to differences in occurrence and concentrations of the compounds in the agricultural environment, differences in physicochemical properties affecting plant uptake, or differences in plant metabolism. Benzothiazoles and benzotriazoles are widely occurring compounds- benzothiazoles are also used as biocides and food flavorings, and benzothiazole has even been reported to occur naturally in products such as tea leaves [195]. In fact, benzothiazole has been detected at similar concentrations to those we report in several food products [28,196], so the high detection frequency of benzothiazole compared to the other tire-derived compounds analyzed could simply be a result of its environmental ubiquity.

Soil sorption can reduce the availability of a compound for plant uptake. All studied benzothiazoles and 5methyl-1H-benzotriazole are neutral in the pH range of most soils and are relatively hydrophilic (log K_{OW} of 1.13 to 2.35, Table S4.2), implying low sorption and high bioavailability to plants. 5methyl-1H-

benzotriazole is known to exhibit low soil sorption [197]. The log K_{OW} values of benzothiazoles and benzotriazole are also within the optimal range for plant uptake and translocation [89,90]. While differential availability for plant uptake may not explain differences in detection frequencies between benzothiazoles and benzotriazoles, the location and functional group properties of benzimidazole and benzotriazole substitutions may alter the extent of plant uptake and metabolism [154]. This study showed that Aridopsis plants rapidly and completely depleted unsubstituted benzotriazole from hydroponic medium, while 2-aminobenzotriazole was not depleted, suggesting that substitutions can substantially reduce the uptake of benzotriazoles [154] and could explain the difference in detection frequency between benzotriazole and 5methyl-1H-benzotriazole. Furthermore, plant metabolism can occur rapidly, and could contribute to lack of detections. Hydroxy-, primary amino-, and mercapto- groups can be sites of direct conjugation in plants [130,198], leading to fast transformation kinetics, which could explain the absence of 2-mercaptobenzothiazole, 2-hydroxybenzothiazole and 2-aminobenzothiazole, while benzothiazole was frequently detected. In a comparative metabolomics experiment, after 24 hours, a substantial proportion of unsubstituted benzimidazole remained in Arabidopsis plants, while substituted benzimidazoles were further metabolized [154]. This pattern likely holds true for benzothiazoles, which share a very similar base structure with benzimidazoles. Although benzothiazole was rapidly metabolized in lettuce plants in a hydroponic experiment [153], it could be more recalcitrant compared to its substituted derivatives in plants grown on the field.

The detection frequencies of the four PPDs analyzed corresponds to usage of the individual compounds. 6PPD (25%), is the most used PPD, followed by IPPD (10.7%), and then the much less used CPPD (3.6 %) and DPPD (not detected)[199]. All four PPDs have detection frequencies > 50% in air, soil, and runoff water, with 6PPD dominating the concentration profile in all sample types [17,21]. Although 6PPD is known to be unstable in aqueous systems [123], it was detected in 25% of leafy vegetable samples suggesting that it may be more stable in terrestrial environments and/or within plants. On the other hand, it could also suggest the presence of tire and road wear particles on the field, which can act as a long-term source of 6PPD to plants, replenishing losses of 6PPD [153]. In general, PPDs were present at very low concentrations (<1 ng/g). PPD-quinones were not detected in any samples, likely due to slightly higher limits of quantification (1.1 – 2.8 ng/g) than their respective PPDs (0.05 – 0.6 ng/g). PPD-quinones have been reported to occur in air, soil, and runoff water at similar or even higher concentrations to their parent PPDs [17,21], however their concentrations in the agricultural environment are unknown. PPDs are relatively hydrophobic (log K_{OW} of 3.28 to 4.93 [17,200]). PPD-quinones are also hydrophobic (log K_{OW} of 2.58 to 4.30[17,200]), and could exhibit specific binding at the quinone moiety. This results in a high sorption potential for 6PPD-quinone [200,201], the same is presumably true for other PPD-quinones. Thus it is likely that PPDs and PPD-quinones in the soil environment would sorb to soil organic matter, which could limit

the availability for plant uptake [78,79]. Hydrophobic compounds are also known to accumulate in plant roots, as has been previously shown for 6PPD and 6PPD-quinone [153], so future work should investigate the occurrence of these tire-derived compounds in root vegetables, such as carrots or potatoes.

HMMM was not detected in any samples, although it was shown to have high uptake into lettuce plants in a hydroponic study [153], and had a low limit of quantification of 1.1 ng/g. This could imply that HMMM was not present in the agricultural environment to begin with, or that it was not available for plant uptake due to sorption, or transformation in soil, or that it was rapidly transformed in the plant. Irreversible loss of HMMM was demonstrated in multiple soils [156], although the mechanism is not currently known. Likewise, DPG was taken up by lettuce under hydroponic conditions [153], but demonstrated high sorption in multiple soils due to its positive charge [156], implying a low availability of DPG for plant uptake. In this study, DPG was detected in 21.4% of samples. Aniline was not detected, which could be an artifact of a higher limit of quantification (27.8 ng/g), low occurrence of aniline in the agricultural environment, or plant metabolism [202].

This study was designed to provide a first overview of tire-derived compound levels in leafy vegetables, not to determine the source of the tire-derived compounds. However, we had assumed that tire-derived compounds are introduced to the agricultural environment predominantly through irrigation with reclaimed wastewater or application of biosolids as fertilizer. We hypothesized that leafy vegetables grown in countries where both practices are prohibited (i.e. Switzerland) would have much lower levels of tire-derived compounds than leafy vegetables from Israel, where all samples were irrigated with reclaimed wastewater. Contrary to our hypothesis, we did not observe any statistical relationships between country of origin, and tire-derived compound levels in the leafy vegetables. Differences in reclaimed wastewater quality originating from different wastewater treatment plants had a large influence on concentrations of pharmaceuticals in edible crops [76,169] and we had hypothesized that the same would be true for tire-derived compounds, but this trend was not observed (Table S4.5). We also did not observe a statistically significant relationship between tire-derived compound levels and the season in which samples from the Israeli sample set were grown, although we had hypothesized that distinct rainy and dry seasons in Israel would lead to different levels of tire-derived compounds entering the agricultural environment. These observations could be due to our small sample size. Additionally, the flux of tire-derived compounds into wastewater treatment plants is associated with road-wash events, which occur irregularly as compared to the relatively constant emissions of pharmaceuticals. Irregular emissions of tire-derived compounds make it hard to compare reclaimed wastewater quality between different wastewater treatment plants, while for pharmaceuticals a clear trend was observed [76,169]. Overall, the lack of relationship between known growth conditions and tire-derived compound levels in leafy vegetables

(Table S4.5) could imply that there are multiple pathways by which tire-derived compounds reach the agricultural environment, and future studies are needed to uncover these pathways in order to guide agriculture policy. We did observe much higher contamination of two samples grown near plastic factories. Although these data are too limited to draw conclusions, future research should investigate whether plastic factories can act as point sources of tire-derived compounds to the agricultural environment.

Other than two tentatively (confidence level 3) identified transformation products, we were unable to identify transformation products in leafy vegetable samples. This is relatively unsurprising - since tire-derived compounds were generally present in leafy vegetable samples at trace levels, it can be expected that their transformation products were also present at very low concentrations. It is a major analytical challenge to prioritize and identify unknown compounds at such low concentrations with non-target mass spectrometry, particularly when the compounds are present in a complex matrix, such as plant extracts. Often, statistical techniques can be employed to overcome this challenge. For example, ratios of signal between exposed plants and controls (non-exposed plants) are typically used to prioritize potential transformation products. In the case of time-series experiments, trends in signal over time can also be used to identify potential transformation products. However, a survey study is ill-suited for such analyses, since there are neither time series data, nor true controls (leafy vegetable samples which definitely do not contain any tire-derived compounds or transformation products). Our expected compounds analysis screened all samples for the exact masses of products resulting from sixteen tire-derived compounds undergoing all possible permutations of nine Phase I and thirty-two Phase II transformations – this search identified 1195 potential matches. Without a way to statistically prioritize these potential transformation products, the results of this analysis could not be used.

On the other hand, molecular networking is a more specific approach, since it groups known compounds (tire-derived compounds) with unknown compounds (transformation products) based on similarities in MS2 fragmentation spectra, which imply structural similarities. However, in non-target high resolution mass spectrometry using data-dependent acquisition, MS2 spectra are only collected for compounds exhibiting the highest signals at a given retention time. In this case, it is likely that tire-derived compound transformation products did not fulfill this criterium. Thus, using molecular networks analysis, we did not identify any unknown compounds with meaningful ($m/z > 60$, minimum 2 related fragments) spectral similarities. This is unsurprising, since endogenous plant molecules are likely to be much more abundant than any tire-derived compound transformation products in leafy vegetable extracts, and thus, data acquired in data-dependent MS2 acquisition mode was poorly suited for molecular network analysis.

These results do not mean that transformation products are not present in consumer produce, or not relevant. On the contrary, it is well established that target monitoring of organic contaminants, including tire-derived compounds, often under-estimates exposure, since a large fraction of organic contaminants in plants are present as transformation products [109,112,116,129,153]. To fill this data gap, mechanistic plant metabolomic studies are needed to identify in-plant transformation products of tire-derived compounds. These studies should be followed up with suspect screenings of identified products in real consumer produce to thoroughly assess exposure to tire-derived compounds via produce consumption.

The above discussion highlights the many open questions regarding the sources and fate processes of tire-derived compounds in the agricultural environment. Future research is needed to fully understand the processes which control the flux of tire-derived compounds from the road to food. As future research directions, we suggest: 1) quantification of tire-derived compounds in biosolids and reclaimed wastewater, 2) investigation of sorption and transformation of tire-derived compounds in agricultural soils, and 3) identification of in-plant transformation products of tire-derived compounds, and development of methods capable of measuring these transformation products in real samples.

Based on mean as well as maximum tire-derived compound concentrations, and mean and 95th percentile leafy vegetable consumption, we provide estimated daily intakes via leafy vegetable consumption ($EDI_{leafy\ vegetable}$) in Table 4.1. For most compounds, the $EDI_{leafy\ vegetable}$ in the maximum scenario exceeds the mean scenario by more than an order of magnitude. This is due to both large differences between the maximum and the mean tire-derived compound concentrations in leafy vegetables, as well as between the 95th percentile and mean leafy vegetable consumption in both the Swiss and Israeli populations. The $EDI_{leafy\ vegetable}$ based on mean concentrations is generally low compared to other exposure pathways, which are also calculated based on mean environmental concentrations. For example, the $EDI_{leafy\ vegetable}$ of DPG in the mean case (0.05 – 0.3 ng/person/day) was lower than via dust ingestion (0.7 – 60.9 ng/person/day)[48], or drinking water (72.1 ng/person/day)[26], although the exposure from drinking water was estimated based on only one water sample. Benzothiazole $EDI_{leafy\ vegetable}$ values in the mean scenario (12 – 52 ng/person/day) are slightly higher than those reported for $\sum_5$ benzothiazole derivatives via inhalation of outdoor air near the workplace (1.4 – 4.2 ng/person/day) and near the home (3.5 – 9.1 ng/person/day)[24], and comparable to EDI from drinking water (36.2 ng/person/day [26]). Total EDI for benzothiazoles has been calculated based on measured concentrations in urine to be 4.8 to 18.2 µg/person/day [57], which would suggest that leafy vegetable consumption, inhalation, and drinking water all contribute as only minor sources to total benzothiazole exposure. Potential explanations for this large discrepancy could be that major exposure routes to benzothiazole are still unknown. Another explanation could be that target analyses have underestimated benzothiazole

uptake, if benzothiazole is mostly taken up in conjugated (or otherwise transformed) form, and subsequently deconjugated in the body [129]. The $EDI_{leafy\ vegetable}$ of 6PPD and other PPD derivatives via leafy vegetable consumption in the mean scenario (0.04 – 0.2 ng/person/day) were in the same range as EDI via inhalation of $\sum_5$PPDs (0.01 – 0.04) [17] for general residents, but much lower than for roadside workers (IPPD: 4.2 ng/person/day, 6PPD: 13.3 ng/person/day) [203]. EDI of PPDs via roadside soil ingestion ($\sum_5$PPDs: 35 – 63 ng/person/day) [17] also much exceeded $EDI_{leafy\ vegetable}$. However, for people regularly consuming high quantities of highly contaminated leafy vegetables, exposure to tire-derived compounds will be much higher ($EDI_{leafy\ vegetable}$ maximum scenario).

$EDI_{leafy\ vegetable}$ for a given single compound and scenario varies up to nearly ten-fold between different the Israeli and Swiss populations (Table 4.1), due to differences in both leafy vegetable consumption and tire-derived compound concentrations. Between different compounds, $EDI_{leafy\ vegetable}$ spans several orders of magnitude – the same is true for $EDI_{leafy\ vegetable}$ for pharmaceuticals in Israel [183]. For the Israeli adult population, the highest $EDI_{leafy\ vegetable}$ of a tire-derived compound was that of benzothiazole (mean scenario 52 ng/person/day), which is comparable to the $EDI_{leafy\ vegetable}$ of nicotine (40 ng/person/day), caffeine (20 ng/person/day), and sulfamethoxazole (20 ng/person/day), but much lower than $EDI_{leafy\ vegetable}$ for carbamazepine (870 ng/person/day) or lamotrigine (570 ng/person/day) [183]. Leafy vegetables tend to accumulate organic contaminants more than other crop types [75,76], so our data can be used as a starting point for estimating tire-derived compound dietary intake, although the total is likely to be higher since leafy vegetables represent only a fraction of total diet.

Currently, there are limited data regarding effects of tire-derived compounds on human health [8,176]. A no observable effect level of 357 mg/day was proposed for benzothiazole based on a rat study [204], which is substantially above $EDI_{leafy\ vegetable}$ for the mean and maximum concentration scenario. 6PPD has demonstrated moderate toxicity in various rodent species [205], while 6PPD-quinone demonstrates a species-specific toxicity in fish [176]. However, human toxicity of tire-derived compounds has not been systematically evaluated, thus, the risk associated with this exposure cannot be assessed. Future work addressing toxicity associated with ingestion of tire-derived compounds should consider mixture toxicity, since we show that multiple compounds co-occur along with pharmaceuticals in leafy vegetable produce. Additionally, uptake and exposure might be higher in other types of produce. Due to a limited number of samples, and a lack of information about their specific growth conditions, we were unable to implicate specific sources of tire-derived compounds. Future work is needed to quantify the fluxes of tire-derived compounds to the agricultural environment, assess the fate of tire-derived compounds in the agricultural environment, and assess the contribution of transformation products to total tire-derived compound

exposure. Future research should also address the biological effects of ingestion of tire-derived compounds.

Chapter 5: The invisible footprint of climbing shoes: high exposure to rubber additives in indoor facilities

-Air Study-

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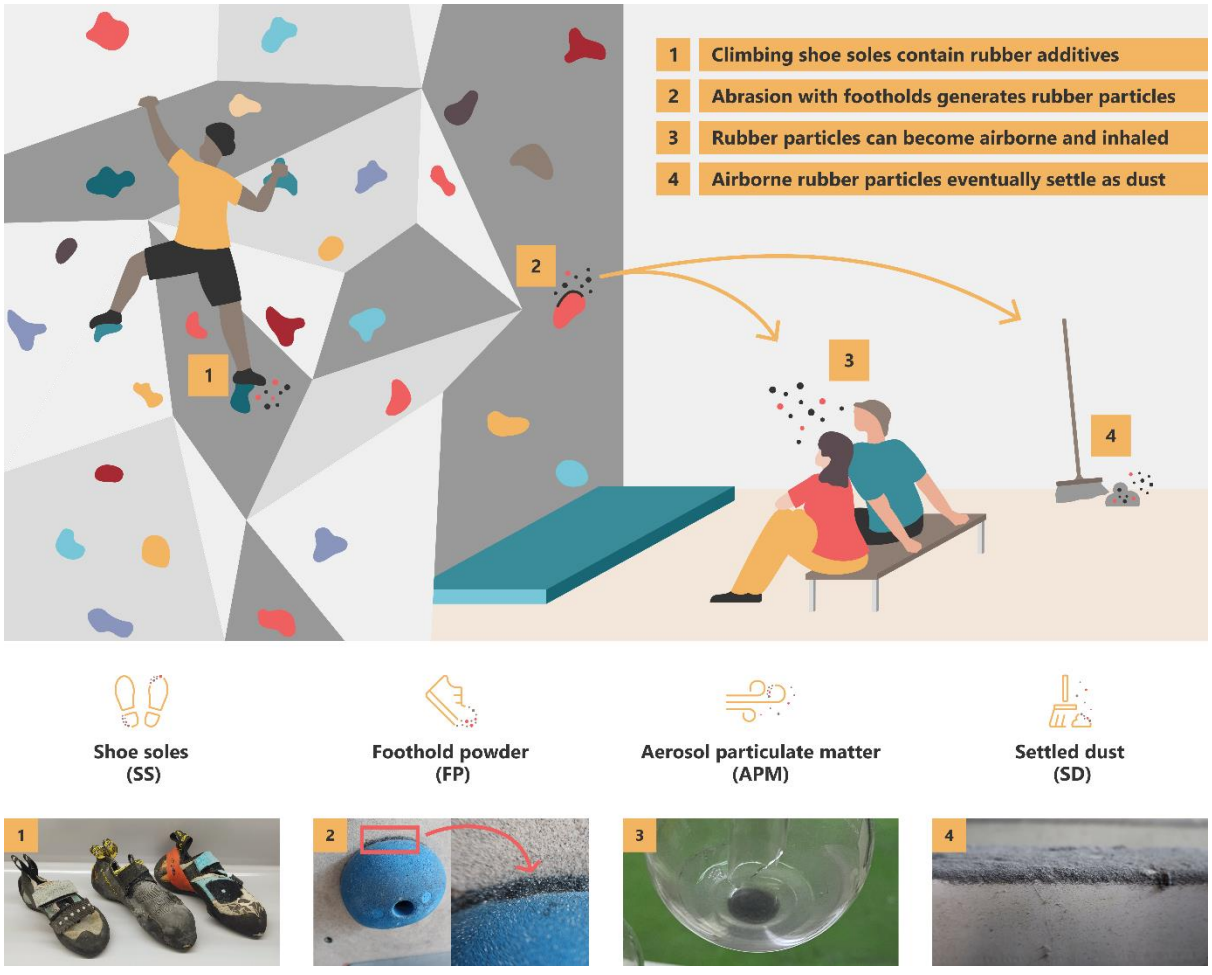
† These authors contributed equally.



Manuscript in review.

5.2 Abstract

There is increasing research focused on rubber additives, predominantly originating from tire and road wear particles. Other consumer products including sports equipment also contain rubber additives and human exposure to these compounds is of concern due to demonstrated toxicity to animal species. Rubber additives are intentionally incorporated into climbing shoes for enhanced performance. We found high concentrations of rubber additives in shoe sole samples, aerosol particulate matter, and settled dust in indoor climbing halls. The estimated daily intake via inhalation/ingestion for climbers and employees in some of these facilities exceeds the intake of rubber additives from previously known sources. Abrasion powder resulting from friction of climbing shoes on the holds is the likeliest source of high concentrations of rubber additives observed in aerosol particulate matter and settled dust, since other emission sources could be excluded. These findings identify a previously unknown human exposure route of rubber additives and emphasize the global problem of the toxicity burden of plastic additives.

5.3 Introduction

Air quality has an important impact on human health. Concentrations of particulate matter are linked to rates and outcomes of cancer[206], respiratory and cardiovascular disease[207], COVID19[208], and ultimately, life expectancy[207,209], especially in densely populated areas[210]. Currently, more than half of the global population lives in cities, with a projected increase to 68% by 2050[211]. In parallel with urbanization, the global population is spending less time outdoors, with the US population estimated to spend 90% of their time indoors[212]. Indoor air quality is a critical and increasing determinant of human health and indoor air quality is relevant not only in the home and workplace, but also in places of recreation. Due to safety, accessibility, and/or practical constraints, outdoor recreation is unrealistic for many living in dense urban centers, so indoor recreational facilities serve as places to exercise, as well as socialize. Recent studies recognize the importance of indoor air quality in recreational sports facilities[213]. Climbing is one increasingly popular form of indoor recreation. In 2018, an estimated 1.5% of the UK population[214], and about 4.4% of the US population[215] visited indoor climbing halls. Of these visitors, about 20% are regulars and spend several hours a day, multiple times a week in climbing halls[214].

Several monitoring studies conducted in indoor climbing gyms reported very high particulate matter concentrations[216,217]. Particulate matter exposure during indoor climbing is hypothesized to be the driver for acute decline in lung function of climbers[217]. The primary source of particulate matter is likely the chalk used by climbers, but other sources may also contribute. In indoor halls, climbers wear specialized climbing shoes, with soles made of highly functionalized rubber. The rubber is chemically engineered to be flexible and sticky. Soles are intentionally designed to slowly abrade during climbing, due to desired friction with climbing holds. This leads to a constant generation of rubber particles, which accumulate on

the climbing footholds. Most climbers own brushes to clean these climbing holds, which results in a constant aerosolization of rubber particles, which may remain airborne long enough to be inhaled.

Plastic additives in general have been identified to increase the global toxicity burden on humans and the environment with over 13,000 substances across a wide range of sectors and product value chains[13]. In tires, which are also highly engineered and abrade during their intended use, rubber additive concentrations are very high[6]. Tire additives and their transformation products, such as 6PPD-quinone, have been found to be responsible for Coho salmon mass mortality[31], as well as toxicity in rainbow trout, brook trout[40], and white-spotted char populations[43]. 6PPD-quinone is also toxic to human lung cells[65,218,219]. Recent studies have detected rubber additives in human urine[48,56] and blood[220], with inhalation hypothesized to be a major source of exposure. Shoe manufactures buy rubber as raw material from the same suppliers as tire manufactures. Due to the highly specialized properties of climbing shoes, we hypothesized that these rubber particles contain high additive concentrations, and that climbing halls are a hot spot of human exposure.

In this study, we explored climbing halls as an indoor source of exposure to rubber additives. We collected air particulate matter samples in five climbing halls and measured the concentrations of rubber-derived compounds therein. Based on these concentrations, we calculated the exposure for employees and recreational visitors to these rubber-derived compounds. We were able to implicate climbing shoes as the source of these rubber-derived compounds.

5.3 Results & Discussion

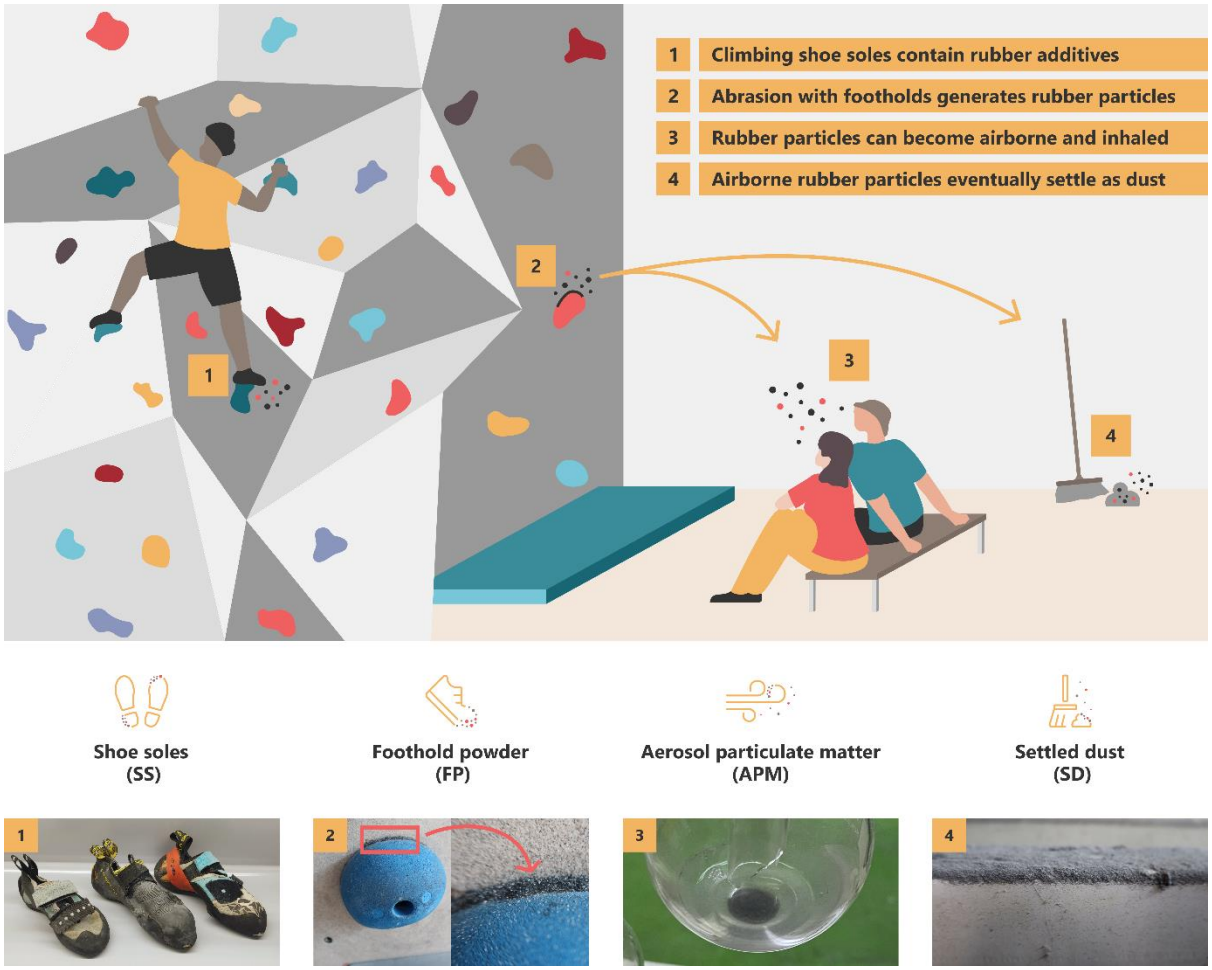


Figure 5.1 Schematic of a climbing hall. Specialized climbing shoes are worn with highly functionalized rubber soles (1 – shoe soles). Friction between these shoe soles and the footholds generates rubber particles (2 – foothold powder). Those can be aerosolized and be inhaled directly upon generation, due to the brushing of holds, or by climbers falling onto mats and resuspending rubber particles which had settled (3 – aerosol particulate matter). Eventually, aerosol particles also settle elsewhere as dust (4 – settled dust).

5.3.1 High exposure to rubber-derived compounds in climbing halls for visitors and employees

Our air sampling campaigns, using a liquid impinger[221,222] to collect aerosols, were carried out during peak activity hours, revealing very high particulate matter (PM) concentrations (Figure 5.1, Table 5.1). Based on the impinger geometry (see supplementary information) and air flow rate used (60 L/min), the collected aerosols could be separated into two fractions: 1) a fraction with predominantly extra-thoracic and tracheobronchial deposition based on the aerodynamic cut off > 6.4 µm (referred to as upper respiratory tract fraction; URT) and 2) a fraction with predominantly bronchial and alveolar deposition (aerodynamic cut off < 6.4 µm; referred to as lower respiratory tract fraction; LRT). The PM

concentrations of aerosols (from four climbing halls) ranged from 590 μg/m^3 to 1,990 μg/m^3 in the URT fraction (>6.4 μm) and from 890 μg/m^3 to 1080 μg/m^3 in the LRT fraction (Table 5.1). Halls 01 and 03 had the highest number of check-ins per hour, which may explain why the highest aerosol PM concentrations were measured in these halls. Halls 02 and 04 had a lower number of check-ins per hour, and slightly smaller hall sizes. Notably, the air of Hall 02 was not ventilated, while the air of Hall 04 was, which could explain the higher aerosol PM concentrations in Hall 02 as compared to Hall 04. Hall 05 had much lower PM concentrations in both the URT and LRT fractions (80 μg/m^3 and 100 μg/m^3 respectively), which can be linked to substantially lower climbing activity in this hall, and the largest hall size. Hall 05 is a recently constructed, three-story hall, with open airflow between the climbing areas on the different floors (Table S5.1). A relationship between activity and PM concentrations has been previously observed in climbing halls[216] and gymnasiums[223]. Despite the different aerodynamic size cut off of the liquid impinger used here compared to other measurement devices, the aerosol concentrations reported in this study are in the same range as PM concentrations previously reported for indoor climbing halls (from 509 to 4,028 μg/m^3, measured using an impactor with an aerodynamic size cut-off of 10 μm; PM_{10})[216]. These values exceed those of most other indoor environments[224] including indoor artificial turf halls (31 to 40 μg/m^3)[101] and other sports environments[213]. They are comparable to maximum reported PM_{10} concentrations in gymnastic facilities where chalk is also used (500 to 900 μg/m^3)[225].

Our measured values are near current limits for occupational exposure to low-toxicity dusts (<4-10 mg/m^3 inhalable fraction; <1.5 - 4 mg/m^3 respirable fraction over an 8-hour time-weighted average exposure; limits are different for different countries)[226]. Low-toxicity dusts are defined as all poorly soluble, nonfibrous dusts with <1% crystalline silica content that have negligible toxic effect on the body but if inhaled in sufficient quantity, accumulate and cause injury in the terminal airways and proximal alveoli, leading to inflammation with subsequent development of lung diseases such as COPD and pneumoconiosis[226].

It is important to note that the presence of leachable rubber-derived compounds (RDCs) in climbing hall dusts may pose a higher health risk than conventional low-toxicity dusts, thereby providing a compelling rationale for further investigations. In Halls 01 – 04, sufficient aerosol PM was collected to perform chemical analyses and quantify 15 rubber-derived compounds (RDCs). Ten RDCs were detected above the limit of quantitation (LOQ) in at least one of these samples (Table 5.2). Substantial variation was observed between the aerosol PM samples from four halls, but a consistent set of RDCs dominated the chemical profile in all samples. Among these, four RDCs were identified as transformation products (TPs) of compounds commonly used in rubber products: 2-hydroxybenzothiazole (2OH-BTZ), benzothiazole (BTZ), as well N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine-quinone (6PPD-

quinone) were detected in the aerosol PM of all climbing halls, and aniline was detected in the aerosol PM of Halls 01 and 02 (Figure 5.2a). Cumulative RDC concentrations in the URT fraction ranged from 0.92 to 28.4 ng/m^3, while RDC concentrations in the LRT fraction ranged from 1.10 to 7.81 ng/m^3.

It has been shown that concentrations in settled dust of organic compounds with similar physico-characteristics as RDCs (phthalate esters) tend to correlate with their concentrations in aerosol PM [99], thus, we quantified RDCs in dust samples from all climbing halls. We detected 14 out of the 15 RDCs (except CPPD-Q) in at least one triplicate sample from nine climbing halls investigated (Table 5.2). Since settled dust samples collected from different areas of a hall contain different amounts of rubber particles, it is unsurprising that substantial variability in total RDC concentrations was observed between triplicate settled dust samples (Figure S5.1, Table S5.2). Overall, the chemical profiles within dust samples from nine halls were consistent except in Hall 02 (Figure 5.5). Although the chemical profile in aerosol PM varied between four halls, the chemical profile was consistently dominated by several chemicals, which also dominated the profile of settled dust samples (Figure 5.5). Overall, cumulative RDC concentrations in settled dust samples were high (1.6 to 55 µg/g). The similarity in chemical profile of settled dust and aerosol particulate matter samples from nine halls in four different countries (France, Switzerland, Spain and Austria) suggests an important source of RDCs which may be ubiquitously present in climbing halls. Although further studies may be required to confirm the levels of these compounds globally, it is unlikely that the results will be significantly different from those reported here, as there are only a limited number of manufacturers of highly specialized climbing footwear, most of which market their products globally.

Table 5.1 Concentrations of aerosol PM measured in Halls 01 through 05

	Hall 01	***Hall 02***	***Hall 03***	***Hall 04***	***Hall 05***
LRT fraction (µg/m^3)	1040	900	1080	890	100
URT fraction (µg/m^3)	1590	1000	1990	590	80
Total aerosol PM (µg/m^3)	2630	1900	3070	1480	180

Several recent studies report concentrations of RDCs in settled dust from outdoor and indoor environments, mostly driven by the increasing research about tire particles. Concentrations of most RDCs in settled dust samples were higher in some halls than those reported from other indoor environments (Figure 5.2, Figure S5.2). In some samples (particularly in Halls 06 and 09), benzothiazole concentrations were one or two orders of magnitudes higher in our samples than in house dust and even road dust [45,46,105]. 6PPDq concentrations were also higher than in most house dust samples collected around the world[19,47,49,51] and were similar to road dust samples[105,227]. Concentrations of DPG were also high (up to 9.65 µg/g) and exceeded most reported DPG concentrations in house dust[48,228](Figure 5.2).

Table 5.2 Rubber-derived compound concentrations measured in bouldering halls. Concentrations measured in aerosol PM and settled dust samples from all bouldering halls are shown as a range (aerosol PM data from Hall 05 excluded).

	LRT fraction (ng/m^3)	***URT fraction (ng/m^3)***	***Total aerosol PM (ng/m^3)***	***Settled dust (ng/g)***
Aniline	<LOQ – 1.31	<LOQ – 2.80	<LOQ – 4.12	<LOQ - 1420
DPG	0.005 – 1.96	<LOQ – 4.97	0.005 – 6.50	58 - 9650
2OH-BTZ	1.07 – 4.41	0.33 – 4.11	1.41 – 7.26	<LOQ - 9500
IPPD	<LOQ – 0.05	<LOQ – 0.14	<LOQ – 0.18	<LOQ – 380
BTZ	<LOQ – 2.47	0.51 – 13.9	0.51 – 16.4	<LOQ - 34450
2amino-BTZ	<LOQ	<LOQ	<LOQ	<LOQ - 510
2SH-BTZ	<LOQ – 0.55	<LOQ – 1.41	<LOQ – 1.71	<LOQ - 4370
HMMM	<LOQ – 0.25	<LOQ – 0.50	<LOQ – 0.75	<LOQ – 17000
CPPD	<LOQ	<LOQ	<LOQ	<LOQ - 8
6PPD	<LOQ – 0.24	0.04 – 0.31	0.04 – 0.49	<LOQ – 300
IPPDq	<LOQ – 0.02	<LOQ – 0.04	<LOQ – 0.06	<LOQ - 42
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ – 47
6PPDq	0.02 – 0.37	0.04 – 0.71	0.05 – 1.08	<LOQ – 580
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	<LOQ	<LOQ – 0.8

Similar to dust, the concentrations of most RDCs in the collected aerosol PM samples were very high, particularly in Halls 01, 02, and 03 compared to other atmospheric environments (Figure 5.2, Figure S5.2 and SI: Excel file containing concentrations of all RDCs). PPD and PPDq concentrations in the LRT fraction of this study were higher than those measured in the $PM_{2.5}$ fraction of aerosols collected in Chinese megacities[17,20] and similar to concentrations determined from $PM_{2.5}$ fractions at roadside sites and city centres in China during air pollution events[21]. Concentrations of DPG, BTZ, and 2OH-BTZ in aerosol PM samples collected in the LRT fraction of the current study were one or two orders of magnitude higher than those determined in the total particulate matter collected in 18 megacities worldwide[18] and BTZ and 2OH-BTZ were up to 10-fold above concentrations measured in PM_{10} fraction of aerosols from industrial areas in Spain[24]. So far, studies reporting RDC concentrations in aerosol PM collected in indoor environments remain very scarce. Dye et al. (2006)[101] investigated 2OH-BTZ, 2-amino-BTZ, 2SH-BTZ and IPPD concentrations in PM_{10} collected in indoor artificial turf halls. They reported higher concentrations of 2-amino-BTZ, 2SH-BTZ and IPPD compared to our climbing hall samples, but lower concentrations of 2OH-BTZ. However, it should be noted that the reported values in the study by Dye et al. were not direct measurements but based on RDC concentrations in ground granulate and estimated concentrations of rubber in the aerosol PM, so these data should be treated with caution.

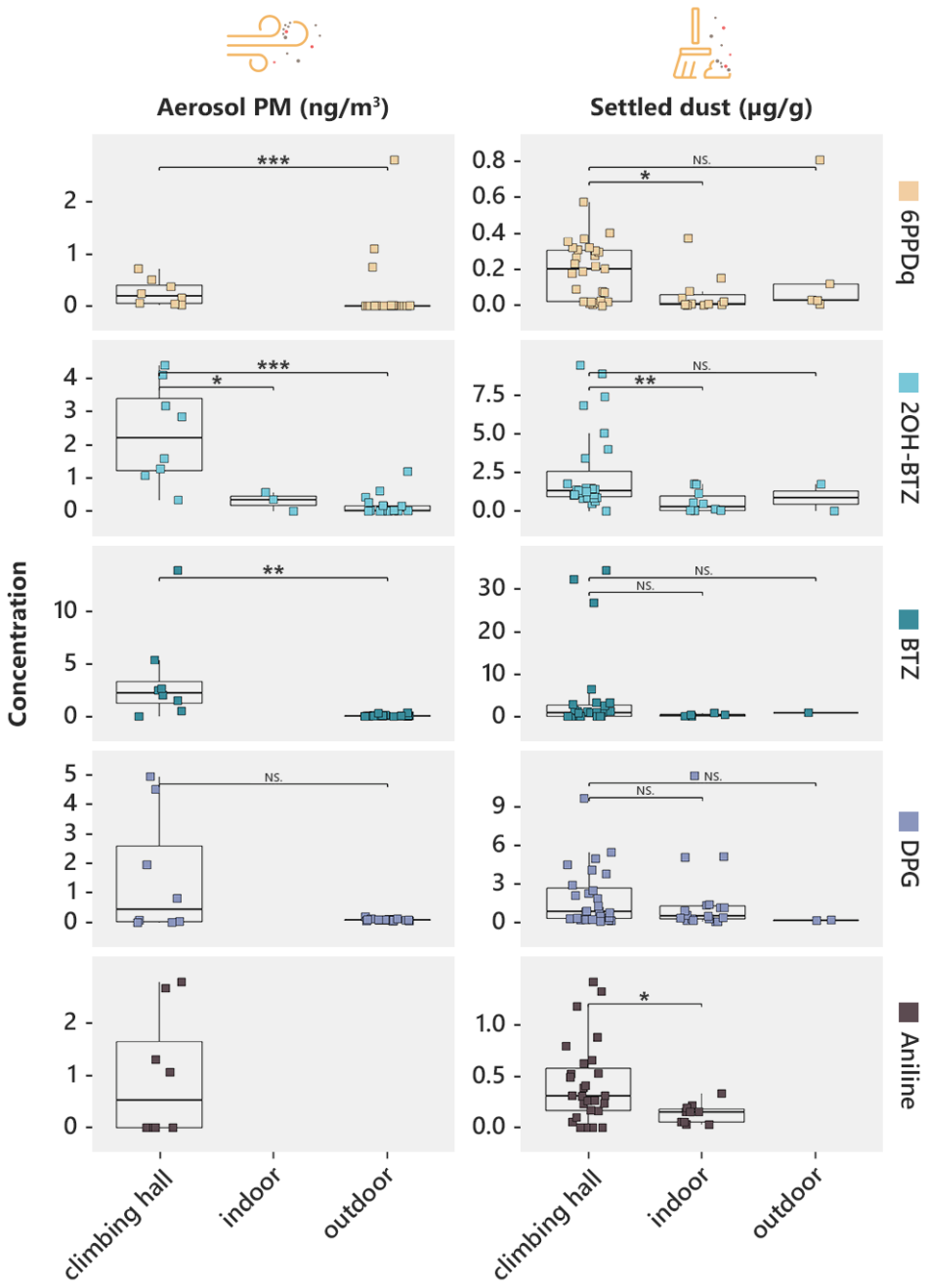


*Figure 5.2 Comparison of rubber-derived compound concentrations in climbing halls and other environments. Rubber-derived compound concentrations measured in the combined LRT and URT fractions of climbing hall aerosol particulate matter (left) and settled dust (right) compared to concentrations reported in the literature for various indoor (houses, vehicles, shopping malls, dormitories, parking lot, sport halls) and outdoor (roadsides, city centers, playgrounds, recycling plants, industrial sites) environments. Details about literature values, including size fractions of particulate matter are provided in the Supporting Information: Excel file. Statistical differences between groups were tested with the Wilcoxon signed-rank test (NS means $p \geq 0.05$; * means $p < 0.05$; ** means $p < 0.01$; *** means $p < 0.001$).*

Our data show that concentrations of RDCs found in the combined LRT and URT fractions of aerosol PM of indoor air of some climbing halls exceeds those of most other environments that have been studied so far. Most climbers spend several hours in indoor climbing hall facilities and usually have a high respiration rate that could increase intake of RDCs. We explored the human exposure to RDCs in climbing halls by calculating the estimated daily intake via inhalation/ingestion ($EDI_{inh/ing}$) for two groups: regular adult climbers and employees working at the halls (Table 5.3; see SI for details). In this calculation we assume that the RDCs in the LRT fraction represent inhalation exposure, while the concentrations measured in the URT fraction represent a combination of inhalation/ingestion exposure, since a high fraction of aerosol PM depositing in the extrathoracic/tracheal region of the URT is quickly cleared to the gastrointestinal tract[229].

Mean $EDI_{inh/ing}$ values estimated for the two sub-groups (adult climbers and employees) showed that employees would be subjected to a higher exposure than climbers due to their longer average exposure time, despite their lower inhalation rate (Tables 5.3, S3). $EDI_{inh/ing}$ for ∑benzothiazoles ranged from 0.04 to 29 ng/kg/day and exceeded $EDI_{inh/ing}$ for ∑PPDs (up to 0.9 ng/kg/day) which were similar to $EDI_{inh/ing}$ for ∑PPDqs (up to 1.5 ng/kg/day). The $EDI_{inh/ing}$ derived for BTZs in this study was up to two orders of magnitude above those estimated for employees near industrial sites in Spain[24] and exceeded dermal exposure through textile (EDI_{dermal} for $\sum_3$BTZs = 244 – 395 pg/kg/day[230]). $EDI_{inh/ing}$ for PPDs and PPDqs were up to 3.1 and 7.8-fold higher than EDI_{inh} for near-roadside workers in Chinese megacities and two orders of magnitudes higher than the EDI_{inh} for the adult population in Hong-Kong[17]. $EDI_{inh/ing}$ for DPG ranged from 0.01 – 8.61 ng/kg/day exceeding in most cases EDI via household dust ingestion in 11 countries (0.0 – 0.9 ng/kg/day)[48] (Table 5.3).

By comparing the EDI obtained in this study with the available EDI from literature derived for other environments, we highlight that exposure from some indoor climbing halls exceeds every other exposure source reported to date, including from such contaminated environments as roadsides in megacities (Table 5.3). Although EDIs derived from our data remain below the estimated tolerable daily intake values for BTZ[231] and aniline[232], our results imply that the contribution of climbing halls to the total daily intake of RDCs is significant for individuals exercising or working in these facilities and would contribute to total exposure from many potential sources (dermal exposure to textiles, house dust ingestion, outdoor air pollution). For all other RDCs investigated, no tolerable daily intake values are available, so conclusions on the risk associated with this exposure cannot be drawn. It is important to note that the EDIs reported in this study are relevant for climbers who visit climbing halls during peak hours. It has been shown that aerosol PM concentrations vary greatly throughout the day, in correlation with the number of visitors

present[216]. Therefore, it can be expected that climbers visiting halls outside of peak hours will have lower exposure to RDCs via inhalation of aerosol PM.

Table 5.3 Estimated daily intake via inhalation/ingestion of rubber-derived compounds. Range of estimated daily intake (ng/kg/day) by inhalation from two sub-groups (adult climbers and employees, age 21 - 31) derived from Halls 01 to 05 and based on aerosol PM data (combined LRT+URT fractions). n.d = Not determined due to aerosol PM concentrations <LOQ. Data are compared with EDI from multiple sources obtained from literature.

EDI (ng/kg/day)	Aniline	BTZ	2OH-BTZ	DPG	HMMM	6PPD	IPPD	6PPDq	IPPDq
$EDI_{inh/ing}$ (Employee)	n.d - 5.45	0.06 - 21.7	1.86 - 7.14	0.01 - 8.61	n.d – 0.99	n.d - 0.65	n.d - 0.24	0.07 - 1.43	n.d - 0.08
$EDI_{inh/ing}$ (Climber)	n.d - 3.50	0.04 – 13.9	1.19 – 6.17	0.01 - 5.53	n.d - 0.63	n.d - 0.42	n.d - 0.16	0.05 - 0.92	n.d - 0.05
EDI by dust ingestion[48] or roadside soil ingestion[17]				0.01 - 0.87[48]		Σ5PPDs 0.5 – 0.9[17]		Σ5PPDqs 0.70 - 1.10[17]	
EDI_{inh} ambient air (worker)		Σ5BTZs 0.02 - 0.06[24]				0.19[21]	0.06[21]	0.13[21]	0.07[21]
EDI_{inh} ambient air (adult residents)						Σ5PPDs 0.0002 - 0.0006[17]		Σ5PPDqs 0.0001 – 0.001[17]	

5.3.2 Background levels of rubber derived compounds and evaluation of potential sources

We performed several tests to eliminate sample contamination as the source of high RDC levels in aerosol particulate matter and settled dust samples. Laboratory blanks, which assessed background levels in solvents, glassware and inadvertent contamination via laboratory aerosols, were mostly clean, except for low amounts of 2SH-BTZ and 2OH-BTZ, which were detected in one out of four blanks (Section S5.1). Collection and storage blanks contained low levels of 2SH-BTZ, 2OH-BTZ, BTZ, DPG, and IPPD (Section S5.1). This is unsurprising, since collection and storage blanks were prepared in the climbing halls, where RDCs were present at high concentrations. RDC levels in these blanks were at least one order of magnitude lower than what was measured in aerosol PM samples (Section S5.1, Table 5.2), which confirms that the majority of RDCs in our samples were not introduced via contamination.

Since levels of several RDCs in aerosol PM and settled dust from some climbing halls greatly exceed those reported in other environments (Figure 5.2), we expected that the high concentrations of these RDCs were specifically due to climbing activity. In reference samples collected in offices of climbing Hall 02 (same building), where climbing is not practiced, we detected 6PPD and IPPD (0.10 and 0.86 ng/m^3 respectively) in the combined LRT + URT samples (Section S5.1). This is to be expected, since the

concentrations of 6PPD and IPPD in these reference samples (offices) are in the range of those reported in other indoor[101] and outdoor[17,19–21,23,24,233] environments (Figure S5.2), implying that these RDCs are present at background levels, and not specifically derived from climbing activity. On the other hand, BTZ, 2OH-BTZ, 6PPDq, IPPDq, DPG, aniline and HMMM were not detected in the reference samples (Section S5.1), and their concentrations in our aerosol PM samples from the more contaminated halls exceed previously reported values (Figure 5.2), indicating that these compounds have a climbing-specific source.

Several potential climbing-related sources of RDCs were identified. IPPD was detected in one climbing hold (66.0 ng/g), and in one of the mats measured (83.1 ng/g). IPPDq was detected in the same mat (5.8 ng/g). In contrast, dust samples contain <LOQ – 380 ng/g IPPD and <LOQ - 42 ng/g IPPDq. Climbing holds and mats are made from durable materials with very low abrasion, so they probably did not contribute substantially to the aerosol PM or settled dust present in climbing halls and no other RDCs were found in these items (Section S5.1). Therefore, we concluded that another climbing related source must be the main contributor to the high concentrations of RDCs measured in settled dust and aerosol PM samples.

5.3.3 Engineered climbing shoes contain high quantities of rubber-derived compounds

Sports products are highly engineered materials, and so are climbing shoes. Their soles are composed of rubber for an optimal friction on the climbing holds. Raw rubber materials for climbing shoes are often sourced from the same type of rubber used by tire manufacturers. Specific rubber formulas have been developed to produce shoes that offer various combinations of softness, flexibility, stiffness, and stickiness. These properties, as well as rubber durability, are typically optimized using additives. Thus, soles of climbing shoes could be responsible for the high RDC levels found in the air and dust samples from climbing halls.

Among the thirty shoe sole (SS) samples screened, all 15 RDCs were found in at least one shoe sole sample. Concentrations were highly variable between shoe models with cumulative RDC concentrations ranging from 25 to 3,405 µg/g (mean: 711 µg/g) (Fig 5.3, Table S5.2). Benzothiazoles exhibited high concentrations with 2SH-BTZ being the main constituent (mean: 538 µg/g) representing on average 67% of the total mass of RDCs detected (Figure S5.3). BTZ, 2OH-BTZ and 2-amino-BTZ were detected in lower concentrations (mean: 58, 53 and 3 µg/g, respectively). These results suggest that, as in other rubber products, 2SH-BTZ is used as a vulcanization accelerator during the curing process. Other benzothiazoles are also present and are typically considered to be impurities or degradation products[100,234]. Unlike benzothiazoles, DPG and aniline were not detected in every shoe sole sample, with concentrations

ranging from <LOQ to 814 µg/g and <LOQ to 225 µg/g, respectively. DPG is another vulcanization accelerator and may be used together with or instead of 2SH-BTZ (S18 and S19; Figure 5.3). *p*-Phenylenediamine compounds were detected in most shoe sole samples in variable concentrations, with 6PPD and IPPD as the compounds with the highest concentrations (mean: 1303 ng/g and 661 ng/g, respectively). This is not surprising, since of the numerous PPDs available, 6PPD and IPPD are the most commonly used rubber antiozonants[199]. CPPD and DPPD were only detected sporadically and at trace levels (Table S5.2). The respective quinone transformation products, 6PPDq and IPPDq, were consistently detected (mean: 23 ng/g and 15 ng/g, respectively) and as expected, their concentration in the samples were correlated to the concentration of the parent compounds. CPPDq and DPPDq were also occasionally detected along with their parent compounds, but at very low concentrations (Table S5.2). Overall, RDC concentrations in shoe sole samples were highly variable and likely due to different compounding strategies used by manufacturers as well as the target product characteristics (i.e., stiffness, durability, performance, adhesiveness). RDC concentrations in shoe sole samples were generally lower (DPG and PPDs) or similar (benzothiazoles) to those in tire tread[100,234] but higher than in other elastomeric consumer products[100].

Foothold powder collected from the top of several climbing footholds (Figures 5.1 and 5.5) was, based on visual inspection, comprised primarily of abraded climbing shoe soles (in contrast to settled dust samples, which was more heterogenous in composition). As these foothold powder (FP) samples were collected in public climbing halls, where visitors wear a variety of different climbing shoe models, these samples should represent the diversity of shoe soles prevalent in climbing hall environments. Indeed, the foothold powder samples were highly representative of the variability found among individual shoe samples, both in terms of RDC concentrations and profile (Figures 5.3, S5.3). Variability in RDC concentrations between triplicate samples collected in one climbing hall was generally lower for foothold powder than for settled dust samples (Figure S5.4), which is unsurprising since foothold powder is a more homogenous material, consisting primarily of rubber. However, some variability was observed, which is most likely due to dilution of the foothold powder samples with chalk or other dust. The abrasion of shoe soles generates fine elongated rubber particles (Figure 5.4) which can become airborne over time, since it is common practice for climbers to brush particles off holds. It has been previously shown that abrasion of particles and fibers containing additives drives the chemical composition of indoor dust[235], and it is highly likely that the fine rubber particles emitted via abrasion were captured by our air sampler. With SEM imaging, we confirmed that elongated particles, visually very similar to those in shoe powder, were present in settled dust samples, including in the respirable size fraction (Figure 5.4). Although some RDCs were indeed found in all four sample types, i.e., shoe soles, foothold powder, settled dust and

aerosol particulate matter, the chemical profile did not fully match across the four sample types (Figure 5.5). We thus decided to explore the possibility that chemical transformations of some RDCs in airborne particles could link the chemical profiles of RDCs in climbing shoes to those in aerosol particulate matter and settled dust samples.

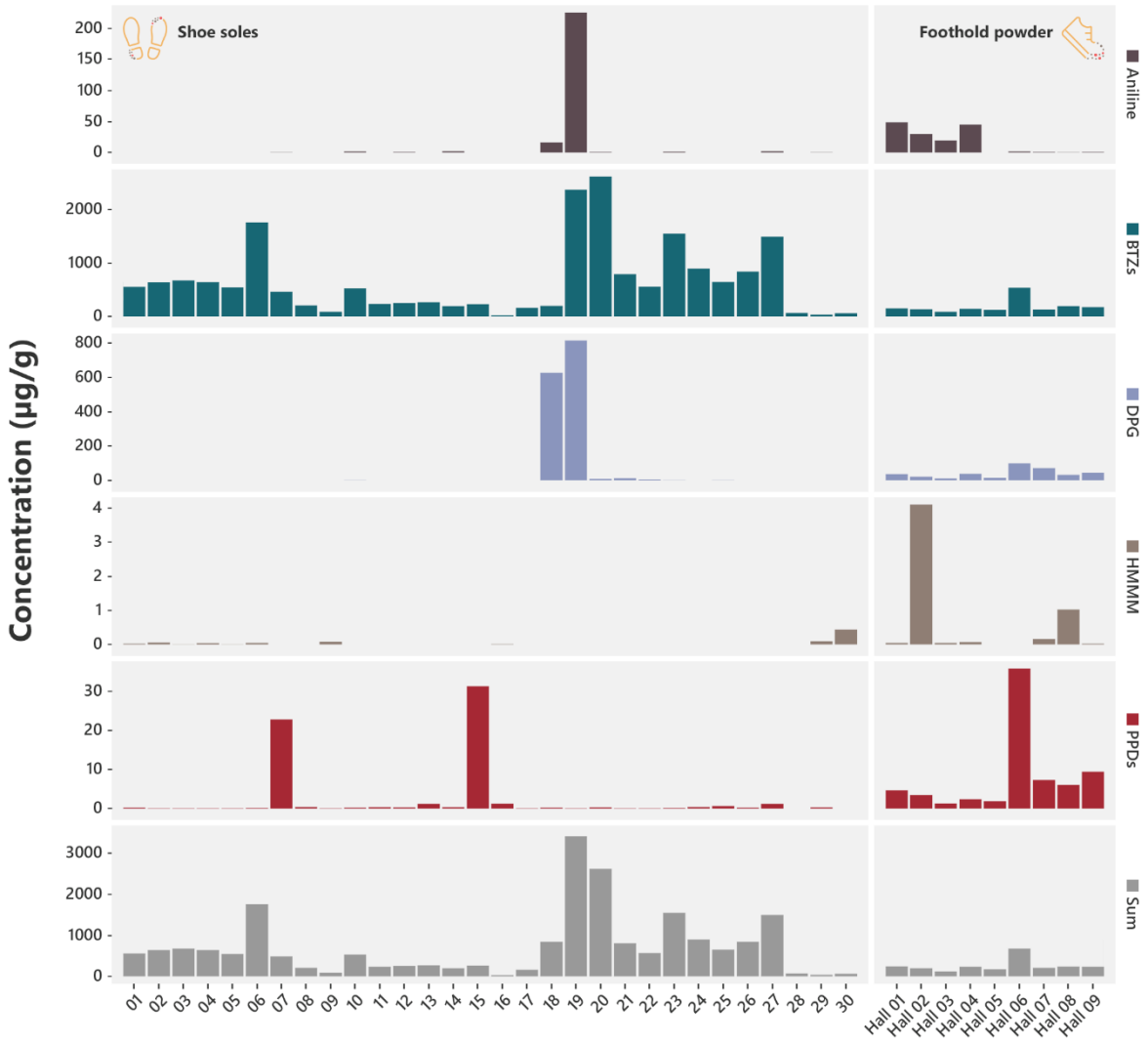


Figure 5.3 Rubber-derived compound concentrations in climbing shoes and foothold powder. RDC concentrations in thirty climbing shoe soles (left), and foothold powder from nine climbing halls (right). Concentrations of RDCs vary substantially between different shoe models. Foothold powder samples are representative of the variety of different shoe models.

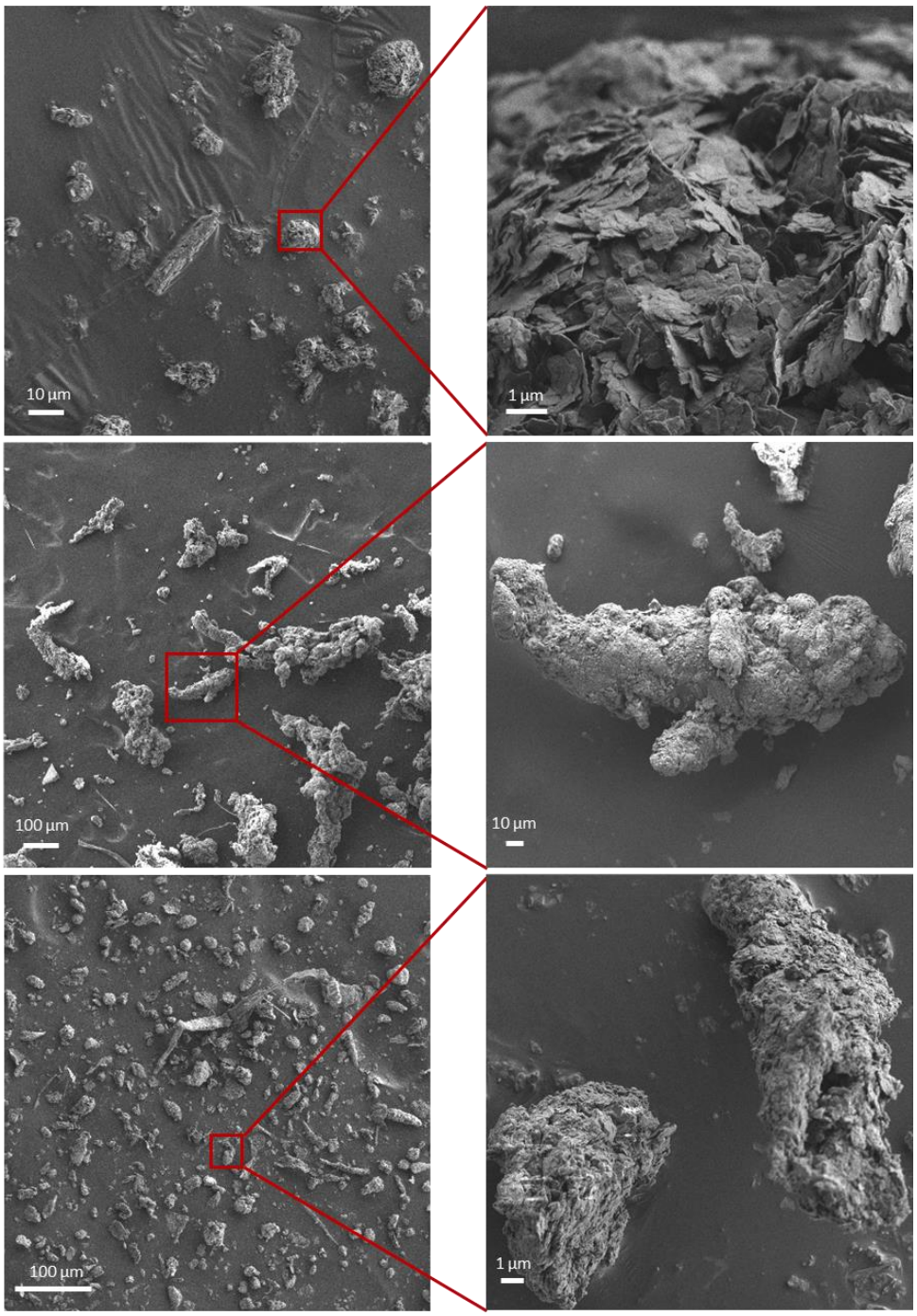


Figure 5.4 Scanning electron microscopy images of rubber particles in settled dust. Representative scanning electron microscopy images of (A) chalk (calcium carbonate powder), (B) a foothold powder sample and (C) a settled dust sample collected in climbing halls. Rubber particles resulting from the abrasion of shoe soles are visible in the foothold powder and settled dust samples (B, C). Rubber particles are distinguishable from chalk particles (A) due to their elongated shape and surface physical characteristics with a smooth carbon-based surface compared to chalk. Surface roughness appears to increase between recently generated rubber particles identified in the foothold powder samples (B) and particles found in settled dust (C).

5.3.4 Transformations in indoor air implicate climbing shoes as the source of rubber-derived compounds

While shoe sole and foothold powder samples were likely comprised mostly of rubber, the aerosol PM and settled dust samples were more heterogeneous, so concentrations of RDCs cannot be directly compared across the sample types. Therefore, we normalized all individual RDC concentrations to ∑RDCs. All settled dust and aerosol PM (combined LRT and URT fractions, Figure S5.4) samples had RDC profiles which differed distinctly from shoe sole and foothold powder samples (Figure 5.5). Strikingly, the mean fraction of 2SH-BTZ dropped from 48.0% in the foothold powder samples to 1.2% in the settled dust and aerosol PM samples. At the same time, the fractions of BTZ and 2OH-BTZ increased from 13.9% to 26.3% and 9.6% to 30.6%, respectively. A similar pattern emerged for the PPDs. The fraction of 6PPD dropped from 1.6% in the foothold powder samples to 1.1% in the settled dust and aerosol particulate matter samples, while the fraction of 6PPDq increased from 0.1% in the foothold powder samples to 4.7% in the settled dust and aerosol particulate matter samples. Likewise, the fraction of IPPD dropped from 0.8% to 0.4%, while IPPDq increased from 0.02% to 0.03% (Figure 5.5, S5.5).

All observed shifts (2SH-BTZ → 2OH-BTZ + BTZ; 6PPD → 6PPDq; IPPD → IPPD-q) are likely the result of transformation reactions on particle surfaces. The rubber particles collected in our aerosol PM samples must by virtue of the collection technique, exhibit small aerodynamic diameters with a correspondingly larger surface area, since larger rubber particles likely do not remain airborne long enough to be inhaled (or captured by our sampling device). Rubber particles present in the collected settled dust samples were also enriched in smaller sized particulates after aerosol transport (Figure 5.4). Such small particles have a high specific surface area, which allows for rapid reactions with reactive species in the surrounding gas phase. Ozone, as well as secondary species, such as the hydroxyl radical and NO_X drive chemical reactions on particle surfaces in indoor air[236,237]. In contrast, the foothold powder samples exhibited a lower specific surface area due to the larger particle sizes (Figure 5.4) and were too pristine to have undergone extensive transformation reactions prior to collection.

The chemical transformations we observed have been previously reported[30,170,238,239]. To confirm that these transformations can also occur in rubber particles from climbing shoes, we conducted fast-ageing experiments on foothold powder samples, using an ozone exposure chamber[240]. After four hours of exposure to a high fast-ageing ozone (1 g/m^3) concentration, the chemical profile of the foothold powder samples shifted substantially and corresponded to the chemical profile of the aerosol PM and settled dust samples (Figure S5.8, S5.9, Table S5.4). It is important to note that the ozone concentrations used in these experiments are significantly higher than what would typically be found in indoor climbing halls and

exceed the WHO's recommended indoor air limit of 50 ppb. Therefore, these experiments do not conclusively demonstrate that the observed transformations result from reactions with ozone. However, they do confirm that the RDCs in settled dust and aerosol PM samples are transformation products of those present in foothold powder.

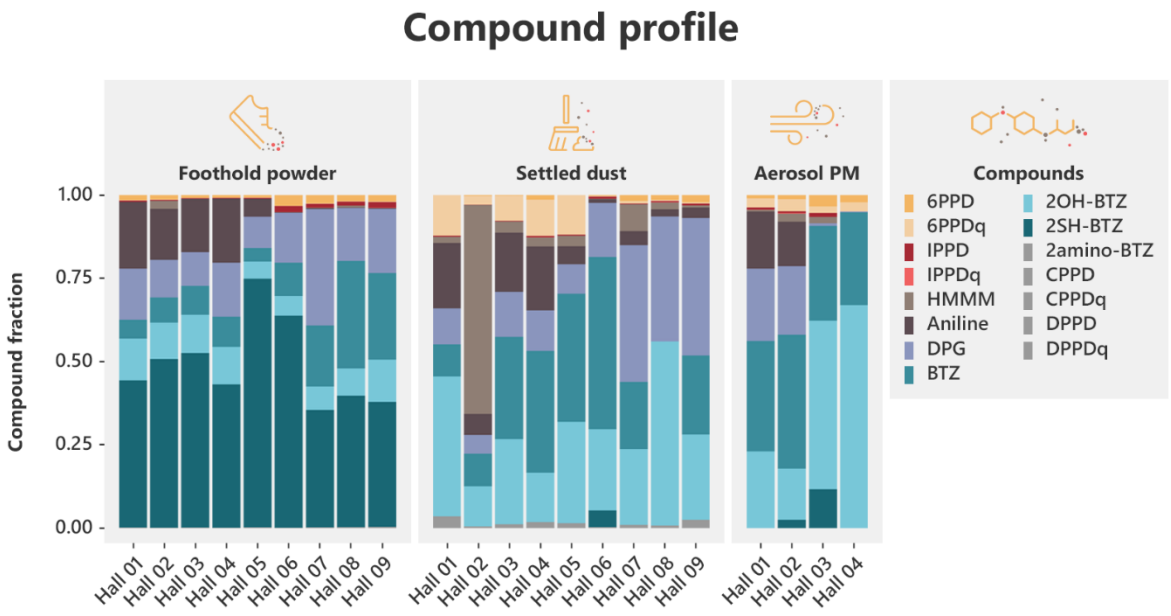


Figure 5.5 Rubber-derived compound profile shift. Rubber-derived compound profile in foothold powder, settled dust, and aerosol PM. Since the three sample types are heterogeneous, and contain different rubber contents, the absolute RDC concentrations cannot be directly compared between sample types. Thus, individual compound concentrations were normalized to the sum of RDC concentrations detected in each sample. Foothold powder and settled dust compound profiles for each hall represent an average of triplicate samples, aerosol PM represents an average of the URT and LRT fractions. Individual, absolute concentrations for each sample are presented in Figures S5.1, S5.6, S5.7 and Table S5.2, and individual compound profiles for all samples are presented in Figure S5.4. A shift in chemical profile was observed from foothold powder to settled dust and aerosol PM samples.

Transformation of 2SH-BTZ is well studied in the aquatic environment[28], where 2OH-BTZ and BTZ are frequently reported as transformation products, including when transformation is induced by ozone[238,239]. In our ozonation experiments, the mean concentration of 2SH-BTZ decreased from 78 µg/g to 23 µg/g, while the mean concentrations of 2OH-BTZ and BTZ increased from 15 µg/g to 17 µg/g, and 13 µg/g to 39 µg/g respectively. Likewise, it is well documented that PPDs react with ozone to form PPD-quinones[30,31,170], and the 6PPDq/6PPD ratio in crumb rubber has been related to the extent of environmental weathering[100]. In our ozonation experiments, the mean 6PPDq/6PPD ratio increased from 0.04 to 0.12, and the mean IPPDq/IPPD ratio from 0.02 to 0.04. These changes were driven by decreasing

6PPD and IPPD concentrations; 6PPDq and IPPDq showed no obvious concentration changes. This likely reflects a balance between continuous formation and further transformation of 6PPDq and IPPDq, as it is known that 6PPDq is not a stable end product of 6PPD ozonation, but is further transformed[123]. The drops in 6PPD and IPPD concentrations likely indicate formation of many transformation products other than 6PPDq and IPPDq[30,123,170]. As our ozonation experiments were carried out at substantially higher ozone concentrations than are expected in climbing halls, the transformations observed in our environmental samples may have been induced by other reactive species, such as NO_x or hydroxyl radicals. These experiments in conjunction with the body of literature reporting these ozone-induced transformations indicate that the observed shift of RDC profile in our samples results from atmospheric transformations of the RDCs after generation of the rubber particles on the climbing holds. This explanation is further supported by the fact that we did not find any significant alternative sources of the RDCs to aerosol PM or settled dust in climbing halls.

5.4 Implications

In indoor climbing halls, concentrations of several RDCs significantly exceed previously reported values. Total daily intake of RDCs for individuals visiting or working in these facilities exceeds exposure via all other known routes. Indoor climbing is becoming increasingly popular, with 6.36 million people participating in the sport in the US in 2023 alone[103]. The climbing gym market was valued at 3 billion dollars in 2023 and is expected to reach 6 billion dollars in 2032, worldwide[241]. By the end of 2023, 785 climbing gyms were operational in the US and Canada[104], indicating that climbing facilities worldwide likely employ many thousands of individuals who may be exposed to high RDC concentrations in the workplace. The occupational exposure of employees in the climbing shoe manufacturing sector also warrants further research.

The majority of the URT fraction of the collected aerosol PM would typically deposit in the nose and upper airways, and is subsequently swallowed[242]. Aerosol PM from the LRT fraction has a higher probability of deposition within deeper regions of the lung[243]. Thus, exposure to RDCs in climbing halls will be via both the gastrointestinal and respiratory systems. In our study, we focussed on the sampling of particle-associated RDCs and used an appropriate sampling device and analysis workflow. Due to the physico-chemical characteristics of some RDCs (relatively high volatility and low octanol/air partition coefficient, Table S5.5), it is possible that some compounds would diffuse from the rubber particles and also be present in the gaseous phase in the air of climbing halls. In outdoor air, RDC partitioning was such that BTZ and 2OH-BTZ were mainly found in the gas phase (70 and 95 %, respectively) but DPG, HMMM and all PPDs/PPDQs were mainly found in the particulate phase (> 75 %)[244]. If this partitioning also holds true in indoor environments, the overall (gas + particles) chemical burden in climbing halls air

would substantially increase for BTZ and 2OH-BTZ and the human exposure to these RDCs would be higher than estimated in our study.

Future research needs to address the leaching kinetics of RDCs from collected aerosol PM, ideally in epithelial lung fluid- and gastrointestinal fluid-mimetics. Further, the bioavailability of RDCs following different exposure routes and the toxicological assessment of RDCs should be investigated in greater depth. So far, there is concerning literature on the toxicity of several RDCs; in particular PM-bound PPDs and PPDqs may contribute to the oxidative potential of PM[66]. Oxidative potential of PM induces oxidative stress and inflammation in the respiratory and cardiovascular systems[245]. Indeed, organic tire extracts and tire wear particles have been shown to induce DNA damage, inflammation, and cell death in human lung cells[65,218,219].

It is possible that RDCs from climbing shoes have environmental implications outside of indoor climbing halls. Wastewater from the washing of climbing holds is likely to be contaminated, and may enter municipal sewer systems, or be directly disposed of into the environment. Likewise, contaminated dust collected by air filters or vacuum cleaners likely enters the municipal waste stream. On a very local scale, RDCs from climbing shoes may also contaminate rocks, soils, and air in areas where outdoor climbing is practiced; however, this is probably very limited due to atmospheric dispersion outdoors.

We have shown that from the time of particle generation to the time of entrance into a target organism or environment, the RDC profile can shift substantially, including towards more toxic species, e.g., 6PPD to 6PPDq. Previous work has relied on concentrations of RDCs in unweathered material to estimate exposure[101] or even toxicity[246], but this approach should be treated with caution. In fact, a recent review has called for a need to understand atmospheric transformations of organic RDCs in tire wear particles[247]. Due to elevated ozone and NO_X levels above the road surface, we expect that tire wear particles will initially undergo similar atmospheric transformation reactions to those we observed. Thus, our findings provide insights which can guide future research on the impact of atmospherically aged tire wear particles.

As recently pointed out by Arp et al.[248], a frequent shortcoming of chemical regulation is that it is reactionary and not precautionary. Substances are added to regulation lists only after exposure and ecological harm have been demonstrated, often long after initial red flags. With a global increase in urbanization, we expect to live, work, and recreate in safe indoor environments. Particularly in indoor sports facilities, where respiration increases, air quality standards need to be robust. In this paper, we therefore point to a potential red flag: human exposure to RDC in sporting goods in indoor environments. Rubber formulations containing toxic RDCs should not be used in products that will further increase

human or environmental exposure. Spurred mostly by the ongoing research about tire wear particles, the tire industry is under pressure to find alternatives for some additives, such as 6PPD. Upcoming research and legislation, including the upcoming fifth session (INC-5) of the UNEA-5.2 Intergovernmental Negotiation Committee on Plastic Pollution, must not overlook consumer products, such as climbing shoes, which contain a high additive content, and dominate the human exposure for a subset of the population. A recent study screening RDCs in a multitude of rubber consumer products found that most products had RDC concentrations much lower than those in climbing shoes[100]. This contrast shows that although rubber is widely used, only highly engineered consumer products, such as climbing shoes and tires, contain a high additive content. This knowledge can focus future work to identify hotspots of RDC exposure, but also highlights the global problem of the toxicity burden of plastic additives. Moreover, our findings may indicate a potential issue within the upstream supply chain. To our knowledge, rubber produced for the tire industry is, in some instances, utilized for sporting goods. Additives that enhance the performance of car tires may not be essential for shoe soles. By reducing these additives to those that are essential for specific product properties, it is possible to reduce unnecessary exposure. Until rubber becomes safer, potential strategies to minimize exposure in climbing halls should also be considered. The variability in RDC concentrations in air between the five halls suggests that hall size, check-ins per hour, and ventilation may directly influence air quality, thus, climbing halls might consider optimal ventilation, active management of visitor flow, or other measures such as more frequent cleaning, mobile and stationary HEPA air filters, or banning of certain shoe models.

5.5 Materials and methods

5.5.1 Sample collection and characterization

Four types of samples were collected: aerosol PM, settled dust, shoe soles, and foothold powder samples (Figure 5.1). Complete sample sets (LRT and URT fractions of aerosol PM, triplicate settled dust, and triplicate foothold powder samples) were collected in five halls in Vienna between March 2023 and April 2024. These five halls vary in size, age, number of visitors, and ventilation (Table S5.1). To assess the levels of RDCs more broadly, additional samples (triplicate settled dust and triplicate foothold powder samples) were collected in four more halls, in Switzerland, France, and Spain. In addition, thirty shoe sole samples representing major brands and models were obtained as donations from climbers, or from a shoe resoler. Details about the climbing halls are provided in Table S5.1. All samples were collected between February 2023 and June 2024.

PM in both aerosol fractions was collected with a standardized glass liquid impinger (Copley Scientific Ltd), which is an active sampling device that separates aerosol PM into aerosols which predominantly deposit in the upper respiratory tract (> 6.4 µm aerodynamic diameter) and the fraction

that deposits predominantly in the lower respiratory tract (< 6.4 µm aerodynamic diameter). Pooled aerosol PM samples (i.e., material collected from five daily 3 h sampling runs processing ~11 m^3 air at each run) were collected in Hall 01 and Hall 02 on five consecutive days in April 2023 during peak activity (5 to 8 p.m.). Pooled aerosol PM samples were collected in Hall 03, 04, and 05 on five consecutive days in April and May 2024 during peak activity (5 to 8 p.m.). Peak activity hours were determined based on hourly visitor check-in data from the climbing halls. The glass liquid impinger was operated as follows: Before the device was assembled, 7 mL of MilliQ-water was introduced into the upper chamber and 475 g (=130 mL) of ceramic beads (9730, ZY-P, 2.6-3.3 mm, SiLibeads, Sigmund Lindner GmbH, Warmensteinach, Germany) and 25 mL of MilliQ-water were introduced into the lower chamber (LRT fraction). The air inlet was set at a height of 142 cm, facing the climbing wall at approximately 3 m distance. The air flow rate was 60 ± 2 L/min, for a total volume of 54 m^3 air per sample in each climbing hall. Temperature, humidity, and liquid levels of the upper and lower chambers were monitored every 15 minutes, and liquids were replenished as needed. At the end of each day of sampling, every part of the device was rinsed three times with 6 mL MilliQ-water, and three times with 6 mL ethanol and collected. MilliQ water and ethanol rinses were stored separately at -20°C in amber glass vials until analysis. Samples from the mouth / throat piece and the upper chamber were combined to represent the URT fraction of aerosol PM; samples from the lower chamber were collected separately to represent the LRT fraction of the aerosol PM. Further details about aerosol sampling are provided in Section S5.2.

Settled dust samples were collected from uncleaned floor and wood surfaces 5 to 10 m from the climbing walls (where rubber particles in dust would most likely have been aerially transported, rather than falling directly from the climbing walls). In each hall, settled dust from three distinct locations was sampled to assess spatial variability within the hall. Dust was collected using a clean metallic spatula and immediately placed in cleaned glass vials, then stored at -20°C until further processing.

Thirty shoe sole samples were collected from both used and new climbing shoes to represent the marketplace. Approximately 1 g of rubber was cut out from the tip of the sole using a ceramic knife. The tip of the shoe sole represents the area most susceptible area to abrasion on the climbing holds during use. Samples were cut into 1 mm^2 pieces and ground into fine powder using cryo-ball milling (MM400, Retsch®) for 2 min at 25 Hz. After grinding, 50 mg powder was immediately suspended in 1 mL dichloromethane to prevent re-agglomeration, and then extracted. Clean climbing hold and mat samples were obtained from climbing Hall 02 and extracted via the same procedure as the shoe sole samples.

A cleaned metal spatula was used to collect 1 – 5 g of rubber powder accumulated in the clefts of climbing footholds (foothold powder samples). These foothold powder samples were immediately placed in cleaned glass vials and stored at -20°C until further processing.

Sub-samples of pure solid chalk, foothold powder samples and settled dust samples were coated with a gold nanolayer (10 nm) and visually characterized with a scanning electron microscope (Gemini SEM 300, Zeiss) at various magnification levels.

5.5.2 Sample extraction and measurement

Liquid was removed from the aerosol particulate matter samples via rotary evaporation (ethanol) or lyophilization (MilliQ-water). The residual particle mass was determined gravimetrically using a high precision balance and samples were then resuspended in ethanol. All samples were then extracted with accelerated solvent extraction (details Section S5.3). The selection of RDCs for analyses was based on several criteria such as common use in the rubber industry, diversity in terms of chemical classes, availability of commercial standards for quantitation and potential toxicity for humans. Therefore, the following RDCs were analyzed in all samples with UPLC-MS/MS: benzothiazole (BTZ), 2-hydroxybenzothiazole (2OH-BTZ), 2-aminobenzothiazole (2-amino-BTZ), 2-mercaptobenzothiazole (2SH-BTZ), aniline, 1,3-diphenylguanidine (DPG), hexa(methoxymethyl)melamine (HMMM), and the phenylenediamine compounds: 6PPD, IPPD, CPPD, DPPD and their associated quinones: 6PPDq, IPPDq, CPPDq, DPPDq. Details are provided in the SI regarding the chemicals and internal standards used (Section S5.4), UPLC-MS/MS methods (Section S5.5) and extraction recovery (Table S5.6) for analyses of all samples.

5.5.3 QA/QC

We investigated contamination that may have arisen from different sources. Collection blanks were prepared to assess contamination that could have originated during aerosol particulate matter sampling, subsequent storage, and laboratory processing; storage blanks were prepared to assess contamination originating from sample storage and laboratory processing; and laboratory blanks were prepared to isolate contamination originating during laboratory processing. Collection blanks were collected before each composite sampling event. The glass liquid impinger was assembled in the climbing hall (without air flow), then immediately rinsed with exactly the same protocol as the samples. Storage blanks were also prepared in the climbing hall by filling amber glass storage vials with MilliQ-water or ethanol, and then opening and closing the vials five times, to simulate the collection of samples. Collection and storage blanks were stored and extracted in the same manner as aerosol particulate matter samples. Laboratory

blanks were prepared in the same manner as samples, beginning with accelerated solvent extraction. The total mass of RDCs measured in collection blanks (representing contamination accumulated during sample collection, storage, and laboratory processing) was subtracted from the mass of RDCs measured in aerosol particulate matter samples.

To investigate background levels of RDCs (not arising from climbing activity), we collected reference samples in an office of climbing Hall 02, which was in the same building, but not connected to the climbing area. Sampling procedure and duration (15 hours) were the same as for the air sampling in the climbing areas. Finally, to account for other potential sources of RDCs in climbing areas, we obtained samples of climbing holds, as well as two types of climbing mats from climbing Hall 02. These control samples were extracted with the same procedure as the shoe sole samples.

5.5.4 Exposure calculations

To determine the human exposure to RDCs in climbing halls, estimated daily intake values via inhalation (and ingestion) were calculated using equation (2) for two types of individuals: regular adult climbers and employees working at the halls:

$$EDI_{inh/ing} = \frac{C_{air}\ IR\ ET\ EF}{BW\ Cf} \quad (2)$$

whereby $EDI_{inh/ing}$ is the estimated daily intake *via* inhalation (and ingestion) (ng/kg/day), C_{air} the concentration of RDCs in the total aerosol PM (ng/m^3), IR the inhalation rate (m^3/hour), ET the exposure time (hours/day), EF the exposure frequency (days/year), BW the body weight (kg) and Cf the number of days per year. Details regarding exposure parameters obtained from the US EPA exposure factor handbook[249] are available in Table S5.3. It should be noted that despite our recognition that the URT fraction of aerosol PM will likely be transported rapidly to the gastrointestinal tract following deposition and therefore ingested, the use of Equation 2 still provides a suitable estimate for total RDC exposure from the aerosol PM, because deposition in the URT is still dependent on the inhalation rate of the subject.

5.5.5 Transformation experiments

Ozonation experiments were performed to investigate whether the compound profile shift from foothold powder to aerosol particulate matter and settled dust could be explained by transformations of

the compounds in foothold powder. Foothold powder was collected from climbing Hall 01 immediately before experiment start and divided into six sub-samples. An ozone chamber[240] was employed to expose three sub-samples to elevated ozone concentrations (1 g/m^3) at room temperature for 4 hours in the dark. Ozone concentrations were substantially higher than realistic indoor concentrations, which is typical for ozonation experiments[30,170], and deemed necessary due to the higher particle size, and lower surface area to volume ratio of foothold powder samples compared to aerosol particulate matter. Elevated ozone concentrations enabled fast aging tests, as is typical in many fields of environmental health and safety. NO_X concentration was also measured to be 9 ppm during the experiment. After ozonation, all sub samples were spiked with internal standards, and extracted and measured as described above.

Chapter 6: General Conclusions and outlook

6.1 Conclusions and Implications of this PhD Thesis

In the detection study (*Chapter 4: Uptake of tire-derived compounds in leafy vegetables and implications for human dietary exposure*), we measured rubber-derived compounds at trace concentrations in edible produce. This provides conclusive evidence that multiple rubber-derived compounds are present in the agricultural environment, and are taken up by edible plants, entering our food chain.

The concentration of rubber-derived compounds which accumulate in leafy vegetables is influenced by uptake and translocation processes, bio-accessibility, and plant metabolism. Of the five rubber-derived compounds we studied in detail, sorption to roots was low (K_D between 0.05 and 0.7 L g−1), indicating that the compounds could move from roots to leaves passively. Partitioning between root tissue and the transpiration stream determines the extent of passive translocation. The translocation factors in the hydroponic study and bioconcentration factors in the soil study follow the order of HMMM>DPG>BTZ>6PPD≈6PPD-quinone, which is inversely related to the compounds' lipophilicity (log K_{OW}), with speciation in various cellular compartments also playing an important role. Although the two uptake studies were conducted under very different experimental conditions, this trend in translocation was consistent, and is probably generally true for leafy vegetables. However, translocation of rubber-derived compounds might vary in other plants due to variations in root lipid contents[250], transpiration rates, or relative contribution of active transport pathways.

Passive translocation via the transpiration stream seems to be the dominant uptake process for rubber-derived compounds, based on evidence from the hydroponic and soil studies. Higher and more variable concentrations of all compounds in outer compared to inner lettuce leaves implicate transpiration-dependent translocation. Furthermore, rubber-derived compound accumulation in lettuce was strongly influenced by bioaccessible rubber-derived compound concentrations in both the hydroponic and soil studies. The amount of rubber-derived compounds which leached from tire wear particles into hydroponic medium determined rubber-derived compound accumulation in lettuce, which differed from lettuce exposed to a single spike of rubber-derived compounds. In the single-spike experiments, concentrations in the plants first increased then decreased, due partly to substrate depletion in the hydroponic medium, while concentrations in plants exposed to tire wear particles increased throughout the experiment due to continuous leaching. In soil-grown lettuce, the formation of non-extractable residues reduced bioaccessibility of HMMM and 6PPD, limiting their accumulation in lettuce, while higher

bioaccessibility of DPG and 6PPD-quinone enabled relatively high accumulation. 6PPD, due to its low bioaccessibility and low translocation factor, was measured at very low concentrations in the plants. The accumulation of all rubber-derived compounds in lettuce was lower in lettuce grown in soils with a higher clay content, due to lower bioaccessibility compared to lettuce grown in sandy soils. Overall, in both the hydroponic and soil studies, rubber-derived compound accumulation in lettuce leaves scaled with bioaccessible rubber-derived compound concentrations (concentration independent uptake, which is characteristic of passive uptake). The roles of passive and active transport pathways in the accumulation of rubber-derived compounds in lettuce could be confirmed with the use of pathway inhibitors[95,115,121], multiple rubber-derived compound dosing levels[115], or transpiration stream quantification[96,115,163].

Plants metabolize the tire-derived compounds we studied. Due perhaps to their secondary amine groups, 6PPD and DPG may be directly conjugated, which contributed to their rapid transformation in the hydroponic study. Indeed, almost all potential transformation products of DPG and 6PPD identified in the hydroponic and soil studies were likely conjugates, as evidenced by their higher mass to charge ratios. On the other hand, almost all potential transformation products of HMMM identified in the hydroponic and soil studies had lower mass to charge ratios and retention times than HMMM, indicative of Phase I transformations. Benzothiazole was also rapidly hydroxylated (a Phase I transformation) in the hydroponic study. While benzothiazole concentration in lettuce plants decreased within several hours, HMMM concentration first decreased after 10 days, which suggests that Phase I transformations might be rate limiting in some cases, but rapid in others. This is likely related to compound structure, and incorporation into existing plant metabolic pathways[96]. Rapid metabolism of benzothiazole could explain why it was not detected in the soil study. In the soil study, smaller, more polar transformation products (Phase I metabolites) were generally collocated with their parent compounds in lettuce, while larger, less polar transformation products (Phase II metabolites) were not. This could be due to the kinetics of Phase III metabolism, or mobility of Phase II metabolites within the plant. While some transformation products observed in the hydroponic study were stable within the plants, a glucose conjugate of 6PPD decreased in concentration after several days, which could be due to excretion or further metabolism.

The findings from the hydroponic and soil studies do not explain the results from the detection study. The rapid metabolism of BTZ observed in the hydroponic study, and the lack of any detectable BTZ in the soil study would suggest that BTZ is not likely to be detected in environmental lettuce samples – however, BTZ was detected in 43% of samples, at up to 238 ng/g dry weight. Low translocation of 6PPD was observed in both the hydroponic and soil studies, and low bioaccessibility of 6PPD was observed in the soil study, however, 6PPD was detected in 25% of samples, albeit at trace concentrations of less than 1 ng/g. Benzothiazole and 6PPD are among the compounds present in highest concentrations in tire

rubber, and benzothiazole has applications beyond rubber. Therefore, the detection of BTZ and 6PPD in environmental samples could be due simply to higher concentrations of these compounds in the agricultural environment. Studies quantifying rubber-derived compounds in agricultural environments are still missing, as are studies investigating the relative importance of the different pathways by which rubber-derived compounds reach the agricultural environment. A further explanation for the discrepancies between the results from the mechanistic and environmental studies could be that alternative uptake pathways, such as foliar uptake, are relevant for these rubber-derived compounds.

Environmental transformations shift the set of rubber-derived compounds which humans are exposed to via food and air, as evidenced in the hydroponic, soil, and air studies. Phase I and Phase II metabolism in plants transformed the rubber-derived compounds which were taken up into lettuce, and atmospheric transformations shifted the rubber-derived compound profile of airborne rubber particles in indoor climbing gyms. In all three plant studies (chapters 2-4), nontarget approaches were used to search for transformation products of rubber-derived compounds in plants, with mixed success. Due to the high concentrations of rubber-derived compounds in the plants in the hydroponic study, many transformation products were identified, and annotated with varying confidence levels. In the soil study, potential transformation products were also identified, but annotations were generally at lower confidence levels. As parent compounds were already in plants at much lower concentrations in the soil study compared to the hydroponic study, it is likely that many transformation products present at trace levels were missed. More replicates could have increased the statistical power of the differential analyses used to identify up-regulated transformation products. Due to the inherent difficulties of nontarget screening of environmental samples (trace concentrations, and lack of controlled experimental design which would enable statistical analyses of different sample groups), the detection study generally failed to identify transformation products of rubber-derived compounds. Based on these studies, it is recommended that hydroponic, or even cell culture studies[132] are used to discover (non-target) transformation products formed in plants. The transformation products identified with such studies should be used to conduct suspect screenings, instead of non-target analyses, to check for transformation products in more realistic experiments and environmental samples. Target analyses can also provide critical insights into transformation processes, as evidenced in the air study. Although many transformation products were likely formed outside of what we observed with our target approach, the increased sensitivity of target screening with a triple quadrupole mass spectrometer was invaluable in measuring transformation products such as 6PPD-quinone in airborne particulate matter samples.

Estimated daily intake of rubber-derived compounds via leafy vegetable ingestion, and via inhalation in indoor climbing gyms were calculated in chapters 4 and 5. The estimated daily intake due to leafy

vegetable ingestion is generally lower than, or comparable to other exposure pathways reported in the literature, such as dust ingestion, drinking water, and inhalation of outdoor air, although leafy vegetables represent only a fraction of total dietary intake. Estimated daily intake via inhalation of rubber-derived compounds for climbers and climbing hall employees exceeds other exposure pathways reported in the literature, including via inhalation for roadside workers[251]. This finding demonstrates that consumer rubber products other than tires may be highly relevant for human exposure to rubber-derived compounds.

In their recent report, the United Nations identifies two criteria which designate a plastic product of particular concern: one, the product must contain chemicals of concern, and two, the product must be used in a way that causes human or environmental exposure[13]. Not all consumer rubber products fulfill the first criteria, because additive content is generally related to the material demands of a product[100]. Highly engineered products, such as climbing shoes, contain high loads of chemical additives, including chemicals of concern, such as 6PPD. Climbing shoes also fulfill the second criteria, since they abrade in an enclosed indoor environment where human exposure is certain. Although sports products are not identified as a priority sector by the United Nations report, the work presented in chapter 5 may inspire future research that could identify other hotspots of rubber-derived compound, or other chemical additive exposure. The findings should have implications for regulation and industry developments – as the tire industry comes under societal and legislative pressure to develop safer rubber formulations, consumer products must also be included in this process. Although consumer products, such as climbing shoes, represent a minor share of the rubber market, they may be of disproportionally large significance regarding human exposure for some of the population.

6.2 Open Research Questions

More detection studies are needed to quantify TRWP and rubber-derived compounds in the agricultural environment. There have been several large-scale screening studies of rubber-derived compounds in urban air[17,18], surface waters[17,33,193,252], and roadside soils[17,253], but as of yet none in the agricultural environment. Detection studies should be complemented with modeling work, to gain an understanding of how TRWP and rubber-derived compounds enter the agricultural environment via different pathways, such as biosolids application[25] and treated wastewater irrigation.

Furthermore, several open questions remain regarding the mechanisms behind bioaccessibility, uptake, distribution and metabolism of rubber-derived compounds within plants. The active and passive uptake processes which drive plant uptake and translocation of rubber-derived compounds should be confirmed experimentally. Foliar uptake of rubber-derived compounds should also be investigated as an alternative uptake route. Further studies of rubber-derived compound transformation pathways within

plants are needed, with emphasis on structural elucidation of transformation products. Studies should also investigate the formation and distribution of Phase III metabolites, potentially employing ^{14}C labelled compounds to quantify non-extractable, bound residues. The uptake, distribution, and metabolism of rubber-derived compounds should be investigated in different plants, including root and fruit crops. Finally, mechanistic studies are needed to elucidate the sorption and transformation processes which reduce bioaccessibility of rubber-derived compounds in soils.

The effects of rubber-derived compounds on exposed plants, or on agricultural soils were not addressed within this thesis. In general, there is still very little research on the effects of TRWP and rubber-derived compounds to soils[254], other than effects to soil fauna.[255,256]. The effects of TRWP and rubber-derived compounds on agricultural soil microbiomes should be investigated, which may also have consequences for plant metabolism and health[257]. Organic contaminants have been shown to disrupt various plant metabolic processes[154,258–260] Metabolic shifts can lead to phenotypic changes in plants- for example, amino acids can be reallocated from protein synthesis to detoxification pathways, reducing the overall protein content of plants[260], and decreased auxin contents can lead to a decrease in biomass[259]. Therefore, plant uptake of rubber-derived compounds may have consequences for the dietary value of edible crops, aside from the obvious concerns associated with contaminant exposure. In general, as global agricultural systems come under more pressure to feed a growing population, it is important to study the effects that global changes, including novel entities pollution, have on the value and productivity of agricultural systems.

The research in this thesis mainly focused on a subset of sixteen rubber-derived compounds – however, there are hundreds of leachable organic rubber-derived compounds[12]. Effect-directed analyses[31,44] may be used to identify compounds which should be prioritized for research. However, these analyses are complicated and time consuming. Transparency from the rubber industry regarding rubber formulations at all levels of the supply chain would greatly facilitate research on the exposure and effects of rubber-derived compounds. However, complete transparency is not sufficient, since non-intentionally added substances, such as transformation products and impurities may also be chemicals of concern[13], as is the case for 6PPD-quinone. Collaboration between the rubber industry, researchers, and regulators may facilitate precautionary identification of non-intentionally added chemicals of concern, and enable the rubber industry to develop safer rubber formulations.

Although human exposure to rubber-derived compounds via dietary and inhalation pathways is evident, little is known about the behavior of rubber-derived compounds in the body. In the air study, we measured rubber-derived compound concentrations in the particulate fraction of inhaled air. Inhaled particles with higher (>6.4 µm) aerodynamic diameters are likely to settle in the upper respiratory tract,

and be swallowed, while particles with a smaller aerodynamic diameter may settle in the lungs. Thus, exposure to particle-associated rubber-derived compounds will be via both the digestive and respiratory tracts. In ongoing work, we are conducting leaching experiments with respirable dust particles in simulated lung fluids, to investigate the release and bioaccessibility of rubber-derived compounds. Follow up work should investigate the same processes within the human digestive system, similar to work which has been conducted in fish[234]. The bioavailability of rubber-derived compounds should also be investigated, by quantifying the amount of accessible compounds which are taken up by cells. Furthermore, the ability of rubber-derived compounds to cross epithelial membranes will determine whether they are translocated from the respiratory and digestive systems into the blood stream. In this case, exposure would be systemic, which could have a wide range of health consequences. Metabolism of rubber-derived compounds within the human body should also be investigated.

Mechanistic investigations should be complemented with robust biomonitoring studies. Since a majority of rubber-derived compounds will likely be transformed in the human body, biomonitoring studies should take transformation products into account in their screenings. Biomarkers such as carboxy products of PPD-quinones[261], may be used to identify exposure to compounds.

The toxicity of rubber-derived compounds, primarily in aquatic organisms, is currently receiving a lot of research attention. The work presented in this thesis contributes to emerging evidence that humans are exposed to rubber-derived compounds, which should inspire more mammalian toxicity studies. There are many ways to investigate toxicity: in ongoing work, we are conducting classic in-vitro experiments to assess the cytotoxicity of lung cells exposed to rubber-derived compounds which are leached from respirable road and climbing hall dust. The toxicity of known leachable rubber-derived compounds may be mechanistically evaluated with standard dose-response experiments; however, the toxicity of unknown rubber-derived compounds, as well as the mixture toxicity of the many leachable rubber-derived compounds must also be considered. Additionally, non-traditional techniques, such as metabolomic screenings, may be employed to uncover biological effects of rubber-derived compounds before phenotypic changes are observed.

Human exposure to rubber-derived compounds may be mitigated via creative development of best-practice solutions. Particularly, case-specific application of biosolids and recycled wastewater irrigation may reduce the risk of introducing contaminants into the food chain. For example, 6PPD and 6PPD-quinone accumulate in lettuce roots, so these practices are not recommended in fields where root vegetables are produced. On the other hand, many organic compounds are not phloem mobile, so are not transported into fruits. Phloem mobility and accumulation of rubber-derived compounds in fruits should be confirmed experimentally, but biosolids application and recycled wastewater irrigation might be of

lower risk in fruit production. Soil properties play an important role in determining the bioaccessible concentrations of rubber-derived compounds for plant uptake – recycled wastewater irrigation should be avoided in sandy soils, where most compounds will be bioaccessible, but might be of less concern in soils with a high clay content. However, it must be considered that non-extractable residues can slowly be re-released and become bioaccessible to plants[158], particularly if the soil matrix is altered, for example by plant roots[166], or changed redox conditions following flooding. Biosolids application and recycled wastewater irrigation are important solutions to maintain agricultural productivity in a changing climate. However, careful evaluation of when and where these practices actually make sense is necessary to mitigate the introduction of wastewater-derived contaminants into our food chain

Likewise, the differences in measured airborne rubber-derived compound concentrations between the different climbing halls in the air study suggest that best practices within climbing halls may reduce rubber-derived compound exposure. In an ongoing citizen science project, volunteer climbers and climbing hall owners are mailing us samples from their climbing gyms, along with a survey about ventilation systems in the gyms, number of visitors, size and age of the gym, etc. With a large data set, we hope to statistically evaluate which factors influence rubber-derived compound concentrations in climbing gyms. In addition, effect-based analyses combined with non-target screenings to identify bioavailable and toxic rubber-derived compounds should be conducted in collaboration with the climbing shoe industry, in order to prioritize chemicals for replacement in rubber formulations.

Supporting information

S2: Supporting Information to Chapter 2

Table S2.1: Physicochemical properties of the TWP-derived compounds investigated in this study, including structure, exact mass (g mol^{-1}), dissociation constant (pK_a), and octanol-water partition coefficient ($logK_{ow}$). N/A: not available

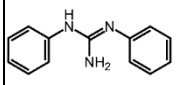
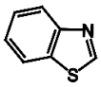
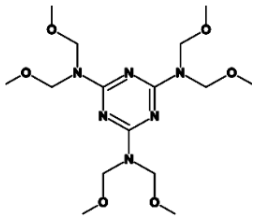
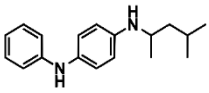
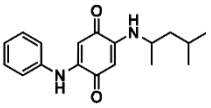
Compound	DPG	BTZ	HMMM	6PPD	6PPD-quinone
Structure					
Exact mass (g mol^{-1})	211.11034	135.01403	390.22129	268.19311	298.16702
pK_a	10.12[262]	7.8[263]	7.01[27]	6.7[168]	N/A
$logK_{ow}$	2.9[262]	2[264]	1.6[109]	5.6[31]	5-5.5[31]

Table S2.2: Extraction test recoveries in % for nutrient solution and plant material

Matrix	Tested concentration [µg L^{-1}]	DPG	BTZ	HMMM	6PPD	6PPD-q
Nutrient solution	50	103	87.2	91.6	89.3	104
	500	91.8	97.9	87.7	91.1	94.3
Plant material	20	96.6	103	92.1	90.8	87.6
	100	95.4	93.5	95.9	96.1	102

Section S2.1: Triple quadrupole-MS analysis

The TWP-derived compounds extracted with acetonitrile were analyzed by ultra-performance liquid chromatography - triple quadrupole mass spectrometry (Agilent 1290 Infinity II - Agilent 6470, hereafter: triple quadrupole-MS) using a C18 column (Acquity HSS T3, 1.8 µm, Waters) in the positive ionization mode (capillary voltage: 2500 V) via multiple reaction monitoring (MRM). The LC flow rate was 0.6 mL min-1 at a column temperature of 40 °C and the injection volume was 1 µl. The mobile phase consisted of ultrapure water (Phase A) and acetonitrile (Phase B), both containing 0.1% formic acid. The eluent gradient was held at 95% Phase A for one minute, then set that the contribution of Phase A decreased from 95 to 5% over 7 min, was held at 5% for 1 min and increased again to 95% over the last 2 min. Electrospray ionization (ESI) ionization was achieved at gas temp 240oC, gas flow 5 Lmin-1. The nebulizer was set to 30 psi; sheath gas to 250oC, 11 Lmin-1. Quantification was achieved by external calibration standards prepared in acetonitrile (0.1 to 1500 µgL-1). Transitions from precursor ions to product ions are specified in Table S2.1.

Table S2.3: Method specifications for triple quadrupole-MS measurements

Compound name	Precursor ion m/z	Product ion m/z	Fragmentor voltage (V)	Collision energy (V)	Cell accelerator voltage (V)	Retention time (min)
6PPD	269	184	150	45	5	3.34
6PPD	269	107	150	45	5	3.34
6PPD	269	93	150	45	5	3.34
6PPD-quinone	299	256.1	112	23	5	5.14
6PPD-quinone	299	241	112	31	5	5.14
6PPD-quinone	299	215	112	15	5	5.14
6PPD-quinone	299	187	112	31	5	5.14
Benzothiazole	136	109	150	31	5	2.72
Benzothiazole	136	77	150	27	5	2.72
Benzothiazole	136	65	150	38	5	2.72
DPG	212	195	150	20	5	1.7
DPG	212	119	150	20	5	1.7
DPG	212	94	150	20	5	1.7
HMMM	391	283	150	15	5	3.17
HMMM	391	253	150	23	5	3.17
HMMM	391	207	150	19	5	3.17
HMMM	391	177	150	35	5	3.17

Section S2.2: Orbitrap-HRMS analysis

The leaf extracts were further analyzed by high-performance liquid chromatography – Orbitrap high resolution mass spectrometry (Thermo Scientific Ultimate 3000 – Thermo Scientific Q Exactive™, hereafter: Orbitrap-HRMS) The liquid chromatography was operated using an LPG-3400SD pump. Phase A was ultrapure water with 0.1% formic acid. Phase B was acetonitrile with 0.1% formic acid. Phase A was held at 95% for one minute, then decreased to 5% over 11 minutes. It was then held at 5% for 2 minutes, before increasing back to 95% over 2 more minutes. 5 minute equilibration time was included between each sample. Autosampling was performed with a WPS-3000 autosampler. The draw speed was 0.5 μLs^{-1}, with a 0.003 s draw delay. Dispense speed was 8.333 μLs^{-1}. The same C18 column (Acquity HSS T3, 1.8 µm, Waters) as from the triple quadrupole-MS was used and maintained at 40 ºC with a TCC-3000 column oven.

The Q Exactive – Orbitrap HRMS (Thermo Scientific, Vienna, Austria) was operated in Full MS / dd-MS^2 mode (exact parameters to be found in Table S2.2). An initial screening run was performed on six extracts of control plants, and on six extracts of the spiked plants harvested after 14 days. The Compound Discoverer software (Thermo Scientific, Vienna, Austria) was used to annotate compounds with an intensity ratio of less than 5 between the control and spiked plants as background compounds. This list of background compounds was exported and used as an exclusion list for the following measurements. All control leaf extracts and spiked leaf extracts from all time points were measured with the Orbitrap-HRMS and analyzed with Compound Discoverer.

Table S2.4: Method specifications for Orbitrap-HRMS measurements

Orbitrap-HRMS Parameters	
General	
Runtime	1.5 to 11 min
Polarity	Positive
Default charge state	1
Full MS	
Resolution	70,000
AGC target	$3x10^6$
Maximum IT	100 ms
Scan range	70 to 1000 m/z
dd-MS^2	
Resolution	17,500
AGC target	$2x10^4$
Maximum IT	120 ms
Loop count	5
TopN	5
Isolation window	0.4 m/z
(N)CE / stepped nce	20, 30, 40
dd Settings	
Minimum AGC target	$1x10^2$
Intensity threshold	$8.3x10^2$
Exclude isotopes	On
Dynamic exclusion	5.0 s
If idle…	Pick others

Section S2.3: Compound Discoverer analysis

Compound Discoverer 3.1.1.12 was used. Two separate workflows were applied, one for the Molecular Networks analysis, and one for the Expected Compounds analysis. Both workflows, and their associated parameters are presented in Figure S5.1 and Figure S5.2, respectively. Molecular networking was used to identify structurally related compounds producing common fragments (see also the main text section 2.4). Transformation products of the parent compounds were then inferred based on the MS2 spectra, and in some cases, by comparison with the list of expected compounds. *For example,* the shared fragments of DPG and its detected transformation products, i.e., $TP_{DPG}266$ (proposed molecular formula: $C_{16}H_{15}N_3O$), $TP_{DPG}268$ (proposed molecular formula: $C_{15}H_{13}N_3O_2$), and $TP_{DPG}270$ (proposed molecular formula: $C_{15}H_{15}N_3O_2$) support their structural relation to DPG. These transformation products had masses higher than the parent compound, implying conjugation reactions. For $TP_{DPG}268$, the exact monoisotopic mass of DPG was found in the MS2 spectra. This implies that the overall structure contained an intact DPG molecule covalently linked to an endogenous biomolecule, which was then fragmented by Orbitrap-HRMS ion fragmentation, producing DPG as a fragment.[132] For $TP_{DPG}270$, the exact precursor mass matches the predicted mass for acetate conjugation, suggesting acetate as the endogenous ligand. Following the same line of reasoning, we suggest that the transformation product of 6PPD that we measured is a 6PPD-glucose conjugate.

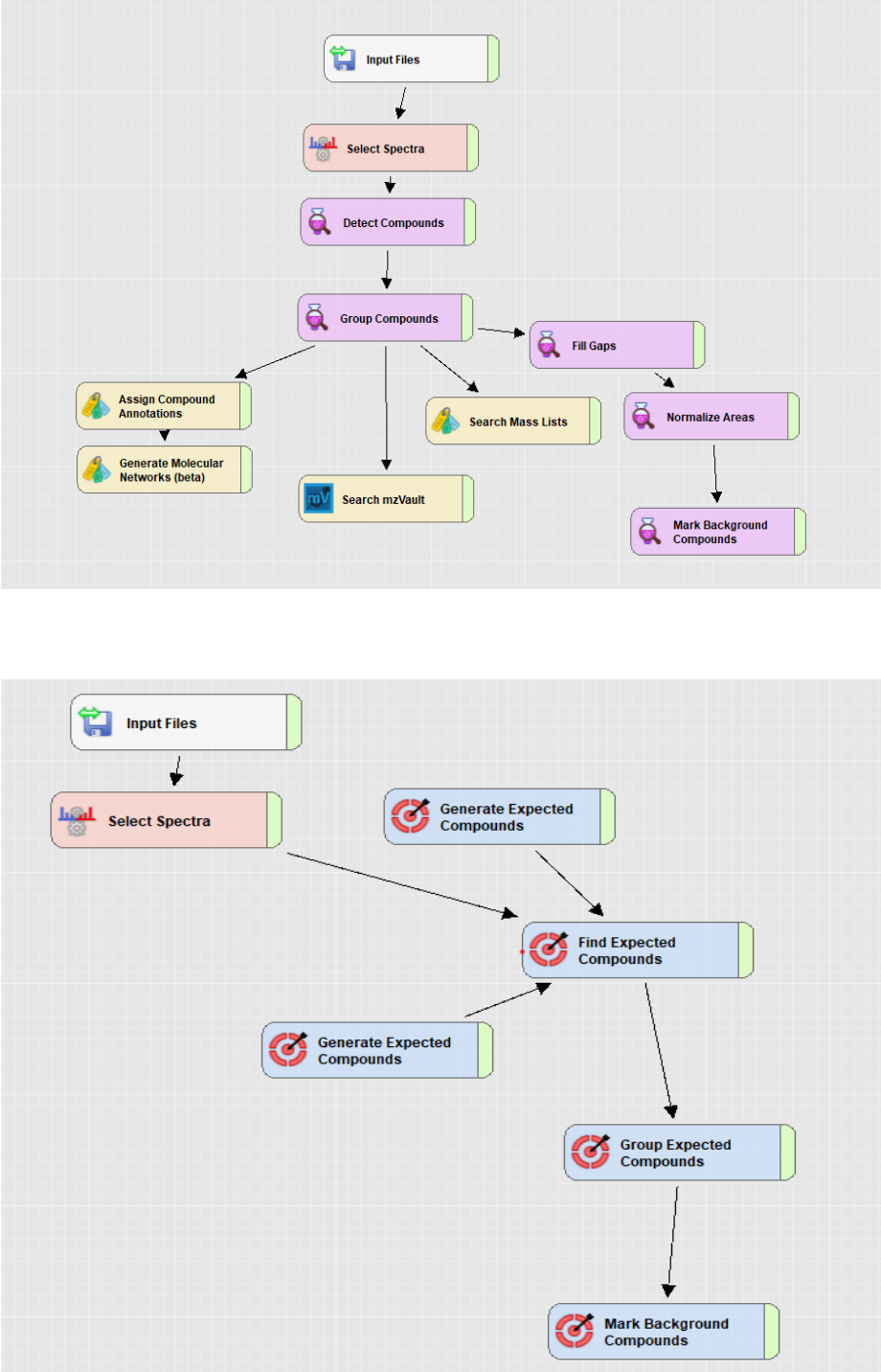


Figure S2.1: Compound Discoverer workflows used for the Molecular Networking approach (top) and Expected Compounds approach (bottom).

Table S2.5: Compound Discoverer parameters

Detect Compounds		**Generate Expected Compounds**	
Mass Tolerance	5 ppm	Max # Phase I	1
Intensity Tolerance	30	Max # All Steps	3
S/N Threshold	4	Phase 1 (all compounds)	H2 O -> H2 -> H O -> H ->
Min. Peak Intensity	100000	Phase 1 (HMMM)	C H3 -> H
Ions	[M+H]+1; [M+K]+1; [M+Na]+1	Phase 2	See Table S2.4
Group Compounds		**Find Expected Compounds**	
Mass Tolerance	10 ppm	Mass Tolerance	5 ppm
RT Tolerance	0.2 min	Intensity Tolerance	30
Fragment Data Selection	[M+H]+1; [M-H]-1	Intensity Threshold	0.1
Fill Gaps		SN Threshold	3
Mass Tolerance	5 ppm	Min # Isotope	2
S/N Threshold	1.5	Min. Peak Intensity	100000
Normalize Areas			
Min. QC Coverage	50%		
Max QC Area RSD	30%		
Mark Background Compounds			
Max. Sample/Blank	5		
Generate Molecular Networks			
Use Full MSn Tree	True		
Match Mass Shift	True		
Match Transformation	True		
Variate Transformation	False		
S/N Threshold	3		
Mass Tolerance	2.5 mmu		
Min. Fragment m/z	50		
Require MS2	True		
Min. MSn Score	20		
Min. MSn Coverage	50		
Min. Matched Fragments	2		

Table S2.6: Transformations for generating expected compounds

Name	Leaving Group	Arriving Group	ΔM [Da]	Phase	Max Occurrence
Acetamide addition		C2 H5 N O	59.03711	Phase2	1
Acetate addition		C2 H3 O2	59.0133	Phase2	1
Acetylation	H	C2 H3 O	42.01056	Phase2	1
Alanine addition		C3 H7 N O2	89.04768	Phase2	1
AQC derivatization		C10 H6 N2 O	170.048	Phase1	1
Arginine Conjugation	H O	C6 H13 N4 O2	156.1011	Phase2	1
C6H10O3 unknown addition		C6 H10 O3	130.063	Phase2	1
CH2O addition		C H2 O	30.01056	Phase2	1
CH4O addition		C H4 O	32.02621	Phase2	1
CO addition		C O	27.99491	Phase2	1
CO+H2O		C H2 O2	46.00548	Phase2	1
CO2 addition		C O2	43.98983	Phase2	1
Cysteine Conjugation 1	H	C3 H6 N O2 S	119.0041	Phase2	1
Cysteine Conjugation 2		C3 H7 N O2 S	121.0198	Phase2	1
Dehydration	H2 O		-18.0106	Phase1	2
Deoxy hexoside		C6 H10 O4	146.0579	Phase2	1
Desaturation	H2		-2.01565	Phase1	3
Formic acid addition		C H2 O2	46.00548	Phase2	1
Gallic acid addition	H2 O	C7 H6 O5	152.011	Phase2	1
Glucoronic acid addition		C6 H10 O7	194.0427	Phase2	1
Glucose addition		C6 H12 O6	180.0634	Phase2	1
Glucose_glucose		C12 H20 O10	324.1057	Phase2	1
Glucoside Conjugation	H	C6 H11 O5	162.0528	Phase2	1
Glucuronide addition		C6 H8 O6	176.0321	Phase2	1
Glucuronide Conjugation	H	C6 H9 O6	176.0321	Phase2	1
Glutamine Conjugation	H O	C5 H9 N2 O3	128.0586	Phase2	1
Glycine addition		C2 H5 N O2	75.03203	Phase2	1
Glycine Conjugation	H O	C2 H4 N O2	57.02146	Phase2	1
GSH Conjugation 1		C10 H15 N3 O6 S	305.0682	Phase2	1
GSH Conjugation 2		C10 H17 N3 O6 S	307.0838	Phase2	1
H2CN addition		C H2 N	28.01872	Phase2	1
HCN addition		C H N	27.0109	Phase2	1
HMMMtrans_demethylation	C H3	H	-14.0157	Phase1	6
HSO3 addition		H O3 S	80.96464	Phase2	1
Ketene addition		C2 H2 O	42.01056	Phase2	1

acetyl-alanine conjugation	H	C5 H8 N O3	129.0426	Phase2	1
173 conjugation	H	C6 H8 N O5	173.0324	Phase2	1
alanine conjugation	H	C3 H6 N O2	87.03203	Phase2	1
C12H13NO6 addition		C12 H13 N O6	267.0743	Phase2	1
Malonyl + glucose		C9 H12 O8	248.0532	Phase2	1
Malonyl + glucosyl		C8 H10 O7	218.0427	Phase2	1
Malonyl addition		C3 H2 O3	86.00039	Phase2	1
Methylation	H	C H3	14.01565	Phase2	1
N-acetilglucosamina		C8 H13 N O5	203.0794	Phase2	1
N-acetylcysteine		C5 H9 N O3 S	163.0303	Phase2	1
NH3 addition		H3 N	17.02655	Phase2	1
NO addition		N O	29.99799	Phase2	1
NO2 addition		N O2	45.9929	Phase2	1
OH removal	H O		-17.0027	Phase1	1
Ornitine Conjugation	H O	C5 H11 N2 O2	114.0793	Phase2	1
Palmitoyl Conjugation	H	C16 H31 O	238.2297	Phase2	1
Reduction	H		-1.00783	Phase1	2
Stearyl Conjugation	H	C18 H35 O	266.261	Phase2	1
Taurine Conjugation	H O	C2 H5 N O3 S	105.9963	Phase2	1
Unknown addition C5H10N2O3		C5 H10 N2 O3	146.0691	Phase2	1
Unknown addition C7H10O3		C7 H10 O3	142.063	Phase2	1
Unknown C10H17N3O6S		C10 H17 N3 O6 S	307.0838	Phase2	1
Unknown C6H9O8S		C6 H9 O8 S	241.0018	Phase2	1
Unknown C8H11O9S		C8 H11 O9 S	283.0124	Phase2	1
Unknown C8H14N2O5S		C8 H14 N2 O5 S	250.0623	Phase2	1
Unknown C8H15NO6		C8 H15 N O6	221.0899	Phase2	1
Unknown C9H14O9		C9 H14 O9	266.0638	Phase2	1
Xylose addition	H2 O	C5 H10 O5	132.0423	Phase2	1

Table S2.7: Concentration of TWP-derived compounds per unit biomass in lettuce leaves: Results of Tukey's HSD multiple comparisons across compounds for each time point. Different lowercase letters indicate statistically significant differences between compounds ($p<0.05$).

a) spiked compounds

	DPG	BTZ	HMMM	6PPD	6PPD-q
3 hrs	cd	a	b	d	bc
6 hrs	d	a	b	cd	c
12 hrs	b	b	a	b	b
1 day	b	b	a	b	b
2 days	b	b	a	b	b
4 days	b	b	a	b	b
7 days	b	d	a	cd	bc
10 days	b	c	a	c	c
14 days	b	b	a	b	b

b) tire leachate

	DPG	BTZ	HMMM	6PPD	6PPD-q
3 hrs	a	b	b	b	b
6 hrs	a	c	b	c	c
12 hrs	a	b	a	b	b
1 day	b	c	a	c	c
2 days	b	b	a	b	b
4 days	b	b	a	b	b
7 days	b	b	a	b	b
10 days	b	b	a	b	b
14 days	b	b	a	b	b

Table S2.8: Concentration of TWP-derived compounds per unit biomass in lettuce leaves: Results of Tukey's HSD multiple comparisons across time points for each compound. Different lowercase letters indicate statistically significant differences between time points ($p<0.05$).

a)spiked compounds

	3 hrs	6 hrs	12 hrs	1 day	2 days	4 days	7 days	10 days	14 days
DPG	d	cd	d	d	cd	bc	a	ab	b
BTZ	ab	a	cd	bc	cde	def	ef	f	f
HMMM	e	e	e	de	cd	bc	ab	a	abc
6PPD	c	c	bc	bc	bc	b	a	bc	c
6PPD-q	b	ab	ab	ab	b	ab	ab	ab	a

b) tire leachate

	3 hrs	6 hrs	12 hrs	1 day	2 days	4 days	7 days	10 days	14 days
DPG	c	c	c	bc	bc	c	bc	b	a
BTZ	ab	a	a	a	a	a	ab	a	b
HMMM	c	c	c	c	c	bc	b	a	a
6PPD	b	b	b	ab	b	b	a	a	a
6PPD-q	c	c	bc	abc	bc	bc	bc	ab	a

Table S2.9: Translocation factors of TWP-derived compounds from lettuce roots to the leaves after a single initial compound spike: Results of Tukey's HSD multiple comparisons across compounds for each time point. Different lowercase letters indicate statistically significant differences between compounds ($p<0.05$). NA indicates insufficient data for statistical comparisons.

	HMMM	BTZ	DPG	6PPD	6PPD-q
3 hrs	a	a	b	b	b
6 hrs	NA	NA	NA	NA	NA
12 hrs	a	a	b	b	b
1 day	a	b	b	b	b
2 days	ab	ab	b	b	b
4 days	a	b	b	b	b
7 days	a	b	b	b	b
10 days	a	b	b	b	b
14 days	a	b	b	b	b

Table S2.10: Ion intensities of TWP-derived compounds and their transformation products over time: Results of Tukey's HSD multiple comparisons across compounds for each time point. Different lowercase letters indicate statistically significant differences between compounds ($p<0.05$).

	3 hrs	6 hrs	12 hrs	1 day	2 days	4 days	7 days	10 days	14 days
DPG	d	cd	d	d	d	bcd	abc	a	ab
$TP_{DPG}266$	b	ab	b	b	b	b	ab	ab	a
$TP_{DPG}270$	c	bc	c	c	c	c	bc	a	ab
$TP_{DPG}268$	b	b	b	b	b	b	a	a	a
BTZ	ab	ab	ab	ab	b	ab	ab	a	ab
$TP_{BTZ}152$	c	bc	c	c	c	bc	b	a	a
HMMM	d	cd	cd	cd	bc	b	b	a	a
$TP_{HMMM}271$	a	a	a	a	a	a	a	a	a
$TP_{HMMM}377$	d	cd	d	d	bcd	bc	b	a	a
$TP_{HMMM}363$	c	bc	bc	bc	bc	bc	b	a	a
$TP_{HMMM}359$	e	cde	de	cde	bcd	bc	b	a	a
$TP_{HMMM}345$	d	cd	d	cd	bcd	bc	b	a	a
$TP_{HMMM}331$	b	b	b	b	b	b	b	a	a
$TP_{HMMM}301$	b	b	b	b	b	b	b	a	a
$TP_{HMMM}303$	b	b	b	b	b	b	b	a	a
$TP_{HMMM}546$	a	a	a	a	a	a	a	a	a
6PPD	c	bc	bc	bc	bc	b	a	bc	bc
$TP_{6PPD}431$	c	abc	bc	ab	a	a	a	abc	c
6PPD-q	b	b	b	b	b	b	b	b	a
$TP_{6PPD\text{-}q}262$	a	a	a	a	a	a	a	a	a
$TP_{6PPD\text{-}q}174$	a	bc	b	bcd	cde	de	ef	ef	f
$TP_{6PPD\text{-}q}214$	a	a	a	a	a	a	a	a	a

Table S2.11: Concentrations of TWP-derived compounds in the nutrient solution with and without lettuce plants: Results of ANOVA testing for each compound and time point. Asterisk (*) indicates statistically significant differences between samples with and without lettuce plants ($p<0.05$).

a) spiked compounds

	0	3 hrs	6 hrs	12 hrs	1 day	2 days	4 days	7 days	10 days	14 days
DPG			*	*	*	*	*	*	*	*
BTZ				*	*	*	*	*	*	*
HMMM				*					*	*
6PPD				*	*	*	*	*		*
6PPD-q		*	*	*	*	*	*	*	*	*

b) tire leachate

	3 hrs	6 hrs	12 hrs	1 day	2 days	4 days	7 days	10 days	14 days
DPG	*		*						*
BTZ			*	*	*	*	*	*	*
HMMM	*	*				*			
6PPD	*	*		*	*			*	
6PPD-q	*			*			*		

Table S2.12: Biomass of lettuce plants at the beginning and end of the experiment.

		Plants exposed to TWP				Plants exposed to initial spike			
		plant weight [g]		weight difference		plant weight [g]		weight difference	
time point	*plant*	*beginning*	*end*	*total*	*%*	*beginning*	*end*	*total*	*%*
1	A	1.95	1.86	-0.09	-4.56	1.50	1.49	-0.01	-0.8
1	B	2.62	2.57	-0.06	-2.10	0.89	0.86	-0.03	-3.5
1	C	2.02	2.01	-0.01	-0.54	1.20	1.17	-0.03	-2.9
2	A	1.99	2.01	0.02	1.11	1.41	1.28	-0.13	-9.4*
2	B	1.29	1.28	-0.01	-0.78	1.49	1.41	-0.08	-5.5*
2	C	1.38	1.34	-0.04	-2.83	2.11	2.09	-0.02	-1.1
3	A	2.12	2.10	-0.02	-0.80	1.89	1.90	0.01	0.3
3	B	1.37	1.35	-0.02	-1.61	1.54	1.49	-0.05	-3.2
3	C	1.68	1.72	0.04	2.08	1.35	1.30	-0.05	-3.9
4	A	1.07	1.07	0.00	-0.09	2.05	2.02	-0.03	-1.4
4	B	1.45	1.44	-0.01	-0.76	1.09	1.06	-0.03	-3.0
4	C	0.95	0.92	-0.03	-3.16	1.45	1.41	-0.04	-2.6
5	A	2.76	2.71	-0.05	-1.74	1.16	1.21	0.05	4.2
5	B	1.47	1.50	0.03	2.11	1.17	1.12	-0.05	-4.5
5	C	1.50	1.48	-0.02	-1.07	1.26	1.28	0.01	1.2
6	A	2.04	N/A	N/A	N/A	1.52	2.17	0.65	42.6
6	B	2.84	N/A	N/A	N/A	0.72	0.70	-0.02	-2.2
6	C	1.9	N/A	N/A	N/A	1.60	2.13	0.53	33.2
7	A	2.13	2.39	0.26	12.30	1.11	1.26	0.15	13.2
7	B	1.24	1.36	0.12	9.44	1.16	0.83	-0.33	-28.3*
7	C	0.91	0.88	-0.03	-3.30	1.145	1.25	0.10	8.9
8	A	2.98	3.34	0.36	12.01	1.64	2.07	0.43	26.2
8	B	2.20	2.55	0.35	15.77	1.81	2.27	0.46	25.6
8	C	0.86	1.02	0.16	18.84	1.55	1.10	-0.45	-28.9*
9	A	2.53	3.12	0.59	23.20	1.60	1.86	0.26	16.1
9	B	1.94	2.13	0.19	9.74	1.51	2.01	0.50	32.8
9	C	1.68	1.88	0.20	12.08	1.37	1.67	0.30	21.6

Plants with biomass loss > 5% (marked with an asterisk) were excluded from further analysis.

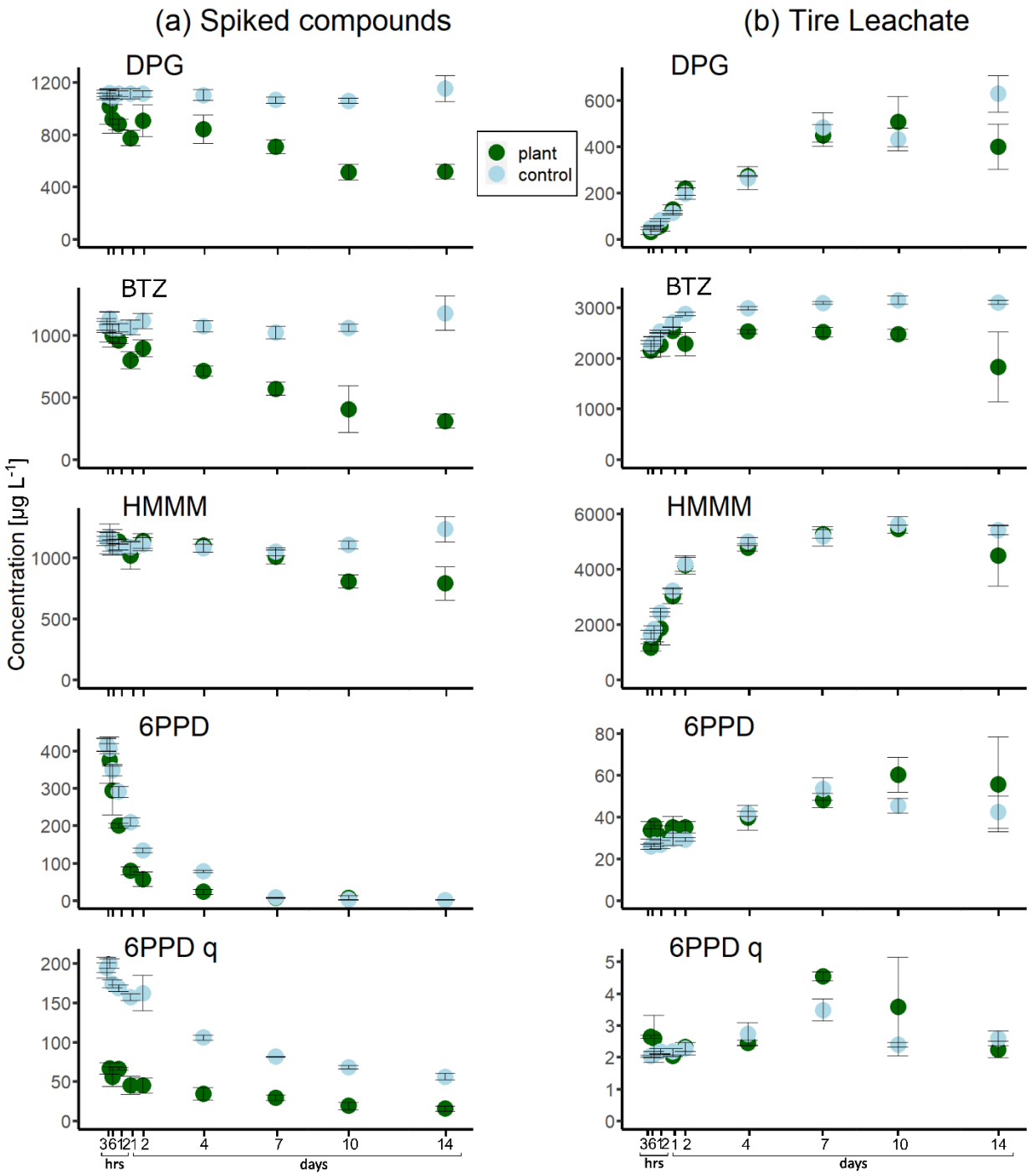


Figure S2.2: Concentrations of TWP-derived compounds in the nutrient solution with lettuce plants (● green dots) or without plants (● blue dots) over 14 days after exposure to (a) a single initial TWP-derived compound spike and (b) continuously replenished leaching from TWP in the nutrient solution. Error bars represent the standard deviation from triplicate measurements.

Table S2.13: K_D values of TWP-derived compounds in lettuce root tissue at initial aqueous concentrations of 50 and 500 µgL^{-1}.

Initial concentration	DPG	BTZ	HMMM	6PPD	6PPD-q
50 µg/L	0.68	0.06	0.10	0.26	N/A
500 µg/L	0.17	0.05	0.05	1.99	N/A

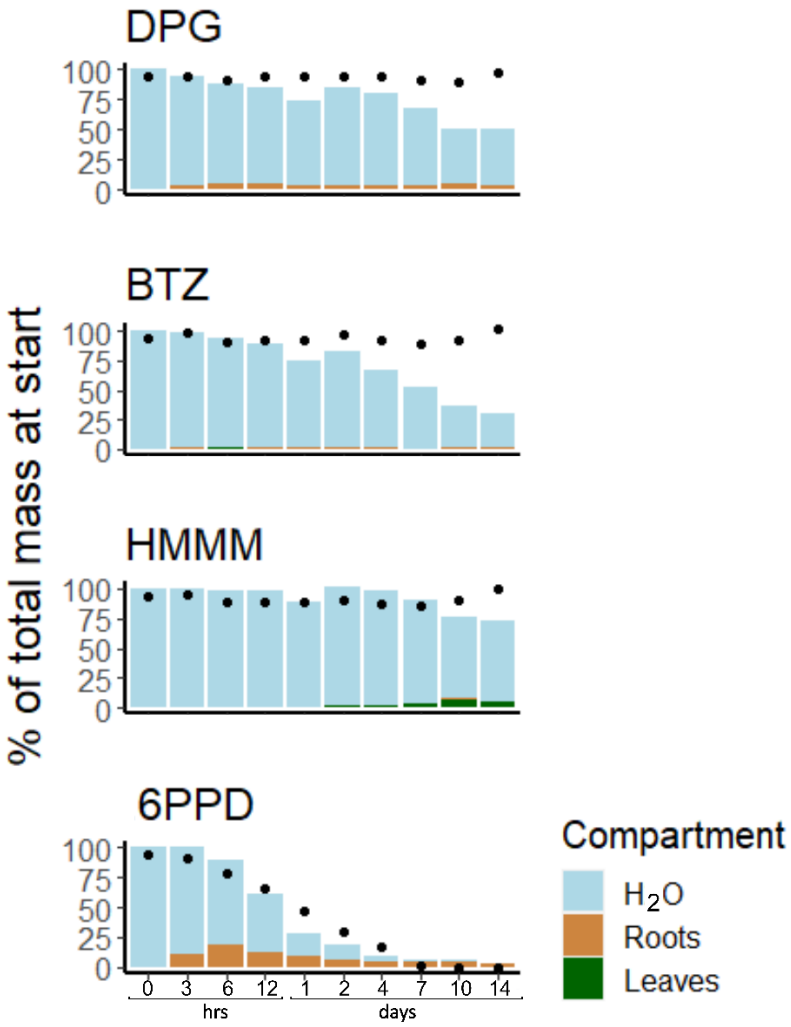


Figure S2.3: Mass of DPG, BTZ, HMMM and 6PPD in the nutrient solution (blue bars ■), roots (brown bars ■) and in lettuce leaves (green bars ■) relative to the total mass added at the start. Abiotic controls without plants are represented as black dots (●).

Section S2.4: Transformation products of TWP-derived compounds

Information for all transformation products, arranged by TWP-derived compounds, are shown in Table S2.7. The naming scheme for the transformation products was adapted from Alhelou et al[27]. All molecular formulas proposed are based on a mass error of less than 5PPM. Fragments marked with an asterisk (*) were used as diagnostic fragments to confirm either a compound's structure, or its relation to its parent compound. Diagnostic fragments are either exact fragment matches, or fragment matches adjusted by the calculated mass shift between the transformation product and the parent compound. Exact fragment matches are based on our measurements or on literature (in this case, appropriate studies are cited). In addition to reporting diagnostic fragments, we report all fragments measured on the Orbitrap-HRMS at an intensity level > $5x10^4$. For many transformation products, we do not propose exact structures but rather highlight the relevant reaction components (e.g. because we were unable to determine the exact site of conjugation). Confidence levels were adapted from Schymanski et al[128] as follows:

1: *Confirmed Structure* with Reference Standard. MS, MS^2, RT matching.

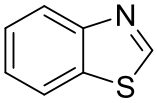
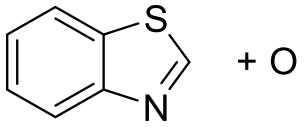
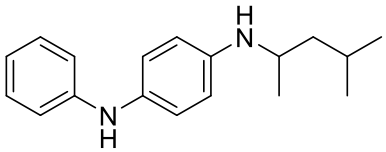
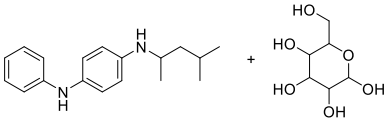
2(a): *Probable Structure* with Library matches. MS, MS^2 matching to literature

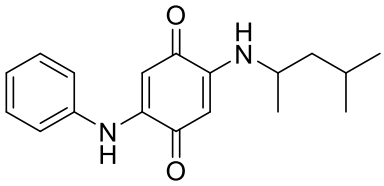
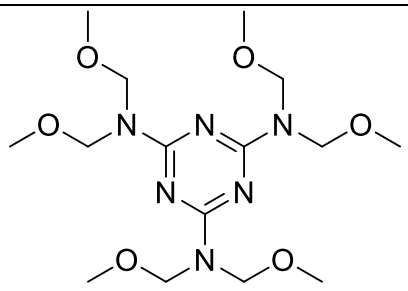
3: Tentative Candidates: MS supports probable reaction. MS^2 confirm relation to parent compound.

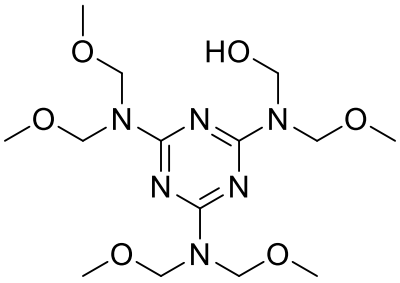
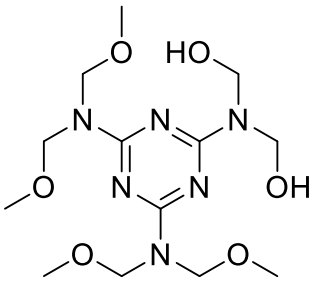
4: Unequivocal Molecular Formula: No structure proposed. Formula based on exact mass, MS^2 confirms relation to parent compound.

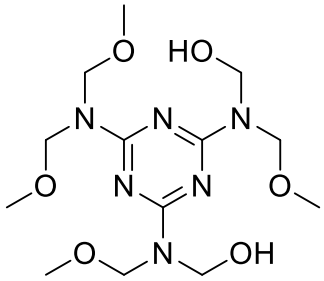
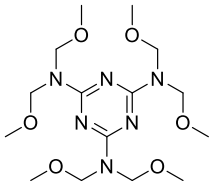
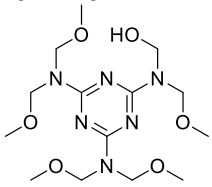
5: Exact mass of interest. More than one possible molecular formula. MS^2 confirms relation to parent compound.

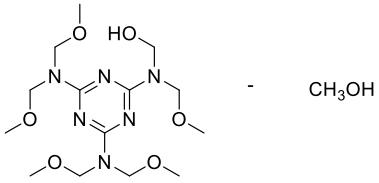
Table S2.14: Transformation products of TWP-derived compounds

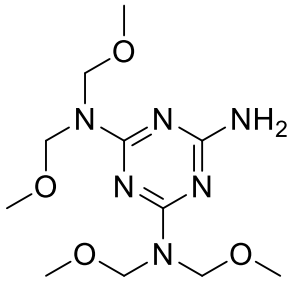
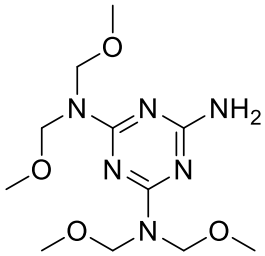
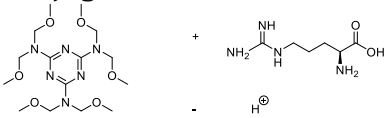
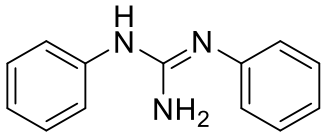
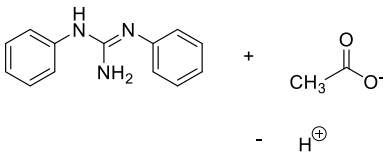
ID	Monisotopic Mass	Δ ppm	Proposed Molecular Formula	Proposed Structure/Reactions	Assigned Confidence Level	RT	Fragments
Benzothiazole							
BTZ	135.01403	1.78	C_7H_5NS		Parent	5.2	72.02843; 83.53615; 136.02126
$TP_{BTZ}152$	151.00888	2.02	C_7H_5NOS		3	3.9	88.02333; 99.5122; 97.02833*; 99.53137*; 152.01601
6PPD							
6PPD	268.19311	3.13	$C_{18}H_{24}N_2$		Parent	6.6	93.05740; 184.09885; 185.10664; 269.19952
$TP_{6PPD}431$	430.24539	3.21	$C_{24}H_{34}N_2O_5$	Glucose Conjugation 	3	5.3	184.09885*; 185.10664;* 209.10664; 268.19205*; 347.15994*; 346.15103*;

							431.25165
6PPD-quinone							
6PPD-q	298.16702	3.72	$C_{18}H_{22}N_2O_2$		Parent	8.9	100.11225; 187.08591; 215.08073; 241.09608; 243.11143; 256.11960; 299.17422
$TP_{6PPD\text{-}q}262$	261.13555	1.533 3.61	$C_{13}H_{17}N_4O_2$ $C_{15}H_{19}NO_3$		5	9.5	206.07991*; 262.14294
$TP_{6PPD\text{-}q}174$	173.12001	2.54	$C_{12}H_{15}N$		4	9.4	118.06526*; 132.08080*
$TP_{6PPD\text{-}q}214$	213.20858	3.09	$C_{11}H_{25}N_4$ $C_{13}H_{27}NO$		5	9.6	57.07044*
HMMM							
HMMM	390.22129	3.57	$C_{15}H_{30}N_6O_6$		Parent	6.2	72.90399; 107.08781; 159.66292; 167.88551; 176.18416; 177.08781; 192.54492; 193.11816;

							207.09831; 253.14046; 283.15042; 301.16147; 329.19138; 359.19931; 373.19775
$TP_{HMMM377}$	376.20581	3.25	$C_{14}H_{28}N_6O_6$	Methoxy hydrolysis 	2a	5.5	163.072142[27,109]*; 177.087786[27,109]*; 207.098366[27,109]*; 225.76080; 239.124244[27,109]*; 253.13982*; 269.134988[27,109]*; 283.150577[27,109]*; 315.17664
$TP_{HMMM363\_1}$	362.19018	3.32	$C_{13}H_{26}N_6O_6$	2x Methoxy hydrolysis 	2a	4.9	163.072133*[27,109]; 177.087722*[27,109]; 207.098244*[27,109]; 239.124111*[27,109]; 253.14032*; 255.118777*[27,109]; 269.1344

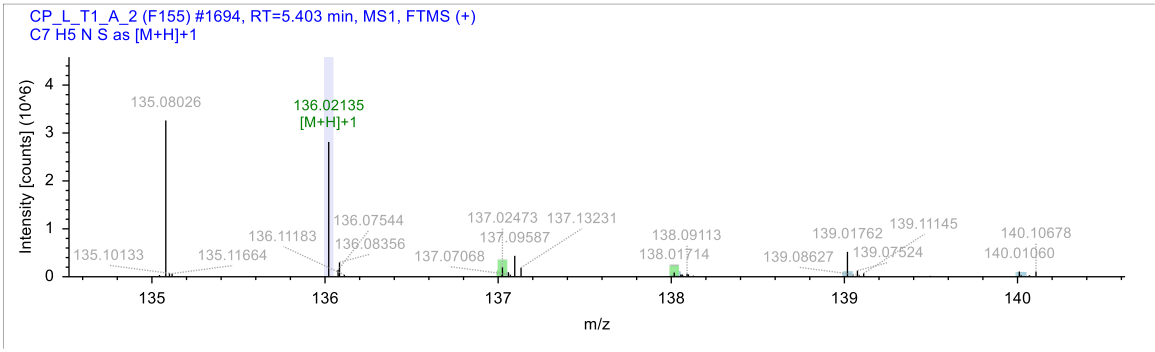
							3; 283.15106*[27,109]
$TP_{HMMM363}$ isomer	362.19018	3.32	$C_{13}H_{26}N_6O_6$	2x Methoxy hydrolysis 	2a	5.5	163.07211*[27,109]; 177.08775*[27,109]; 193.08316*[109]; 207.09827*[27,109]; 225.10896*[109]; 239.12392*[27,109] 253.14059*; 269.13474*[109]; 283.14984*
$TP_{HMMM359}$	358.19526	3.37	$C_{14}H_{26}N_6O_5$	Methanol loss  - CH_3OH	3	6.2	69.75610; 75.14574; 77.06249; 177.08778*; 193.11906*; 207.09814*; 218.30775; 249.23236; 253.13989*; 283.15073*
$TP_{HMMM345}$	344.17975	3.10	$C_{13}H_{24}N_6O_5$	Methanol loss and methoxy hydrolysis  - CH_3OH	3	5.5	163.07211*; 177.08771*; 207.09827*; 253.1401

							4*; 283.15060*; 163.07211*
$TP_{HMMM}345$ isomer	344.17975	3.10	$C_{13}H_{24}N_6O_5$	Methanol loss and methoxy hydrolysis 	3	6.2	163.07220*; 177.08781*; 207.09836*; 253.14020*; 283.15073*; 163.07220*
$TP_{HMMM}331$	330.16416	3.05	$C_{12}H_{22}N_6O_5$	Methoxy hydrolysis of $TP_{HMMM}345$	3	4.9	163.07211*; 177.08774*; 207.09825*; 253.14140*; 163.07211*
$TP_{HMMM}301$	300.1537	3.011 1.451 1.46	$C_{11}H_{20}N_6O_4$ $C_{10}H_{24}N_2O_8$ $C_9H_{18}N_9O_3$		5	4.8	163.07201*; 165.08778; 177.08760*; 195.09839; 207.09785*; 225.10893;

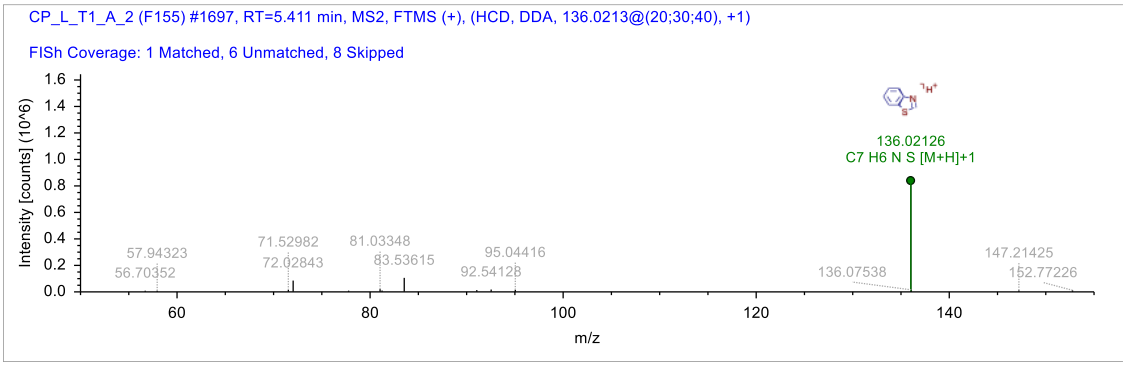
$TP_{HMMM}303$ (Tetra-MMM)	302.16928	3.22	$C_{11}H_{22}N_6O_4$		2a	4.5	165.08766*[27,133]; 195.09811*[27,133]; 241.13931*[27]; 271.15005*[27,133];
$TP_{HMMM}271$	270.14313	3.36	$C_{10}H_{18}N_6O_3$	Methanol loss from TetraMMM  -	3	4.5	165.08775*; 195.09837*; 241.13919*; 271.1504*
$TP_{HMMM}546$	545.31827	4.22	$C_{21}H_{41}N_{10}O_7$	Reduction (-H), Arginine Conjugation 	3	8.7	107.08588*; 177.12720*
DPG							
DPG	211.11034	2.88	$C_{13}H_{13}N_3$		Parent	4.0	94.06531; 119.06019; 195.09102
$TP_{DPG}266$	265.12065	3.25	$C_{16}H_{15}N_3O$		4	4.0	195.09077*
$TP_{DPG}268$	267.09992	3.21	$C_{15}H_{13}N_3O_2$		4	2.3	94.06536*; 119.06019*; 122.05982; 195.09108*; 212.11693*
$TP_{DPG}270$	269.11550	3.44	$C_{15}H_{15}N_3O_2$	-H, acetate addition 	3	2.2	119.06023*; 195.09142*

A) BTZ

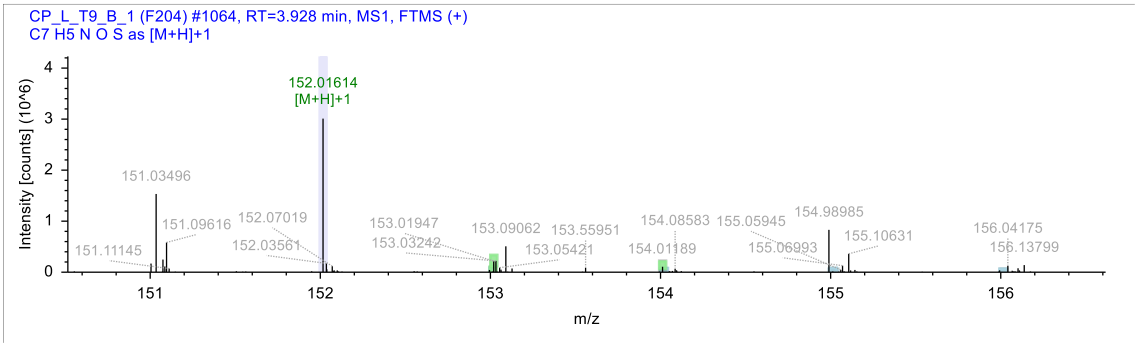
BTZ MS1 Spectrum



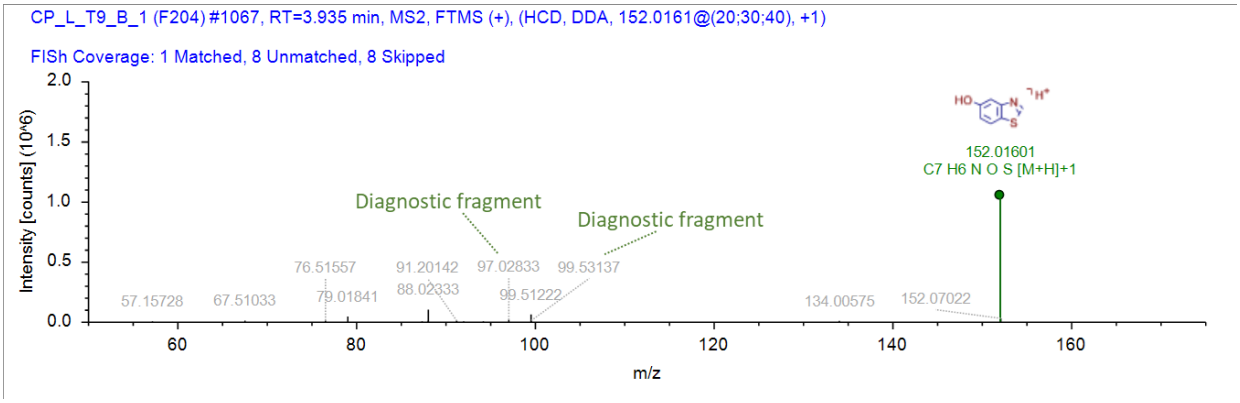
BTZ MS2 Spectrum



BTZ $TP_{BTZ}152$ MS1 Spectrum

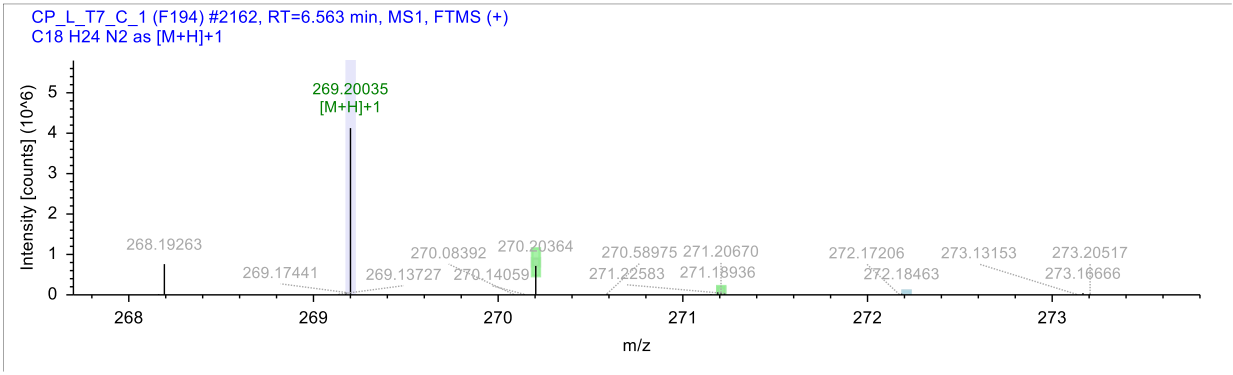


BTZ TP_{BTZ}152 MS2 Spectrum

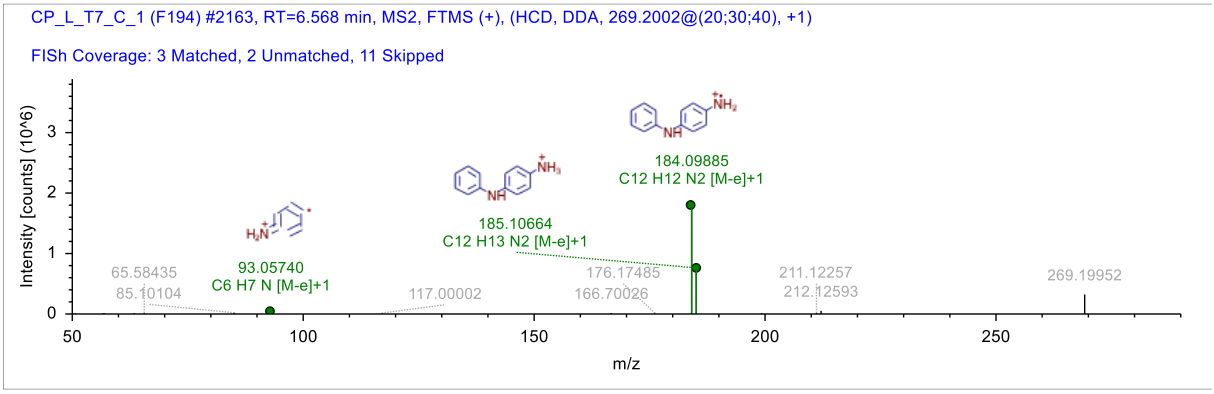


B) 6PPD

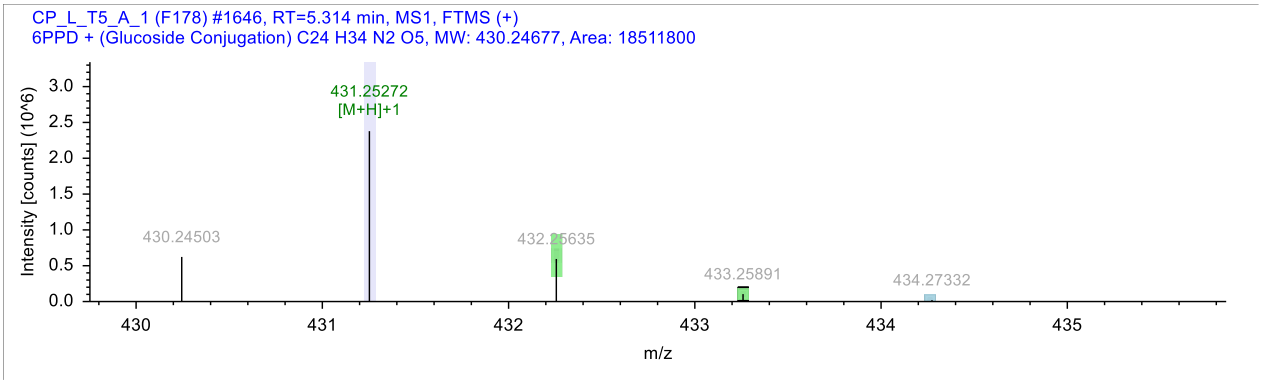
6PPD MS1 Spectrum



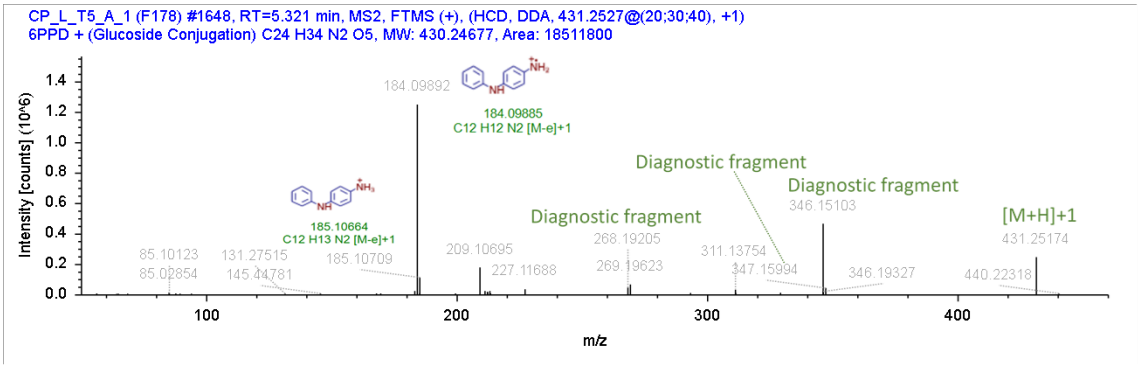
6PPD MS2 Spectrum



6PPD TP_{6PPD}431 MS1 Spectrum

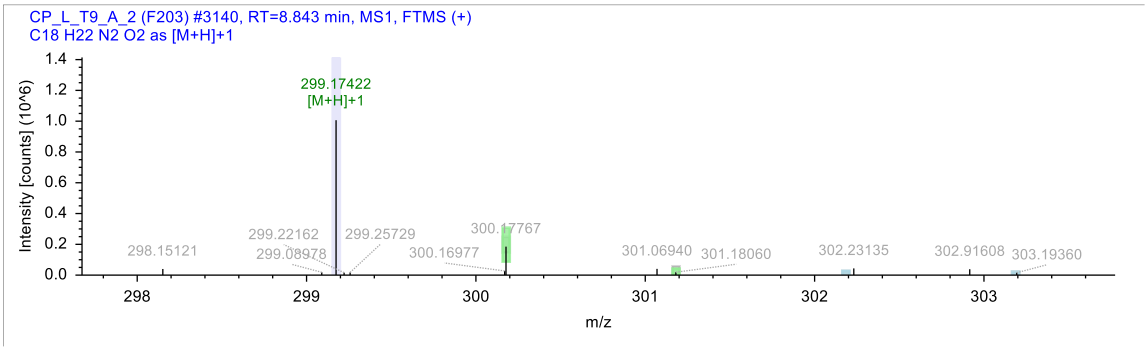


6PPD TP_{6PPD}431 MS2 Spectrum

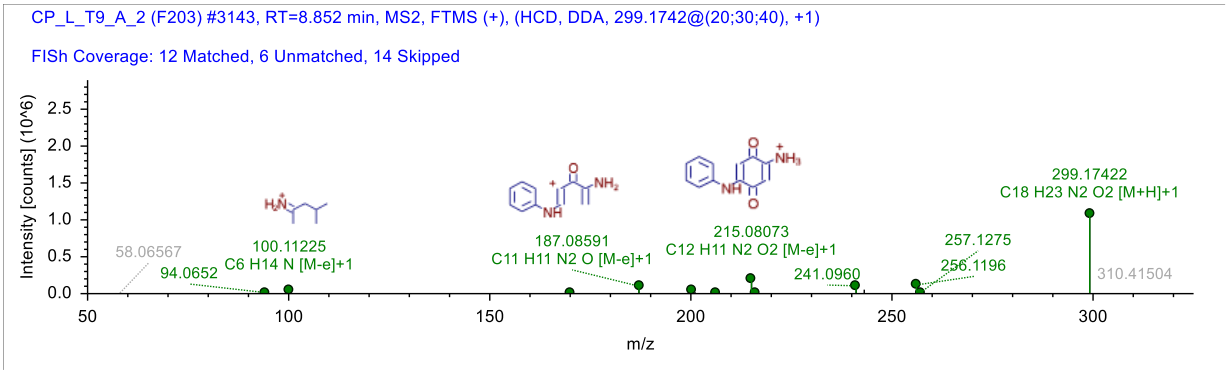


C) 6PPD-q

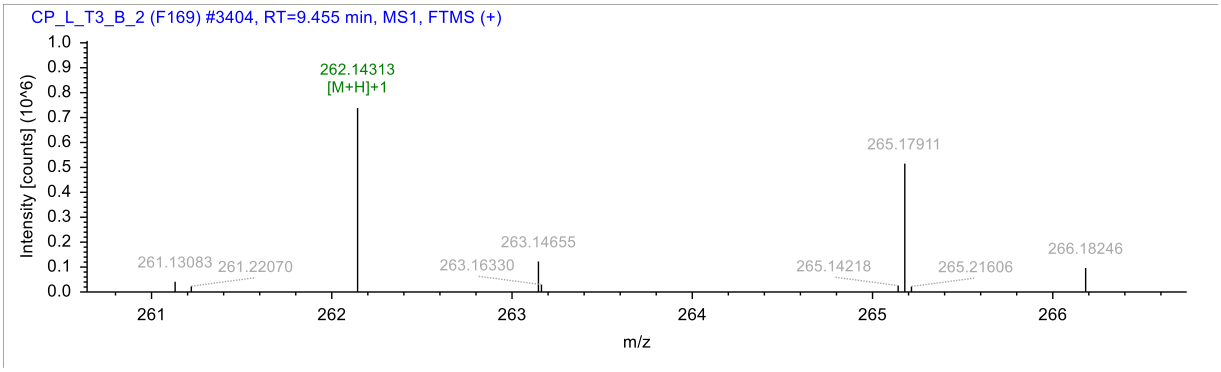
6PPD-q MS1 Spectrum



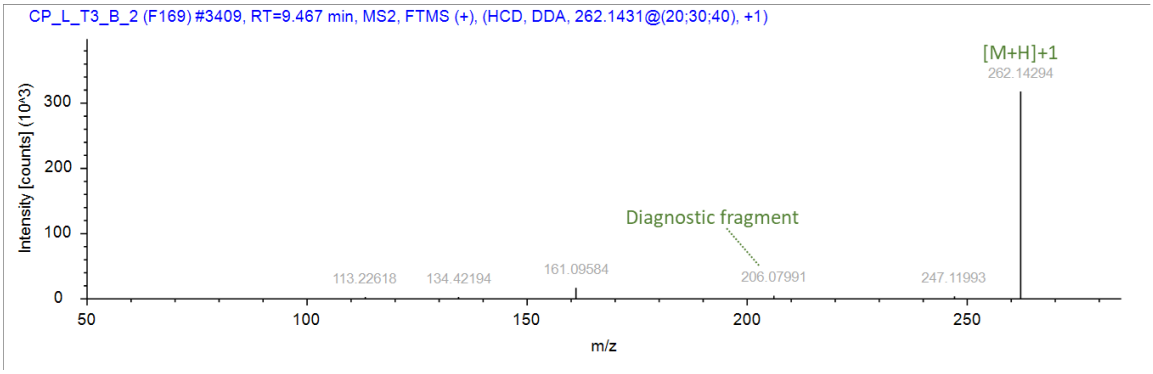
6PPD-q MS2 Spectrum



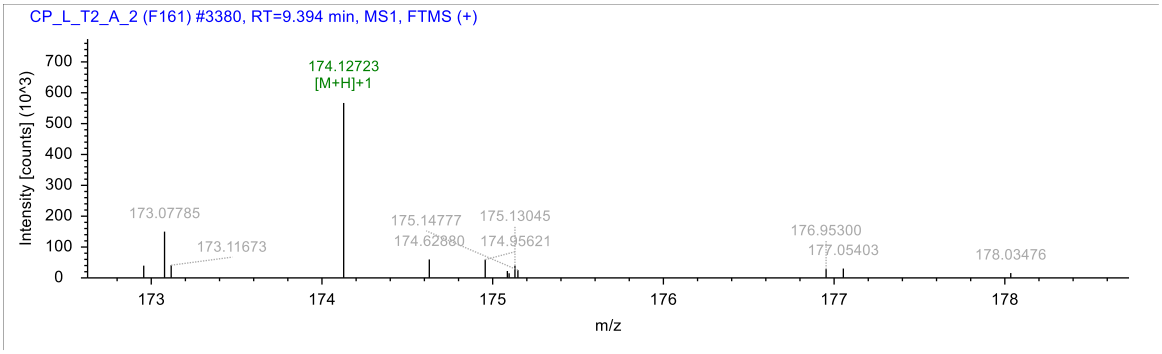
6PPD-q $TP_{6PPD\text{-}q}$262 MS1 Spectrum



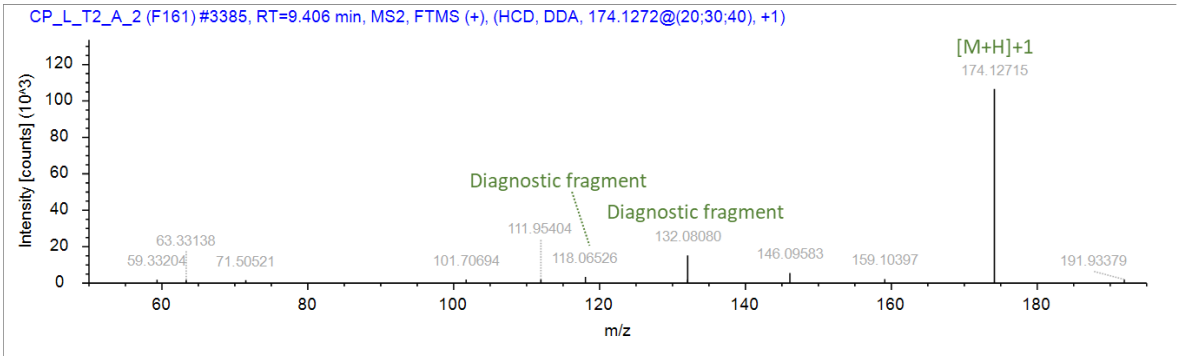
6PPD-q $TP_{6PPD\text{-}q}$262 MS2 Spectrum



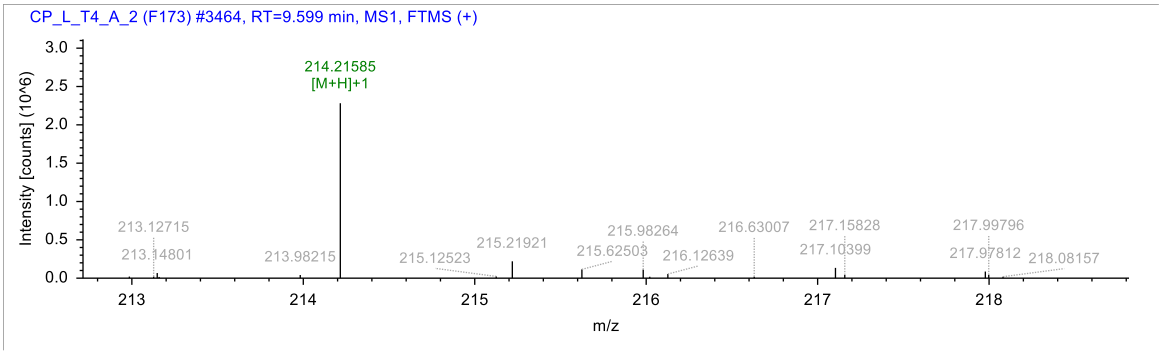
6PPD-q $TP_{6PPD\text{-}q}$174 MS1 Spectrum



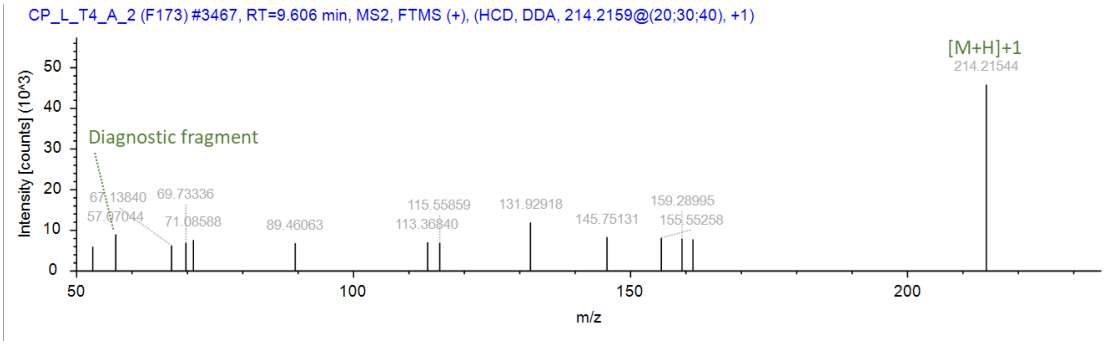
6PPD-q $TP_{6PPD\text{-}q}$174 MS2 Spectrum



6PPD-q $TP_{6PPD\text{-}q}$214 MS1 Spectrum

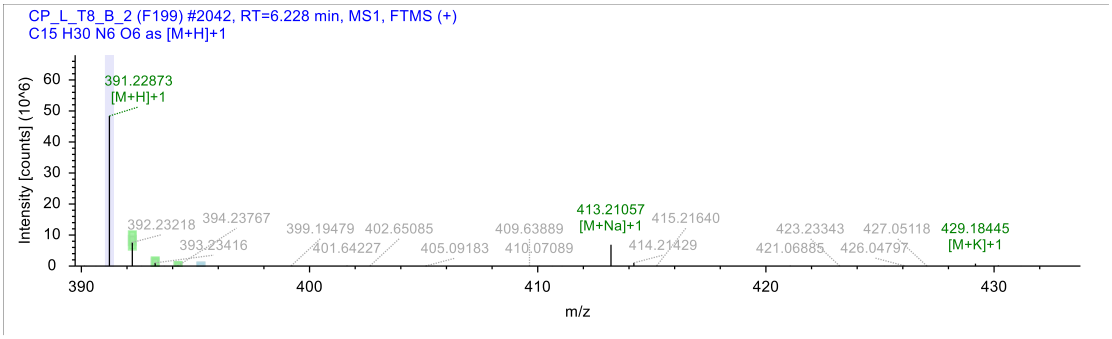


6PPD-q $TP_{6PPD\text{-}q}$214 MS2 Spectrum

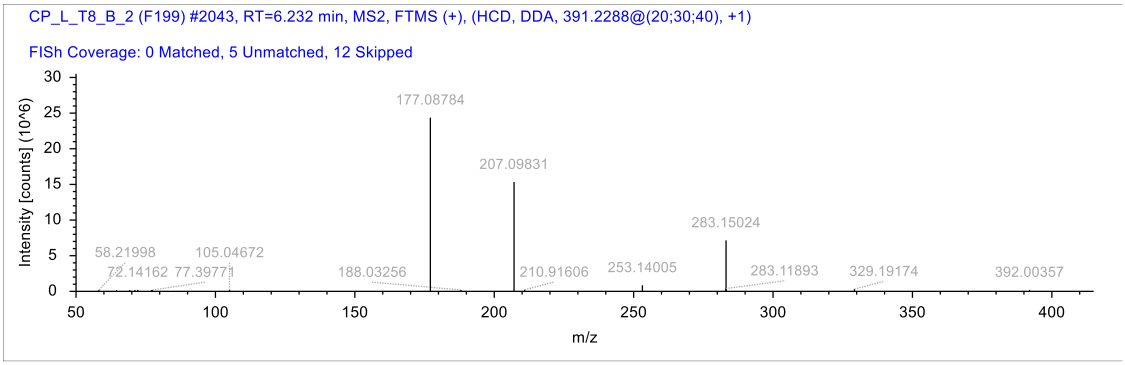


D) HMMM

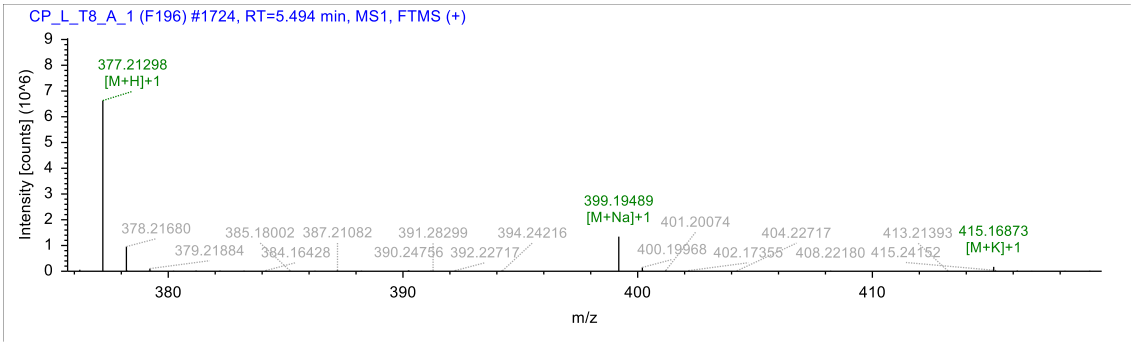
HMMM MS1 Spectrum



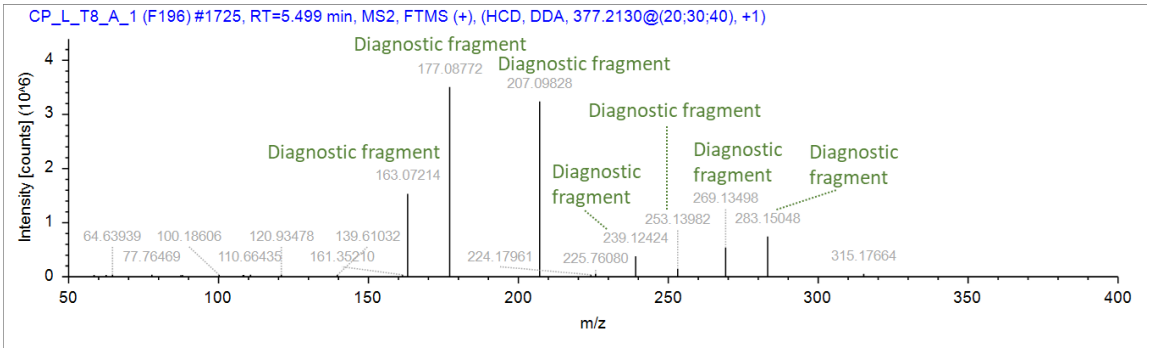
HMMM MS2 Spectrum



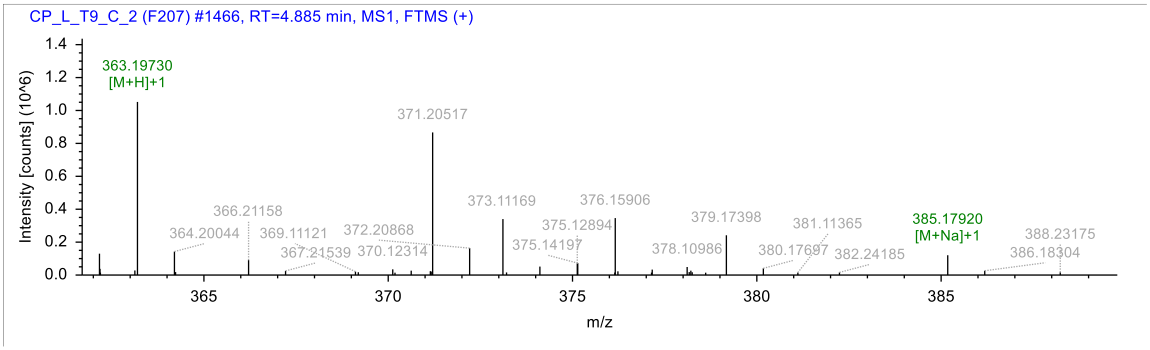
HMMM $TP_{HMMM}377$ MS1 Spectrum



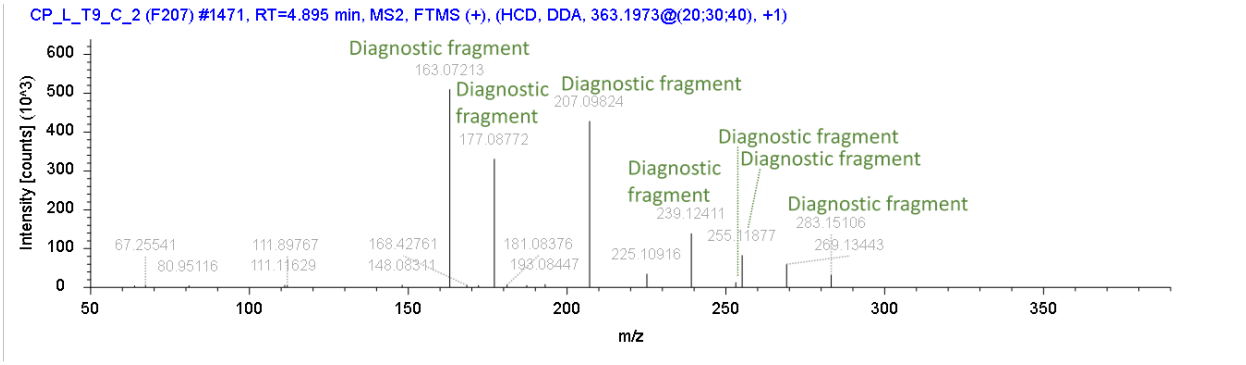
HMMM $TP_{HMMM}377$ MS2 Spectrum



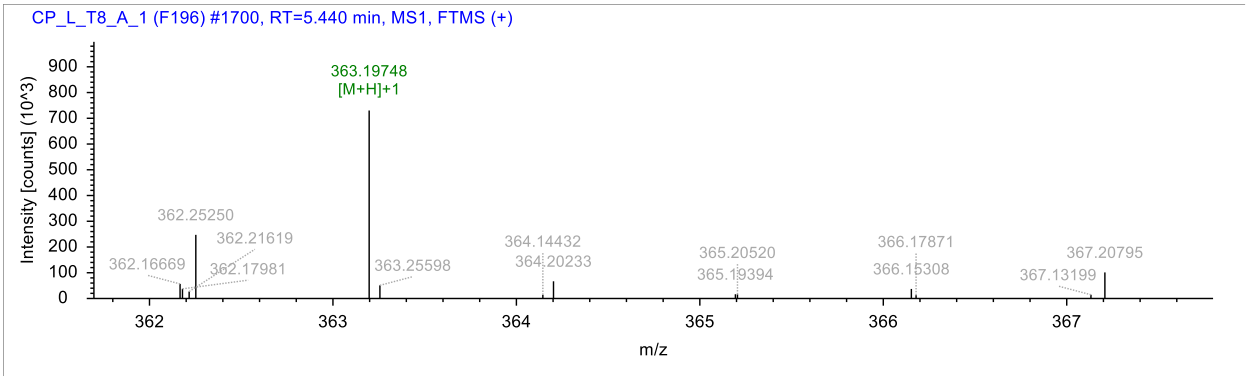
HMMM $TP_{HMMM}363\_1$ MS1 Spectrum



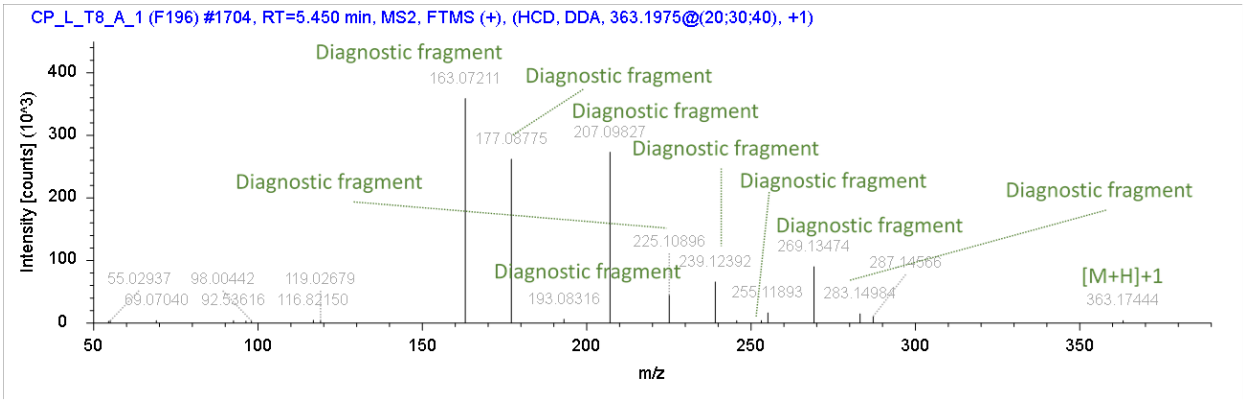
HMMM $TP_{HMMM}363\_1$ MS2 Spectrum



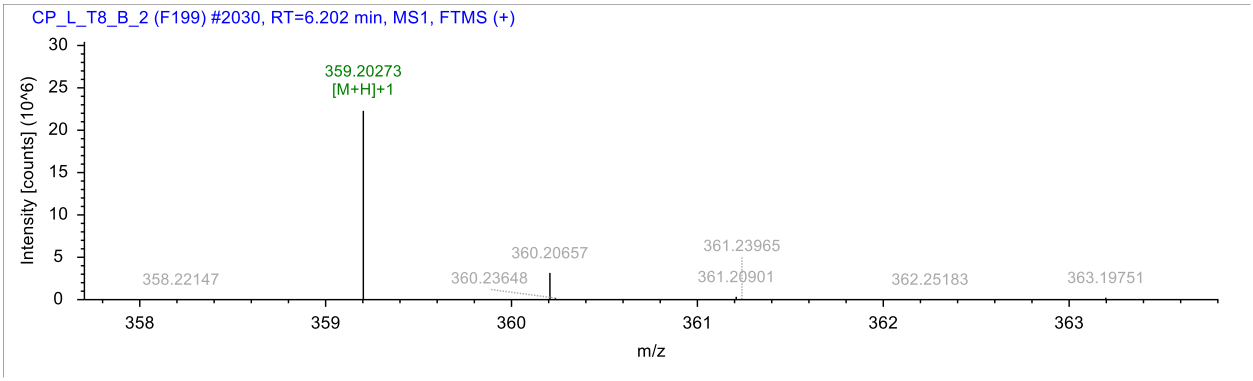
HMMM $TP_{HMMM}363$ isomer MS1 Spectrum



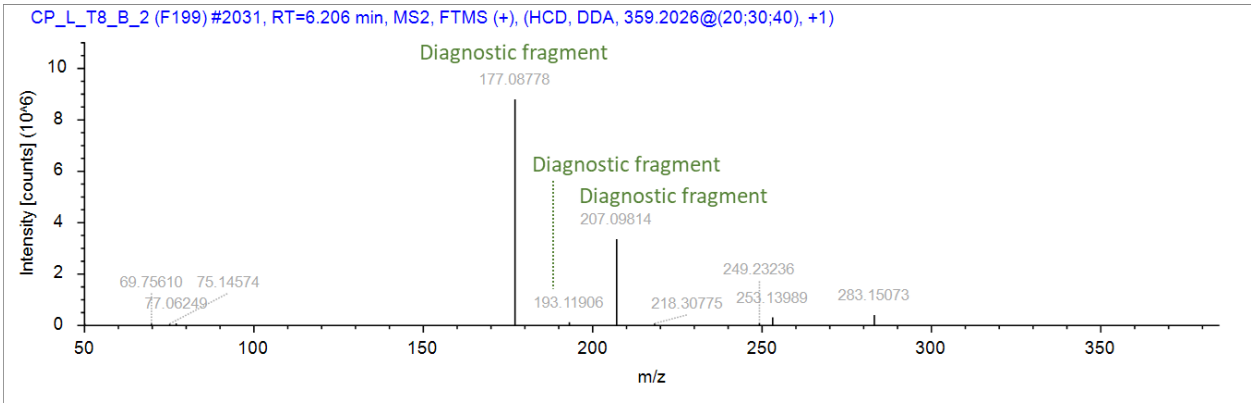
HMMM $TP_{HMMM}363$ isomer MS2 Spectrum



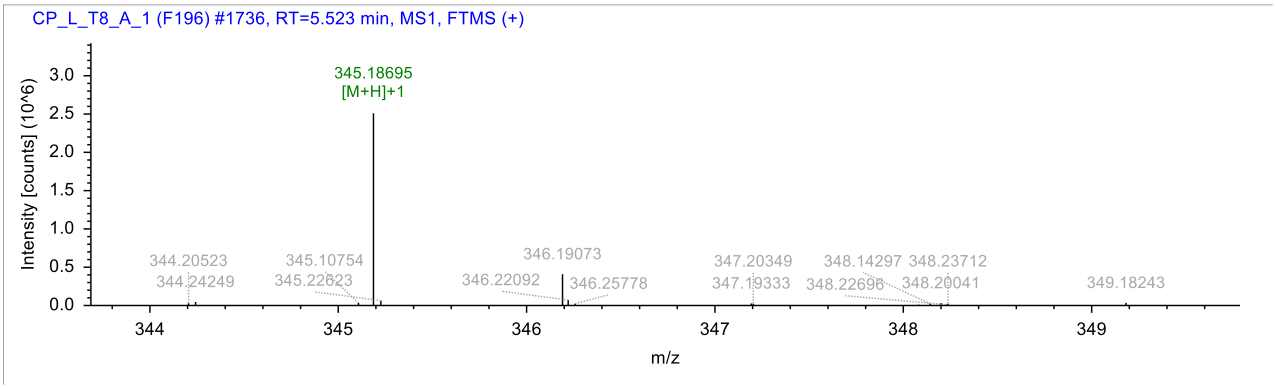
HMMM $TP_{HMMM}359$ MS1 Spectrum



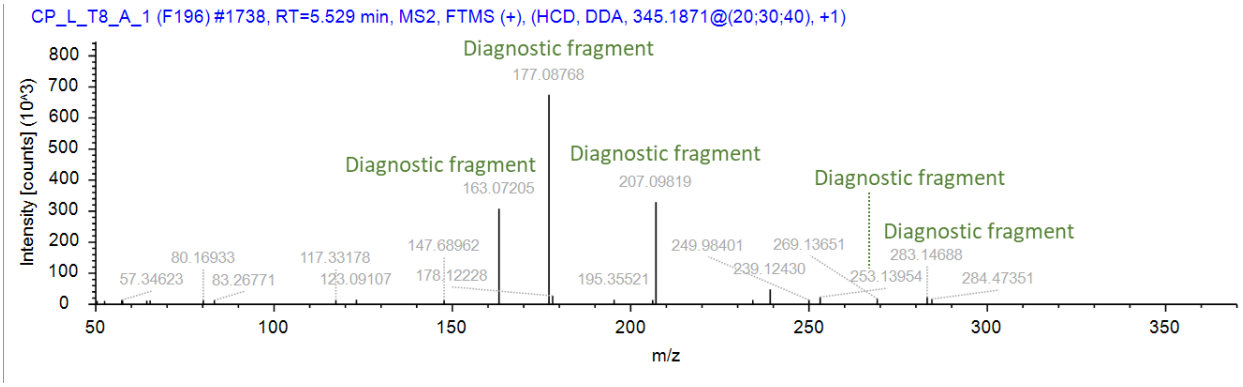
HMMM $TP_{HMMM}359$ MS2 Spectrum



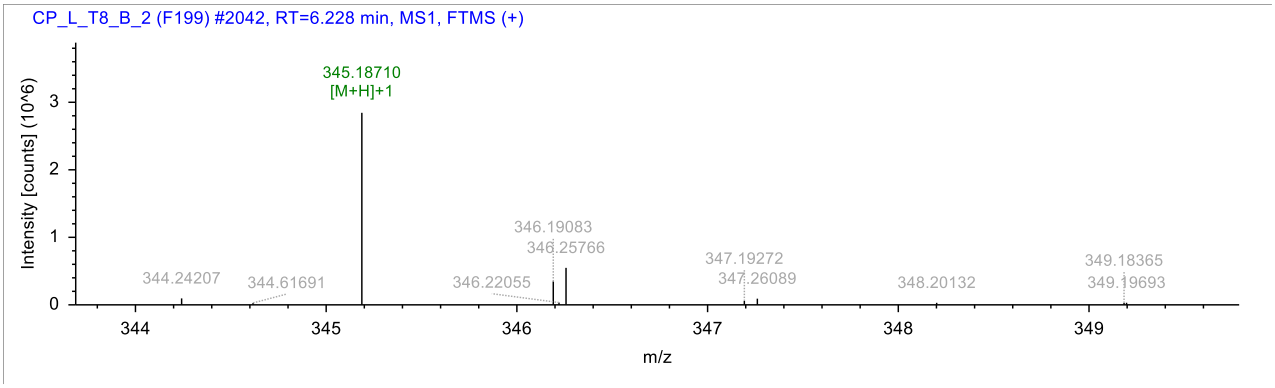
HMMM $TP_{HMMM}345$ MS1 Spectrum



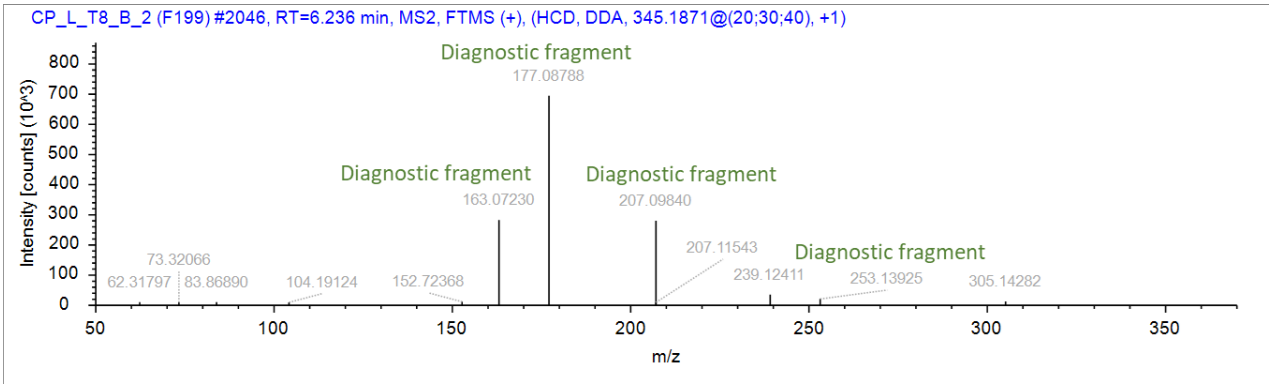
HMMM $TP_{HMMM}345$ MS2 Spectrum



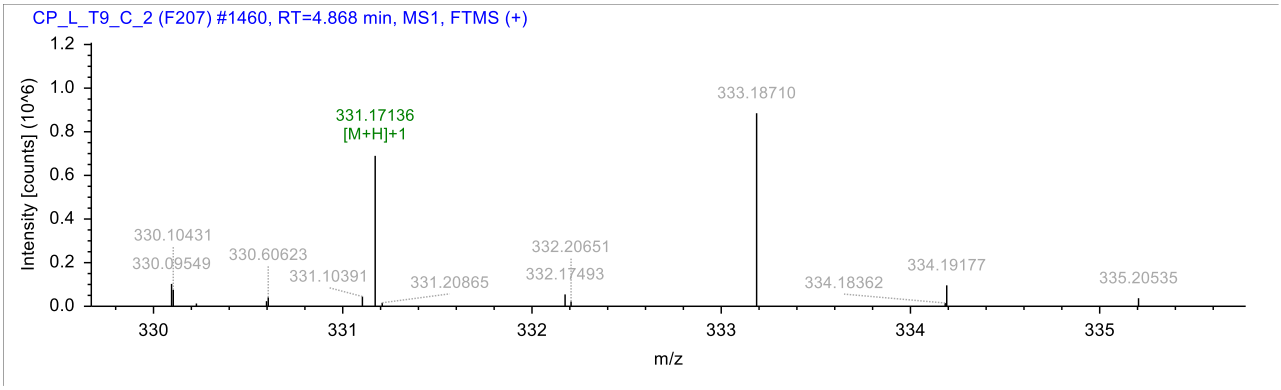
HMMM $TP_{HMMM}345$ isomer MS1 Spectrum



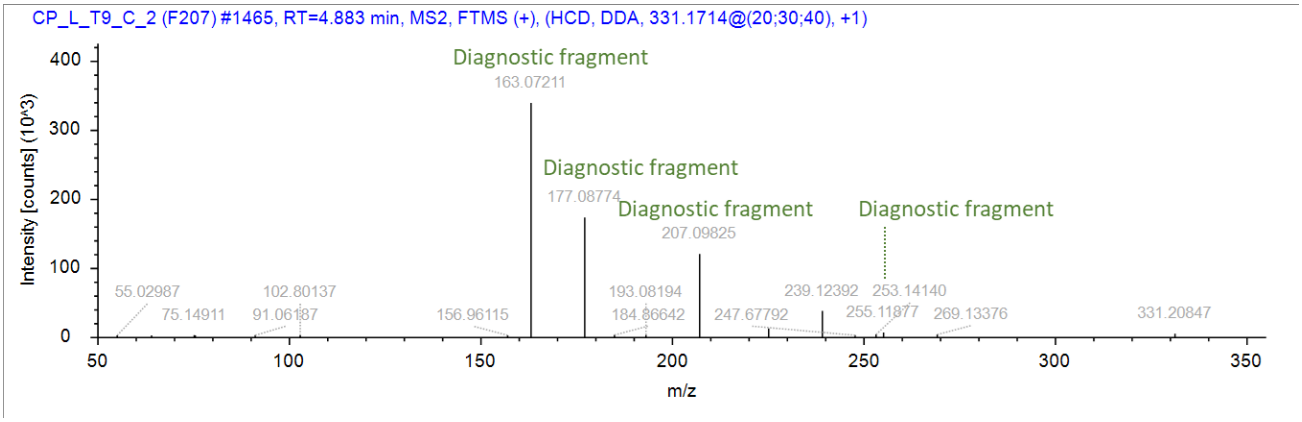
HMMM $TP_{HMMM}345$ isomer MS2 Spectrum



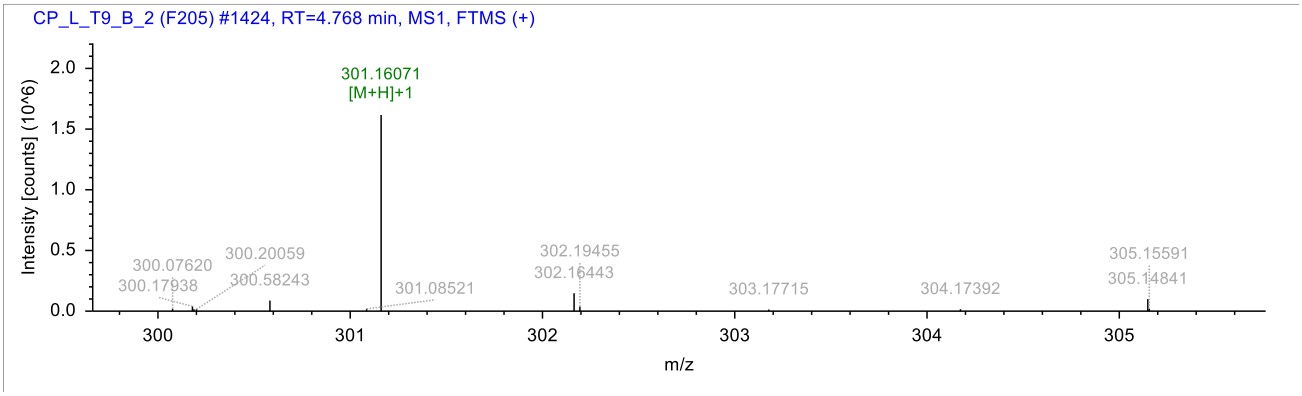
HMMM $TP_{HMMM}331$ MS1 Spectrum



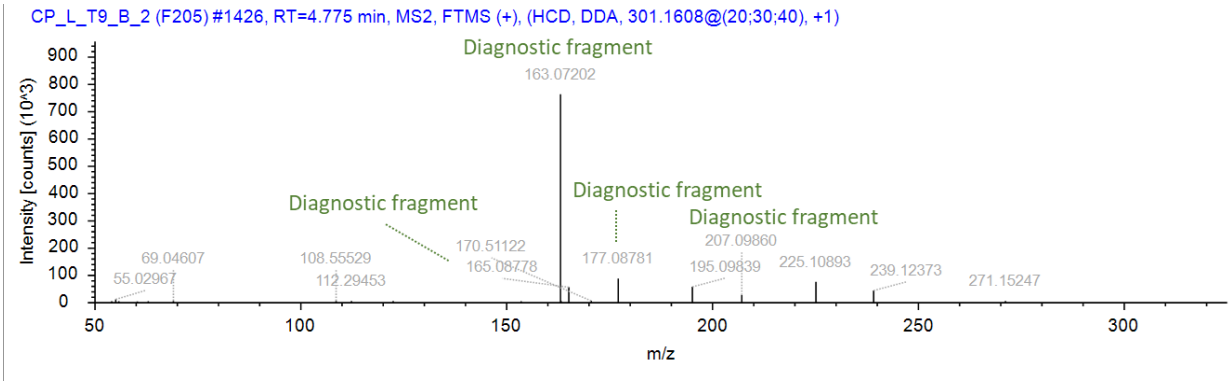
HMMM $TP_{HMMM}331$ MS2 Spectrum



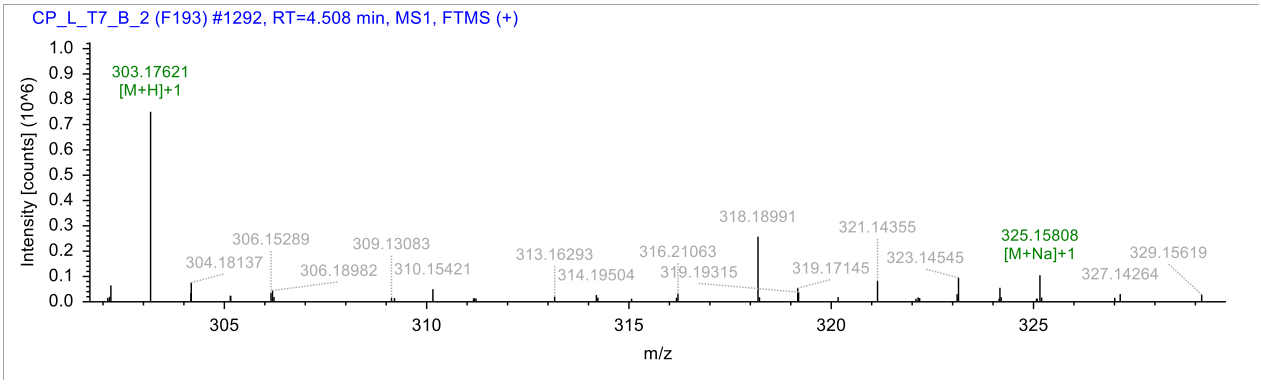
HMMM $TP_{HMMM}301$ MS1 Spectrum



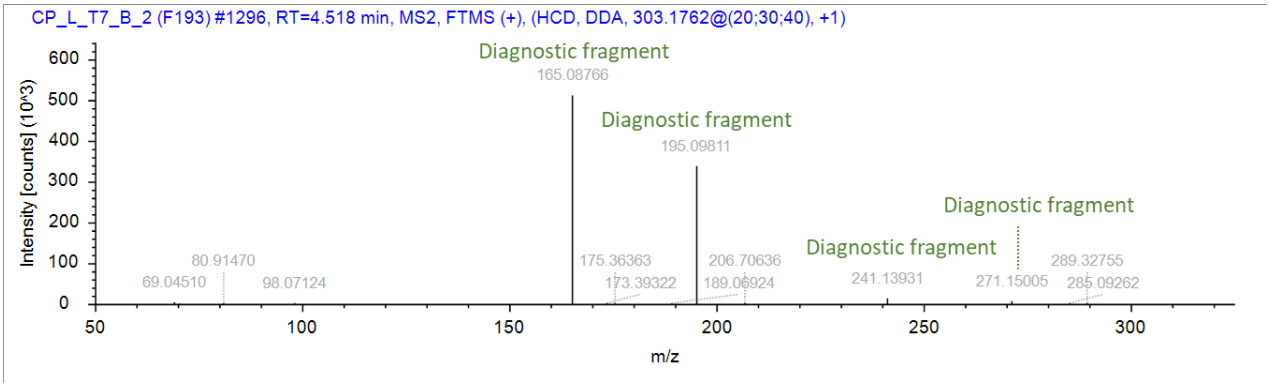
HMMM $TP_{HMMM}301$ MS2 Spectrum



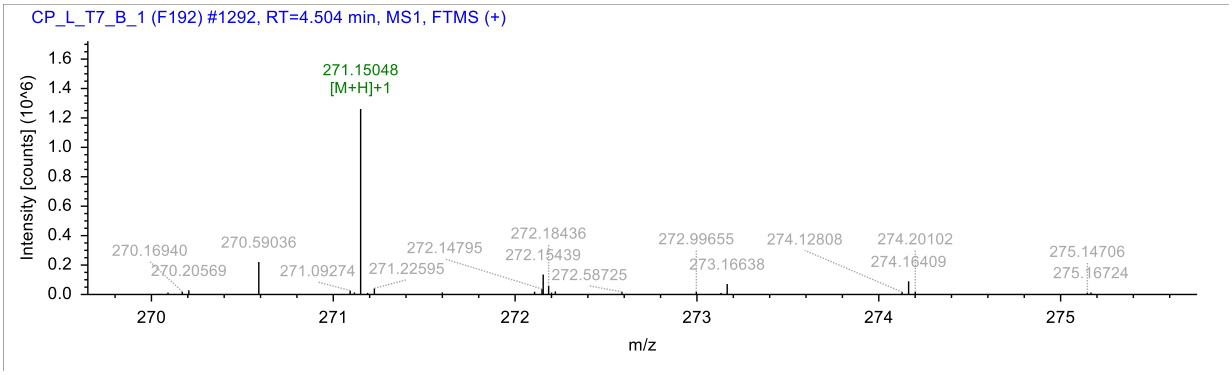
HMMM $TP_{HMMM}303$ MS1 Spectrum



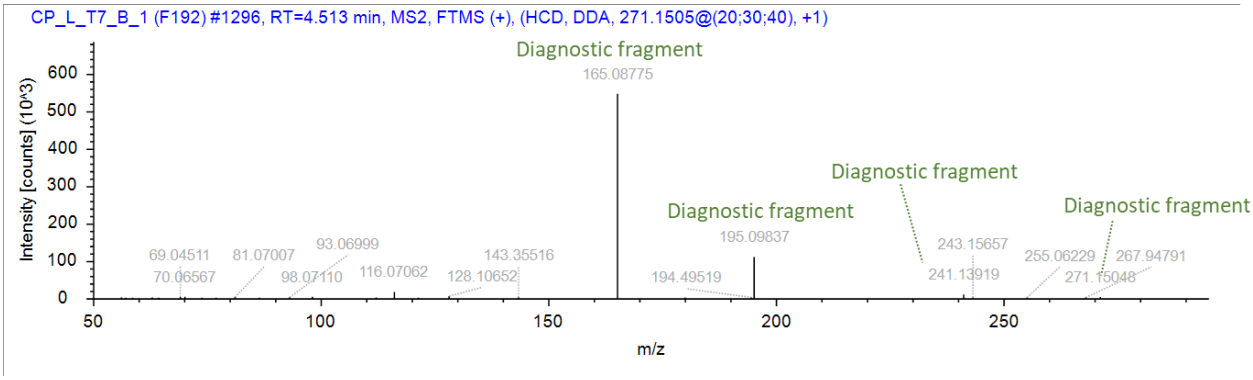
HMMM $TP_{HMMM}303$ MS2 Spectrum



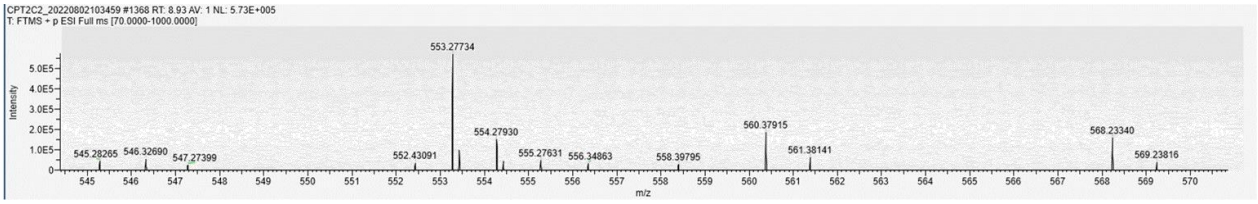
HMMM $TP_{HMMM}271$ MS1 Spectrum



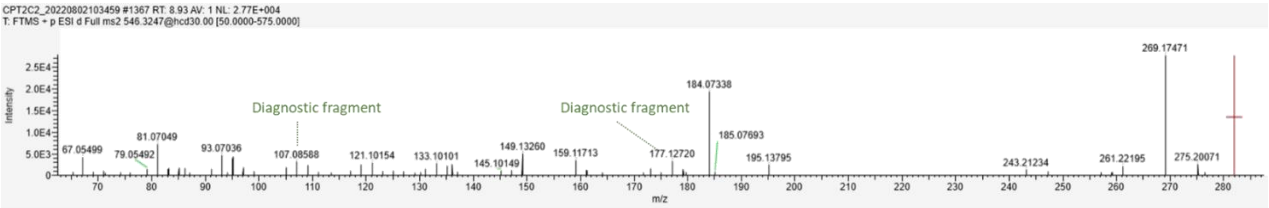
HMMM $TP_{HMMM}271$ MS2 Spectrum



HMMM $TP_{HMMM}546$ MS1 Spectrum

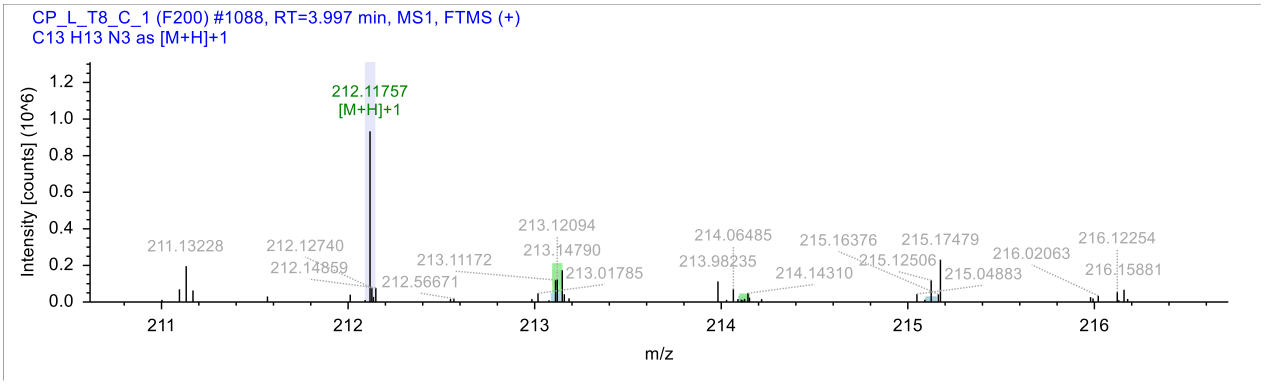


HMMM $TP_{HMMM}546$ MS2 Spectrum

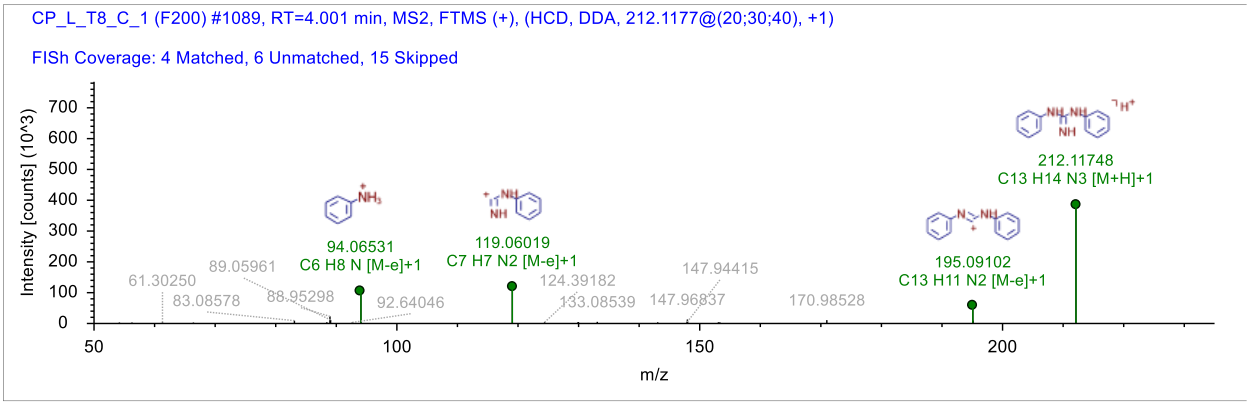


E) DPG

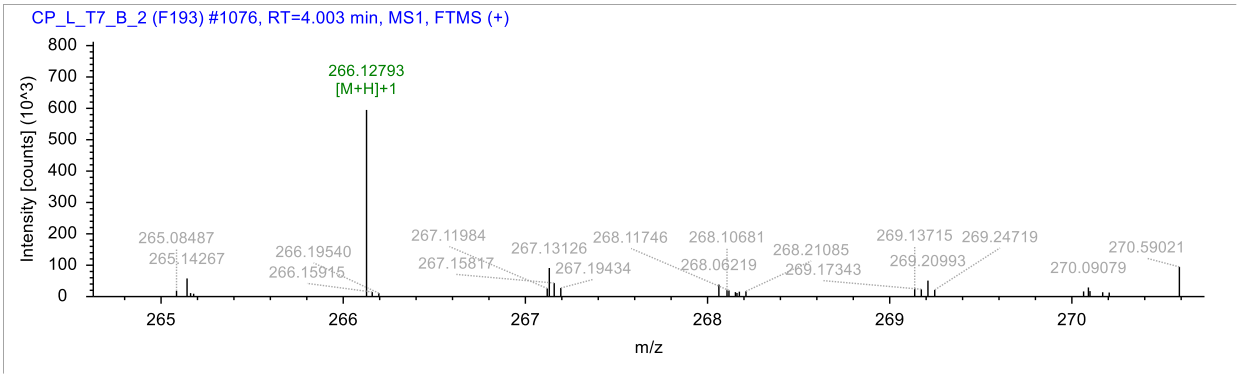
DPG MS1 Spectrum



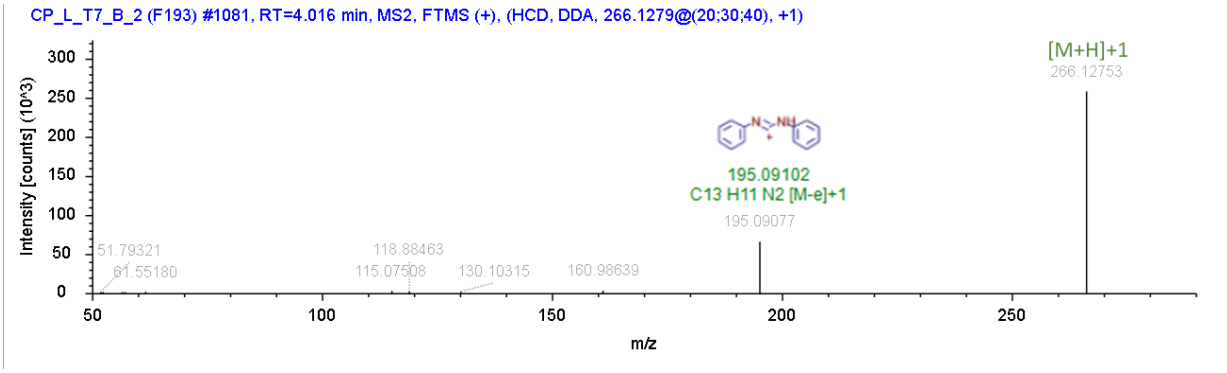
DPG MS2 Spectrum



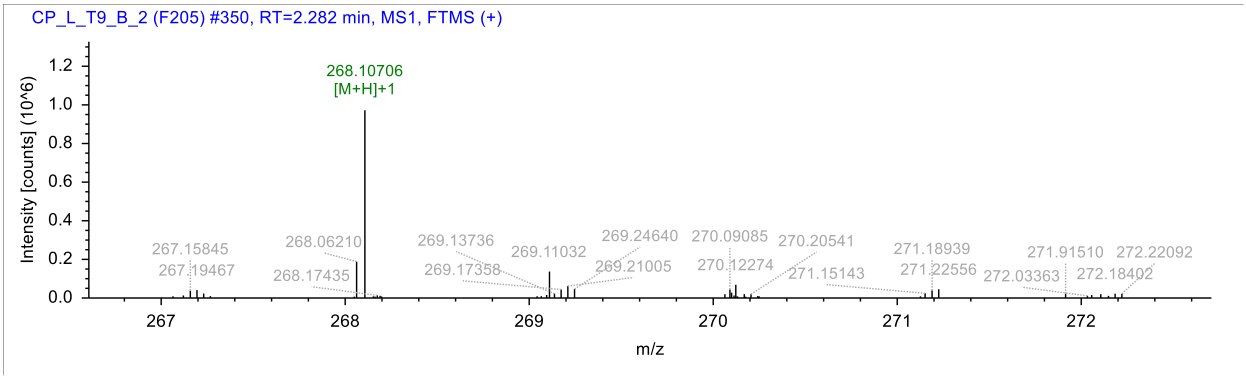
DPG $TP_{DPG}266$ MS1 Spectrum



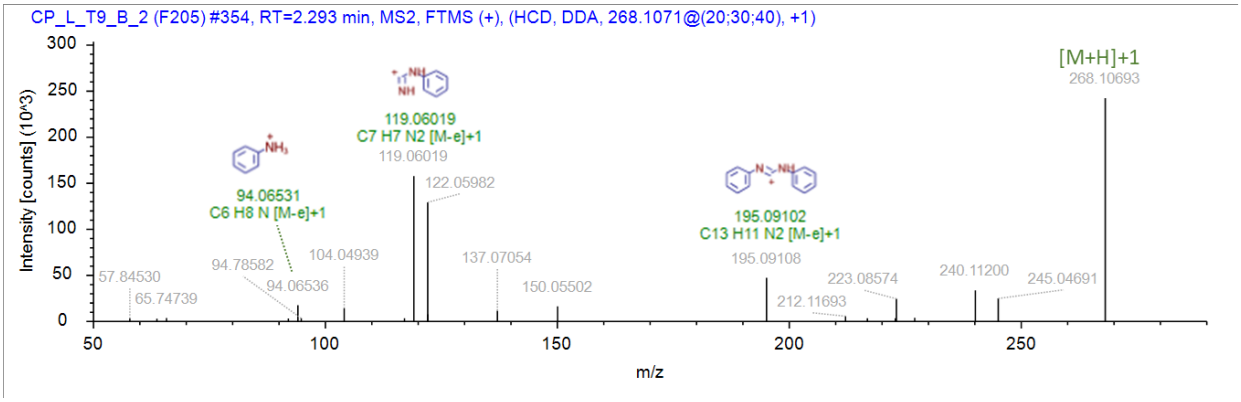
DPG $TP_{DPG}266$ MS2 Spectrum



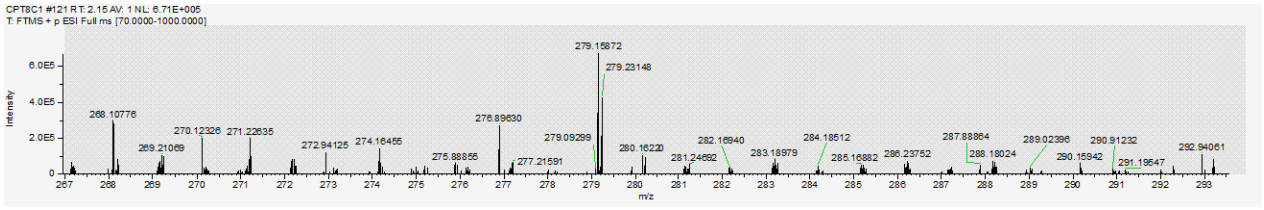
DPG $TP_{DPG}268$ MS1 Spectrum



DPG $TP_{DPG}268$ MS2 Spectrum



DPG $TP_{DPG}270$ MS1 Spectrum



DPG $TP_{DPG}270$ MS2 Spectrum

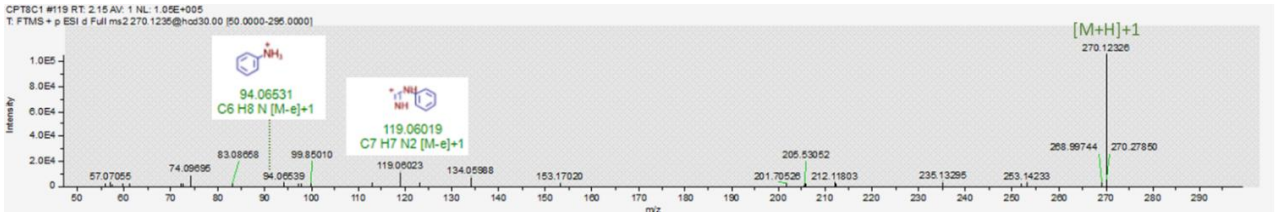


Figure S2.4: HRMS Spectra parent compounds and transformation products for A)BTZ, B)6PPD, C)6PPD-q, D)HMMM, and E)DPG.

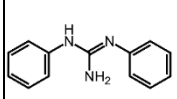
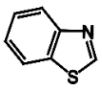
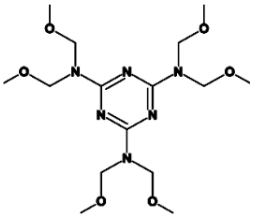
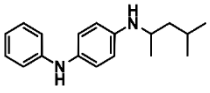
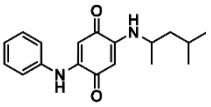
S3: Supporting Information to Chapter 3

Table S3.1: Soil Properties

	Ein Hashlosha	**Sa'ad**	**Nir Oz**
Sand (%)	47	25	82
Silt (%)	40	48	14
Clay (%)	13	27	4
Soil organic matter (%)	2.64 ± 0.01	2.27 ± 0.07	1.04 ± 0.05
$CaCO_3$ (%)	8	16	4
Bulk density (g/cm^3)	1.27 ± 0.01	1.24 ± 0.01	1.38 ± 0.01
Cation exchange capacity (meq/100g)	16	21	11
pH	7.5	7.6	7.5

Soil properties according to Ben Mordechay et al, 2023 [158].

Table S3.2: Compound Properties

Compound	DPG	BTZ	HMMM	6PPD	6PPD-quinone
Structure					
Exact mass ($g\ mol^{-1}$)	211.11034	135.01403	390.22129	268.19311	298.16702
pK_a	10.12[262]	7.8[263]	7.01[27]	6.7[168]	-4.0[170]
$logK_{ow}$	2.9[262]	2[264]	1.6[109]	5.6[31]	4.30[200]
logD*	0.31	1.54	1.49	5.54	4.30

*logD was approximated at the mean soil pH (7.53) with the following equation:

$$\log D = \log K_{OW} + \log \frac{1}{1 + 10^{pKa - pH}}$$

Table S2.3: Soil Recovery

Selected method (no buffer or Ca^{2+}):

	DPG	BTZ	HMMM	6PPD	d5-6PPD-quinone
No soil	94 ± 9 %	99 ± 3 %	98 ± 2 %	61 ± 3 %	97 ± 2 %
Nir Oz	107 ± 0 %	37 ± 3 %	7 ± 1 %	43 ± 1 %	96 ± 0 %
Sa'Ad	99 ± 2 %	56 ± 10 %	17 ± 2 %	24 ± 3 %	83 ± 2 %
Ein Hashlosha	99 ± 5 %	45 ± 2 %	20 ± 10 %	23 ± 1 %	87 ± 5 %

Buffer addition:

	DPG	BTZ	HMMM	6PPD	d5-6PPD-quinone
No soil	120 ± 10 %	99 ± 4 %	24 ± 27 %	18 ± 18 %	74 ± 9 %
Nir Oz	104 ± 3 %	31 ± 2 %	6 ± 5 %	29 ± 10 %	78 ± 11 %
Sa'Ad	96 ± 3 %	53 ± 4 %	12 ± 1 %	25 ± 6 %	77 ± 14 %
Ein Hashlosha	106 ± 5 %	49 ± 1 %	10 ± 3 %	21 ± 8 %	85 ± 15 %

Ca^{2+} addition:

	DPG	BTZ	HMMM	6PPD	d5-6PPD-quinone
No soil	99 ± 2 %	95 ± 10 %	95 ± 6 %	48 ± 10 %	78 ± 20 %
Nir Oz	108 ± 5 %	35 ± 5 %	7 ± 6 %	42 ± 5 %	88 ± 8 %
Sa'Ad	99 ± 2 %	55 ± 6 %	20 ± 13 %	27 ± 2 %	83 ± 5 %
Ein Hashlosha	100 ± 7 %	50 ± 5 %	13 ± 2 %	20 ± 10 %	78 ± 23 %

Buffer and Ca^{2+} addition:

	DPG	BTZ	HMMM	6PPD	d5-6PPD-quinone
No soil	116 ± 3 %	100 ± 1 %	15 ± 17 %	13 ± 16 %	73 ± 21 %
Nir Oz	112 ± 4 %	37 ± 11 %	12 ± 2 %	31 ± 8 %	82 ± 11 %
Sa'Ad	100 ± 4 %	48 ± 3 %	13 ± 1 %	24 ± 8 %	79 ± 14 %
Ein Hashlosha	99 ± 2 %	43 ± 10 %	11 ± 2 %	20 ± 3 %	84 ± 9 %

Section S3.1: Orbitrap Measurements

Auto sampling was performed with an Vanquish Horizon autosampler (Thermo Fisher Scientific, VH-A10-A). Since tire-derived compounds and their transformation products are unstable in aqueous solution, samples were stored in 100% acetonitrile. A custom injection program was employed to dilute samples to 50% ultrapure water before injection into the column, in order to improve peak shape of early eluting analytes. The injection needle was washed, then 2 µL of ultrapure water were drawn, the needle was washed again, then 4 µL of sample were drawn. The needle was washed again, and then 2 µL of ultrapure water were drawn. The ultrapure water and sample were mixed by drawing and dispensing the 8 µL within the needle ten times, then the needle was washed a final time, and the mixed sample was injected.

The liquid chromatography was operated using a Vanquish Horizon pump (Thermo Fisher Scientific, VH-P10-A). Phase A was ultrapure water with 0.1% formic acid. Phase B was methanol with 0.1% formic acid. Phase A was held at 95% for two minutes, then decreased to 5% over 22 minutes. It was then held at 5% for 2 minutes, before increasing back to 95% over 1 more minute. 4 minutes equilibration time was included between each sample. A Waters Acquity™ Premier HSS T3 column (100 mm, 1.8 µm pore size, 2.1 mm diameter) was used and maintained at 60°C with a Vanquish Horizon column compartment (Thermo Fisher Scientific, VH-C10-A).

The Orbitrap Exploris 240 (Thermo Fisher Scientific) was operated with AcquireX data acquisition software (Thermo Fisher Scientific). For transformation product identification, an inclusion composite sample was prepared by mixing 10 µL of all lettuce samples which had been exposed to tire-derived compounds. An exclusion composite sample was prepared by mixing 10 µL of all lettuce samples which had not been exposed to tire-derived compounds. Both samples were measured in full scan mode to generate an inclusion list of compounds present in the inclusion sample but not in the exclusion sample. Then, the inclusion sample was injected four times, while the Orbitrap was operated under Full MS / dd-MS^2 with Targeted Mass Inclusion and Exclusion, to collect MS^2 spectra of all compounds on the inclusion list. This workflow was repeated once with low Normalized Collision Energy (HCD Collision Energies: 10%, 25%, 40%), and once with high Normalized Collision Energy (HCD Collision Energies: 50%, 70%, 90%). Then, all samples were measured with the Orbitrap operated under Full MS mode with maximum resolution (240,000). Detailed method parameters are provided in the tables below.

Orbitrap Parameters: Full Scan only	
General	
Runtime	0 to 26 min
Polarity	Positive
Default charge state	1
Mild Trapping	True
Lock Mass Correction	EASY-IC™, Scan-to-Scan mode, for Full Scan only
Full Scan	
Resolution	240,000
Scan range	90 to 900 m/z
RF Lens	50%

Orbitrap Parameters: Full Scan – dd MS^2	
General	
Runtime	0 to 26 min
Polarity	Positive
Default charge state	1
Mild Trapping	True
Lock Mass Correction	EASY-IC™, Scan-to-Scan mode, for Full Scan only
Full Scan	
Resolution	60,000
Scan range	90 to 900 m/z
RF Lens	70 %
dd-MS^2	
Resolution	15,000
Isolation window	0.4 m/z
Normalized Collision Energies	10%,25%,40% or 50%,70%,90%
Minimum AGC target	Standard
Maximum Injection Time	Auto
Dynamic exclusion	Exclude after 3 times if occurs within 12 s for 60 s
Dynamic exclusion mass tolerance	5 ppm

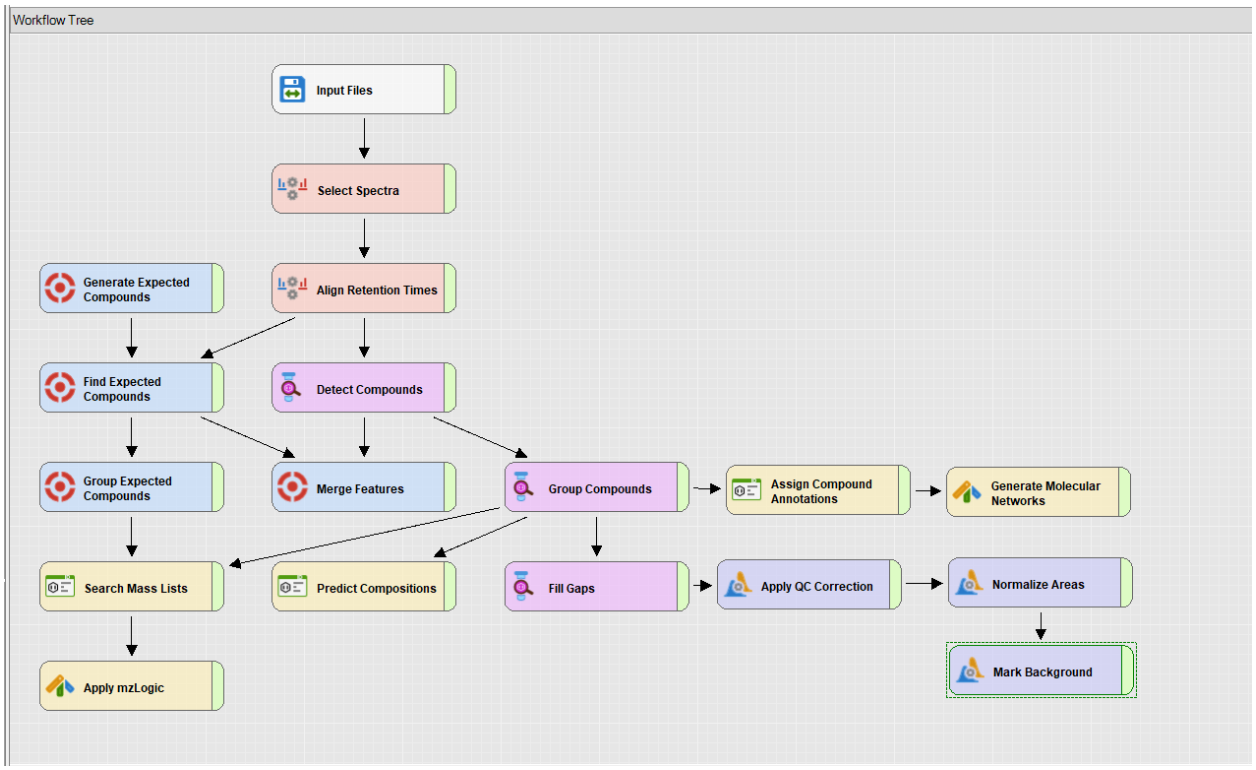


Figure S3.1: Compound Discoverer data processing workflow

Table S3.4: Compound Discoverer Parameters

Align Retention Times	
Alignment Model	Adaptive curve
Maximum Shift	0.5
Mass Tolerance	5ppm
Detect Compounds	
Mass tolerance	5ppm
Min. Peak Intensity	100000
Chromatographic S/N	3
Remove Baseline	False
Ions	[M+H]+1, [M+Na]+1
Group (Expected) Compounds	
Mass tolerance	5ppm
RT tolerance	0.2 min
Align Peaks	True (False)
Preferred Ions	[M+H]+1
Area Integration	All Ions
Peak Rating Threshold	5
Number of Files	1
Fill Gaps	
Mass Tolerance	5ppm
S/N Threshold	1.5
Apply QC Correction	
Min. QC Coverage	50
Max. QC Area	30
Max. Corrected QC Area RSD (%)	25
Normalize Areas	
Normalization Type	Constant Median
Exclude Blanks	True
Mark Background	
Max Sample/Blank	5
Generate Expected Compounds	
Apply Dealkylation	True
Apply Dearylation	True
Max # Steps	2
Min. Mass [Da]	120
Max. # Phase II	1
Max. # All Steps	3
Ions	[M+H]+, [M+Na]+

Find Expected Compounds	
Mass Tolerance	5ppm
Intensity Tolerance	30%
Intensity Threshold	0.1%
Min. # Isotopes	1
Use Most Intense Isotope only	True
Min. Peak Intensity	100000
Chromatographic S/N	3
Remove Baseline	False
Generate Molecular Networks	
Use Full MSn Tree	True
Match Mass Shift	True
Match Transformations	True
Variate Transformations	True
S/N Threshold	3
Mass Tolerance	5 ppm
Min. Fragment m/z	50
Require Transformations	False
Require MSn	True
Min. MSn Score	10
Min. MSn Coverage	10
Min. Fragments	3
Predict Compositions	
Mass Tolerance	5 ppm
Min. H/C	0.1
Max. H/C	3.5
Isotope Intensity Tolerance	30 %
Isotope Intensity Threshold	0.1 %
S/N Threshold	3
Use Fragments Matching	True
Mass Tolerance	5 ppm
S/N Threshold	3

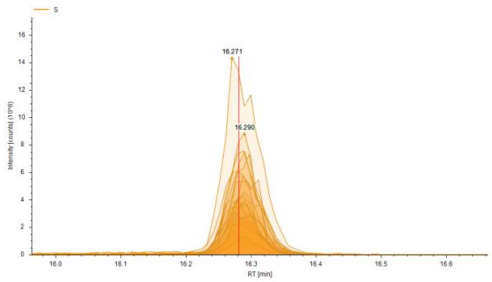
Section S3.2: Potential Transformation Products

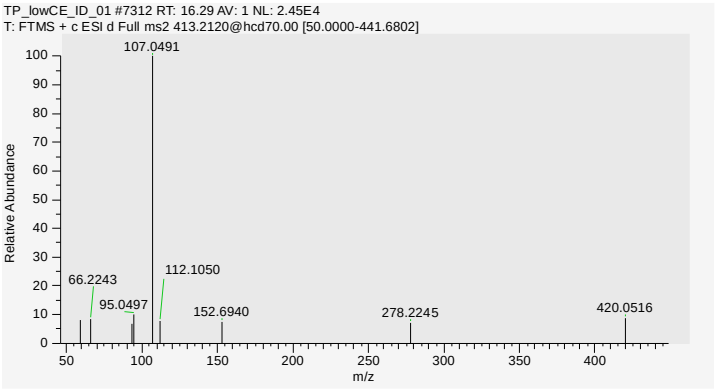
Note: Representative MS2 spectra are shown for each compound. In some cases, multiple MS2 spectra were acquired (including at different collision energies), and fragment lists were combined for comparison with parent compounds. Thus, some fragments listed may not be shown in the representative spectrum shown here. All raw data files are available from the authors upon request.

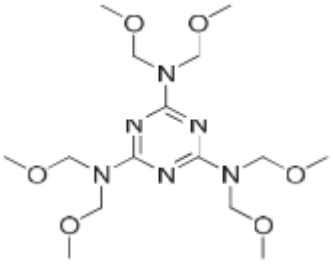
HMMM

ID	m/z	Ion	Molecular formula	Transformation	Confidence Level	RT (min)	Fragments matching parent	References
HMMM	391.22995 413.2120	[M+H]+ [M+Na]+	$C_{15}H_{30}N_6O_6$ (-0.03 Δppm)		1	16.3	Diagnostic: 391.2; 177.1; 207.1; 345.2; 359.2; 153.9; 163.1	
TP_HMMM_377 (Hexamethylmelamine pentamethyl ether)	377.21424	[M+H]+	$C_{14}H_{28}N_6O_6$ (-0.18 Δppm)	Methoxy hydrolysis	2a	14.4	177.1; 207.1; 153.9; 163.1; 345.2; 359.2	[27,109,133,153]
TP_HMMM_361	361.18296	[M+H]+	$C_{13}H_{24}N_6O_6$ (-0.13 Δppm)	2 x methoxy hydrolysis, oxidation to aldehyde	2a	15.1	163.1	[27,133]
TP_HMMM_303 (Tetra-MMM)	303.17746	[M+H]+	$C_{11}H_{22}N_6O_4$ (-0.23 Δppm)	2 x methoxy hydrolysis, formaldehyde elimination to free amine	2a	11.6	207.1; 163.1	[27,133,153]

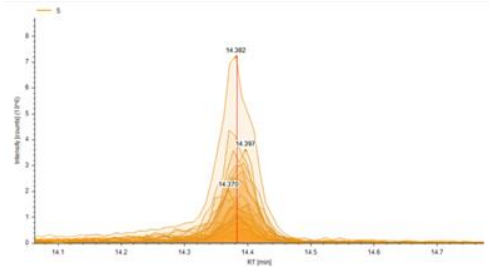
HMMM

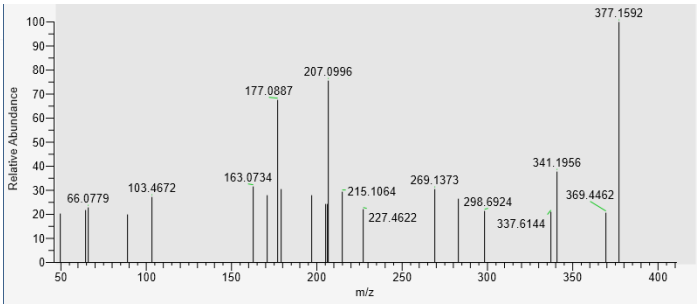


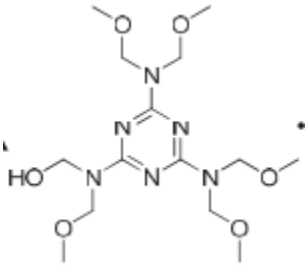




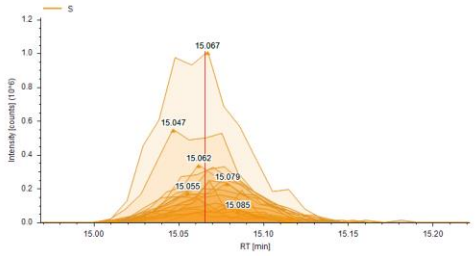
TP_HMMM_377

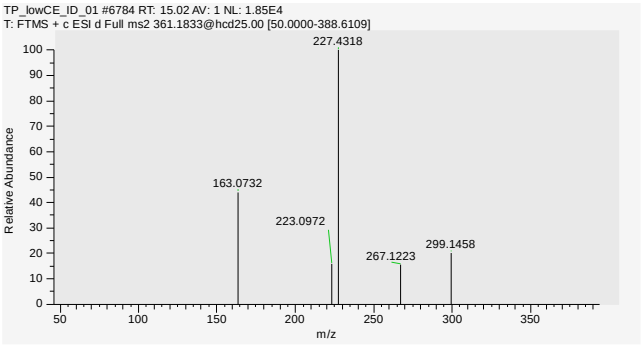


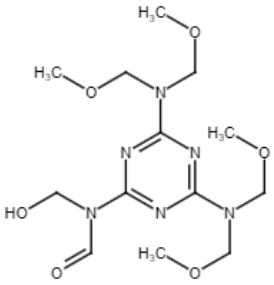




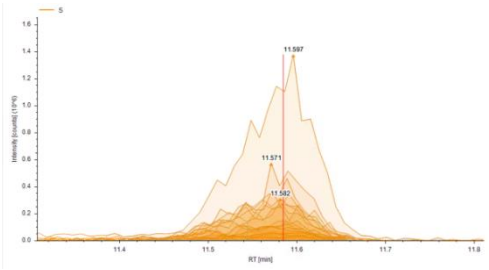
TP_HMMM_361

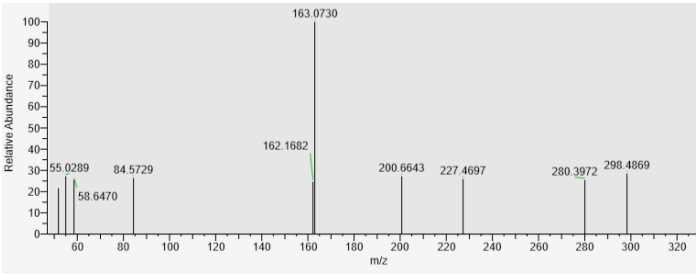


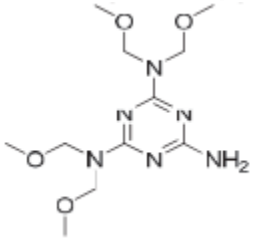




TP_HMMM_303



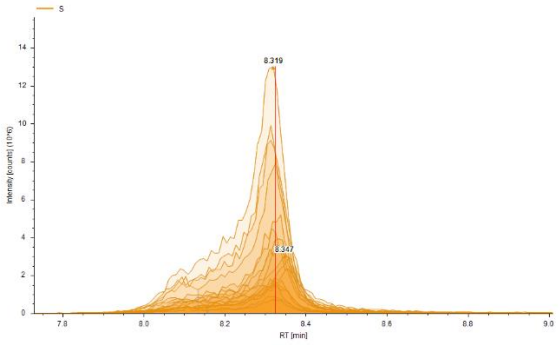


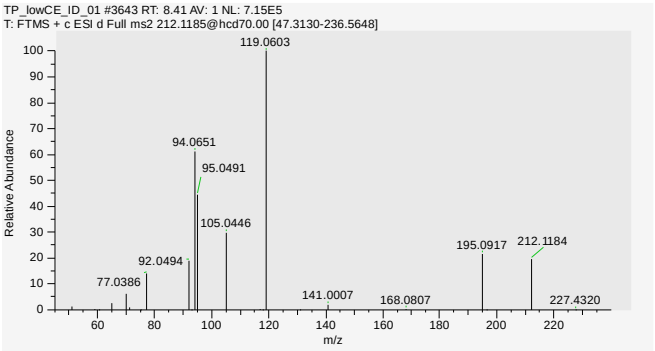


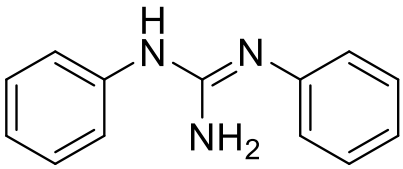
DPG

ID	m/z	Ion	Molecular formula	Transformation	Confidence Level	RT (min)	Fragments matching parent	References
DPG	212.11842	[M+H]+	$C_{13}H_{13}N_3$		1	8.3	Diagnostic: 212.1; 195.1; 141.0; 119.1; 94.1; 95.1; 105.0	
TP_DPG_393	393.29991	[M+H]+			5	24.2	195.1; 119.1; 95.1; 105	
TP_DPG_381	381.26108	[M+H]+	$C_{18}H_{32}N_6O_3$ (0.56 Δppm)		4	20.7	119,1; 94.1; 105	
TP_DPG_379	379.24543	[M+H]+	$C_{18}H_{30}N_6O_3$ (0.57 Δppm)		4	20.6	119.1	
TP_DPG_345	345.23990	[M+Na]+			5	23.3	195.1; 119.1; 95.1	
TP_DPG_315	315.17770	[M+H]+	$C_{12}H_{22}N_6O_4$ (0.54 Δppm)		4	17.0	119.1; 95.1	
TP_DPG_288 (P20)	288.14948	[M+H]+	$C_{19}H_{17}N_3$	rearrangement	2a	12.6	195.1; 119.1¸94.1	[36]
TP_DPG_210	210.20144	[M+H]+			5	11.1	141; 105	

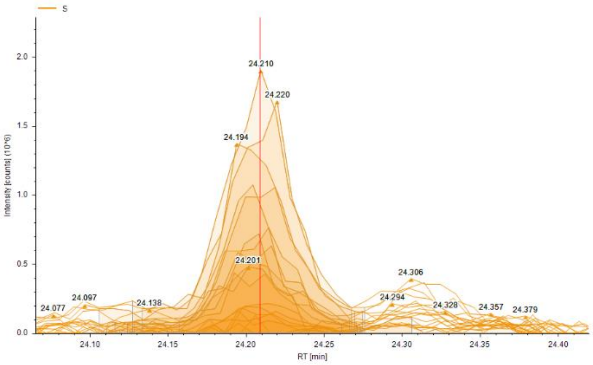
DPG

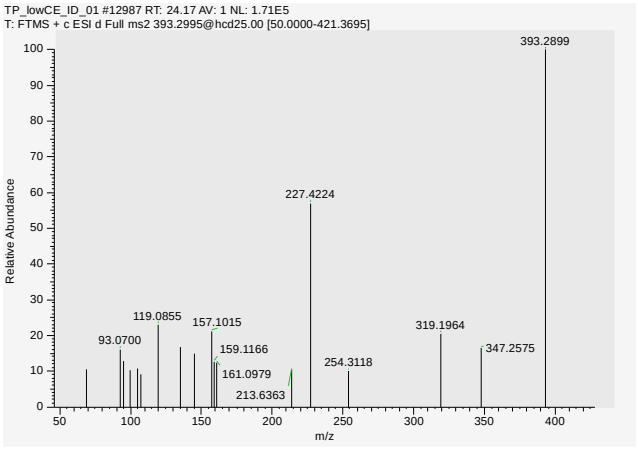






TP_DPG_393





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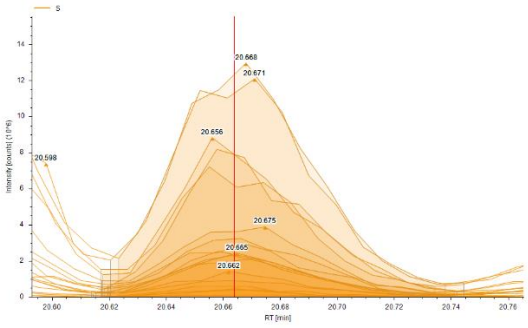

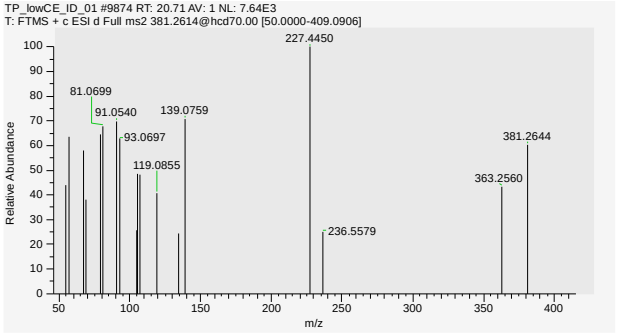

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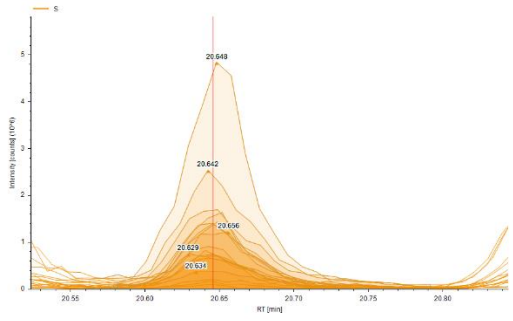
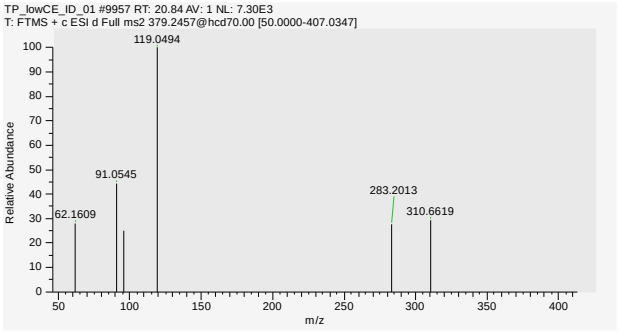
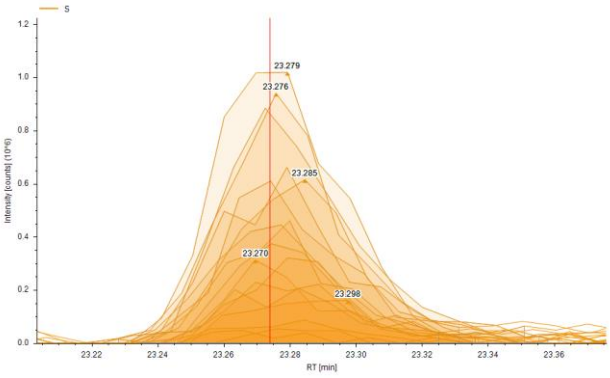
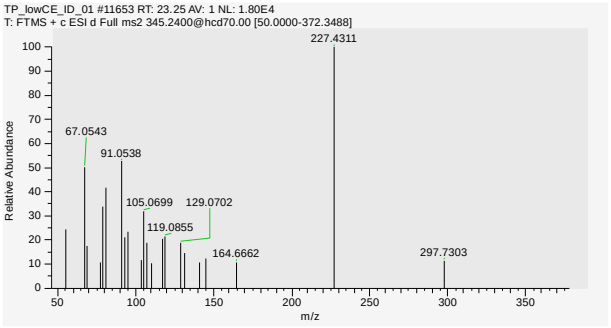


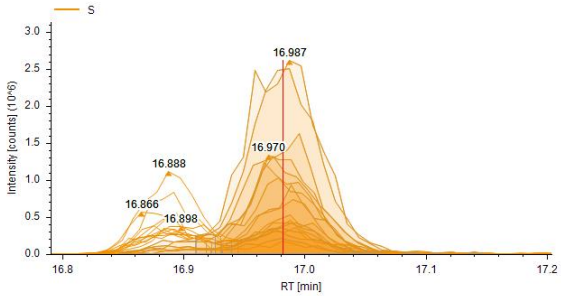


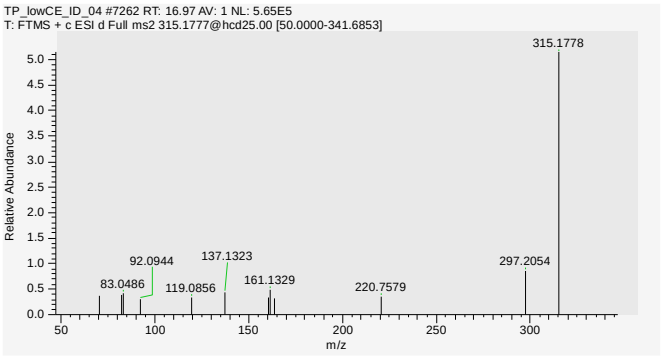
TP_DPG_345



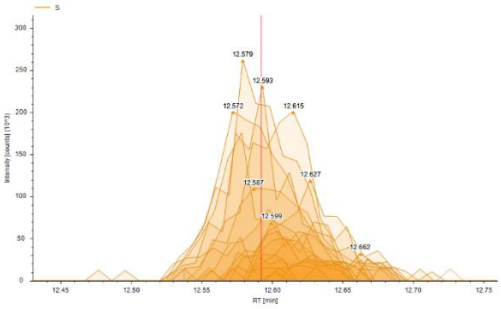


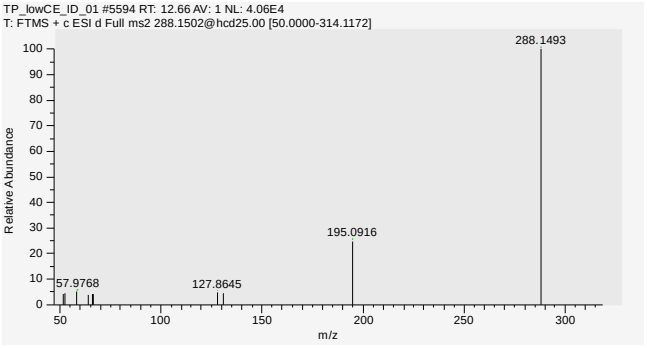
TP_DPG_315

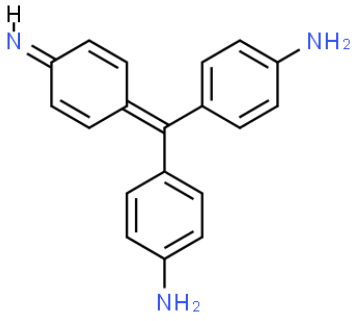




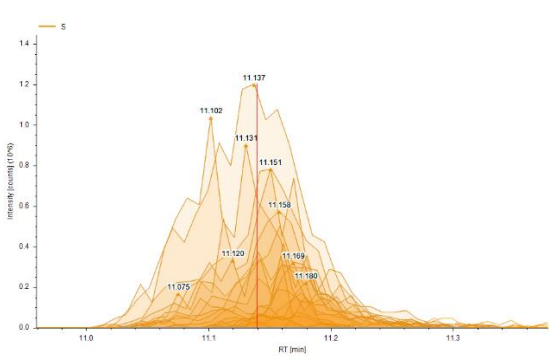
TP_DPG_288

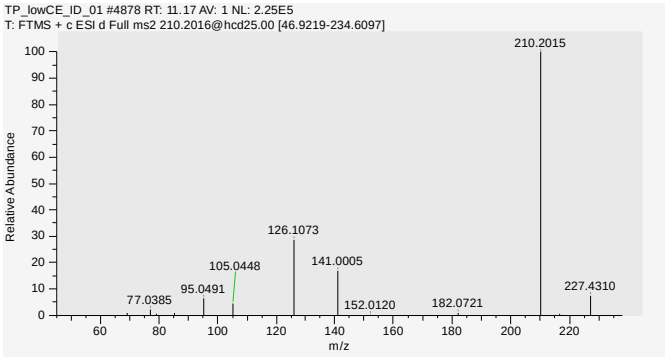






TP_DPG_210

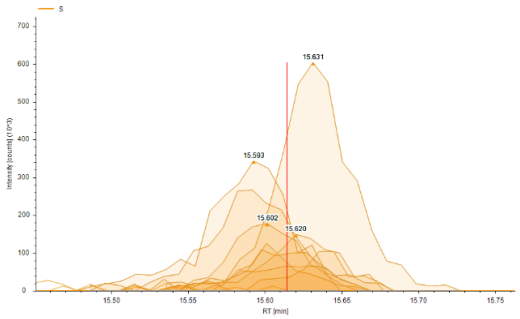


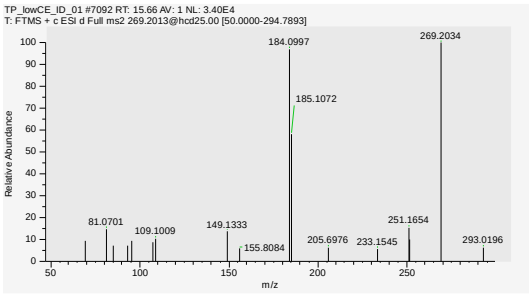


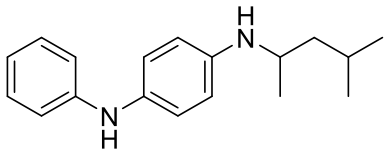
6PPD

ID	m/z	Ion	Molecular formula	Transformation	Confidence Level	RT (min)	Fragments matching parent	References
6PPD	269.20121	[M+H]+	$C_{18}H_{24}N_2$			15.6	Diagnostic: 269.2; 184.1; 185.1	
TP_6PPD_361	361.23489	[M+H]+	$C_{18}H_{28}N_6O_2$ (0.66 Δppm)		4	22.1	185.1	
TP_6PPD_329	329.15949	[M+H]+			5	23.0	269.2; 185.1	
TP_6PPD_267	267.19544	[M+H]+	$C_{16}H_{26}O_3$ (-0.11 Δppm)		4	21.4	185.1	
TP_6PPD_231	231.19540	[M+H]+	$C_{13}H_{26}O_3$ (-0.21 Δppm)		4	20.3	185.1	
TP_6PPD_205	205.16995	[M+H]+	$C_{13}H_{20}N_2$ (0.10 Δppm)		4	11.2	185.1*	

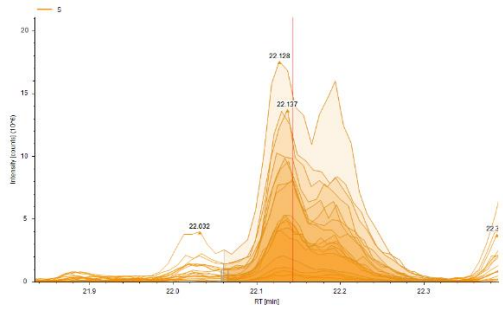
6PPD

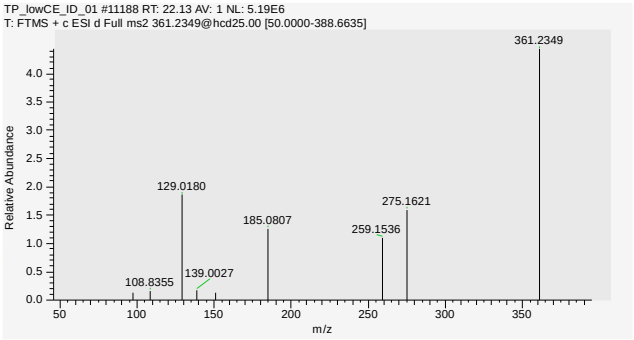




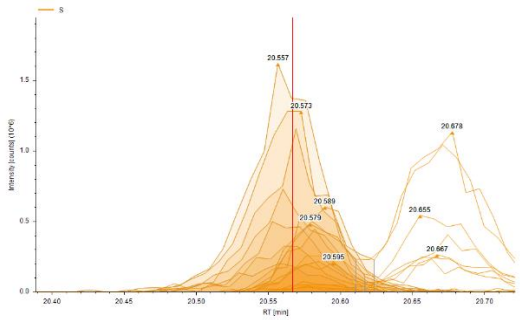


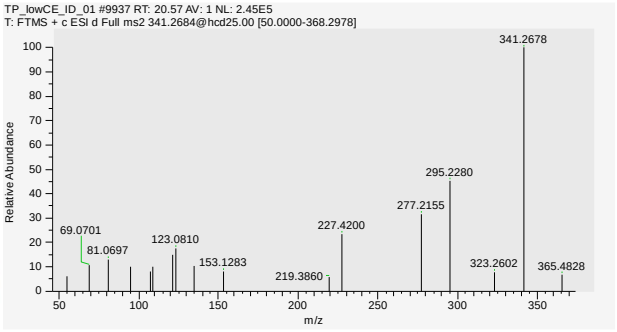
TP_6PPD_361



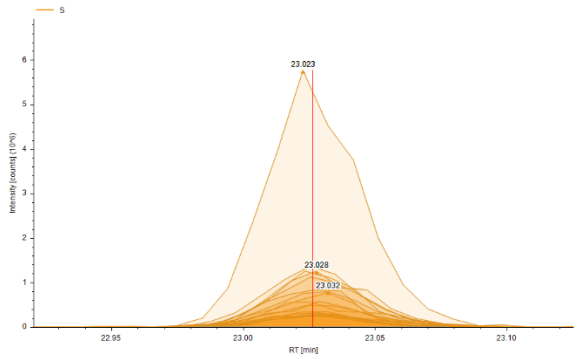


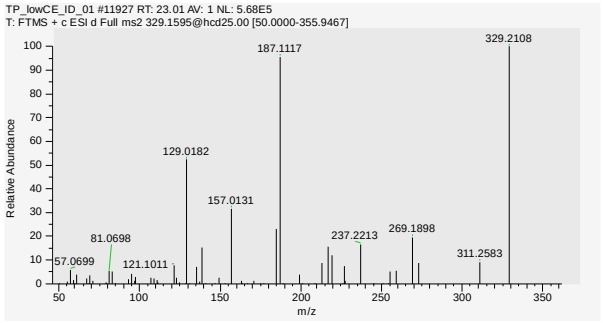
TP_6PPD_341



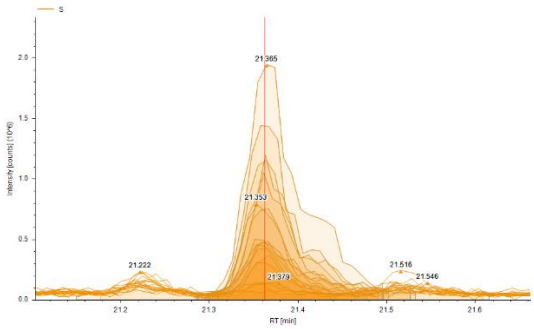


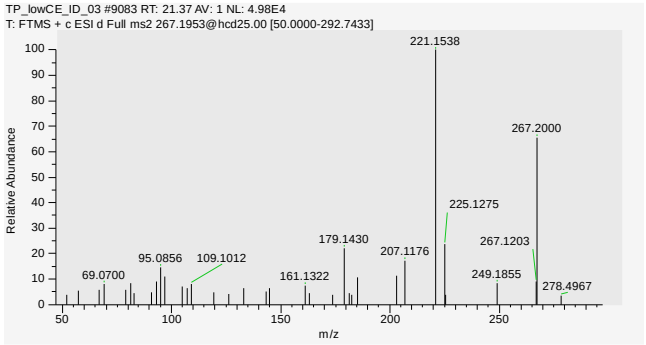
TP_6PPD_329



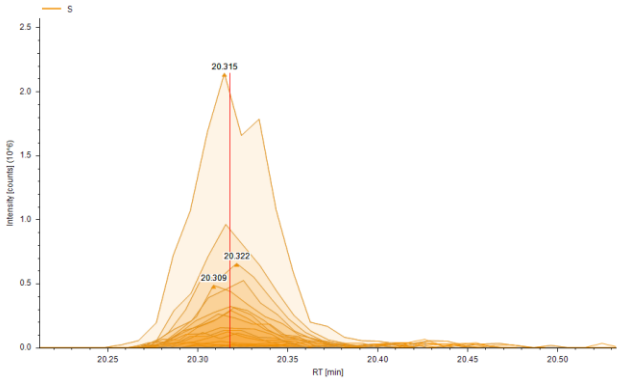


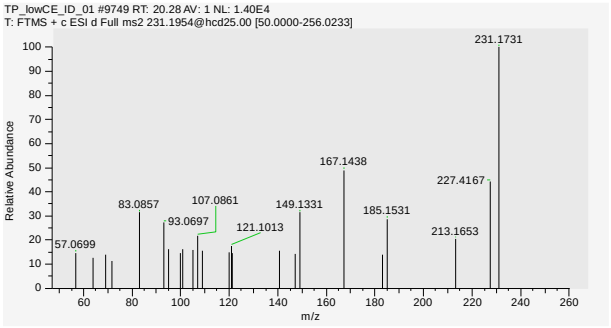
TP_6PPD_267



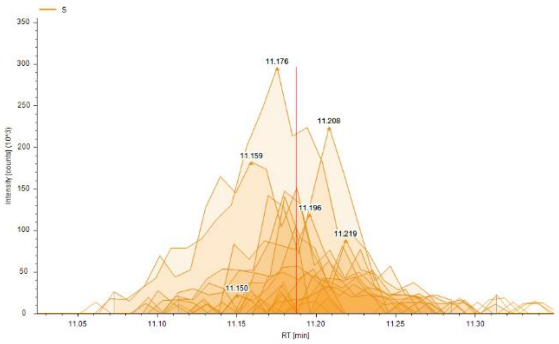


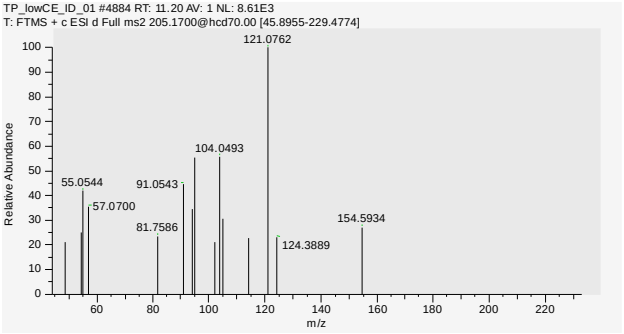
TP_6PPD_231





TP_6PPD_205



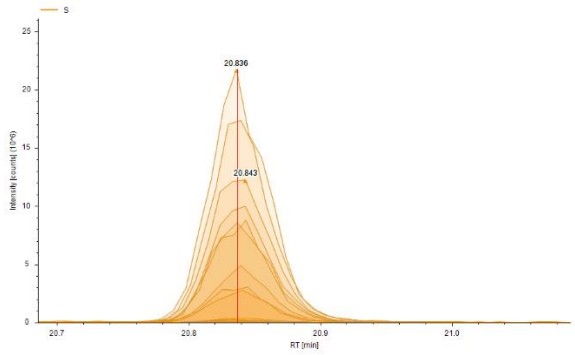


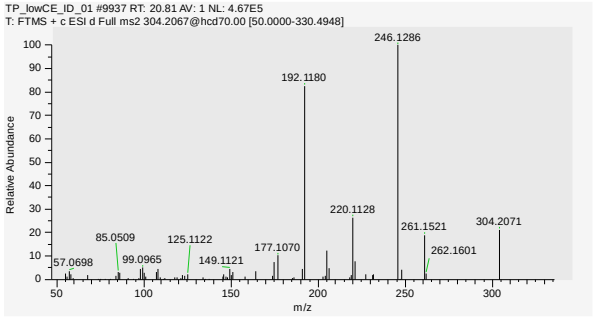
d5-6PPD-quinone

ID	m/z	Ion	Molecular formula	Transformation	Confidence Level	RT (min)	Fragments matching parent	References
d5-6PPD-quinone	304.20673	[M+H]+	$C_{18}H_{17}D_5N_2O_2$ (-0.19 Δppm)		1	20.8	Diagnostic: 304.2; 220.1; 192.1; 246.1	
TP_d56PPDq_379	379.24545	[M+Na]+			5	20.1	220.1	
TP_d56PPDq_210	210.20144	[M+H]+			5	11.1	220.1*; 192.1*	
TP_d56PPDq_205	205.16995	[M+H]+	$C_{13}H_{20}N_2$ (0.10 Δppm)		4		220.1*; 192.1*	

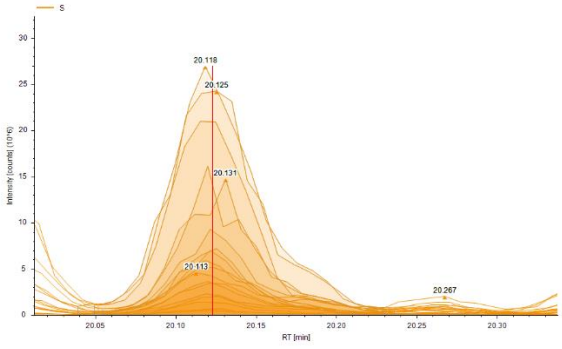
*indicates fragments which were matched after mass shift transformation

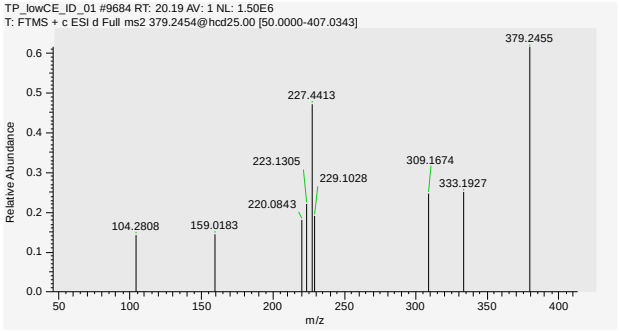
d5-6PPD-quinone



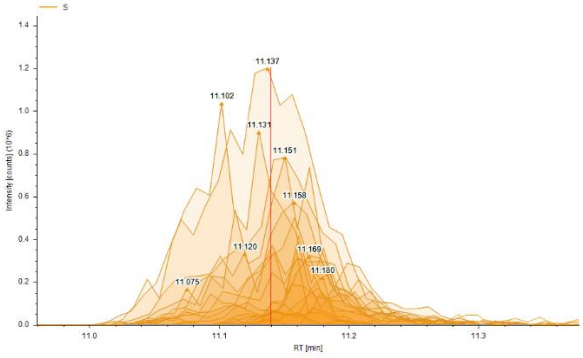


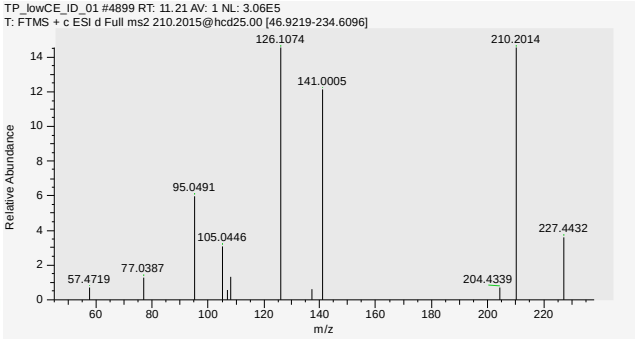
TP_d56PPDq_379



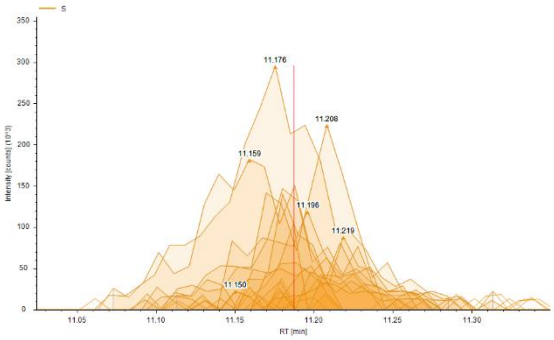


TP_d56PPDq_210





TP_d56PPDq_205



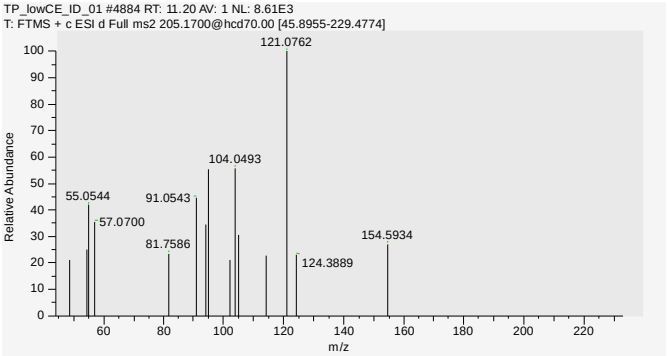


Table S3.5: Limits of quantifications (ng/g dry weight) of all tire-derived compounds in leaves and soil

	Soil		Leaves	
	Analytical (ng/mL)	Biomass (ng/g dry weight)	Analytical (ng/mL)	Biomass (ng/g dry weight)
HMMM	0.1	0.2	0.1	0.5
DPG	0.1	0.2	1.0	5.0
d5-6PPDq	0.1	0.2	0.1	0.5
6PPD	0.1	0.2	0.1	0.5
BTZ	7.5	18	10.0	50.0

Table S3.6: Concentrations in control soil (ng/g dry weight)

6PPD	d56PPDq	DPG	HMMM	Soil	Replicate	Top/Bottom
<LOQ	<LOQ	3.3	<LOQ	EH	1	Bottom
<LOQ	<LOQ	2.8	<LOQ	EH	1	Top
<LOQ	<LOQ	3.8	<LOQ	EH	2	Bottom
<LOQ	<LOQ	2.8	<LOQ	EH	2	Top
<LOQ	<LOQ	2.8	<LOQ	EH	3	Bottom
<LOQ	<LOQ	2.6	<LOQ	EH	3	Top
<LOQ	<LOQ	<LOQ	<LOQ	NO	1	Bottom
<LOQ	<LOQ	<LOQ	<LOQ	NO	1	Top
<LOQ	<LOQ	2.5	<LOQ	NO	2	Bottom
<LOQ	<LOQ	<LOQ	<LOQ	NO	2	Top
<LOQ	<LOQ	<LOQ	<LOQ	NO	3	Bottom
<LOQ	<LOQ	<LOQ	<LOQ	NO	3	Top
<LOQ	<LOQ	3.0	<LOQ	SA	1	Bottom
<LOQ	<LOQ	2.9	<LOQ	SA	1	Top
<LOQ	<LOQ	3.0	<LOQ	SA	2	Bottom
<LOQ	<LOQ	4.1	<LOQ	SA	3	Bottom
<LOQ	<LOQ	3.0	<LOQ	SA	2	Top
<LOQ	<LOQ	2.9	<LOQ	SA	3	Top

S4: Supporting Information to Chapter 4

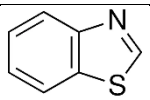
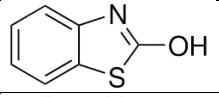
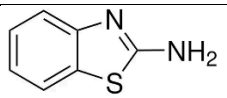
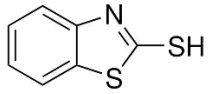
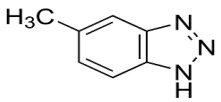
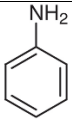
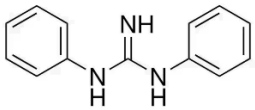
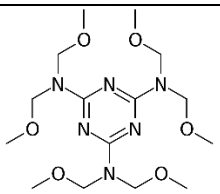
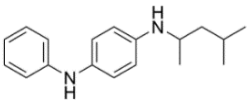
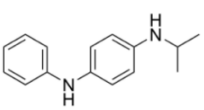
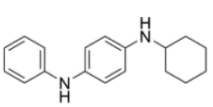
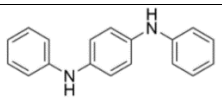
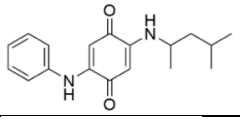
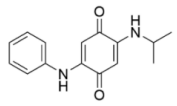
Table S4.1: Sample Information

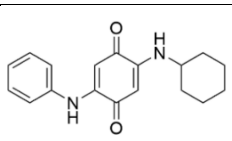
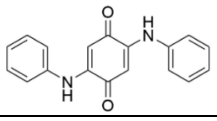
Sample ID	Sample set	Growth country	Crop type	WWTP for irrigation	Compost application
IL-01	Israel	Israel	lettuce	Haifa	
IL-02	Israel	Israel	parsley	Haifa	No
IL-03	Israel	Israel	Mint	Jerusalem (Sorek)	Yes
IL-04	Israel	Israel	Mint	Haifa	Yes
IL-05	Israel	Israel	parsley	Shafdan	yes
IL-06	Israel	Israel	Coriander	Shafdan	no
IL-07	Israel	Israel	Parsley	Shafdan	yes
IL-08	Israel	Israel	Parsley	Haifa	yes
IL-09	Israel	Israel	Celery	Shafdan	yes
IL-10	Israel	Israel	Dill	Jerusalem (Sorek)	yes
IL-11	Israel	Israel	Parsley	Shafdan	Yes
IL-12	Israel	Israel	Beetroot leaves	Shafdan	Yes
IL-13	Israel	Israel	Beetroot leaves	Haifa	
CH-01	Switzerland	Switzerland	Chicory		
CH-02	Switzerland	Switzerland	Lamb's lettuce		
CH-03	Switzerland	Switzerland	Lamb's lettuce		
CH-04	Switzerland	Switzerland	Lettuce		
CH-05	Switzerland	Switzerland	Lamb's lettuce		
IT-01	Switzerland	Italy	Lettuce		
IT-02	Switzerland	Italy	Lettuce		
IT-03	Switzerland	Italy	Lettuce		
IT-04	Switzerland	Italy	Spinach		
IT-05	Switzerland	Italy	Arugula		
ES-01	Switzerland	Spain	Lettuce		
ES-02	Switzerland	Spain	Lettuce		
ES-03	Switzerland	Spain	Lettuce		
ES-04	Switzerland	Spain	Lettuce		
ES-05	Switzerland	Spain	Lettuce		

Blank cells indicate no information available to us.

For samples from Israel, detailed information about sampling locations, wastewater treatment plants, and sampling procedure are provided in Ben Mordechay et al 2021.[76]

Table S4.2: Physiochemical properties of the tire-derived compounds

Compound	Structure	Log K_{OW}	pKa
Benzothiazole		2.17[28]	1.2[265] (conjugate acid)
2-hydroxybenzothiazole *		2.35[28]	8.9[266]
2-aminobenzothiazole		2.00[28]	3.58** (conjugate acid)
2-mercaptobenzothiazole		1.83[28]	7.1[266]
5-methyl-1H-benzotriazole		1.13[197]	8.5[266]
Aniline		0.91[69]	4.6[267] (conjugate acid)
1,3-diphenylguanidine		3.61[156]	9.74[156] (conjugate acid)
HMMM		1.26[156]	3.60[156] (conjugate acid)
6PPD		4.47[17]	6.46[17] (conjugate acid)
IPPD		3.28[17]	6.42[17] (conjugate acid)
CPPD		4.64[17]	6.42[17] (conjugate acid)
DPPD		4.93[17]	2.42[17] (conjugate acid)
6PPD-quinone		3.98[17]	0.61[17]
IPPD-quinone		2.58[17]	0.87[17]

CPPD-quinone		3.94[17]	0.62[17]
DPPD-quinone		3.46[17]	0.47[17]

*2-hydroxybenzothiazole is a tautomer of 2-benzothiazolinone and the two forms exist in equilibrium.

**pKa modelled using Chemicalize software (Chemaxon)

Table S4.3: UPLC-MS/MS Method Details

All samples were analyzed by ultra-performance liquid chromatography - triple quadrupole mass spectrometry (Agilent 1290 Infinity II - Agilent 6470) using a C18 column (Acquity HSS T3, 1.8 µm, Waters, 50 mm) in the positive ionization mode (capillary voltage: 2500 V) via multiple reaction monitoring (MRM). The LC flow rate was 0.6 mL min^{-1} at a column temperature of 40 °C and the injection volume was 5 µL. The mobile phase consisted of ultrapure water (Phase A) and acetonitrile (Phase B), both containing 0.1% formic acid. The eluent gradient was held at 95% Phase A for one minute, then the contribution of Phase A decreased from 95 to 5% over 9 min, was held at 5% for 2 min and increased again to 95% over the last 1 min. Column was then re-equilibrated at 95% Phase A for one minute. Electrospray ionization (ESI) was achieved at gas temp 240°C, gas flow 5 L min^{-1}. The nebulizer was set to 30 psi; sheath gas to 250°C, 11 L min^{-1}. Transitions from precursor ions to product ions are specified in the following table. At least 2 product ions were monitored per compound, quantifier ions are indicated in bold.

Compound name	Surrogate standard	Precursor ion m/z	Product ion m/z	Fragmentor voltage (V)	Collision energy (V)	Cell accelerator voltage (V)	t_R (min)
2-aminobenzothiazole	**d4-BTZ**	**151**	**109**	**150**	**30**	**5**	**1.7**
2-aminobenzothiazole		151	65	150	38	5	1.7
2-hydroxybenzothiazole	**d4-BTZ**	**152**	**124**	**140**	**22**	**4**	**3.6**
2-hydroxybenzothiazole		152	92	140	26	4	3.6
2-mercaptobenzothiazole	**d4-BTZ**	**168**	**135**	**135**	**28**	**5**	**4.2**
2-mercaptobenzothiazole		168	124	135	24	5	4.2
2-mercaptobenzothiazole		168	109	135	28	5	4.2
6PPD	**d5-6PPD-q**	**269**	**184**	**150**	**45**	**5**	**5.0**
6PPD		269	107	150	45	5	5.0
6PPD		269	93	150	45	5	5.0
6PPD-quinone		299	241	150	53	5	7.1
6PPD-quinone		299	215	150	30	5	7.1
6PPD-quinone	**d5-6PPD-q**	**299**	**187**	**150**	**30**	**5**	**7.1**
Aniline	**d4-BTZ**	**94**	**77**	**100**	**22**	**4**	**2.8**
Aniline		94	51	100	23	4	2.8
Aniline		94	50	100	41	4	2.8
Benzothiazole	**d4-BTZ**	**136**	**109**	**150**	**31**	**5**	**3.9**
Benzothiazole		136	77	150	27	5	3.9
Benzothiazole		136	65	150	38	5	3.9
Benzothiazole-d4		140	113	150	31	5	3.8
Benzothiazole-d4		140	81	150	19	5	3.8
Benzothiazole-d4		**140**	**69**	**150**	**36**	**5**	**3.8**
CPPD	**d5-6PPD-q**	**267.2**	**184**	**100**	**30**	**5**	**4.7**
CPPD		267.2	107	100	50	5	4.7
CPPD		267.2	93.1	100	46	5	4.7
CPPD-quinone		297.1	215	130	18	5	6.8
CPPD-quinone	**d5-6PPD-q**	**297.1**	**187**	**130**	**34**	**5**	**6.8**
CPPD-quinone		297.1	55.2	130	50	5	6.8
d5-6PPD-quinone		**304.2**	**246.1**	**110**	**36**	**4**	**7.0**
d5-6PPD-quinone		304.2	220.1	110	36	5	7.0
d5-6PPD-quinone		304.2	192.1	110	36	5	7.0
DPG		212	195	150	20	5	2.8
DPG	**d5-6PPD-q**	**212**	**119**	**150**	**20**	**5**	**2.8**
DPG		212	94	150	20	5	2.8
DPPD	**d5-6PPD-q**	**260.1**	**183**	**130**	**38**	**5**	**7.3**
DPPD		260.1	167	130	46	5	7.3
DPPD		260.1	156	130	46	5	7.3

DPPD-quinone	**d5-6PPD-q**	**291.1**	**263**	**150**	**22**	**5**	**6.4**
DPPD-quinone		291.1	235.2	150	34	5	6.4
DPPD-quinone		291.1	144	150	38	5	6.4
HMMM		391	283	150	15	5	4.7
HMMM		391	253	150	23	5	4.7
HMMM		391	207	150	19	5	4.7
HMMM	**d5-6PPD-q**	**391**	**177**	**150**	**35**	**5**	**4.7**
IPPD	**d5-6PPD-q**	**227.2**	**184**	**100**	**18**	**5**	**3.8**
IPPD		227.2	118	100	46	5	3.8
IPPD		227.2	107	100	46	5	3.8
IPPD-quinone		257.2	216	110	30	5	5.6
IPPD-quinone	**d5-6PPD-q**	**257.2**	**187**	**110**	**30**	**5**	**5.6**
IPPD-quinone		257.2	170	110	34	5	5.6
5M-1H-BTR	**d4-BTZ**	**134.1**	**77.1**	**108**	**31**	**5**	**3.2**
5M-1H-BTR		134.1	79.1	108	23	5	3.2
5M-1H-BTR		134.1	51.2	108	45	5	3.2

Section S4.1: Peak Picking Settings

Raw data from LC-MS/MS measurements were processed with Agilent MassHunter Quantitative Analysis software. Peaks were selected within a window of 0.15 minutes to the left and right of the retention time of the highest calibration standard. Noise regions were set at 0.5 minutes to the left and right of the peak, and noise value was calculated using the ASTM method (ASTM E685-93). A signal to noise threshold of 3 was set for peak detection. Peak integration was performed using the Agile2 algorithm.

Section S4.2: Orbitrap-HRMS analysis

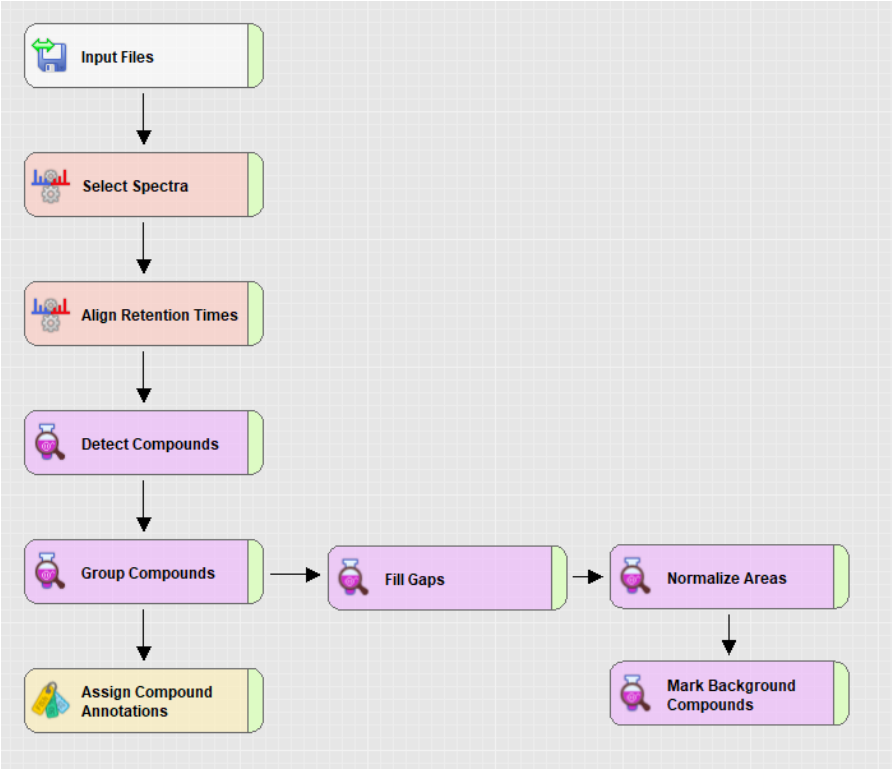
All samples were analyzed with high-performance liquid chromatography – Orbitrap high resolution mass spectrometry (Thermo Scientific Ultimate 3000 – Thermo Scientific Q Exactive™, hereafter: Orbitrap-HRMS) The liquid chromatography was operated using an LPG-3400SD pump. Phase A was ultrapure water with 0.1% formic acid. Phase B was acetonitrile with 0.1% formic acid. Phase A was held at 95% for one minute, then decreased to 5% over 10 minutes. It was then held at 5% for 2 minutes, before increasing back to 95% over 30 seconds. 2.5 minute equilibration time was included between each sample. Autosampling was performed with a WPS-3000 autosampler. Injection volume was 5µL. The draw speed was 0.5 µL s^{-1}, with a 0.003 s draw delay. Blanks were injected every ten samples. Dispense speed was 1 µL s^{-1}. The same C18 column (Acquity HSS T3, 1.8 µm, Waters 5mm) as from the triple quadrupole-MS was used and maintained at 40 ºC with a TCC-3000 column oven.

The Q Exactive – Orbitrap HRMS (Thermo Scientific, Vienna, Austria) was operated in Full MS / dd-MS^2 mode (exact parameters below). Internal standard d4-BTZ was used as a lock mass during its elution time (4.5 to 6.0 min). Two Method blanks (determined to contain no target analytes) and three positive controls (samples determined to contain target analytes) were measured first, and Compound Discoverer was used to annotate compounds with a maximum signal ratio between the signals and blanks less than five to background compounds. This list of background compounds was used as an exclusion list in all further measurements. The expected compounds feature of compound discoverer was additionally used to generate a list of potential transformation products – this was used as an inclusion list in further measurements.

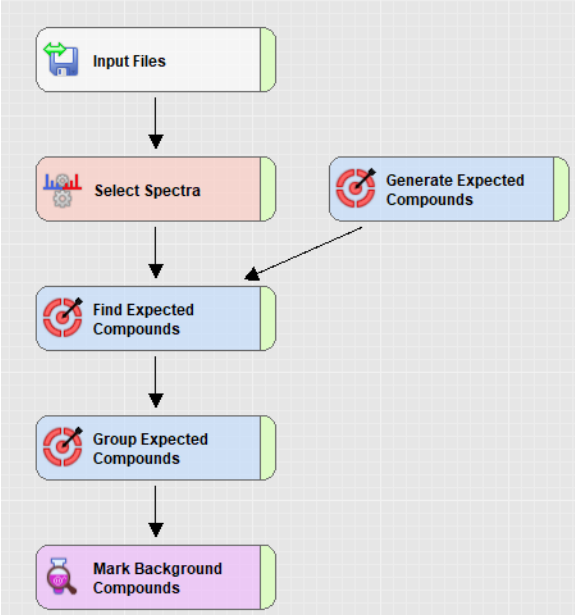
Orbitrap-HRMS Parameters	
General	
Runtime	1.19 to 11 min
Polarity	Positive
Default charge state	1
Full MS	
Resolution	70,000
AGC target	$1x10^5$
Maximum IT	50 ms
Scan range	70 to 1000 m/z
dd-MS^2	
Resolution	17,500
AGC target	$1x10^5$
Maximum IT	50 ms
Loop count	5
TopN	5
Isolation window	0.4 m/z
(N)CE / stepped nce	20, 30, 40
dd Settings	
Minimum AGC target	$8x10^3$
Intensity threshold	$1.6x10^5$
Dynamic exclusion	5.0 s
If idle…	Pick others

Section S4.3: Non-target workflow specifications using Compound Discoverer software

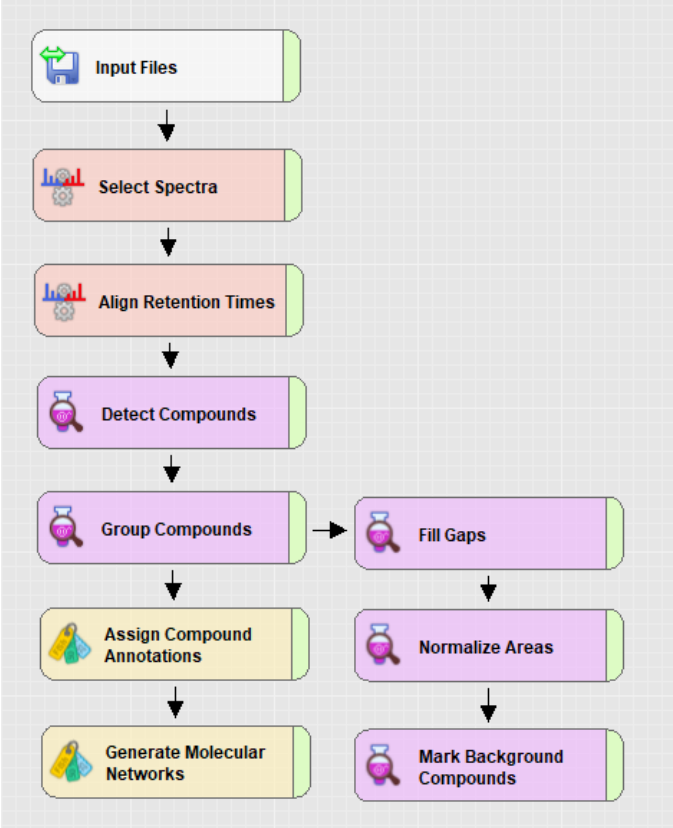
Suspect screening workflow:



Expected Compounds workflow:



Molecular Networks workflow:



Workflow parameters by node

Align Retention Times	
Alignment Model	Adaptive curve
Maximum Shift	0.5
Mass Tolerance	5ppm
Detect Compounds	
Mass tolerance	5ppm
Min. Peak Intensity	100000
Chromatographic S/N	1.5
Remove Baseline	False
Ions	$[M+H]^+$, $[M+Na]^+$
Group (Expected) Compounds	
Mass tolerance	5ppm
RT tolerance	0.2 min
Align Peaks	True
Preferred Ions	$[M+H]^+$
Area Integration	Most Common Ion
Peak Rating Threshold	5
Number of Files	2
Fill Gaps	
Mass Tolerance	5ppm
S/N Threshold	1.5
Normalize Areas	
Normalization Type	Constant Median
Exclude Blanks	True
Generate Expected Compounds	
Apply Dealkylation	True
Apply Dearylation	False
Max # Steps	1
Min. Mass [Da]	100
Max. # Phase II	1
Max. # All Steps	2
Ions	$[M+H]^+$, $[M+Na]^+$
Find Expected Compounds	
Mass Tolerance	5ppm
Intensity Tolerance	30%
Intensity Threshold	0.1%
Min. # Isotopes	2
Use Most Intense Isotope only	True
Min. Peak Intensity	100000
Chromatographic S/N	3
Remove Baseline	False
Generate Molecular Networks	
Use Full MSn Tree	True
Match Mass Shift	True
Match Transformations	True
Variate Transformations	False

S/N Threshold	3
Mass Tolerance	5 ppm
Min. Fragment m/z	90
Require Transformations	False
Require MSn	True
Min. MSn Score	20
Min. MSn Coverage	20
Min. Fragments	0

Table S4.4: Limit of quantification (LOQ) and recovery for each compound.

Compound	LOQ ng/g	Recovery % at 1 ng/g (SD)	Recovery % at 10 ng/g (SD)	Recovery % at 100 ng/g (SD)
DPG	0.2	57% (12)	86% (14)	69% (6)
2OH-BTZ	55.6	<LOQ	112% (24.2)	78% (8.7)
BTZ	8.3	<LOQ	182% (30)	117% (4)
6PPD	0.1	104%	151% (5)	125% (5)
IPPD	0.05	133% (12)	73% (14)	124% (4)
CPPD	0.3	<LOQ	141% (5)	124% (7)
DPPD	0.6	113% (5)	122% (7)	11% (1)
2amino-BTZ	1.1	105% (3)	105% (8)	111% (2)
Aniline	27.8	<LOQ	<LOQ	78% (13)
5M-1H-BTR	2.8	<LOQ	121% (14.6)	107% (2.5)
2SH-BTZ	75.6	<LOQ	<LOQ	152% (4)
HMMM	1.1	96% (16)	106% (3)	99% (7)
IPPDq	1.1	121% (11)	109% (4)	108% (2)
DPPDq	2.8	<LOQ	147% (8)	178% (2)
CPPDq	1.1	108% (4)	112% (5)	105% (1)
6PPDq	2.8	125% (5)	109% (6)	105% (1)

Note: LOQ, calculated as described in Section 2.3, can vary slightly between analytical runs based on variations in instrument sensitivity or background contamination. All samples discussed in this study were quantified during a single analytical run, and the corresponding LOQ values are presented in the above table. Recovery tests were measured in a separate analytical run, in which LOQ values were slightly lower, enabling the quantification of recovery at concentrations which are in some cases lower than the LOQ reported from the samples measurement.

It must also be noted that the results of this spiking test may not reflect the extraction efficiency of compounds which are chemically bound within plant material, or associated with tire and road wear particles which were themselves taken up into plant material. Real recovery rates might be lower, and therefore actual concentrations of tire-derived compounds in leafy vegetable samples might be higher than what is reported.

Section S4.4: Discussion of outlier samples

IL-01 was a lettuce sample grown in a small agricultural community in the north of Israel (precise location is protected under a non-disclosure agreement). A thermoplastic factory is the only industry located in this community. Images from the factory's website show agricultural fields surrounding manufacturing buildings. The factory produces standard and customized thermoplastics.

CH-04 was a lettuce sample from Feldhof Gemüse (Oberriet, Switzerland), which is a vegetable distributor located near the Rhine River, on the Swiss-Austrian border[268]. Directly next to the Feldhof Gemüse headquarters is a production site of SAX Polymers, a plastic manufacturer[269], as well as the warehouse of Lenor Plastics[270] (circled in yellow). It is unclear whether the lettuce samples we obtained were grown directly on site, or simply distributed through the Feldhof Gemüse warehouse. In addition to the plastics factory, a major highway (A13) is located adjacent to Feldhof Gemüse.

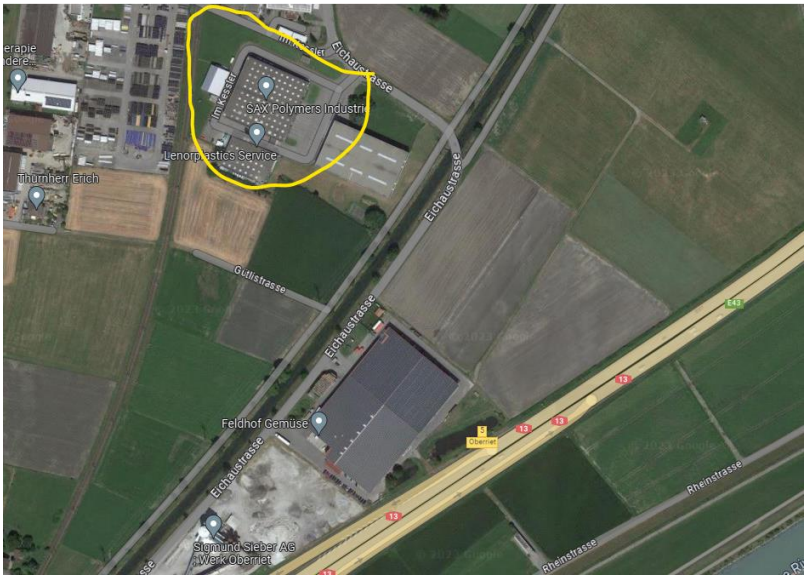


Table S4.5: ANOVA results for tire-derived compounds (TDC) and pharmaceuticals (PPCP)

Comparison	**p-value (TDC)**	**p-value (PPCP)**
Number of detects vs. growth country	0.68	NA
Cumulative concentration vs. growth country	0.45	NA
Number of detects vs. leafy vegetable type[a]	0.09	0.12
Cumulative concentration vs. leafy vegetable type[a]	0.34	0.72
Number of detects vs. compost application[b]	0.70	0.26
Cumulative concentration vs. compost application[b]	0.17	0.30
Number of detects vs. WWTP[b]	0.40	0.002
Cumulative concentration vs. WWTP[b]	0.23	0.03
Number of detects vs. Season[b]	0.82	0.05
Cumulative concentration vs. Season[b]	0.32	0.16

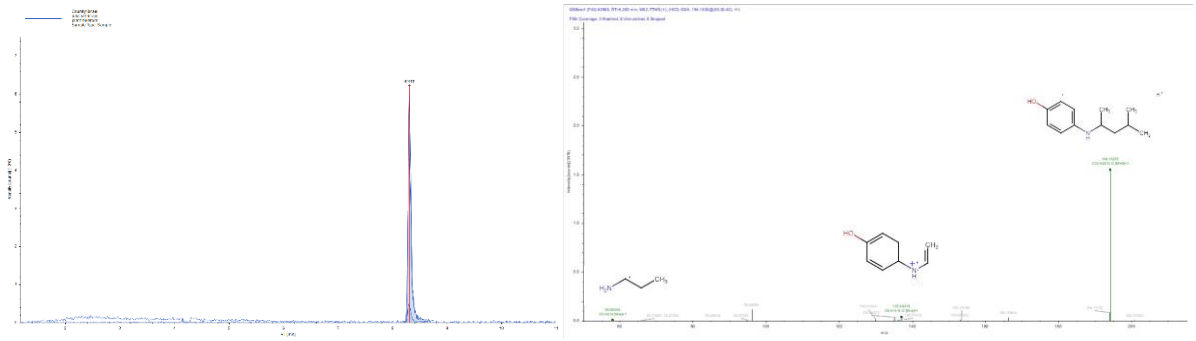
[a]ANOVA included only leafy vegetable types for which we had at least three samples (lettuce, lamb's lettuce, and parsley)

[b]ANOVA included only samples from Israel, for which information about compost application, wastewater treatment plant of irrigation water origin, and season was available.

Section S4.5: Transformation Products detected

Compound Identifier	Molecular formula	Retention Time	Exact mass of ion	Mass error of molecular ion (Δppm)	Diagnostic Fragments	Confidence level
HMMM TP 546	$C_{21}H_{41}N_{10}O_7$	8.53	546.32391	1.21	107.08522; 177.12689	3
6PPD TP194	$C_{12}H_{19}NO$	8.31	194.15316	-4.03	137.08319; 58.06564	3

Representative chromatogram and MS2 spectrum 6PPD TP194



Representative chromatogram and MS2 spectrum HMMM TP546

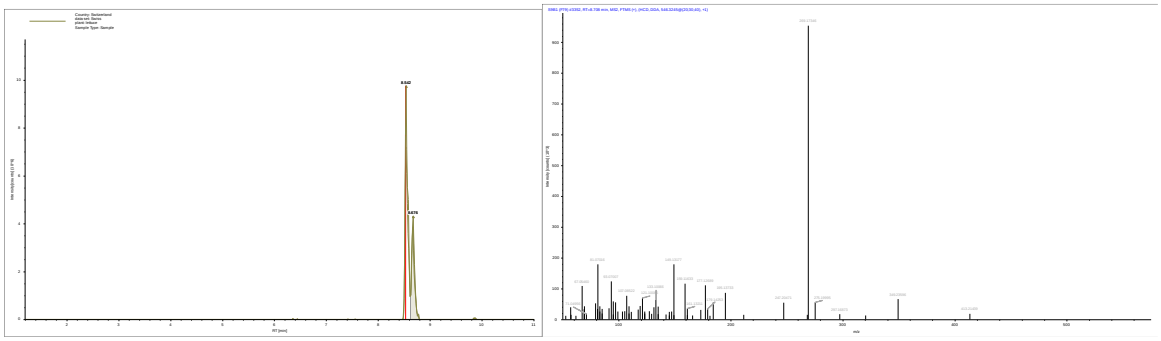


Table S4.6: Pharmaceuticals in leafy vegetable samples (ng/g dry weight)

	Lamotrigine	Carbamazepine	Epoxide carbamazepine	Dihydroxy carbamazepine	Gabapentin	Atenolol	Nicotine	Venlafaxine	Crotamiton	Benzotriazole	Sotalol	Thiabendazole	Enrofloxacin	Metoprolol	Bisoprolol
IL-01	216.9	866.5	391	32.51	6.33	0	12.7	4.3	0	7.06	0	0	0	0	0
IL-02	130.5	1343	612.5	5.52	0	0	0	1.2	0	0	0	0	0	0	0
IL-03	26.8	118.9	196.7	9.05	1.14	15.75	4.4	9.4	0	0	0	1.5	0	0.62	0.44
IL-04	67.1	525.8	674.9	5.4	0	0	13.6	2.1	0	0	0	0	0	0	0
IL-05	53.7	1.9	2.5	0	0	0	1.4	0	0	2.85	0	0	0	0	0
IL-06	90.6	2.1	4	4.33	0	0	12.2	0	0	0	0	0	0	0	0
IL-07	30.4	23.4	27.7	0.939	0	0	1.4	0	1.5	0	0	0	0	0	0
IL-08	49.6	190.5	72.7	0.786	0	0	0	0.4	0	0	0	0	1.2	0	0
IL-09	32.2	11.4	3.8	0.3	0	0	1.4	0.1	7.40	0	0	0	1.2	0	0
IL-10	1474	71.6	68.1	11.76	20.14	2.39	1.4	3.9	0	0	1.5	0	0	0.68	0.59
IL-11	36.6	2.8	6.1	0	0	0	1.4	0	0	0	0	0	0	0	0
IL-12	351.4	1.5	6.4	0.99	0.97	0.5	9.8	0	0	2.85	0	0	0	0	0
IL-13	0	283.8	568.4	38.19	0.26	0	1.4	0.9	0	0	0	0	0	0	0

S5: Supporting Information to Chapter 5

Table S5.1: Bouldering Hall information

	Checkins per hour	Ground area (m^2)	Hall age (years)	Climbing wall area (m^2)	Ventilation	Location
Hall 01	50.1	1200	7	N.A	Yes	Austria
Hall 02	37.7	Not available	19	1100	No	Austria
Hall 03	69.1	1100	4	1300	Yes	Austria
Hall 04	36.0	1100	7	1100	Yes	Austria
Hall 05	16.2	1300	3	1400	Yes	Austria
Hall 06	N.A	1800	N.A	N.A	Yes	France
Hall 07	N.A	2200	2	1500	No	Switzerland
Hall 08	N.A	N.A	N.A	1500	Yes	Switzerland
Hall 09	N.A	N.A	N.A	1000	Yes	Spain

Note: Not all data were available for all halls.

Table S5.2: Concentration of all rubber derived chemicals in all samples from every hall (n=9) and in shoe samples (n=30)

Hall 01 (ng/g)	**FP A**	**FP B**	**FP C**	**SD A**	**SD B**	**SD C**	**URT APM**	**LRT APM**
Aniline	38700	61650	45670	531	307	311	1681	1020
DPG	36190	45410	27970	329	115	183	3117	790
2OH-BTZ	30370	29580	29360	479	1155	1199	1990	1515
IPPD	888	1556	417	6.59	<LOQ	14.3	69.0	26.1
BTZ	1963	21780	21030	<LOQ	<LOQ	925	3354	1927
2amino-BTZ	476	425	376	47.9	64.0	142	<LOQ	<LOQ
2SH-BTZ	79880	102100	142600	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
HMMM	30.9	51.3	46.9	<LOQ	52.7	96.7	56.0	31.9
CPPD	13.8	16.3	10.9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPD	4677	2978	2401	<LOQ	<LOQ	<LOQ	93.0	54.5
IPPDq	30.9	54.7	24.2	<LOQ	<LOQ	<LOQ	4.78	5.58
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	293	279	201	179	312	307	316	148
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Hall 02 (ng/g)	FP A	FP B	FP C	SD A	SD B	SD C	URT APM	LRT APM
Aniline	38360	18800	32650	884	<LOQ	<LOQ	2804	1459
DPG	33430	12470	21380	721	203	58.0	4539	2182
2OH-BTZ	25410	12980	25870	1086	1262	1063	4112	1419
IPPD	512	375	832	7.98	8.25	6.91	133	56.0
BTZ	18610	8674	17430	1190	757	<LOQ	13890	2746
2amino-BTZ	326	155	317	40.4	60.3	52.5	<LOQ	<LOQ
2SH-BTZ	95560	77900	116400	<LOQ	<LOQ	<LOQ	1409	<LOQ
HMMM	324	6642	5326	441	17000	15180	498	275
CPPD	7.19	16.8	16.3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPD	1493	1379	4849	30.5	<LOQ	<LOQ	305	116
IPPDq	35.4	17.2	56.6	<LOQ	<LOQ	<LOQ	42.5	21.2
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	224	115	326	267	206	190	714	408
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Hall 03 (ng/g)	FP A	FP B	FP C	SD A	SD B	SD C	URT APM	LRT APM
Aniline	17630	26450	14920	412	1424	1185	<LOQ	<LOQ
DPG	11980	12590	11750	297	1235	793	40.8	36.4
2OH-BTZ	11610	16000	14530	1019	1471	1443	1430	4075
IPPD	114	191	357	8.45	10.6	<LOQ	71.6	24.9
BTZ	7568	14060	10610	861	1477	2825	1326	1393
2amino-BTZ	224	212	249	47.7	62.2	70.2	<LOQ	<LOQ
2SH-BTZ	51910	72690	67060	<LOQ	<LOQ	<LOQ	581	509
HMMM	39.0	50.5	42.3	178	186	111	76.2	118
CPPD	<LOQ	5.94	10.0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPD	1069	612	1106	<LOQ	31.1	<LOQ	125	224
IPPDq	4.03	4.60	6.34	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	134	130	124	371	323	405	118	51.5
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Hall 04 (ng/g)	FP A	FP B	FP C	SD A	SD B	SD C	URT APM	LRT APM
Aniline	39640	61250	33220	1329	389	629	<LOQ	<LOQ
DPG	26270	61430	28350	855	262	362	<LOQ	5.98
2OH-BTZ	27270	24550	23970	1499	<LOQ	845	566	1207
IPPD	255	364	471	20.5	11.3	12.1	<LOQ	<LOQ
BTZ	20450	22840	18550	2497	788	1122	872	<LOQ
2amino-BTZ	290	311	354	73.3	44.6	48.6	<LOQ	<LOQ
2SH-BTZ	85890	98470	108400	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
HMMM	60.7	92.7	67.7	178	58.5	86.3	<LOQ	<LOQ
CPPD	7.75	12.0	7.60	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPD	1550	1971	1600	48.6	38.7	43.3	67.9	<LOQ
IPPDq	23.6	26.1	21.0	8.17	<LOQ	<LOQ	<LOQ	<LOQ
DPPDq	<LOQ	<LOQ	<LOQ	46.8	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	233	241	163	356	219	576	60.0	20.7
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Hall 05 (ng/g)	FP A	FP B	SD A	SD B	SD C
Aniline	<LOQ	20910	<LOQ	526	<LOQ
DPG	15370	15990	137	431	168
2OH-BTZ	<LOQ	19890	812	857	642
IPPD	238	678	6.60	10.8	9.60
BTZ	<LOQ	16030	813	964	1136
2amino-BTZ	573	316	37.5	44.1	32.4
2SH-BTZ	128100	118900	<LOQ	<LOQ	<LOQ
HMMM	<LOQ	49.5	53.3	85.8	102
CPPD	<LOQ	14.0	<LOQ	<LOQ	<LOQ
6PPD	901	1430	<LOQ	<LOQ	<LOQ
IPPDq	<LOQ	11.2	<LOQ	<LOQ	<LOQ
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	308	88.6	280	323	298
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Hall 06 (ng/g)	FP A	FP B	FP C	SD A	SD B	SD C
Aniline	1237	1633	1906	237	659	493
DPG	118000	93820	87500	4463	4927	9652
2OH-BTZ	41090	33310	42970	8945	9502	6879
IPPD	22590	7439	8907	163	384	307
BTZ	73030	59430	66550	6398	34450	32390
2amino-BTZ	627	478	527	131	105	86.3
2SH-BTZ	553000	357000	383800	542	4369	2294
HMMM	<LOQ	<LOQ	<LOQ	36.9	57.9	33.9
CPPD	167	67.8	90.9	<LOQ	<LOQ	<LOQ
6PPD	33840	13640	19350	30.8	178	165
IPPDq	159	96.9	96.3	<LOQ	<LOQ	41.7
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	299	185	324	23.7	91.5	73.5
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	27.2	12.0	15.8	0.40	<LOQ	0.80

Hall 07 (ng/g)	FP A	FP B	FP C	SD A	SD B	SD C
Aniline	744	861	740	232	797	262
DPG	69410	82330	65950	4053	2223	3743
2OH-BTZ	14400	15030	14080	1394	4021	1340
IPPD	1429	1660	3604	20.8	<LOQ	18.1
BTZ	40940	36820	35140	<LOQ	3243	3206
2amino-BTZ	457	715	429	73.0	171	40.0
2SH-BTZ	74020	112000	43480	<LOQ	<LOQ	<LOQ
HMMM	92.4	231	160	207	2224	333
CPPD	11.6	13.4	10.2	2.10	7.60	<LOQ
6PPD	3712	3302	7355	273	33.3	62.6
IPPDq	41.7	56.2	34.7	<LOQ	<LOQ	<LOQ
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	243	233	190	27.2	234	13.4
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	2.33	3.94	3.05	<LOQ	<LOQ	<LOQ

Hall 08 (ng/g)	FP A	FP B	FP C	SD A	SD B	SD C
Aniline	294	394	204	312	100	54.7
DPG	28910	41110	28110	5431	2069	838
2OH-BTZ	22230	13890	18020	3432	5068	1775
IPPD	1577	4804	708	38.0	33.0	4.86
BTZ	99690	62080	57860	<LOQ	<LOQ	<LOQ
2amino-BTZ	1100	641	354	<LOQ	<LOQ	64.1
2SH-BTZ	203100	47480	59430	<LOQ	<LOQ	<LOQ
HMMM	234	865	1957	256	65.6	75.0
CPPD	13.1	7.00	14.3	<LOQ	<LOQ	<LOQ
6PPD	1332	6141	2869	296	20.4	25.0
IPPDq	109	70.2	25.4	7.20	28.8	<LOQ
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	179	232	140	78.6	<LOQ	6.34
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	1.92	1.50	<LOQ	<LOQ	<LOQ

Hall 09 (ng/g)	FP A	FP B	FP C	SD A	SD B	SD C
Aniline	442	540	1111	172	165	264
DPG	32510	36590	67940	2861	1829	2445
2OH-BTZ	26810	30290	27920	1412	877	7454
IPPD	2330	2610	7454	35.2	22.6	20.2
BTZ	51760	60320	63930	<LOQ	<LOQ	26840
2amino-BTZ	628	841	1009	164	82.5	507
2SH-BTZ	76880	66250	119500	<LOQ	<LOQ	<LOQ
HMMM	24.1	24.4	23.8	41.0	31.0	67.3
CPPD	15.2	11.3	25.8	2.00	<LOQ	<LOQ
6PPD	2985	3113	7574	131	112	77.0
IPPDq	103	122	426	<LOQ	0.45	0.92
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	230	277	915	16.5	22.4	22.3
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	6.58	7.21	13.0	<LOQ	<LOQ	<LOQ

Shoe soles (ng/g)	SS 01	SS 02	SS 03	SS 04	SS 05	SS 06	SS 07
Aniline	266	62.0	204	71.8	89.9	230	406
DPG	75.4	105	39.1	143	78.5	61.6	245
2OH-BTZ	66470	71960	38850	43730	60340	266900	32920
IPPD	100	22.4	7.40	23.2	8.60	50.7	10120
BTZ	77050	70820	54710	38980	42830	57070	50560
2amino-BTZ	2061	2563	1267	1439	1043	607	668
2SH-BTZ	407800	489300	576500	556300	437400	1431000	379000
HMMM	24.0	57.8	7.10	38.9	7.70	46.4	3.00
CPPD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	210
6PPD	40.0	4.20	<LOQ	5.80	<LOQ	4.80	11710
IPPDq	13.9	9.10	7.60	9.90	6.60	2.10	343
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	34.90	<LOQ
6PPDq	22.2	13.3	9.90	19.4	8.80	1.10	371
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	2.80	3.90
DPPD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	18.4	18.7
	SS 08	**SS 09**	**SS 10**	**SS 11**	**SS 12**	**SS 13**	**SS 14**
Aniline	<LOQ	<LOQ	1653	<LOQ	965	<LOQ	2050
DPG	<LOQ	<LOQ	2061	22.9	<LOQ	170	<LOQ
2OH-BTZ	10450	7366	9954	9719	5540	101500	8478
IPPD	12.6	11.3	11.8	12.5	13.3	719	16.3
BTZ	23150	20050	44410	20670	12350	28580	43240
2amino-BTZ	16470	<LOQ	<LOQ	2988	<LOQ	19200	<LOQ
2SH-BTZ	155700	64160	470900	198600	233500	116000	142600
HMMM	<LOQ	78.7	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CPPD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPD	361	<LOQ	<LOQ	207	183	464	278
IPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	129	98.3	<LOQ	<LOQ	<LOQ
	SS 15	**SS 16**	**SS 17**	**SS 18**	**SS 19**	**SS 20**	**SS 21**
Aniline	<LOQ	87.4	225	16460	224700	840	237
DPG	<LOQ	223	5.60	625000	813600	9289	13600

2OH-BTZ	14280	8957	90520	79140	77000	42270	12430
IPPD	8301	<LOQ	5.90	<LOQ	<LOQ	18.9	5.80
BTZ	78550	8137	61610	100200	228100	201700	56920
2amino-BTZ	<LOQ	17.7	109	369	647	<LOQ	<LOQ
2SH-BTZ	138600	5889	8173	19150	2061000	2366000	719000
HMMM	<LOQ	15.0	<LOQ	0.10	3.10	2.80	1.00
CPPD	180	<LOQ	<LOQ	132	8.80	156	<LOQ
6PPD	22600	1123	2.20	1.50	1.30	1.40	0.40
IPPDq	54.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	83.6	89.1	10.7	1.30	1.00	1.10	12.8
CPPDq	<LOQ	<LOQ	<LOQ	6.70	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	<LOQ	<LOQ	3.3	19.4	2.10
	SS 22	**SS 23**	**SS 24**	**SS 25**	**SS 26**	**SS 27**	**SS 28**
Aniline	219	1214	183	271	262	1903	32.5
DPG	6796	1511	73	2169	423	364	58.3
2OH-BTZ	17190	33590	19380	13540	372600	33500	15330
IPPD	2.50	1.20	2.30	5.20	17.6	202	0.90
BTZ	60120	37410	6924	52370	5108	112400	28130
2amino-BTZ	28	378	45	<LOQ	534	1031	130
2SH-BTZ	483300	1472000	804500	580700	415800	1342000	24510
HMMM	1.20	1.20	3.10	1.70	0.70	2.60	0.80
CPPD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPD	0.30	11	301	614	133	896	2.90
IPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	9.90	9.50	2.40	4.80	6.40	15.9	1.40
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	14.1	<LOQ	<LOQ	54.9	<LOQ

	SS 29	**SS 30**
Aniline	555	<LOQ
DPG	117	12.0
2OH-BTZ	8520	3281
IPPD	148	7.39
BTZ	8482	14300
2amino-BTZ	16550	<LOQ

2SH-BTZ	5392	46580
HMMM	90.2	429
CPPD	<LOQ	<LOQ
6PPD	46.2	<LOQ
IPPDq	15.4	<LOQ
DPPDq	<LOQ	<LOQ
6PPDq	<LOQ	<LOQ
CPPDq	<LOQ	<LOQ
DPPD	<LOQ	<LOQ

Table S5.3: Parameters used in equation (2) for calculation of $EDI_{inh/ing}$. IR values were determined for low intensity activities (employees) and moderate intensity (adult climbers) for individuals aging 21 – 31 yrs according to US EPA[249]*. Calculations were performed for employees working 8h/day, 5days/week and for adult climbers visiting the facilities for 3h/day, 3 days/week. IR = Inhalation rate, ET = exposure time per day, EF = exposure frequency, BW = average body weight, Cf = conversion factor from year to day.*

	Employees	Adult climbers
IR (m^3/day)	17.3	37.4
ET (h/day)	8	3
EF (day/years)	228	156
BW (kg)	63	63
Cf	365	365

Table S5.4: Ozonation Experimental Results

	No ozonation (µg/g)			**4 hours ozonation (µg/g)**		
Aniline	34.5	29.5	30.9	12.1	12.1	8.9
DPG	36.9	29.4	29.1	12.4	11.6	8.4
2OH-BTZ	16.7	15.7	13.3	21.7	18.9	10.2
IPPD	5.4	2.5	2.1	2.3	1.2	1.1
BTZ	13.4	11.0	15.0	44.4	31.7	41.5
2amino-BTZ	0.2	0.2	<LOQ	0.2	0.2	<LOQ
2SH-BTZ	101.2	68.3	64.2	30.9	26.7	11.3
HMMM	0.05	0.05	0.06	0.04	0.04	0.02
CPPD	0.1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPD	7.9	2.5	2.4	1.5	0.8	0.6
IPPDq	0.08	0.06	0.05	0.06	0.04	0.05
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	0.2	0.1	0.1	0.1	0.1	0.1
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table S5.5: Physico-chemical characteristics of Rubber derived chemicals

Compound	**CAS**	**Log K_{OA}**	**Modelled % on particle phase at equilibrium**	**Measured particle-bound fraction (%) in ambient $PM_{2.5}$**[244]
Aniline	62-53-3	5.3[271]	/	/
BTZ	95-16-9	6.0[272]- 6.8[247]	0.0[247]	25
2amino-BTZ	136-95-8	10.3[247]	10.4[247]	55
2OH-BTZ	934-34-9	8.4[247]	0.1[247]	5
2SH-BTZ	149-30-4	11.0[247]	48.6[247]	/
6PPDq	2754428-18-5	15.3[273]	35 – 100[273]	98
DPG	102-06-7	12.4[247]	94.3[247]	95
6PPD	793-24-8	9.3[247]	1.2[247]	100
HMMM	3089-11-0	/	/	75
CPPD	101-87-1	9.3[247]	1.4[247]	/
CPPDq	68054-78-4	/	/	/
IPPD	101-72-4	10.5[247]	17.7[247]	100
IPPDq	68054-73-9	/	/	75
DPPD	74-31-7	12.5[274] - 12.1[247]	87.8[247]	/
DPPDq	3421-08-7	/	/	/

Table S5.6: Extraction Recovery

Recovery tests were conducted by spiking target compounds into an empty (without sample) ASE cell and extracting and measuring as described in the Materials and Methods section. In Vienna, target compounds were spiked at 500 ng/mL (benzothiazoles) or 100 ng/mL (all other compounds), to mimic the concentrations observed in most samples. Absolute recovery is the measured concentration as a percentage of the spiked concentration. Relative recovery is the absolute recovery normalized by its respective internal standard. The spiking of deuterated internal standard on the samples before extraction accounted for potential losses during extraction and matrix effects.

Compound	**Extraction recovery (%) (mean ± sd)** Lausanne	**Extraction recovery (%) (mean ± sd)** Vienna	
	relative	absolute	relative
Aniline	81 ± 8	62 ± 29	66 ± 22
BTZ	75 ± 14	96 ± 3	98 ± 4
2amino-BTZ	91 ± 3	100 ± 4	98 ± 4
2OH-BTZ	96 ± 3	87 ± 4	89 ± 5
2SH-BTZ	42 ± 23	29 ± 11	32 ± 14
6-PPDq	90 ± 7	73 ± 24	97 ± 6
DPG	82 ± 15	65 ± 26	76 ± 20
6PPD	57 ± 44	73 ± 20	87 ± 10
HMMM	109 ± 10	107 ± 5	145 ± 34
CPPD	32 ± 27	82 ± 23	101 ± 7
CPPDq	92 ± 5	78 ± 16	109 ± 9
IPPD	33 ± 31	77 ± 19	90 ± 9
IPPDq	91 ± 4	84 ± 11	117 ± 18
DPPD	67 ± 23	102 ± 21	132 ± 7
DPPDq	86 ± 36	62 ± 27	74 ± 21

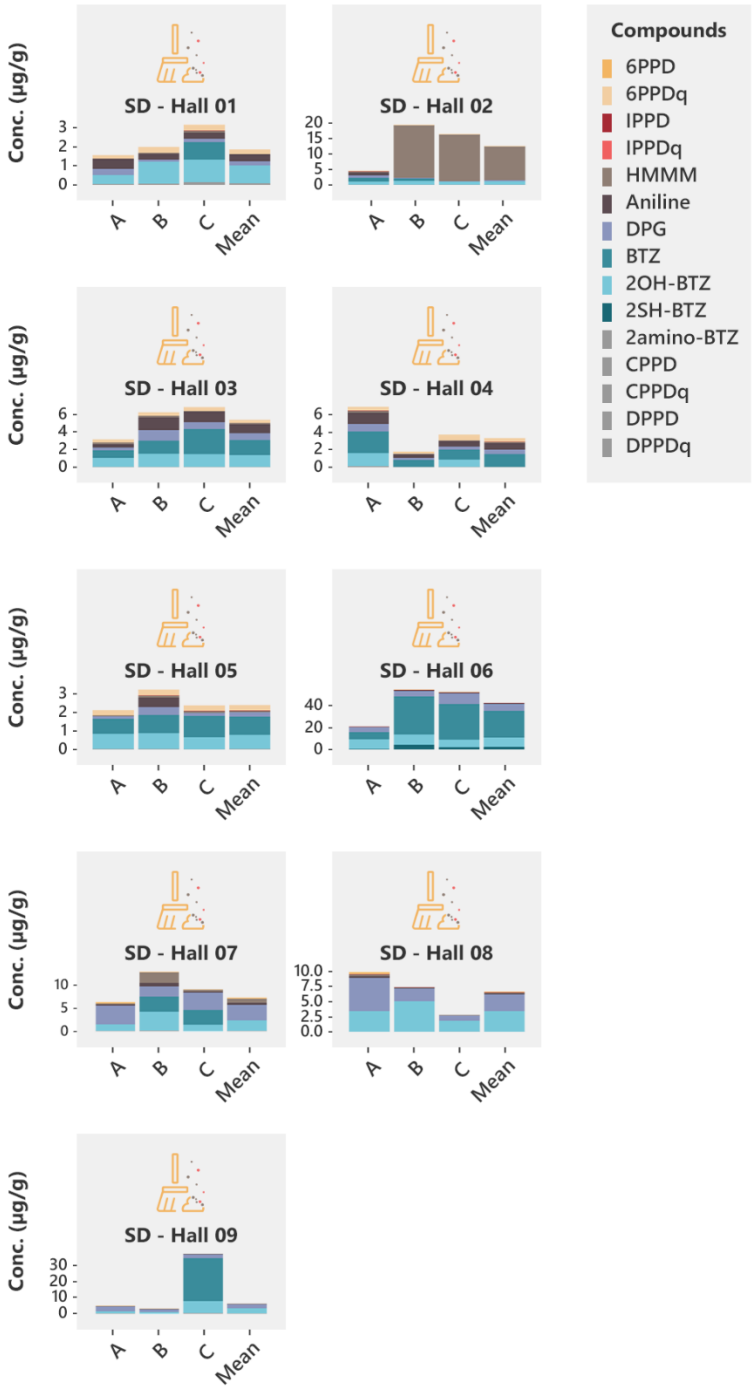


Figure S5.1: Rubber-derived chemical concentrations in triplicate (A-C) settled dust samples for Halls 01 - 09. Compounds in light gray were either below limit of quantification or very sporadically detected at trace concentrations.

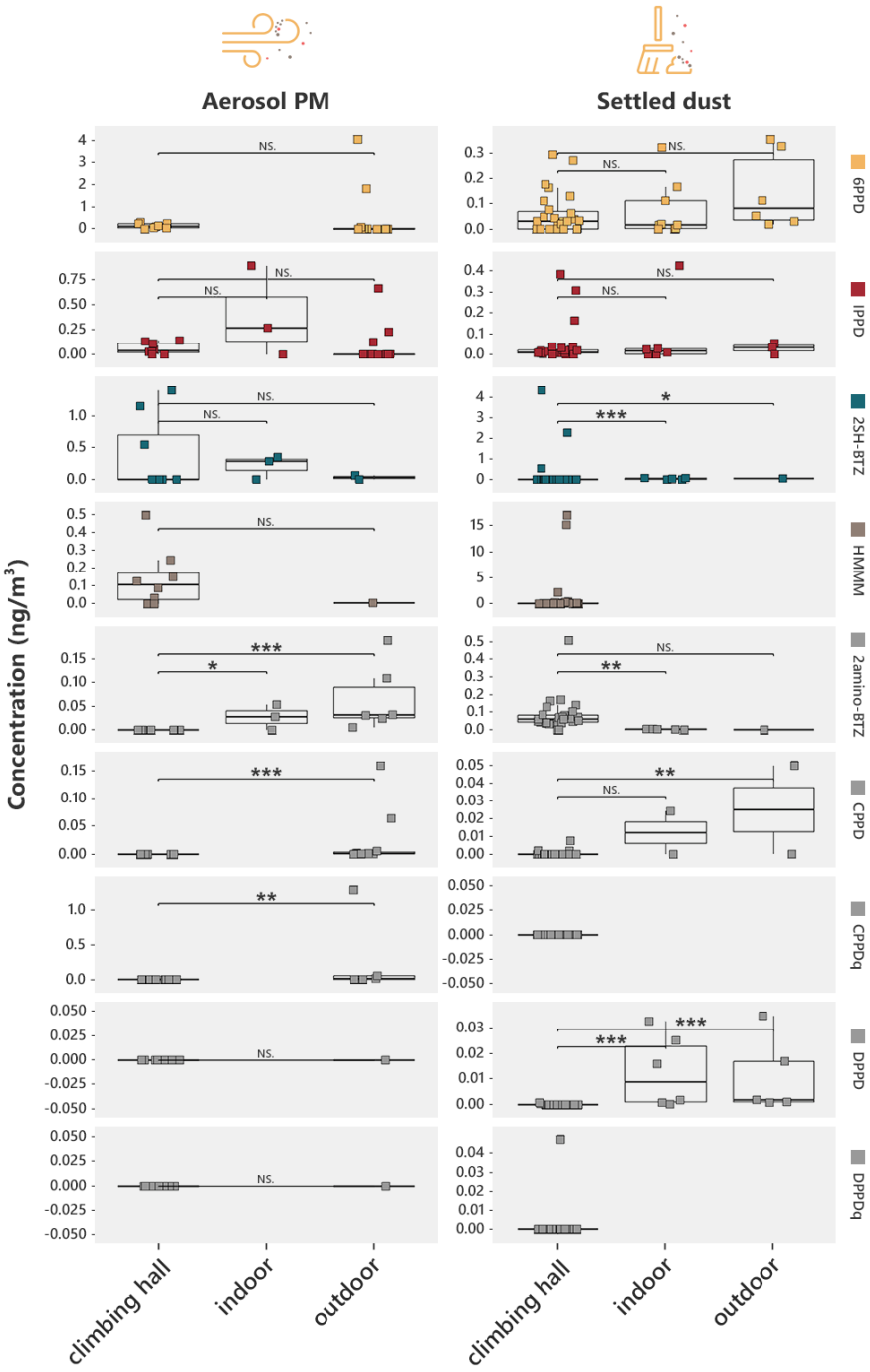


*Figure S5.2: Literature comparison of RDCs in aerosol particulate matter and settled dust samples, which based on our data, do not necessarily arise uniquely from climbing activity. Compounds in light gray were either below limit of quantification or very sporadically detected at trace concentrations. Details about literature values provided in the Supporting Information: Excel file. Statistical differences between groups were tested with the Wilcoxon signed-rank test (NS means $p \geq 0.05$; * means $p < 0.05$; ** means $p < 0.01$; *** means $p < 0.001$).*

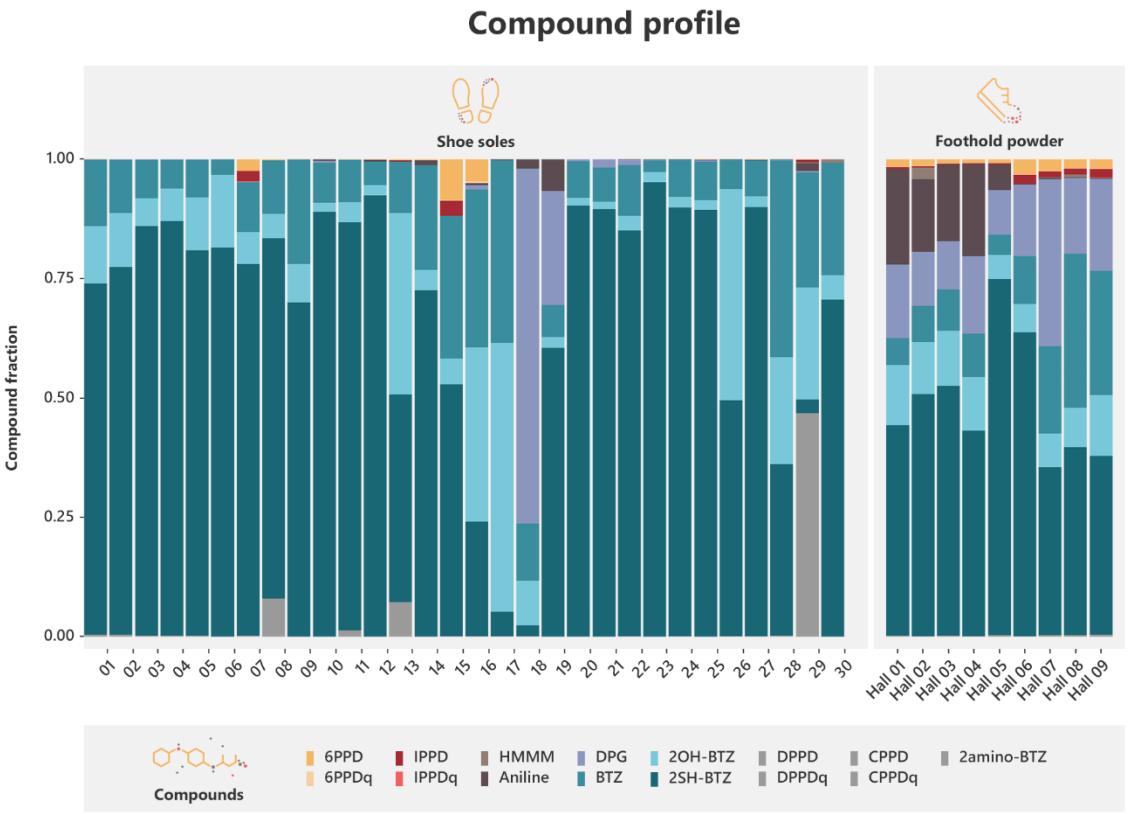
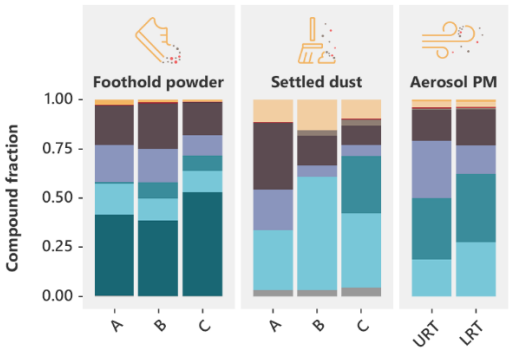


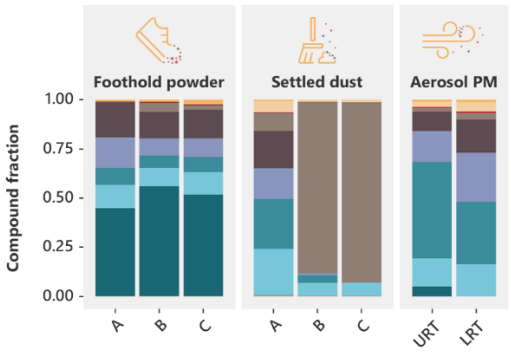
Figure S5.3: Rubber-derived compound profile in shoe soles and foothold powder

Profile showing [compound$_i$]/∑15[compounds] for 15 rubber-derived compounds in 30 shoe sole samples, and foothold powder from nine climbing halls. The rubber-derived compound profile varies between shoe sole models, reflecting the variety of targeted properties in different climbing shoes (adhesiveness, softness, durability, flexibility). The foothold powder samples have a rubber-derived compound profile which represents an average of the different shoes on the market, and is relatively consistent between halls.

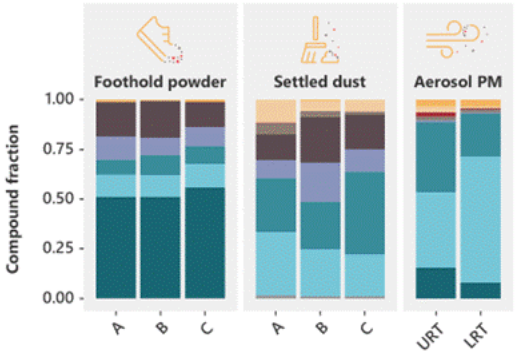
Compound profile - Hall 01



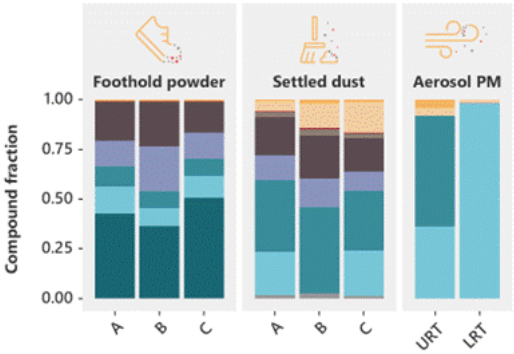
Compound profile - Hall 02



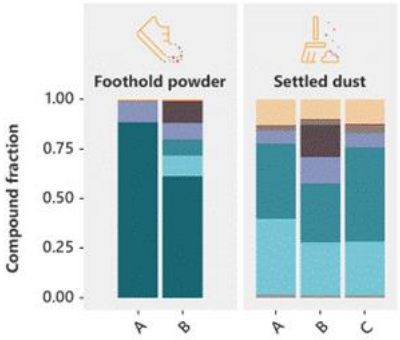
Compound profile - Hall 03



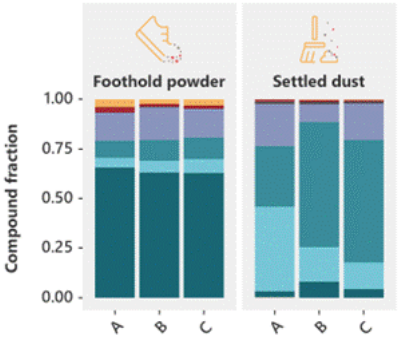
Compound profile - Hall 04



Compound profile - Hall 05



Compound profile - Hall 06



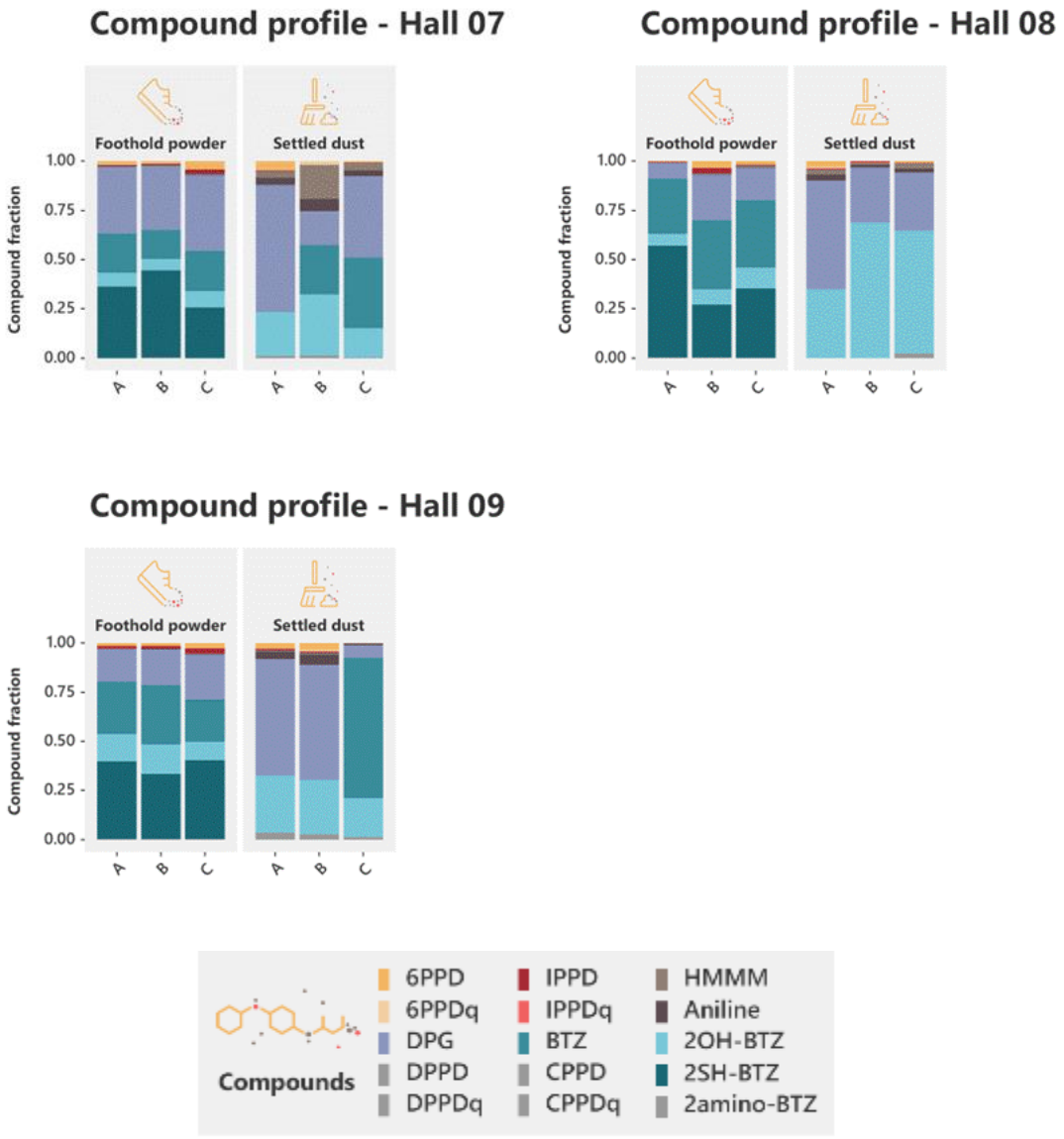


Figure S5.4: Paired comparison within halls for triplicates samples (A, B, C) and both URT and LRT fraction of the aerosol particulate matter samples


Foothold powder
Settled dust
Aerosol PM
2-Mercaptobenzothiazole (2SH-BTZ)
2-Hydroxybenzothiazole (2OH-BTZ)
Benzothiazole (BTZ)
6PPD
6PPD-quinone (6PPDq)
Concentration (%)

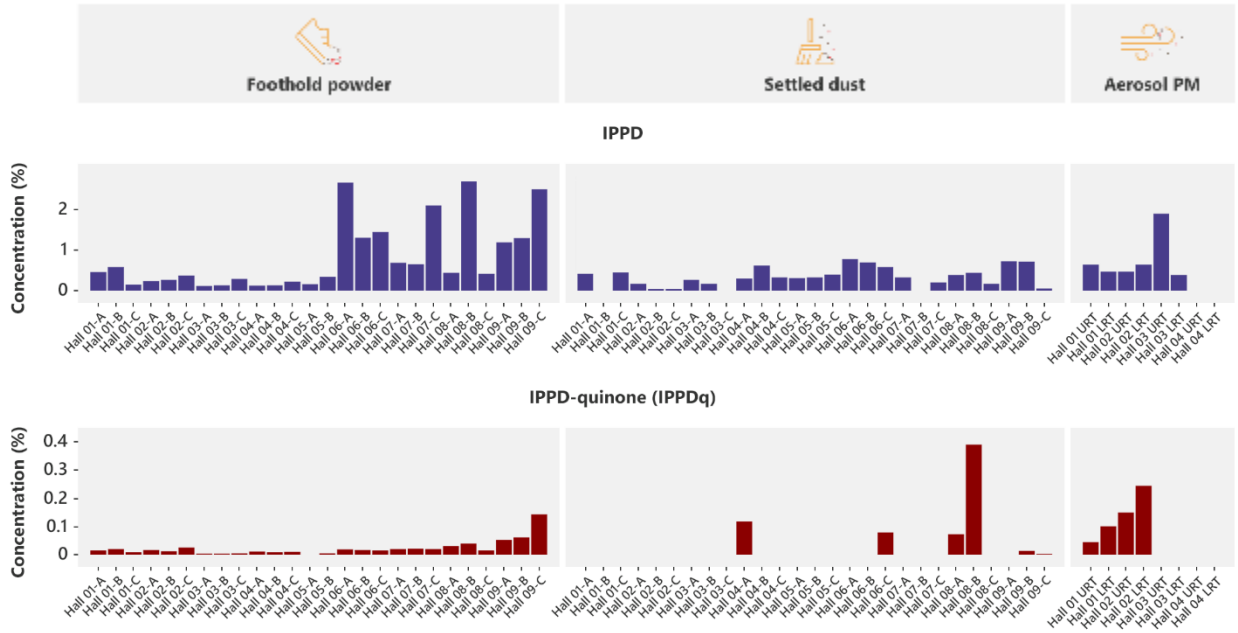


Figure S5.5: Rubber-derived compound concentration shifts.

Concentration % (calculated as $[compound_i]/\sum_{15}[compounds]\times100$) for 2-mercaptobenzothiazole, 2-hydroxybenzothiazole, benzothiazole, 6PPD, 6PPD-quinone, IPPD, and IPPD-quinone in foothold powder (FP), settled dust (SD), and aerosol particulate matter (APM) samples. Compounds shown in blue are most likely included in climbing shoe formulation as additives, while compounds shown in red are most likely transformation products of those additives. Relative concentrations of 2-mercaptobenzothiazole, 6PPD, and IPPD decrease from foothold powder to settled dust and aerosol particulate matter samples, likely due to transformation. Meanwhile, relative concentrations of products 2-hydroxybenzothiazole, benzothiazole, 6PPD-quinone, and IPPD-quinone increase.

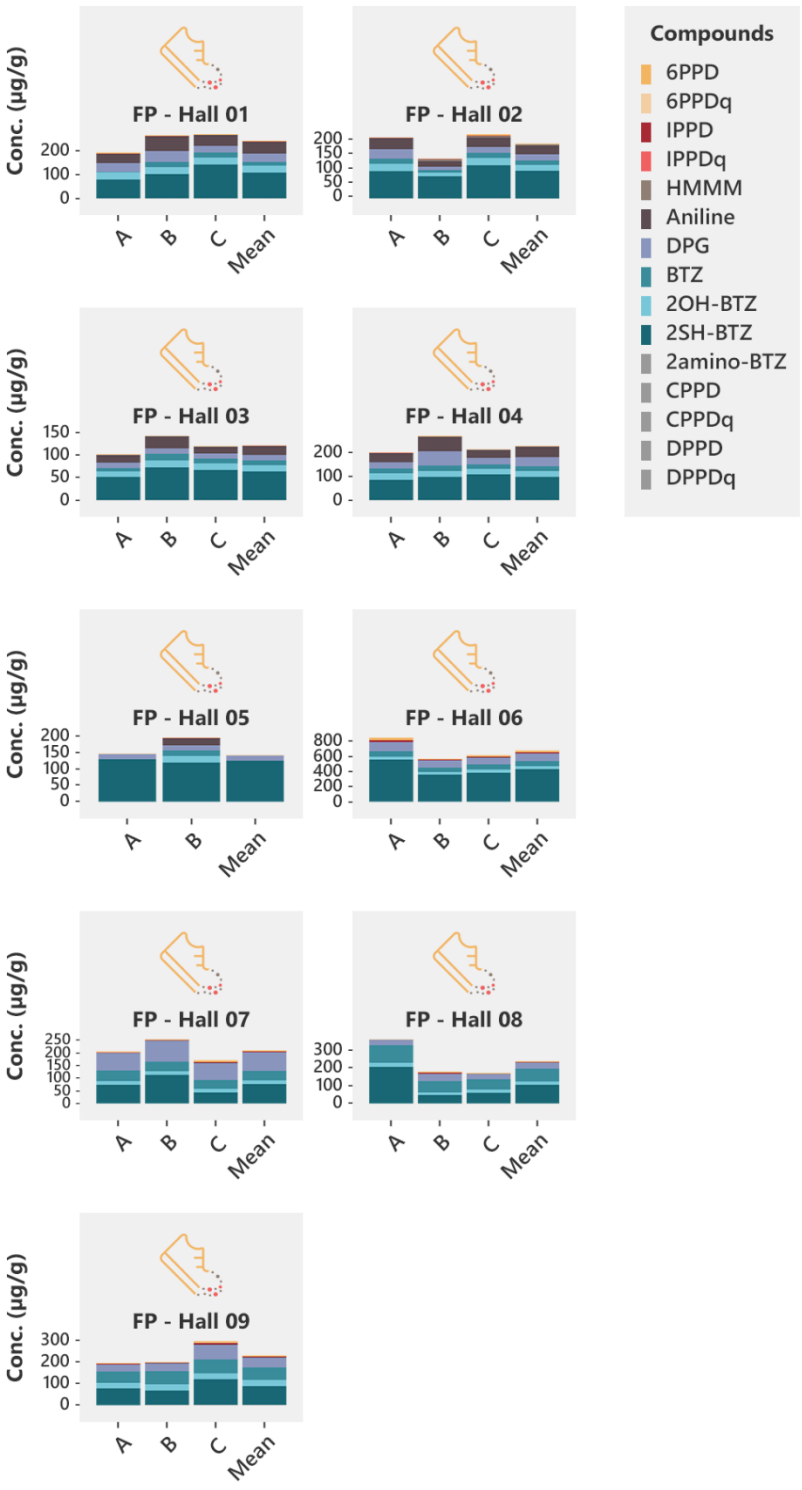


Figure S5.6: Variability within halls for triplicates samples (A, B, C) of foothold powder samples and the associated mean values

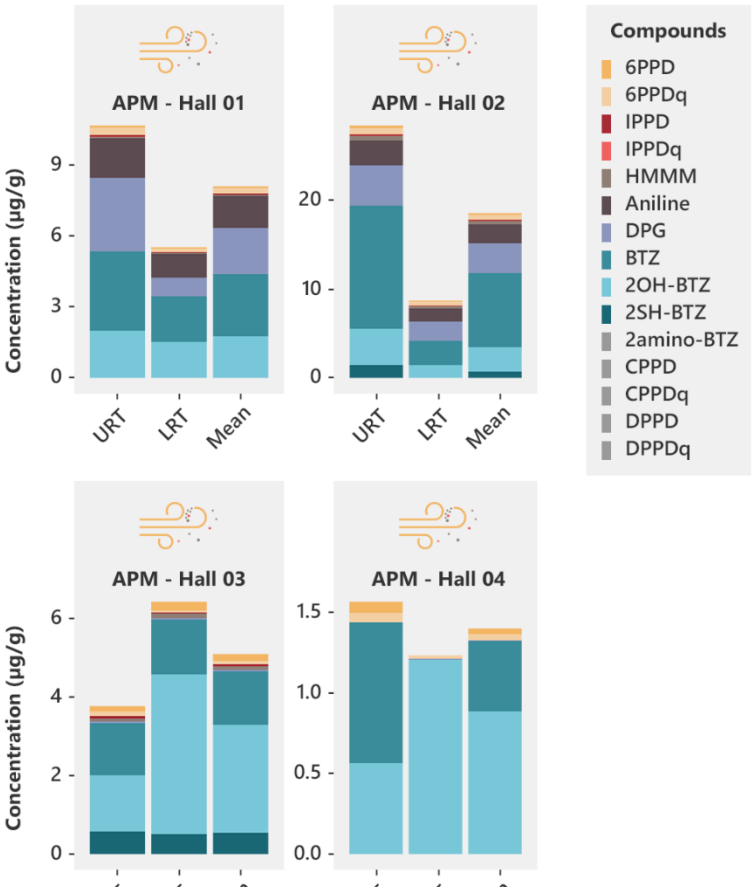
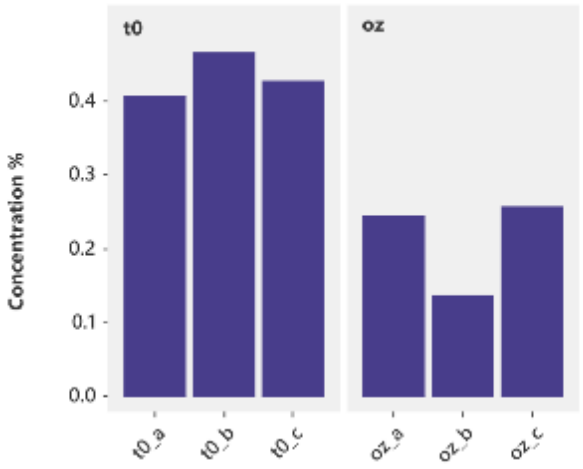
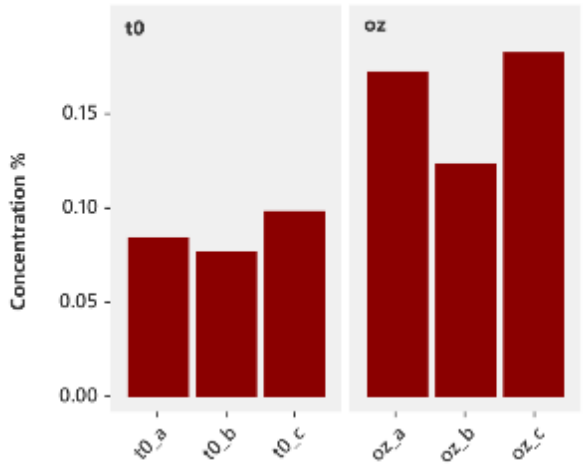


Figure S5.7: Rubber derived concentrations in URT and LRT fractions of aerosol particulate matter samples from Halls 01 – 04, as well as their mean values.

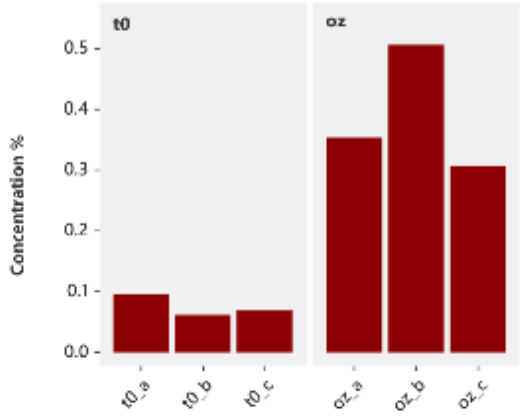
2-mercaptobenzothiazole



2-hydroxybenzothiazole



Benzothiazole



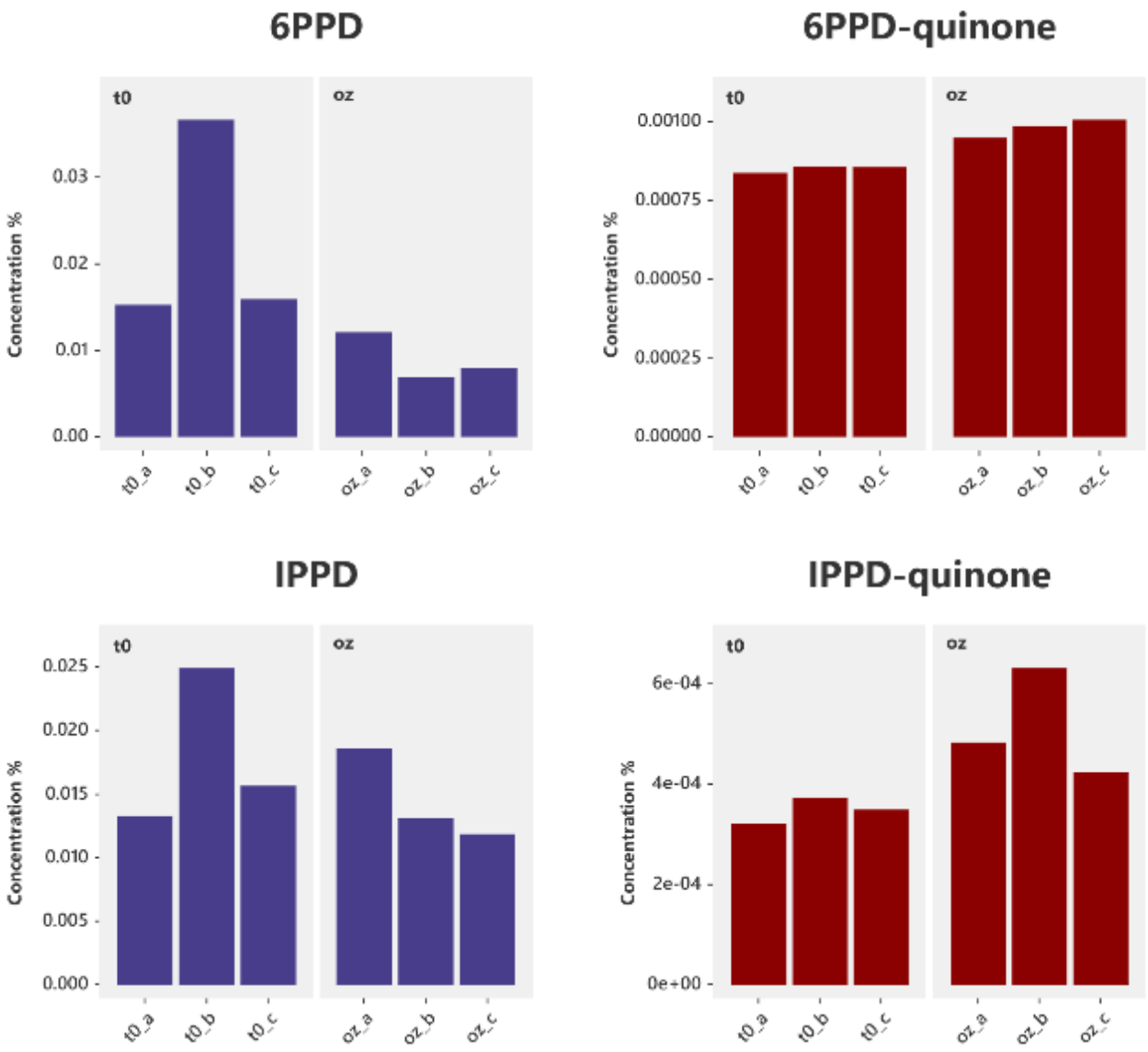


Figure S5.8: Rubber derived compound concentration shifts ozonation experiments

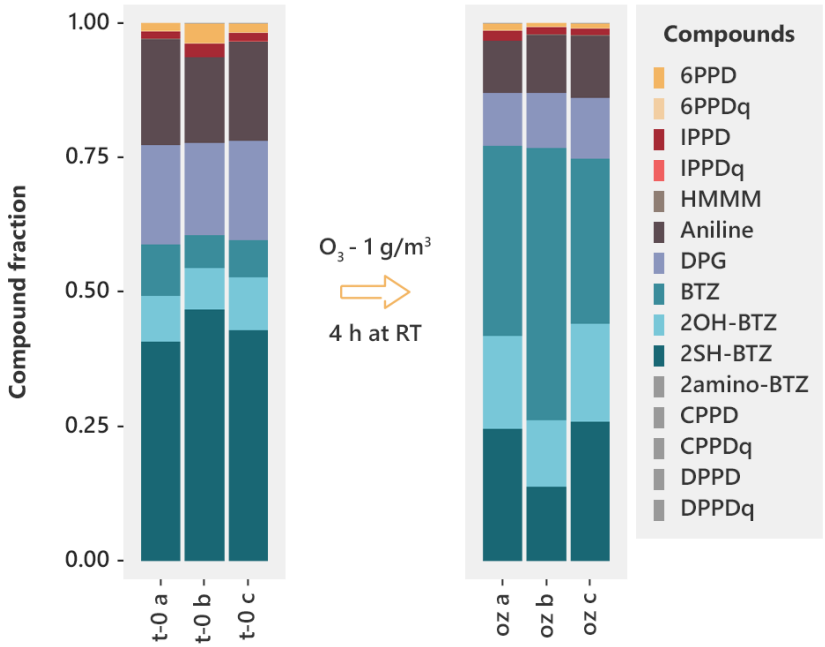


Figure S5.9: Ozonation experiment results

Supplementary Text S5.1: Results from blanks and reference samples

In the following tables, the terms URT and LRT fractions are used to denote samples where aerosols were actively collected from the air sampling devices, while the terms upper and lower chambers are used when blank measurements were collected from the device without active air sampling. Total particle mass in collection and storage blanks was determined gravimetrically. In the blanks taken for the upper and lower chambers of the air sampling device in each climbing hall, particle masses ranging from –3.9 to 0.3 mg were detected, reflecting the error of the analytical balance.

Total particles detected:

Blank	Particle Mass (mg)
Storage blank	-3.9
Collection blank – Hall 01 upper chamber	-2.4
Collection blank – Hall 01 lower chamber	-2.9
Collection blank – Hall 02 upper chamber.	0.3
Collection blank – Hall 02 lower chamber	-1.7
Collection blank – Hall 03 upper chamber	1.9
Collection blank – Hall 03 lower chamber	0.0
Collection blank – Hall 04 upper chamber	2.8
Collection blank – Hall 04 lower chamber	1.8
Collection blank – Hall 05 upper chamber	0.5
Collection blank – Hall 05 lower chamber	7.2

Rubber-derived compounds detected in blanks (ng):

Name	LOQ	Laboratory blanks				Storage blank	Collection blanks			
						Hall 1	Upper chamber hall 1	Upper chamber hall 2	Lower chamber hall 1	Lower chamber hall 2
Aniline	50	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPG	0.73	<LOQ	<LOQ	<LOQ	<LOQ	1.3	<LOQ	1.0	0.88	2.81
2OH-BTZ	3.7	78.2	<LOQ	<LOQ	<LOQ	6.3	9.3	10.5	25.9	20.8
IPPD	0.55	<LOQ	<LOQ	<LOQ	<LOQ	0.7	0.55	0.81	0.81	0.63
BTZ	20	<LOQ	<LOQ	<LOQ	<LOQ	35	42.9	43.4	45.4	50.6
2amino-BTZ	150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2SH-BTZ	29.5	1621.6	<LOQ	<LOQ	<LOQ	<LOQ	32.4	30.2	<LOQ	<LOQ
HMMM	0.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CPPD	0.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPD	2.9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IPPDq	0.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPDq	5.9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	0.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CPPDq	2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Name	Collection blanks			
	Upper chamber hall 3	Lower chamber hall 3	Upper chamber hall 4	Lower chamber hall 4
Aniline	<LOQ	<LOQ	<LOQ	<LOQ
DPG	<LOQ	<LOQ	<LOQ	<LOQ
2OH-BTZ	<LOQ	16.3	32.5	<LOQ
IPPD	<LOQ	2.28	<LOQ	<LOQ
BTZ	<LOQ	<LOQ	<LOQ	<LOQ
2amino-BTZ	<LOQ	<LOQ	<LOQ	<LOQ
2SH-BTZ	<LOQ	<LOQ	<LOQ	<LOQ
HMMM	<LOQ	<LOQ	<LOQ	<LOQ
CPPD	<LOQ	<LOQ	<LOQ	<LOQ
6PPD	<LOQ	<LOQ	<LOQ	<LOQ
IPPDq	<LOQ	<LOQ	<LOQ	<LOQ
DPPDq	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	<LOQ	4.12	<LOQ	<LOQ
CPPDq	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	<LOQ	<LOQ	<LOQ	<LOQ

Rubber-derived compounds in reference samples from an office and investigated alternative sources (ng).

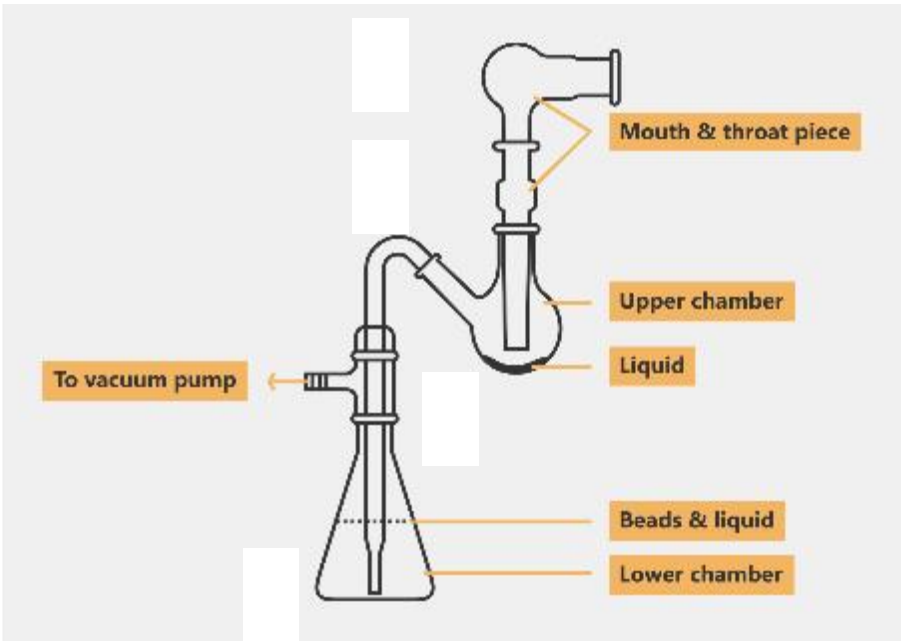
Name	LOQ	Mat-fiber	Mat-smooth	Hold	Reference sample URT fraction	Reference sample LRT fraction
Aniline	50	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPG	0.73	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2OH-BTZ	3.7	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
IPPD	0.55	<LOQ	4.75	3.05	14.2	32.2
BTZ	20	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2amino-BTZ	150	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2SH-BTZ	29.5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
HMMM	0.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CPPD	0.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPD	2.9	<LOQ	<LOQ	<LOQ	<LOQ	5.2
IPPDq	0.2	<LOQ	0.33	<LOQ	<LOQ	<LOQ
DPPDq	5.9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6PPDq	0.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CPPDq	2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
DPPD	5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Supplementary Text S5.2: Aerosol particulate matter sampling details

Aerosol particulate matter (PM) is a complex mixture of airborne particles. Particles are classified by their aerodynamic diameter (AD), which takes the size, shape and density of the particles into account and therefore describes possible deposition patterns of airborne particles in different regions of the lung[275,276]. WHO differentiates between resle particles (AD < 100 µm) – which usually deposit in the nose and upper airways and are subsequently swallowed[242,277]– and respirable particles (AD < 10 µm), where the probability of particle deposition in the peripheral lung is dependent on AD[243,278]. WHO guidelines focus on $PM_{2.5}$ and PM_{10} which are defined as particles with an AD below 2.5 µm and 10 µm respectively[275]. They are of special interest because associations between short- and long-term exposure and adverse health effects have been shown[279–281].

First described by Hallworth & Westmoreland[282], the glass liquid impinger geometry and constant airflow of 60 ± 5 L/min, within the range of human respiration rates, leads to the separation of particles by their AD. Particles above 6.4 µm AD (upper respiratory tract - URT fraction) will impact at the mouth and throat piece or upper chamber, whereas particles below that size (lower respiratory tract - LRT fraction) will deposit in the lower chamber.

By introducing ceramic beads into the collection liquid of the lower chamber (granular fluid bed filtration), the entrapment efficiency of ultrafine particles increases[283]. During the current study, the liquid level was replenished regularly as water evaporation during the collection time has been shown to result in a poorer entrapment efficiency[284].



Compartment A & B: Mouth and throat piece (URT fraction part I)
Compartment C: Upper chamber (URT fraction part II)
Compartment D: Lower chamber (LRT fraction)

Vial preparation

Amber sample vials (250 mL) and liquid containers (Duran bottles, 250 mL and 500 mL) were muffled at 550°C for 5 h after removing all plastic parts. Vial lids were cleaned by rinsing three times with ethanol before closure and liquid containers were covered with tinfoil. For every day of sampling, a fresh bottle of

MilliQ-water and ethanol (96% v/v, filtered 0.22 µm) was prepared to avoid contamination by ambient particles.

Sampling procedure

A glass liquid impinger was used as described in the European Pharmacopoeia (Ph. Eur.). Before the device was assembled, 7 mL of MilliQ-water was introduced into the upper chamber and 475 g (=130 mL) of ceramic beads (9730, ZY-P, 2.6-3.3 mm, SiLibeads, Sigmund Lindner GmbH, Warmensteinach, Germany) and 25 mL of MilliQ-water were introduced into the lower chamber.

The device was elevated to place the mouthpiece at 142 cm above ground level and it was aligned to face the climbing wall at approximately 3m distance. Subsequently, the vacuum pump (LCP6, Copley Scientific Ltd., Nottingham, UK) was activated and the airflow was set to 60 ± 2 L/min, under constant monitoring using a flowmeter (5300-1, TSI Inc). During the sampling period of 3 h, the temperature, humidity and liquid levels of the upper and lower chambers were checked periodically (every 15 min) and liquids were replenished as needed. Collected material from multiple consecutive sampling periods at a single hall were combined during the washing procedure described below to be able to determine analytes of interest above the detection limit of the analytical method. In total, APM from 54 m³ air was collected in each climbing hall (5 days × 3 h × 60 min × 60 L/min).

After switching off the pump, every part of the device was rinsed three times with 6 mL MilliQ-water and collected. The beads were rinsed with the wash fluid of the lower chamber, collected and subsequently washed three times more with 6 mL MilliQ-water. This procedure was repeated with ethanol. All rinse fluids were collected, whereby discrete vials for MilliQ water and ethanol were used, and stored at -20°C.

The samples of the mouth and throat piece and the upper chamber were combined and represent the URT fraction of aerosol particulate matter; whereas the fluids from the lower chamber were collected in a separate vial to represent the LRT aerosol particulate matter fraction. Liquid was removed from the vials containing ethanol samples by evaporation, whereas water was removed from the corresponding sample vials by lyophilization. The contents from the two dried vials corresponding to the upper chamber (i.e. water and ethanol wash) were collected in solvent and combined before extraction to obtain single values for the URT particulate matter concentration. Likewise, the contents from the two dried lower chamber vials (water and ethanol) were collected in solvent and combined before extraction to obtain single values for the LRT particulate matter concentrations (Tables 5.1-5.2, Table S5.2).

	Sampling times	**Average humidity**	**Average temperature**
Hall 01	17.5.2023 18:08-20:48	56%	19°C
	18.5.2023 17:23-20:26	65%	19°C
	19.5.2923 17:16-20:56	61%	18°C
	20.5.2023 17:25-20:29	62%	19°C
	21.5.2023 17:07-20:07	53%	21°C
Hall 02	23.04.2023 19:03-20:35	51%	21°C
	25.04.2023 16:48-21:05	52%	23°C
	26.04.2023 16:45-19:53	46%	22°C
	27.04.2023 17:03-20:59	43%	22°C
	28.04.2023 16:31-19:00	54%	20°C
Hall 03	15.04.2024 16:43-20:01	61%	22°C
	16.04.2024 16:55-21:06	58%	21°C
	17.04.2024 16:55-20:03	60%	22°C

	18.04.2024 17:21-20:29	54%	22°C
	19.04.2024 16:57-19:03	53%	22°C
Hall 04	22.04.2024 17:39-20:49	29%	19°C
	23.04.2024 17:16-20:29	37%	22°C
	24.04.2024 17:00-20:12	37%	22°C
	25.04.2024 17:01-20:15	34%	21°C
	26.04.2024 17:02-20:12	33%	22°C
Hall 05	29.04.2024 17:59-21:14	49%	21°C
	20.04.2024 16:53-19:59	50%	21°C
	01.05.2024 17:06-	54%	22°C
	02.05.2024 17:00-20:09	54%	22°C
	03.05.2024 17:13-19:17	55%	21°C

Cleaning

The glass liquid impinger was washed with MilliQ and ethanol after each day of sample collection and dried overnight before further use. Before sampling at the next location, the glass liquid impinger was cleaned thoroughly via sonication.

Supplementary Text S5.3: Accelerated solvent extraction method details

50 mg of all samples (shoe sole, foothold powder, settled dust and aerosol particulate matter samples) were transferred into an accelerated solvent extraction cell (ASE, Thermo-Fischer) filled with glass beads and internal standards were spiked. ASE extraction consisted of three static cycles of 5 minutes at 80°C using 100% acetonitrile as solvent. Extracts were concentrated under N_2 blowdown to approximately 0.5 mL, then reconstituted to 2 mL with acetonitrile, and filtered through 0.2 μm nylon filters before analysis.

Supplementary Text S5.4: Chemicals used

1. Vienna

Dichloromethane (99.9%) was purchased from Thermo Scientific. Acetonitrile (LCMS Grade) and ethanol (99.8%) were purchased from Sigma-Aldrich. Formic acid for HPLC was purchased from Merck.

IPPD, CPPD, DPPD, 6PPDq, IPPDq, DPPDq, DPPDq, and d5-6PPDq were from HPC Standards®. DPG, benzothiazole, aniline, 2-mercaptobenzothiazole, 2-hydroxybenzothiazole, 2-aminobenzothiazole, and 6PPD were purchased from Sigma-Aldrich. HMMM was purchased from TCI Europe. D4-Benzothiazole was purchased from LGC Standards.

2.Lausanne

Acetonitrile was purchased from Biosolve® and dichloromethane were from Carlo Erba®. All solvents were UPLC grades.

Standards of aniline, Aniline d5, benzothiazole, Benzothiazole-d4, 2-hydroxybenzothiazole, 2-mercaptobenzothiazole were from Sigma-aldrich®. 1,3-diphenylguanidine and 1,3 diphenylurea-d10 were from Chemie Brunschwig AG®. Hexa(methoxymethyl)melamine, 6PPD, IPPD, CPPD, DPPD, 6PPDq, IPPDq, CPPDq and DPPDq were from HPC Standards®. Standards quality was always >95%

Supplementary Text S5.5: UPLC-MS/MS method details

All samples were measured with ultra-high pressure liquid chromatography coupled with triple quadrupole mass spectrometry, in either Vienna Austria or Lausanne Switzerland. Below are the method details as used in each laboratory.

1.1 Vienna

All samples measured in Vienna were analyzed by ultra-performance liquid chromatography - triple quadrupole mass spectrometry (Agilent 1290 Infinity II - Agilent 6470) using a C18 column (Acquity HSS T3, 1.8 µm, Waters) in the positive ionization mode (capillary voltage: 2500 V) via multiple reaction monitoring (MRM). The LC flow rate was 0.6 mL/min at a column temperature of 40 °C and the injection volume was 5 µL. The mobile phase consisted of ultrapure water (Phase A) and acetonitrile (Phase B), both containing 0.1% formic acid. The eluent gradient was held at 95% Phase A for one minute, then set that the contribution of Phase A decreased from 95 to 5% over 9 min, was held at 5% for 2 min and increased again to 95% over the last 1 min. Column was then re-equilibrated at 95% Phase A for one minute. Electrospray ionization (ESI) was achieved at gas temp 240ºC, gas flow 5 L min^{-1}. The nebulizer was set to 30 psi; sheath gas to 250ºC, 11 L min^{-1}. Quantification was achieved by external calibration standards prepared in acetonitrile (0.01 to 500 µg L^{-1}). Transitions from precursor ions to product ions are specified in the following table. At least 2 product ions were monitored per compound, quantifier ions are indicated in bold.

Compound name	**ISTD**	**Precursor ion m/z**	**Product ion m/z**	**Fragmentor voltage (V)**	**Collision energy (V)**	**Cell accelerator voltage (V)**	**RT (min)**
2-aminobenzothiazole	**d4-BTZ**	**151**	**109**	**150**	**30**	**5**	**5.4**
2-aminobenzothiazole		151	65	150	38	5	5.4
2-hydroxybenzothiazole	**d4-BTZ**	**152**	**124**	**140**	**22**	**4**	**3.6**
2-hydroxybenzothiazole		152	92	140	26	4	3.6
2-mercaptobenzothiazole	**d4-BTZ**	**168**	**135**	**135**	**28**	**5**	**3.1**
2-mercaptobenzothiazole		168	124	135	24	5	3.1
2-mercaptobenzothiazole		168	109	135	28	5	3.1

6PPD	**d5-6PPD-q**	**269**	**184**	**150**	**45**	**5**	**3.8**
6PPD		269	107	150	45	5	3.8
6PPD		269	93	150	45	5	3.8
6PPD-quinone		299	241	150	53	5	5.8
6PPD-quinone		299	215	150	30	5	5.8
6PPD-quinone	**d5-6PPD-q**	**299**	**187**	**150**	**30**	**5**	**5.8**
Aniline	**d4-BTZ**	**94**	**77**	**100**	**22**	**4**	**2.6**
Aniline		94	51	100	23	4	2.6
Aniline		94	50	100	41	4	2.6
Benzothiazole	**d4-BTZ**	**136**	**109**	**150**	**31**	**5**	**2.8**
Benzothiazole		136	77	150	27	5	2.8
Benzothiazole		136	65	150	38	5	2.8
Benzothiazole-d4		140	113	150	31	5	4.5
Benzothiazole-d4		140	81	150	19	5	4.5
Benzothiazole-d4		**140**	**69**	**150**	**36**	**5**	**4.5**
CPPD	**d5-6PPD-q**	**267.2**	**184**	**100**	**30**	**5**	**3.8**
CPPD		267.2	107	100	50	5	3.8
CPPD		267.2	93.1	100	46	5	3.8
CPPD-quinone		297.1	215	130	18	5	6
CPPD-quinone		297.1	187	130	34	5	6
CPPD-quinone	**d5-6PPD-q**	**297.1**	**55.2**	**130**	**50**	**5**	**6**
d5-6PPD-quinone		**304.2**	**246.1**	**110**	**36**	**4**	**6.5**
d5-6PPD-quinone		304.2	220.1	110	36	5	6.5
d5-6PPD-quinone		304.2	192.1	110	36	5	6.5
DPG		212	195	150	20	5	1.8
DPG	**d5-6PPD-q**	**212**	**119**	**150**	**20**	**5**	**1.8**
DPG		212	94	150	20	5	1.8
DPPD		260.1	183	130	38	5	6.4
DPPD	**d5-6PPD-q**	**260.1**	**167**	**130**	**46**	**5**	**6.4**
DPPD		260.1	156	130	46	5	6.4
DPPD-quinone	**d5-6PPD-q**	**291.1**	**263**	**150**	**22**	**5**	**5.5**
DPPD-quinone		291.1	235.2	150	34	5	5.5
DPPD-quinone		291.1	144	150	38	5	5.5
HMMM		391	283	150	15	5	3.5

HMMM		391	253	150	23	5	3.5
HMMM		391	207	150	19	5	3.5
HMMM	**d5-6PPD-q**	**391**	**177**	**150**	**35**	**5**	**3.5**
IPPD	**d5-6PPD-q**	**227.2**	**184**	**100**	**18**	**5**	**3**
IPPD		227.2	118	100	46	5	3
IPPD		227.2	107	100	46	5	3
IPPD-quinone		257.2	216	110	30	5	4.8
IPPD-quinone	**d5-6PPD-q**	**257.2**	**187**	**110**	**30**	**5**	**4.8**
IPPD-quinone		257.2	170	110	34	5	4.8

1.2 Lausanne

UPLC-MSMS analyses

UPLC-MSMS analyses were carried out with an ACQUITY UPLC system that was coupled to a Xevo TQ MS mass spectrometer equipped with an electrospray ionization source (ESI) (Waters®). Chromatographic separation was performed with an ACQUITY UPLC HSS T3 column (100 × 2.1 mm, 1.8 µm) at a flow rate of 0.40 mL min^{-1} and a column temperature of 30 °C. The mobile phase consisted of (A) 0.1 % formic acid in 95 % water and 5 % acetonitrile and (B) 0.1 % formic acid in 95 % acetonitrile and 5 % water. The elution gradient used is presented in table 5.1.

Step	Time (min)	Flow (mL/min)	%A	%B
1	0.00	0.40	95.0	5.0
2	0.50	0.40	95.0	5.0
3	5.00	0.40	5.0	95.0
4	9.00	0.40	5.0	95.0
5	10.00	0.40	95.0	5.0
6	12.00	0.40	95.0	5.0

Table 1: Elution gradient of the liquid chromatographic column with solvents A: 0.1 % formic acid in 95 % water and 5 % acetonitrile and B: 0.1 % formic acid in 95 % acetonitrile and 5 % water

The MS parameters were set as follows; electrospray in positive mode, source temperature: 150°C, desolvation temperature 600°C, cone gas flow (N_2): 50 L/h, desolvation gas flow: 1000 L/h, collision gas flow: 0.15 mL /min, ion spray voltage: 1500 V, cone voltage: 30 V.

The optimal instrumental parameters for each analyte were obtained by tuning using direct infusion. Quantitative analyses were performed in MS/MS mode. Identification of the analytes was based on comparison of two MRM transitions and retention times. The analyte specific parameters are presented in Table 5.2. Data was acquired and processed with MassLynx V4.2 (Waters®).

Compound	RT (min)	Parent ion (m/z)	Daughter ion (m/z)	Daughter Ion	Dwell time (s)	Cone voltage (V)	Collision voltage (V)
Aniline	0.97	94.10	76.96	Confirmation	0.078	32	16
			94.01	Quantifier			
Aniline d5	0.97	98.87	54.32	Confirmation	0.078	36	22
			82.00	Quantifier			
Diphenylguanidine	2.50	211.97	93.98	Confirmation	0.077	30	20
			119.02	Quantifier			
Benzothiazole	3.53	136.10	64.93	Confirmation	0.031	40	22
			108.90	Quantifier			
Benzothiazole d4	3.52	139.86	68.83	Confirmation	0.031	44	24
			112.31	Quantifier			
2-hydroxybenzothiazole	3.28	152.10	79.94	Confirmation	0.031	44	21
			123.96	Quantifier			
2-mercaptobenzothiazole	3.68	168.03	91.97	Confirmation	0.031	36	21
			135.08	Quantifier			
6PPD	3.71	269.05	106.99	Confirmation	0.050	24	42
			184.09	Quantifier			
Diphenylurea d10	4.16	222.95	81.17	Confirmation	0.050	28	28
			99.24	Quantifier			
6PPD Quinone	5.37	299.00	187.00	Confirmation	0.050	28	30
			241.00	Quantifier			
6PPD Quinone d5	5.35	304.01	192.04	Quantifier	0.050	28	28
HMMM	3.50	391.28	177.05	Confirmation	0.01	12	6
			359.22	Quantifier			
IPPD	3.01	227.16	107.04	Confirmation	0.05	25	38
			184.13	Quantifier			
IPPD-Q	4.33	257.14	187.12	Confirmation	0.04	28	23
			215.08	Quantifier			

CPPD	3.50	267.20	92.90	Confirmation	0.05	24	16
			184.80	Quantifier			
CPPD-Q	5.12	297.20	187.12	Confirmation	0.03	30	16
			215.08	Quantifier			
DPPD	5.28	260.14	167.16	Confirmation	0.03	38	30
			183.09	Quantifier			
DPPD-Q	4.80	291.14	143.90	Confirmation	0.03	32	20
			263.12	Quantifier			

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