



Organic and aqueous extraction of lipids from birch pollen grains exposed to gaseous pollutants

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Abstract

The lipid fraction of birch pollen grains (BPGs) is not yet fully described, although pollen lipid molecules may play a role in the allergic immune response. The mechanisms by which atmospheric pollutants modify allergenic pollen grains (PGs) are also far from being elucidated despite high potential effects on allergic sensitization. This work is a contribution to a better description of the lipid profile (both external and cytoplasmic) of BPGs and of alterations induced by gaseous air pollutants. Several lipid extractions were performed using organic and aqueous solvents on BPGs following exposure to ozone and/or nitrogen dioxide and under conditions favoring the release of internal lipids. Ozone reacted with alkenes to produce aldehydes and saturated fatty acids, while nitrogen dioxide was shown to be unreactive with lipids. NO₂ exhibited a protective effect against the reactivity of alkenes with ozone, probably by competition for adsorption sites. The decreased reactivity of ozone during simultaneous exposure to NO₂/O₃ raised the possibility of a Langmuir-Hinshelwood mechanism. Oxidation reactions induced by exposure of BPGs to ozone did not substantially modify the extraction of lipids by aqueous solvent, suggesting that the bioaccessibility of lipids was not modified by oxidation. On the contrary, the rupture of PGs appeared to be a key factor in enhancing the bioaccessibility of bioactive lipid mediators (linoleic and α -linolenic acids) in an aqueous solution. The internal lipid fraction of BPGs has specific characteristics compared with external lipids, with more abundant hexadecanoic acid, tricosanol, and particularly unsaturated fatty acids (linoleic and α -linolenic acids). Several mechanisms of action of gaseous pollutants on allergenic pollen were identified in this study: gaseous air pollutants can (i) modify the external lipid fraction by reactivity of alkenes, (ii) adsorb on the surface of PGs and be a source of oxidative stress after inhalation of PGs, and (iii) promote the release of cytoplasmic bioactive lipids by facilitating pollen rupture.

Keywords Ozone · Nitrogen dioxide · Bioaccessibility · Pollen cytoplasmic granules · Pollen rupture · Asthma

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Introduction

Birch pollen grain (BPG) is one of the most abundant tree pollen in Europe and a major cause of allergenic rhinitis and asthma attacks (Biedermann et al. 2019). Seasonal allergic asthma has been linked to pollen aeroallergens even though the majority of pollen grains (PGs) are too large in size (> 20 μ m in diameter) to reach the lower respiratory airways where asthmatic reactions occur (Schäppi et al. 1997a; Taylor et al. 2004; D'Amato et al. 2007; Taylor et al. 2007; Abou Chakra et al. 2011a; Caillaud et al. 2014; D'Amato et al. 2016). Birch pollen allergens have been detected in inhalable aerosol fractions (particulate matter of less than 10 μ m in diameter), particularly on days of light rainfall during the birch pollen season (Schäppi et al. 1996, 1997b; Grote et al. 2003; Taylor et al. 2004). Pollen cytoplasmic granules (PCGs) are microscopic particles such as starch granules, polysaccharide

particles, and other cytoplasmic pollen fragments; PCGs released from the pollen cytoplasm are capable of triggering allergic asthmatic reactions (Abou Chakra et al. 2011a, b, 2012). Several mechanisms are proposed in the literature for the release of PCGs which could be the result of pollen grain bursting due to sporoderm breakage, to an abortive germination (growth and burst of the pollen tube), or to the ejection of PCGs from the grain aperture after pollen immersion in an aqueous solution (Suphioglu et al. 1992; Grote et al. 2001; Taylor et al. 2002; Motta et al. 2006; Rogerieux et al. 2007; Ghiani et al. 2012; Smiljanic et al. 2016, 2019).

PCGs from allergenic PGs are a source of allergenic proteins and NAD(P)H oxidases in the respirable fine fraction of atmospheric aerosols, which can cause allergic airway inflammation (Bacsi et al. 2005, 2006; Boldogh et al. 2005). Immunological studies have highlighted the potential immunological role of pollen lipid molecules in allergic immune response regulation (Behrendt et al. 2001; Traidl-Hoffmann et al. 2003; Gilles et al. 2012; Bublin et al. 2014; Dahl 2018; Aglas et al. 2018). However, lipid molecules in allergenic airborne pollen fragments of respirable size are still understudied (Farah et al. 2020a). The lipid fraction of mature pollen is distributed between the external and internal lipids constituting respectively the extracellular pollen wall and the cytoplasm (Evans et al. 1990, 1991; Piffanelli et al. 1997, 1998; Pacini and Hesse 2005). The sporoderm of PGs act as barriers for the extraction of cytoplasmic lipids and PGs must therefore be fractured in order to release their internal lipids (Evans et al. 1991). Among external lipids extracted from allergenic BPGs, the following chemical families have been identified: alkanes, alkenes, alcohols, aldehydes, fatty acids (saturated and unsaturated), aldehydes, ethers, amino-alcohols, and phenolic compounds (Henricsson et al. 1996; Traidl-Hoffmann et al. 2002; Bashir et al. 2013; Mueller et al. 2016; Zhu et al. 2018). To the best of our knowledge, the internal lipid composition of birch pollen has not been previously described in the literature. In *Phleum pratense* PGs, large differences were detected between external and internal lipid fractions, in particular for unsaturated fatty acids (Farah et al. 2020a).

The interactions of atmospheric pollutants with pollen allergens can worsen respiratory allergy and asthma (Sénéchal et al. 2015; Frank and Ernst 2016). For example, in Denmark, peaks in ozone concentrations were observed in spring concomitantly with peaks in BPGs concentrations (Ørby et al. 2015). Ozone has been associated with a worsening of allergic symptoms during birch pollen season (Berger et al. 2020) but also with increased Bet v 1 allergen content in BPGs (Beck et al. 2013). Moreover, pollen-associated lipid mediators (PALMs) were extracted in higher quantities in BPGs from areas with higher ozone exposure (Beck et al. 2013). Chemical modifications of external lipids have been highlighted for BPGs exposed to ozone in laboratory

conditions (Zhu et al. 2018). Moreover, Bet v 1 nitration by exposure to polluted air induced an increased potency to trigger and enhance an immune allergic reaction (Franze et al. 2005; Reinmuth-Selzle et al. 2014). The co-exposure of birch pollen extracts to both NO₂ and O₃ gases highlighted a synergistic effect between those pollutants (Franze et al. 2003). In addition, the exposure to atmospheric pollutants is a facilitating factor for pollen rupture in the atmosphere (Motta et al. 2006; Sénéchal et al. 2015; Puc et al. 2016). The molecular mechanisms behind gaseous pollutant (NO₂ or O₃) effects on PGs are largely unknown (Frank and Ernst 2016). Moreover, tropospheric ozone concentrations are predicted to rise in polluted areas during the twenty-first century as a result of climate change (Lu et al. 2019). In addition, the concentrations of BPGs have been observed to rise in various locations in recent decades, notably because of the increase in atmospheric concentrations of carbon dioxide (Frei and Gassner 2008; Hoebeke et al. 2017; Besancenot et al. 2019). Thus, individual exposure to the ozone-BPG cocktail is already affecting the health of allergic patients, and this harmful co-exposure is expected to increase further in the coming decades (Berger et al. 2020).

Lipidic fractions (both external and internal) of allergenic PGs are still understudied. In this work, BPGs were exposed in the laboratory to O₃, NO₂, and a mixture of both gases. Lipid extractions were performed with both organic and aqueous solvents in order to discuss the bioaccessibility of lipids and their potential modification by oxidation reactions. As the pollen sporoderm is an efficient barrier against solvent extraction, BPGs were also artificially ruptured in order to gain insights about the internal lipids. This work contributes to a better description of the lipid profile of BPGs and its alteration by gaseous urban air pollutants in the perspective of an increase of birch pollen allergy in the twenty-first century.

Materials and methods

Sampling of birch pollen grains

Male catkins were harvested during birch (*Betula pendula*) pollination period in March 2017 on the campus of the University of Lille (France). Fresh PGs were sieved at 40 µm in the laboratory, placed in a closed container under a dry atmosphere for 24 h, and then stored hermetically in sealed vials at 4 °C until used. Microscopic observations of birch pollen after this drying step shows that PGs were all intact (no broken PG was observed). Broken pollen grains were artificially produced in the laboratory by grinding pollen grains with a magnetic stirrer for 4 h at 1000 rpm (Farah et al. 2020a).

The water content of stored BPGs was estimated to less than 5% by weighing BPGs at different temperatures up to

150 °C. All experiments were therefore carried out with dry pollen. In the text below, ‘intact PGs’ refers to dried pollen grains without any further treatment and ‘broken PGs’ refers to dried pollen grains artificially broken (crushed by grinding) in the laboratory.

Exposure of pollen grains to ozone and nitrogen dioxide

PGs were deposited between two sheets of cellulose filter paper (GE Healthcare, Life Sciences Whatman™, diameter 55 mm) and exposed to gaseous pollutant flow into a stainless-steel fluidized bed reactor. Exposures were performed for 16 h under dry conditions and laboratory temperature (20 ± 1 °C). PGs were exposed separately to 130 ± 5 ppb of O_3 and to 100 ± 5 ppb of NO_2 . PGs were also exposed to a mixture of NO_2 and O_3 at concentrations of 100 ± 5 ppb for each gas. Ozone was generated from synthetic air by a UV generator at a flow rate of $1.35 \text{ L} \cdot \text{min}^{-1}$. O_3 concentration was measured at the outlet of the filter holder every 10 s with a detection limit of 1 ppb using an ozone analyzer (Thermo Fisher Scientific, model Megatec 49i). Nitrogen dioxide was generated from a flow ($2.5 \text{ L} \cdot \text{min}^{-1}$) of nitric oxide using a NO_x generator (Ansycos, SYCOS KT-GPTM). NO_x concentration was measured at the reactor outlet by a nitrogen oxide analyzer (Thermo Fisher Scientific, Model Megatec 42i) performing a measurement every 60 s with a detection limit of 0.4 ppb.

Organic extraction of external and internal lipids

The extraction procedure for external lipids without disrupting pollen cell-wall integrity was described elsewhere (Zhu et al. 2018; Farah et al. 2020a, 2020b). In brief, external molecular lipids were extracted from 50 mg of either intact or broken PGs with 5 mL of methylene chloride ($\geq 99.8\%$; Sigma-Aldrich). The organic extract was then dried under a stream of nitrogen. Finally, the residue was solubilized in 50 μL of methylene chloride and derivatized by reconstituting with 50 μL of 99% of BSTFA - 1% TMCS (N, O-bis(trimethylsilyl) trifluoroacetamide, trimethylchlorosilane; Sigma-Aldrich).

Chemical analyses were carried out with a gas chromatograph coupled to a mass spectrometer (GC/MS) detector for identification and with a flame ionization detector (GC/FID) for quantification. Chromatographic parameters were as follows: injection of 1 μL , helium column flow of $1 \text{ mL} \cdot \text{min}^{-1}$, split ratio of 1:10, injector temperature of 250 °C, oven temperature initially at 50 °C and raised at $4 \text{ }^\circ\text{C} \cdot \text{min}^{-1}$ to reach 310 °C (hold for 19 min). The MS ionization was done by electronic impact at 70 eV. Lipid molecular species were quantified using carbon equivalent number as previously described (Scanlon and Willis 1985; Zhu et al. 2018) excepted for unsaturated fatty acids (linoleic acid and α -linolenic acid)

which were directly quantified using their FID response factor determined by injection of standard solutions. For each lipid extraction condition, three separate extractions and separate GC analyses were performed. Every single sample was treated as an independent variable and the error bars for mass concentrations were expressed as the maximum deviation for those three analyses around the mean value. The mass of lipids A_i in internal fraction was calculated from the mass of A_i calculated from extracts of broken and intact PGs following eq. 1:

$$m(A_i)_{\text{internal}} = m(A_i)_{\text{extraction of broken PGs}} - m(A_i)_{\text{extraction of intact PGs}} \quad (1)$$

Negatives values calculated for $m(A_i)_{\text{internal}}$ and values inferior to the maximum deviation were set to zero.

Total dry extractible mass

In order to estimate the total mass of internal extractable matter by organic solvent, 1 g of broken and intact PGs was extracted with 5 mL of methylene chloride. The total amount of extractable lipids was then weighed with a precision balance ($\pm 0.1 \text{ mg}$) after complete evaporation of the solvent.

Aqueous lipid extraction

PGs were extracted with phosphate buffered saline (PBS) solution (Thermo Fisher Scientific) to be close to physiological conditions. The aqueous pollen extraction steps have been described elsewhere (Farah et al. 2020b). In brief, 50 mg of PGs were extracted for 2 h by suspension in 2 mL of PBS solution under different conditions: intact PGs with no agitation or with magnetic stirring agitation (1000 rpm), laboratory-ruptured PGs deposited in PBS without agitation, and laboratory-ruptured PGs with gentle agitation. In order to identify the lipid molecular species released in the aqueous phase from broken pollen, PGs were fractured during 4 h using mechanical agitation with a magnetic stirrer (Farah et al. 2020a) followed by extraction with 2 mL of PBS in two ways: suspension without agitation or gentle agitation by rotation of extraction tubes.

Pollen lipids extracted by PBS were then transferred to organic solvent by the following steps: centrifugation at 1800g for 2 min after PBS extraction, filtration of the supernatant, liquid-liquid extraction (PBS-methylene chloride) by adding 2 mL of organic solvent, hand-shake for 2 min, centrifugation at 1800g for 2 min, and recovery of the organic phase (repeated three times) followed by evaporation to dryness of solvent. The dry residue was finally solubilized in 50 μL of methylene chloride and 50 μL of BSTFA-TMCS.

Scanning electron microscopy (SEM)

BPGs were observed by SEM to determine the extent of physical damage due to thorough mechanical stirring. BPGs were spread over a conductive carbon tape and gold-coated prior to SEM imaging using a field-emission gun scanning electron microscope (Hitachi S4700 FEG-SEM) operated at 3 kV.

Results

Description of external and internal lipid composition of birch pollen grains

A total dry mass of $27 \pm 1 \text{ mg.g}^{-1}$ of extractable lipids from BPGs collected in 2017 was obtained; such mass is identical to the total mass extracted from BPGs collected in 2016 on the same site (Zhu et al. 2018). Identified and quantified lipids from BPGs collected in 2017 (this work) summed up for 3591 ng.mg^{-1} (i.e. about 13% of the total dry extract). Extractable lipids can be classified in 6 chemical families (Table 1 and Fig. 1): alkenes ($1655 \pm 167 \text{ ng.mg}^{-1}$), alcohols ($1052 \pm 123 \text{ ng.mg}^{-1}$), fatty acids ($671 \pm 81 \text{ ng.mg}^{-1}$), alkanes ($164 \pm 19 \text{ ng.mg}^{-1}$), aldehydes ($30 \pm 5 \text{ ng.mg}^{-1}$) and unsaturated fatty acids ($19 \pm 3 \text{ ng.mg}^{-1}$). Total masses per family were very similar between organic extracts of BPGs from the 2016 and 2017 pollination seasons (Zhu et al. 2018). Unsaturated fatty acids were not identified and quantified in the work of Zhu et al. (2018).

The total dry residue of the extract from crushed BPGs weighed 33 mg.g^{-1} corresponding to the sum of extractable organic matter from both internal and external parts of PGs. The total mass of identified lipids was almost equally distributed between internal (4181 ng.mg^{-1}) and external fractions (3591 ng.mg^{-1}). The same lipids were identified between both external and internal regions, only their mass fractions differed. Mass fractions of unsaturated fatty acids were most different between external and internal lipids of BPGs: more than 99% of unsaturated fatty acids were recovered from broken BPGs (i.e. internal lipids). Hexadecanoic acid and tricosanol were also more abundant in internal lipids (respectively 97% and 98%). The internal lipid fraction of BPGs was described for the first time in this work and it has great similarities with that of *Phleum pratense* PGs (Farah et al. 2020a).

Modification of external lipids by O_3 and NO_2

Aldehydes and saturated fatty acids are produced by the reactions between ozone and alkenes from the external lipid fraction of BPGs (Zhu et al. 2018). This reactivity induces a decrease in alkenes mass fractions (from 1655 to 542 ng.mg^{-1}) and an increase from 671 to 960 ng.mg^{-1} for saturated fatty acids and from 30 to 413 ng.mg^{-1} for aldehydes (Table 2).

Mass fractions of alkenes are similar between BPGs samples from pollination period 2017 and 2016 (respectively 1655 and 1651 ng.mg^{-1}). The consumption of alkenes by ozone is however much more important for BPGs from 2017 (decrease of 70%) compared with 2016 (decrease of 30%) despite exposure of pollen samples to very close doses of ozone (2017: 16 h at $130 \pm 5 \text{ ppb}$; 2016: 16 h at $115 \pm 5 \text{ ppb}$) (Zhu et al. 2018).

Exposure of BPGs to NO_2 alone (100 ppb, 16 h) had no observable effects on the lipid molecular species identified in this work (Table 2). New compounds were not identified consecutively to NO_2 exposure. Alkenes consumption was less marked when BPGs were exposed simultaneously to NO_2 and O_3 : observation of a decrease of 70% for alkenes after exposure to ozone alone and of 30% after exposure to both NO_2 and O_3 (Table 2).

Aqueous extraction after ozone exposure

Lipid extraction from BPGs with organic solvent does not allow the estimation of the bioaccessibility of pollen lipids under physiological conditions. Hence, several lipid extraction methods were used with phosphate buffer solution (PBS) as the aqueous solvent. Different levels of mechanical stress were applied on PGs in order to break the sporoderm of pollen and enable internal (cytoplasmic) lipid extraction. PGs were severely damaged by 4 h of magnetic stirring (Fig. 2); after 4 h of mechanical grinding, no intact pollen grain could be observed by scanning electron microscopy. Aqueous extraction of unsaturated fatty acids was enhanced by a factor of almost a hundred (Fig. 3) when lipids were extracted from broken BPGs with thorough magnetic stirring (1000 rpm) compared with lipid extracted from intact BPGs with gentle agitation (linoleic acid: compared 2260 ± 211 to $18 \pm 4 \text{ } \mu\text{g.mg}^{-1}$; α -linolenic acid: 1750 ± 172 compared with $15 \pm 4 \text{ } \mu\text{g.mg}^{-1}$). Aqueous extraction of unsaturated fatty acids from broken PGs with magnetic stirring was even more efficient than that with organic solvent (4010 ± 383 compared with $1704 \pm 135 \text{ } \mu\text{g.mg}^{-1}$). However, the use of PBS as the extraction solvent resulted in less alkanes, alcohols, and alkenes extracted than using methylene chloride (Table 1).

Oxidation reactions induced by ozone exposure of BPGs did not substantially modify the extraction by aqueous solvent (Table 2). The total mass of extractable lipids was not significantly different between non-exposed ($5576 \pm 662 \text{ ng.mg}^{-1}$) and O_3 -exposed BPGs ($4948 \pm 417 \text{ ng.mg}^{-1}$) (Table 2). Only unsaturated fatty acids were less extracted with PBS for O_3 -exposed BPGs ($3359 \pm 157 \text{ ng.mg}^{-1}$) compared with non-exposed ones ($4010 \pm 383 \text{ ng.mg}^{-1}$).

Discussion and conclusion

The internal lipid fraction of BPGs has specific characteristics compared with external lipids with more abundant

Table 1 Mass fractions of lipids extracted from birch pollen grains either with organic (methylene chloride) or with aqueous solvent (PBS: phosphate buffer solution)

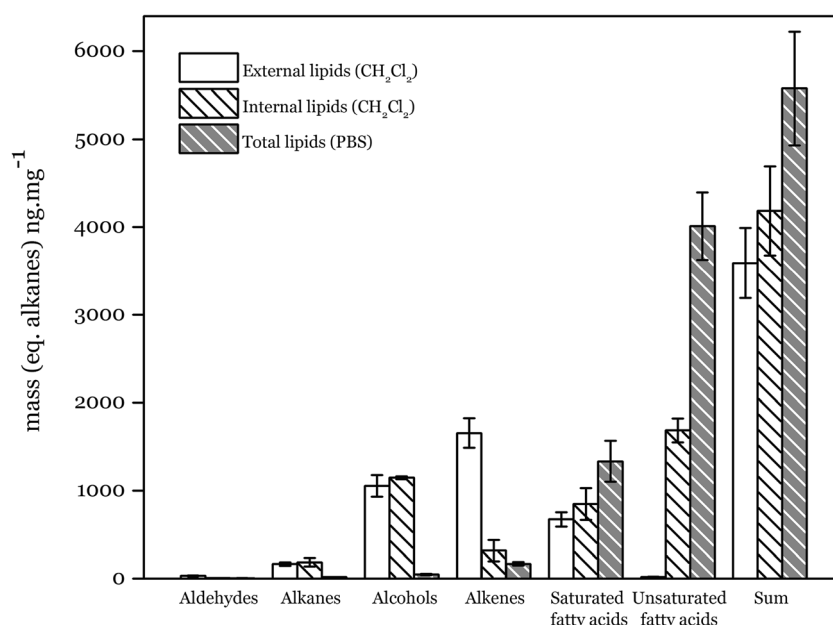
ng.mg ⁻¹	CH ₂ Cl ₂ extraction			PBS extraction
	External lipids	Internal lipids	Total lipids	Total lipids
Alkanes				
Heneicosane	8±1	—	6±1	2±1
Tricosane	20 ± 3	1±1	21±3	2±1
Pentacosane	58 ± 5	1±1	59±8	6±1
Heptacosane	78 ± 10	183±49	261±49	7±1
Sum	164 ± 19	185±51	347±61	17±4
Alcohols				
Hexadecanol	1 ± 1	1±1	2±1	5±1
Octadecanol	2 ± 1	5±2	7±1	1±1
Eicosanol	12 ± 1	—	10±2	1±1
Heneicosanol	7 ± 1	—	7±1	1±1
Docosanol	861 ± 92	—	816±66	31±5
Tricosanol	21 ± 4	1136±12	1157±12	1±1
Tetracosanol	103 ± 18	—	102±11	3±1
Hexacosanol	27 ± 3	—	24±3	2±1
Octacosanol	18 ± 2	—	15±2	1±1
Sum	1052 ± 123	1142±15	2140±99	46±13
Alkenes				
Decene	4 ± 1	—	4±1	1±1
Pentacosene	52 ± 4	13±8	65±8	11±1
Heptacosene	824 ± 73	304±113	1128±113	83±10
Nonacosene	596 ± 62	—	622±87	55±7
Hentriacontene	179 ± 27	—	183±27	17±2
Sum	1655 ± 167	317±121	2003±236	167±21
Fatty acids				
Hexanoic acid	2 ± 1	—	2±1	1±1
Heptanoic acid	1 ± 1	2±1	3±1	1±1
Octanoic acid	2 ± 1	10±2	12±2	4±1
Nonanoic acid	13 ± 2	5±3	18±3	3±1
Dodecanoic acid	2 ± 1	5±2	7±2	1±1
Hexadecanoic acid	16 ± 3	598±43	614±43	1185±209
Octadecanoic acid	13 ± 2	26±11	39±11	47±7
Eicosanoic acid	8 ± 1	9±3	17±3	11±2
Heneicosanoic acid	3 ± 1	2±1	5±1	6±1
Docosanoic acid	238 ± 25	54±43	292±43	40±7
Tricosanoic acid	46 ± 4	14±11	60±11	2±1
Tetracosanoic acid	308 ± 36	120±61	428±61	29±5
Hexacosanoic acid	19 ± 3	—	22±4	2±1
Sum	671 ± 81	845±181	1519±186	1332±244
Unsaturated fatty acids				
Linoleic acid	7 ± 1	984±81	991±81	2260±211
α-Linolenic acid	12 ± 2	701±54	713±54	1750±172
Sum	19 ± 3	1685±135	1704±135	4010±383
Aldehydes				
Nonanal	14 ± 2	—	9±1	1±1
Octadecanal	6 ± 1	6±2	12±2	1±1
Eicosanal	4 ± 1	—	—	—
Docosanal	6 ± 1	1±1	5±1	2±1
Sum	30 ± 5	7±3	27±4	4±3
Total sum	3591 ± 398	4181±506	7738±721	5576±668

hexadecanoic acid, tricosanol, and particularly unsaturated fatty acids (linoleic and α-linolenic acids). The most abundant lipids extracted from BPGs indeed originated from the internal fraction: linoleic acid 2.26 μg.mg⁻¹ and α-linolenic acid 1.75 μg.mg⁻¹. Rupturing of the membrane of BPGs is a crucial mechanism to account for the dispersion of allergens in the fine fraction of the atmospheric aerosol (Schäppi et al. 1997a; Grote et al. 2003; Taylor et al. 2004; Visez et al. 2015). Inhalation of broken BPGs and/or their PCGs therefore

exposes the immune system simultaneously to internal lipids and to the protein allergens.

Understanding of the bioaccessibility of biochemical compounds carried by PGs in physiological conditions needs to be improved. Pollen rupture seems indeed a crucial mechanism for the release of potentially bioactive lipids. PBS used as an aqueous solvent proved to be a very poor solvent for lipid extraction from BPGs, including the most hydrophilic lipids (saturated and unsaturated fatty acids), except under

Fig. 1 Mass fractions of the main chemical families extracted from birch pollen grains with organic solvent (methylene chloride) and aqueous solvent (PBS: phosphate buffer solution)



conditions of extraction with strong stirring. To be effective, the extraction must be carried out with mechanically ruptured BPGs. Pollen rupture seems to be a prerequisite to enhance the bioaccessibility of certain lipids in an aqueous solution, especially for unsaturated fatty acids mostly found in the cytoplasm of the BPGs. Inhalation of broken BPGs may be common, at least on specific meteorological conditions, as the fraction of BPGs without their cytoplasm has been estimated to be up to 9% in an atmospheric environment (Puc et al. 2016). Investigations of immune response reactions should take into account this cytoplasmic-specific lipid fraction in order to determine whether inhalation of broken pollens grains fragments (or PCGs) may play a role in allergic sensitization or in exacerbation of allergic symptoms. Moreover, the biochemical composition of BPGs is not completely elucidated, which limits our knowledge of the biochemical microenvironment surrounding a PG deposited on the nasal epithelium or a PCG deposited on the lower airways.

Chemical alterations following BPG exposure to ozone do not seem to be able to modify lipid bioaccessibility in the aqueous phase, at least for the lipids identified in this study. Unsaturated fatty acids are less extracted in the aqueous phase after pollen exposure to ozone: extraction for intact pollen was 4010 ng.mg⁻¹ as compared with 3359 ng.mg⁻¹ for pollen exposed to ozone. The release of these compounds may be impaired after exposure to ozone as it was previously observed for some proteins after exposure to pollutants (Behrendt and Becker 2001). Unsaturated fatty acids may have also been degraded by ozone although the expected products during ozonolysis of α -linolenic acid (nonanedioic acid for example) were not detected. The degradation of lipidic compounds by ozone in the pollen cytoplasm raises the question of ozone

diffusion through the sporoderm, while chemical reactions on solid particles are essentially expected at their surface (Shiraiwa et al. 2011a). It has been shown that NO can diffuse into the BPG cytoplasm but in experimental conditions with excessively high doses of pollutant exposure (48 h at 1 to 5% of NO) (Lhuissier et al. 2000). Conversely, nitrogen dioxide only reacted on the surface of BPGs and the chemical alterations did not pass through the pollen wall for exposures ranging from 1 to 400 ppm day (Goschnick and Schuricht 1995, 1996). Following ozone exposures, SDS/PAGE analyses of water-soluble BPGs proteins showed no changes in the polypeptide profiles suggesting protection of proteins within the PG cytoplasm (Cuinica et al. 2013, 2014). Proteins extracted and then exposed outside the BPGs underwent for their part post-translational modifications (Franze et al. 2003, 2005). More work is clearly needed to unravel the diffusion of gaseous pollutants into the pollen cytoplasm.

Alkenes are strongly consumed by ozone during laboratory exposure of timothy and birch PGs (Zhu et al. 2018; Farah et al. 2020b). It is not possible to easily extrapolate this reactivity to atmospheric conditions because the distribution in residence time of the PGs in the atmosphere is not known at all. The residence time of BPGs in the atmosphere can be long enough to allow them to travel considerable distances (Sofiev et al. 2006; Skjoth et al. 2007; Sofiev 2016). However, most tree pollen probably has much shorter travel distances but potentially with periods of deposition and resuspension that are poorly described in the literature (Proctor et al. 1996). PGs can also be exposed to pollutants directly on the plant. A dose of 130 ppb of ozone for 16 h probably

Table 2 Mass fractions of external lipids extracted from birch pollen grains with organic solvent after exposure to gaseous pollutants (NO₂ and/or O₃) for 16 h. Mass fractions of total lipids extracted from birch pollen grains with aqueous solvent after exposure to O₃ for 16 h

ng.mg ⁻¹	Organic extraction (external lipids)				Aqueous extraction (total lipids)	
	No exposure	NO ₂ (100 ppb)	NO ₂ + O ₃ (100 ppb each)	O ₃ (130 ppb)	No exposure	O ₃ (130 ppb)
Alkanes						
Heneicosane	8±1	10 ± 2	10 ± 1	11 ± 2	2±1	2±1
Tricosane	20±3	18 ± 4	17 ± 3	20 ± 3	2±1	2±1
Pentacosane	58±5	62 ± 11	62 ± 7	61 ± 12	6±1	5±1
Heptacosane	78±10	78 ± 12	81 ± 6	75 ± 12	7±1	6±1
Sum	164±19	168 ± 29	170 ± 17	167 ± 29	17±4	15±4
Alcohols						
Hexadecanol	1±1	1 ± 1	1 ± 1	1 ± 1	5±1	4±2
Octadecanol	2±1	2 ± 1	2 ± 1	2 ± 1	1±1	1±1
Eicosanol	12±1	12 ± 1	14 ± 1	14 ± 2	1±1	1±1
Heneicosanol	7±1	7 ± 1	8 ± 2	8 ± 1	1±1	1±1
Docosanol	861±92	826 ± 101	787 ± 144	821 ± 84	31±5	30±5
Tricosanol	21±4	22 ± 4	21 ± 3	19 ± 2	1±1	1±1
Tetracosanol	103±18	99 ± 9	115 ± 13	86 ± 25	3±1	4±1
Hexacosanol	27±3	26 ± 2	27 ± 4	25 ± 2	2±1	2±1
Octacosanol	18±2	18 ± 3	17 ± 2	18 ± 2	1±1	1±1
Sum	1052±123	1013 ± 123	992 ± 171	994 ± 120	46±13	45±14
Alkenes						
Decene	4±1	4 ± 1	2 ± 1	1 ± 1	1±1	1±1
Pentacosene	52±4	51 ± 6	31 ± 4	22 ± 3	11±1	4±1
Heptacosene	824±73	817 ± 70	560 ± 81	316 ± 53	83±10	53±16
Nonacosene	596±62	527 ± 44	368 ± 53	145 ± 22	55±7	30±10
Hentriacontene	179±27	155 ± 15	86 ± 9	58 ± 4	17±2	7±3
Sum	1655±168	1554 ± 136	1047 ± 148	542 ± 83	167±21	95±31
Fatty acids						
Hexanoic acid	2±2	2 ± 1	3 ± 1	4 ± 1	1±1	1±1
Heptanoic acid	1±1	1 ± 1	2 ± 1	2 ± 1	1±1	1±1
Octanoic acid	2±1	3 ± 1	3 ± 1	3 ± 1	4±1	3±1
Nonanoic acid	13±2	15 ± 1	118 ± 14	148 ± 19	3±1	7±1
Dodecanoic acid	2±1	1 ± 1	2 ± 1	4 ± 1	1±1	1±1
Hexadecanoic acid	16±3	15 ± 2	14 ± 1	18 ± 3	1185±209	1252±164
Octadecanoic acid	13±2	20 ± 2	36 ± 9	58 ± 6	47±7	46±9
Eicosanoic acid	8±1	8 ± 1	48 ± 9	68 ± 11	11±2	12±2
Heneicosanoic acid	3±1	2 ± 1	7 ± 1	10 ± 1	6±1	6±3
Docosanoic acid	238±25	228 ± 16	268 ± 19	254 ± 20	40±7	39±9
Tricosanoic acid	46 ± 4	42 ± 4	48 ± 4	45 ± 5	2±1	2±1
Tetracosanoic acid	308±36	303 ± 25	307 ± 23	326 ± 35	29±5	29±10
Hexacosanoic acid	19±3	20 ± 1	21 ± 2	20 ± 3	2±1	5±1
Sum	671±82	660 ± 57	877 ± 86	960 ± 111	1332±238	1404±204
Unsaturated fatty acid						
Linoleic acid	7±1	6 ± 1	6 ± 1	4 ± 1	2260±211	1903±63
α-Linolenic acid	12±2	11 ± 2	8 ± 2	7 ± 1	1750±172	1456±94
Sum	19±3	17 ± 3	14 ± 3	11 ± 2	4010±383	3359±157
Aldehydes						
Nonanal	14±2	21 ± 3	139 ± 25	181 ± 32	1±1	6±2
Octadecanal	6±1	9 ± 1	66 ± 11	97 ± 16	1±1	14±3
Eicosanal	4±1	7 ± 1	71 ± 11	97 ± 18	—	—
Docosanal	6±1	5 ± 1	28 ± 3	38 ± 6	2±1	10±2
Sum	30±5	42 ± 6	304 ± 52	413 ± 72	4±3	30±7
Total sum	3591±400	3454 ± 354	3404 ± 477	3087 ± 417	5576±662	4948±417

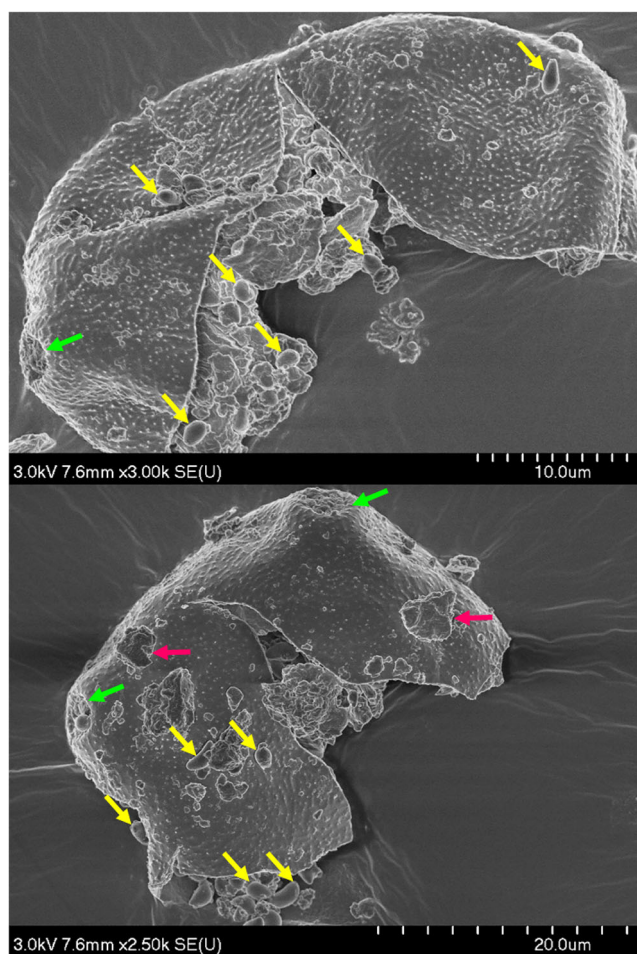


Fig. 2 Birch pollen grain (BPG) observed after thorough magnetic stirring under dry conditions with shredded sporoderms letting out micrometer-sized pollen cytoplasmic granules (PCGs). Internal lipids are no longer enclosed in BPGs. Yellow arrows point to PCGs. Green arrows mark BPG apertures. Pink arrows indicate fragments of pollen wall

corresponds to a high pollen exposure value in comparison with real atmospheric conditions. There is a lack of analysis of PGs collected in the atmosphere and analyzed at the individual grain level for molecular tracers of exposure to pollutants. Aldehydes are, for example, excellent tracer molecules for the exposure of birch and timothy grass PGs to ozone (Zhu et al. 2018; Farah et al. 2020b). These chemical modifications, or their absence, could provide information on the degree of PG exposure to air pollution during the airborne lifetime of pollen.

Synergistic effects have been described between ozone and nitrogen dioxide resulting in an enhanced nitration of bet v 1 allergen after O_3 and NO_2 simultaneous exposure compared with NO_2 exposure alone (Franze et al. 2003). On the contrary, NO_2 has in this work a protective effect towards alkenes reactivity with ozone. Ozone reactivity may proceed via two reaction pathways, either ozone first adsorbs before reacting

or either gas-phase ozone reacts directly with adsorbed alkenes (mechanisms 1 and 2) (Baxter and Hu 2002).

Mechanism 1: Langmuir-Hinshelwood type

$O_3(g) = O_3(\text{adsorbed})$

$O_3(\text{adsorbed}) + \text{alkene}(\text{adsorbed}) = \text{fatty acid}(\text{adsorbed}) + \text{aldehyde}(\text{adsorbed})$

Mechanism 2: Eley-Rideal type

$O_3(g) + \text{alkene}(\text{adsorbed}) = \text{fatty acid}(\text{adsorbed}) + \text{aldehyde}(\text{adsorbed})$

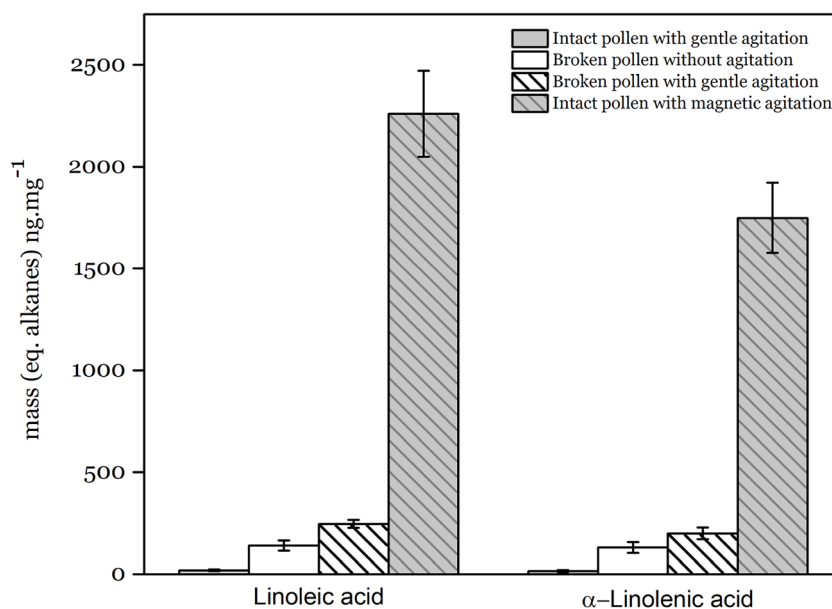
The decreasing reactivity of ozone during simultaneous NO_2/O_3 exposure suggests a reactivity following the Langmuir-Hinshelwood mechanism. Indeed, competition for adsorption on the PG surface between NO_2 and O_3 could result in a decreased surface concentration of adsorbed ozone resulting in a lower probability of reaction with alkenes. Direct measurements of ozone adsorption have already been performed and an estimate of 29 ng of adsorbed ozone per mg of BPG has been proposed (Alebic-Juretic et al. 1997; Zhu et al. 2018). Washing of BPGs with organic solvent has also resulted in a decrease in ozone adsorption which was tentatively attributed to the loss of ozone adsorption on lipids (Zhu et al. 2018). The washing with organic solvent could also change adsorbing properties of the surface resulting in a lowered ozone adsorption.

Another aspect in controlling pollutant adsorption and reactivity is the potential competition with adsorbed water. In this work, pollutant exposures were performed in a dry atmosphere with desiccated BPGs. With the Langmuir-Hinshelwood mechanism, gaseous and adsorbed water indeed contribute to competitive adsorption with reactive species (Kolb et al. 2010; Chu et al. 2019). The strong observed consumption of alkenes with ozone in dry laboratory conditions may be a limit case illustrating maximum reactivity not limited by competitive adsorption. The involvement of water molecules in oxidative reactivity is probably much more complex as reactivity occurs between H_2O and NO_2 to produce acids (HNO_2 , HNO_3) on the pollen surface (Okuyama et al. 2007; Chassard et al. 2015).

Whereas two BPG samples with the same mass fractions of alkenes were exposed to the same amount of ozone, an important increase in reactivity was observed for BPGs harvested in 2017 compared with those harvested in 2016. Different properties of pollen surfaces regarding ozone adsorption may tentatively explain this differential reactivity. Reactivity of pollutants on the pollen surface is known to be species-specific, dose-dependent and this specific example shows that reactivity could also be different for PGs coming from different batches collected on different years at the same location.

Besides surface reactivity, adsorption of pollutants on the PG surface may have a role in allergic symptom

Fig. 3 Extraction of unsaturated fatty acids with phosphate buffer solution (PBS) under different conditions: gray = gentle agitation of birch pollen grains (BPGs); white = broken BPGs without agitation; white dashed = broken BPGs with gentle agitation; gray dashed = broken BPGs with magnetic stirring



exacerbations. Adsorbed pollutants could be transferred to nasal and ocular mucous membranes and become a source of pollen-associated oxidative stress with subsequent inflammatory processes (Okuyama et al. 2007; Shiraiwa et al. 2011b, 2012). Transfer of adsorbed pollutants to the immune system via inhaled PGs is to our opinion understudied especially since PGs have been demonstrated to possess high adsorption capabilities (Thio et al. 2011; Zhang et al. 2016). Ragweed PGs are for example adsorbent as strong as activated carbon for removing hydrophobic organic compounds from aqueous solutions (Thio et al. 2011). Adsorption of acid and basic pollutants (HNO_2 , HNO_3 , HCl , and NH_3) were quantified in concentrations of up to $1 \mu\text{g.mg}^{-1}$ in cedar PGs (Okuyama et al. 2007). Cedar pollen has adsorption capability for these pollutants higher than other tested particles of biogenic origins (humic acid and yellow sand) (Okuyama et al. 2007).

Conclusion

Knowledge of the lipid fraction of birch pollen grains is still incomplete. Only a fraction of the material extractable by organic or aqueous solvents has been identified and quantified. The bioaccessibility of pollen lipids under physiological conditions is largely unknown and constrains our understanding of their potential role in the allergic reaction. Moreover, pollen rupture is a key factor in the bioaccessibility of allergens and lipids. Pollen lipid fraction can be modified by air pollution at least during its airborne journey. Some lipids are indeed modified in laboratory conditions by ozone, but it is difficult to extrapolate to atmospheric conditions because competition for surface adsorption between several gaseous species and

ozone could greatly limit the reactivity. It is also not clear whether gaseous pollutants can pass through the sporoderm of intact pollen grains to reach the cytoplasm and react with internal lipids and allergens. The adsorption of gaseous pollutants (NO_2 or O_3) on the pollen surface allows their transfer to the mucous membranes upon inhalation where they can produce oxidative stress or modify the acidity of the environment. The role of these gaseous pollutants adsorbed on the pollen surface in the allergic reaction is largely unknown for both health effects and germination-related processes.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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