



Influence of *Phleum pratense* pollen grains rupture on lipids extraction

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Abstract In this work, we completed the first identification and quantification of internal lipids for allergenic *Phleum pratense* pollen grains (PGs). We have extracted PGs with aqueous solvent under different extraction procedures and observed release of extra amount of unsaturated fatty acids (α -linolenic and linoleic acids) when PGs were broken during extraction. We showed that rupture of PGs is necessary for the release of higher quantity of bioactive compounds. Organic solvent extraction of crushed PGs confirmed the predominance of unsaturated fatty acids in the inner parts of PGs as well as a different distribution of external and internal lipids. The total mass of extractable matter by organic solvent was determined at $22 \pm 1 \text{ mg g}^{-1}$ for intact pollen and at $48 \pm 1 \text{ mg g}^{-1}$ for broken pollen. This finding brings new insight into the physical degradation of PGs by air pollutants during atmospheric transport. We

hypothesize that the presence of bioactive lipids in pollen cytoplasm gives further importance to asthma triggered by cracked or broken allergenic PGs. The rupturing of PGs in the atmosphere may expose the population to the risk of inhaling micrometer-sized aerosols (cytoplasmic granules) containing not only allergens but also bioactive lipids.

Keywords Pollen allergy · Lipidomic · Timothy grass · Allergenic pollen · Cytoplasmic granules

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1 Introduction

Pollen grains (PGs) from grasses are an important trigger of asthmatic attacks (D'Amato et al. 2008, 2016; Idroze et al. 2019; Knox 1993; Newson et al. 1997; Porcel Carreño et al. 2019; Simunovic et al. 2020). Grass PGs are considered too large (25–60 µm) to reach the lower respiratory system where asthmatic responses occur (Wilson et al. 1973). Asthma attacks induced by grass PGs have been linked to pollen cytoplasmic granules (PCGs) with size ranging from 0.6 to 2.5 µm (Marks et al. 2001; Suphioglu 1998). The release of PCGs could occur after rupture of pollen walls by osmotic shock, abortive germination or mechanical stress, with a suspected enhancement of rupture induced by air pollutants (D'Amato et al. 2016; Grote et al. 2001; Motta et al. 2006; Sénéchal et al. 2015; Suphioglu 1998; Suphioglu et al. 1992; Taylor et al. 2002; Visez et al. 2015). When immersed in water, a single grass PG can release 700 to 1000 PCGs (Schäppi et al. 1999; Suphioglu et al. 1992). Release of pollen allergens after pollen rupture in the atmosphere, in particular in the case of grass pollen, is currently the strongest hypothesis for occurrence of thunderstorm asthma (D'Amato et al. 2008; Knox 1993; Lee et al. 2017; Sutherland et al. 2017; Thien et al. 2018).

Major allergens of *Phleum pratense* pollen such as Phl p 1, 4, 5, 6 and 13 were found in PCGs (Behrendt et al. 1999; Fischer et al. 1996; Grote et al. 2001; Swoboda et al. 2004; Taylor et al. 2002; Vrtala et al. 1999). Isolated PCGs have elicited a positive skin prick test in grass pollen-sensitized asthmatics, as well as an earlier bronchial response in asthma attacks (D'Amato et al. 2007; Suphioglu 1998; Suphioglu et al. 1992). Moreover, grass PCGs induced high humoral and cellular responses in PCG-sensitized rodents (Chakra et al. 2011; Motta et al. 2004a, b). The influence of pollen lipids on induction or exacerbation phase of allergenic reaction has been deeply investigated, and the picture of PGs as a sole carrier of allergens is outdated (Aglas et al. 2018; Behrendt et al. 2001; Bublin et al. 2014; Dahl 2018; Gilles et al. 2009, 2012; Plötz et al. 2004; Traidl-Hoffmann et al. 2002, 2005). Despite the significant role of pollen lipids on allergic immune response, little is known about internal lipidomic composition of allergenic PGs; lipidomic compounds that could be released

consecutively upon expulsion of cytoplasmic content of PGs.

Pollen lipids could be external lipids located on the extracellular pollen wall and internal lipids present in pollen cytoplasm. The PG walls and plasma membrane act as a barrier to the extraction of cytoplasmic lipids, and pollen rupturing is therefore required in order to release internal lipids (Evans et al. 1990, 1991; Piffanelli et al. 1997). In earlier studies, lipid analyses were performed on intact allergenic PGs, extracted with organic solvent and the following chemical families identified: saturated and unsaturated fatty acids, alkanes, alkenes, alcohols, aldehydes, ethers, amino alcohols, phenols, sterols and terpenes (Abed El Azim et al. 2015; Bashir et al. 2013a, b; Farah et al. 2019; González Roldán et al. 2019; Henricsson et al. 1996; Naas et al. 2016; Nicolson and Human 2012; Zhu et al. 2018). Unsaturated acids were also identified and quantified in allergenic pollen by aqueous extraction but the state (ruptured or not) of PGs was not studied in these works (Plötz et al. 2004; Traidl-Hoffmann et al. 2002). Identification of internal lipids has been only conducted on nonallergenic pollen of *Brassica napus*; unsaturated fatty acids including hexadecatrienoic acid (C16:3), α -linolenic acid (C18:3) and linoleic acid (C18:2) were predominantly extracted from internal lipids after pollen rupturing (Evans et al. 1990, 1991; Piffanelli et al. 1997, 1998).

Whether the lipids are present on the external surface or in the cytoplasm of the PGs could change their bioavailability, and it therefore questions the state (intact, cracked or broken) of PGs being actually inhaled. Mechanisms of rupture of PGs were thoroughly investigated as well as the role of lipids in allergic responses; however, each aspect was explored independently and the influence of PG rupture in the lipid release is a missing piece. It is therefore important to take into account the lipid distribution between external and internal parts of PGs. As example, aqueous pollen extracts (APEs) are research standards of interest as regards the health effects of lipids (Plötz et al. 2004; Traidl-Hoffmann et al. 2002). APEs are prepared by incubating PGs in aqueous medium (Roswell Park Memorial Institute medium or Phosphate Buffered Saline) generally at temperature close to 37 °C for 30 min followed by centrifugation and filtration (Plötz et al. 2004; Traidl-Hoffmann et al. 2002). The final state of PGs after APE preparation is

not checked so that influence of PGs rupture in the release of lipids cannot be evaluated. In other words, are APEs representative of intact or of broken PGs extraction? Large discrepancies observed between studies for mass concentrations of octadecenoic, linoleic and α -linolenic acids could be explained by rupture of PGs affected by pollen freshness, solvent use and steps of extraction procedure like agitation or centrifugation (Farah et al. 2019; Plötz et al. 2004; Traidl-Hoffmann et al. 2002). In this work, we have extracted *Phleum pratense* PGs with organic and aqueous solvent under different extraction procedures and under different states for PGs (intact or broken). We aimed to unravel the distribution of lipids between external and internal parts of *Phleum pratense* PGs and to establish the importance of pollen rupture for laboratory extractions of lipids of pollen.

2 Materials and methods

2.1 Pollen sources

Phleum pratense (Timothy grass) belonging to the family of grass was chosen as a model for grass allergenic pollen. Batches of *Phleum pratense* pollen were purchased nondefatted (collected in Bohemia Czech Republic in 2007) and were stored hermetically in glass vials at 4 °C until use. Pollen grains (PGs) were processed for observation by scanning electron microscopy (SEM) to ensure their purity and to check that PGs were nonbroken (intact) prior to laboratory use.

2.2 Chemicals

The following chemical compounds were used: methylene chloride and methanol $\geq 99.8\%$ (Sigma-Aldrich); BSTFA-TMCS 99:1 (*N,O*-bis(trimethylsilyl) trifluoroacetamide, trimethylchlorosilane) (Sigma-Aldrich); linear alkanes mix from C8 to C40 carbon atoms (Sigma-Aldrich); linoleic acid (cis-9, cis-12-octadecadienoic acid, Sigma-Aldrich); α -linolenic acid (Z,Z,Z-9,12,15-octadecatrienoic acid, Fisher Scientific); and phosphate-buffered saline (PBS) (Thermo Fisher Scientific).

2.3 Extraction of lipids with organic solvent

External lipids were extracted from intact PGs with organic solvent following the lipidomic extraction procedure previously described (Farah et al. 2019). In brief, 50 mg of pollen was inserted upon cotton wool in a Pasteur Pipette and soaked with methylene chloride corresponding to a total volume of 5 mL. Organic extract was then dried under a stream of nitrogen. Finally, the residue was reconstituted and derivatized by adding 50 μ L of BSTFA/TMCS and 50 μ L of methylene chloride.

In order to release internal lipids, PGs were crushed by mechanical agitation with a magnetic stirrer for 4 h (Evans et al. 1990, 1991). Lipids were then extracted with the same protocol as for external lipids. After quantification, internal lipids were obtained by the difference between the total lipids and the external lipids extracted, respectively, from broken and intact PGs.

In order to estimate the total mass of external and internal extractable lipids, 1 g of intact or broken PGs was extracted with 5 mL of methylene chloride. The residue obtained after solvent evaporation was then weighed on a precision scale (± 0.1 mg).

2.4 Extraction of lipids with aqueous solvent

Experimental conditions for the PGs extraction are summarized in Fig. 1. PGs were extracted by phosphate-buffered saline (PBS) in three different experimental conditions of suspension in PBS (Fig. 1): (a) immersed in the solvent for 22 h without stirring; (b) with a gentle stirring by slow rotation of extraction tubes for 2 h; and (c) magnetic stirring at a speed of 1000 rpm for 2 h. PGs were also crushed by mechanical agitation (magnetic stirrer) for 4 h and then extracted for a duration of 2 h by suspension into PBS without stirring. Fifty milligrams of PGs were extracted in 2 mL of PBS for each of the three experimental conditions. Lipids extracted by PBS were then transferred to organic solvent as follows: centrifugation at 1800 g for 2 min after PBS extraction, filtration of the supernatant, addition of 2 mL of methylene chloride, hand-shake for 2 min, centrifugation at 1800 g for 2 min, recovery and filtration of organic phase (repeated three times) followed by evaporation to dryness of solvent. The dry residue is

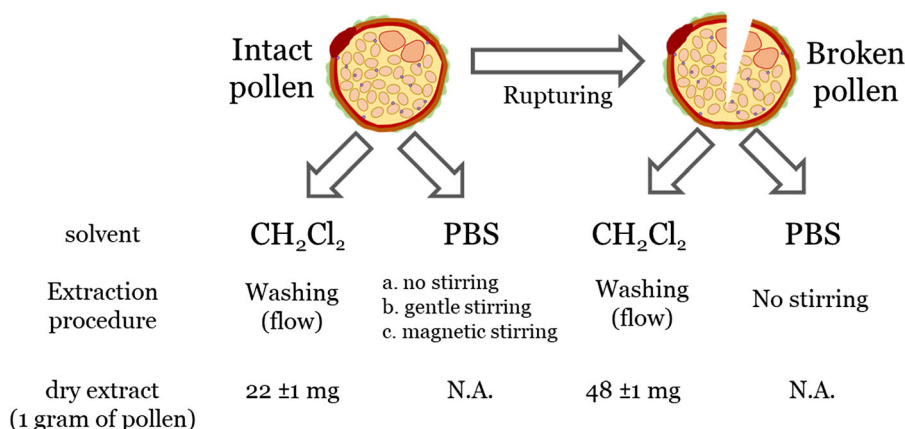


Fig. 1 Experimental conditions of *Phleum pratense* pollen grains extraction (PBS phosphate buffer solution, CH_2Cl_2 methylene chloride, N.A. not available). Lipids extraction by PBS was carried out under different conditions: **a** immersed in the solvent for 22 h without agitation, **b** with gentle agitation by

slow rotation of extraction tube for 2 h and **c** with mechanical agitation (magnetic stirring) for 2 h. The mass of dry extract corresponds to the final mass extracted on 1 g of pollen by CH_2Cl_2 after evaporation of the organic solvent

finally solubilized in 50 μL of methylene chloride and 50 μL of BSTFA-TMCS.

2.5 Gas chromatography analysis

Analyses were carried out with a gas chromatograph coupled to mass spectrometer detector (GC/MS) 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 mL min^{-1} , split ratio of 1:10, injector temperature of 250 $^{\circ}\text{C}$ and the oven temperature fixed initially at 50 $^{\circ}\text{C}$ and then raised to 4 $^{\circ}\text{C min}^{-1}$ to reach 310 $^{\circ}\text{C}$ for 19 min. The MS ionization was done by electronic impact of 70 eV. Lipid molecular species were quantified using carbon equivalent number as previously described (Scanlon and Willis 1985; Zhu et al. 2018) except for unsaturated fatty acids (linoleic acid and α -linolenic acid) which were directly quantified by 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 statistically 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.

2.6 Scanning electron microscopy imaging

Phleum pratense PGs (intact and broken) were spread over a conductive carbon tape and gold-coated for scanning electron microscopy (SEM) imaging using a Hitachi FlexSEM-1000 microscope operated at 5 kV under high vacuum.

3 Results

3.1 Extraction with methylene chloride

Pollen grains were crushed in laboratory by using a magnetic stirrer for 4 h. The rupture of pollen was checked by SEM imaging (Fig. 2). After magnetic stirring, most of the PGs observed were broken (> 99%) proving the efficiency of this method for laboratory pollen rupture. The total mass of residue extracted with methylene chloride from 1 g of *Phleum pratense* PGs was determined to 22 ± 1 mg for pollen without treatment (intact) and to 48 ± 1 mg for broken (crushed) pollen. Molecules identified in this residue by GC/MS on an HP5 column were classified into seven chemical families: alkanes, alkenes, aldehydes, alcohols, saturated and unsaturated fatty acids and sterols. Our MS identifications of lipids for *Phleum pratense* were compared to the ones in the literature in a previously published paper (Farah et al. 2019).

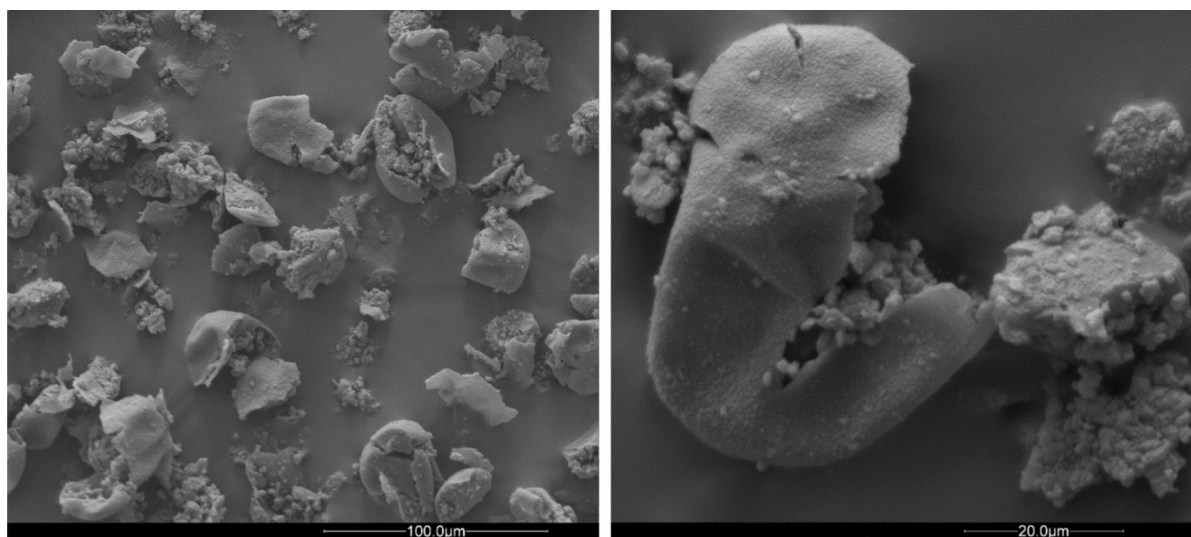


Fig. 2 SEM images of *Phleum pratense* pollen after mechanical crushing

The compounds extracted with methylene chloride were identical between intact and broken pollen, and only the mass of extracted quantities was different (Table 1). The mass distribution of alkanes (intact: $1828 \pm 105 \text{ ng mg}^{-1}$; broken $1897 \pm 136 \text{ ng mg}^{-1}$), alkenes (intact: $1445 \pm 118 \text{ ng mg}^{-1}$; broken: $1605 \pm 245 \text{ ng mg}^{-1}$); and aldehydes (intact: $33 \pm 6 \text{ ng mg}^{-1}$; broken: $33 \pm 6 \text{ ng mg}^{-1}$) was in the same range for intact and broken pollen. A small difference in mass distribution of alcohols was observed (intact: $26 \pm 3 \text{ ng mg}^{-1}$; broken: $60 \pm 7 \text{ ng mg}^{-1}$), whereas large differences were obtained for saturated fatty acids (intact: $434 \pm 33 \text{ ng mg}^{-1}$; broken: $1398 \pm 194 \text{ ng mg}^{-1}$) and for unsaturated fatty acids (intact $54 \pm 5 \text{ ng mg}^{-1}$; broken: $1243 \pm 287 \text{ ng mg}^{-1}$). In particular, the amount of α -linolenic acid was more than 20 times superior in broken PGs, making it the most abundant extracted compound from broken PGs ($1191 \pm 274 \text{ ng mg}^{-1}$).

The sum of quantified compounds (Table 1) for intact and broken PGs was, respectively, of 3820 ± 270 and $6236 \pm 875 \text{ ng mg}^{-1}$; those masses account for 18% of total extractable mass for intact pollen ($22 \mu\text{g mg}^{-1}$) and for 13% for broken pollen ($48 \mu\text{g mg}^{-1}$). Indeed, only lipids eluted on a gas chromatograph equipped with an HP5 column and identifiable by mass spectrometry were analyzed in this work.

3.2 Extraction with phosphate buffer solution

The experimental conditions of contact between PGs and phosphate buffer solution (PBS) changed drastically the amount of extracted lipids (Fig. 3). When magnetic stirring of the PBS–pollen mixture was carried out, the lipid extraction efficiency (Fig. 3c) was higher than conditions with gentle agitation (Fig. 3b) or without agitation (Fig. 3a). PGs were observed ruptured in PBS solution after 2 h of magnetic stirring, while they were intact using other conditions of extraction (Fig. 4).

The influence of rupture of PGs on the efficiency of extraction by PBS was confirmed by extracting PGs that were beforehand ruptured in laboratory by mechanical crushing. The efficiency of the unsaturated acids extraction procedure with crushed PGs was indeed similar to the one of PBS extraction with magnetic stirring (Table 2).

The compounds extracted by PBS with magnetic stirring were similar to the one extracted with methylene chloride but differed in quantity (Table 3). In particular, aldehydes were not extracted with PBS. No new compound was identified by changing the extraction solvent. The total mass of extracted compounds with PBS was smaller ($906 \pm 229 \text{ ng mg}^{-1}$) than with organic solvent for intact ($3819 \pm 270 \text{ ng mg}^{-1}$) or broken PGs ($6237 \pm 875 \text{ ng mg}^{-1}$) (Tables 1, 3). The mass of unsaturated acids extracted by PBS with magnetic agitation ($406 \pm 120 \text{ ng mg}^{-1}$)

Table 1 Compounds identified and quantified in lipids extractable with methylene chloride on *Phleum pratense* pollen grains without treatment (intact) or broken (crushed in laboratory)

	Intact pollen	Broken pollen
Alkanes (ng mg⁻¹)		
Heptadecane	5 ± 1	3 ± 1
Nonadecane	3 ± 1	3 ± 1
Heneicosane	9 ± 14	4 ± 1
Docosane	5 ± 1	4 ± 1
Tricosane	78 ± 9	74 ± 8
Tetracosane	19 ± 3	55 ± 6
Pentacosane	338 ± 21	339 ± 7
Hexacosane	38 ± 4	48 ± 7
Heptacosane	656 ± 3	673 ± 54
Octacosane	28 ± 3	30 ± 4
Nonacosane	317 ± 21	339 ± 32
triacontane	21 ± 2	17 ± 4
Hentriacontane	311 ± 22	309 ± 10
Sum	1828 ± 105	1897 ± 136
Alkenes (ng mg⁻¹)		
Decene	5 ± 1	4 ± 1
Pentacosene	41 ± 6	46 ± 5
Heptacosene	464 ± 47	526 ± 81
Octacosene	58 ± 8	53 ± 13
Nonacosene	297 ± 18	351 ± 65
triacontene	39 ± 7	40 ± 8
Hentriacontene	541 ± 31	585 ± 72
Sum	1445 ± 118	1605 ± 245
Aldehydes (ng mg⁻¹)		
Nonanal	1 ± 1	1 ± 1
Octadecanal	3 ± 1	3 ± 1
Eicosanal	5 ± 1	5 ± 1
Heneicosanal	5 ± 1	5 ± 1
Docosanal	10 ± 1	10 ± 1
Tetracosanal	9 ± 1	9 ± 1
Sum	33 ± 6	33 ± 6
Alcohols (ng mg⁻¹)		
Hexadecanol	12 ± 1	26 ± 3
Octadecanol	6 ± 1	24 ± 3
Docosanol	8 ± 1	10 ± 1
Sum	26 ± 3	60 ± 7
Saturated fatty acids (ng mg⁻¹)		
Pentanoic acid	1 ± 0	2 ± 1
Hexanoic acid	6 ± 1	11 ± 2
Heptanoic acid	3 ± 0	4 ± 1
Octanoic acid	3 ± 0	12 ± 2

Table 1 continued

	Intact pollen	Broken pollen
Nonanoic acid	4 ± 0	6 ± 1
Decanoic acid	1 ± 0	1 ± 1
Undecanoic acid	2 ± 0	2 ± 1
Dodecanoic acid	2 ± 0	5 ± 1
Tetradecanoic acid	10 ± 0	35 ± 11
Pentadecanoic acid	1 ± 0	3 ± 1
Hexadecanoic acid	160 ± 13	686 ± 85
Heptadecanoic acid	3 ± 0	9 ± 1
Octadecanoic acid	159 ± 16	366 ± 52
Nonadecanoic acid	5 ± 1	11 ± 1
Eicosanoic acid	41 ± 0	82 ± 6
Heneicosanoic acid	8 ± 0	8 ± 0
Docosanoic acid	16 ± 1	149 ± 25
Tetracosanoic acid	5 ± 0	3 ± 1
Octacosanoic acid	4 ± 1	3 ± 1
Sum	434 ± 33	1398 ± 194
Unsaturated fatty acids (ng mg⁻¹)		
Linoleic acid	3 ± 1	52 ± 13
α-Linolenic acid	51 ± 4	1191 ± 274
Sum	54 ± 5	1243 ± 287

Masses were given as equivalent alkane with the carbon equivalent number methodology except for unsaturated acids that were quantified with standard solutions

or from broken pollen (476 ± 114 ng mg⁻¹) was also smaller than the mass extracted with methylene chloride for broken pollen (1243 ± 287 ng mg⁻¹).

4 Discussion

The extraction of lipids from *Phleum pratense* pollen grains (PGs) with phosphate buffer solution (PBS) was proven ineffective when carried out in the absence of mechanical agitation, even if the contact time of PGs with PBS was particularly long (22 h). Migration of pro-inflammatory substances from *Phleum pratense* PGs was reported to occur in less than 10 min when intact PGs were incubated with PBS; the concentration of eicosanoids LTB₄ reached about 0.04 ng mg⁻¹ (Behrendt et al. 2001). Those concentrations are below GC/MS detection limits and cannot therefore be

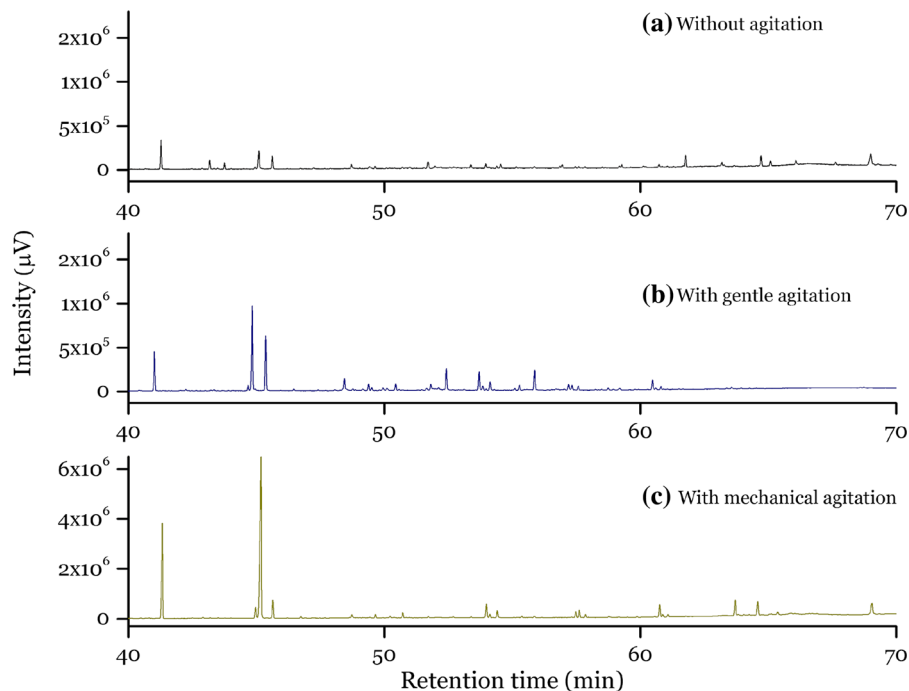


Fig. 3 Chromatograms of lipids extracted from *Phleum pratense* pollen by PBS under different conditions: **a** without agitation, immersed in solvent for 22 h, **b** with gentle agitation

by slow rotation of extraction tube for 2 h and **c** with mechanical agitation (magnetic stirring) for 2 h

detected in this work. When a PG reaches a humid environment, a very small proportion of lipids may diffuse, but the majority of lipids remains inaccessible. On the contrary, lipids extraction was possible in large quantities ($906 \pm 229 \text{ ng mg}^{-1}$) when PGs were crushed prior to PBS extraction.

Only external lipids of allergenic PGs have been identified so far in the literature (Bashir et al. 2013a; Farah et al. 2019; Henricsson et al. 1996; Zhu et al. 2018). Indeed, PGs extractions for lipidomic analysis were carried out with organic solvents known to extract mainly surface lipids (Evans et al. 1991). In this work, we showed that the quantities extracted from crushed pollen (when the internal lipids are easily accessible by a solvent) are twice more than those obtained from lipid extraction of intact pollen (48 mg g^{-1} compared to 22 mg g^{-1}). In other words, pollen lipids are distributed in relatively close proportions between the external parts of the pollen (22 mg g^{-1}) and the cytoplasm (26 mg g^{-1}). Aqueous pollen extracts (APEs) were commonly used in the literature to reveal the bioactive properties of pollen lipids (Bashir et al. 2013a; Plötz et al. 2004; Traidl-Hoffmann et al. 2002). However, the state of

pollen (intact or broken) at the end of the APE preparation procedure is not provided in the literature and it is therefore impossible to conclude whether lipids were extracted only from outer part of PGs or from both outer and inner parts. This was reflected by the discrepancies in extraction of unsaturated fatty acids between studies (Table 4). We showed that methylene chloride extracted only 51 ng mg^{-1} of α -linolenic acid and that higher extraction efficiency (1191 ng mg^{-1}) required crushing of PGs. We can hypothesize that high extraction efficiencies of α -linolenic acid ($1058\text{--}5012 \text{ ng mg}^{-1}$) found in the literature are the result of cytoplasmic expulsion (Plötz et al. 2004; Traidl-Hoffmann et al. 2002). During APE preparation, PGs rupture may indeed occur in water or during centrifugation. Similarly, pollen coat may have a cloud condensation nuclei activity and extraction of pollen coating is a critical step for the purpose of laboratory hygroscopicity experiments (Mikhailov et al. 2019). As for APE, PGs are frequently centrifuged for the laboratory preparation of pollen extracts and therefore the integrity of PGs (intact or ruptured) must be systematically checked in order to

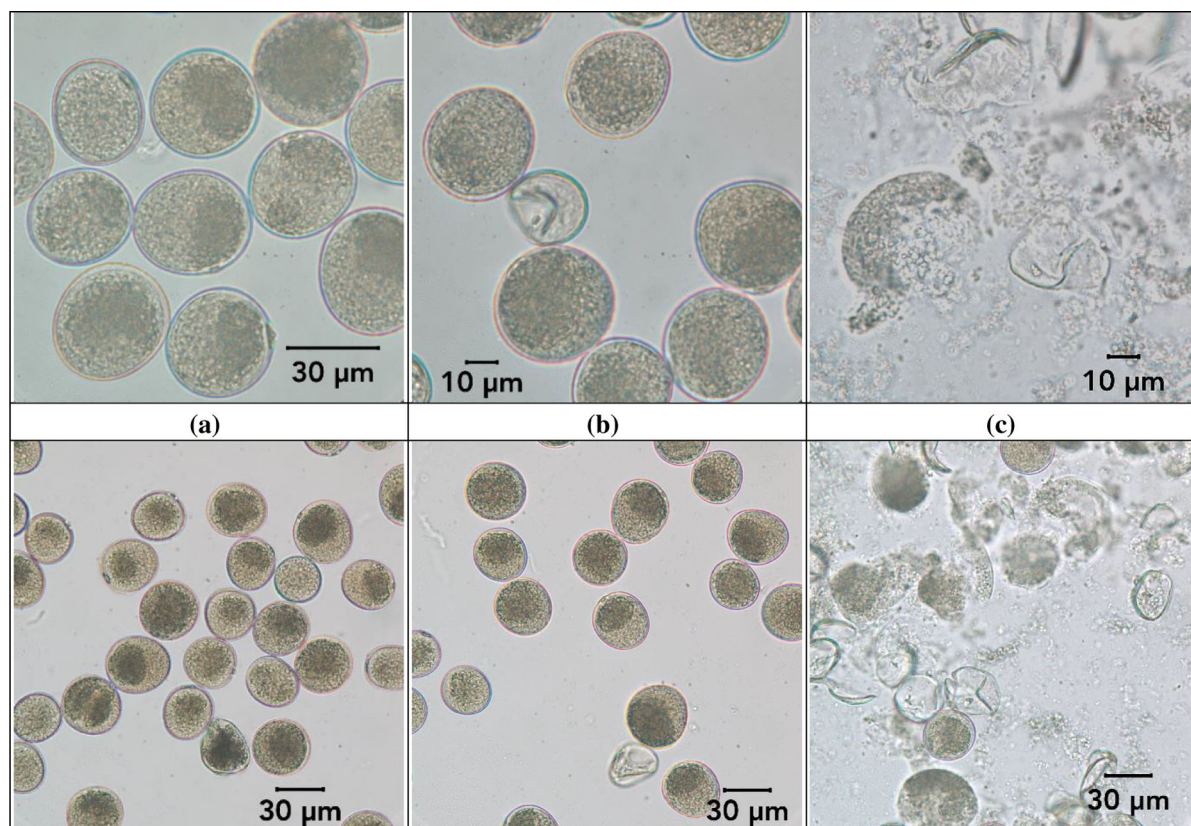


Fig. 4 *Phleum pratense* pollen grains observed in phosphate buffer solution: **a** without agitation, immersed in solvent for 22 h; **b** with gentle agitation by slow rotation of extraction tube for 2 h; **c** solution after 2 h of magnetic stirring

Table 2 Mass fraction of unsaturated linoleic and α -linolenic fatty acids extracted with phosphate buffer solution from *Phleum pratense* pollen grains under different extraction conditions: (a) without agitation, (b) gentle agitation,

(c) mechanical agitation by magnetic stirring and (d) pollen grains were first crushed with magnetic stirring (without solvent) and then extracted with PBS without agitation

ng mg ⁻¹	Without agitation (a)	Gentle agitation (b)	Magnetic agitation (c)	Without agitation but with broken pollen grains (d)
Linoleic acid	1 ± 1	3 ± 2	21 ± 7	23 ± 10
α -Linolenic acid	9 ± 5	50 ± 7	385 ± 113	453 ± 104

guarantee a surface extraction of pollen coat only (Prisle et al. 2019).

PGs rupture in the atmosphere is possible through direct contact with water (Solomon 2002), through exposition to high humidity (Taylor et al. 2004) and in the case of mechanical stress (Visez et al. 2015). Moreover, air pollutants can trigger the release of PCGs from *Phleum pratense* PGs in humid conditions

(Motta et al. 2006). PGs rupture has been extensively studied for the release of cytoplasmic granules and the distribution of allergens in the fine (respirable) fraction of the atmospheric aerosol. Although the nature of lipids is identical between external lipids and cytoplasmic lipids, a much higher proportion of saturated and unsaturated fatty acids are present in the cytoplasm. Considering on the one hand the more efficient

Table 3 Compounds identified and quantified in lipids extractable with PBS by magnetic stirring (2 h) on *Phleum pratense* pollen grains

Alkenes (ng mg ⁻¹)	
Pentacosene	4 ± 1
Heptacosene	39 ± 9
Octacosene	4 ± 1
Nonacosene	19 ± 5
Hentriacontene	39 ± 9
Sum	105 ± 25
Alkanes (ng mg ⁻¹)	
Tricosane	1 ± 1
Pentacosane	13 ± 3
Heptacosane	18 ± 5
Nonacosane	7 ± 2
Hentriacontane	8 ± 2
Sum	47 ± 13
Alcohols (ng mg ⁻¹)	
Dodecanol	2 ± 1
Hexadecanol	3 ± 1
Octadecanol	1 ± 1
Sum	6 ± 3
Fatty acids (ng mg ⁻¹)	
Heptanoic acid	1 ± 1
Octanoic acid	3 ± 1
Nonanoic acid	3 ± 1
Decanoic acid	1 ± 1
Dodecanoic acid	1 ± 1
Tetradecanoic acid	10 ± 2
Pentadecanoic acid	2 ± 1
Hexadecanoic acid	258 ± 50
Heptadecanoic acid	1 ± 1
Octadecanoic acid	51 ± 5
Nonadecanoic acid	1 ± 1
Eicosanoic acid	8 ± 2
Docosanoic acid	2 ± 1
Sum	342 ± 68
Unsaturated fatty acids (ng mg ⁻¹)	
Linoleic acid	21 ± 7
α-Linolenic acid	385 ± 113
Sum	406 ± 120

Masses were given as equivalent alkane with the carbon equivalent number methodology except for unsaturated acids directly quantified with standard solutions

extraction of lipids from broken pollen by an aqueous solvent and on the other hand the predominance of

linoleic and α-linolenic acids on the cytoplasmic content, it can be hypothesized that inhalation of broken (or cracked) PGs instead of intact PGs can have different health consequences due to their lipidic fractions. The presence of bioactive lipids within the cytoplasm is another parameter, yet not studied, that may explain the higher prevalence of allergy in cities since exposure to ruptured PGs should be higher due to increased fragility caused by air pollution.

α-Linolenic and linoleic acids were found predominantly in the cytoplasm of *Phleum pratense* PGs. These polyunsaturated acids are recognized as biological regulators and able to modulate the immune response with a generally beneficial effect in a variety of inflammatory disorders (Stulnig 2003). Linoleic acid and its isomers have the ability to modulate inflammation and immunity (Viladomiu et al. 2016; Yang et al. 2015). Medical supplementation of conjugated linoleic acids to birch-allergic patients before and during the birch pollen season induces improvements in symptoms (Turpeinen et al. 2008). Several acids found predominant in cytoplasm are regulators of cytokine release in peripheral lymphocytes (Karsten et al. 1994). The abundant presence of saturated and unsaturated fatty acids in the pollen cytoplasm calls for future research effort in order to fully understand their health-related implications.

The composition of lipidic fraction of allergenic PGs is in fact largely unknown as well as the repartition between external and cytoplasmic fractions. Quantitative lipidomic studies are indeed rare, and lipidomic analysis of lipids of allergenic PGs was performed on intact (nonbroken) PGs with organic solvent (Bashir et al. 2013a; Farah et al. 2019; Henriksen et al. 1996; Zhu et al. 2018). Those lipidomic studies performed with organic solvent were attempts for a better description of pollen (bio)chemical composition and not studies on the bioavailability of lipids. Moreover, while extensive works were carried out on the internal lipids for *Brassica napus* (Evans et al. 1990, 1991; Piffanelli et al. 1997, 1998), less is known about allergenic pollen internal chemical fraction. This work is the first attempt to quantify the internal lipid fraction for an allergenic pollen, but only 18% of the external lipid fraction and 13% of the internal lipid fraction were identified and quantified. Bioavailability of these lipids when PGs or PCGs are in contact with pulmonary epithelium needs to be quantified in future studies. Lipids could have

Table 4 Comparison of saturated (hexadecanoic and octadecanoic), monounsaturated (hexadecenoic and octadecenoic) and polyunsaturated fatty acids from different works (Plötzet al. 2004; Traidl-Hoffmann et al. 2002) and for different conditions of extraction of *Phleum pratense* pollen grains

Unsaturated fatty acids (ng mg ⁻¹)	This work external fraction	This work ext + internal fraction	This work PBS extract	Traidl-Hoffmann et al. (2002)	Plötz et al. (2004)
Extraction solvent	Methylene chloride	Methylene chloride	PBS	RPMI	RPMI
Method and duration of extraction	Washing 5 min	Grinding and washing 5 min	Magnetic stirring (120 min)	Centrifugation (500 g) 30 min	Centrifugation (500 g) 30 min
Hexadecanoic acid	160	686	258	–	1243
Octadecanoic acid	159	366	51	–	1207
Octadecenoic acid	–	–	–	–	233
Linoleic acid	3	52	21	230	813
Linolenic acid	51	1191	385	1058	5012
Sum	373	2295	715	1288	8508

PBS phosphate buffer solution, RPMI Roswell Park Memorial Institute medium

bioactivity properties only if their bioavailability proves to be efficient, both in terms of extractible quantities but also in kinetic of release before PGs clearance.

More generally, the biochemical composition of PG remains unknown for most allergenic pollen species. In addition, many pollutants can modify this chemical composition: pesticides, polycyclic aromatic hydrocarbons, reactive oxygen species, ... (Hierro 2016; Sénéchal et al. 2015). In the context of a likely increase in the exposure of the general population to PGs due to the increase in atmospheric carbon dioxide concentration (Albertine et al. 2014), there is a growing importance to better understand the nature, bioavailability and bioactivity of compounds released concomitantly with pollen allergens.

5 Conclusion

In this work, extraction of lipids from the allergenic pollen of *Phleum pratense* by an organic solvent (methylene chloride) was compared against lipid extraction by an aqueous solvent (PBS: phosphate buffer solution). It was logically found that methylene chloride is a more effective solvent than PBS for lipid extraction. The aqueous solvent only allows lipid extraction when a significant stirring was applied to the solution, thus questioning the bioavailability of

lipids in an aqueous medium. It is necessary to better understand the bioavailability of allergenic pollen lipids, in particular by studying the extraction of lipids by solvents with physicochemical properties as close as possible to physiological fluids.

The comparison of lipid extraction was also carried out for intact and broken pollen in order to unravel the distribution of lipids between the external and cytoplasmic parts of *Phleum pratense* PGs. The major finding of this work is the enhanced extraction of unsaturated fatty acids when PGs are broken. Indeed, unsaturated fatty acids were only extracted by PBS when PGs suffered rupture. Rupture of pollen has been extensively studied for its importance in the release of allergens in the respirable fraction of the aerosols and explanation of pollen-related asthma attacks. Pollen allergens were indeed mainly stored in the cytoplasm and were detected on the surface of PCGs. The rupturing of PGs in the atmosphere exposes the population to a micrometer-sized aerosol (cytoplasmic granules) containing allergens and bioactive lipids. This work questions further how important the rupture of grass pollen grains in the atmosphere contributes to allergy and asthma.

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