



Extractable lipids from *Phleum pratense* pollen grains and their modifications by ozone exposure

Jinane Farah · Marie Choël · Patricia de Nadaï · Sylvie Gosselin ·
Denis Petitprez · Moomem Baroudi · Nicolas Visez 

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Abstract Grass pollen grains are an important source of aeroallergens eliciting respiratory allergic diseases worldwide. In the field of allergology, *Phleum pratense* pollen is considered as a model for other grass taxa. Upon contact with the aqueous phase of mucosa membranes, lipids are co-delivered from pollen grains with allergens. Lipid molecular species have pro-allergic, pro-inflammatory and immunomodulatory effects on the cells of the allergic immune response. The quantitative analysis of *Phleum*

pratense pollen lipids is missing in the literature. In this work, the total mass of lipids extractable by methylene chloride was determined to be $22 \pm 1 \mu\text{g}$ per mg of *Phleum pratense* pollen. Eighteen percent of the lipidic mass was quantified and identified by gas chromatographic analysis. Identified lipids included alkanes, alkenes, saturated and unsaturated fatty acids, aldehydes and alcohols. Pollen samples harvested at different times and locations showed a very similar lipidic pattern, both qualitatively and quantitatively. Laboratory ozone exposure of pollen substantially modified its lipidic composition by reactions of ozone with alkenes leading to the production of fatty acids and aldehydes. A deeper knowledge of the lipids released by pollen grains, including pollen from polluted areas, is essential for a better understanding of the chemical environment at the cellular level where allergic reactions take place.

Keywords Pollen · Allergy · Lipidomics · Timothy grass · Air pollution

J. Farah · S. Gosselin · D. Petitprez · N. Visez (✉)
Univ. Lille, CNRS, UMR 8522 - PC2A - Physicochimie
des Processus de Combustion et de l'Atmosphère,
59000 Lille, France
e-mail: nicolas.visez@univ-lille.fr

J. Farah · M. Baroudi
Faculty of Public Health (FSP III), Water & Environment
Science Laboratory, Lebanese University, Tripoli,
Lebanon

M. Choël
Univ. Lille, CNRS, UMR 8516 - LASIR - Laboratoire de
Spectrochimie Infrarouge et Raman, 59000 Lille, France

P. de Nadaï
Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de
Lille, U1019 - UMR 8204 - CIIL - Center for Infection
and Immunity of Lille, 59000 Lille, France

N. Visez
Association Pour La Prévention de La Pollution
Atmosphérique, Parc Eurasanté, 235 avenue de la
Recherche, 59120 Loos, France

1 Introduction

Allergenic pollen grains from grass (Poaceae/Gramineae) family are considered as the prevalent airborne biological pollutants and one of the major causes of pollen allergy worldwide (García-Mozo 2017). Due to

the similarity and cross-reactivity of *Phleum pratense* (timothy grass) pollen allergens with other grasses species, *Phleum pratense* extracts are used as a model in skin prick test for the diagnosis of allergy to grass pollen (Andersson and Lidholm 2003; White and Bernstein 2003). The total amount of proteins in *Phleum pratense* pollen extracts was determined between 10 and 60 µg per mg of pollen (Vrtala et al. 1993; Behrendt et al. 1999; Rogerieux et al. 2007). Among those proteins, the concentration of one of the main allergens Phl p 5 is in the range of micrograms per milligram of pollen (Behrendt et al. 1999; Albertine et al. 2014; Buters 2015; Jochner et al. 2015). For the last two decades, researches on the allergenicity of pollen were extended to the analysis of adjuvant mediators (including pollen-associated lipid mediators, PALMs) released from pollen with allergens upon hydration (Smith et al. 1992; Traidl-Hoffmann et al. 2002; Gilles et al. 2009b). Lipid molecular species have pro-allergic, pro-inflammatory and immunomodulatory effects on the cells of the allergic immune response (Dahl 2018). As an example, Blume et al. (2015) found that lipid mediators released from *Phleum pratense* pollen cause alteration in bronchial epithelial barrier functions and induce polarized release of mediators GM-CSF, CCL20, IL-8 and TNF- α . Signaling molecules secreted by dendritic cells (DCs) and natural killer T (NKT) cells are also up-regulated by *Phleum pratense* pollen lipid extracts (Bashir et al. 2013a). This regulation induced a possible stimulation of the development of allergic and inflammatory immune responses (Bashir et al. 2013a). Moreover, lipid mediators of grass pollen act as chemoattractants and activators of neutrophils and eosinophils (Traidl-Hoffmann et al. 2002; Plötz et al. 2004). Apart from their effects on polymorphonuclear granulocytes, pollen lipids also favor a Th2-dominating response of the immune system (Gilles et al. 2009a, b). A more recent study shows that PALMs induce a maturation pathway that prepares DCs for the recognition of glycolipids co-delivered with PALMs to mucosal surfaces (González Roldán et al. 2019). Furthermore, lipids are located, at least in part, on the external layer of pollen grains (Piffanelli et al. 1997; Pacini and Hesse 2005; Murphy 2006; Quilichini et al. 2015), giving lipids the chance of being early recruiters of inflammatory mediators (Traidl-Hoffmann et al. 2002; Bashir et al. 2013a), but also to be exposed to air pollutants. *Phleum pratense* pollen

grains have been found to release higher amount of pro-inflammatory eicosanoid-like substances when collected near roads with heavy traffic (Behrendt et al. 2001a). The modification of the composition of the lipidic fraction by ozone has been shown to occur through different chemical reaction pathways according to pollen types. In *Pinus halepensis* pollen, ozone reacts both with coumaric monomers of the sporopollenin and with the unsaturated monocarboxylic acids (Naas et al. 2016), while in *Betula pendula* pollen, ozone reacts with alkenes only (Zhu et al. 2018).

Despite the potential influence of pollen-derived lipids as adjuvants of the allergic reactions, studies on the identification and quantification of the lipidic fraction of allergenic pollen are scarce in the literature (Henricsson et al. 1996; Bashir et al. 2013a; Mueller et al. 2016; Naas et al. 2016; Zhu et al. 2018). The most extensive pollen lipidomic study to date covers 22 pollen samples from allergenic plants; however, only relative peak areas were given for 41 lipid molecular species identified in chloroform extract of *Phleum pratense* pollen grains (Bashir et al. 2013a). In this work, we provide a step toward a better understanding of the lipid fraction of *Phleum pratense* pollen grains. This study aims at a quantitative description of extractable lipids from the highly allergenic pollen of *Phleum pratense*. Lipids were extracted with organic solvent to ensure a highly efficient extraction so that the total mass fraction of lipids in pollen could be determined. Several commercial pollen samples were compared to ascertain that our results were not specific to a single batch only. Identification of lipids was carried out by gas chromatography coupled to mass spectrometry (GC–MS), and quantification was performed by gas chromatography equipped with a flame ionization detector (GC–FID). Finally, the influence of ozone pollution on the lipid fraction of *Phleum pratense* pollen was investigated by means of controlled laboratory exposures.

2 Materials and methods

Pollen grains were purchased from three commercial suppliers (Bonapol, Pharmallerga, Greer) and were harvested in different locations, respectively, Czech Republic (Bohemia) in 2018 and 2007, Czech Republic (Moravia) in 2016 and the USA (Minnesota) in 2014. Suppliers were specifically requested to provide

non-defatted batches of pollen. Samples were stored hermetically in glass vials at 4 °C until use. Samples of pollen grains collected in Czech Republic in 2007 (50 mg for each experiment) were also exposed to ozone inside a stainless-steel reactor for 16 h at a concentration of 100 ppb (flow 1.35 L.min⁻¹, laboratory temperature 20 ± 1 °C, dry conditions). Ozone exposure (time x concentration) was chosen relatively high in order to reveal more easily oxidation products by GC–MS analysis. Ozone was generated by an UV generator at a wavelength of 254 nm and measured by an ozone analyzer at the reactor outlet.

For extraction and chromatographic analysis, 50 mg of pollen grains was washed with 5 mL of methylene chloride (99.8%). A second successive extraction of pollen grains has demonstrated that only a trace amount of lipids was still extractable after the first washing of pollen grains. Organic extract was evaporated to dryness under a flow of pure nitrogen and finally solubilized in 50 µL of methylene chloride and 50 µL of a solution of 99% BSTFA/1% TMCS. Samples were injected into a gas chromatograph coupled to a mass spectrometer (scan mode 45–500 uma). 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 °C; the oven temperature was fixed initially at 50 °C for 1 min, and then it was raised by 4 °C/min to reach 310 °C (final temperature held for 19 min). Identifications of extracted lipids were performed by comparison of experimental spectra with NIST 2008 mass spectra library (Version 2.2.0) and/or based on the comparison of retention times of standards. Retention times were also compared within a chemical class for additional checking of compounds identifications. As an example, retention times of the straight chain fatty acids are given as a function of number of carbon atoms in Fig. 1.

Samples collected in Czech Republic in 2007 were also injected for the purpose of quantification into a gas chromatograph equipped with a flame ionization detector. Equivalent carbon numbers (ECNs) were used for quantification of the lipids. Response factors for alkanes (R_{alkanes}) were determined using flame ionization detection for linear alkanes from C₈ to C₄₀ in the range of 6–36 µg g⁻¹. Mass ratios of lipids were calculated with the following formula (Scanlon and Willis 1985):

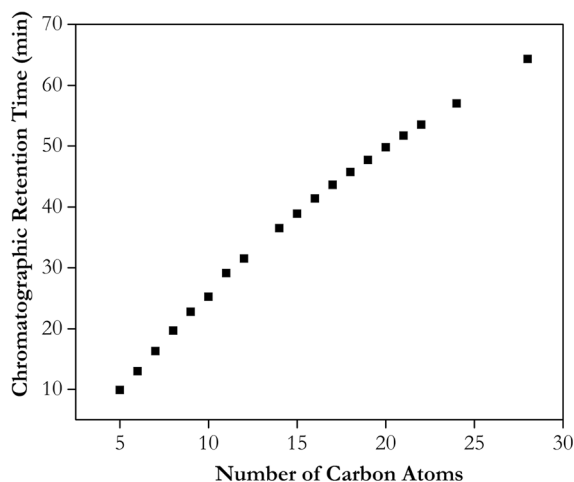


Fig. 1 Retention times (GC-FID) of saturated fatty acids as a function of the number of carbon atoms. The relationship between both values was used to check identifications of extracted lipids

$$Y(A_i) = \frac{ECN(alkane)}{ECN(A_i)} \cdot \frac{MW(A_i)}{MW(alkane)} \cdot R_{alkane} \cdot \frac{chromatographic\ area(A_i)}{mass\ of\ extracted\ pollen} \quad (1)$$

where $y(A_i)$ is the mass ratio (µg g⁻¹ of pollen) of specie A_i , $ECN(alkane)$ is the ECN for the linear alkane with the same carbon number, MW is the molecular weight, and R_{alkane} is the response factor determined for alkanes (µg per chromatographic area unit). Unsaturated acids were for their part directly calibrated by injection of standard solutions. Extraction of each pollen sample was performed three times and was separately analyzed by GC-FID. The error bars for mass concentrations were expressed as the maximum deviation of the three analyses around the mean value.

In order to compare the total mass of extractable lipids with mass concentrations calculated from chromatographic analysis, 1 g of pollen (Czech Republic, 2007) was extracted with 5 mL of methylene chloride and the final extraction residue was weighed (± 0.1 mg) after solvent evaporation to dryness.

Intact (without treatment) and defatted *Phleum pratense* pollen grains were examined by SEM to determine whether their physical integrity was altered during the pollen processing steps in laboratory. Commercial pollen grains from Czech Republic (2007) were spread over a conductive carbon tape

and gold-coated prior to SEM imaging using a FlexSEM-1000 microscope operated at 5 kV under high vacuum. About thirty pollen grains were individually observed.

3 Results

Intact pollen (without treatment) and defatted (solvent-washed) pollen were observed by SEM to assess any damages suffered by pollen grains during solvent extraction. No visible differences were detected between intact and defatted pollen (Fig. 2); the solvent extraction has no visible effect on the external structure of pollen grains.

Quantitative analysis was performed on pollen collected in Czech Republic (Bohemia) in 2007 for reasons of availability of samples at the time of analysis. However, samples of *Phleum pratense* pollen grains from different batches (different collection dates and harvesting locations) were compared to check sample-to-sample differences in lipid fraction. The sum of chromatographic areas (GC-FID) corresponding to the analysis of each sample (Fig. 3) was in the same range (chromatographic areas from 31,532 to 37,290 mV min, average $34,411 \pm 9\%$). Each pollen sample has a similar amount of lipids extracted by methylene chloride.

A total organic solvent-extractable mass of lipids of $22 \pm 1 \mu\text{g}$ per mg was determined by weighting for pollen of *Phleum pratense* collected in Czech Republic in 2007. Fifty lipids belonging to 6 chemical families have been identified (Fig. 4) and quantified in pollen grains of *Phleum pratense* (Czech Republic in 2007): n-alkanes and alkenes (Table 1), saturated and

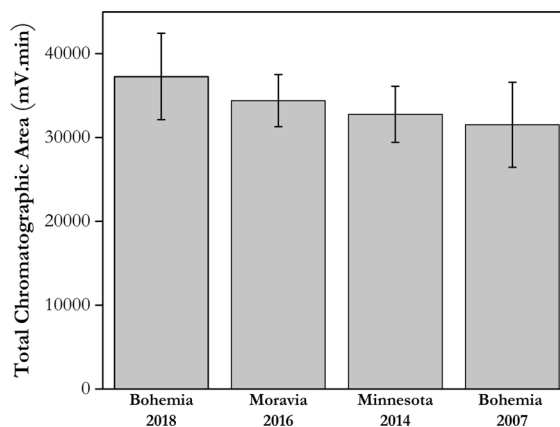


Fig. 3 Comparison between the sums of chromatographic areas of methylene chloride extracts of *Phleum pratense* pollen grains harvested at different dates and locations

unsaturated fatty acids (Table 2), aldehydes and alcohols (Table 3). The sum of masses of identified compounds per lipid class is as follows (as alkane equivalent masses): $1828 \pm 105 \text{ ng mg}^{-1}$ of alkanes (13 compounds), $1445 \pm 118 \text{ ng mg}^{-1}$ for alkenes (7 compounds), $434 \pm 46 \text{ ng mg}^{-1}$ for saturated fatty acids (19 compounds), $54 \pm 5 \text{ ng mg}^{-1}$ for unsaturated fatty acids (2 compounds), $33 \pm 6 \text{ ng mg}^{-1}$ for aldehydes (6 compounds) and $26 \pm 3 \text{ ng mg}^{-1}$ for alcohols (3 compounds). The total mass of lipids quantified by gas chromatography is $3.8 \pm 0.3 \mu\text{g mg}^{-1}$ of pollen (compared to a total extractable mass of $22 \pm 1 \mu\text{g mg}^{-1}$ of pollen).

The lipid fraction of *Phleum pratense* pollen grains includes an important fraction of alkanes with odd number of carbon atoms (C23 to C31 with C27 as the major alkane extracted: $656 \pm 3 \text{ ng mg}^{-1}$) and also an important fraction of alkenes with C27

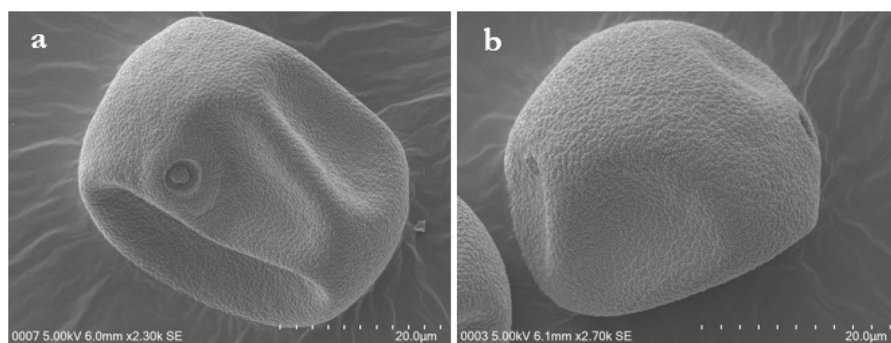


Fig. 2 SEM images of *Phleum pratense* pollen harvested in Bohemia, Czech Republic in 2007 (from Bonapol supplier). **a** Intact pollen grain without treatment and **b** pollen defatted with methylene chloride

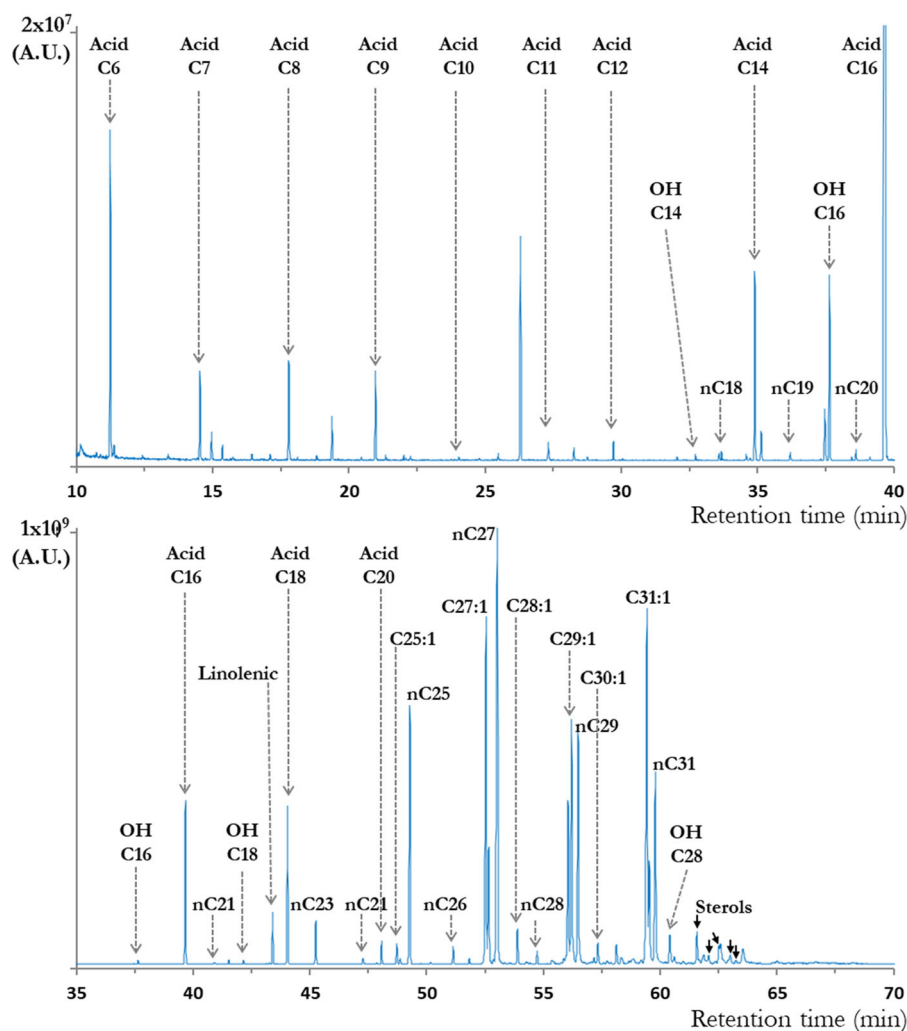


Fig. 4 Chromatogram of methylene chloride extract of *Phleum pratense* pollen grains (abbreviations: Acid saturated fatty acid, OH alcohol, CXX:1 alkene, nCXX alkane)

(heptacosene), C29 (nonacosene) and C31 (hentriacontene) as the most important alkenes. Monocarboxylic acids C16 (hexadecanoic acid) and C18 (octadecanoic acid) were extracted in high quantities (respectively, 160 ± 13 and 159 ± 16 ng mg⁻¹). Among fatty acids, saturated compounds were predominantly extracted (434 ± 33 ng mg⁻¹) as only 51 ± 4 ng mg⁻¹ of α -linolenic acid and 3 ± 1 ng mg⁻¹ of linoleic acid were extracted.

One study in the literature reports lipidomics of *Phleum pratense* pollen extracted with organic solvent (Bashir et al. 2013a), but only semiquantitative data were supplied in this study (relative chromatographic areas). Compounds that were identified in both studies

are mentioned in Tables 1, 2 and 3. Twenty compounds identified from our work were confirmed by Bashir et al. (2013a), representing about 70% of the mass identified in this work confirmed by literature data. Bashir et al. (2013a) have identified 18 compounds not confirmed in this work and belonging to the following families: alkane, unsaturated monocarboxylic acid, dicarboxylic acid, alcohol, terpene, sterol and others (benzoic acid, glucitol, arabinitol, phosphoric acid). Most of these compounds have very weak chromatographic areas, and it is one likely reason explaining the failure to confirm identifications in this work. Among these compounds, four have important chromatographic areas: butenedioic acid,

Table 1 Identification and quantification of n-alkanes and alkenes (alkane mass equivalent) extracted with methylene chloride from *Phleum pratense* pollen grains collected in Czech Republic in 2007 (T_R = retention time)

Alkanes	T_R (min)	Mass (ng mg ⁻¹) (eq. alkanes)	% of family	
			This work	Bashir et al.
Hexane	–	–	–	0.2
Heptadecane	32.6	5 ± 1	0.3	–
Nonadecane	37.8	3 ± 1	0.2	–
Heneicosane	42.5	9 ± 14	0.5	–
Docosane	44.8	5 ± 1	0.3	–
Tricosane	46.8	78 ± 9	4	3
Tetracosane	48.9	19 ± 3	1	0.3
Pentacosane	50.8	338 ± 21	18	18
Hexacosane	52.7	38 ± 4	2	2
Heptacosane	54.6	656 ± 3	36	37
Octacosane	56.3	28 ± 3	2	1
Nonacosane	58.3	317 ± 21	17	19
Triacontane	59.6	21 ± 2	1	1
Hentriacontane	61.3	311 ± 22	17	19
Sum		1828 ± 105	100	100
Alkenes				
Decene	27.5	5 ± 1	0.3	–
Pentacosene	50.4	41 ± 6	3	15
Hexacosene	–	–	–	1
Heptacosene	54.2	464 ± 47	32	–
Octacosene	56.2	58 ± 8	4	–
Nonacosene	57.3	297 ± 18	21	84
Triacontene	59.0	39 ± 7	3	–
Hentriacontene	60.9	541 ± 31	37	–
Sum		1445 ± 118	100	100

Comparison with data from the literature (Bashir et al. 2013a)

propanetricarboxylic acid, heptacosanol and stigmasterol. Butenedioic and propanetricarboxylic acids have too short retention times for being analyzed in our chromatographic conditions. Sterols have been observed in this work, but isomers have not been identified. Mass spectrum of heptacosanol was surprisingly not observed in our data.

We have identified: a continuous series of alkene (C25 to C31), whereas Bashir et al. (2013a) have reported a discontinuous series (C25, 26 and 29); a continuous series for fatty acids (C5 to C28), whereas Bashir et al. (2013a) have obtained a discontinuous one (C14–C17, C19–C20 and C22); a series of aldehydes (C9 to C24) not confirmed by Bashir et al. (2013a); and a series of alcohols (C16, 18 and 22) also not confirmed by Bashir et al. (2013a) (C17 observed but not confirmed in this work).

The relative proportion of extractable lipids was modified by an exposure to 100 ppb of ozone for 16 h (Fig. 5). Ozone has reacted with alkenes to produce fatty acids and aldehydes. Alkenes were consumed with a mass concentration decrease from 1445 ± 118 to 910 ± 62 ng mg⁻¹; fatty acids and aldehydes were produced with a mass concentration increase from, respectively, 434 ± 33 to 756 ± 64 ng mg⁻¹ and from 33 ± 6 to 269 ± 38 ng mg⁻¹. The two unsaturated fatty acids identified were not reactive toward ozone in our experimental conditions: The mass concentrations of α -linolenic acid (C18:3) and linoleic acid (C18:2) were, respectively, 3 ± 1 ng mg⁻¹ and 51 ± 4 ng mg⁻¹ in the intact pollen extract and 2 ± 1 ng mg⁻¹ and 41 ± 4 ng mg⁻¹ in the pollen sample exposed to ozone.

Table 2 Identification and quantification of saturated and unsaturated fatty acids extracted with methylene chloride from *Phleum pratense* pollen grains collected in Czech Republic in 2007 (T_R = retention time)

Saturated fatty acids	T_R (min)	Mass (ng mg ⁻¹) (eq. alkanes)	% of family	
			This work	Bashir et al.
Pentanoic acid	9.9	1 ± 1	0.2	–
Hexanoic acid	13.0	6 ± 1	1	–
Heptanoic acid	16.3	3 ± 1	1	–
Octanoic acid	19.7	3 ± 1	1	–
Nonanoic acid	22.8	4 ± 1	1	–
Decanoic acid	25.2	1 ± 1	0.2	–
Undecanoic acid	29.1	2 ± 1	1	–
Dodecanoic acid	31.5	2 ± 1	0.5	–
Tetradecanoic acid	36.5	10 ± 1	2	4
Pentadecanoic acid	38.9	1 ± 1	0.2	0.4
Hexadecanoic acid	41.4	160 ± 13	37	74
Heptadecanoic acid	43.6	3 ± 1	1	2
Octadecanoic acid	45.7	159 ± 16	37	–
Nonadecanoic acid	47.7	5 ± 1	1	1
Eicosanoic acid	49.8	41 ± 1	10	15
Heneicosanoic acid	51.7	8 ± 1	2	–
Docosanoic acid	53.5	16 ± 1	4	4
Tetracosanoic acid	57.0	5 ± 1	1	–
Octacosanoic acid	64.3	4 ± 1	1	–
Sum		434 ± 46	100	100
Unsaturated fatty acids				
Hexadecenoic acid	–	–	–	0.4
Octadecenoic acid	–	–	–	2
Linoleic acid	44.7	3 ± 1	6	7
α -Linolenic acid	45.6	51 ± 4	94	90.6
Sum		54 ± 5	100	100

Comparison with data from the literature (Bashir et al. 2013a)

4 Discussion

The total organic solvent-extractable mass of pollen lipids for *Phleum pratense* pollen has been determined to $22 \pm 1 \mu\text{g mg}^{-1}$. A large portion of extractable lipids of *Phleum pratense* pollen is still unknown; in this work, 50 species were identified and quantified in the extractable lipids, and those species accounted for about one-fifth of the total mass of extractable lipids. A better knowledge of the lipidic composition of allergenic pollen grains is needed to identify all molecules potentially associated with adjuvant potency to the allergic reaction.

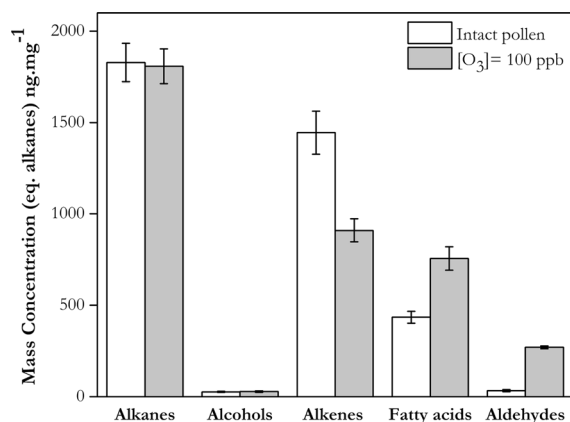
The variation of lipids among commercial pollen batches (Fig. 3) seems low compared to variation reported in the literature for proteins: from 10 to 60 μg per mg of pollen (Vrtala et al. 1993; Behrendt et al.

1999; Rogerieux et al. 2007). The pollen samples harvested in three different geographical locations show a similar distribution of lipids with total chromatographic areas in the range of 31,532–37,290 mV.min. The n-alkanes distribution obtained in this work was also almost identical to the one previously published (Bashir et al. 2013a) (Table 1). For comparison with this relative constancy in lipids extracted, one of the main allergens Phl p 5 has concentrations varying from 8.6 to 32.2 pg per pollen grain for pollen samplings from 2012 to 2014 (Plaza et al. 2016). On the contrary, large discrepancies appear between studies for the quantification of octadecenoic, linoleic and α -linolenic acids (Table 4). As example, α -linolenic acid was determined to be $51 \pm 1 \text{ ng mg}^{-1}$ in this work and between 1058 ng mg^{-1} to 5012 ng mg^{-1} in the literature

Table 3 Identification and quantification of aldehydes, alcohols and other compounds extracted with methylene chloride from *Phleum pratense* pollen grains collected in Czech Republic in 2007 (T_R = retention time)

	T_R (min)	Mass (ng mg ⁻¹) (eq. alkanes)	% of family	
			This work	Bashir et al.
Aldehydes				
Nonanal	14.1	1 ± 1	3	–
Octadecanal	40.8	3 ± 1	9	–
Eicosanal	45.3	5 ± 1	15	–
Heneicosanal	47.5	5 ± 1	15	–
Docosanal	49.6	10 ± 1	30	–
Tetracosanal	53.4	9 ± 1	27	–
<i>Sum</i>		33 ± 6	100	
Alcohols				
Hexadecanol	39.4	12 ± 1	46	–
Octadecanol	43.8	6 ± 1	24	–
Docosanol	51.8	8 ± 1	30	–
Heptacosanol	–	–	–	100
<i>Sum</i>		26 ± 3	100	
Other compounds*				
Butenedioic acid	–	–	–	7
Propanetricarboxylic acid	–	–	–	81
Stigmasterol	–	–	–	12
<i>Sum</i>			0	100

Comparison with data from the literature (Bashir et al. 2013a). *Only compounds with peak ratio area > 1 are shown

**Fig. 5** Main chemical families extracted from *Phleum pratense* pollen grains collected in Czech Republic in 2007 without treatment (intact) and exposed in laboratory to 100 ppb of ozone for 16 h

(Traidl-Hoffmann et al. 2002; Plötz et al. 2004). The high discrepancies reported here can have several

explanations: errors in quantification in one or several studies; discrepancies between pollen batches; higher extraction efficiencies of RPMI (Roswell Park Memorial Institute medium) solvent used by Traidl-Hoffmann et al. (2002) and Plötz et al. (2004) compared to methylene chloride; rupture of pollen and release of cytoplasmic content during extraction (Evans et al. 1990; Piffanelli et al. 1997, 1998). Methylene chloride is known to extract only external lipids (Evans et al. 1990, 1991; Henricsson et al. 1996; Piffanelli et al. 1997; Bashir et al. 2013a). Only rupture of pollen grains during laboratory handling could explain the extraction of internal lipids with this solvent. The SEM observations in this work showed that pollen grains were not broken after organic extraction. Unfortunately, the integrity of the pollen structure and the absence of ruptured pollen after solvent extraction process were not systematically checked in the literature. The pollen wall can be fragilized by a mechanical stress such as sonication or centrifugation,

Table 4 Comparison of concentrations of unsaturated fatty acids quantified in *Phleum pratense* pollen grains. RPMI: Roswell Park Memorial Institute medium (Traidl-Hoffmann et al. 2002; Plötz et al. 2004)

Unsaturated fatty acids ng mg ⁻¹	This work (eq. alkane)	Traidl-Hoffmann et al. (2002)	Plötz et al. (2004)
Extraction solvent	Methylene chloride	RPMI	RPMI
Method and duration of extraction	Washing 5 min	Centrifugation (500 g) 30 min	Centrifugation (500 g) 30 min
Hexadecanoic acid			1243
Hexadecenoic acid	—	—	—
Octadecanoic acid	159	—	1207
Octadecenoic acid	—	—	233
Linoleic acid	3	230	813
Linolenic acid	52	1058	5012
Sum	214	1288	7265

and the cytoplasmic content could be released or more easily reachable by solvent (Hideux 1971). Thus, pollen may rupture in the case of extraction methods using, for example, centrifugation, leading to higher extractable lipid contents and explaining discrepancies in data reported in the literature.

Differential distribution of lipids between external and internal parts of pollen was shown for *Brassica napus* pollen with an important concentration of fatty acids (hexadecanoic, linoleic and α -linolenic) in pollen cytoplasm (Evans et al. 1991, 1992; Piffanelli et al. 1997); this differential distribution of lipids is not yet known for *Phleum pratense* pollen.

Another essential question is whether or not lipids are released in biological conditions. Organic solvents were used in this study and in the work of Bashir et al. (2013a) for the purpose of identification and quantification of lipids giving an extent of the maximum quantity of extractable lipids. Bioavailability depends on localization of lipids in pollen (hence on the physical state of pollen: intact or broken) and on chemical affinities between lipids and extraction solvent. Aqueous solvent will likely not be as efficient as methylene chloride to extract lipidic compounds, but it gives a more real-life approach and moreover pollen rupture could occur by osmotic shock or abortive germination in aqueous solution (Wang et al. 2012). To the best of our knowledge, pollen rupture has never been mentioned in the literature to occur during immersion of pollen grains into organic solvent.

Bioavailability could also be modified by ozone exposure as oxygenated compounds are likely to be

more soluble in aqueous solution. The distribution of lipids was modified by artificial ozone exposure: alkenes reacted with ozone (consumption from 1445 to 910 ng mg⁻¹) to form aldehydes and saturated carboxylic acids (aldehydes produced from 33 to 269 ng mg⁻¹ and fatty acids from 434 to 756 ng mg⁻¹). This alkenes reactivity has already been observed for birch pollen grains in the same experimental conditions (Zhu et al. 2018), but it differs from the reactivity observed with *Pinus halepensis* where unsaturated fatty acids and monomers from the sporopollenin reacted with ozone (Naas et al. 2016). The average decay of alkenes with ozone exposure was particularly important ($38 \pm 9\%$) for three alkenes: heptacosene (C27), nonacosene (C29) and hentriacontene (C31). We hypothesize that those compounds may act as antioxidants, thus offering protection from ozone to more important molecules involved in reproduction processes. The unsaturated fatty acids were for their part not reactive toward ozone in our experimental conditions; those compounds may not be easily reachable by ozone or may have a much slower kinetic of reactivity compared to alkenes. The inflammatory potential of saturated fatty acids extracted from pollen grains is documented in the literature (Lernhardt 1990; Karsten et al. 1994; Bashir et al. 2013a, b). The effect of an increase in the mass concentrations of fatty acids during ozone exposure could influence the release of cytokines by peripheral lymphocytes and DC/NKT stimulation. The chemical micro-environment where a polluted pollen grain deposits in the pulmonary tract could be

therefore influenced by the total ozone exposure of pollen before inhalation.

5 Conclusion

In the present state of knowledge, the potential allergenicity of a grain relies on the amount of allergens, the nature and quantity of lipids with adjuvant effects, the ability of pollen to release cytoplasmic granules by rupture and to the chemical, biological and physical modifications induced by environmental pollutants. While the role of lipids in allergic reaction has been extensively studied (Dahl 2018), little is known about the amount and nature of lipids in allergenic pollen. In this work, we estimate the total extractable lipids of *Phleum pratense* pollen to 22 ± 1 µg per mg of pollen of which 18% have been quantified and identified. The modification of pollen lipids by atmospheric pollution is expected (Behrendt et al. 2001a; Naas et al. 2016; Zhu et al. 2018), and we have shown here that ozone can modify the distribution of alkenes, aldehydes and fatty acids. More effects of pollution on lipidic fraction could be suspected like the adsorption of volatile organic compounds on pollen surface (Behrendt et al. 2001b; Huss-Marp et al. 2008) as well as the adhesion of particulate matter bringing organic compounds like polycyclic aromatic hydrocarbons (Shiraiwa et al. 2012), phthalates (Kim et al. 2018) and pesticides (Padilla et al. 2017). The lipid composition of allergenic pollen clearly needs to be further investigated: A large fraction of lipids is still to be identified and quantified (around 80% for *Phleum pratense*), the distribution of lipids in cytoplasmic and external parts of pollen needs to be unraveled, and bioavailability of lipids must be better understood. Further studies should also be carried out to find whether lipid fraction modified by atmospheric transport could make polluted pollen grains stronger immune stimulants.

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