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Manuscript Draft

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Title: Extraction and quantification of Ole e 1 from atmospheric air samples: an optimized protocol

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Section/Category: Toxicology and Risk Assessment

Keywords: allergens; aerobiology; olive pollen

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Abstract: Olive pollen is the main cause of pollinosis in Mediterranean countries. The immunological analysis of Ole e 1, the major allergen of Olea europaea, has usually carried out by means of ELISA (Enzyme-Linked ImmunoSorbent Assay). However, most published works only specify the methodology related to antigen quantifications, but not the related to protein extraction. Furthermore, the results obtained are not compared with different buffers or modifications of them. The main aim of this study is to obtain an optimized and reproducible ELISA protocol for quantifications of Ole e 1 in the atmosphere. The study of Ole e 1 allergen and olive pollen in the atmosphere of Malaga (Spain) was carried out by means of an automatic multi-vial cyclonic sampler and a Hirst volumetric pollen trap, respectively. ELISA was tuned up on the basis of previously published protocols to quantify this allergen. Variations in the concentrations of capture and detection antibodies, as well as in the buffers used to carry out the extraction, were evaluated. The highest protein extraction was obtained when a modified buffer was applied. The correlation analysis between daily pollen concentrations and allergen quantifications showed highly significant values. The ELISA protocol, together with the buffer combination proposed in this work, considerably reduced the concentrations of capture and detection antibodies used for quantifying Ole e 1 in the atmosphere, allowing detect this allergen even in days in which the airborne pollen concentration was very low or null.

Dear Editor,

We are sending you a manuscript entitled “Extraction and quantification of Ole e 1 from atmospheric air samples: an optimized protocol” for its possible publication in Chemosphere.

All the results and conclusions presented in this work were part of the doctoral thesis presented by Dr García-Sánchez. The writing of this article has been carried out by this author, supervised by Dr Trigo, who was his thesis supervisor and by Dr Recio, researcher of the Aerobiology Group of the University of Malaga, who contributed with her remarkable experience in the field of statistics for the analysis of the results of this work.

The authors of the manuscript declare there are not conflict of interest in publishing this paper.

Best regards

Marta Recio, José García Sánchez and Mª del Mar Trigo

*Title Page

Title: Extraction and quantification of Ole e 1 from atmospheric air samples: an optimized protocol

Author names: José García-Sánchez, Mª del Mar Trigo, Marta Recio

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Abstract

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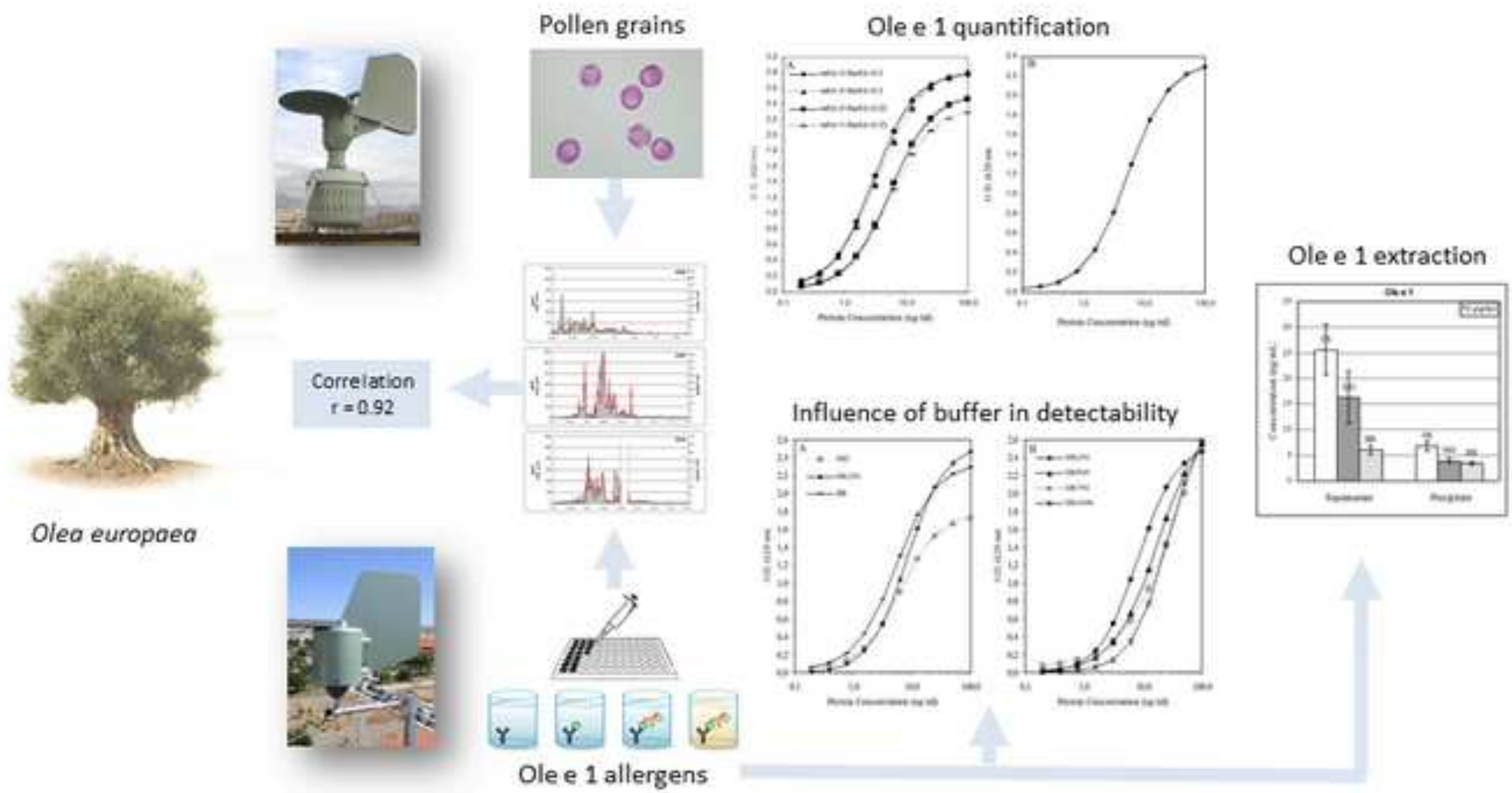
Keywords: allergens, aerobiology, olive pollen

Graphical Abstract



***Highlights (3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

Highligth

Olive pollen is one the main cause of pollinosis in Mediterranean countries.

This paper offer a detailed methodology to extract and quantify the Ole e 1 allergen.

Antibodies concentrations used have been reduced considerably, reducing also the cost.

The proposed protocol allows quantify Ole e 1 even in days with zero record of pollen.

This research shows different alternatives for extracting Ole e 1 from air samples.

Figure 1
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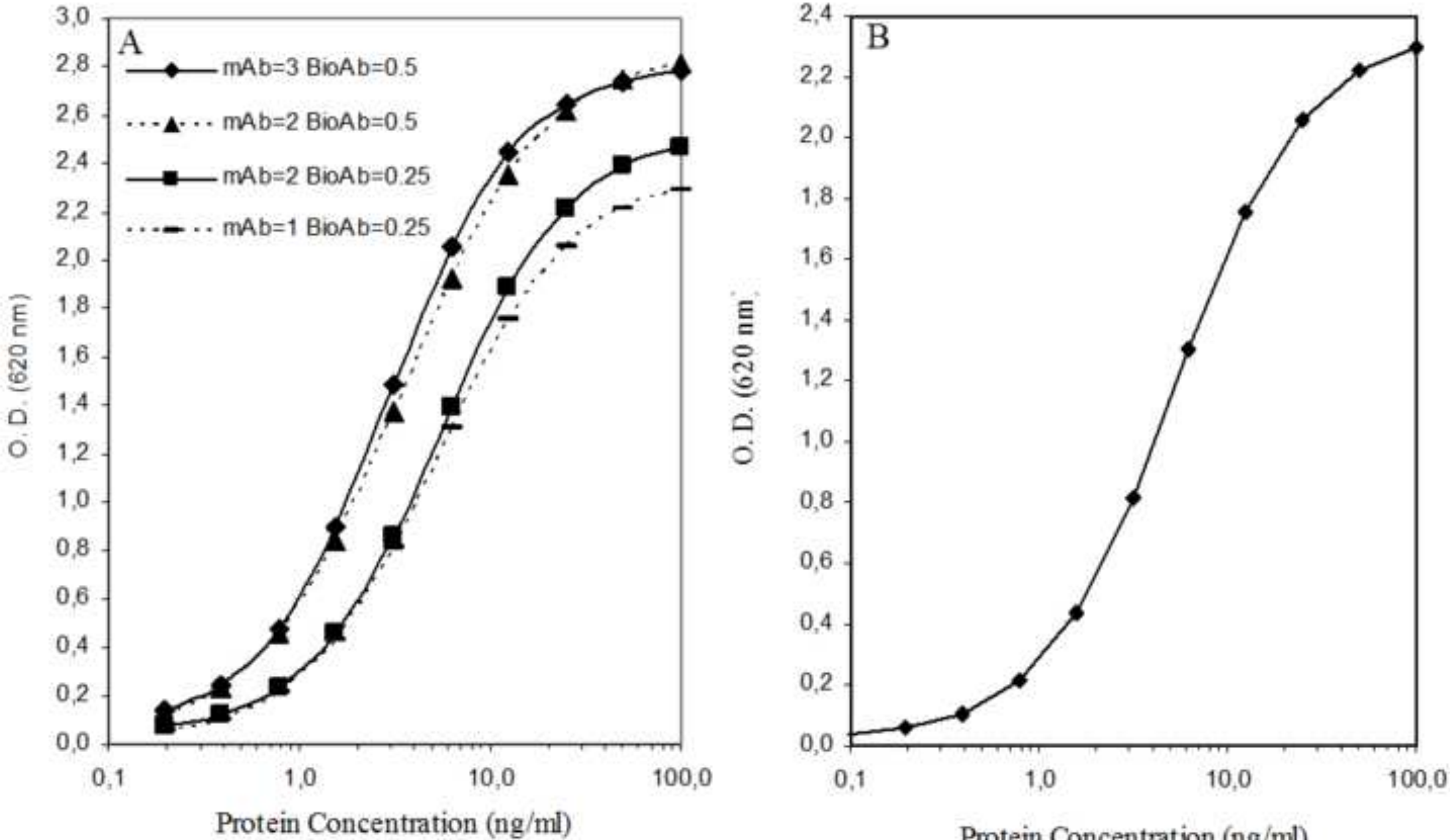

Figure 2
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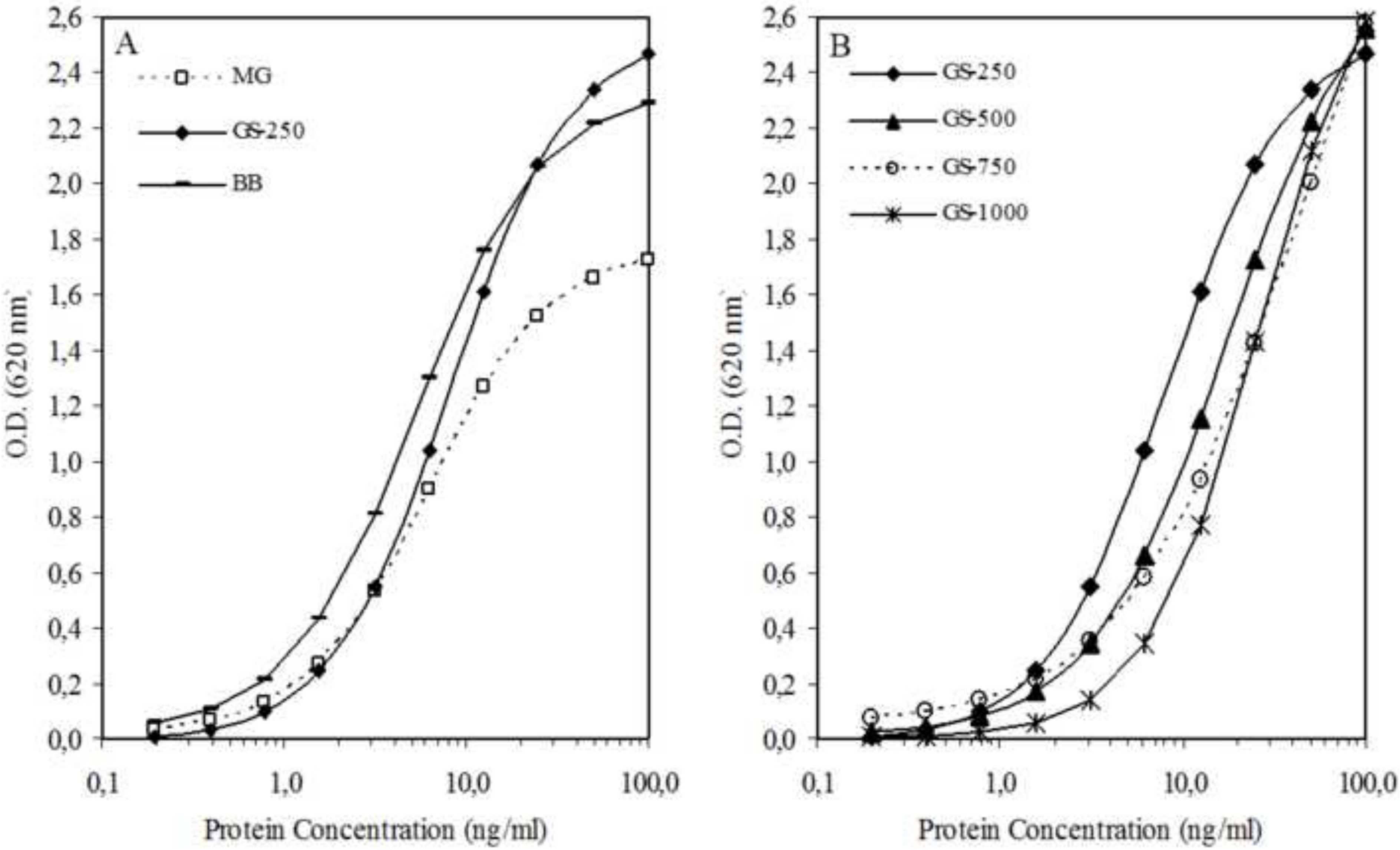

Figure 3
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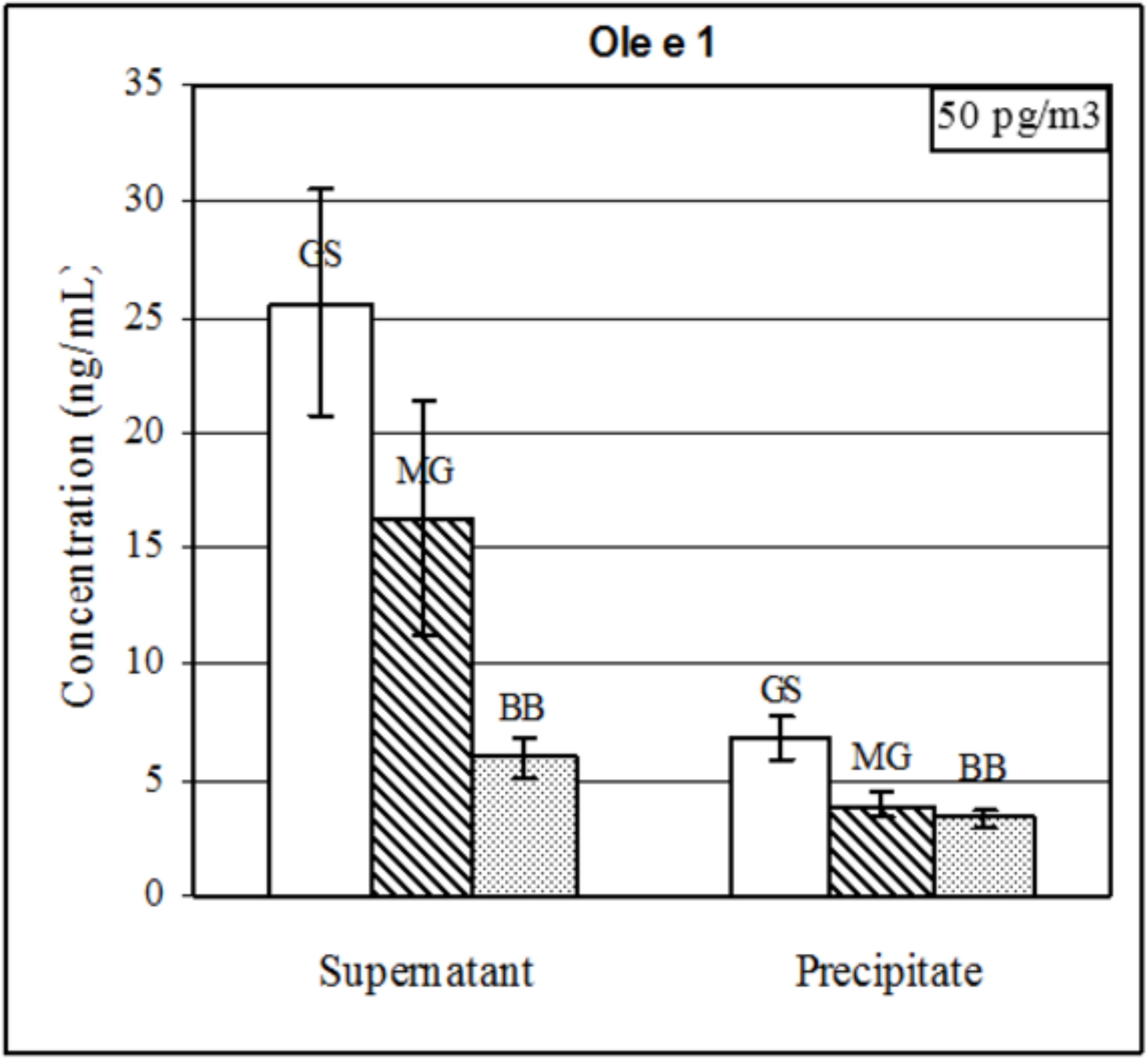

Figure 4
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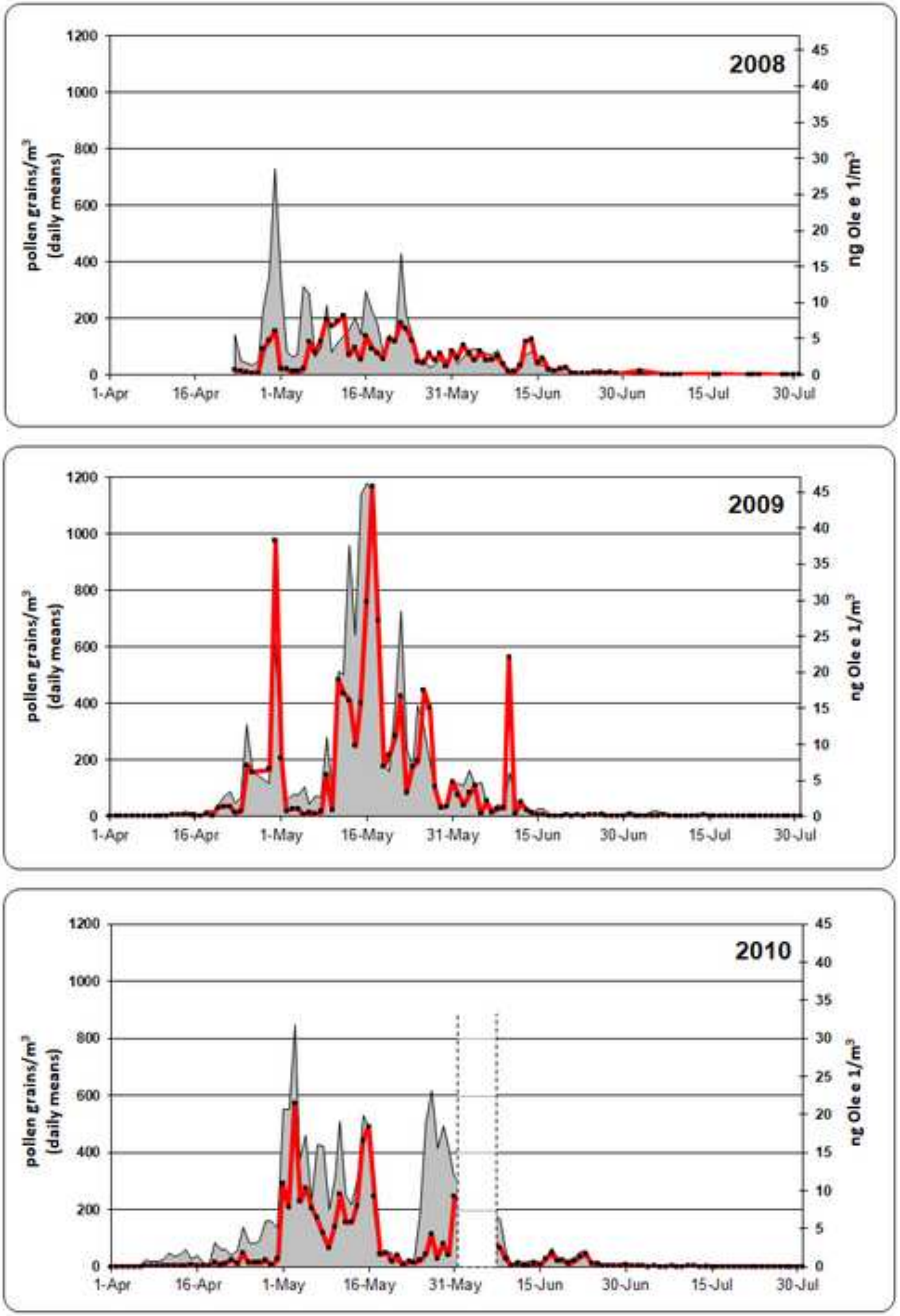

Figure 5
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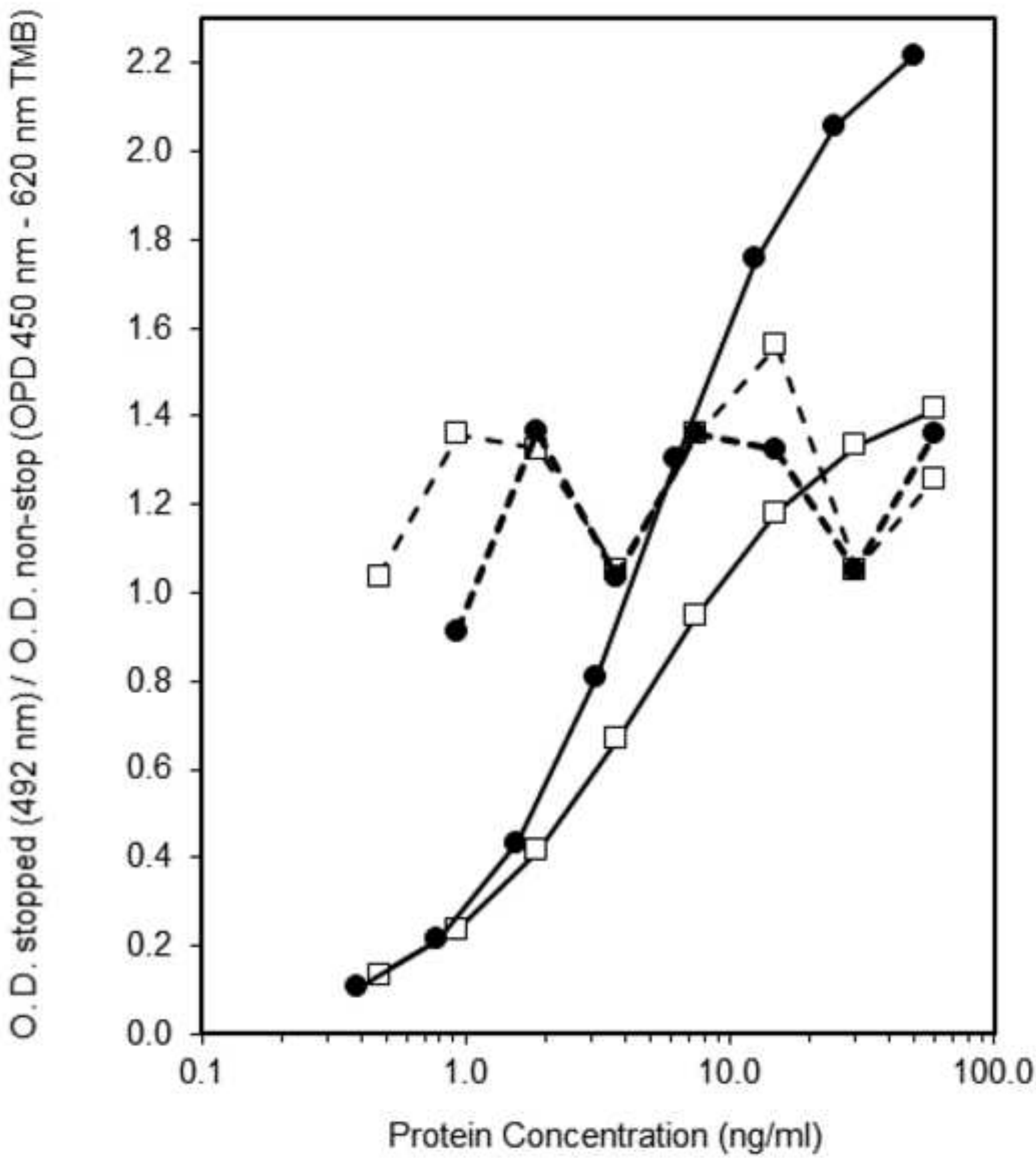

Table 1
Click here to download Table: Table1_Garcia-Sanchez_et_al.doc

Table 1

Period		April 23th-July 31st 2008	April 1st-July 31st 2009	April 1st-July 31st 2010
Pollen (pg/m^3)	Sum	7391	14756	12405
	Peak Day	730 (April 30th)	1179 (May 16th)	847 (May 3rd)
Allergen (ng/m^3)	Sum	173.63	439.93	213.12
	Peak Day	8.26 (May 12th)	45.69 (May 17th)	21.39 (May 3rd)
Spearman correlation	Total	0.872*** (89)	0.922*** (121)	0.911*** (115)
	MPS	0.706*** (64)	0.916*** (70)	0.804*** (62)

***Response to reviewers/editor in question & answer format (word file)**

Dear editor,

Thank you very much for accepting our manuscript with moderate revision.

You can find below the responses (blue text) to all the comments made by the reviewers.

Reviewer #1: The paper "Extraction and quantification of Ole e 1 from atmospheric air samples: an optimized protocol" is an original article because it permit to have a look on the difference of some results due to the protocol used.

For me, this article is accepted with some minor revisions:

- lines 75-78: even if it described in the references, it will be nice that the authors add 3 to 6 lines about the equipment and devices used for the study.

The devices used for this study have been described and cited in the manuscript in the section "2.4 Pollen and airborne allergen monitoring" (page 8, lines 160-163). In addition, its work is also discussed on page 6, lines 121-123. We think that describing the devices again in the section proposed by the reviewer would be a little redundant.

- line 168:it is written that in 2008, the sampler started later, it may be preferable that in the figure 4 all the three graphs begin ad stop at the same dates. Why on the graph of 2010, some data at the beginning of June are missing?

Done. Now, all the graphs begin and stop at the same dates and the missing data have been explained in the figure caption (page 20, line 447). In addition, minor errors have been corrected in Figure 4: text of the Y axis and months in the X axis.

- line 255: I don't find in the bibliography the reference Mahendra K. Agarwall et al 1984!

Changed to Agarwall et al., 1984.

Reviewer #2: Manuscript Number: CHEM59126

Title: Extraction and quantification of Ole e 1 from atmospheric air samples: an optimized protocol.

The paper aims to obtain an optimized and reproducible protein extraction protocol for the quantification of Ole 1 in the atmosphere by means ELISA. The manuscript deserves publication as it is well planned, structured, well written and discussed. After a careful revision, I detected several very specific weaknesses:

1. The references used in the Introduction chapter are quite old. Please to update.

Done

2. To avoid refer to Figures and Tables in the Discussion chapter, please delete.

We have added references to figures and tables in the discussion chapter because due to the complexity and variety of the experiments and results, we thought that it would greatly facilitate the reader to understand and interpret them. We would like to keep those references. But, if the editor does not consider suitable to include them, we will proceed to remove them.

3. In the lines 264 to 266 the authors comment: "Therefore, we decided to use TMB in the assays instead OPD, but without stopping the reaction, since it was the combination with which the best results were obtained (data not shown)."

Please to include some data and a figure supporting this affirmation.

This paragraph has been modified and a new figure has been added to illustrate these experiments (page 12, line 266-273). This is figure 5 and its correspondent caption has been incorporated to the ms. (page 20, line 448-451)

4. The reference Mahendra K. Agarwal et al. 1984 is cited in the text but not in the reference chapter.

Changed to Agarwall et al., 1984.

5. Please to delete "F. T." from the references "F. T. Spieksma et al. 1995; F. T. M. Spieksma et al. 1990" in the line 257 of the text

Done

Title: Extraction and quantification of Ole e 1 from atmospheric air samples: an optimized protocol

Author names: José García-Sánchez, Mª del Mar Trigo, Marta Recio

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Abstract

Olive pollen is the main cause of pollinosis in Mediterranean countries. The immunological analysis of Ole e 1, the major allergen of Olea europaea, has usually carried out by means of ELISA (Enzyme-Linked ImmunoSorbent Assay). However, most published works only specify the methodology related to antigen quantifications, but not the related to protein extraction. Furthermore, the results obtained are not compared with different buffers or modifications of them. The main aim of this study is to obtain an optimized and reproducible ELISA protocol for quantifications of Ole e 1 in the atmosphere. The study of Ole e 1 allergen and olive pollen in the atmosphere of Malaga (Spain) was carried out by means of an automatic multi-vial cyclonic sampler and a Hirst volumetric pollen trap, respectively. ELISA was tuned up on the basis of previously published protocols to quantify this allergen. Variations in the concentrations of capture and detection antibodies, as well as in the buffers used to carry out the extraction, were evaluated. The highest protein extraction was obtained when a modified buffer was applied. The correlation analysis between daily pollen concentrations and allergen quantifications showed highly significant values. The ELISA protocol, together with the buffer combination proposed in this work, considerably reduced the concentrations of capture and detection antibodies used

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Keywords: allergens, aerobiology, olive pollen

1. Introduction

Currently, more than 25% of the population in industrialized countries has Type I allergic disorders such as rhinitis, atopic rhinoconjunctivitis and bronchial asthma (Asher et al. 2014; Beasley et al. 1998; Burney et al. 1996; Ojeda et al. 2018). Pollen grains and other small airborne particles are some of the main agents responsible for these diseases (D’Amato et al. 1998, 2016; D’Amato and Spieksma 1990; Jalbert and Golebiowski 2015; Solomon and Mathews 1978). Traditionally, the allergenic potential of the atmosphere has been studied with the aid of different types of particles samplers, being the Hirst and Rotorod types the most used (Hirst 1952; Ogden and Raynor 1967). Once the samples are obtained, pollen grains and spores are counted and their results transformed into “number of biotic particles per cubic meter of air”. However, the antigenic content of these particles may vary highly and be influenced by atmospheric and transport conditions. For this reason, it is essential to use optimal and standardized methodologies for detection and quantification of the main aeroallergens capable of causing pollinosis in the population.

One of the first studies carried out in order to detect airborne pollen allergens was conducted on ragweed in 1972 by means of “passive transfer antigen neutralization techniques” (Busse et al. 1972). Later on, in 1981, ragweed allergens were again studied, as well as *Alternaria* spore allergens but, this time, using RAST-type analysis (Agarwal et al. 1981). It was not until 1994 when the first studies about pollen allergens in the atmosphere were published by using enzyme-linked immunosorbent assay (ELISA). These studies were carried out against *Betula* allergens (Pehkonen and Rantio-Lehtimäki 1994; Rantio-Lehtimäki et al. 1994). Two years later, in 1996, the first studies made with Sandwich ELISA, a test specially designed for the quantification of specific allergens, were presented against Bet v 1 and Phl p 5 allergens (Schäppi et al. 1996). Thenceforth, many studies have been carried out in order to quantify pollen allergens in the atmosphere (Jato et al. 2010; Moreno-Grau et al. 2006; Plaza et al. 2016; Schäppi et al. 1997). The main goal of this study was to obtain an optimized and reproducible

ELISA protocol for the quantification of Ole 1 in the atmosphere, which has a high incidence in the Mediterranean region and some North America areas (Bousquet et al. 1985).

An important and very common issue to deal with when we try to find a detailed and proper methodology to process the samples, is the isolation and preservation of the proteins they contain. Usually, most of the published works only specify the methodology related to the antigen quantifications, but not that related to the protein extraction from the samples. Additionally, when the extraction methodology is specified, the results obtained are not compared with different buffers or modifications of them, which does not offer possible alternatives, depending on the allergens we want to study, since not all buffers affect in the same way the extraction and stability of proteins.

2. Materials and Methods

In this paper, different alternatives for extraction and quantification of the allergen Ole e 1 have been tested. For this purpose, a Sandwich ELISA test for Ole e 1 was developed, estimating what antibody concentrations were the most adequate to determinate the corresponding antigenic concentration in aerobiological samples.

Protein extractions were performed from controls of purified olive pollen by means of the use of different extraction buffers, in order to optimize the extraction of the allergen Ole e 1 from pollen grains contained in the air samples. In addition, the effect of these buffers on the behaviour of the ELISA standard curve was studied, and the most appropriate alternative was proposed, depending on the variations observed in the detectability and sensitivity of the assays.

The air samples for protein quantification were taken from the atmosphere of Malaga (southern Spain) during the years 2008, 2009 and 2010 and carried out by using a cyclonic sampler, which is an optimal device for this type of samplings, previously used in numerous studies (Fernández-González et al. 2013; Jato et al. 2010; Plaza et al. 2016; Takahashi et al. 2001). These results were compared with *Olea europaea* pollen counts, obtained by means of a Hirst type volumetric pollen trap during the same period of study.

2.1. Ole e 1 allergen quantification by means of Sandwich ELISA

In order to quantify the allergen Ole e 1 by sandwich ELISA, monoclonal antibodies (mAb), as capture antibodies, and biotinylated polyclonal antibodies (BioAb), as detection antibodies, have been used. Besides this, sensitivity, detectability and antigenic determination range at different concentrations of these antibodies have been compared. Both, antibodies and purified antigens used for the calibration curves, were supplied by Bial-Arístegui (Bilbao, Spain).

Protocol:

The different concentrations of the capture and detection antibodies (for standard curve) tested for the determination of Ole e 1 was the following:

- 3 μg/mL mAb and 0.5 μg/mL BioAb
- 2 μg/mL mAb and 0.5 μg/mL BioAb
- 2 μg/mL mAb and 0.25 μg/mL BioAb
- 1 μg/mL mAb and 0.25 μg/mL BioAb

The concentration range of the standard curve for Ole e 1 allergen was from 100 to 0.195 ng/mL.

Initially, 100 μl of the capture antibody in phosphate buffered saline (PBS), pH 7.4, are added to flat bottom 96-well plates (Costar® 3590, Corning Incorporated, USA) and then incubated covered and sealed with parafilm for at least 12 hours at 4°C in humidity chamber. The liquid is then removed from the plate and 200 μl of blocking buffer (BB), pH 7.4, are added. BB is constituted by PBS, 0.05% Tween 20 and 1% bovine serum albumin (BSA, Amersham RPN412, GE Healthcare, UK). Then, the plate is incubated for one hour at 37°C.

The Ole e 1 allergen standard curve was obtained with this purified protein diluted in BB. Purified proteins of Ole e 1 were diluted sequentially 1:2 in a concentration range of 100 to 0.195 ng/mL and incubated for 2 hours at 37°C.

Subsequently, three washes are carried out with PBS plus Tween 20 (PBS-T) for 2 minutes each. After that, 100 μL of biotinylated antibody in BB buffer are added and incubated for 1 hour at 37° C. Three additional washes are performed with PBS-T and then 100 μL of

Streptoavidin-Peroxidase (SIGMA S5512, Sigma-Aldrich®, USA) diluted in BB buffer at a concentration of 0.25μg/mL are added, followed of incubation for 1 hour at 37° C. Finally, 3 more washes with PBS-T are performed and 100 μL of 3,3 ', 5,5'-tetramethylbenzidine (TMB, SIGMA T0440, Sigma-Aldrich®, USA) added, and the well-plates incubated for 30 minutes in darkness at room temperature. Absorbance at 620 nm was measured by using a microplate reader (Thermo, Multiskan EX), without stopping the reaction and avoiding pH changes.

2.2. Protein extraction from pollen grains

In order to study the extraction of the antigen Ole e 1, known concentrations of purified pollen, supplied by Iber-Polen (Jaen, Spain), were used. For this, dilutions of pure pollen stocks of *Olea europapea* L. were prepared to set homogeneous samples that simulated a day in which 50 pollen grains/m^3 of air were detected in the atmosphere, this concentration being equivalent to one day with moderate pollen concentration in the Mediterranean region. Therefore, we can compare, in a uniform way, the protein concentrations obtained with any extraction buffer.

For this work, the simulation of a 24-hour sampling, performed with a Cyclone trap (Holmes and Emberlin 1994) was used as reference. The total volume of air aspirated in 24 hours by this device is 23.76 m^3, since it sucks 16.5 L of air per minute. For the analysis, the whole sample remaining deposited in an Eppendorf tube is resuspended in 1 mL of extraction buffer. Therefore, the pollen concentration used to perform the protein extractions in one day with a concentration of 50 grains of pollen/m^3 of air was 1188 pollen grains/mL (50 pollen grains x 23.76 m^3 of air).

The homogeneous pollen samples of 1188 pollen grains/mL to which compare the effect of the different extraction buffers were obtained by adding 1 mg of purified pollen in 1 mL of distilled water with a 2% of Tween 20 (to avoid the pollen grains form aggregates) and then counting the pollen grains with the aid of a Neubauer Chamber. Therefore, we obtained the number of pollen grains per mL that, subsequently, was adjusted by dilution to the concentration of 1188 pollen grains/mL in order to perform the extractions.

The extractions were performed with three different types of buffers, which are called in this work as MG, GS and BB.

- MG buffer: proposed by Moreno-Grau et al. (2006). It is composed by 50 mM phosphate buffer, pH 7.4, 125 mM (NH) 4HCO 3, 150 mM NaCl, 3 mM EDTA and 0.005% Tween 20. The extraction methodology proposed by the aforementioned authors was followed in its entirety.

- GS buffer: it has the same composition as MG buffer, but with the following modifications: 250 mM (NH) 4HCO 3, 1% Tween 20 and 0.5% BSA.

- BB buffer (blocking buffer): consisting of PBS, 0.05% Tween 20 and 1% BSA.

All the incubations were made in a total volume of 1 mL, in Eppendorf tubes, for 2 hours, at room temperature and shaking the tubes every 30 minutes. They were then centrifuged at 8000 g for 10 minutes in the case of MG and at 13800 g for 15 minutes in the cases of GS and BB. Both the supernatant and the precipitate were stored in the freezer at -20°C.

The estimation of Ole e 1 concentration by the proposed ELISA, when extracting with a determined buffer, was carried out by three replicates and always extrapolating to the standard curves, incubated with the same extraction buffer.

2.3. Influence of buffers on detectability and sensitivity of the ELISA

For determining the viability of MG, GS and BB buffers in ELISAs, its effects on the detectability and sensitivity were evaluated. In the case of GS buffer, four variants were tested in which concentrations of $(NH)_4HCO_3$ were progressively increased, these being 250, 500, 750 and 1000 mM. The antigens used in each ELISA were Ole e 1 purified proteins, supplied by the company Bial-Arístegui.

2.4. Pollen and airborne allergen monitoring

The study of quantification of pollen and allergens in the atmosphere of Malaga was carried out with two volumetric samplers. Airborne pollen samplings were performed by using a Hirst-type volumetric spore trap (Hirst 1952) by Lanzoni, model VPSS 2000 (Bologna, Italia). Airborne allergen samplings were performed with the aid of an automatic multi-vial Cyclone sampler (Holmes and Emberlin 1994) by Burkard® (Burkard Manufacturing Co Ltd, Hertfordshire, England).

Both samplers were located on the roof of the Biology building of the Faculty of Sciences, University of Malaga, at coordinates 30SUF6847564395 (Datum ETRS89), at a height of 60 meters above sea level and 15 meters above ground level.

The sampling period for both devices took place from April to July, both inclusive, (olive flowering season) of the years 2008, 2009 and 2010. Due to technical problems, the Cyclone sampler started to work a little later, on April 23th 2008 and, occasionally, we do not have samplings for a few days during the rest of the studied period either.

For the preparation, assembly, conservation and analysis of the airborne pollen samples, taken with the Hirst-type sampler, we followed the methodology proposed by the Spanish Aerobiology Network, REA in its acronym in Spanish, in its Management and Quality Manual (Galán et al. 2007)

In order to observe the relationship between pollen concentrations and their respective allergen levels, the results obtained during the entire study period, as well as during the Main Pollen Season or MPS (95% accumulated pollen in the year) (Pathirane 1975), was compared by using the Spearman correlation test since the data did not fit a normal distribution. The Kolmogorov-Smirnov test was used to check the normality of the data.

3. Results

3.1. Ole e 1 quantification by means of ELISA

When comparing the effect of different concentrations of capture and detection antibodies, it can be observed that the most important factor in the detectability of the assay is biotinylated antibodies, the detectability being greater the higher the concentration of the antibodies is (Fig. 1A). Variations in ELISA sensitivity were hardly observed when the concentrations of capture antibodies varied (Fig. 1A).

In the dose-response standard curve of Ole e 1 at concentration of 1 µg/mL for the capture monoclonal antibody, and 0.25 µg/mL for the detection biotinylating polyclonal antibody (Fig. 1B), we obtained a maximum intra-assay coefficient of variation (CV) of 6.6% (n= 16) and a maximum inter-assay CV of 9.5% (n= 10) in the linear range 0.78-25 ng/mL. This combination of antibody concentrations was used to carry out the quantification of the allergen Ole e 1 in the atmosphere of Malaga.

The estimated detection limit (calculated as the mean of blank values plus 3 times the standard deviation) was 0.24 ng/mL.

3.2. *Influence of buffers on detectability and sensitivity of ELISA*

It can be observed that the standard curve that most affinity and detectability provides is that in which the BB buffer has been used, the other ones in which GS-250 and MG were used, are the following more sensitive alternatives but with the peculiarity that GS-250 offers lower saturation at high concentrations, thus offering a higher determination range (Fig. 2A). The sensitivity is the same in all cases in values lower than 3 ng/mL, GS-250 being the most sensitive above this concentration, and MG the least one. It can be also observed that when the concentrations of $(NH)_4HCO_3$ increase in the GS buffer, the affinity and detectability of the calibration curves decrease considerably (Fig. 2B).

3.3. Extraction of antigens in equivalent samples to 50 gp/m^3 of air

When protein quantifications of Ole e 1 were carried out with the three proposed buffers in equivalents of 50 gp/m^3 (Fig. 3), we observed that, in both, supernatants and precipitates, the highest concentrations were obtained when the GS buffer was applied, followed by MG and, finally, BB, with 38% and 71% less, respectively, in relation to GS. By means of ANOVA, it was observed significant differences among the extractions of the three buffers ($\alpha = 0.01$).

3.4. Quantification and correlation analysis of olive pollen and its allergen Ole e 1 in the atmosphere of Malaga

The concentrations of the allergen Ole e 1 and *Olea europaea* pollen during the olive flowering season recorded in Malaga between 2008 and 2010 are shown in Table 1 and Fig. 4. The highest concentrations of Ole e 1 were registered during 2009, 2008 being the year in which the concentrations of this aeroallergen were the lowest. Regarding the pollen counts, they followed exactly the same behaviour, although not in the same proportion, 2009 being the year in which the highest proportion of aero-allergens per pollen grain was detected and 2010 the lowest. Something worthy to be mentioned is the fact that, although they do not necessarily have to coincide in time, the peak days for allergens and pollen were coincident in 2009 and 2010.

Regarding the correlation analysis between daily pollen concentrations and allergen quantifications carried out by means of Spearman test, during the whole study period, as well as during the main pollen season (MPS) (95%), the coefficients showed very high and significant values (α=0.001) during the three years studied (Table 1).

4. Discussion

In this work, the quantification protocol for the protein Ole e 1 (olive tree major allergen (Rodríguez et al. 2001)) by means of double antibody sandwich ELISA was optimized. In addition, the effect of three different buffers (MG, GS and BB), on both ELISAs (Fig. 2) and protein extraction process (Fig. 3), has been studied. The extraction buffer we have named MG, published by Moreno-Grau et al. (2006), is a modification of the buffer proposed by Takahashi et al. (2001). This buffer has been compared with the buffer BB, which is the same buffer commonly used as coating buffer in ELISAs, which has been used to perform protein extractions from pollen grains for the first time in this type of studies. The third buffer we have compared, the so-called GS, is a combination of the two previous buffers.

When carrying out studies of quantification of pollen allergens in the atmosphere of a given territory, we will have to face two fundamental problems that will determine the methodology to use when carrying out the tests in the laboratory. The first one, which is applicable almost globally, is the budgetary shortage affecting certain scientific disciplines, reason why it is necessary to optimize the concentrations of antibodies. In our case, this fact markedly determined the final concentrations of antibodies that were used to carry out the protein quantifications in the aerobiological samples. The second one, which is particular for each area of study, is to choose the extraction buffer as well as the most appropriate methodology, depending on the different allergens that we want to simultaneously study during a given period. For the ELISAs developed in this study, we used the same combinations of antibodies that have been used previously in numerous research papers (Arilla et al. 2002; Moreno-Grau et al. 2006; Plaza et al. 2016). These anti-Ole e 1 antibodies, produced by the company Bial-Arístegui, have proved to be highly recommendable for quantifying Ole e 1, since they have provided high detectability, sensitivity and reproducibility and, therefore, high reliability.

Regarding the budgetary shortage, one of the main advantages of the protocol proposed is that the concentrations of capture and detection antibodies used have been considerably reduced, which cheapen the cost of each ELISA without altering the results. On the other hand, detectability has been the same or higher than in previous studies performed on Ole e 1 in which monoclonal antibodies were used (Moreno-Grau et al. 2006; Plaza et al. 2016; van Ree et al. 2000). This allowed to quantify Ole e 1 in days in which airborne concentrations were of only one pollen grain per cubic meter of air or, even, on days with zero records of pollen grains within the MPS. This demonstrates the fact that may exist pauci- or sub-micronic particles in the atmosphere containing pollen aeroallergens as unveiled by several authors (Agarwal et al. 1984; Busse et al. 1972; Cabrera et al. 2002; Fernandez-Caldas et al. 1989; Linares et al. 2007; Rantio-Lehtimäki et al. 1994; Schäppi et al. 1997; Spieksma et al. 1990, 1995)

~~The achievement of these more favourable results is related in part to the use of TMB versus OPD (o-phenylenediamine) as peroxidase substrates. It was observed that, stopping the OPD colorimetric reaction with acid, the absorbance levels obtained decreased considerably. However, when acid was applied to stop the TMB reaction, the opposite effect was observed: absorbance levels increased considerably, although detectability and reproducibility were negatively affected. Therefore, we decided to use TMB in the assays instead OPD, but without stopping the reaction, since it was the combination with which the best results were obtained (data not shown).~~

The achievement of these more favourable results is related in part to the use of TMB versus OPD (o-phenylenediamine) as peroxidase substrates. It was observed that, stopping the OPD and TMB colorimetric reaction by adding an acid, the absorbance levels obtained were altered considerably, the detectability and reproducibility being negatively affected. Therefore, we decided to use TMB instead OPD in the assays, but without stopping the reaction, since those were the conditions under the best results were obtained (Fig. 5).

As regards the extraction buffer, in order to decide which methodology and which extraction buffer use to carry out the allergen quantification when several pollen types are involved in the study, at the same time, in a certain area, it would be necessary to previously carry out all the tests proposed here for each one of these allergens. This allows to stablish which buffer is the most suitable for the combination of allergens to quantify. In addition, it must be taken into account the pollen concentrations of these pollen types registered in the atmosphere of the study area during the MPS. Based on these considerations, in this work, although only the results for Ole e 1, the major allergen of *Olea europea* L., are presented, the decision to use the buffer BB as mean of extraction and quantification was due to the fact that the studied samples contained also other important aeroallergens. In fact, Par j 1-Par j 2, the major allergens of *Parietaria judaica* L. and Lol p 1, grasses group I, the major allergen of *Lolium perenne* L. (data not shown) are present in the atmosphere of Malaga at the same time that Ole e 1 and they were much more favoured by the use of this extraction buffer. Therefore, based on the results obtained in the protein extraction tests (Fig. 3), if we are going to study in a sample only the allergen Ole e 1, it would be advisable to use the buffer GS-250 since we obtained almost 40% and 70% more extraction, on average, in comparison to the buffers MG and BB respectively.

However, in the case the atmosphere of Malaga, the buffer BB was used on the basis of three fundamental reasons: it was the buffer that provided the best detectability among the three buffers tested (Fig. 2A); because it was a priority buffer to study the allergens Lol p 1, Par j 1 and Par j 2 during the same period (data not shown); and, finally, in spite of not being the most efficient option for Ole e 1, in terms of extraction, in days with a single pollen grain of olive per cubic meter of air, as said above, we obtained positive values, within the estimated detection limit for the allergen Ole e 1. All these are factors that should be taken into account in any similar study.

By way of conclusion, on the basis of the results obtained (Table 1 and Figure 4), the ELISA protocol described in the present paper, together with the buffer BB used to carry out the extractions of the antigen Ole e 1, have proved to be fully adequate to carry out the antigenic analysis of aerobiological samples, being a great tool for the study of a pollen allergen as important in the Mediterranean region such as Ole e 1 (Bousquet et al. 1985; Wheeler 1992).

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Table 1

Title: Pollen and allergen results and statistical analysis.

Footnote: Total pollen and peak day of *Olea europaea* and Ole e 1 allergen quantification obtained by Hirst type and Cyclone samplers, respectively, during study period. Spearman rank correlation coefficients obtained between pollen concentrations and allergen quantifications during the study period (Total) and the main pollen season (MPS). $^{***}\alpha \leq 0.001$. In brackets date of peak day for pollen and allergen and number of data for Spearman correlation

Figure captions

Fig. 1 Dose-response curves in two-site sandwich ELISA of Ole e 1 purified protein. (A): Comparison between several concentrations of antibodies. (O.D.) optical density. (B): Curve with 1 µg/mL of capture antibody and 0.25 µg/mL of detection antibody.

Fig. 2 Effects of different buffers in the detectability and sensitivity of the ELISA for Ole e 1. (A): Comparison of buffers MG, GS-250 and BB. (B): Comparison of buffers GS-250, GS-500, GS-750 and GS-1000.

Fig. 3 Extractions of Ole e 1 antigens in equivalent of 50 pollen grains/m^3 using buffers GS-250, MG and BB.

Fig. 4 Concentrations of Ole e 1 allergen (line) and *Olea europea* pollen (shading) during the olive grove blooming between 2008 and 2010 obtained by Cyclone and Hirst type samplers respectively. The gap in 2010 was due to technical problems.

Fig. 5 Comparison of the dose-response curves of the ELISA for Ole e 1 by using of OPD (white square) and TMB (black circle) as peroxidase substrate, stopping the colorimetric reaction by means of acid (dashed line) and without stopping the reaction and reading the results after 30 minutes of reaction (continuous line).

Title: Extraction and quantification of Ole e 1 from atmospheric air samples: an optimized protocol

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Abstract

Olive pollen is the main cause of pollinosis in Mediterranean countries. The immunological analysis of Ole e 1, the major allergen of Olea europaea, has usually carried out by means of ELISA (Enzyme-Linked ImmunoSorbent Assay). However, most published works only specify the methodology related to antigen quantifications, but not the related to protein extraction. Furthermore, the results obtained are not compared with different buffers or modifications of them. The main aim of this study is to obtain an optimized and reproducible ELISA protocol for quantifications of Ole e 1 in the atmosphere. The study of Ole e 1 allergen and olive pollen in the atmosphere of Malaga (Spain) was carried out by means of an automatic multi-vial cyclonic sampler and a Hirst volumetric pollen trap, respectively. ELISA was tuned up on the basis of previously published protocols to quantify this allergen. Variations in the concentrations of capture and detection antibodies, as well as in the buffers used to carry out the extraction, were evaluated. The highest protein extraction was obtained when a modified buffer was applied. The correlation analysis between daily pollen concentrations and allergen quantifications showed highly significant values. The ELISA protocol, together with the buffer combination proposed in this work, considerably reduced the concentrations of capture and detection antibodies used

for quantifying Ole e 1 in the atmosphere, allowing detect this allergen even in days in which the airborne pollen concentration was very low or null.

Keywords: allergens, aerobiology, olive pollen

1. Introduction

Currently, more than 25% of the population in industrialized countries has Type I allergic disorders such as rhinitis, atopic rhinoconjunctivitis and bronchial asthma (Asher et al. 2014; Beasley et al. 1998; Burney et al. 1996; Ojeda et al. 2018). Pollen grains and other small airborne particles are some of the main agents responsible for these diseases (D'Amato et al. 1998, 2016; D'Amato and Spieksma 1990; Jalbert and Golebiowski 2015; Solomon and Mathews 1978). Traditionally, the allergenic potential of the atmosphere has been studied with the aid of different types of particles samplers, being the Hirst and Rotorod types the most used (Hirst 1952; Ogden and Raynor 1967). Once the samples are obtained, pollen grains and spores are counted and their results transformed into "number of biotic particles per cubic meter of air". However, the antigenic content of these particles may vary highly and be influenced by atmospheric and transport conditions. For this reason, it is essential to use optimal and standardized methodologies for detection and quantification of the main aeroallergens capable of causing pollinosis in the population.

One of the first studies carried out in order to detect airborne pollen allergens was conducted on ragweed in 1972 by means of "passive transfer antigen neutralization techniques" (Busse et al. 1972). Later on, in 1981, ragweed allergens were again studied, as well as *Alternaria* spore allergens but, this time, using RAST-type analysis (Agarwal et al. 1981). It was not until 1994 when the first studies about pollen allergens in the atmosphere were published by using enzyme-linked immunosorbent assay (ELISA). These studies were carried out against *Betula* allergens (Pehkonen and Rantio-Lehtimäki 1994; Rantio-Lehtimäki et al. 1994). Two years later, in 1996, the first studies made with Sandwich ELISA, a test specially designed for the quantification of specific allergens, were presented against Bet v 1 and Phl p 5 allergens (Schäppi et al. 1996). Thenceforth, many studies have been carried out in order to quantify pollen allergens in the atmosphere (Jato et al. 2010; Moreno-Grau et al. 2006; Plaza et al. 2016; Schäppi et al. 1997). The main goal of this study was to obtain an optimized and reproducible

ELISA protocol for the quantification of Ole 1 in the atmosphere, which has a high incidence in the Mediterranean region and some North America areas (Bousquet et al. 1985).

An important and very common issue to deal with when we try to find a detailed and proper methodology to process the samples, is the isolation and preservation of the proteins they contain. Usually, most of the published works only specify the methodology related to the antigen quantifications, but not that related to the protein extraction from the samples. Additionally, when the extraction methodology is specified, the results obtained are not compared with different buffers or modifications of them, which does not offer possible alternatives, depending on the allergens we want to study, since not all buffers affect in the same way the extraction and stability of proteins.

2. Materials and Methods

In this paper, different alternatives for extraction and quantification of the allergen Ole e 1 have been tested. For this purpose, a Sandwich ELISA test for Ole e 1 was developed, estimating what antibody concentrations were the most adequate to determinate the corresponding antigenic concentration in aerobiological samples.

Protein extractions were performed from controls of purified olive pollen by means of the use of different extraction buffers, in order to optimize the extraction of the allergen Ole e 1 from pollen grains contained in the air samples. In addition, the effect of these buffers on the behaviour of the ELISA standard curve was studied, and the most appropriate alternative was proposed, depending on the variations observed in the detectability and sensitivity of the assays.

The air samples for protein quantification were taken from the atmosphere of Malaga (southern Spain) during the years 2008, 2009 and 2010 and carried out by using a cyclonic sampler, which is an optimal device for this type of samplings, previously used in numerous studies (Fernández-González et al. 2013; Jato et al. 2010; Plaza et al. 2016; Takahashi et al. 2001). These results were compared with *Olea europaea* pollen counts, obtained by means of a Hirst type volumetric pollen trap during the same period of study.

2.1. Ole e 1 allergen quantification by means of Sandwich ELISA

In order to quantify the allergen Ole e 1 by sandwich ELISA, monoclonal antibodies (mAb), as capture antibodies, and biotinylated polyclonal antibodies (BioAb), as detection antibodies, have been used. Besides this, sensitivity, detectability and antigenic determination range at different concentrations of these antibodies have been compared. Both, antibodies and purified antigens used for the calibration curves, were supplied by Bial-Arístegui (Bilbao, Spain).

Protocol:

The different concentrations of the capture and detection antibodies (for standard curve) tested for the determination of Ole e 1 was the following:

- 3 μg/mL mAb and 0.5 μg/mL BioAb
- 2 μg/mL mAb and 0.5 μg/mL BioAb
- 2 μg/mL mAb and 0.25 μg/mL BioAb
- 1 μg/mL mAb and 0.25 μg/mL BioAb

The concentration range of the standard curve for Ole e 1 allergen was from 100 to 0.195 ng/mL.

Initially, 100 μl of the capture antibody in phosphate buffered saline (PBS), pH 7.4, are added to flat bottom 96-well plates (Costar® 3590, Corning Incorporated, USA) and then incubated covered and sealed with parafilm for at least 12 hours at 4°C in humidity chamber. The liquid is then removed from the plate and 200 μl of blocking buffer (BB), pH 7.4, are added. BB is constituted by PBS, 0.05% Tween 20 and 1% bovine serum albumin (BSA, Amersham RPN412, GE Healthcare, UK). Then, the plate is incubated for one hour at 37°C.

The Ole e 1 allergen standard curve was obtained with this purified protein diluted in BB. Purified proteins of Ole e 1 were diluted sequentially 1:2 in a concentration range of 100 to 0.195 ng/mL and incubated for 2 hours at 37°C.

Subsequently, three washes are carried out with PBS plus Tween 20 (PBS-T) for 2 minutes each. After that, 100 μL of biotinylated antibody in BB buffer are added and incubated for 1 hour at 37° C. Three additional washes are performed with PBS-T and then 100 μL of

Streptoavidin-Peroxidase (SIGMA S5512, Sigma-Aldrich®, USA) diluted in BB buffer at a concentration of 0.25µg/mL are added, followed of incubation for 1 hour at 37° C. Finally, 3 more washes with PBS-T are performed and 100 µL of 3,3 ', 5,5'-tetramethylbenzidine (TMB, SIGMA T0440, Sigma-Aldrich®, USA) added, and the well-plates incubated for 30 minutes in darkness at room temperature. Absorbance at 620 nm was measured by using a microplate reader (Thermo, Multiskan EX), without stopping the reaction and avoiding pH changes.

2.2. Protein extraction from pollen grains

In order to study the extraction of the antigen Ole e 1, known concentrations of purified pollen, supplied by Iber-Polen (Jaen, Spain), were used. For this, dilutions of pure pollen stocks of *Olea europaea* L. were prepared to set homogeneous samples that simulated a day in which 50 pollen grains/m^3 of air were detected in the atmosphere, this concentration being equivalent to one day with moderate pollen concentration in the Mediterranean region. Therefore, we can compare, in a uniform way, the protein concentrations obtained with any extraction buffer.

For this work, the simulation of a 24-hour sampling, performed with a Cyclone trap (Holmes and Emberlin 1994) was used as reference. The total volume of air aspirated in 24 hours by this device is 23.76 m^3, since it sucks 16.5 L of air per minute. For the analysis, the whole sample remaining deposited in an Eppendorf tube is resuspended in 1 mL of extraction buffer. Therefore, the pollen concentration used to perform the protein extractions in one day with a concentration of 50 grains of pollen/m^3 of air was 1188 pollen grains/mL (50 pollen grains x 23.76 m^3 of air).

The homogeneous pollen samples of 1188 pollen grains/mL to which compare the effect of the different extraction buffers were obtained by adding 1 mg of purified pollen in 1 mL of distilled water with a 2% of Tween 20 (to avoid the pollen grains form aggregates) and then counting the pollen grains with the aid of a Neubauer Chamber. Therefore, we obtained the number of pollen grains per mL that, subsequently, was adjusted by dilution to the concentration of 1188 pollen grains/mL in order to perform the extractions.

The extractions were performed with three different types of buffers, which are called in this work as MG, GS and BB.

- MG buffer: proposed by Moreno-Grau et al. (2006). It is composed by 50 mM phosphate buffer, pH 7.4, 125 mM (NH) 4HCO 3, 150 mM NaCl, 3 mM EDTA and 0.005% Tween 20. The extraction methodology proposed by the aforementioned authors was followed in its entirety.

- GS buffer: it has the same composition as MG buffer, but with the following modifications: 250 mM (NH) 4HCO 3, 1% Tween 20 and 0.5% BSA.

- BB buffer (blocking buffer): consisting of PBS, 0.05% Tween 20 and 1% BSA.

All the incubations were made in a total volume of 1 mL, in Eppendorf tubes, for 2 hours, at room temperature and shaking the tubes every 30 minutes. They were then centrifuged at 8000 g for 10 minutes in the case of MG and at 13800 g for 15 minutes in the cases of GS and BB. Both the supernatant and the precipitate were stored in the freezer at -20°C.

The estimation of Ole e 1 concentration by the proposed ELISA, when extracting with a determined buffer, was carried out by three replicates and always extrapolating to the standard curves, incubated with the same extraction buffer.

2.3. Influence of buffers on detectability and sensitivity of the ELISA

For determining the viability of MG, GS and BB buffers in ELISAs, its effects on the detectability and sensitivity were evaluated. In the case of GS buffer, four variants were tested in which concentrations of $(NH)_4HCO_3$ were progressively increased, these being 250, 500, 750 and 1000 mM. The antigens used in each ELISA were Ole e 1 purified proteins, supplied by the company Bial-Arístegui.

2.4. Pollen and airborne allergen monitoring

The study of quantification of pollen and allergens in the atmosphere of Malaga was carried out with two volumetric samplers. Airborne pollen samplings were performed by using a Hirst-type volumetric spore trap (Hirst 1952) by Lanzoni, model VPSS 2000 (Bologna, Italia). Airborne allergen samplings were performed with the aid of an automatic multi-vial Cyclone sampler (Holmes and Emberlin 1994) by Burkard® (Burkard Manufacturing Co Ltd, Hertfordshire, England).

Both samplers were located on the roof of the Biology building of the Faculty of Sciences, University of Malaga, at coordinates 30SUF6847564395 (Datum ETRS89), at a height of 60 meters above sea level and 15 meters above ground level.

The sampling period for both devices took place from April to July, both inclusive, (olive flowering season) of the years 2008, 2009 and 2010. Due to technical problems, the Cyclone sampler started to work a little later, on April 23th 2008 and, occasionally, we do not have samplings for a few days during the rest of the studied period either.

For the preparation, assembly, conservation and analysis of the airborne pollen samples, taken with the Hirst-type sampler, we followed the methodology proposed by the Spanish Aerobiology Network, REA in its acronym in Spanish, in its Management and Quality Manual (Galán et al. 2007)

In order to observe the relationship between pollen concentrations and their respective allergen levels, the results obtained during the entire study period, as well as during the Main Pollen Season or MPS (95% accumulated pollen in the year) (Pathirane 1975), was compared by using the Spearman correlation test since the data did not fit a normal distribution. The Kolmogorov-Smirnov test was used to check the normality of the data.

3. Results

3.1. Ole e 1 quantification by means of ELISA

When comparing the effect of different concentrations of capture and detection antibodies, it can be observed that the most important factor in the detectability of the assay is biotinylated antibodies, the detectability being greater the higher the concentration of the antibodies is (Fig. 1A). Variations in ELISA sensitivity were hardly observed when the concentrations of capture antibodies varied (Fig. 1A).

In the dose-response standard curve of Ole e 1 at concentration of 1 µg/mL for the capture monoclonal antibody, and 0.25 µg/mL for the detection biotinylating polyclonal antibody (Fig. 1B), we obtained a maximum intra-assay coefficient of variation (CV) of 6.6% (n= 16) and a maximum inter-assay CV of 9.5% (n= 10) in the linear range 0.78-25 ng/mL. This combination of antibody concentrations was used to carry out the quantification of the allergen Ole e 1 in the atmosphere of Malaga.

The estimated detection limit (calculated as the mean of blank values plus 3 times the standard deviation) was 0.24 ng/mL.

3.2. *Influence of buffers on detectability and sensitivity of ELISA*

It can be observed that the standard curve that most affinity and detectability provides is that in which the BB buffer has been used, the other ones in which GS-250 and MG were used, are the following more sensitive alternatives but with the peculiarity that GS-250 offers lower saturation at high concentrations, thus offering a higher determination range (Fig. 2A). The sensitivity is the same in all cases in values lower than 3 ng/mL, GS-250 being the most sensitive above this concentration, and MG the least one. It can be also observed that when the concentrations of $(NH)_4HCO_3$ increase in the GS buffer, the affinity and detectability of the calibration curves decrease considerably (Fig. 2B).

3.3. Extraction of antigens in equivalent samples to 50 gp/m^3 of air

When protein quantifications of Ole e 1 were carried out with the three proposed buffers in equivalents of 50 gp/m^3 (Fig. 3), we observed that, in both, supernatants and precipitates, the highest concentrations were obtained when the GS buffer was applied, followed by MG and, finally, BB, with 38% and 71% less, respectively, in relation to GS. By means of ANOVA, it was observed significant differences among the extractions of the three buffers ($\alpha = 0.01$).

3.4. Quantification and correlation analysis of olive pollen and its allergen Ole e 1 in the atmosphere of Malaga

The concentrations of the allergen Ole e 1 and *Olea europaea* pollen during the olive flowering season recorded in Malaga between 2008 and 2010 are shown in Table 1 and Fig. 4. The highest concentrations of Ole e 1 were registered during 2009, 2008 being the year in which the concentrations of this aeroallergen were the lowest. Regarding the pollen counts, they followed exactly the same behaviour, although not in the same proportion, 2009 being the year in which the highest proportion of aero-allergens per pollen grain was detected and 2010 the lowest. Something worthy to be mentioned is the fact that, although they do not necessarily have to coincide in time, the peak days for allergens and pollen were coincident in 2009 and 2010.

Regarding the correlation analysis between daily pollen concentrations and allergen quantifications carried out by means of Spearman test, during the whole study period, as well as during the main pollen season (MPS) (95%), the coefficients showed very high and significant values (α=0.001) during the three years studied (Table 1).

4. Discussion

In this work, the quantification protocol for the protein Ole e 1 (olive tree major allergen (Rodríguez et al. 2001)) by means of double antibody sandwich ELISA was optimized. In addition, the effect of three different buffers (MG, GS and BB), on both ELISAs (Fig. 2) and protein extraction process (Fig. 3), has been studied. The extraction buffer we have named MG, published by Moreno-Grau et al. (2006), is a modification of the buffer proposed by Takahashi et al. (2001). This buffer has been compared with the buffer BB, which is the same buffer commonly used as coating buffer in ELISAs, which has been used to perform protein extractions from pollen grains for the first time in this type of studies. The third buffer we have compared, the so-called GS, is a combination of the two previous buffers.

When carrying out studies of quantification of pollen allergens in the atmosphere of a given territory, we will have to face two fundamental problems that will determine the methodology to use when carrying out the tests in the laboratory. The first one, which is applicable almost globally, is the budgetary shortage affecting certain scientific disciplines, reason why it is necessary to optimize the concentrations of antibodies. In our case, this fact markedly determined the final concentrations of antibodies that were used to carry out the protein quantifications in the aerobiological samples. The second one, which is particular for each area of study, is to choose the extraction buffer as well as the most appropriate methodology, depending on the different allergens that we want to simultaneously study during a given period. For the ELISAs developed in this study, we used the same combinations of antibodies that have been used previously in numerous research papers (Arilla et al. 2002; Moreno-Grau et al. 2006; Plaza et al. 2016). These anti-Ole e 1 antibodies, produced by the company Bial-Arístegui, have proved to be highly recommendable for quantifying Ole e 1, since they have provided high detectability, sensitivity and reproducibility and, therefore, high reliability.

Regarding the budgetary shortage, one of the main advantages of the protocol proposed is that the concentrations of capture and detection antibodies used have been considerably reduced, which cheapen the cost of each ELISA without altering the results. On the other hand, detectability has been the same or higher than in previous studies performed on Ole e 1 in which monoclonal antibodies were used (Moreno-Grau et al. 2006; Plaza et al. 2016; van Ree et al. 2000). This allowed to quantify Ole e 1 in days in which airborne concentrations were of only one pollen grain per cubic meter of air or, even, on days with zero records of pollen grains within the MPS. This demonstrates the fact that may exist pauci- or sub-micronic particles in the atmosphere containing pollen aeroallergens as unveiled by several authors (Agarwal et al. 1984; Busse et al. 1972; Cabrera et al. 2002; Fernandez-Caldas et al. 1989; Linares et al. 2007; Rantio-Lehtimäki et al. 1994; Schäppi et al. 1997; Spieksma et al. 1990, 1995)

The achievement of these more favourable results is related in part to the use of TMB versus OPD (o-phenylenediamine) as peroxidase substrates. It was observed that, stopping the OPD and TMB colorimetric reaction by adding an acid, the absorbance levels obtained were altered considerably, the detectability and reproducibility being negatively affected. Therefore, we decided to use TMB instead OPD in the assays, but without stopping the reaction, since those were the conditions under the best results were obtained (Fig. 5).

As regards the extraction buffer, in order to decide which methodology and which extraction buffer use to carry out the allergen quantification when several pollen types are involved in the study, at the same time, in a certain area, it would be necessary to previously carry out all the tests proposed here for each one of these allergens. This allows to stablish which buffer is the most suitable for the combination of allergens to quantify. In addition, it must be taken into account the pollen concentrations of these pollen types registered in the atmosphere of the study area during the MPS. Based on these considerations, in this work, although only the results for Ole e 1, the major allergen of *Olea europea* L., are presented, the decision to use the buffer BB as mean of extraction and quantification was due to the fact that the studied samples contained also other important aeroallergens. In fact, Par j 1-Par j 2, the major allergens of *Parietaria judaica* L. and Lol p 1, grasses group I, the major allergen of *Lolium perenne* L. (data not shown) are present in the atmosphere of Malaga at the same time that Ole e 1 and they were much more favoured by the use of this extraction buffer. Therefore, based on the results obtained in the protein extraction tests (Fig. 3), if we are going to study in a sample only the allergen Ole e 1, it would be advisable to use the buffer GS-250 since we obtained almost 40% and 70% more extraction, on average, in comparison to the buffers MG and BB respectively.
However, in the case the atmosphere of Malaga, the buffer BB was used on the basis of three fundamental reasons: it was the buffer that provided the best detectability among the three buffers tested (Fig. 2A); because it was a priority buffer to study the allergens Lol p 1, Par j 1

and Par j 2 during the same period (data not shown); and, finally, in spite of not being the most efficient option for Ole e 1, in terms of extraction, in days with a single pollen grain of olive per cubic meter of air, as said above, we obtained positive values, within the estimated detection limit for the allergen Ole e 1. All these are factors that should be taken into account in any similar study.

By way of conclusion, on the basis of the results obtained (Table 1 and Figure 4), the ELISA protocol described in the present paper, together with the buffer BB used to carry out the extractions of the antigen Ole e 1, have proved to be fully adequate to carry out the antigenic analysis of aerobiological samples, being a great tool for the study of a pollen allergen as important in the Mediterranean region such as Ole e 1 (Bousquet et al. 1985; Wheeler 1992).

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Table 1

Title: Pollen and allergen results and statistical analysis.

Footnote: Total pollen and peak day of *Olea europaea* and Ole e 1 allergen quantification obtained by Hirst type and Cyclone samplers, respectively, during study period. Spearman rank correlation coefficients obtained between pollen concentrations and allergen quantifications during the study period (Total) and the main pollen season (MPS). ***$\alpha \leq 0.001$. In brackets date of peak day for pollen and allergen and number of data for Spearman correlation

Figure captions

Fig. 1 Dose-response curves in two-site sandwich ELISA of Ole e 1 purified protein. (A): Comparison between several concentrations of antibodies. (O.D.) optical density. (B): Curve with 1 μg/mL of capture antibody and 0.25 μg/mL of detection antibody.

Fig. 2 Effects of different buffers in the detectability and sensitivity of the ELISA for Ole e 1. (A): Comparison of buffers MG, GS-250 and BB. (B): Comparison of buffers GS-250, GS-500, GS-750 and GS-1000.

Fig. 3 Extractions of Ole e 1 antigens in equivalent of 50 pollen grains/m^3 using buffers GS-250, MG and BB.

Fig. 4 Concentrations of Ole e 1 allergen (line) and *Olea europea* pollen (shading) during the olive grove blooming between 2008 and 2010 obtained by Cyclone and Hirst type samplers respectively. The gap in 2010 was due to technical problems.

Fig. 5 Comparison of the dose-response curves of the ELISA for Ole e 1 by using of OPD (white square) and TMB (black circle) as peroxidase substrate, stopping the colorimetric reaction by means of acid (dashed line) and without stopping the reaction and reading the results after 30 minutes of reaction (continuous line).