To editor and reviewers,

We thank very much the reviewers for their effort and their time to read our manuscript and provide very valuable comments. After carefully reading all the comments of the two referees, we understand that our manuscript could create confusion. Our main intention was to describe an approach to advance ecological research with all the necessary steps, including sampling, extraction of metabolites in liquid phase, analyses with 3 different instruments, data mining and analysis, to obtain the metabolomic fingerprints from particles in suspension in the lower atmosphere. We expect this method will be useful for addressing novel questions in ecology and other related disciplines. Therefore, it is a methodologic manuscript oriented especially to ecological research and is not intended to target atmospheric chemistry studies, although we recognize that this is more of the focus for the AMT journal. Since this method describes all the details necessary to characterize the metabolomes of aerosols, we thought that AMT was a suitable target journal. However, while we think that our methodology does provide very valuable information for atmospheric scientists, we recognize that this method is aimed mainly to assist the ecological community and now acknowledge that the scope of AMT is more oriented to publish research focused in "advances in remote sensing, as well as in situ and laboratory measurement techniques for the constituents and properties of the Earth's atmosphere". After carefully reading all the reviewer comments we believe that our manuscript would probably fit better to an ecological journal but since our manuscript was still considered for revision and will be published in the online discussion, we have now modified the text clarifying the aims of the method. Note that it does address any of the issues related to the field of aerosol chemistry, as this was never our intention. We hope the aims of this methodological manuscript are now clearer.

Anonymous Referee #1

Received and published: 7 October 2016 Referee report for

"Atmo-metabolomics: a new measurement approach for investigating aerosol composition and ecosystem functioning" by Albert Rivas-Ubach et al., submitted to AMT This manuscript describes the organic analysis of ambient aerosols with three techniques, GC-MS, LC-MS and direct infusion MS.

The focus of this manuscript is not clear at all. The title seems to suggest that a new technique is described but all that is provided are analyses techniques that are used in the community since years. So, I cannot see what the new aspect of this paper is. Creating a new word for existing analysis strategies is not helpful.

It is not clear what the focus of the paper should be. The actual results seem to suggest that tracers of PBAP are the main focus but then the manuscript often mentions that the aim is to determine the overall particle composition, which is clearly dominated by many other sources ad not only PBAP.

As mentioned previously, our manuscript is focused on the ecological community. While these techniques have been available to the scientific community, these metabolomic techniques have not been used to characterize the metabolomes of aerosols. The aim is to provide ecologists and environmental scientists with a tool to assess the ecosystem status and stress levels through the atmospheric detection of biochemical compounds. To demonstrate our method, we collected aerosol samples from two distinct seasons to test our methodology but without attempting a deep analysis of the differences between the seasons. We simply chose seasonality for comparison purposes and to test the sensitivity of the instruments to detect differences between the metabolomic fingerprints; other factors such as different ecosystems could have been chosen too.

Metabolomics techniques, which include all the steps from the sampling to the analysis of the data, have been widely used to measure the metabolomes from living systems. However, metabolomics can also be applied to obtain metabolic signatures from any sample contaning natural organic matter (NOM). We acknowledge that diverse mass spectrometry techniques such as GC-MS have been used for years in the atmospheric

research, especially to detect and quantify volatile species such as BVOCs. Nonetheless, our purpose in this manuscript was never to improve or replace those well-defined approaches. With the approach that we present in this manuscript, researchers should be able to detect metabolites in aerosols directly linked with the main physiological processes occurring in living organisms. Moreover, our manuscript provides a good synthesis of the main techniques used for metabolomic analyses, including FT-ICR-MS, which we believe it is especially useful for those researchers interested to introduce metabolomics approaches to further understand the link between the atmosphere and ecosystems

Figure 1-3 are to a large extent trivial and would be better suited in a review rather than in a research paper.

The main intention of our manuscript was to explain in detail a methodology of sampling particles in suspension.

Figure 1 shows the most common sources of compounds and particulates as well as their major roles and their interaction with ecosystems, humans and climate. We think it is useful to provide a general background, especially to the readers from ecological and other environmental disciplines, of the main sources and processes of compounds in the atmosphere.

Figure 2 describes the sampling method; we consider this to be important for a methodological paper; however, following the referee's comment, we have now moved this figure to the supporting information (Figure S1). This figure provides a general picture of how the sampling was performed and how the cassettes should hold the filters for a homogeneous sampling; important information for researchers that are not familiar with aerosol sampling.

Figure 3 (now Figure 2) describes step by step how to extract the metabolites from the filters into a solution. We think showing this figure in the main text of a methodological article for metabolomics analyses is necessary.

p. 4 looks to me more like a conclusion section rather than text for an introduction.

The prupose of this section is to make the case for why this approach would be useful for ecologists and other disciplines. It is not based on the results of our study but just shows the need and the potential value of demonstrating this approach.

The results (e.g. in Figure 4-8) show some interesting findings but overall they are hardly discussed and compared to existing, up to date literature. For all the applied techniques (GC, LC, and direct infusion high resolution MS) there are many current publications, which need to be discussed.

The main objective of our methodological article is to provide the proof that the "atmo-metabolomes" (metabolomic fingerprints) of spring and summer differ statistically between them. Additionally, the filters were analyzed with three different instruments (GC-MS, LC-MS, FTICR) to test whether they were sensitive enough to detect significant changes ($P < 0.05$) between seasons. To discuss all the details obtained from each instrument would shift the aim of this manuscript and it would considerably lengthen the text. One of the main aims of the study was to provide a method able to discern the differences in aerosol metabolomes between two different seasons with three different instruments.

We acknowledge the large aerosol bibliography available using the MS techniques. However, as discussed above, this article is not intended to be a review of all the bibliography or a revision of current atmospheric sampling techniques. We agree that it is important to show a certain properly referenced background directly related with our instruments and results. In the new version of the manuscript we restructured the introduction providing more information related to MS studies.

The space in a journal is limited and the section providing discussion of the results from the three mass spectrometry instruments, which is not the central focus of the article, is already almost 800 words. For this reason, we have not extended this part in order to keep the aim of the study clear in a relatively concise manuscript.

Aerosol sampling (p. 8). It is not clear why the commercial filter holders were modified. This should be clearly motivated.

Filter cassettes do not require modification if they already ensure a homogeneous distribution of the air-flow along the filter surface during the sampling. The commercial cassettes we used in our study were designed in a way that the air did not flow properly along the entire surface of the filters, so they had to be slightly modified to achieve that homogeneous distribution of the air-flow. We wanted to provide all the sampling details in the manuscript but we finally decided to delete those sentences from the M&M section to avoid any confusion.

Filter sampling is used in aerosol sciences since decades and it is a standard method. However, much of the sampling description seems to suggest that a new technique is presented, which is not the case. Aerosol was collected without any upper size cut as it is standard practice in aerosol science. This is a serious short-coming and brings the severe risk that large biological material is collected that would not be transported over significant distances due their large size. Collecting aerosol within a certain size range is absolutely essential for any aerosol sampling and analysis. Therefore, the results of this study cannot claim to represent atmospheric aerosols.

We agree that our sampling procedures do not differ substantially from the ones used in atmospheric sciences. As a methodological article, its aim is to explain in detail all the main steps in order to obtain the metabolomic fingerprints of the particles in suspension in the low atmosphere with different analytical instruments. In this article we put together in a comprehensive way, especially for the ecological and plant science community, all those steps and we are convinced that a detailed description of our sampling method is necessary. However, contrary to many aerosol sampling methods, our methodology is very flexible, portable and economic, and probably the most important aspect: very simple. As mentioned above, we wanted to put all the details together in a single manuscript and we expect it to be valuable for the research community.

Furthermore, as mentioned by the referee, our sampling method can collect large biological material. While an upper cut size can be employed to increase the footprint of the ecosystem represented, the approach used here is suitable for characterizing the ecosystem of the immedidate surrounding area which was our objective for this study.

As stated before, the aim of this article was not to provide answers on the chemistry processes occurring in the atmosphere in a specific moment but to explain a method to obtain the metabolomic profiles of the particles in suspension in the atmosphere. For this reason, it is not necessary to sample a specific size range but that sample can include all particles to obtain a general picture of which molecular compounds are present in the particle fraction in the lower atmosphere. We are convinced that this methodological approach provides very valuable information for the ecological community.

GC and LC results. LC results report 18 identified compounds. GC analysis mention 14 compounds. Most comprehensive aerosol analyses presented in the literature using these techniques identify many more compounds. It is not clear why in the study presented here only a small number of compounds was identified. There is no evidence given how these compounds were identified. Simply mentioning "Library identification" is not sufficient. More details would need to be given.

The number of compounds identified and verified in a sample depends mainly on three factors: i) the solvents used for the extraction of metabolites, ii) the concentration of metabolites in the samples and iii) the specific metabolites present in the metabolite databases.

Typically, un-targeted metabolomics techniques have been applied to obtain the metabolic fingerprints and profiles from living organisms. For this reason the metabolite databases include metabolites from living organisms and mainly from their primary metabolism. The focus of our method are compounds known to be metabolites coming from living organisms. According to the metabolite databases used for this study we assigned a bit more than 30 compounds combining both GC and LC-MS methodologies that are directly linked to the metabolism of organisms, likely from plants. Additionally, sampling was performed in an area with very low biological activity compared to more forested areas and organic volatile compounds derived from plants or anthropogenic emissions could not be identified by our libraries since those compounds are simply not normally listed in our metabolomic libraries. As we mentioned in the manuscript: "The techniques to characterize the gas phase component of atmo-metabolomes are well described elsewhere (Smith and Španěl,

2011; Tholl et al., 2006). Our purpose here is to describe an atmo-metabolomic method for sampling aerosols and characterize the particle phase of the atmo-metabolomes.".

In the past version of the manuscript we also mentioned that "Metabolite assignation with LC-MS was performed by our metabolite library with more than 200 typical metabolites usually present in plants and fungi including products from primary and secondary metabolism"; so we made it clear which kind of compounds we were targeting. Our intention was never to reproduce a method to sample all atmospheric organic compounds but to measure with LC-MS and GC-MS compounds present in solid particles coming from living systems.

We have rewritten the section regarding the metabolite identification by LC-MS. It now reads: "Metabolite assignation with LC-MS was performed by our metabolite library with more than 200 typical metabolites usually present in plants and fungi including products from primary and secondary metabolism. Assignation were performed separately for each ionization mode (positive and negative) and using the exact mass of metabolites and RT. ESI do not typically fragment all ions, however, in some molecules we could still detect some fragments which were also considered for the metabolite assignation and relative quantification. According to Sumner et al., 2007, our LC-MS metabolite assignment is putative since it was based on total exact mass of the metabolite and RT of standard measurements in the instrument. However, the use of high MS resolution achieved with Orbitrap technology and RT reduces substantially the number of false positive assignations. For more detailed information regarding the metabolite assignation see Rivas-Ubach et al., (2016b). RT and m/z values of metabolite matching for LC-MS are shown in Table S2."

Figure 7 and 8 show interesting results but more discussion would be needed.

Already responded above.

Section 4.1 is mostly trivial discussion and can be shortened a lot. The same applies to much of section 4.3.

Following the referee's advice, we have now shortened the section 4.1 and deleted the section 4.3 re-organizing some content into the introduction.

Anonymous Referee #2

Received and published: 2 October 2016

Overview: This manuscript describes a metabolic-approach for the analysis of atmospheric aerosol. The approach includes GC/MS, LC/MS and direct injection FT-ICRMS measurements. To demonstrate the potential for this method to contribute toward an improved understanding of natural metabolites associated with aerosol, the authors studied the composition of aerosol collected in the spring and the summer. Key results include: the finding that plant-related metabolites (namely organic acids and carbohydrates) are higher in the spring than summer; the summer samples included metabolites associated with oxidative stress; and summer aerosol composition included a higher fraction of high molecular weight compounds than spring with a higher O/C ratio. The manuscript contains very valuable laboratory method information that is well referenced. However, the details about the advanced statistical analysis are deficient. The introduction and methods sections are well-written, but the results and discussion section seems to be presented poorly. Given the inadequate description of the statistical approach, I found the results section to be especially difficult to understand. Another aspect for further consideration is placing this work into the context of the current literature on aerosol chemistry. There's quite a bit of similar work without a so-called "metabolomics" approach that is relevant.

Many thanks for the positive evaluation on the interest of the study and for considering very valuable the laboratory method information. *We have now rewritten the introduction, focusing it on the ecological applications of the study of the metabolomic fingerprint of ecosystems on atmospheric aerosols. And to address the referee's concerns, we have now clarified the statistical methods section and combined the results and discussion. We hope that now the text is clearer.*

We acknowledge that GC-MS and other mass spectrometry techniques have been widely used in the atmospheric research. Nonetheless, as in our response to the previous referee, our purpose for this manuscript was not to improve or replace those well-defined approaches or to investigate the chemistry of the atmosphere. We present an approach that is novel and useful for the ecological community by enabling researchers to detect aerosol metabolites that may be directly linked with the main physiological and ecological processes of living organisms.

Specific suggestions:

The literature review of atmospheric aerosol composition is weak and outdated. Since the authors claim to be the first to apply metabolomics techniques to aerosol, which are not necessarily different from other composition measurements, it would be nice if they would acknowledge the vast literature of GC/MS, LC/MS and FT-ICR-MS results aimed at understanding aerosol composition.

Our manuscript aims to describe in enough detail the set of necessary procedures to obtain the metabolome profiles from aerosols. As mentioned above, this method is mainly focused to detect signatures directly linked to the main physiological and ecological processes of organisms; metabolites which are not volatile but are also part of many particles in suspension in the atmosphere. We have modified the introduction more clearly focusing the aims on the ecological aspects.

Lines 102 - 106: How important is the carbon and nutrient deposition of aerosols to ecological systems?

We have expanded the section in the introduction about the aerosol deposition on ecosystems.

Lines 145 - 148: The atmospheric system is quite complex and the goals of this manuscript are quite broad. I suggest some refinement of the manuscript goals with a focus on a well-defined portion of the atmospheric system, since this work doesn't address larger spatial sampling, research flight measurements, or multiphase measurements.

As mentioned above, we have rewritten the introduction section. We have now better focused our manuscript and hope the purposes of our method are clearer.

Line 187: I often see this statement in manuscripts, but it is not a realistic resolving power for environmental samples. Can the authors cite a paper demonstrating the successful measurement of a complex mixture with a resolving power and actual resolution of 1,000,000?

We reviewed the capability of FT-ICR-MS in the manuscript as an introduction of this analytical method. Therefore, we have to report the maximum resolving power that FT-ICR-MS can achieve. However, we did not state that such resolving power is currently used in environmental study. We stated the actual resolving power (~400,000 at 400 m/z) for our samples.

The organization of sections 2.3 - 2.5 is a little bit strange. Specifically, a description of the GC/MS sample prep (in 2.3) is given followed by LC/MS analysis (2.4), which is in turn followed by the GC/MS analysis (2.5).

We understand that this may create some confusion, however, we wanted to be consistent and we have followed the same order for the methods and results along the article; LC-MS, GC-MS and FTICR consecutively.

The section 2.3 described the extraction of metabolites from the quartz filters which is common for all the three MS techniques (LC, GC and ICR). However, differently to LC-MS and DI-FT-ICR extracts, samples for GC-MS require an additional step; the derivatization of metabolites. This step is also indicated in the Figure 3 and it is clearly linked to the extraction of metabolites. We considered the derivatization should not be in the following section of GC-MS analyses (2.5). However, we could consider moving this section if required.

After the section for sample preparation (2.3)(common for the three techniques), we have described the parameters used for each one of the MS instruments separately according to the order established (2.4 for LC-MS, 2.5 for GC-MS and 2.6 for DI-FTICR).

Following the instrument analysis sections, the next 3 sections (2.7, 2.8 and 2.9) provide the details to obtain the numerical data from each of the instruments. Also, these 3 sections follow the same order established, so 2.7 for LC-MS, 2.8 for GC-MS and 2.9 for FT-ICR.

So, our logic for the description of the methods was:

1. Extraction of metabolites. (2.3)

2. Data acquisition by each MS instrument (2.4, 2.5 and 2.6)

3. Processing of MS chromatograms/spectra from each instrument. (2.7, 2.8 and 2.9)

We think that this order is comprehensive; however, we can change the distribution of the methods if required. An option would be to include all the instruments in a single "Data acquisition" and "Processing of chromatograms/spectra" section by using subtitles.

Line 346: How were both positive and negative ionization performed with LC/MS? Were they done in separate runs or using fast polarity switching?

The LTQ Orbitrap Velos cannot switch ionization polarities quickly. Only the most recent Q-Exactive and the new LUMOS Orbitrap versions can operate with fast polarity switch. So samples were first injected in positive mode and then in negative mode. We now have indicated this detail in the manuscript and it can be read as: "All samples were first analyzed in positive (+) ionization mode and later in negative (-) ionization mode."

Line 371: Was negative mode ESI performed? Why was negative ESI not performed for atmospheric aerosol characterization?

Analyses in FT-ICR-MS were performed exclusively in negative mode as already mentioned in the manuscript: "Samples were directly infused into the mass spectrometer using a standard Bruker electrospray ionization (ESI) in negative mode at a flow rate of 3.0 µL/min through an Agilent 1200 series pump (Agilent Technologies, Santa Clara, CA, USA ."

FT-ICR-MS in negative mode is the most used method to investigate natural organic matter. While positive ESI mode could increase the compound coverage, we opted to use negative mode only as our instrument was optimized under ESI(-) for organic matter exploration.

Lines 381 - 383: Both fragment ions and exact mass were used to assign metabolites. Were these measurements made in single runs LTQ MS/MS and FT-MS in tandem or something else?

Although we already referenced a manuscript where the metabolite assignation is well described, we agree that this section should be more detailed, especially for a methodological article like the present one. We have extended this section of the

manuscript. It now reads: "Metabolite assignation with LC-MS was performed by our metabolite library with more than 200 typical metabolites usually present in plants and fungi including products from primary and secondary metabolism. Assignation were performed separately for each ionization mode (positive and negative) and using the exact mass of metabolites and RT. ESI do not typically fragment all ions, however, in some molecules we could still detect some fragments which were also considered for the metabolite assignation and relative quantification. According to Sumner et al., 2007, our LC-MS metabolite assignment is putative since it was based on total exact mass of the metabolite and RT of standard measurements in the instrument. However, the use of high MS resolution achieved with Orbitrap technology and RT reduces substantially the number of false positive assignations. For more detailed information regarding the metabolite assignation see Rivas-Ubach et al., (2016b). RT and m/z values of metabolite matching for LC-MS are shown in Table S2."

Line 418: Why was S/N > 7 used as a threshold? How was the S/N determined?

S/N was determined in the Bruker Data Analysis software, which was assessed based on baselines near each peak. S/N of 3 and 5 are often used in natural organic matter exploration as that range is considered as the mínimum detection limit (Riedel and Dittmar 2014). We chose S/N>7 for a more conservative measure.

Lines 473 - 476: How many data points were used for this analysis? How were the sub-sets of data selected for analysis? Some discussion on the QA filtering procedures and selection of data for statistical analysis is greatly needed.

Each analytical technique generated their own data that were posteriorly analyzed separately. All the data (metabolomic fingerprints) from each instrument were used to perform the PERMANOVAs. PERMANOVAs were performed separately.

As this manuscript was not especially focused on the understanding of the metabolomes or chemical signatures between summer and spring aerosols, we did not include the number of features we observed and used for the statistical analyses. We can include this information if you think it necessary.

We have added some more text in the material and methods section explaining the data filtering in more detail. Now the text reads: "For each season (spring and summer) and dataset (LC-MS, GC-MS and FT-ICR-MS), the variables present in less than 50% of the samples were excluded for the statistical analyses. The signal values measured in the experimental blanks in each of the instruments were subtracted from the datasets. Each of the variables from metabolome fingerprints obtained from each MS instrument were posteriorly submitted to Levene's and Shapiro tests to assess homogeneity of variances and normality, respectively. Variables that did not comply with those statistical assumptions were removed from the datasets. Outlier measurements were replaced for missing values and were defined as those measurements of a specific variable with values three-fold higher than the third quartile or three-fold lower than the first quartile of each season. For FT-ICR-MS datasets we have been very conservative and only the formula assigned features that presented less than 0.3ppm of error were used although cutoff values up to 0.5ppm are typically used (Osterholz et al., 2016)."

Line 487: In what sense is the statistical significance?

As typically used in the vast majority of environmental studies, the alpha error or type I error is maintained at 5%. The term "statistical significance" is widely used for P values lower than 0.05 for a given test. So, alpha error (type I error), the probability of rejecting the null hypothesis when is true, was maintained at 5%.

Lines 489-496: What do these compounds indicate? How were they identified?

In the results section we only indicate which compounds increased significantly (P <0.05) or marginally significantly (P <0.1) in the spring samples. Some of the metabolites identified are briefly discussed in the discussion section (4.4). We did not discuss all the results obtained with each of the instruments since it would be out of the main aim of the study. This article is just a methodological article and we have focused the discussion on the major results and it was not our intention to investigate all of the differences between the seasons.

Those compounds were identified according to our LC-MS database of metabolites of plants and fungi, however, as already mentioned above we have now extended the section of metabolite identification and provided more details.

501-504: This approach from Kim et al. is highly speculative. It's also not an appropriate approach for atmospheric aerosol. Did you extra proteins? How did you verify protein-like components?

We highly agree with the referee. Although the compound classification obtained from van Krevelen (vK) diagrams (O:C vs. H:C) provides a certain approximation of the composition of the samples, we also think that their use should be limited. However, vK diagrams are widely used to understand the chemical changes in samples and this classification is still widely used to represent the FTICR data. Because this compound classification is a widely used method to understand organic matter composition, our intention was to show this to the readers. However, it should be noted that even in the previous manuscript version we only briefly mentioned this classification. In fact, we are already working on another manuscript reviewing this commonly used compound classification for FTICR data. For this reason, in the new version of the manuscript we finally decided to retain the review of the existence of such classification but we have deleted the previous Figure 7.

Lines 517 - 520: What is the meaning of this observation?

Here we mention that particles in summer showed significantly higher intensities in features with higher O/C ratios. This result is briefly discussed in the discussion method, however, as the aim of the article is solely methodological, we did not discuss each of the results in depth. We simply chose two seasons to test if we could detect statistically significant differences between the "atmo-metabolomes" between the two seasons. Different factors could be chosen for this test, like two different ecosystems but we considered that seasonality was more a feasible and comprehensive factor to test.

Aerosol sampling information is vague and seems to imply that the authors are unfamiliar with standard sampling techniques for atmospheric chemistry. How did you assess the total carbon concentrations, filter artifacts, and other recovery issues?

Our intention was not to reproduce a standard atmospheric chemistry sampling technique as we recognize that there are numerous researchers focused on the chemistry transformations in the atmosphere and for that reason many specific protocols are typically used. However, our simple method is suitable for characterizing the metabolome of the atmosphere.

The aim of metabolomics is to compare relatively different groups of samples. Since it is practically impossible to obtain a full metabolome in terms of absolute concentrations for each of the detected metabolites, as long as the sample preparation is performed equally for all the samples we can perform a relative comparison between groups of samples. Filter artifacts were coped with experimental blanks that were injected to all instruments and any signal obtained from those blanks was posteriorly subtracted from the original samples. The use of blanks is a standard procedure for any metabolomics study. We have now included more details in the material and methods to respond to those concerns.

Sampling flow rates are expected to change with diurnal cycles (e.g., temperature & pressure); how was this recorded or accounted for?

Each filter was sampled exactly for the same amount of time and in the same time range as described in the material and methods section: "The pump was working daily during 18 consecutive hours and pumped air at 30 L per minute through each filter. Filters were replaced manually before 09:00am and the pump started working automatically at 09:00am and stopped automatically at 03:00am the following day. Filters were stored at -80°C until metabolite extraction. Filters were sampled on a tower at 8 meters height."

Lines 535 - 537: The purpose of the study was to assess the sensitivity of different mass spectrometry instruments. But, I didn't understand how that was accomplished? Did you

define method detection limits or find any limitations in your approach? More discussion on this would be appreciated.

We rely on the statistical analyses to test the sensitivity of the used techniques to detect changes between seasons. We sampled in an area with a very low primary producer activity and still we were able to detect significant differences in the overall atmo-metabolomes between spring and summer. The significance obtained in the PERMANOVA test proves that each of the techniques was sensitive enough to detect changes between those samples. The principal component analyses (PCAs) for each of the instruments also prove that the instruments were able to detect significantly different overall composition in the spring vs. summer samples. In order to clarify this concern, we have modified the text properly in different sections.

How does you approach differ from the existing approaches to canopy measurements or other ecological studies focused on atmospheric-biosphere exchange?

In this article we explained, and put together, the different steps to obtain the metabolomic fingerpints (or metabolomic signatures) from particles sampled in the lower atmosphere. As far as we know, no other approach for analyzing aerosol metabolomes has been published.

Similar sampling methods can be performed in other ways with different pumps and filters, however, the method we propose is more portable (lower weight and volume), flexible (can be easily manipulated in different ways) and more economic than the commonly commercialized prototypes for aerosol filter sampling. Also as a methodological article we provided detailed information on how our sampling was designed and performed. As discussed in the manuscript, the main idea is to obtain the minimum values in the filter-size/pump-flow ratio to concentrate as much as possible the filters. Our objective was not to perform a comparative study with all the available sampling methods. We just described a very simple and flexible method that samples particles in suspension efficiently and at a low cost. In addition, researchers can choose the filter size they require while many commercial systems are compatible only with a unique filter size.

Lines 584-587: Which solvents did you use to sequentially extract the filters? How did you evaluate the results of various solvent combinations?

We did not perform a sequential extraction in this study. We used methanol:water (80:20) as one of the most widely used solvent mix for extraction of metabolites. We cite different studies and methods where the number of extractions and recovery is discussed. We did not attempt to use a whole variety of extraction methods; we only aimed to show a generally used extraction method to investigate whether the analytical techniques can differenciate statistically the metabolomes between spring and summer aerosols. We also mention that this extraction method is not exclusive but suggested and indicate that different extraction methods can be also used. As widely discussed in several analytical chemistry articles, different extraction methods obtain different range of metabolites based mainly on their polarity.

Lines 590-591: What was quantified in your study?

In this study we performed a relative quantification of the metabolomic fingerprints for comparative analyses between spring and summer.

Lines 596 - 600: How was the absorption extract recovery assessed?

We measured how much volume of solvent was recovered (after the extraction procedures) with respect to the initial solvent added. In the text we mention that we can get an extraction recovery of 89% which indicates that we recover 0.89mL per each 1mL added to the tubes with the filters to perform the extraction. We did not think that it was necessary to incorporate this information in the methods section. However, we can introduce the explanation if the referee thinks it is necessary.

Line 623: "match" or assign?

We appreciate you made us notice this, we agree “assign” is more suitable than “match” in this sentence. We have changed the word in the manuscript.

Lines 706-710: Please clarify how the "metabolic fingerprint" was defined/classified?

We have added a clarification of metabolomic fingerprints and metabolomic profiles in the introduction section. Now the text reads: "The first step to characterize a metabolome profile is to obtain the chemical signature of the sample (metabolomic fingerprint) without further molecular identification (Sardans et al., 2011). The identification of specific metabolites can be further obtained by the information present in the metabolomic fingerprints. In this study, we describe the different procedures to obtain the metabolomic fingerprints and identify molecular compounds from aerosols. This atmo-ecometabolomics methodology is a potential tool to shed light in novel questions in ecology, especially for the ecosystem-atmosphere interface."

Table 1: Fingerprint information is unclear. Please add some explanation in the body of the paper.

See comment above.

Figure 1: What about aqueous phase processing of VOCs or aerosol?

We modified the figure but the different atmospheric VOCs transformations are not presented in detail since it was never our intention to address that issue in this manuscript.

Figure 3: How were common inorganic ions removed from the samples before DI-FT- ICR-MS?

It should be noted that most of the inorganic ions are at much lower mass range than our FTICR-MS analytical window (100-1200 m/z). Thus, unless those ions generate clusters that would interfere with the FT-ICR-MS measurements, such as sodium and chloride, removal of inorganic ions were not necessary. In addition, such a problem is more evident in direct infusion positive ion mode, which was not considered in this study.

Figure 5: I assume this is the list of "metabolic fingerprint" species. Please clarify.

As mentioned before we have included the definition of what a metabolic fingerprint is. The list of metabolites does not represent the entire fingerprint of the different seasons but only the portion that has been identified/assigned. We hope it is now clearer.

Figure 7: How were the species in (a) subsetted from the whole dataset?

As explained before, we did not use a subset of the datasets but the whole amount of detected features. However, we have now deleted this figure from the new manuscript version.

Atmo-ecometabolomics: a new measurement approach for further investigate the link of atmospheric particles composition with ecosystem functioning.

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Abstract.

Aerosols directly and indirectly play crucial roles in the processes controlling the composition of the atmosphere and the functioning of ecosystems. Gaining a deeper understanding of the chemical composition of aerosols is beginning to be recognized as important for ecological research. A comprehension of the chemical composition of aerosol particles chemistry can potentially provide valuable information to further understand the link between aerosols and ecosystems. In this study, we used mass spectrometry (MS) coupled to liquid chromatography (LC-MS), gas chromatography (GC-MS) and Fourier transform ion cyclotron resonance (FT-ICR-MS) to describe step by step an efficient method to characterize the chemical composition of aerosols , namely the atmo-metabolome, from two distinct seasons: spring and summer. We used the data to test statistically whether the analytical platforms were sensitive enough as to detect overall differences between season atmo-metabolomes. Our results showed that our sampling and extraction methods are suitable for aerosol chemical characterization with any of the analytical platforms used in this study. The three datasets obtained from these individual platforms showed significant differences of the overall atmo-metabolome between spring and summer. LC-MS and GC-MS analyses identified several metabolites that can be attributed to pollen and other plant-related aerosols. Spring samples exhibit higher concentrations of metabolites linked to higher plant activity while summer samples had higher concentrations of metabolites that may reflect certain oxidative stresses. FT-ICR-MS analysis showed that summer aerosols were generally higher in molecular weight and with higher O/C ratios, indicating higher oxidation levels and condensation of compounds relative to spring. Our method represents advanced novel approach to study the link between the composition of aerosols and ecosystems.

1. Introduction

1.1 Atmo-ecometabolomics.

Aerosols are solids and/or liquids in suspension typically derived from both biogenic and anthropogenic sources (Canagaratna et al., 2007). Primary biological aerosol particles (PBAP) are directly released from organisms and include cells such as pollen, spores, or whole microorganisms as well as fragments from plants and animal debris (Després et al., 2012). Primary producers also produce large amounts of volatile organic compounds (VOCs) which are emitted into the atmosphere and together with anthropogenic sources, such as the combustion of fossil fuels, are oxidized and then condense forming secondary organic aerosols (SOA) (Després et al., 2012; Fuzzi et al., 2006; Kirkby et al., 2016; Pandis et al., 1992) (Figure 1). To date, most research have focused on how aerosols affect climate system and atmospheric processes (Andreae and Crutzen, 1997; Ayers and Gras, 1991; Baustian et al., 2012; Carlton et al., 2010; Després et al., 2012; Jokinen et al., 2015; Ramanathan et al., 2001; Zhang et al., 2004). However, the components of the biosphere, such as plants, are in constant interaction with aerosols and can play important roles in aquatic and terrestrial ecosystems at different levels (Baker et al., 2003; Gu et al., 2002; Mahowald et al., 2005; Seco et al., 2007). For example, aerosols can serve as important carbon and nutrient sources for the phyllosphere, which is the microbial communities coexisting in plant leaves (Arnold et al., 2000; Lindow and Brandl, 2003; Vorholt, 2012). The microbial diversity of the phyllosphere can produce a variety of effects on their hosts and therefore can affect the ecosystems (Peñuelas and Terradas, 2014; Whipps et al., 2008). Plants also can absorb deposited particles from the atmosphere (Fageria et al., 2009; Seco et al., 2007; Uzu et al., 2010; Wedding et al., 1975) but the effects of plant particle uptake has been mainly focused for for trace metals (Achotegui-Castells et al., 2013; Feng et al., 2011; Uzu et al., 2010; Xiong et al., 2014) and other significant nutrients for agricultural purposes (Fernández and Brown, 2013). In aquatic ecosystems, much research has focused on the aerosol deposition as the nutrient source for phytoplantkon (Baker et al., 2003; Paerl, 1997; Paytan et al., 2009; Wang et al., 2015). Aerosol deposition represents thus an important source of nutrients for ecosystems (Baker et al., 2003; Wang et al., 2015). It is has been widely studied that the different nutrient proportions, mainly C, N and P, can determine the ecosystem structure and function (Elser et al., 1996; Sterner and Elser, 2002) and any significant change in the composition of aerosols may produce significant to produce significant shits in ecosystems (Carnicer et al., 2015; Peñuelas et al., 2012; Sardans et al., 2012a).

679 Studies on VOCs have already addressed several atmosphere-ecosystem interface
680 questions at chemical level (Kantsa et al., 2015; Seco et al., 2007, 2015). However, other low
681 molecular weight metabolites (~80-1000 Da), directly derived from diverse primary and
682 secondary physiological processes from living organisms, are not commonly identified or taken
683 into account in aerosol particles and may play important roles in the ecosystem functioning.
684 Metabolomics aims to study the metabolome of entire organisms or specific cells or tissues
685 and includes the all the used procedures for sample collection, metabolite extraction, extract
686 analysis and data analysis (Figure 2). A metabolome consists of the thousands of small (< 1,000
687 Da) compounds (metabolites) present in an organism at a given time (Fiehn, 2002). Such
688 molecules include the substrates and products of cellular primary metabolism such as sugars,
689 amino acids, and nucleotides as well as the plant and fungi secondary metabolism compounds
690 such as polyphenolics. Those metabolites are all involved in a great variety of complex
691 physiological processes to maintain the organisms' homeostasis, growth and responses to
692 biotic and non-biotic stressors (Peñuelas and Sardans, 2009). Metabolomic techniques have
693 been widely applied in biomedicine (Claudino et al., 2007; Walsh et al., 2006), human nutrition
694 (Gibney et al., 2005; Wishart, 2008), plant physiology (Hirai et al., 2004; Kaplan et al., 2004),
695 and more recently in ecology (ecometabolomics)(Bundy et al., 2008; Rivas-Ubach et al., 2012;
696 Sardans et al., 2011) to understand how flexible are the metabolomes change under certain
697 circumstances or stressors situations (Gargallo-Garriga et al., 2014; Rivas-Ubach et al., 2016a,
698 2016c, Sardans et al., 2011, 2014). The first step to characterize a metabolome profile is to
699 obtain the chemical signature of the sample (metabolomic fingerprint) without further
700 molecular identification (Sardans et al., 2011). The identification of specific metabolites can be
701 further obtained by the information present in the metabolomic fingerprints. In this study, we
702 describe the different procedures to obtain the metabolomic fingerprints and identify
703 molecular compounds from aerosols: atmo-ecometabolomics. This methodology is a potential
704 tool to shed light in novel questions in ecology, especially for the ecosystem-atmosphere
705 interface.
706 In this study, we propose atmo-ecometabolomics as a novel tool to detect molecular
707 signatures directly related to stress (biomarkes) at a very large environmental scale. Recent
708 climate projections predict an enhancement of extreme climatic events such as warming and
709 drought which will lead to increases in plant stress and BVOC emissions (Peñuelas and Staudt,
710 2010). Plants have shown large chemical composition shifts when exposed to environmental
711 stressors (Leiss et al., 2009; Macedo, 2012; Rivas-Ubach et al., 2014, 2016b; Sardans et al.,
712 2011). Several stress biomarkers have been already identified (Glauser et al., 2008; Guy et al.,
713 2008; Henry J. Thompson et al., 2005; Keltjens and van Beusichem, 1998; Shulaev et al., 2008)

and could also be reflected in aerosols as indirect indicator of the stress status of ecosystems. Moreover, significant shifts in phenology in ecosystems have been detected during the last decades (Menzel et al., 2006; Parmesan, 2006; Parmesan and Yohe, 2003; Walther et al., 2002). According to the relationship between the phenological stage of ecosystems and the metabolic signatures in aerosols; long temporal atmo-ecometabolomics studies can also potentially provide crucial information of the phenological changes of ecosystems. Moreover, each ecosystem should present specific metabolomic signatures in aerosols which long temporal atmo-ecometabolomics studies could also provide important information of the succession or recession of ecosystems. Additionally, the large variety of compounds forming part to aerosol particles could be also of great interest because of their importance for human health including lung diseases and allergies (D'Amato et al., 2002; Després et al., 2012; Pope et al., 1995) (Figure 1). Therefore, atmo-ecometabolomics may serve as a powerful tool to assess stress and phenological changes at ecosystem and larger scales through the characterization and quantification of metabolomic signatures and specific biomarkers. The aim of this study is to layout detailed procedures to define the metabolome from particles in suspension in the low atmosphere. Ecologists can thus benefit from this approach for investigating further the link between aerosol composition and ecosystems.

1.2 Atmo-ecometabolomic analytical instruments.

Mass spectrometry (MS) coupled to liquid or gas chromatographs (LC-MS and GC-MS respectively) are recently the most common instruments for metabolomic analyses (Fiehn, 2002; Sardans et al., 2011; Zhang et al., 2012) demonstrating high performance and sensitivity (Pan and Raftery, 2007). LC-MS and GC-MS techniques provide a similar data format (or dimension) in metabolomic studies; i.e. in both techniques, metabolites are first separated through chromatography (liquid or gas) resulting in two independent and orthogonal values; mass-to-charge ratio (m/z) and retention time (RT) relative to each of the ions detected which are used to further improve the metabolite assignation (Sumner et al., 2007). Generally, in metabolomic studies, GC-MS is suitable for detecting compounds such as carbohydrates, fatty acids, essential oils, carotenoids and also organic acids (Gullberg et al., 2004). GC analyses present excellent reproducibility with minimal RT shifts between samples; however, GC-MS requires sample derivatization which increases the labor time in sample preparation and provides indirect detection of the metabolites that complicates the elucidation of novel metabolites. LC-MS can cover plant secondary metabolites such as flavonoids, alkaloids, phenolic acids, and saponins together with primary metabolites such as amino acids, carbohydrates and organic acids (De Vos et al., 2007). LC techniques often show greater RT

749 shifts between samples but provides a direct detection of the metabolites since derivatization
750 is not required. Nonetheless, no single mass spectrometry technique can cover all metabolite
751 classes (Ding et al., 2007; Zhang et al., 2012), and the combination of platforms is a common
752 approach in metabolomics to increase the number of metabolites measured in the
753 metabolomes (Hall, 2006).
754 Mass resolving power of the spectrometers is an important factor to consider in
755 metabolomics. The high-resolution of Orbitrap mass spectrometers reduces the error of
756 metabolite matching considerably when using high-resolution metabolite libraries (Rivas-
757 Ubach et al., 2016b). FT-ICR-MS affords the highest mass resolving power (up to 1,000,000)
758 and thus enabling formula assignment of a wide range of detected compounds (Marshall et al.,
759 1998). Although FT-ICR-MS can be coupled to liquid chromatography, direct infusion ESI (DI) is
760 the most common method to analyze samples with this technique. DI- FT-ICR-MS provides
761 ultrahigh mass resolution (< 1 ppm mass error after internal calibration) that enables accurate
762 elemental formula assignments to most of the detected compounds based on their exact mass
763 alone (Klein et al., 2006; Kujawinski, 2002). As such, FT-ICR-MS provides powerful means to
764 understand the global characteristics of any complex organic samples (Kim et al., 2003;
765 Reemtsma, 2009; Roullier-Gall et al., 2014; Schmitt-Kopplin et al., 2012; Sleighter and Hatcher,
766 2007; Tfaily et al., 2015). It should be noted that exact mass provided DI-FT-ICR-MS alone is not
767 sufficient for putative metabolite identification, and peak intensity measured with such a
768 method is only semi-quantitative (Kujawinski, 2002; Liu et al., 2015). However, it is possible to
769 assess the diversity of molecular species with different essential nutrients such as nitrogen,
770 phosphorus or sulfur. This is especially interesting to understand how the elemental
771 assignation in aerosols shifts in response to environmental changes; an important issue for
772 ecological stoichiometry studies (Rivas-Ubach et al., 2012; Sardans et al., 2012b; Sterner and
773 Elser, 2002). DI-FT-ICR-MS acquisition time is significantly shorter (typically between 5-15
774 minutes) than MS coupled to a LC or GC which it can take over 40 minutes per sample.
775 Visualization of FT-ICR data using van Krevelen diagrams (vK) based on O:C and H:C
776 ratios of the assigned features have been used in numerous studies to understand chemical
777 compositions of diverse complex organic matrices (Kim et al., 2003; van Krevelen, 1950;
778 Schmitt-Kopplin et al., 2012). vK diagrams provide important information of the main chemical
779 reactions such as methylation, demethylation, hydrogenation, hydration, condensation,
780 oxidation or reduction of the detected ions (Kim et al., 2003). Additionally, plotting O/C vs. H/C
781 ratios of all of the assigned formulas can also provide an approximation of the compound
782 classes present in the samples (Kim et al., 2003; Minor et al., 2014; Sleighter and Hatcher,
783 2007). However, compounds in the environment can easily be transformed or degraded, and

784 thus change their O:C and H:C ratios compared to their original form. Consequently, while this
785 classification can still provide a general idea of the organic compound compositions in aerosols,
786 any compound classification based on stoichiometric constraints should be used with caution.
787 C number versus mass (CvM) can also be used to represent FT-ICR-MS data and provides
788 crucial information on oxidation processes or molecular weight shifts when comparing two or
789 more systems (Reemtsma, 2009). Therefore, FT-ICR-MS is a useful tool to obtain high-
790 resolution metabolomic profiles and to gain a better understanding of the aerosol sources as
791 well as their chemical transformation in the atmosphere.

792

793 **1.3 Testing atmo-ecometabolomics.**

794 The present article aims to describe step by step a method for sampling and characterize the
795 particle phase of the atmo-metabolomes by contrasting two distinct seasons: spring and
796 summer. We designed a simple aerosol sampling method and collected total aerosol particles
797 (without any size cutoff) in spring and summer of 2015 at the Pacific Northwest National
798 Laboratory campus (Richland, WA, USA). We used those samples to adapt the existing
799 metabolomics protocols to extract the metabolites from aerosols in solvents to posteriorly
800 analyze them with; i) LC-MS, ii) GC-MS and iii) DI-FT-ICR-MS. The generated data with each of
801 the instruments was analyzed following some basic statistical approximations typical for
802 ecometabolomics and chemical characterization studies. The aerosol sampling method, the
803 metabolite extraction procedures and some major metabolomic differences between spring
804 and summer aerosols are detailed and discussed. The techniques to characterize the gas phase
805 component of atmo-metabolomes are well described elsewhere (Smith and Španěl, 2011;
806 Tholl et al., 2006). The application of atmo-ecometabolomics in natural ecosystems represents
807 a new approach in ecology to shed light in the understanding of the link between metabolic
808 composition of aerosols and ecosystems. This novel method in ecological sciences allows
809 understanding deeply recent research issues related with ecosystem stress, phyllosphere,
810 ecological stoichiometry, ecosystem phenology, global change, among others.

811

812 **2. Experimental details.**

813

814 **2.1 Study site.**

815 Sampling was conducted at the Pacific Northwest National Laboratory (PNNL) campus (46° 34´
816 N, 119° 28´ W) located in the north side of the city of Richland (Washington, USA). Nearby
817 landscape is a desert mainly covered by shrubs and steppes with *Ericameria nauseosa,*

Chrysothamnus viscidiflorus, Purshia tridentate, Grayia spinose, Artemisia tripartita, Sarcobatus vermiculatous, *Salsola tragus* and, *Tamarix romosissima* as some of the common species. The PNNL campus is covered by lawn and introduced planted tree species such as *Platanus sp.* The surrounding metropolitan area has a population of about 250,000 and the economy and land use is dominated by agriculture and the nearby Hanford nuclear reservation. The climate is semi-arid desert with a mean annual precipitation ranging between 180 and 220 mm per year. Annual thermic amplitude is large with an average maximum annual temperature around 32°C, with peaks reaching up to 42-45°C and the average minimum annual temperature is -2°C with lowest peaks reaching temperatures of -20°C.

2.2 Aerosol sampling.

To represent the spring season, we sampled aerosols in 2015 from May 7th to 20th, both inclusive (14 consecutive days). For the summer season, samples were collected in 2015 from July 15th to 30th, both inclusive (16 consecutive days). According to weather conditions reported by the US National Weather Service at the local airport (KPSC), the May sampling period had daily average (maximum) temperature ranging from 11 to 21°C (14 to 29°C) and daily average (maximum) humidity ranging from 49 to 78% (72 to 100%) while the July sampling period had daily average (maximum) temperature ranging from 19 to 29°C (28 to 40°C) and daily average (maximum) humidity ranging from 35 to 50% (57 to 86%). Total precipitation of 28.2 mm was reported for the May sampling period and no precipitation was reported for the July sampling period. For the aerosol collection, we designed a simple and portable aerosol sampling system that allows the sampling of multiple filters at once (Figure S1). Aerosol particles were collected on Whatman QM-A 37mm high-purity quartz filters (Whatman International Ltd, Maidstone, UK), which were precombusted for 5hrs at 450°C to minimize any impurity (Schmitt-Kopplin et al., 2012). Two filters were simultaneously collected each day. A precombusted quartz filter was inserted into a filter cassette. Filter cassettes were connected to the pump by using PVC flexible tubing of 0.6 cm diameter. The pump was working daily during 18 consecutive hours and pumped air at 30 L per minute through each filter. Filters were replaced manually before 09:00am and the pump started working automatically at 09:00am and stopped automatically at 03:00am the following day. Filters were stored at -80°C until metabolite extraction. Filters were sampled on a tower at 8 meters height.

One of the objectives of this study was to describe an operational protocol to extract the metabolites from aerosols to posteriorly analyze with the corresponding instruments. The extraction of metabolites was mainly sonication-based, so an additional aerosol sampling was

performed in late spring to test different sonication times during the extraction of polar and semi-polar metabolites and analyzed by LC-MS and GC-MS analyses. For that, we sampled 3 filters during two consecutive days at a flow rate of 30L per minute (18 hours of sampling per day) (hereafter test-filters). We sampled 6 rounds of test-filters (3 filters x 6 rounds = 18 filters). The pump started sampling at 09:00am and stopped at 03:00am each day. Sampling was performed from June 5th to the 16th (12 days). Filters were also stored at -80ºC until metabolite extraction.

2.3 Metabolite extraction for mass spectrometry analysis.

Three different tube sets were labeled; set A (8mL glass tubes) to perform the extractions, set B (15 mL polypropylene centrifuge tubes) to keep the extracts and set C (2 mL glass tubes) to keep the concentrated extract. Each filter was carefully rolled (Figure 3.1) and introduced into the corresponding tube of set A (Figure 3.2). Five mL of MeOH/H_2O (80:20) was added as an extraction solvent (this volume of extract was enough to cover the 37mm filters but it may vary depending on the diameter of the set A tubes) (Figure 3.3) and samples were sonicated for 10 min at 24ºC (Figure 3.4). For each tube of set A, 4 mL of the extract was transferred to the corresponding 15 mL centrifuge tubes of set B (Figure 3.4.1). These procedures were repeated on the same filters to perform two extractions but adding 4 mL of MeOH/H_2O (80:20) as fresh extract and the resulting extract was thus combined with the initial one (Figures 3.5, 3.5.1). All extracts in tubes of set B were then dried with an ultra-high purity nitrogen evaporator (Figure 3.6) and 1 mL of fresh extraction solvent was posteriorly added to each tube and vortexed for 30 s to ensure the correct dissolution of the extract (Figure 3.7). Tubes of set B were thus centrifuged for 5 min at 4,000 x g (Figure 3.8) and supernatants were transferred into the set C of 2 mL glass tubes (Figure 3.9). Samples were then stored at -80 ºC until the mass spectrometry analysis (Figure 3.10).

The extracts were analyzed by LC-MS (Orbitrap mass analyser), GC-MS (single quadrupole mass analyzer) and DI-FT-ICR-MS (12T) (Figure 3.11). For DI and LC-MS analyses; the extracts from all samples were directly introduced into a labeled HPLC vial set with inserts (Figure 3.12). We typically add 200 µL of extract in the HPLC but this volume may be varied for other studies.

GC-MS required a pre-treatment of the samples prior to the instrumental analyses; the dried extracted metabolites were chemically derivatized to their trimethylsilyl ester forms as previously described (Kim et al., 2015). For the derivatization, first 500 µL of each extract from the set of tubes C (Figure 2.10) were placed into a set of glass vials and dried down in a vacuum evaporator. Once dried, 20 µL of methoxyamine in pyridine (30 mg/mL) was added to

each sample. All vials were vortexed for 30 seconds and incubated at 37°C in a Thermomixer (Eppendorf AG, Hamburg, Germany) for 90 min with shaking at 1000 rpm to protect carbonyl groups. After the first incubation, all samples were centrifuged for 15 seconds and 80 µL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) was added to each vial. Vials were then vortexed for 10 seconds and again incubated for 30 min at 37°C with shaking (1,000 rpm) to derivatize hydroxyl, carboxyl and amine groups. After the second incubation, vials were centrifuged for 15 seconds and extracts were transferred into clean labeled glass vials with 200 µL inserts by using Pasteur pipettes. A cap with septum was then tightened onto each of the vials.

The description of the method used to test different sonication times during metabolite extraction is detailed in the supporting information (Supplementary Text).

2.4 LC-MS analysis.

LC-MS chromatograms were obtained using a Vanquish ultra-high pressure liquid chromatography (UHPLC) system coupled to an LTQ Orbitrap Velos high-resolution mass spectrometer equipped with a heated electrospray ionization (HESI) source (Thermo Fisher Scientific, Waltham, Massachusetts, USA). A reversed-phase C18 Hypersil gold column (150 × 2.1 mm, 3µ particle size; Thermo Scientific, Waltham, Massachusetts, USA) at 30 ºC was used. The mobile phases consisted of acetonitrile (A) and water (0.1% acetic acid) (B). Mobile phases were filtered and degassed for 15 min in an ultrasonic bath prior to use. At a flow rate of 0.3 mL per minute, the elution gradient initiated at 10% A (90% B) and was held for 5 min, then the gradient linearly changed to 10% B (90% A) for the next 15 min. The initial proportions (10% A; 90% B) were thus linearly recovered over the next 5 min, and the column was washed and stabilized for 5 more minutes. The injection volume of the samples was 5 µL. All samples were first analyzed in positive (+) ionization mode and later in negative (-) ionization mode. The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) full-scan mode with a mass range of 50-1000 m/z at 60,000 resolving power. Blank samples were analyzed during the sequence and a mixture of standards at known concentration were injected every 15 samples to test instrument sensitivity and mass accuracy.

2.5 GC-MS analyses.

After derivatization, samples were cooled down to room temperature and posteriorly analyzed by an Agilent GC 7890A coupled with MSD 5975C mass spectrometer (Agilent Technologies, Santa Clara, CA). Separations were performed on a HP-5MS column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies). The injection mode was split-less, and the injection port

temperature was held at 250°C. The column oven was initially maintained at 60°C for 1 min and then ramped to 325°C by 10°C/min, followed by a 10 min hold at 325°C. Blank controls and mixture of fatty acid methyl esters (FAMEs; C8-C28) were analyzed prior to sample analysis.

2.6 DI-FT-ICR-MS analyses.

Aerosol extracts were analyzed on a 12 Tesla Bruker SolariX Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker daltonics Inc, Billerica, MA, USA). Samples were directly infused into the mass spectrometer using a standard Bruker electrospray ionization (ESI) in negative mode at a flow rate of 3.0 µL/min through an Agilent 1200 series pump (Agilent Technologies, Santa Clara, CA, USA). The ESI source was equipped with a fused silica tube (30 µm i.d.). The ion accumulation time was optimized for all samples (0.1s). All samples were analyzed at a resolving power of 400,000 ($m/\Delta m_{50\%}$ at m/z 400). Experimental conditions were as follows: needle voltage, +4.4 kV; Q1 set to 50 m/z; and the heated resistively coated glass capillary operated at 180 °C. Blanks were injected every 10 samples.

2.7 Processing of LC-MS chromatograms.

The LC-MS files were processed by MZmine 2.17 (Pluskal et al., 2010). Chromatograms of both positive and negative modes were separately baseline corrected, deconvoluted, aligned and metabolites were autoassigned before the numerical database was exported in CSV format. The parameters used for the extraction of the data are given in Table. S1.

Metabolite assignation with LC-MS was performed by our metabolite library with more than 200 typical metabolites usually present in plants and fungi including products from primary and secondary metabolism. Assignation were performed separately for each ionization mode (positive and negative) and using the exact mass of metabolites and RT. ESI do not typically fragment all ions, however, in some molecules we could still detect some fragments which were also considered for the metabolite assignation and relative quantification. According to Sumner et al., 2007, our LC-MS metabolite assignment is putative since it was based on total exact mass of the metabolite and RT of standard measurements in the instrument. However, the use of high MS resolution achieved with Orbitrap technology and RT reduces substantially the number of false positive assignations. For more detailed information regarding the metabolite assignation see Rivas-Ubach et al., (2016b). RT and m/z values of metabolite matching for LC-MS are shown in Table S2.

2.8 Processing of GC-MS chromatograms.

GC-MS data was processed with two different software; MZmine and Metabolite Detector. MZmine 2.17 (Pluskal et al., 2010) was specifically used to obtain the metabolomic fingerprints from the additional sampled filters to test the sonication time and be thus more consistent with the LC-MS data. Parameters to get the numerical datasets with MZmine are shown in Table S3.

Metabolite Detector 2.5 (Hiller et al., 2009) was used to process the GC-MS raw data files from the spring and summer. First, "Agilent .D" files were converted to netCDF format using Agilent Chemstation and posteriorly converted to "bin" files using Metabolite Detector. Chromatograms were deconvoluted, aligned and the metabolites were autoassigned before exporting the datasets in CSV format. Briefly, retention indices (RI) of detected metabolites were calculated based on the analysis of the FAMEs mixture, followed by their chromatographic alignment across all analyses after deconvolution. Metabolites were initially identified by matching experimental spectra to PNNL increased version of FiehnLib (Kind et al., 2009), containing spectra and validated retention indices for over 850 metabolites, with probability threshold of 0.8. NIST14 GC-MS library was also used to cross-validate identification of metabolites by matching fragmented spectra. All metabolite identifications were manually validated to reduce deconvolution errors during automated data-processing and to eliminate false identifications. Parameters used in Metabolite detector are shown in table S4. Metabolite matching information in GC-MS is shown in Table S5.

2.9 Processing of DI-FT-ICR spectra.

The mass spectrum for each sample was averaged over 144 individual scans and then internally calibrated using an organic matter homologous series separated by 14 Da (–CH2 groups). The mass measurement accuracy was typically within 1 ppm for singly charged ions across a broad *m*/*z* range (100-1100 *m*/*z*). DataAnalysis software (BrukerDaltonik version 4.2) was used to convert raw spectra to a list of m/z values applying FTMS peak picker with signal to noise (S/N) of 7, which is above the minimum detection limit for FT-ICR-MS for NOM (Riedel and Dittmar, 2014) and absolute intensity threshold of 100. Chemical formulas, containing C, H, O, N, S, and P, were then assigned using an in-house built software following the Compound Identification Algorithm (CIA), described by Kujawinski and Behn (2006). Chemical formulas were assigned based on the following criteria: S/N >7, mass measurement error <1 ppm. All observed ions in the spectra were singly charged as confirmed by the 1.0034 Da spacing found between isotopic forms of the same molecule (i.e., between $^{12}C_n$ and $^{12}C_{n-1}$–$^{13}C_1$).

2.10 Statistical analyses.

For each season (spring and summer) and dataset (LC-MS, GC-MS and FT-ICR-MS), the variables present in less than 50% of the samples were excluded for the statistical analyses. The signal values measured in the experimental blanks in each of the instruments were subtracted from the datasets. Each of the variables from metabolome fingerprints obtained from each MS instrument were posteriorly submitted to Levene's and Shapiro tests to assess homogeneity of variances and normality, respectively. Variables that did not comply with those statistical assumptions were removed from the datasets. Outlier measurements were replaced for missing values and were defined as those measurements of a specific variable with values three-fold higher than the third quartile or three-fold lower than the first quartile of each season. For FT-ICR-MS datasets we have been very conservative and only the formula assigned features that presented less than 0.3ppm of error were used although cutoff values up to 0.5ppm are typically used (Osterholz et al., 2016).

The metabolome fingerprints from aerosols obtained from each instrument (3 independent datasets; LC-MS, GC-MS and DI-FT-ICR-MS) were tested by PERMANOVAs using the Bray Curtis distance to test for overall metabolomic differences between spring and summer (Table 1). The permutations were set at 10,000. Posteriorly, each metabolome fingerprint was also subjected to principal component analysis (PCA) to show in two dimensions the natural variability among the samples (van den Berg et al., 2006; Kim et al., 2010) (Figure 4).

Heat-map plots for the assigned variables with LC-MS and GC-MS were plotted to show the relative concentration change of specific metabolites between spring and summer (Figure 5). Each assigned variable was also submitted to t-student test with season as the categorical factor to test for statistical significance (Table S6).

We counted the proportions of formula classes from the FT-ICR-MS dataset (CHO, CHNO, CHOS, CHNOS, CHNOSP, CHOSP, CHOP, CHNOP, CHNOPS and CHOPS) for each sample. All calculated proportions were transformed using *arcsin(rootsquare)* before submitting them separately to t-student tests with season (spring and summer) as the categorical factor to assess for statistical significance (Figure 6). A t-test was also performed on the O/C ratios of detected features in the FT-ICR-MS with season as the categorical factor to determine whether the oxidation status of the molecular compounds statistically change significantly between spring and summer (Figure 7).

The PERMANOVAs, PCAs, heat maps and t-tests were performed with R (R Core Team, 2013). The PERMANOVA analysis was conducted with the *adonis* function in the package

"vegan" (Oksanen et al., 2013). The PCAs were performed by the *pca* function of the "mixOmics" package of R (Dejean et al., 2013). Heat maps were performed by the *heatmap.2* function of the "gplots" package (Warnes et al., 2016). T-tests were performed with the function *t.test* in the package "stats" (R Core Team, 2013). All graphs were obtained by R and graphically treated by Adobe Illustrator CS6.

The value obtained from the deconvoluted peaks in LC-MS and GC-MS are directly related to the concentration of the corresponding variable even though they do not represent the real concentration in the sample in terms of mg of metabolite per weight of sample. However, the use of those values are suitable for metabolomic comparative analyses as previously demonstrated in other studies (Gargallo-Garriga et al., 2014; Lee and Fiehn, 2013; Leiss et al., 2013; Mari et al., 2013; Rivas-Ubach et al., 2014, 2016c). In this study, we use the term *relative abundance* when referring to the relative concentration of metabolites.

FT-ICR data is typically not directly quantifiable (Wozniak et al., 2008), however although not as robust than LC-MS or GC-MS techniques, using the intensity of the detected ions by FT-ICR is still a good proxy of their relative concentration (Kellerman et al., 2014; Spencer et al., 2015). We used the measured ion intensity for the specific vK and CvM representations, for those purposes the measured intensity of each individual ion detected in each of the samples was divided by the total intensity of the spectra (Kellerman et al., 2014; Spencer et al., 2015).

Chromatograms and spectra from LC-MS and FT-ICR-MS, respectively, of samples corresponding to days 16th and 30th June showed signs of contamination and were thus not considered in the corresponding datasets for statistical analyses.

3. Results and discussion.

3.1 Aerosol sampling in filters and study site.

Optimal flow rates for the aerosol collection is important; excessive flow rates may collapse the filters and low flow rates will not collect enough particles for a good detection of compounds. We used 37mm quartz filters that performed well without collapsing at flow rates of 50 L/min, however, after the internal tubing friction associated with the extension of the tubing and the sampling of two simultaneous filters caused a decrease in in the flow rates at the aerosol collection point and we achieved flow rates of 30 L/min. Larger tube diameters (>0.65cm diameter) could be considered when higher flow rates are necessary.

Our sampling method allows sampling different number of biological replicates at the same time for statistical purposes. Furthermore, sampling can be performed at different heights on a tower or mast by extending tubing if the pump performance is able to keep enough flow rates at the sampling point. So, the experimental design (number of replicates,

filter material, length and diameter of tubing) and the pump performance are key elements to consider in atmo-ecometabolomics.

Our aerosol collection was performed in a semi-urban area surrounded by landscapes dominated by large and diverse agricultural cropland and a large desert shrubland with low biological activity, so we expected to detect a complex variety of molecules that complicate finding the atmospheric/ecological interpretation of the data. However, the obtained results were equally useful to describe the main steps to obtain the atmo-metabolomes and to test the sensitivity of different mass spectrometry techniques (LC-MS, GC-MS, FT-ICR-MS) to characterize the atmo-metabolomes in low activity ecosystems and assess their potential for detecting overall statistically significant changes between seasons.

3.2 Metabolite extraction in organic solvents.

Organic solvents combined with water are typically used for metabolomics analyses allowing the extraction of a good range of semi-polar and non-polar metabolites (Kim et al., 2010; Lin et al., 2006; Rivas-Ubach et al., 2013; t'Kindt et al., 2008). Solvents such as methanol, acetonitrile or chloroform interact with plastics, especially under sonication, and chromatograms may show contaminant features when using plastic tubes for metabolite extraction (Figure S2). Our results showed that the use of silanized glass tubes is highly recommended during the sonication step (Figure 3.4) to avoid artifacts. Combusted glassware for 5 hours at 450ºC or higher is also recommended to prevent from any organic contaminants. If plastic tubes are finally used during the extraction, especially during sonication, an initial test to detect any potential plastic contaminant is recommended.

Methanol/water (80:20) solution typically used in metabolomics studies showing a wide recovery of polar and semi-polar metabolites compared to other organic solvents (t'Kindt et al., 2008), however, the use of other solvents recover different matrices of compounds. We performed two extractions with the same solvent on the same sample to ensure higher metabolite recovery from the aerosol samples (Böttcher et al., 2007; Nikiforova et al., 2005; Rivas-Ubach et al., 2013, 2014) (Figure 3.5).

The filter size is also an important factor to consider for atmo-ecometabolomic analyses. On one hand, the lower the ratio of *filter size/pump flow rate* is, the more concentrated the samples will be. On the other hand, smaller filters are easier to handle in the laboratory during extractions allowing also higher extract recovery. Quartz filters absorb high volumes of extract that cannot be easily recovered. Our protocol with 37mm diameter filters recovered the of 89% initial solvent volume. Larger filters complicate the extraction of

metabolites (more filter handling, larger tubes and larger volumes of extract are required) and decrease considerably the recovery of extracts due the large solvent absorption.

3.3 Testing atmo-ecometabolomics contrasting two distinct seasons.

Even sampling in low productive ecosystems, PERMANOVAs of all atmo-metabolome fingerprints generated from each analytical instrument (LC-MS, GC-MS and DI-FT-ICR-MS) showed significant differences between spring and summer (Pseudo-F = 2.96, $P < 0.05$; Pseudo-F = 4.41, $P < 0.0001$; and Pseudo-F = 6.46, $P < 0.001$; respectively) (Table 1).
We also found clear separation of spring and summer samples in all the performedPCAs (Figure 4). The principal component 1 (PC1) and PC2 of the PCA performed with LC-MS data explained 13.0% and 10.3% respectively of the total metabolomic variance among samples. The PC1 and PC2 of the PCA performed with GC-MS data explained 24.2% and 13.2% respectively of the total variance. The PC1 and PC2 from DI-FT-ICR-MS PCA explained 28.2 and 12.9% respectively of the total variance of metabolomes among samples. All PCAs performed with each mass spectrometry technique showed similar values for the axis that separate mainly spring and summer cases, being the PC1 for LC-MS Orbitrap (13.0%) and PC2 for GC-MS and DI-FT-ICR-MS techniques (13.2% and 12.9% respectively). Those results indicate that all the analyzed chemical fractions from the samples changed significantly between seasons. However, it is important to note that each technique is not exclusive but complementary since they provide different information (Ding et al., 2007; Zhang et al., 2012).

Student t-tests showed statistical significance between spring and summer in several of the assigned metabolites with LC-MS and GC-MS (Figure 5 and Table S6). For the dataset generated by LC-MS, we found that spring had significantly higher relative abundance ($P < 0.05$) of α-ketoglutaric acid, adonitol, sorbitol-Mannitol, malic acid and marginally higher relative abundance ($P < 0.1$) of proline, d-tocopherol and hexoses (Figure 5a). Summer had higher relative abundance of isoleucine ($P < 0.05$) and marginally higher relative abundance of phenylalanine and coumaric acid ($P < 0.1$). The analyses on the dataset generated by GC-MS showed that spring had significantly higher relative abundances of glucose and galactose ($P < 0.05$) and marginally higher concentrations of trehalose ($P < 0.1$). Fumaric acid was found in marginally higher relative abundance in the summer ($P < 0.1$) (Figure 5b). Hexoses, glucose, galactose, trehalose and several other organic acids related to the tricarboxylic acid cycle such as ketoglutaric acid, malic acid and citric acid (Figure 5) are good indicators of growth activity in plants (Rivas-Ubach et al., 2012) and atmospheric pollination (Roulston and Cane, 2000). Those results are in agreement with the DI-FT-ICR data showing significantly higher proportions of CHOP ($P < 0.05$) and marginally significant CHNOSP ($P < 0.1$) molecular formulas

in spring (Figure 6). Phosphorus and sugars have been typically related to higher activity in plants (Rivas-Ubach et al., 2012; Sterner and Elser, 2002) although sugars can play other functions such as stress tolerance (Ingram and Bartels, 1996; Rivas-Ubach et al., 2014, 2016c). LC-MS showed that atmo-metabolomes in summer had higher relative abundance of coumaric acid and acacetin but also of phenylalanine and shikimic acid tended to be slightly higher in summer (Figure 5a). Shikimic acid is the precursor of several secondary metabolites such as flavonoids, tannins and other phenolic metabolites with strong antioxidant activity through phenylalanine and other routes (Ghasemzadeh and Ghasemzadeh, 2011; Seigler, 1998; Talapatra and Talapatra, 2015). Antioxidants protect cell membranes from peroxidation (Kim et al., 2005; Rice-Evans et al., 1996) and have been typically reported to be in higher concentrations in plants under oxidation stressors such as drought (Peñuelas et al., 2004). Summer is the driest season in the sampled area receiving up to 3 times less precipitation than spring, for this reason we expect higher antioxidant activity in plants facing drought stress (Rivas-Ubach et al., 2014, 2016c).

GC-MS also detected several fatty acid compounds in the extracts (Figure 5b). Fatty acids are present in pollen as up to 20% of their dry weight depending on the plant species (Roulston and Cane, 2000) and arachidic acid and linoleic acid, among others, are typical fatty acids found in pollen (Solberg and Remedios, 1980). Even though none of the identified fatty acids showed statistically significant changes between seasons ($P > 0.05$), their relative abundance clearly tended to increase in spring (Figure 5b); the most active season for plants.

Summer aerosols presented significantly higher proportions ($P < 0.05$) of CHO features than spring aerosols (Figure 6) and, in addition, we generally measured higher relative intensities in high-mass features in summer aerosols with respect to spring aerosols which presented higher relative intensities in lower-mass features (Figure 7a). In a CvM plot, at a given carbon number, the increase of nominal mass is contributed by heteroatoms (e.g. N, S, and O). We observed that summer had higher relative intensities of features with higher-mass than spring but with the same number of C (see region between dashed lines in Figure 7a). In addition, T-test on the O/C values of the formula-assigned features with season as categorical factor showed how summer had significantly higher relative intensities in features with higher O/C ratios (more oxidized compounds) than spring (Figure 7b, c). This result is in accordance with the higher compound masses found in summer respect to spring for a same C-number (Figure 7a) suggesting that aerosol components in summer have higher oxidation rates. This trend could be related to higher levels of photochemical oxidants associated with warm sunny conditions and increased atmospheric photo-oxidation of aerosols (Obee and Hay, 1997). Moreover, we also found higher relative intensities in high-mass aerosol compounds (over 500

Da) in summer (Figure 7a) which may suggest higher rates of polymerization or aerosol condensation. These observations point to one of the major challenges in utilizing atmo-ecometabolomic data which is the confounding effects of atmospheric processing of the original biogenic emissions.

3.4 Conclusions and future perspectives.

· Although the sampling was performed in a complex region with an urban area surrounded by a rural desert landscape with relatively low biological activity, all mass spectrometry techniques (LC-MS, GC-MS and DI-FT-ICR-MS) still detected significant differences between the spring and summer aerosol metabolomes though the methanol/water (80:20) extraction.

· There is no unique analytical technique able to characterize the whole metabolome fingerprint of aerosols. LC-MS and GC-MS and the use of metabolite libraries allow us to detect specific molecular compounds in aerosols while DI-FT-ICR-MS allows obtaining quickly a high-resolution metabolic fingerprint providing the elemental composition of aerosol compounds

· Coupling environmental variables with atmo-ecometabolomics would allow a more precise interpretation of the link between biological systems and the aerosol composition.

· Long term atmo-ecometabolomic experiments in natural ecosystems would improve understanding of the seasonal and interannual shifts of the composition of aerosols, directly linking atmospheric composition with plant phenology and physiology, along natural gradients or environmental changes.

· The use of atmo-ecometabolomic techniques ecological sciences could improve the detection, identification and quantification of any molecular compound related with environmental stressors (biomarkers) providing important information of the general status of the ecosystems. A good description of such biomarkers and other relevant metabolites would allow the creation of aerosol compound libraries which could be applied to understand the status of ecosystems and provide a relatively simple and quick environmental assessment and monitoring tool.

· The study of the impacts of aerosols on the phyllosphere and/or the stoichiometry of ecosystems could be significantly improved by the understanding of the composition of aerosols.

· New modern instruments such as GC-MS Orbitrap should be implemented in atmo-ecometabolomic studies to enable high performance for both RT and m/z resolution. Advances in methodologies for metabolomic analyses, such as Ion Mobility Spectrometry coupled to mass spectrometers (IMS-MS), could potentially improve significantly the number of detected metabolites in aerosols from the current tens and hundreds to thousands.

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1583 **Table 1.** PERMANOVAs of the atmo-metabolome fingerprints generated by LC-MS, GC-MS and

1584 FT-ICR instruments for overall metabolome comparison between seasons.

LC-MS		Sum of Squares	Mean Square	F	*P*
Season	1	0.65	0.65	4.41	0.0001
Residuals	26	3.82	0.15		
Total	27	4.47			
GC-MS		Sum of Squares	Mean Square	F	*P*
Season	1	0.18	0.18	6.46	0.0003
Residuals	28	0.77	0.03		
Total	29	0.94			
FT-ICR	Df	Sum of Squares	Mean Square	F	*P*
Season	1	0.1145	0.11	2.96	0.0285
Residuals	26	1.01	0.04		
Total	27	1.12			

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Figure 1. Schematic diagram showing the emissions of aerosols and posterior deposition on ecosystems.

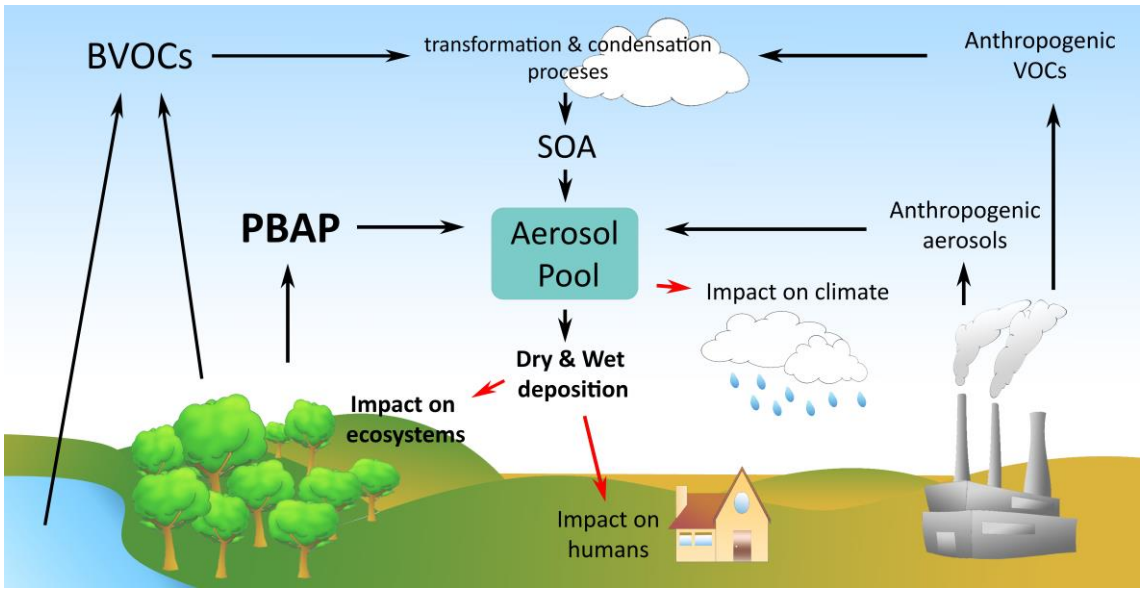


Figure 2. Diagram of the main procedures of a general ecometabolomic study combined with complementary measurements.

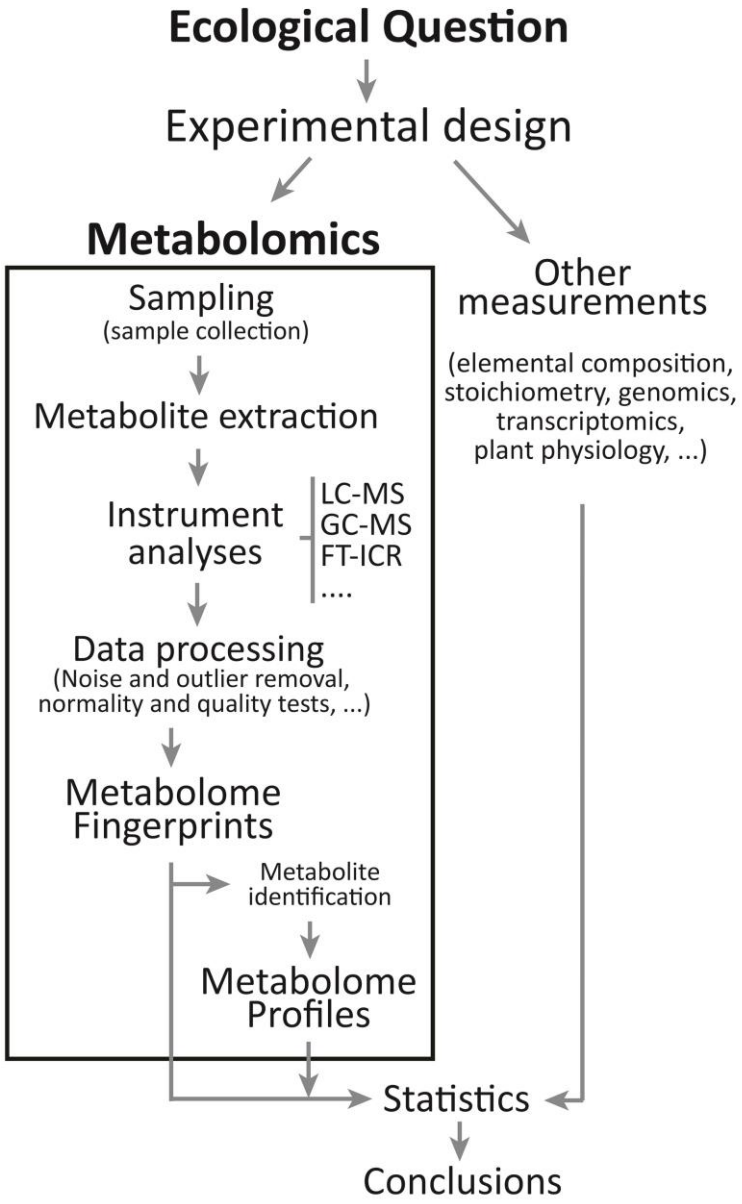


Figure 3. Experimental procedures performed on quartz filters to obtain the semi-polar extracts from aerosols and posteriorly analyze with mass spectrometry techniques.

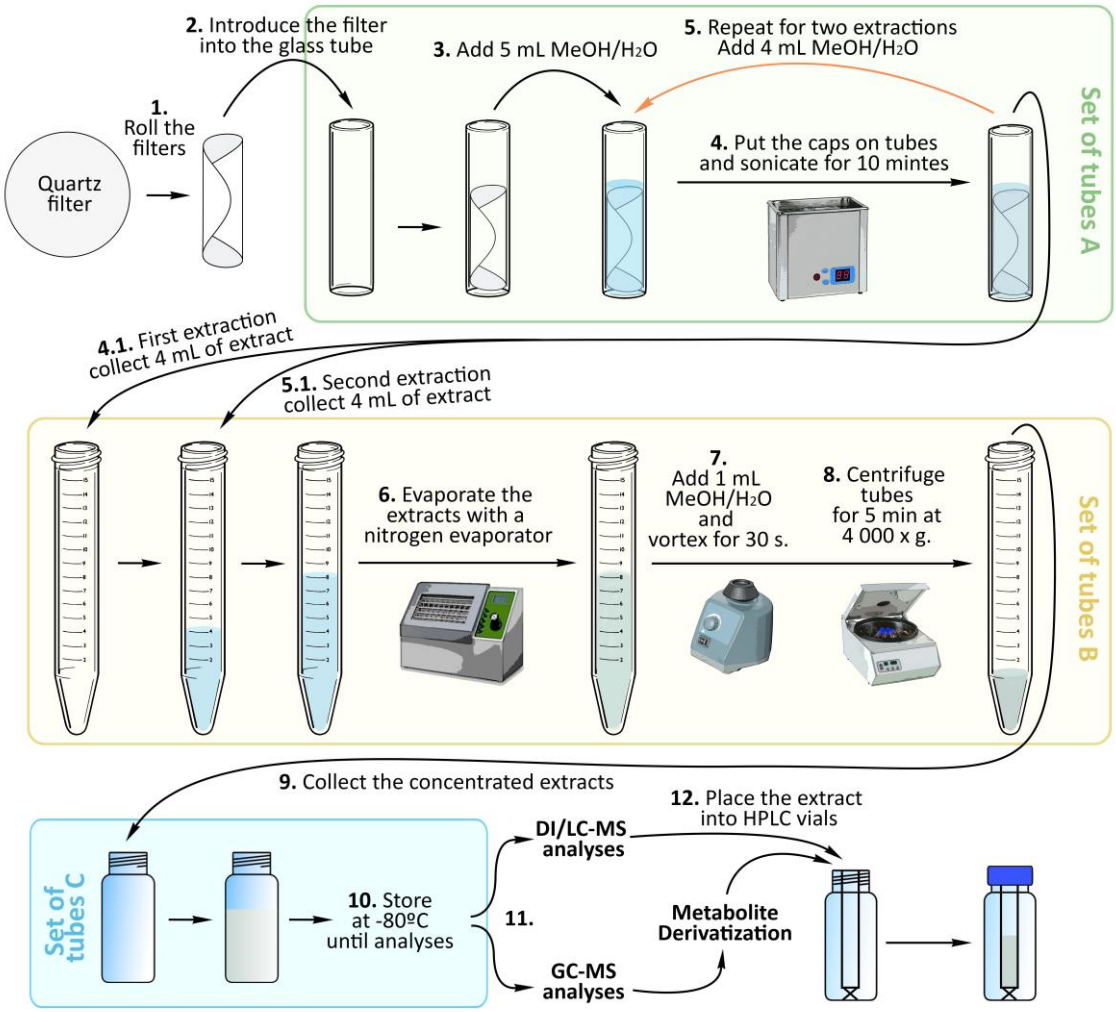


Figure 4. Case plots of the PC1 versus the PC2 of the PCAs conducted from metabolomic fingerprints of aerosols obtained by LC-MS Orbitrap (LC-MS), GC single quadrupole (GC-MS) and direct infusion DI-FT-ICR-MS. Each day of sampling correspond to a different point for each of the graphs. Aerosol metabolomes of spring days are represented by blue triangles and summer days are represented by red circles.

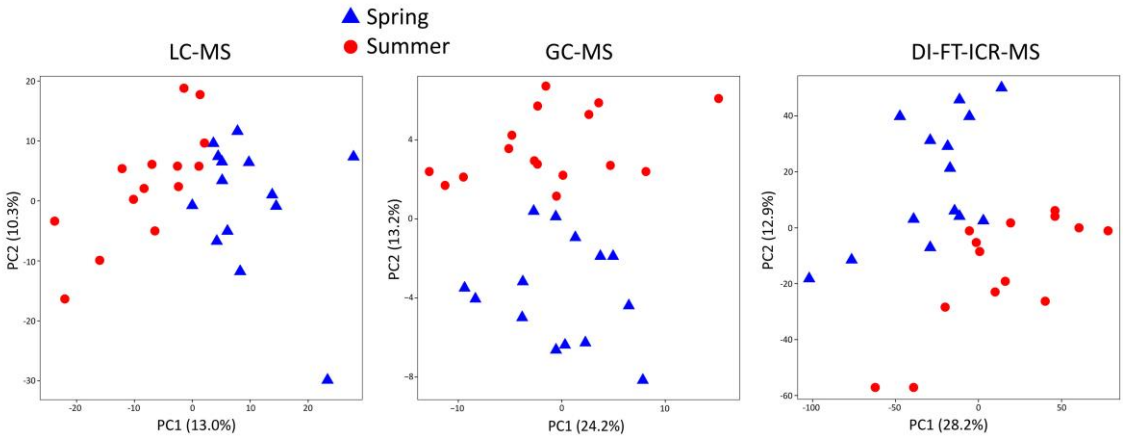


Figure 5. Heat maps of the assigned metabolomic data from the fingerprints obtained from LC-MS Orbitrap (LC-MS) (a) and GC-MS single quadrupole (GC-MS) (b) for the two sampled seasons (spring and summer). The colors represent the relative abundance of the metabolite between seasons. Red represents the highest relative abundance. Metabolites marked by an asterisk or a cross presented differences ($P < 0.05$) or marginally significant differences ($P < 0.1$) between seasons after t-test.

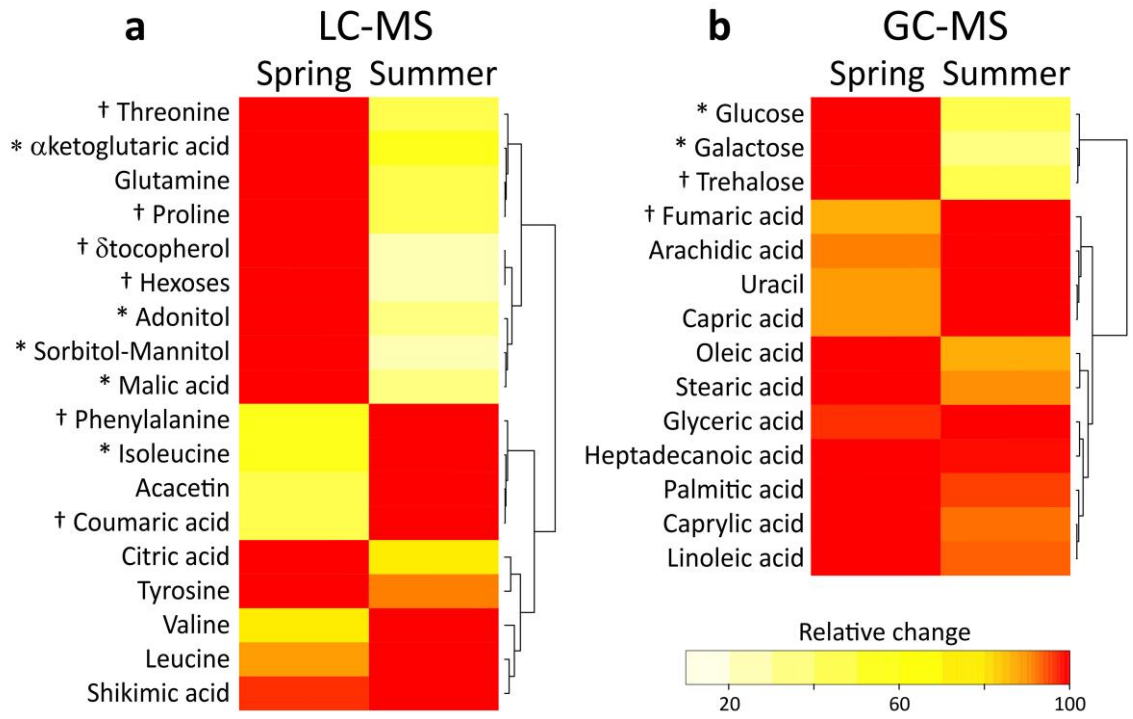


Figure 6. Box plots for the proportion (%) of the CHNO, CHO and CHOS (a) and CHNOS, CHNOP, CHOSP, CHNOSP and CHOP (b) formula classes for spring and summer. Box plots show median values of each feature. Extreme values are shown in open dots. Asterisks denote statistical significance between spring and summer for each comparison ($P < 0.05$ (*); $P < 0.0001$ (****)), and black dots denote marginal significance ($P < 0.1$).

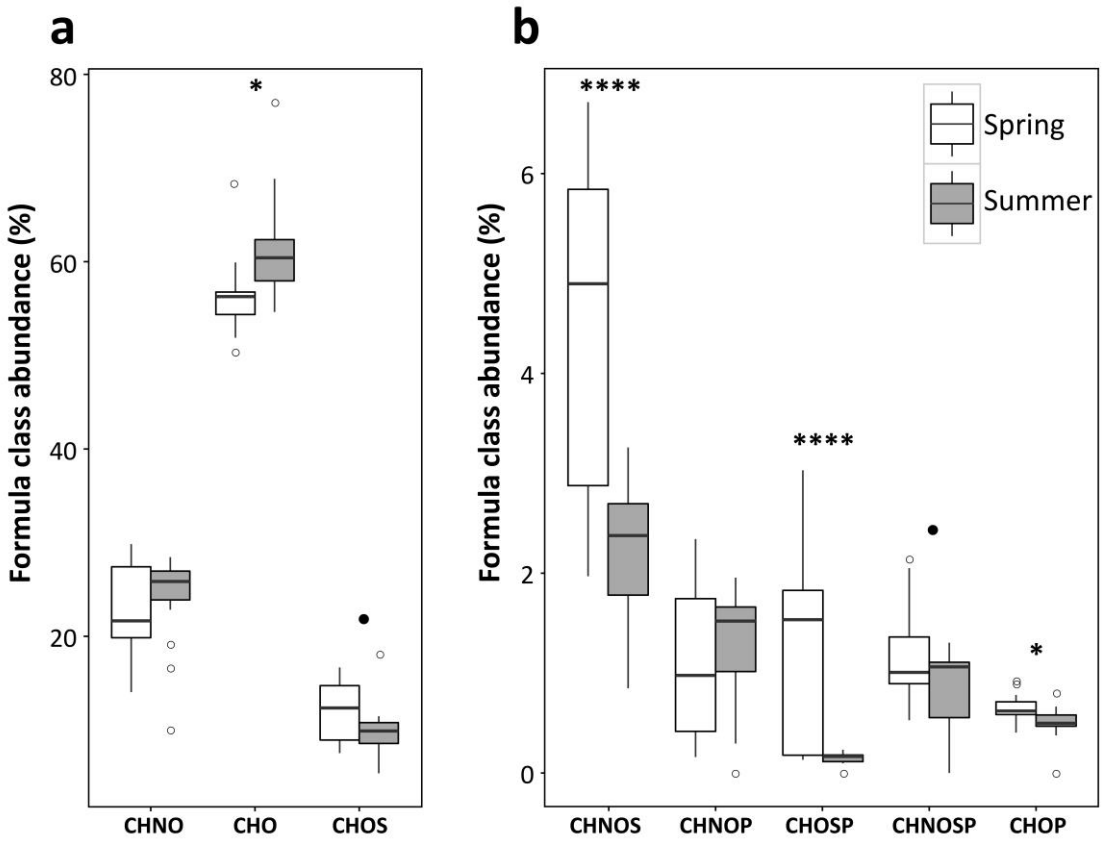
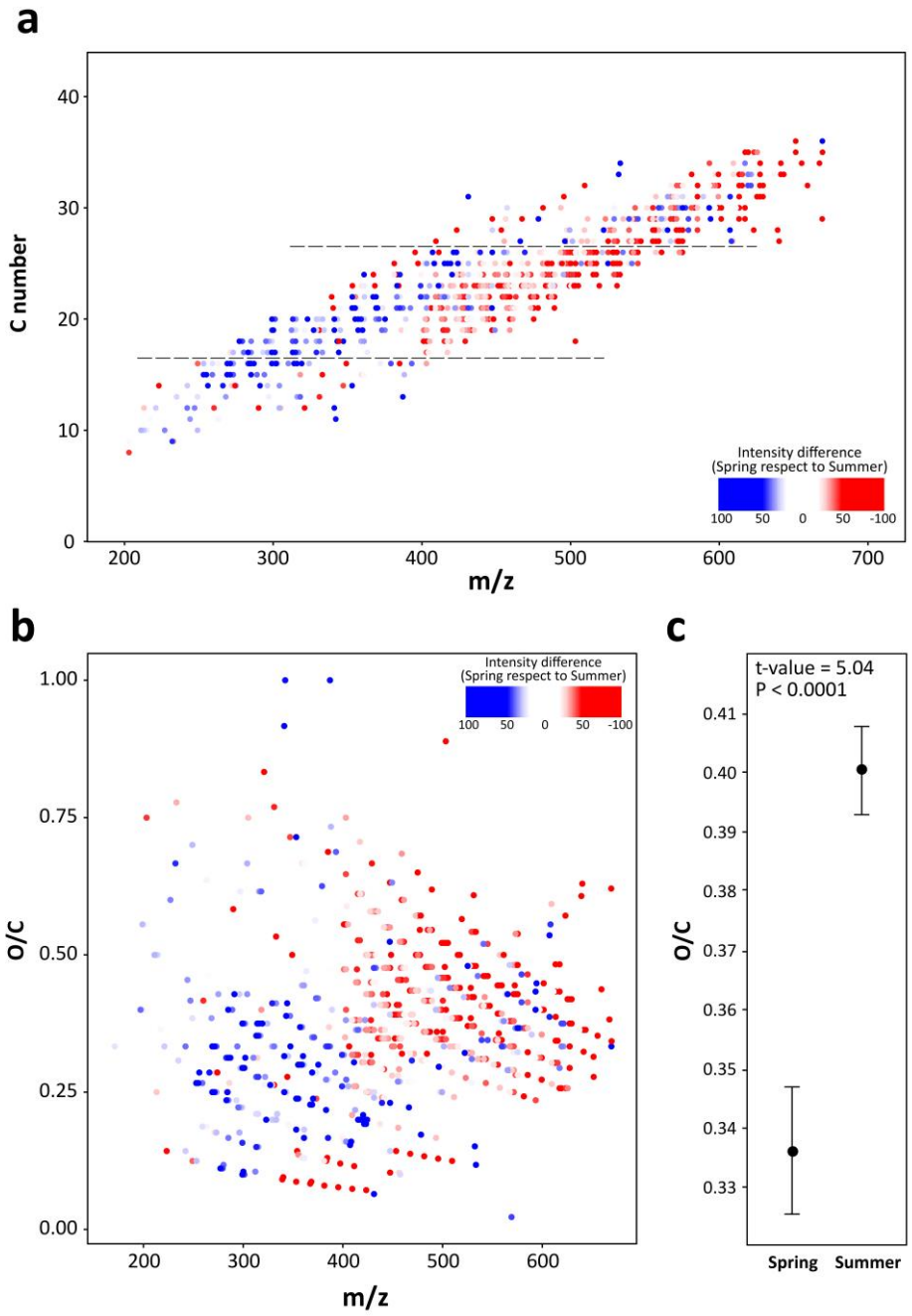


Figure 7. Carbon number versus m/z (CvM) (a) and Oxygen/Carbon ratio versus mass (b) diagrams preformed with DI-FT-ICR data and represented by the relative intensity of spring relative to summer. Darker blue dots represent higher relative intensity in spring and darker red dots represent higher relative intensity in summer. Mean (±SE) of Oxygen/Carbon of the features detected in spring and summer aerosols (c). Statistic-t and P values are shown in the graph.



SUPPORTING INFORMATION.

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Supplementary text. Sonication optimization test.

For the sonication time optimization, the 18 test-filters (sampled for 2 consecutive days) were cut by 2 equal parts (n = 36) and each piece followed the procedures explained above but varying the sonication time (12 pieces were sonicated for 10 minutes, 12 pieces for 15 minutes and 12 pieces for 20 minutes). After testing different sonication times on the test-filters from the additional sampling, the results showed that more than 10 minutes of sonication did not increase the concentration of the extracts.

To test for differences among different sonication times during the extraction of polar and semi-polar metabolites, separately we performed PERMANOVAs for the metabolomic fingerprints obtained by LC-MS and GC-MS. PERMANOVAs were performed with sonication time as fixed factor for each of the sampling rounds of two days to avoid any possible variability from different days (Tables S7 and S8). PERMANOVAs were conducted using the Bray Curtis distance and setting the permutations at 10000.

PERMANOVA performed for each of the sampled days with "sonication time" as dependent categorical variable showed that sonication time did not significantly vary the relative abundances of the extracts in any of the sampled days for datasets generated by LC-MS ($P > 0.05$) (Table S7). Same PERMANOVAs applied to the GC-MS datasets did not show differences between sonication times ($P > 0.05$) with the exception of the test corresponding to 5th and 6th June 2015 ($P = 0.02$) (Table S8).

Because we did not detect significant variation in the concentration of the detected ions among different sonication times (Tables S7 and S8), we considered that 10 minutes of sonication was enough to extract the metabolites in methanol/water (80:20) and get the metabolomic fingerprints of the aerosols.

Table S1. Description of the processes and parameters applied to LC-MS chromatograms with MZMine 2.17 (Pluskal et al., 2010) to obtain the metabolomic fingerprintings of the aerosol extracts from both positive and negative ionization modes. All LC-MS fingerprints including seasonal sampling and additional sampling for sonication test were obtained with the same parameters as shown in the table.

		(+H) Chromatograms		(-H) Chromatograms
1	**Baseline correction – RollingBall baseline corrector**			
	Chromatogram type	TIC		TIC
	Use m/z bins	No		No
	wm	10		12
	ws	8		8
2	**Mass detection** (exact Mass)			
	Noise level	7×10^4		5×10^3
3	**Chromatogram builder**			
	Minimum time span	0.03		0.03
	Minimum height	1×10^4		1×10^3
	m/z tolerance	0.0005		0.0005
4	**Smoothing**			
	Filter width	5		5
5	**Chromatogram deconvolution (local minimum search)**			
	Chromatographic threshold	30%		30%
	Search minimum in RT range (min)	0.1		0.1
	Minimum relative height	5%		5%
	Minimum absolute height	1000		1000
	Minimum ratio of peak top/edge	2		2
	Peak duration range	0-0.5		0-0.5
6	**Chromatogram alignment (join alignment)**			
	m/z tolerance	0.0005		0.0005
	Weight for m/z	80		80
	RT tolerance	0.3		0.3
	Weight for RT	20		20
7	**Gap filling (Peak Finder)**			
	Intensity tolerance	30%		30%
	m/z tolerance	0.0005		0.0005
	Retention time tolerance	0.3		0.3
	RT correction	Yes		Yes
8	**Metabolite Assignation**			
	m/z tolerance	0.0005		0.0005
	RT tolerance	0.3		0.3
9	**Data Exported**	Peak Area		Peak Area
10	**Ions excluded from database**	81.519 84.079 102.032 140.000 146.018 158.995 180.97 181.027 200.022		59.014 119.036 141.018 155.003 217.003 223.020 293.179 311.169

RT, retention time; m/z, mass to charge ratio

Table S2. Retention time (RT) and mass to charge ratio (m/z) of the deconvoluted ions in both negative and positive ionization modes assigned to metabolites with MZmine v.2.17 for LC-MS chromatograms. The assignment of the metabolites was based on the exact mass and RT of standards. RT, m/z and peak range of the standards are shown in the table. Malic acid typically present two peaks for the chromatographic method used. Ions representing fragments of the molecular compound are marked. Error of m/z and RT of assigned ions to metabolites respect the m/z and RT of standars are shown.

		m/z and RT of each ion assigned in MZmine v.2.17			m/z, RT, Peak range of ions from standards measured in the LC-MS Orbitrap system				Error of m/z and RT (deconvoluted ions vs. Standard ions)		
Ionization mode	Hits	Name	m/z	RT	m/z	RT	Fragment	Double peak	Absolutem/z	m/z (ppm)	RT
-H	30	a.ketoglutaric acid	145.01569	1.81	145.01495	1.65			0.00074	5.12	0.16
+H	30	Acacetin	285.07461	16.79	285.07553	16.85			-0.00092	-3.22	-0.06
-H	30	Adonitol (Ribitol)	151.06272	1.36	151.06195	1.42			0.00077	5.12	-0.06
-H	30	Citric acid	191.02057	1.78	191.01945	1.75			0.00112	5.86	0.03
-H	30	Coumaric acid	163.04150	11.15	163.04065	11.3			0.00085	5.24	-0.15
-H	30	δ-tocopherol	401.12877	1.32	401.12906	1.35			-0.00029	-0.72	-0.03
+H	30	Glutamine	147.07610	1.54	147.07630	1.46			-0.00020	-1.36	0.08
+H	30	Glutamine	130.04889	1.75	130.04900	1.46	Yes		-0.00011	-0.86	0.29
-H	30	Hexoses	179.05610	1.33	179.05595	1.43			0.00015	0.84	-0.10
+H	23	Isoleucine	86.09582	1.74	86.09600	1.7	Yes		-0.00018	-2.07	0.04
+H	30	Isoleucine	132.10199	1.74	132.10190	1.7			0.00009	0.69	0.04
+H	30	Leucine	132.10086	1.92	132.10160	1.81			-0.00074	-5.64	0.11
-H	30	Malic acid	133.01570	1.40	133.01560	1.51		Yes	0.00010	0.73	-0.11
-H	30	Malic acid	115.00522	1.62	115.00490	1.51	Yes	Yes	0.00032	2.81	0.11
-H	30	Malic acid	133.01570	1.77	133.01560	1.71		Yes	0.00010	0.79	0.06
-H	30	Malic acid	115.00527	1.85	115.00490	1.71	Yes	Yes	0.00037	3.22	0.14
+H	23	Phenilalanine	166.08657	1.86	166.08640	1.91			0.00017	1.01	-0.05
+H	30	Proline	116.06988	1.61	116.07030	1.49			-0.00042	-3.59	0.12
+H	30	Proline	116.06990	1.65	116.07030	1.49			-0.00040	-3.46	0.16
-H	30	Shikimic acid	173.04573	1.70	173.04553	1.63			0.00020	1.16	0.07
-H	30	Sorbitol - Mannitol	181.07308	1.36	181.07222	1.4			0.00086	4.76	-0.04
+H	30	Threonine	120.06499	1.63	120.06500	1.43			-0.00001	-0.08	0.20
+H	30	Tyrosine	182.08139	1.74	182.08140	1.77			-0.00001	-0.03	-0.03
+H	30	Valine	118.08581	1.35	118.08610	1.53			-0.00029	-2.44	-0.18

RT, retention time
m/z, mass to charge ratio
ppm, parts per million
Hits, number of samples where the metabolite was detected

Table S3. Description of the processes and parameters applied to GC-MS chromatograms with MZMine 2.17 to obtain the metabolomic fingerprintings for the sonication test.

			(-H) Chromatograms
1	**Baseline correction – RollingBall baseline corrector**		
	Chromatogram type		TIC
	Use m/z bins		No
	wm		12
	ws		8
2	**Mass detection** (exact Mass)		
	Noise level		5×10^3
3	**Chromatogram builder**		
	Minimum time span		0.03
	Minimum height		1×10^3
	m/z tolerance		0.0005
4	**Smoothing**		
	Filter width		5
5	**Chromatogram deconvolution (local minimum search)**		
	Chromatographic threshold		30%
	Search minimum in RT range (min)		0.1
	Minimum relative height		5%
	Minimum absolute height		1000
	Minimum ratio of peak top/edge		2
	Peak duration range		0-0.5
6	**Chromatogram alignment (join alignment)**		
	m/z tolerance		0.0005
	Weight for m/z		80
	RT tolerance		0.3
	Weight for RT		20
7	**Gap filling (Peak Finder)**		
	Intensity tolerance		30%
	m/z tolerance		0.0005
	Retention time tolerance		0.3
	RT correction		Yes
8	**Metabolite Assignation**		
	m/z tolerance		0.0005
	RT tolerance		0.3

RT, retention time; m/z, mass to charge ratio

Table S4. Description of the parameters applied to GC-MS chromatograms with Metabolite Detector 2.5 for the obtaining of the spring and summer metabolomic profilings.

Tool settings		
Centroid	Threshold begin	10
	Peak threshold end	-5
	Maximal baseline	30
	FWHM	0.1
Deconvolution	Peak threshold	10
	Minimum peak height	10
	Deconvolution width (scans)	8
Identification	Max RI difference	20
	Cutoff score	0.6
	Pure/Impure	0.6
	Scaled lib	Yes
	Combined score	Yes
Quantification	Minimal distance	0.5
	Minimal required quality index	1
	Exclude	72.5 to 73.5 146.5 to 147.5
Batch quantification Settings		
Compound matching	ARI	20
	Pure/Impure	0.6
	Req. Score	0.8
	RI+Spec	OK
Identification	ARI	20
	Pure/Impure	0.6
	RI+Spec	OK
Other settings	Compound reproducibility	0
	Max. Peak drisc. index	100
	S/N	15
	Number of ions	4
	Extended SIC Scan	Yes

Table S5. Score, retention index (RI), retention time (RT) and signal to noise ratio (S/N) of matched metabolites in GC-MS chromatograms processed with Metabolite Detector 2.5. The number of hits found after chromatogram alignment is indicated. Metabolites matches in less than 70% of the samples were not considered for the study case.

	Score	Quantification Ions	Avg. RI	Avg. RT (Min)	Avg. S/N	Hits	Considered for the study case
palmitic acid	0.95	75 117 313	1717.8	18.89	354.51	30	Yes
D-trehalose	0.83	191 361 362	2478.0	25.17	238.80	30	Yes
stearic acid	0.94	75 117 341	1913.6	20.69	315.26	30	Yes
linoleic acid	0.86	55 67 75	1885.0	20.44	49.92	30	Yes
oleic acid	0.91	55 75 129	1896.1	20.54	20.11	30	Yes
fumaric acid	0.97	73 147 245	1023.7	10.93	31.80	30	Yes
glyceric acid	0.80	73 189 292	1015.1	10.82	62.23	30	Yes
caprylic acid	0.91	75 131 201	938.9	9.78	25.08	30	Yes
capric acid	0.88	55 117 229	1133.8	12.36	30.84	30	Yes
D-glucose	0.80	160 205 319	1602.3	17.77	193.74	30	Yes
D-galactose	0.82	73 205 319	1622.9	17.97	32.98	30	Yes
uracil	0.85	99 131 241	1021.5	10.90	39.39	30	Yes
arachidic acid	0.93	75 132 369	2109.3	22.36	33.91	30	Yes
heptadecanoic acid	0.94	117 327 328	1813.7	19.81	48.14	30	Yes
maltose	0.82	73 204 217	2418.2	24.74	7.52	27	Yes
3-hydroxybutyric acid	0.87	73 117 147	839.2	8.41	9.64	6	No
glycerol	0.80	73 133 205	957.1	10.03	70.75	5	No
L-homoserine	0.81	73 174 218	1122.1	12.21	2.93	4	No
L-serine	0.84	73 204 218	1045.7	11.22	18.84	3	No
1-indanol	0.87	156 205 206	1030.5	11.02	0.72	2	No
4-hydroxypyridine	0.86	73 152 167	832.6	8.32	0.77	2	No

Score, Score value obtained for each metabolite matching with the library.
Avg. RI, Average Retention Index
Avg. RT (min), Average Retention Time (minutes)
Avg. S/N, Average Signal to Noise
Hits, number of samples where the metabolite was detected

Table S6. Student t-tests for each of the assigned metabolites with season as the categorical tested factor. Mean (representing the peak area of ion chromatograms), standard error, statistic t and *P* value for each assigned metabolite are shown for each of the seasons.

LC-MS						
	Spring		**Summer**			
	Mean	**SE**	**Mean**	**SE**	**t**	***P***
a-ketoglutaric acid	48316.99	5099.042	25008.78	4212.44	3.52	0.00165
Acacetin	50809.67	13413.09	105584	35227.62	-1.45	0.17324
Adonitol	87878.02	12553.98	30032.83	4370.85	4.35	0.00049
Citric acid	1976793	179888.1	1574644	244477.2	1.32	0.19773
Coumaric acid	14540.78	3816.208	29699.1	6321.345	-2.05	0.08066
d-tocopherol	266205.7	110367.1	54500.64	8547.013	1.91	0.07784
Glutamine	2467791	1261357	1208597	250593.8	0.98	0.34903
Hexoses	179901.1	75768.74	37733.15	5521.39	1.87	0.08373
Isoleucine	179199.1	29111.56	355432.6	69281.29	-2.35	0.03339
Leucine	621572.5	84248.74	703402.2	152227.1	-0.47	0.64535
Malic acid	417618.5	120080.4	130458.8	36979.81	2.29	0.03681
Phenilalanine	185597.5	25034.1	346194.1	79907.79	-1.92	0.07526
Proline	305332.3	59165.33	151847.2	47975.5	2.01	0.05887
Shikimic acid	303799.8	75605.58	313967.9	108839.2	-0.08	0.93960
Sorbitol/Mannitol	368652.3	67777.98	106951.3	13302.26	3.79	0.00199
Threonine	1047720	178312.1	479188.3	98874.14	2.79	0.01386
Tyrosine	170745.7	45699.96	155459	33666.26	0.27	0.79024
Valine	49486.37	8290.428	63038.64	28060.53	-0.46	0.64978
GC-MS						
	Spring		**Summer**			
	Mean	**SE**	**Mean**	**SE**	**t**	**P**
Palmitic acid	10200493	421999	9742948	374890.5	0.81	0.42282
D-trehalose	8783896	2820577	3608147	344275.8	1.95	0.06154
Stearic acid	8111280	464659.7	7312959	207154.6	1.64	0.11238
Linoleic acid	625152.6	20056.59	582749.8	22021.17	1.41	0.17012
Oleic acid	407369	34730.99	350753.1	28752.97	1.27	0.21586
Fumaric acid	2736172	179111.4	3181144	163342.6	-1.84	0.07657
Glyceric acid	2594000	155801.8	2692241	215277	-0.36	0.72130
Caprylic acid	449961.4	91906.22	417070.9	51513.41	0.32	0.74945
Capric acid	281892.7	20643.83	314941.2	30600.84	-0.87	0.39218
D-glucose	6252214	1203738	2681638	338921.9	3.02	0.00528
D-galactose	2598398	435407.4	1023059	138967.8	3.64	0.00109
Maltose	212970	64761	142173	40001	0.94	0.35548
Uracil	960735	107724.2	1084987	118906.4	-0.77	0.45027
Arachidic acid	212423.2	27627.51	232219.6	35000.68	-0.44	0.66688
Heptadecanoic acid	440164.8	67015.78	435406.3	49581.21	0.06	0.95416

Table S7. PERMANOVA tables for sonication time of the LC-MS Orbitrap fingerprints of each of test samples.

5th & 6th June 2015 (Test sample 1)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.052	0.052	0.92	0.40
Residuals	4	0.226	0.057	0.81	
Total	5	0.278	1		
7th & 8th June 2015 (Test sample 2)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.011	0.011	1.23	0.31
Residuals	4	0.036	0.009	0.77	
Total	5	0.046	1		
9th & 10th June 2015 (Test sample 3)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.018	0.018	1.04	0.49
Residuals	4	0.068	0.017	0.79	
Total	5	0.085	1		
11th & 12th June 2015 (Test sample 4)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.005	0.005	2.00	0.11
Residuals	4	0.010	0.002	0.67	
Total	5	0.015	1		
13th & 14th June 2015 (Test sample 5)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.004	0.004	0.15	0.91
Residuals	4	0.101	0.025	0.96	
Total	5	0.105	1		
15th & 16th June 2015 (Test sample 6)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.024	0.024	0.96	0.38
Residuals	4	0.099	0.025	0.81	
Total	5	0.122	1		

Table S8. PERMANOVA tables for sonication time of the GC-MS Orbitrap fingerprints of each of test samples.

5th & 6th June 2015 (Test sample 1)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.494	0.494	3.21	0.02
Residuals	4	0.616	0.154	0.55	
Total	5	0.494	0.494	3.21	
7th & 8th June 2015 (Test sample 2)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.171	0.171	1.38	0.27
Residuals	4	0.495	0.124	0.74	
Total	5	0.666	1		
9th & 10th June 2015 (Test sample 3)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.179	0.179	2.972	0.13
Residuals	4	0.240	0.060	0.57	
Total	5	0.419	1		
11th & 12th June 2015 (Test sample 4)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.303	0.303	6.29	0.07
Residuals	4	0.193	0.048	0.39	
Total	5	0.496	1		
13th & 14th June 2015 (Test sample 5)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.034	0.034	0.29	0.80
Residuals	4	0.465	0.116	0.93	
Total	5	0.500	1		
15th & 16th June 2015 (Test sample 7)	Df	Sum of Squares	Mean Square	F	*P*
Sonication time	1	0.282	0.282	2.38	0.16
Residuals	4	0.474	0.119	0.63	
Total	5	0.756	1		

Figure S1. Schematic representation of the system used for sampling aerosols.

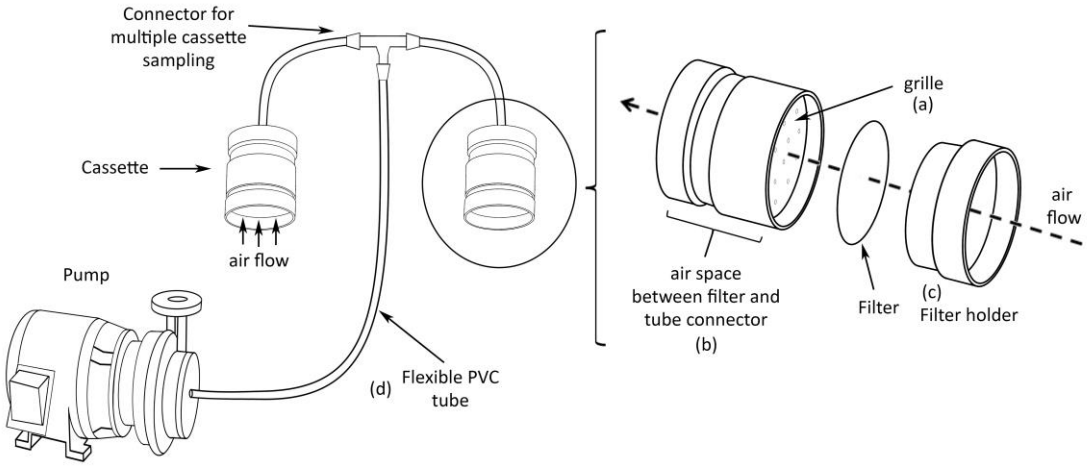

Figure S2. Example of two chromatograms from 14 to 25 minutes of Methanol:Water (80:20) on the LC-MS Orbitrap instrument. Red chromatogram represents the mixture of solvents after 2 hours into a plastic tube. Blue chromatogram represents the solvents after 20 minutes sonication into a plastic tube.