Ecometabolomics: Optimized NMR-based method.

Running Title: Optimized NMR-based Procedures: A tool for Ecology field metabolomic studies

Rivas-Ubach, A.[1,2*], Pérez-Trujillo, M.[3*], Sardans, J.[1,2], Gargallo-Garriga, A.[1,2,3], Parella, T.[3], Peñuelas, J.[1,2].

[1]CREAF, Cerdanyola del Vallès 08193, Catalonia, Spain.

[2]CSIC, Global Ecology Unit CREAF-CEAB-CSIC-UAB, Cerdanyola del Vallès 08193, Catalonia, Spain.

3. Servei de Ressonàcia Magnètica Nuclear, Universitat Autònoma de Barcelona, Cerdanyola del Vallès 08193, Catalonia, Spain.

* Authors contributed equally to this manuscript

Summary

1. Metabolomics is allowing great advances in biological sciences. During the last few years, an increasing number of ecological studies are using a metabolomic approach to answer ecological questions (ecometabolomics). Ecometabolomics is becoming a powerful tool which allows following the responses of the metabolome of an organism to biotic and abiotic environmental changes and the comparison of populations. Some NMR-based protocols have been published for metabolomics analyses oriented to other disciplines such as biomedicine but there is a lack of a description of a detailed protocol applied to ecological studies.
2. Here we propose a NMR-based protocol for ecometabolomic studies that provides an unbiased overview of the metabolome of an organism, including polar and nonpolar metabolites. This protocol is aimed to facilitate the analysis of many samples, as typically required in ecological studies. In addition to NMR fingerprinting, it identifies metabolites for generating metabolic profiles, mainly through the analysis of complex mixtures by NMR experiments, applying strategies of elucidation of small molecules typically used in natural-product research, and allowing the identification of secondary and unknown metabolites. We also provide a detailed description to obtain the numerical data from the ^{1}H-NMR spectra needed to perform statistical analyses such as PCAs, PLS-DAs or PERMANOVAS.
3. We tested and optimized this protocol by using two field plant species (*Erica multiflora* and *Quercus ilex*) sampled once per season. Both species showed high levels of polar compounds such as sugars and amino-acids during the spring, the growing season. *E. multiflora* was also experimentally submitted to drought and the NMR analyses were sensitive enough to detect some compounds related to the avoidance of water loses.

4. This protocol has been designed for studying the metabolome of wild plants but can also be used with animals. It identifies changes in the compositions of metabolites between individuals and detects and identifies biological markers associated with environmental changes. It is effective for targeted and untargeted studies.

Key-words: metabolomics, ecometabolomics, NMR, plant physiology, ecology, protocol

INTRODUCTION

Ecometabolomics

Metabolomics as a new research approach has been widely used in biomedicine (Nicholson, Lindon & Holmes 1999; Wishart *et al.* 2007; Urban *et al.* 2010) , toxicology (Robertson 2005; Alam *et al.* 2010; Hasegawa *et al.* 2010), and plant biology (Fiehn *et al.* 2000; Hirai *et al.* 2004; Weckwerth *et al.* 2004; Scott *et al.* 2010), and its application is increasing in ecological studies (Peñuelas & Sardans 2009a; Peñuelas & Sardans 2009b; Sardans, Peñuelas & Rivas-Ubach 2011). The metabolome is the entirety of molecules present in an organism as the final expression of its genotype at a particular moment (Fiehn 2002; Peñuelas & Sardans 2009a) and can be considered a molecular picture of biological diversity because each living species has its own metabolic profile (Gromova & Roby 2010). In ecological studies, metabolomics (ecometabolomics) has become a promising tool for following the responses of the metabolome of organisms to biotic and abiotic environmental changes (Sardans, Peñuelas & Rivas-Ubach 2011). The metabolome is the chemical phenotype of organisms and is the first to respond to internal and external stressors to maintain physiological homeostasis (Fiehn *et al.* 2000; Peñuelas & Sardans 2009a; Leiss *et al.* 2011; Sardans, Peñuelas & Rivas-Ubach 2011).

An optimized NMR-based protocol for ecometabolomics. Scope of application

Several protocols to explore the metabolome in humans and animals (Beckonert *et al.* 2007; Jukarainen *et al.* 2008; Want *et al.* 2010; Martineau *et al.* 2012) and in microorganisms (Smart *et al.* 2010; Roberts *et al.* 2012) have recently been published. Plants have received less attention, but some methods for conducting metabolomic

analyses of laboratory subjects based on Nuclear Magnetic Resonance (NMR) spectroscopy have been described (Kruger, Troncoso-Ponce & Ratcliffe 2008; Gromova & Roby 2010; Kim, Choi & Verpoorte 2010; Kim & Verpoorte 2010).The field of ecology currently lacks a standard protocol for the analysis of the metabolome of wild plants, which are sampled from the field under very heterogeneous environmental conditions. The objective of ecology is to explain general patterns of structure, function, and evolution in ecosystems. This task is complicated by the large number of factors interacting concurrently and by the resulting high variability at all levels. Individuals within the same species present large differences in elemental composition, metabolism, phenology, and life style and sample size for a consistent statistical analysis is often large. A protocol is thus needed to provide an overall analysis of the main metabolites in field samples, including secondary and nonpolar compounds, and which allows the detection and identification of those metabolites that play a key role in an organism's response to environmental change. This protocol must also be reproducible and amenable to robust statistical analyses.

Among the different analytical techniques, NMR spectroscopy has the advantage of providing an unbiased overview of all the small molecules in a solution. Its analyses require minimal sample preparation, are relatively quick, non-destructive and highly reproducible and robust. ^{1}H NMR signals are directly and linearly correlated to metabolite abundance, leading to a straightforward quantification that avoids the need for calibration curves of standards. Besides, NMR spectroscopy is the key technique for the elucidation of unknown metabolites, allowing the identification of most of the molecules detected and the differentiation between structural isomers, diastereoisomers (Pérez-Trujillo *et al.* 2010; Ellis *et al.* 2012), and even enantiomeric molecules (Ellis *et al.* 2012).

We describe an optimized NMR-based protocol for ecometabolomic studies applied to wild plants, going from sample collection to the identification of biomarkers and that includes the analysis of polar and nonpolar metabolites. The protocol covers sample storage and preparation, the acquisition of NMR fingerprint data of all samples, the identification of metabolites for providing a metabolic profile (based on the analysis of complex mixtures by NMR spectroscopy, which can also identify secondary and unknown metabolites), and the statistical analyses of the NMR data (Fig. 1). These procedures have been focused on the study of wild plants but can also be applied to the study of other organisms such as zooplankton (*Daphnia*), insects, annelids, and molluscs, among others.

PROCEDURES AND MATERIALS

The complete procedure (Fig. 2) is divided into five main categories: **A**. Sample collection and storage, **B**. Metabolite extraction and NMR sample preparation, **C.** Acquisition of NMR fingerprint data, **D**. NMR experiments for metabolite identification, and **E**. Statistical data analysis. (The grinding time of samples, the amount of sample, the sonication test, the repeated extractions, the recovery and the step by step procedure is explained in the Supporting Information with more details).

A. Sample collection and storage (Steps 1-3 of Fig.2).

Fresh plant materials were collected and rapidly packed, labelled, and frozen *in situ* in a container of liquid nitrogen. It prevented the degradation of metabolites (Kim & Verpoorte 2010). Plant physiological processes vary throughout the day, so individual subjects were sampled within a narrow range of time and under a constant environment

(from 11:00hrs to 15:00hrs). The frozen plant material were lyophilized and kept in plastic cans in desiccators or frozen. Lyophilization avoid the hydrolysis of metabolites since maintains inactive any enzymatic reaction. The rapid freeze-drying is thus a crucial step in ecometabolomics. Samples were ground with a ball miller (*Mikrodismembrator-U* (B. Braun Biotech International)). Excessive grinding was avoided to prevent heating the samples; 3-10 minutes at 1600 rpm were enough time to grind lyophilized plant material but it depended on the species and/or organ.

B. **Metabolite extraction and NMR sample preparation (Steps 4-20.a/16.b of Fig.2).**

For each sample, one hundred of dried powder was added to a 50 mL centrifuge tube. 6 mL of a water-methanol (1/1) mixture and 6 mL of chloroform were added to each tube and all samples were votexed (15 s) and sonicated (2 min) Samples were centrifuged at 3 000 rpm (15 min). The two liquid phases were collected separately.

For the aqueous extraction; for each sample, 4 mL of the aqueous extract was collected by micropipette in another centrifuge tube series. Then, the steps 5 to 10 of Fig.2 were repeated in the same tubes. After the two extractions, 25 mL of water were added to each tube to reduce the methanol concentration and allowing lyophilization and samples were kept at -80ºC. Once frozen, samples were lyophilized with caps loosened. Four mL of water were added to each sample and they were vortexed (15 s) to resuspend all the content. Samples were centrifuged at 10 000 rpm for 3 min to concentrate the content at the bottom and frozen again at -80ºC. Again, the samples were lyophilized with caps loosened. Once totally dried, 1 mL of phosphate buffer in D_2O + 0.01% TSP was added to each sample and vortexed for 5 s. All the content was resuspended with micropipette and transferred to Eppendorf tubes. Eppendorfs were

centrifuged at 12 000 rpm (3 min) and 0.6 mL of the supernatant were transferred to NMR tubes.

Finally, the recovery of two polar metabolites (glucose and alanine) was tested.

For the organic extraction; for each sample, 4 mL of the organic extraction was collected by crystal syringes in crystal jars avoiding any collection of pellet. Then, the steps 5 to 10 of Fig.2 were repeated in the same tubes. The organic fractions were placed into 25 mL round-bottom evaporation flasks and were dried in a rotary vacuum evaporator. To each flask, 1 mL of chloroform D containing 0.03% TMS was added and closed. After 10 minutes of waiting, the flasks were mixed and their content was transferred into Eppendorf tubes, centrifuged at 12 000 rpm (3 min) and 0.6 mL of the supernatant were transferred to NMR tubes. The use of special Eppendorf tubes for organic solvents is recommended for the nonpolar samples to avoid any interaction with plastic polymers.

C. Acquisition of NMR fingerprint data

The NMR fingerprint of a sample consists of a quantitative NMR spectrum of it. The spectrum has unassigned signals that correspond to the different metabolites in the sample, and the signal intensities directly correlate to metabolite concentrations (Viant, Ludwig & Günther 2008). The spectra are obtained under specific, defined conditions. This ecometabolomic protocol is based on ^{1}H NMR, but the procedures described may be helpful when using other nuclei (Palomino-Schätzlein *et al.* 2011).

All experiments must be acquired at a constant, known temperature. For this, the temperature of the equipment is calibrated (using a standard methanol sample) and maintained constant at 298.0 K, applying an equilibration delay once the tube is in the core of the magnet and prior to the acquisition (2-4 min).

Samples of the polar and nonpolar fractions are analysed and compared separately. Initially, the NMR fingerprint experiment for polar and for nonpolar samples are set up in the spectrometer (Avance 600 NMR spectrometer equipped with a triple inverse 5-mm tube Z-gradient TBI probe and with a temperature control unit BCU-X; NMR software package for data acquisition and processing Topspin, Bruker Biospin) in order to analyze later all samples using identical conditions . Polar samples are analyzed using a standard quantitative 90º pulse-acquisition ^{1}H NMR experiment with solvent suppression (Zheng & Price 2010). The standard water presaturation experiment, a conventional composite 90° ^{1}H pulse sequence with suppression of the residual water signal (Bax 1985), or a 90° ^{1}H NOE enhanced pulse sequence commonly termed 1D NOESY-presat (Nicholson *et al.* 1995) are adequate (Table 1). Nonpolar samples are analyzed with a standard 90° ^{1}H pulse-acquisition NMR experiment. Acquisition parameters (such as pulse sequence, spectral width, offset frequency, number of transients, etc.) and processing parameters (such as application of a window function, phase correction, baseline correction, etc.) must be set up adequately (detailed in the Supporting Information).

Next, the automation is configured (autosampler BACS60 for tube NMR and ICONNMR software application, Bruker Biospin). The automation controls processes such as insertion/ejection of the sample into the magnet, waiting delay, automatic locking onto the signal of the deuterated solvent, tuning and/or adjusting the tune frequency to the Larmor frequency of the nuclei measured, homogenization of the magnetic field, adjustment of the receiver gain of the sample, and the execution of the experiment (parameter set configured previously), which includes the acquisition of the FID, its Fourier transformation, and the preprocessing of the spectrum. The most

convenient way to proceed is to first run one set of samples, for example the polar ones and then the others.

Finally, before starting the analysis of one set (polar or nonpolar), the correct 90° ^{1}H pulse length must be determined on a representative sample of the set; for polar samples, the offset of the water signal must also be determined for its optimal suppression. These values must be saved and used for the complete set of experiments. Polar and nonpolar samples are then analyzed separately running automatically the set-up experiments. All spectra must then be visually inspected, and those that are poorly phased or baseline corrected must be corrected manually. Finally, the spectra must be calibrated using the signal of the internal calibrating reference added to the sample.

D. **NMR-based metabolite identification. NMR metabolic profile**

The NMR metabolic profile of a sample is obtained when each peak of the NMR spectrum is assigned to its corresponding metabolite. The profile gives the NMR signals a biomolecular meaning (Viant, Ludwig & Günther 2008). This analysis is usually performed on a single representative sample, which could be any of the NMR samples of the fingerprinting procedure or one originating from different samples of the study and prepared following the same procedure described. The differences observed among samples are mainly due to differences in metabolite concentrations. Qualitative differences, however, may occur. The assignment of a specific peak is sometimes not possible (for example, when peaks overlap or when signals are of low intensity). In these cases, the analysis of another sample can be helpful. A visual inspection of all spectra of the fingerprinting can help to find a better sample for the elucidation of a specific signal.

The assignment of the ^{1}H NMR signals is conducted following two approaches. First, by comparison of the resonance frequencies (chemical shifts, δ) and line shapes (multiplicity and coupling constants, *J*) of the spectrum to bibliographical data ((Breitmaier, Haas & Voelter 1979; Gunstone 1995; Fan 1996; Sacchi, Addeo & Paolillo 1997; Vlahov 1999; Fan & Lane 2008; Berger & Sicker 2009)) and NMR spectral databases (BMRB (Ulrich & Zhulin 2007), MDL (Lundberg *et al.* 2005), HMDB (Wishart *et al.* 2009), MMCD (Cui *et al.* 2008), or the AOCS Lipid Library). Second, by the structural elucidation of the mixture (sample) through the performance of a suite of 2D NMR experiments (Table 1) and the concerted analyses of the data obtained. Basic NMR strategies followed for the structural characterization of natural products are applied for the elucidation of complex mixtures of small biological molecules (metabolites), instead of to isolated molecules as in the case of natural products research (Robinette *et al.* 2012). Briefly, protons connected by three to five chemical bonds are identified using 2D ^{1}H-NMR homonuclear COSY and TOCSY correlations. ^{1}H-^{1}H NOESY experiments determine connections between different parts of a same molecule, and heteronuclear ^{1}H-^{13}C HSQC and HMBC methods identify the carbon skeleton of a molecule. This approach, the structural elucidation of the mixture by NMR spectroscopy, is particularly helpful for the identification of secondary metabolites, since less published NMR data of them is available (Table 1). These experiments are time-consuming, but they only need to be performed once. 1D-selective ^{1}H experiments can be complementary to the 2D experiments, depending on the problem requiring elucidation. They are less time-consuming than the 2D correlations but retain maximum resolution and are used to get specific information of a chosen NMR signal of the 1D ^{1}H spectrum. The spectrum is much simpler to analyse and only shows the correlation information for the selected peak. 1D-selective ^{1}H experiments

are valuable tools for elucidating and confirming problematic molecules (Ellis *et al.* 2012). NMR experiments are performed at the same experimental temperature used for the NMR fingerprint spectra (298.0 K) and using the same spectrometer. However, to use the same NMR spectrometer is not necessary, since NMR data are fully comparable independently of the spectrometer used. The use of cryoprobes will also increase the sensitivity and considerably reduce the experimental time needed.

After the sample has been inserted into the magnet and has reached the set temperature (2-4 min) and before the experiment starts, the probe should be tuned, the sample locked, and the homogeneity of the magnetic field optimized. 2D NMR experiments will be performed under standard conditions, and only some basic parameters such as spectral width, offset, and number of transients should be optimized. The version of the experiment with water-signal presaturation is recommended for polar samples. For the 1D ^{1}H-selective experiments, the offset frequency should be adjusted according to the signal of interest to saturate. The acquisition of an initial 1D ^{1}H spectrum will help for the adjustment of these parameters and will also provide information about the concentration of the sample, which will be useful for the adjustment of the number of transients.

Each specific ecometabolomic study and assignment problem will require the performance of some or all of the experiments indicated in Table 1. In this protocol, we describe the most common and useful NMR experiments that provide structural information, but many other NMR experiments are available in the spectrometric libraries that can be useful for the elucidation of specific problems.

E. **Statistical data analysis**

The NMR data from fingerprint spectra were adequately processed before conducting the statistical analyses. The bucketing process consists in obtaining the integral numeric value of the selected regions of the spectra (buckets) directly correlated with the molar concentration by its relationship to the initial concentration of the internal standard (TMS or TSP). For ecometabolomics, variable-size bucketing is highly recommended over regular-size bucketing (Kim, Choi & Verpoorte 2010) for reducing the number of variables for statistical analyses. First, a pattern for each kind of spectrum (polar and nonpolar) was created. The pattern is determined from identifying exactly where an NMR signal (peak) begins and ends for all peaks in the spectrum, and then the bucketing process can be executed based on this pattern. All empty areas (without peaks) of spectra can also be introduced into the pattern to detect any qualitative differences between samples. We used the variable-size bucketing option of AMIX (Bruker Biospin, Rheinstetten, Germany), scaling the buckets relative to the internal standard (TMS or TSP), although other programmes can be used. The output was a data set containing the integral values for each ^{1}H-NMR spectral peak accounted for in the described pattern.

The bucketed data sets from the NMR fingerprint spectra can be analysed directly (without a previous assignment of the metabolites), because rapidly classifying samples according to their origin or their ecological or ecophysiological relevance is sufficient (Sardans, Peñuelas & Rivas-Ubach 2011) (Fig. 3 and Fig. 4). This last approach does not attempt to identify all metabolites but provides the metabolomic signature of the organism and allows detection of any shift or anomaly in its metabolism (Fig. 3 and Fig.4).

Statistical analyses of metabolomic profiles (where the assignments of the NMR peaks are considered) can be performed two main ways when the ^{1}H-NMR spectra have

been treated by variable-size bucketing. (i) All ^{1}H-NMR spectral peaks can be used as individual variables. Here the result is a data set where the number of variables is equivalent to the number of buckets in the bucketing process. (ii) The peaks (buckets) corresponding to the same molecular compound can be added up. The final number of variables in the data set is highly reduced, and the statistical results are easier to interpret. We used this second option.

Multivariate ordination analysis is one of the commonest statistical analyses in metabolomic studies for detecting the most interesting of many variables. Principal Components Analysis (PCA) and Partial Least Squares Discriminant analysis (PLS-DA) are commonly used (Ramadan *et al.* 2006). PLS-DA uses a linear model to discriminate the different tested groups and to project the variables and cases onto new axes, showing the discrimination of the most variable cases by the corresponding variables and PCA shows the most variable cases without taking into account the different groups tested.

Finally, all metabolites combined can also be analysed using permutational MANOVAs (PERMANOVAs) (Anderson, Gorley & Clarke 2008) when the data for all metabolites are non-normal or when a better accommodation of random effects and interaction terms is needed. ANOVA/MANOVA assumes normal distributions and, implicitly, Euclidean distance, but PERMANOVA works with any distance measure appropriate to the data and uses permutations to make it distribution free.

RESULTS AND DISCUSSION

Optimization of the procedure

Different steps of the metabolite extraction procedure were tested in order to optimize the time and obtain reliable results for the statistical analyses.

Sonication time: After testing different times of sonication our experiments showed that the optimum extraction of metabolites was obtained with two minutes of sonication (Step 7 of Fig. 2) (Table S1 and Figures S1, S2). The use of chloroform during extraction will dissolve cellular membranes and thereby reduce the time of sonication. Longer times for sonication reduce the signal strength of metabolites in ^{1}H NMR spectra, perhaps due to the heating of samples and the formation of metabolite artefacts (t'Kindt *et al.* 2008). In other NMR protocols of polar metabolites where chloroform extraction is not performed, sonication for 10 min has been recommended (Kim, Choi & Verpoorte 2010).

Repeated extractions: Our tests showed that two extraction procedures were the optimum. A first extraction from *E. multiflora* and *Q. ilex* leaves yielded 78.5% and 85.6% metabolites, respectively, relative to a second extraction, although the differences were not statistically significant (one-way ANOVA test of the global concentration of metabolites; p=0.32 for *E. multiflora* and p=0.26 for *Q. ilex*). Three extractions from these species showed no differences to two extractions (one-way ANOVA test of the global concentration of metabolites; p=0.97 for *E. multiflora* and p=0.89 for *Q. ilex*); more than 98% of metabolites from the third extraction had already been extracted after the second extraction (See supporting information for more details, *F.sylvatica* was also tested). The importance of repeated extractions has been discussed in protocols based on LC-MS metabolomic analyses (Nikiforova *et al.* 2005; t'Kindt *et al.* 2008). For our protocol, by two extraction procedures the NMR samples presented the maximum concentration of metabolites in the extracts (Supporting information; Table S2).

Recovery: it was tested for two polar metabolites; glucose and alanine. Our results showed a recovery of 92.8% for alanine and of 86.4% for glucose (Figures S3 and S4). It will allow performing the statistical analyses comparing several samples showing a minimum error.

Anticipated results with wild plants.

The ^{1}H NMR metabolic profiles (polar and nonpolar) of the leaves of *E. Multiflora* and *Q. ilex* are shown in Fig. 5. A typical ^{1}H NMR metabolic profile of a polar extract from a wild plant in ecometabolomics shows the presence of primary metabolites, such as sugars, amino acids, organic acids, hydroxyacids, alcohols, and nucleic acids, as well as secondary metabolites characteristic of the particular species or family. These molecules can be completely elucidated and identified as discrete molecules (Fan 1996; Fan & Lane 2008). Nonpolar extracts contain fatty molecules (such as free fatty acids; fatty alcohols; and mono-, di-, and triglyceraldehydes) and nonfatty molecules (such as polyphenols and terpenes). Nonfatty molecules, as with polar molecules, can be completely elucidated as discrete molecules. Fatty molecules, however, are qualitatively analysed as a group, identifying and quantifying the presence of mono- and polyunsaturated fatty chains; mono-, di-, or triglyceraldehydes; free fatty alcohols; and/or free fatty acids (Gunstone 1995; Vlahov 1999; Engelke 2007; Fan & Lane 2008).

A PCA of the foliar metabolic fingerprint of *E. multiflora* throughout the seasons of the year is represented in Fig. 6, The seasonal principal component analysis (PCA) resulted in a first principal component (PC1) separating the foliar metabolome in the different seasons. Spring leaves presented the highest concentrations of polar metabolites, such as alanine, glutamine, asparagine, threonine, α-glucose, β-glucose, and sucrose. In contrast, they had the lowest concentrations of lipids and secondary

metabolites, such as terpene compound 1 and derivatives of *p*-coumaric acid. The results were more discuseed in detail in Rivas-Ubach *et al.,* 2012. The ecometabolomic seasonal analyses showed the highest concentrations of sugars and amino acids in spring (the growing season), directly linked to growth.Other analyses performed on *E. multiflora* leaves have found the lowest stoichiometric N/P ratios to also occur in this season (Rivas-Ubach *et al.* 2012). These results are directly related to the high level of RNA translation that occurs for anabolism during spring, which is the growing season of Mediterranean plants. These findings support the Growth Rate Hypothesis (GRH) (Elser *et al.* 1996), one of the central paradigms of ecological stoichiometry, at the metabolomic level. The GRH proposes that growing organisms must increase their allocation of P to RNA for meeting the elevated demands for the synthesis of proteins required for growth.

Additionaly, *E. multiflora* plants were experimentally stressed by conditions of drought throughout the year. A PCA was performed with only those variables presenting significant differences between control and droughted plants in summer (Fig. 7), even though differences among all seasons were detected (results explained in detail in Rivas-Ubach *et al.* 2012). Mainly the foliar metabolomes of droughted plants presented higher concentrations of quinic acid, tartaric acid, lipids and terpenes ahowing that our protocol was also sensitive enough to detect shifts in the metabolomes as a response to climatic changes (Rivas-Ubach *et al.* 2012). Quinic acid and tartaric acid are compounds with antioxidant function and their higher concentration in droughted plants are in accordance with the known increase of oxidative stress in plants that have endured drought (Peñuelas *et al.* 2004).

A NMR fingerprinting data analyses were performed for *Q. ilex* (Fig.4). Data were classified in the different regions (Fig. 3). The results revealed higher

concentrations of polar compounds directly correlated to higher growth rates, such as sugars and amino acids in spring individuals as expected in other studies (Rivas-Ubach *et al.,* 2012). Also, summer (the warmest season) samples presented lower concentrations of nonpolar compounds than the rest of the seasons in agreement with other experimental warming studies on plants (Livonen *et al.* 2004), chlorophyta (Fuschino *et al.* 2011), and zooplankton (Gladyshev *et al.* 2011).

CONCLUSIONS

Here we presented a new optimized NMR-based protocol for application of metabolomics to field ecology. It has been specially developed to reduce the experimental errors and to be applied to a large number of samples, as often required in ecology. It thus allows performing more accurate statistical analyses. It demonstrated to be sensitive enough to detect the differences in metabolomes of plants across different seasons and among different experimental climatic treatments. This protocol has been designed for studying the metabolome of wild plants but can also be used with animals and it is effective both for targeted and untargeted studies. It will help to increase the knowledge in the shifts of the wild organism's metabolomes across environmental gradients and it will allow making a step forward in the understanding of the role of metabolism driving the ecosystem structure and function.

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Figure captions

Fig. 1 General NMR-based procedures for an ecometabolomic study.

Fig. 2 Experimental procedure for the preparation of plant tissue to obtain polar and nonpolar extracts for NMR analyses.

Fig. 3 Example of the ^{1}H NMR spectra of the polar (water–methanol) and nonpolar (chloroform) extracts of *Erica multiflora* leaves. The main families of compounds observed in the different regions of the spectra are indicated by: **A**, typically amino acids; **B**, mainly sugars; **C**, aromatic region; **D**, fatty chains; **E**, typically tri-, di-, and monoacylglycerides; **F**, polyphenol region; **G**, aldehyde region.

Fig. 4 PCA plots conducted from ^{1}H NMR fingerprinting data from *Quercus ilex* leaves. **A)** Panel of loadings of PC1 and **B)** panel of loadings of PC2. Loadings of the different spectral regions are represented by different colors as indicated. **C)** Panel of individuals categorized by season and differentiated by color as indicated in the figure. Arrows outside the plot indicate the mean PC score for each season. The statistically significant differences between seasons were detected by Bonferroni post hoc tests and are indicated by lowercase letters ($p < 0.05$).

Fig. 5 Typical ^{1}H NMR metabolic-profile spectra of polar (water–methanol) and nonpolar (chloroform) extracts of *E. multiflora* and *Q. ilex* leaves. **Polar metabolites** (assigned signals indicated with numbers): 1, α-glucose (αG); 2, β-glucose (βG); 3, sucrose (Suc); 4, alanine (Ala); 5, asparagine (Asp); 6, glutamine (Gln); 7, leucine (Leu); 8, isoleucine (Ile); 9, threonine (Thr); 10, 6-deoxypyranose; 11, 4-hydroxyphenylacetate; 12, malate; 13, maleate; 14, citrate; 15, 3-amino-4-

hydroxybutyrate; 16, N-acetyl group; 17, quinic acid (Q.ac); 18, tartaric acid (T.ac); 19, arbutin (Arb); 20, choline (Ch); 21, 1,2-propanediol; 22, γ-hydroxybutyrate; 23, lactate; 23, valine (Val); 24, quercitol (Quer); 25, N-acetyl group (Ac); 26, formate (For); 27, catechin derivative (U1). **Nonpolar metabolites** (assigned signals/regions indicated with letters): A, C, D, and F, fatty acid spectral regions; B, linoleyl fatty acid region; E and L, unsaturated fatty acid regions; G, free fatty acid region; H, polyunsaturated fatty acid region; I, diacylglycerid and triacylglycerid region; J, triacylglycerid 2 region; K, triacylglycerid 1 region; M, 1,2 diacylglycerid region; N, polyphenol region; O, aldehyde group region; Ac, acetyl group; DGA, 1,2-diacylglycerid; FAI, fatty alcohols; Lin, linolenyl chain; P1, polyphenol derivative of *p*-coumaric acid 1; P2, polyphenol derivative of *p*-coumaric acid 2; Ter, terpene compound 1; TGA1, triacylglycerid 1; TGA2, triacylglycerid 2; U1, unknown compound 1; U2, unknown compound 2 (data from *Erica multiflora* modified from (Rivas-Ubach *et al.* 2012)).

Fig. 6 Plots of the first principal component (PC1) versus the third principal component (PC3); loadings and scores resulting from PCA conducted through ^{1}H NMR metabolomic variables in *E. multiflora* leaves. (**A**) Panel of metabolomic variables. Variables are indicated by different colours: blue, polar metabolites from primary metabolism; red, glucose and sucrose; orange, polar metabolites from secondary metabolism; black, nonpolar compounds. Different metabolic families are separated by clusters in different colours: blue, sugars; yellow, amino acids; green, compounds related to amino acids and sugar metabolism in plants (RCAAS); violet, lipids. Variable labels are described in Fig. 4, and variables from 30 to 55 represent overlapped signals: 30, 11+15; 31, 5+11; 32, 5+11+13; 33, 6+21; 3, 12+unknown; 35, 15+22; 36, 16+19; 37, 3+16+19; 38, 6+16; 39, 1+2+3+16+18; 40, 1+2+3+5+16+18; 41, 1+2+3+16; 42, 11+14; 43, 9+14+22; 44, 13+14; 45, 1+2+9; 46, 1+2+3+18; 47, 1+2+3+18+19; 48,

1+2+3+18+19+20; 49, 1+2+3+9+18+21; 50, 1+2+3+unknown; 51, 1+2+18; 52, 1+2+9+18; 53, 1+2+6+18; 54, 2+20; 55, 2+3+18+19. (**B**) Panel of samples categorized by season. Seasons are indicated by different colors (red, summer; yellow, autumn; blue, winter; and green, spring). Arrows outside the plots indicate the mean PC score for each season. The statistically significant differences between seasons were detected by Bonferroni post hoc tests and are indicated by lowercase letters ($p < 0.05$). (Adapted from (Rivas-Ubach *et al.* 2012)).

Fig. 7 Plots of the PCAs conducted on the ^{1}H NMR metabolomic variables of the *Erica multiflora* analyses that presented different responses to experimental climatic treatments in summer. Variable labels are described in Fig. 4. Treatment is indicated by color: green, control; yellow, drought. Arrows outside the plots indicate the mean PC score for each treatment. The statistically significant differences are indicated by arrows with lowercase letters ($p < 0.05$). (Adapted from (Rivas-Ubach *et al.* 2012)).

Fig. 1

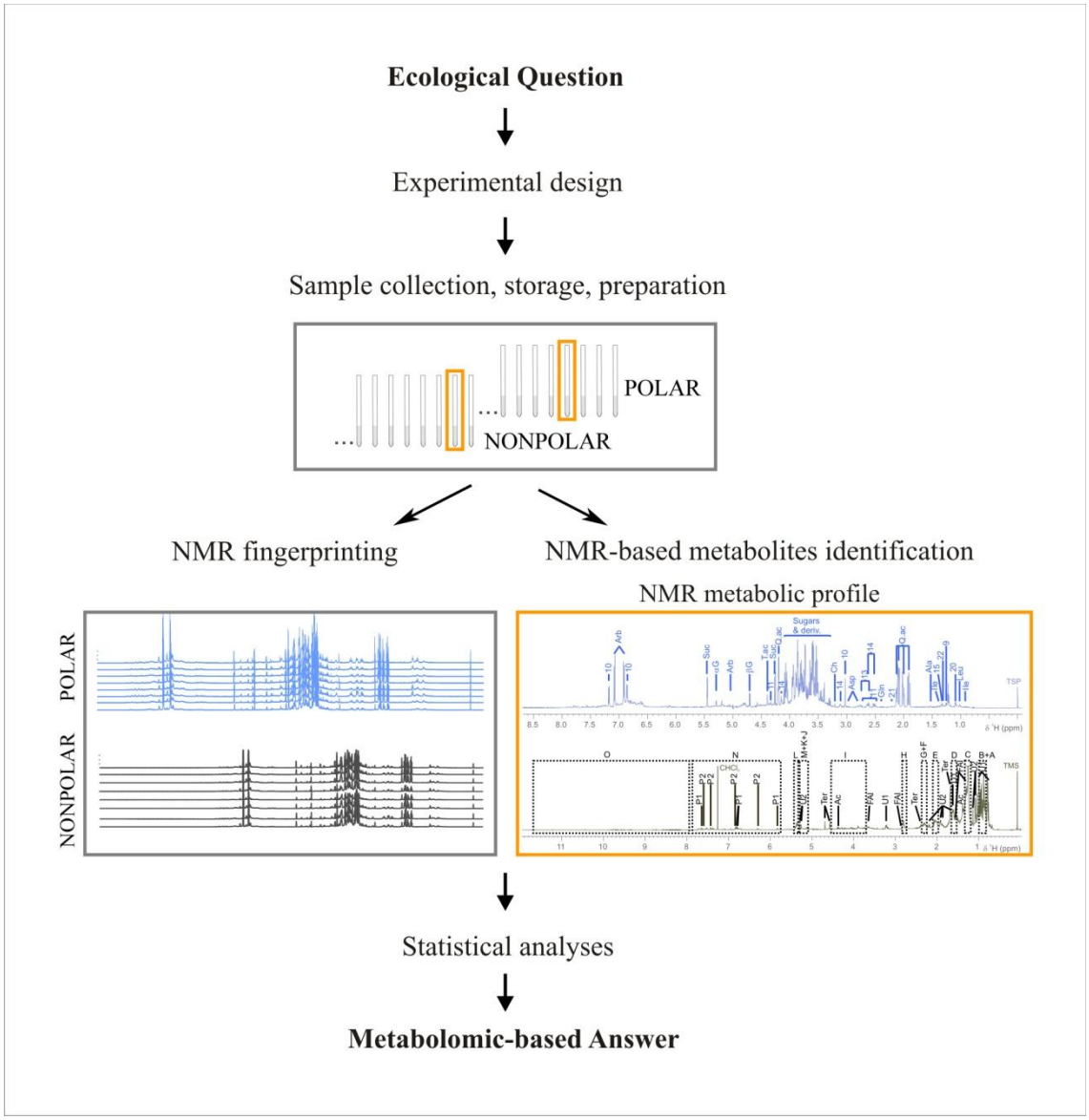
Ecological Question
Experimental design
Sample collection, storage, preparation
POLAR
NONPOLAR
NMR fingerprinting
NMR-based metabolites identification
NMR metabolic profile
POLAR
NONPOLAR
Statistical analyses
Metabolomic-based Answer

Fig. 2

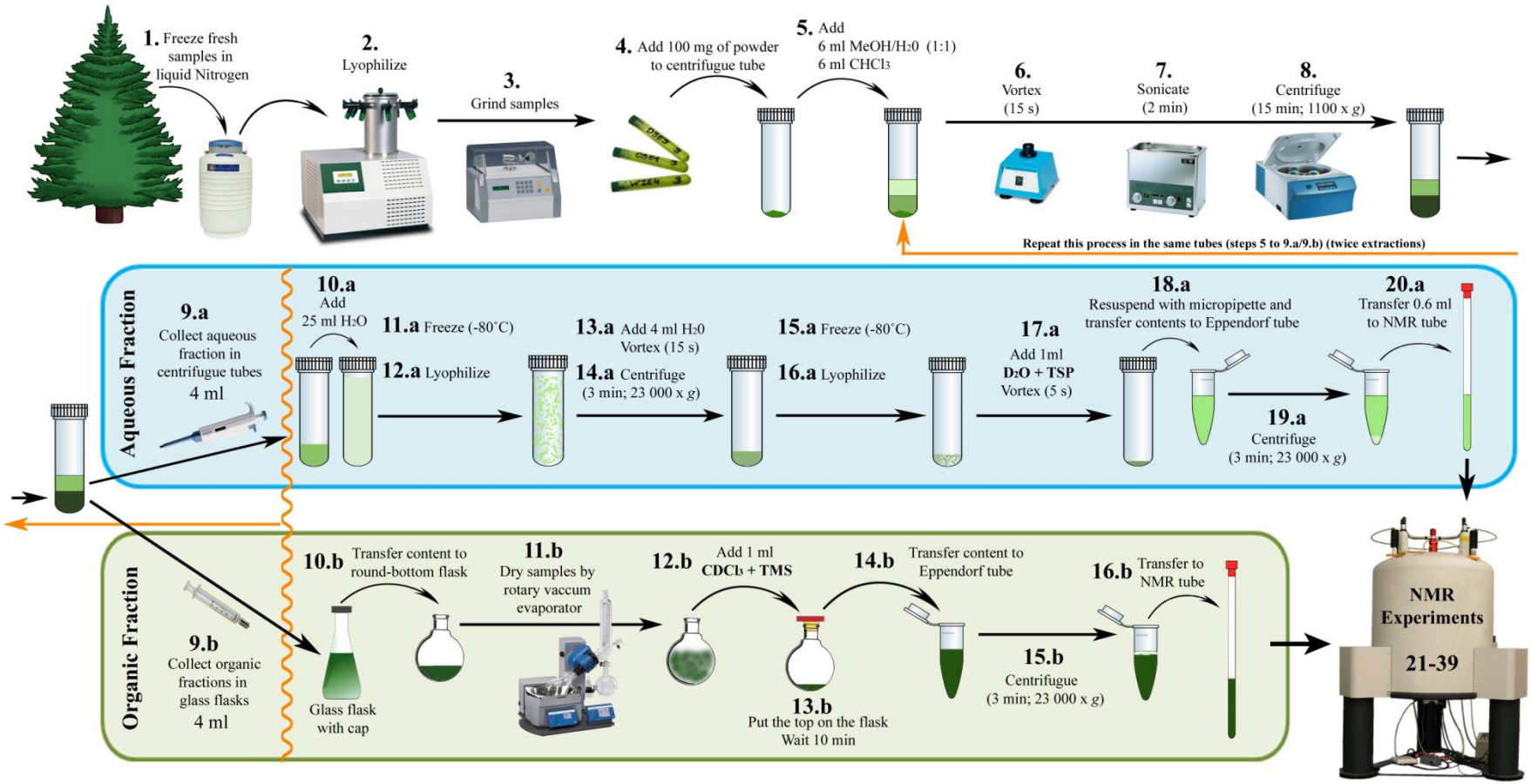
1. Freeze fresh samples in liquid Nitrogen
2. Lyophilize
3. Grind samples
4. Add 100 mg of powder to centrifugue tube
5. Add 6 ml MeOH/H20 (1:1) 6 ml CHCl3
6. Vortex (15 s)
7. Sonicate (2 min)
8. Centrifuge (15 min; 1100 x g)
Repeat this process in the same tubes (steps 5 to 9.a/9.b) (twice extractions)
Aqueous Fraction
9.a Collect aqueous fraction in centrifugue tubes 4 ml
10.a Add 25 ml H2O
11.a Freeze (-80°C)
12.a Lyophilize
13.a Add 4 ml H2O Vortex (15 s)
14.a Centrifuge (3 min; 23 000 x g)
15.a Freeze (-80°C)
16.a Lyophilize
17.a Add 1ml D2O + TSP Vortex (5 s)
18.a Resuspend with micropipette and transfer contents to Eppendorf tube
19.a Centrifuge (3 min; 23 000 x g)
20.a Transfer 0.6 ml to NMR tube
Organic Fraction
9.b Collect organic fractions in glass flasks 4 ml
10.b Transfer content to round-bottom flask
Glass flask with cap
11.b Dry samples by rotary vaccum evaporator
12.b Add 1 ml CDCl3 + TMS
13.b Put the top on the flask Wait 10 min
14.b Transfer content to Eppendorf tube
15.b Centrifuge (3 min; 23 000 x g)
16.b Transfer to NMR tube
NMR Experiments
21-39

Fig. 3

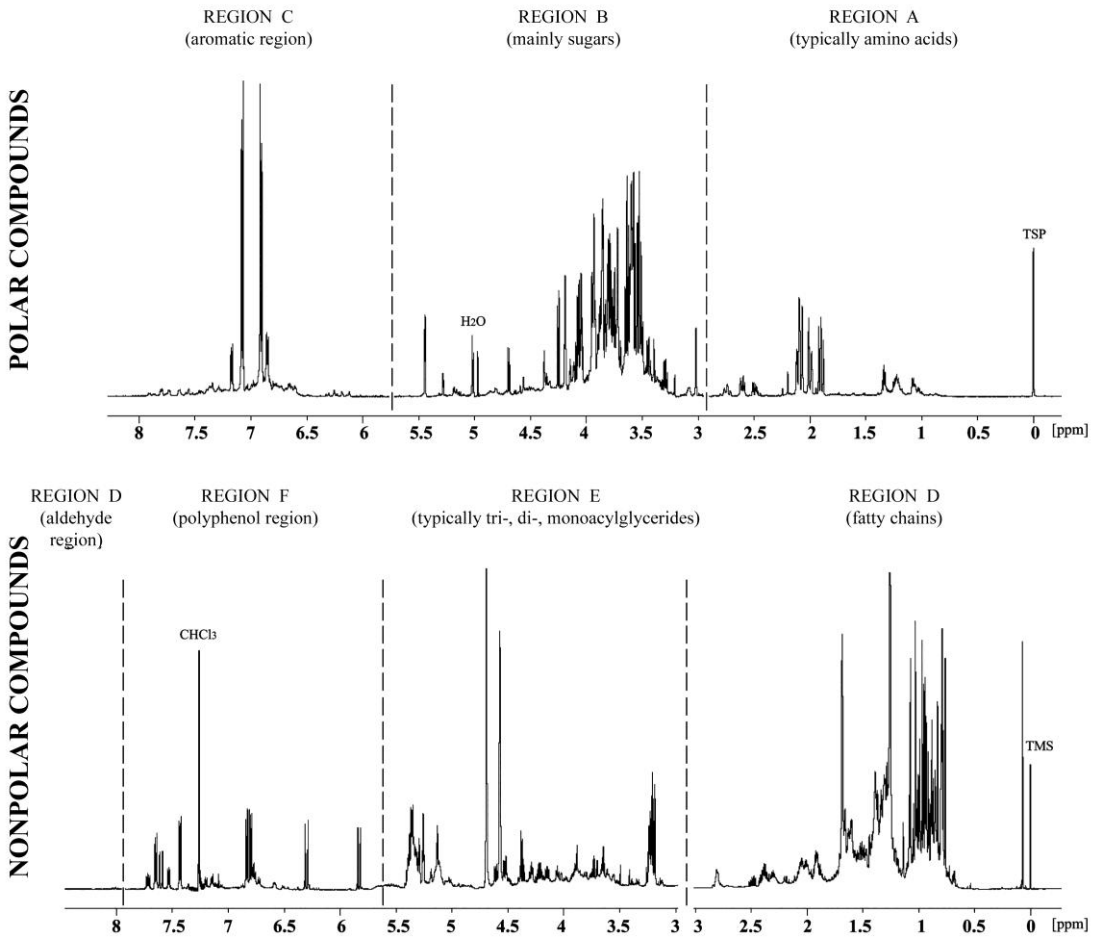
REGION C
(aromatic region)
REGION B
(mainly sugars)
REGION A
(typically amino acids)
POLAR COMPOUNDS
H2O
TSP
8 7.5 7 6.5 6 5.5 5 4.5 4 3.5 3 2.5 2 1.5 1 0.5 0 [ppm]
REGION D
(aldehyde
region)
REGION F
(polyphenol region)
REGION E
(typically tri-, di-, monoacylglycerides)
REGION D
(fatty chains)
NONPOLAR COMPOUNDS
CHCl3
TMS
8 7.5 7 6.5 6 5.5 5 4.5 4 3.5 3 3 2.5 2 1.5 1 0.5 0 [ppm]

Fig. 4

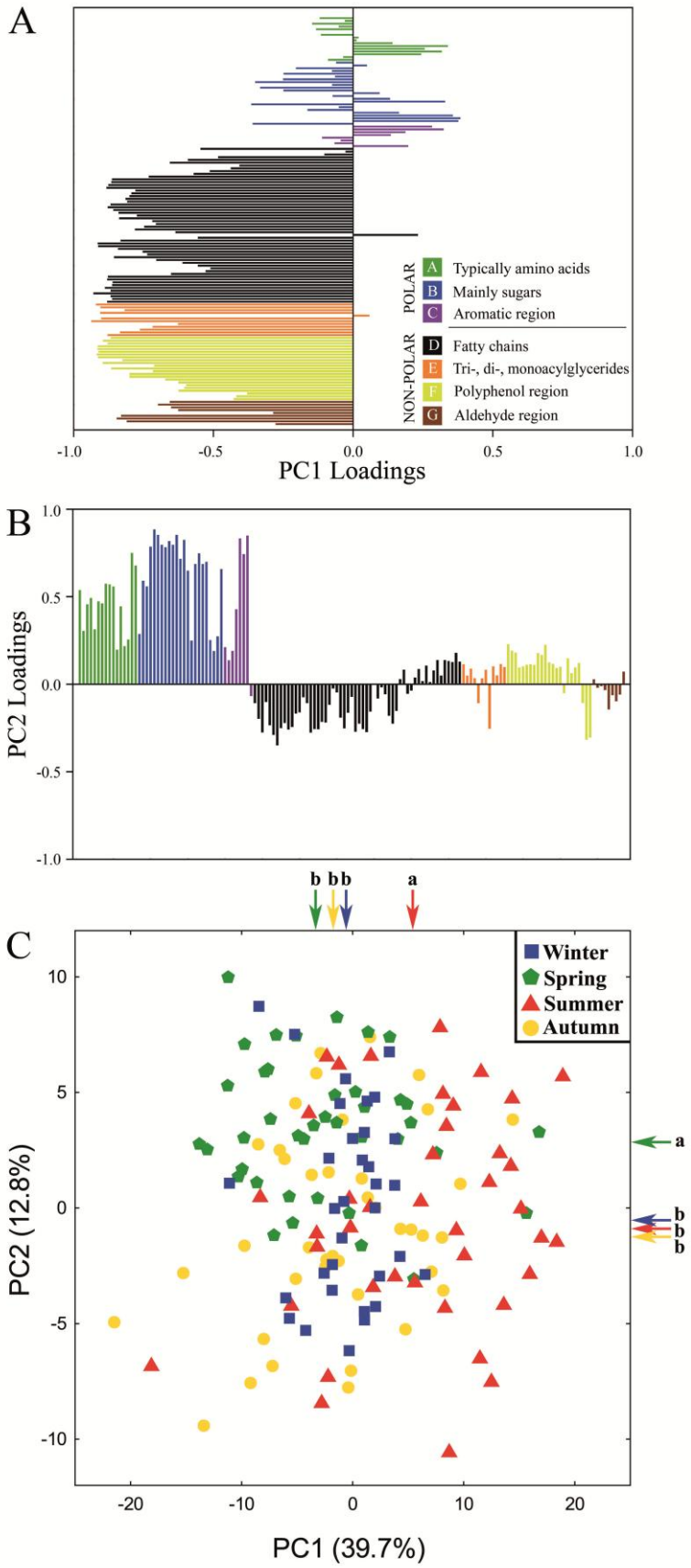
A
POLAR
A Typically amino acids
B Mainly sugars
C Aromatic region
NON-POLAR
D Fatty chains
E Tri-, di-, monoacylglycerides
F Polyphenol region
G Aldehyde region
-1.0
-0.5
0.0
0.5
1.0
PC1 Loadings
B
PC2 Loadings
1.0
0.5
0.0
-0.5
-1.0
b b b
a
C
Winter
Spring
Summer
Autumn
PC2 (12.8%)
10
5
0
-5
-10
-20
-10
0
10
20
PC1 (39.7%)
a
b
b
b

Fig. 5

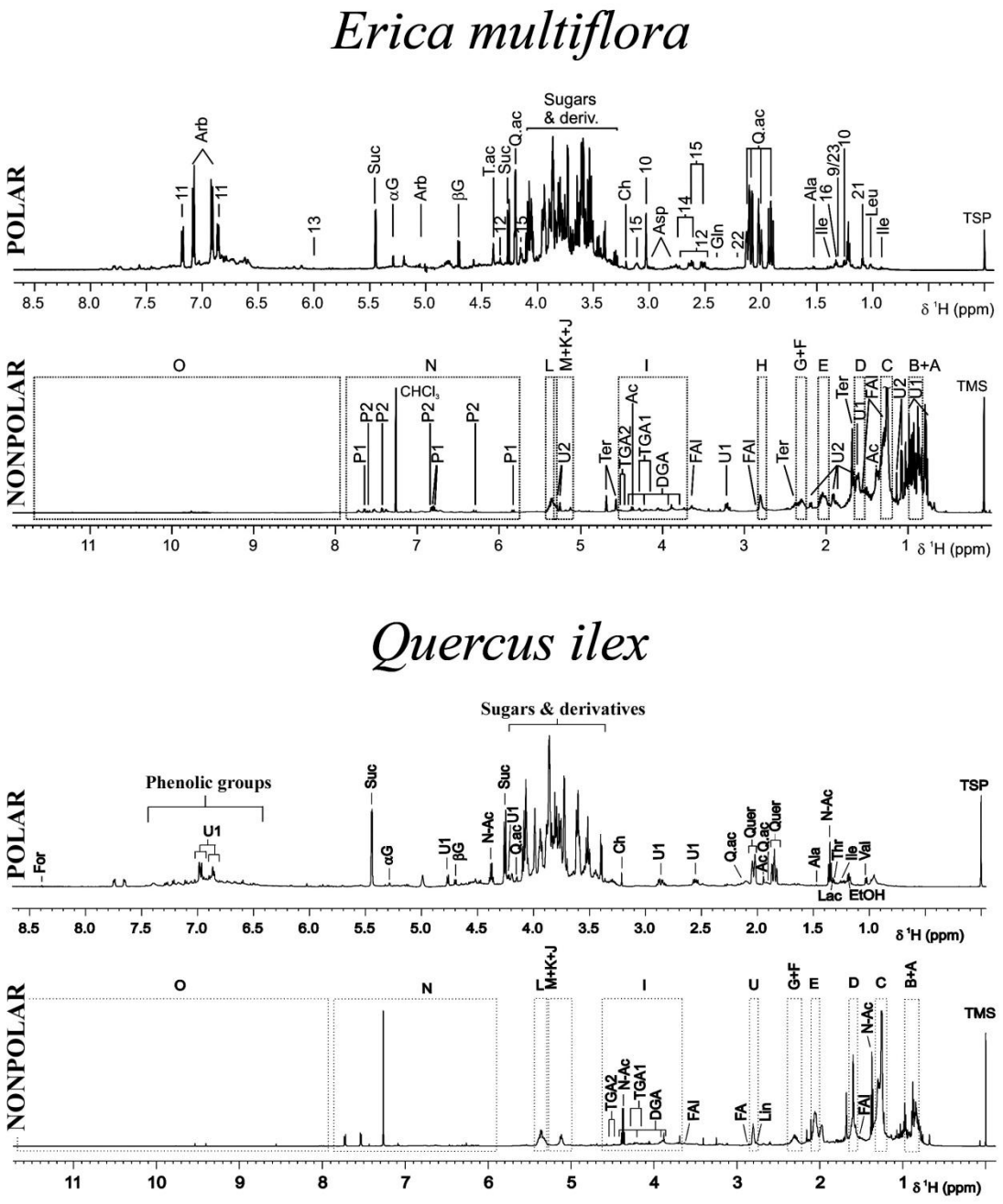
Erica multiflora
POLAR
Arb
Suc
αG
βG
T.ac
Q.ac
Sugars & deriv.
Ch
Asp
Gln
Ala
Ile
Leu
TSP
δ ¹H (ppm)
NONPOLAR
O
N
L
M+K+J
I
H
G+F
E
D
C
B+A
TMS
CHCl3
P1
P2
U1
U2
Ter
TGA2
TGA1
DGA
FAl
Ac
Quercus ilex
Sugars & derivatives
Phenolic groups
For
U1
Suc
αG
βG
N-Ac
Q.ac
Ch
Quer
Ala
Thr
Ile
Val
Lac
EtOH
TSP
δ ¹H (ppm)
NONPOLAR
O
N
L
M+K+J
I
U
G+F
E
D
C
B+A
TMS
TGA2
N-Ac
TGA1
DGA
FAl
FA
Lin

Fig.6

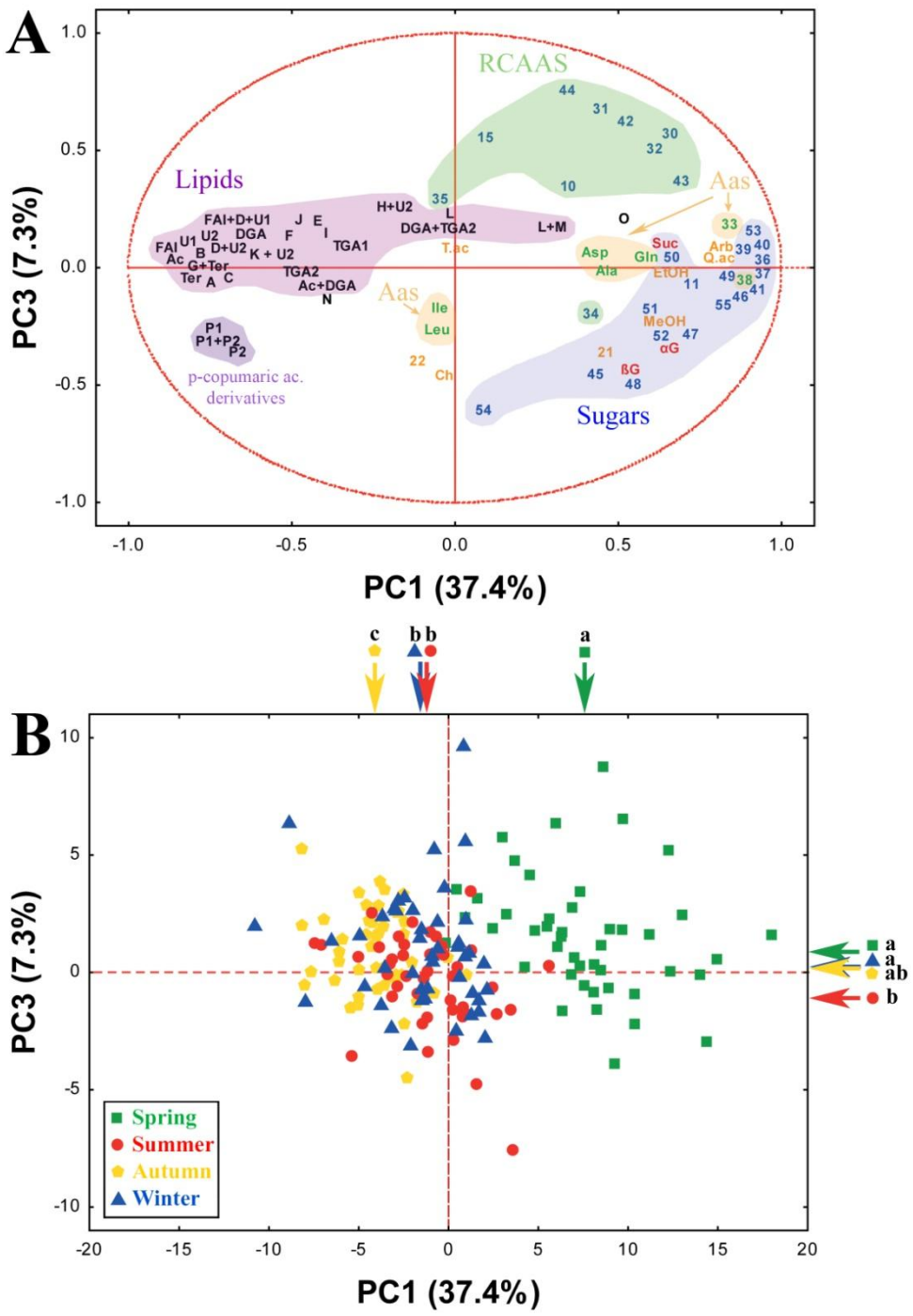
A
RCAAS
Lipids
Aas
Aas
Sugars
p-copumaric ac. derivatives
PC1 (37.4%)
PC3 (7.3%)
B
c
b b
a
a
a
ab
b
Spring
Summer
Autumn
Winter
PC1 (37.4%)
PC3 (7.3%)

Fig. 7

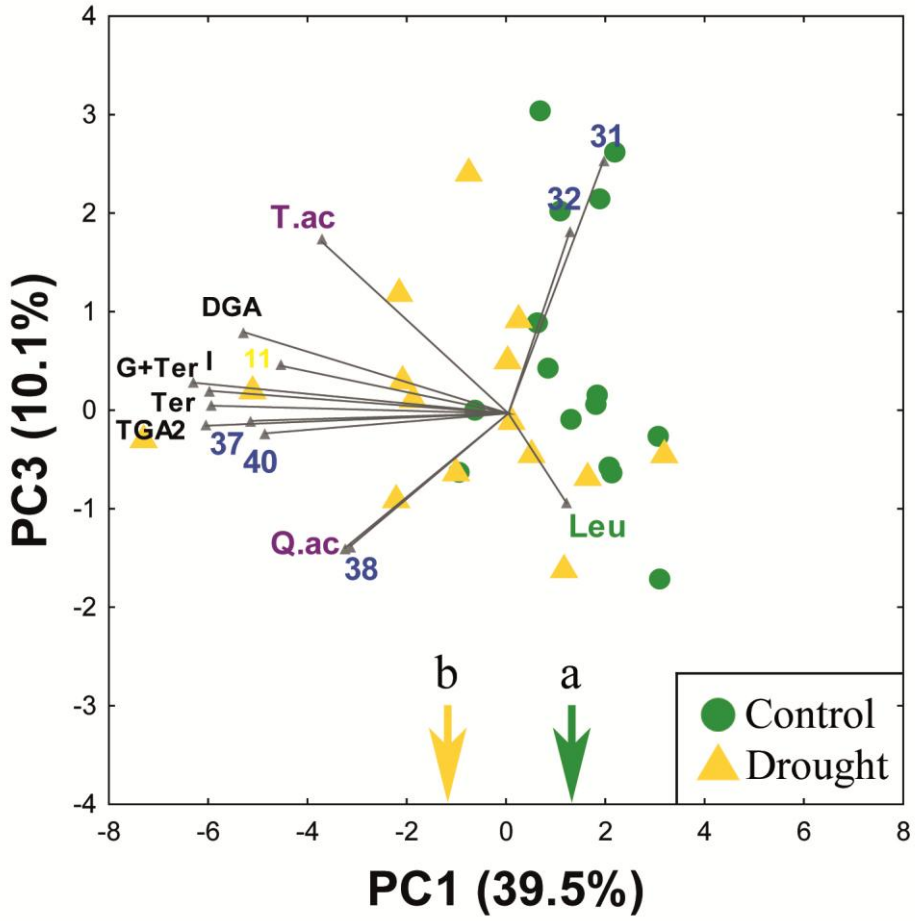
PC3 (10.1%)
PC1 (39.5%)
T.ac
DGA
G+Ter I
Ter
TGA2
11
37
40
31
32
Leu
Q.ac
38
b
a
Control
Drought

Table 1. Most common NMR experiments for identification of metabolites. Standard versions and a brief description of their application for structural elucidation problems are indicated.

Experiment	Version (pulse sequence[a])	Description[b]
1D ^{1}H	· Conventional pulse-acquisition (zg) · With solvent presaturation[c] (zgpr or zgcppr), also 1D NOESY with presaturation (noesypr1d)	Standard experiment routinely used for fingerprinting, identification of metabolites, and determining chemical shifts (d) and coupling-constant (*J*) values. Also used for quantification.
2D ^{1}H-^{1}H COSY	· Gradient selection (cosygpqf) (Aue, Bartholdi & Ernst 1976; Nagayama *et al.* 1980) · With solvent presaturation (cosypgqfpr)	Homonuclear Correlation Spectroscopy based on ^{1}H-^{1}H scalar coupling. Routinely used for the identification of metabolites, it correlates spin systems separated through chemical bonds.
2D ^{1}H-^{1}H TOCSY	· Conventional (mlevph)(Braunschweiler & Ernst 1983; Bax 1985) · With solvent presaturation (mlevphpr) · Selective 1D mode (selmlgp.2)(Bax 1985; Kessler *et al.* 1986; Stonehouse *et al.* 1994; Stott *et al.* 1995)	Total Correlation Spectroscopy. Based on homonuclear ^{1}H-^{1}H scalar coupling. It correlates spin subsystems within the same molecule.
2D ^{1}H-^{1}H NOESY	· Conventional (noesygpph) (Jeener *et al.* 1979; Wagner & Berger 1996) · With solvent presaturation (noesygpphpr) · Selective 1D mode (selnogp) (Kessler *et al.* 1986; Stonehouse *et al.* 1994; Stott *et al.* 1995)	Nuclear Overhauser Effect Spectroscopy. Based on homonuclear ^{1}H-^{1}H through-space interactions. Routinely used for the identification of metabolites, it provides information about which protons are close together in space (≤ 4Å).
2D ^{1}H-^{13}C HSQC	· Conventional using adiabatic ^{13}C pulses (hsqcetgpsisp) (Palmer *et al.* 1991; Kay, Keifer & Saarinen 1992; Schleucher *et al.* 1994)	Heteronuclear Single Quantum Correlation. Based on heteronuclear one-bond ^{1}H-^{13}C scalar coupling. Routinely used for the identification of metabolites, it correlates protons to their directly bonded carbon atom.
2D ^{1}H-^{13}C HMBC	· Conventional using low-pass *J*-filter (hmbcgplpndqf) (Bax & Summers 1986; Bax & Marion 1988)	Heteronuclear Multiple Bond Correlation. Based on heteronuclear long-range ^{1}H-^{13}C scalar coupling. Routinely used for the identification of metabolites, it correlates protons to carbon atoms separated by multiple (usually 2,3) bonds.

[a] According to Bruker nomenclature.

[b] Extensive and updated description of the vast library of NMR experiments and their differents versions is collected in the NMR Guide and Enciclopedia of Bruker.

[c] Experiments for suppression of the signal of the residual water.