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1 **Title:**
2 **Bias in leaf dry mass estimation after oven-drying isoprenoid-storing**
3 **leaves**

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Abstract

Foliage isoprenoid content or essential oil content is a key plant characteristic important in plant science, but also for food, cosmetic and pharmaceutical industry. Commonly, the amount of foliage chemicals is normalized with respect to leaf dry mass (content per dry mass). However, the protocols for foliage drying have received little attention. In particular, volatile and semi-volatile isoprenoids and other compounds with low boiling point may partly volatilize during drying, reducing both isoprenoid and leaf dry matter contents, thereby potentially underestimating the content of volatiles and overestimating that of non-volatile constituents.

Three leaf preservation (flash freezing in liquid nitrogen, freezing at -82°C and at -20°C , and cooling at 4°C) and two leaf desiccation (freeze-drying at -54°C and 1.2 mbar, and air-drying at 30, 40, 60, 70, 80, 100, 110, 125, 150, 200, and 300°C) procedures were applied to replicate leaves, and the leaf dry matter content as well as the leaf isoprenoid content was measured afterwards. The results of this experiment suggested that freeze-drying method (FD) performed the best leaf isoprenoid preservation followed by FF method, whilst air-drying methods performed a dramatic isoprenoid loss in the leaf samples, as well as preserving the leaves at freezing and cooling temperatures.

Leaf isoprenoid content and leaf dry to fresh mass ratio were correlated and there were evidences of leaf dry matter content (LDMC) miscalculation in isoprenoid storing species when using traditional air-drying methods and preservation of samples, possibly related to the isoprenoid loss as well as other volatile compounds. In addition, losses of high C content volatiles and respiration of sugars and carbohydrate pyrolysis with low carbon content may differently affect the bulk leaf C content.

Key words: *Abies balsamea*; *Betula pendula*; leaf dry matter content; *Pinus mugo*; *Quercus ilex*; volatile organic compounds;

Key message:

Air-drying, cooling and freezing leaf samples rich in isoprenoid content evaporates isoprenoids and consequently causes an underestimation of the leaf dry matter content.

Introduction

In plant science, several key leaf traits, including specific leaf area and amounts of foliage chemical elements such as nitrogen, phosphorus and carbon as well as foliage chemical compounds such as isoprenoids, flavonoids, etc. are commonly expressed per leaf dry mass unit. Furthermore, the rate of plant physiological processes such as photosynthetic capacity is often expressed per unit dry mass to characterize the physiological activity of unit mass (Wright et al. 2004). 'Dry mass' as an expression basis is favored over fresh mass, because foliage fresh mass can change during the day and among the days due to changes in leaf water status in transpiring leaves (Correia et al. 2001; Davey and Ellis-Evans 1996; Flexas and Medrano 2002; Hinckley and Bruckerhoff 1975; Medina and Francisco 1994; Tuba et al. 1996; Vos and Groenwold 1988). At the extreme, in desiccation-tolerant species, water content can vary more than an order of magnitude (Davey and Ellis-Evans 1996; Tuba et al. 1996). Thus, dry mass is commonly considered as a more reliable expression basis than fresh mass (Garnier et al. 2001; Niinemets et al. 2011; Zeiller et al. 2007). On the other hand, dry to water saturated fresh mass ratio itself can be a relevant trait (Castrillo et al. 2005; Niinemets et al. 2007; Vile et al. 2005) in leaf economics and leaf anatomy, again emphasizing the importance of reliable estimation of "dry" mass. But estimating leaf dry mass carries itself potential problems.

Even in "dry" plant material, there is a residual amount of water in equilibrium with humidity in the air (Zeiller et al. 2007). This adsorbed water is gradually released with increasing temperature or reducing water vapor concentration in ambient air, but increasing temperatures can also result first in evaporation of compounds with low boiling point (such as volatile and semi-volatile isoprenoids). With further increases in temperature, pyrolysis of certain leaf constituents may occur (Font et al. 2009). On the other hand, drying at physiological temperatures (e.g. 30 °C) can be long-lasting and result in mass loss due to respiration. Some plant tissues, such as foliage of CAM plants, cannot be dried under physiological temperatures without a loss of bulk of dry matter. Volatilization of the studied compounds during drying can lead to major losses of the compound, and accordingly to significant underestimation of their content, even when expressed per unit leaf area.

Foliage in species with specialized isoprenoid storage structures such as in conifers or species

from the families Lamiaceae (e.g., *Mentha*), Myrtaceae (e.g., *Eucalyptus*) and Rutaceae (e.g. *Citrus*), can contain large amounts of isoprenoids: between 1 to 10% of total foliage dry mass (Peñuelas and Llusà 2004). During leaf drying, the effects of compound volatilization will be higher with the increasing of heating temperature and the volatile compounds content in the leaf. On the other hand, mass loss or variation in the content of residual water will affect the concentration of other non-volatile compounds, and estimation of mass-based content of chemical elements. Furthermore, if the mass lost has different carbon content than the bulk of leaf matter (e.g. monoterpene carbon content is 88%), leaf carbon content can even be affected more strongly (Niinemets 1997; Poorter 1994).

A wide range of methods are available for the measurement of leaf dry matter content (*LDMC*); also used previous leaf isoprenoid content determination. They can be summarized in: (1) placing leaves under a low relative humidity desiccating atmosphere (i.e. desiccation with silica gel) (Whittaker et al. 2001), (2) increasing the wind speed (air-drying with a fan) (Maisuthisakul et al. 2007), (3) increasing the air temperature in order to create a low relative humidity environment as well as increasing leaf temperature and evaporate the leaf water rapidly (incubation and sun-drying) (Akah et al. 2003), (4) heating selectively the water molecules in the leaf until evaporation (with a microwave) (Jeni et al. 2010), (5) rapid cryogenic treatment of leaves which avoids water crystallization and allows water sublimation (flash freezing in liquid nitrogen), and (6) freezing the leaves and reducing the surrounding pressure to sublimate the frozen water (vacuum freeze-drying, also called lyophilization or cryodesiccation) (Ormeño et al. 2007; Ormeño et al. 2011).

The most widely used method for *LDMC* determination unites the air-drying (2) and heating desiccation (3) procedures. The time and temperatures used vary largely: commonly 60-80 °C during 48 hours. The widespread protocol for measuring *LDMC* by Garnier et al. (2001) uses 60 °C during at least two days. In the last decades, methods like freeze-drying, flash freezing and microwave drying have won popularity. Freeze-drying at higher or lower pressure is used in food industry to preserve flavor quality, and performs better essential oils retention during the drying process than oven-drying (Díaz-Maroto et al. 2003). On the other hand, microwave drying has been presented as a good alternative to freeze-drying for the determination of organic compounds

108 (Popp et al. 1996), also because microwave drying is less expensive and faster technique than
109 freeze-drying.

110 This experiment is focused on the effect of different drying methods on the leaf isoprenoid
111 content and the consequences on the determination of leaf isoprenoid content per dry matter
112 content. The main hypothesis is that conifer leaves (which contain a large isoprenoid content),
113 when highly manipulated, stored or oven-dried, will dramatically loose volatile isoprenoids
114 before the chemical determination. Furthermore, when leaves are dried at high temperature, this
115 will significantly mislead the calculation of dry matter content as a consequence of isoprenoid
116 loss.

Materials and Methods

Plant material

Foliage from two evergreen conifers, *Abies balsamea* (L.) Mill. and *Pinus mugo* Turra, an evergreen (*Quercus ilex* L.) and a deciduous (*Betula pendula* Roth) broad-leaved species was studied. All the four species are known to be significant isoprenoid emitters, especially the conifers and *Q. ilex* are strong constitutive emitters (Keenan et al. 2009; Kesselmeier and Staudt 1999). The constitutive emissions of *B. pendula* are weaker, but this species can be triggered for high isoprenoid emissions by stress events (Hakola et al. 2001). Leaves of *A. balsamea* (Régimbal and Collin 1994) and *P. mugo* (Tsitsimpikou et al. 2001) store isoprenoids in resin ducts, while *Q. ilex* (Alessio et al. 2008) and *B. pendula* (Haapanala et al. 2009) leaves lack specialized isoprenoid storage structures and can store these compounds non-specifically in only small amounts (Loreto et al. 1998; Niinemets and Reichstein 2002).

The mature coniferous (height 10-12 m for *Abies* and 3-4 m for *Pinus*) and *B. pendula* trees (height 10-15 m, age ca. 30 years) were located in the campus of the Estonian University of Life Sciences (58.39° N, 26.70° E, elevation 41 m), while the 7-yr-old *Q. ilex* trees had been grown from seed (seed source Biotopo di Marocche, Drena, Trentino, Italy (45.97° N, 10.94° E)) in 10 L pots and regularly cut back not to exceed the height of 1 m. The plants were grown in a growth chamber at 25 °C day/20 °C night temperature, 60% of relative air humidity, and light intensity of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by Philips HPI-T Plus 400 W metal halide lamps for 12-hour photoperiod.

Fully mature current-year foliage was harvested in late summer. Branches selected were harvested between 8:00-9:00 morning hours. The branches were re-cut under water, the cut end was maintained in water and the branches were immediately transported to the laboratory. Leaves were rapidly removed from the branch by tweezers. In deciduous species, petioles were excised and only leaf lamina was used, while in *P. mugo* the non-photosynthetic part of the needle enclosed in the needle sheath was excised and only green needle parts were used. A total of ca. 1500 leaves (ca. 950 needles of *A. balsamea* needles, ca. 380 needles of *P. mugo* needles, 70 leaves of *B. pendula* leaves, and 100 leaves of *Q. ilex*) were harvested.

Morphological traits

Leaf fresh mass was measured to the nearest 0.0001 g (ALJ 220-4NM, Kern & Sohm GmbH, Balingen, Germany). Leaf thickness for all species and width and length for needle-leaved species was measured with a digital caliper (Mitutoyo CD-15DC, Mitutoyo Ltd, Hampshire, UK) to the nearest 0.01 mm. Five measurements in different parts of the leaves were taken and averaged to obtain one mean value. In broad-leaved species, major veins were avoided during the thickness measurements.

The leaves of broad-leaved species were scanned at a resolution of 300 dpi. The 24-bit (RGB) color images were converted to grayscale bitmaps corresponding to each RGB channel (red/green/blue), and the gray-scale image of the blue channel was selected and transformed to a black and white image. This transformation minimized pale areas and shadows as the green color is essentially black in the blue channel (Niinemets et al. 2004). The projected leaf area was estimated using Inkscape v.0.91 software (www.inkscape.org). For needles, the projected leaf area was estimated by multiplying the length and width of the needles.

Treatment protocols

All foliage for given species was pooled, and random samples of ca. 3 g were taken for different treatments as explained in Fig. 1. Five different treatment types were applied, including various fresh sample processing methods and drying procedures. Different treatment temperatures were applied for some treatment types, resulting in altogether 15 different treatments (Fig. 1). Fresh sample (as is common for analyses of foliage isoprenoid contents) treatment procedures included flash freezing in liquid N₂ (FF), freezing at –82 °C and –20 °C (F-82, F-20) for 48 h, and cooling at 4 °C (C) for 48 h. Foliage desiccation procedures were freeze-drying (FD) and air-drying in forced-convection drying oven at 30, 40, 60, 70, 80, 100, 110, 150, 200 and 300 °C until a constant mass (designated as AD30, AD40, AD60, etc.). AD30 and AD40 lasted usually 72 h, AD60 to AD110 about 48 h, and AD200 and AD300 about 8 h.

FF, F, and C treatments are not procedures for drying the leaf samples, but they simulate common practices to preserve leaf properties or stop metabolic reactions when samples cannot be analyzed directly (e.g. during field work).

— In the case of flash freezing in liquid nitrogen (FF), leaf samples are submerged into liquid

174 nitrogen and leaf water is frozen within seconds, essentially immediately stopping any biological
175 activity. Depending on plant tissues, extracellular ice crystal growth may draw water from the
176 cytosol during freezing (Buchner and Neuner 2011; Wisniewski et al. 2003). This may
177 potentially lead to loss of some leaf water by sublimation. Nevertheless, intra- and extracellular
178 ice formation are expected to occur rapidly in non-frost acclimated samples, and thus, the
179 distribution of water between different compartments is expected to be maintained under flash
180 freezing.

181 — In the case of freezing (F), leaf temperature is reduced more slowly, and accordingly, the
182 redistribution of water from cytosol to extracellular compartments is more likely to happen than
183 during FF treatment. On the other hand, deep supercooling and vitrification (glassy cellular
184 solutions) reducing ice formation can also occur (Buchner and Neuner 2011; Wisniewski et al.
185 2003).

186 — Cooling (C) slows down the rate of metabolic processes and results in no ice formation,
187 but certain loss of leaf mass due to respiration and loss of water due to transpiration inevitably
188 occurs during cooling. In the case of F and C treatments, the samples were kept in air-tightly
189 sealed polyethylene bags to minimize water evaporation.

190 — Freeze-drying (FD) is a leaf drying process based on sublimation of ice from frozen
191 samples in low air pressure conditions to speed up sublimation. As metabolic activity is rapidly
192 stopped, and also the key plant volatiles have low saturated vapor pressure at typical freeze-
193 drying temperatures, it is supposed to be the most effective drying method preserving both
194 foliage dry mass and volatiles. Freeze drying at -54°C was conducted under 1.2 mbar air
195 pressure for 5 days using a freeze dryer ALPHA 1-2 LD (Martin Christ
196 Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

197 — Air-drying (AD) is a routine method for estimation of leaf dry mass in plant science
198 (Garnier et al. 2001; Zeiller et al. 2007). Typically, dry mass is estimated at temperatures between
199 $50\text{--}80^{\circ}\text{C}$, but in some cases as high as 105°C is used (Purahong et al. 2014). We applied drying
200 treatments at 30, 40, 60, 70, 80, 100, 110, 150 and 200°C in a forced convection ventilated drying
201 oven (100-800, Memmert GmbH+Co.KG, Germany). For drying treatment at 300°C , a muffle
202 furnace was used (LE 4/11/R6, Nabertherm GmbH, Germany). In each case, the drying lasted

until a constant mass at given temperature was achieved (e.g., typically 72 hours at 30 °C and 48 hours at 60 °C). The foliage was cooled at a desiccator with fresh silica gel and dry mass estimated.

Leaf isoprenoid content estimation

After the treatments, foliage isoprenoids were extracted. A random foliage sample of 70-100 mg (average $\pm$ SE of 80 $\pm$ 14 mg of dry mass across all samples) was taken, put into ceramic mortar together with liquid N₂, and homogenized with a ceramic pestle into fine powder (Fig. 1). After trying several ways of sample homogenization and grinding to fine powder, this was the method which performed a better and faster result. Samples from all treatments were homogenized as described.

The homogenized plant material powder was mixed with 1 mL GC-grade hexane (Sigma Aldrich, St. Louis, MO, USA) and left at 4 °C for 24 h. The supernatant was filtered using a PTFE syringe filter (0.45 μ m pore size, VWR International, Radnor, PA, USA) and immediately injected into a Shimadzu gas chromatograph mass-spectrometer (GC-MS) QP2010 Plus instrument equipped with an auto-injector AOC-20i (Shimadzu Corporation, Kyoto, Japan) and with a ZB5-MS (0.25 mm i.d. $\times$ 30 m, 0.25 μ m film Zebron, Phenomenex, Torrance, CA, USA) column. The MS was operated in electron-impact mode (EI) at 70 eV, and the mass scan range was m/z 33-400. The GC oven program, transfer line and ion-source temperature were the same as in Noe et al. (2012).

Isoprenoid compounds were quantified for *A. balsamea* and *P. mugo* by the comparison of their mass spectra with the data from the NIST library (National Institute of Standards and Technology) and from Adams (2001). For calibration, commercial authentic standards were used (Sigma-Aldrich, St. Louis, MO, USA) following the procedures as described in detail in (Copolovici et al. 2009; Niinemets et al. 2013). Isoprenoids detected and quantified included monoterpenes, oxygenated monoterpenes, sesquiterpenes and oxygenated sesquiterpenes; a total of 53 compounds in *A. balsamea* and 51 compounds in *P. mugo*. Leaf isoprenoid concentration was expressed as mg or μ g compound per leaf dry mass as follows: after the isoprenoid extraction, the dry mass of the isoprenoid-free powder ($D_{M,IF}$) was estimated by drying in its original vial under a forced convection hood for several days at room temperature. The mass of

isoprenoids, I , was added to $D_{M,IF}$ to calculate the total dry mass, D_M . The volatile isoprenoid content per dry mass, I_M , was calculated as:

$$I_M = \frac{I}{D_M} = \frac{I}{D_{M,IF} + I}. \quad (1)$$

Theoretical estimation of mass loss during treatments

The drying treatments (AD treatments) can affect both the content of residual water that remains at given temperature T ($M_{RW,T}$), but also the amount of leaf dry matter ($M_{D,T}$) due to differences in mass loss as the result of respiration and loss of volatiles. Leaf respiration rate increases exponentially with temperature up to a temperature of ca. 50 °C and decreases with further increases in temperature due to cessation of leaf physiological activity (e.g., Hüve et al. (2011)), but respiration rate also declines with leaf desiccation, which is expected to occur faster at higher temperature. In contrast, evaporation of volatiles is a purely physical process and is expected to increase monotonically with increasing temperature. Furthermore, at higher temperatures, beyond 100 °C, pyrolysis of several leaf constituents such as sucrose and starch may occur, thereby further reducing leaf dry mass. Thus, leaf dry to fresh mass ratio corresponding to the drying temperature T ($D_{F,T}$) is given as:

$$D_{F,T} = \frac{M_{D,T} + M_{RW,T}}{M_D + M_W}, \quad (2)$$

where M_W is the mass of water and M_D is the water-free leaf mass (dry mass). Moreover, leaf fresh mass, M_F , is defined as:

$$M_F = M_D + M_W. \quad (3)$$

Combining equations (2) and (3),

$$D_{F,T} M_F = M_{D,T} + M_{RW,T}. \quad (4)$$

Since the FD procedure was supposed to keep better the isoprenoid content and therefore give a better estimate of M_D than the AD procedures, the leaf traits measured by freeze drying the sample were considered the reference for comparison to other analytical procedures. Leaf control dry to fresh mass ratio D_F (FD treatment) is defined as:

$$D_F = \frac{M_D}{M_D + M_W} = \frac{M_D}{M_F}. \quad (5)$$

For AD treatments, the ratio of leaf masses estimated after drying at T_1 and T_2 , $R_{T1,T2}$ is:

$$R_{T1,T2} = \frac{M_{D,T1} + M_{RW,T1}}{M_{D,T2} + M_{RW,T2}}, \quad (6)$$

depending both on the differences in the residual water content as well as on possible differences in dry mass loss upon drying.

Theoretical estimation of isoprenoid loss after treatments

In the case of fresh sample extracts (i.e., FF, F and C treatments), a sub-sample of foliage was dried at 60 °C to estimate leaf dry to fresh mass ratio. As with bulk leaf dry matter, volatile isoprenoid content per dry mass (I_M) depends on residual leaf water content, overall loss of dry mass during the treatment, and particularly strongly on the possible loss of isoprenoid content due to evaporation. Thus, I_M at given temperature T is given as:

$$I_{M,T} = \frac{I - I_{L,T}}{M_{D,T} + M_{RW,T}}, \quad (7)$$

where I is the isoprenoid amount in the leaf sample (mg) and $I_{L,T}$ the possible isoprenoid loss at given temperature (mg):

$$I_{L,T} = I - I_{M,T}(M_{D,T} + M_{RW,T}). \quad (8)$$

In the case of isoprenoid content expressed per unit leaf dry mass (I_M , i.e. estimated for the freeze drying treatment):

$$I_M = \frac{I}{M_D}. \quad (9)$$

Substituting I in equation (8) using equation (9),

$$I_{L,T} = I_M M_D - I_{M,T}(M_{D,T} + M_{RW,T}). \quad (10)$$

Combining equations (10) and (4)

$$I_{L,T} = I_M M_D - I_{M,T} D_{F,T} M_F. \quad (11)$$

To express the isoprenoid loss at given temperature per gram of sample (mg g^{-1}), dividing by M_F and combining with equation (5),

$$I_{L,T} = I_M D_F - I_{M,T} D_{F,T}. \quad (12)$$

Carbon analysis

Total C content of freeze-dried and air dried leaf samples were determined by dry combustion method with a Vario MAX CNS elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). As with the bulk dry mass, C contents can change due to residual water content as

well as due to loss of dry mass. In addition, losses of high C content volatiles and respiration of sugars and carbohydrate pyrolysis with low carbon content may differently affect the bulk leaf C content. Carbon content at a drying temperature (g g^{-1}) is defined as:

$$C_{M,T} = \frac{M_D}{M_{D,T}} [C - C_L(M_D - M_{D,T})], \quad (13)$$

where the term $M_D/M_{D,T}$ corresponds to the ratio between freeze-dried leaf dry mass and the measured dry mass at given air-drying temperature, C is the carbon content of freeze-dried sample (g g^{-1}), the term $M_D - M_{D,T}$ corresponds to potential biomass loss during drying and C_L (g g^{-1}) is the fraction of carbon in the biomass lost, around 0.88 g g^{-1} in purely monoterpene-based biomass.

Statistical analysis

Two types of statistical tests were used to identify significant differences between groups of independent replicates. We firstly applied a Shapiro-Wilk normality test with a $P > 0.05$ significance threshold to the data, which was passed in the majority of cases. Even having some cases where the normality test failed, we prioritized the use of parametric tests for further analyses. We accepted statistical differences between two groups if $P < 0.05$ after running a two-tailed Student's t-test. For pairwise multiple comparisons between several groups, we used a One-way ANOVA test with a post-hoc analysis by Holm-Sidak test ($P < 0.05$) (e.g. differences in leaf traits between the four species studied in Table 1, differences in the leaf terpene content between six drying and preserving treatments in Figure 2a, etc.).

In the case of AD treatments, we did not analyze them by testing statistical differences between groups, but we used a non-linear regression (two-parameter exponential decay equation) or a Pearson's linear regression analysis to find a correlation based on the drying temperature.

Results

Leaf water content

Freeze-drying leaf samples as well as air-drying at temperatures above 60 °C fully removed the leaf water content. On the other hand, the sample preservation treatments FF, F-82, F-20 and C, did not significantly change the initial leaf water content ($P = 0.98$, $P = 0.98$, $P = 0.97$, $P = 0.83$, respectively; Student's t-test two-tailed, all species pooled). In all species, AD30-40 reached a "constant mass" state during the treatment, but the water content was not likely fully removed from the leaf samples as explained theoretically (Eqn. 2). After 48 h of drying at 30 °C *A. balsamea* and *P. mugo* leaves contained $45.3 \pm 3.1\%$ and $48.8 \pm 2.4\%$ of residual water content, and $21.3 \pm 1.8\%$ and $3.4 \pm 1.1\%$ after drying at 40 °C, respectively, compared to further drying the leaves at 60 °C (zero water content).

Leaf isoprenoid content

Freeze-dried samples preserved more isoprenoid content per leaf dry mass in both isoprenoid storing species than any other drying method or fresh sample preservation procedure (Fig. 2a and c) (Holm-Sidak pairwise test; $P < 0.05$). The average ($\pm$ SE) maximum isoprenoid contents measured were 23.38 ± 0.66 and 29.49 ± 0.22 mg g⁻¹ in *A. balsamea* and *P. mugo*, respectively.

Compared to the FD treatment, the leaf isoprenoid content decreased dramatically in *A. balsamea* leaves after the FF (36%), F-20 (37%), F-82 (73%), C (33%) and AD70 (82%) treatments (Fig. 2a). A similar isoprenoid loss effect occurred for *P. mugo* leaves in relation to FD treatment: FF (36%), F-20 (80%), F-82 (84%), C (79%) and AD70 (92%) (Fig. 2c). For both species, the leaf isoprenoid loss in relation to the increase of drying temperature (AD treatments) was fitted to a two-parameter exponential decay function (Fig 2b and d).

Air drying at 30 and 40 °C preserved more isoprenoids than the more usually used temperatures 60 to 80 °C in both isoprenoid-storing species ($P < 0.0001$ for both analyses; Student's t-test).

Leaf isoprenoid content of *B. pendula* and *Q. ilex* leaves was very low, in the range of 1 to 10 µg g⁻¹. The isoprenoid compounds were hardly differentiable from the baseline in the chromatograms, and thus, the leaf isoprenoid content was considered close to zero.

Dry to fresh mass ratio

The log-transformed leaf isoprenoid content data was correlated positively to the dry to fresh mass ratio data (Fig. 3): the leaves which preserved more leaf isoprenoid content after the treatment applied accordingly preserved more dry mass per initial fresh mass.

Considering the dry to fresh mass ratio of FD samples as a reference for minimally disturbed samples during drying process, air drying temperatures of 60 to 80 °C applied to isoprenoid-storing species significantly underestimated the value by 10% in *A. balsamea* and by 4.5% in *P. mugo* ($P < 0.001$; two-tailed t-test), reflected also in Fig. 4a and c, respectively, in the pairwise comparisons with AD70. The leaf dry to fresh mass ratio did not change significantly from the FD treatment to the air-dried samples at 60 to 80 °C in the non-storing species: $P = 0.136$ in *B. pendula* and $P = 0.256$ in *Q. ilex*. Also noticeable in the pairwise comparisons in Figure 4e and g, respectively.

Interestingly, air drying at 40 °C resulted in a decrease of the dry to fresh mass ratio compared to drying at 30 °C in the non-storing species ($P < 0.05$; Holm-Sidak pairwise comparisons) (Fig. 4f and h).

Leaf dry to fresh mass ratio in relation to drying temperature (AD treatments) showed a highly significant linear relationship in the isoprenoid storing species (Fig. 4b and d), contrarily than for the non-storing species (Fig 4f, and h). For *A. balsamea* and *P. mugo*, the increase of drying temperature underestimated the dry mass per initial fresh mass.

Discussion

Analysis of leaf isoprenoid content

The analysis of leaf isoprenoid content used needs of a liquid extraction with hexane. To optimize the isoprenoid extraction to the liquid phase, the leaf sample must be ground to a fine powder to increase the sample contact surface area with the extraction liquid. When the sample size is relatively small, a grinder machine might not be successful in powdering the sample therefore we manually ground the samples with a ceramic mortar and a pestle. In fresh samples, fresh tissues will stick together, fibers would remain flexible and difficult to grind. Therefore we concluded that helping the manual grinding procedure with liquid N₂ would help to dry and brittle the tissues. In fact, the frozen samples cracked easily into smaller pieces, and facilitated the processing into fine powder. But as this technique produces shrinkage of tissues and sublimation of some leaf water content, there is some potential isoprenoid loss during the grinding. Nevertheless, we believe that the importance of reaching a fine powder format for a liquid extraction overrides the disadvantage of potential isoprenoid loss during the process.

During the calculation of the leaf isoprenoid concentration, considering the isoprenoid content, I (Eqn. 1), as part of the dry matter content made the calculation valid for isoprenoid and non-isoprenoid-storing species, where I is essentially zero. Furthermore, the calculation of isoprenoid concentration per dry mass was not dependent on the potential residual water content of leaves undergoing the preservation or drying methods AD30 and AD40. This was because whether the leaf sample powder contained moist or not, the isoprenoid content was always referred to the dry mass, D_M .

Leaf isoprenoid content preservation

FF, F and C treatments are used to store leaf samples for further isoprenoid content determination. We hypothesized that these preservation methods would be effective and therefore result in similar isoprenoid content values than the reference method FD. However, the leaf isoprenoid content remaining after FF, F and C methods was significantly lower compared to FD treated samples (Fig. 2a and c):

— The FF method is used to prevent enzymatic reactions and vaporization losses of

isoprenoids (Ormeño et al. 2011), although from our experience, FD method performed a better leaf isoprenoid conservation (Fig. 2a and c). The reason could be that freeze drying under vacuum does not cause shrinkage of the leaf sample, contrarily to FF.

— We theorize that preserving leaf samples at -82 and -20 °C forms ice crystals inside the leaf tissues. These might have broken cell structures and tissues and consequently release part of the isoprenoids stored. In fact, after the freezing treatment, when the air-tight plastic bags containing the leaves were opened, the volatile compounds smell was evident; a sign that these had been released out of the leaf storing organs during the freezing.

— Storing the leaf samples at 4 °C (C treatment) and air-drying near to ambient temperatures (AD30 and AD40) did not stop completely metabolic processes and leaves continued losing the isoprenoids stored as a natural process of leaf decay (Fig. 2). For AD30 and AD40 leaves, because of the favorable temperature for metabolic processes and long-lasting drying period, there was presumably a loss of carbon through the respiration of sugars.

Leaf drying at 60 to 80 °C, performed a very low leaf isoprenoid content preservation compared to FD treatment.

We conclude that the best method to store the samples for further analysis is to freeze-dry these under vacuum, or dipping these in liquid N_2 in the absence of a vacuum freeze-drier.

The leaf isoprenoid content in the broad-leaved species was very low in any case, as found by (Alessio et al. 2008) in *Q. ilex*, and by (Haapanala et al. 2009) in *B. pendula*.

Leaf dry matter content and leaf isoprenoid content

Our coniferous leaves contained 2-3% of total dry mass in form of volatile isoprenoids, which were mostly evaporated from the leaf after usual leaf drying procedures (i.e. air-drying at 60 to 80 °C). This fact can lead to a systematic error in the leaf dry matter determination for leaves containing a substantial storage of volatile compounds, as theorized previously. Consequently, the underestimation of the leaf dry matter content further overestimates the results of other leaf traits and chemical analyses when divided by the calculated dry mass.

In Figure 4b and d, the dry to fresh mass ratio dropped more than 3%, as it would be expected from isoprenoid content evaporation. We hypothesize that as well as stored isoprenoids, other compounds with high volatility such as fats, oils, waxes, phenols, alcohols, etc. could also be

412 evaporated, decreasing the remaining leaf dry matter after the drying treatment. Nevertheless,
413 we did not detect a noticeable decrease in the dry to fresh mass ratio in relation to these non-
414 isoprenoid potential volatiles in *B. pendula* (Fig. 4f) and *Q. ilex* (Fig. 4h). For this reason, more
415 research is needed to investigate the semi-volatile compound content in these species and, in
416 general, the potential sources of mass loss when preserving or drying leaf samples.

417 Shrinkage of broad leaves of *B. pendula* and *Q. ilex* during FF treatment was stronger than
418 for needle-like leaves of *A. balsamea* and *P. mugo*. Therefore we assume that the significant drop
419 in dry to fresh mass ratio after flash freezing in liquid N₂ (Fig. 4e and g) was caused by
420 evaporation of volatile compounds after the rapid shrinkage and breakage of tissues.
421 Analogously, we also believe that in broad leaves, the transpiration was higher than in needles
422 at low drying temperatures (30 and 40 °C): the low dry to fresh mass ratio observed after air-
423 drying at 40 °C (Fig. 4f and h) is probably related to the respiration of sugars during the long-
424 lasting drying procedure. In addition, the dark respiration is maximum at around 40 °C (Sharkey
425 and Bernacchi 2012). Nevertheless, the variation of dry to fresh mass ratio in non-storing species
426 across the treatments FF, FD, F, C and AD30-AD110 was smaller than for the terpenoid-storing
427 leaves (Fig. 4).

428 There are several evidences to confirm the hypothesis exposed on biased dry mass estimation
429 when using preserving or drying methods that release the leaf isoprenoid content in isoprenoid-
430 storing leaves. The application of preserving and drying treatments (specially AD) resulted in a
431 dramatic decrease of isoprenoid content (Fig. 2) as well as dry mass content (Fig. 4) in *A.*
432 *balsamea* and *P. mugo*, stronger than for the non-storing species. Moreover, we found a positive
433 correlation between the isoprenoid content and the dry to fresh mass ratio (Fig. 3) which suggests
434 that both parameters are related. These relationships are to be taken in consideration by
435 researchers using highly isoprenoid-storing species because there is a potential bias of the dry
436 mass estimation and further variables related to that.

437 ***Recommendations on leaf sampling, handling, and isoprenoid extraction***

438 Leaf isoprenoid content determination remains a delicate issue. From the moment of leaf
439 sample collection, the manipulation of the samples starts releasing volatile compounds.
440 Meticulous and gently care should be given to the samples in order to measure the leaf isoprenoid

441 content, as well as other traits including VOCs, like VOCs emission. The following
442 recommendations optimize the estimation of leaf isoprenoid content:

443 (1) Sampling. We suggest that leaves should not be sampled directly from the tree, but a
444 branch should be cut off and transported to the lab. In this way, the leaves are kept as better as
445 possible in their original environment, preserving the branch-leaf water continuum. This will
446 reduce the leaf water loss or evapotranspiration, and analogously the potential volatile compound
447 loss. Leaf samples should be handled with tweezers to prevent warming up the samples with
448 empty hands. The isoprenoid extraction must be done as soon as possible after the sampling.

449 (2) Transport to the lab. If the plant sampled is not in the lab and requires a short transport
450 (minutes), this can be done by placing the branch in a jar in contact with water at ambient
451 temperature, or inside a plastic or vinyl bag at cooling conditions if longer time. If the leaves
452 must wait longer for analysis, leaves can be freeze-dried preferably or nitrogen cooled and kept
453 in paper bags with silica gel. In any case, the researcher must avoid to mechanically perturb the
454 samples.

455 (3) Storing. Storing fresh leaf samples before isoprenoid extraction should be avoided.

456 (4) Drying the leaves. Freeze-drying or submerging in liquid N₂ fresh leaf samples is the best
457 option. Samples can be kept for several days into paper bags with silica gel before the isoprenoid
458 extraction. In addition, FD was the method which performed better conservation of the leaf
459 isoprenoid content, followed by FF. These procedures will also prepare the samples for the
460 homogenization step.

461 (5) Leaf sample homogenization before extraction. This is a key step in the leaf isoprenoid
462 content determination. It is when the leaf material and volatile molecules get exposed to
463 oxidation, cell structures are broken, and other stable conditions for isoprenoid storage disappear.
464 The isoprenoid volatilization or transformation can be done very quickly in some cases, and
465 especially if the homogenization is slow and difficult to perform. Our experience is that dried
466 samples are easier, faster and handy to homogenize. Flash freezing during that process helps it.

467 **Conclusion**

468 The results of this experiment suggested that freeze-drying method (FD) performed the best
469 leaf isoprenoid conservation because of its gentleness to the leaf samples during the drying
470 process. Flash freezing preserved isoprenoids in a lower level. Air-drying methods performed a
471 dramatic isoprenoid loss in the leaf samples, as well as storing the leaves at freezing and cooling
472 temperatures.

473 Leaf isoprenoid content and leaf dry to fresh mass ratio were correlated, moreover generating
474 a bias in the dry mass estimation after leaf preserving or drying methods due to the loss of
475 isoprenoid content.

476 Researchers must take in account the potential dry mass loss from isoprenoid-storage when
477 choosing the leaf drying method. Thus, to gain precision in highly isoprenoid-storing species,
478 freeze-drying under vacuum can be the best option to estimate the leaf dry mass.

479 **Author contribution statement**

480 ÜN: research hypotheses; MPE and LC: conducted the data collection; All authors
481 contributed to interpretation of results, writing, and edits.

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491 **Conflict of interest**

492 The authors declare that they have no conflict of interest.

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493 **Table 1.**

494 Leaf morphological and chemical traits (mean $\pm$ standard error) of freeze-dried leaves at -54
 495 $^{\circ}\text{C}$ and 1.2 mbar for 5 days and fresh leaves ^(#) of four tree species. Superscript letters denote
 496 significant differences between species after a Holm-Sidak pairwise comparison test ($P < 0.05$).
 497 The differences in the isoprenoid content between the two isoprenoid-storing species were tested
 498 by a two-tailed Student's t-test ($P < 0.05$).

	Species			
	<i>Abies balsamea</i>	<i>Pinus mugo</i>	<i>Betula pendula</i>	<i>Quercus ilex</i>
Morphological traits				
Fresh mass (mg) [#]	23.44 $\pm$ 0.43 ^a	58.3 $\pm$ 2.0 ^b	217 $\pm$ 5 ^c	446 $\pm$ 20 ^d
Dry mass (mg)	15.67 $\pm$ 0.33 ^a	21.0 $\pm$ 0.6 ^b	86.3 $\pm$ 2.2 ^c	238 $\pm$ 13 ^d
Water content (%)	46.3 $\pm$ 0.9 ^a	50.9 $\pm$ 1.6 ^a	60.10 $\pm$ 0.31 ^b	49.0 $\pm$ 0.5 ^a
Dry to fresh mass ratio (%)	53.7 $\pm$ 0.9 ^a	49.2 $\pm$ 1.6 ^b	39.91 $\pm$ 0.29 ^c	51.90 $\pm$ 0.49 ^{ab}
Thickness (μm) [#]	511 $\pm$ 7 ^a	752 $\pm$ 8 ^b	176.4 $\pm$ 1.5 ^c	195.5 $\pm$ 2.9 ^d
Width (μm) [#]	1863 $\pm$ 16 ^a	1396 $\pm$ 15 ^b	-	-
Length (mm) [#]	30.23 $\pm$ 0.23 ^a	63.6 $\pm$ 1.5 ^b	-	-
Projected leaf area (mm^2) [#]	56.3 $\pm$ 0.6 ^a	88.9 $\pm$ 2.3 ^b	1291 $\pm$ 29 ^c	2470 $\pm$ 100 ^d
Leaf dry mass per area (g m^{-2})	260 $\pm$ 6 ^a	313 $\pm$ 13 ^b	66.9 $\pm$ 0.8 ^c	94.0 $\pm$ 2.3 ^d
Chemical composition				
Carbon content (%)	47.347 $\pm$ 0.038 ^a	47.34 $\pm$ 0.06 ^a	45.8 $\pm$ 1.4 ^a	47.18 $\pm$ 0.25 ^a
Nitrogen content (%)	1.090 $\pm$ 0.012 ^a	1.190 $\pm$ 0.046 ^a	2.647 $\pm$ 0.046 ^b	2.177 $\pm$ 0.083 ^c
Monoterpene content (mg g^{-1})	17.9 $\pm$ 0.7 ^a	8.920 $\pm$ 0.045 ^b	< 0.01 ^c	< 0.01 ^c
Oxygenated monoterpene content (mg g^{-1})	1.49 $\pm$ 0.05 ^a	0.040 $\pm$ 0.021 ^b	< 0.01 ^c	< 0.01 ^c
Sesquiterpene content (mg g^{-1})	3.73 $\pm$ 0.20 ^a	15.66 $\pm$ 0.06 ^b	< 0.01 ^c	< 0.01 ^c
Oxygenated sesquiterpene content (mg g^{-1})	0.228 $\pm$ 0.038 ^a	4.87 $\pm$ 0.07 ^b	< 0.01 ^c	< 0.01 ^c
Total isoprenoid content (mg g^{-1})	23.38 $\pm$ 0.66 ^a	29.49 $\pm$ 0.22 ^b	< 0.01 ^c	< 0.01 ^c

499

Figure captions

Figure 1. Scheme of five types of treatments applied to fresh leaves of four tree species: coniferous *Abies balsamea* and *Pinus mugo*, evergreen broad-leaved *Quercus ilex*, and deciduous broad-leaved *Betula pendula*. The treatments were flash freezing in liquid nitrogen (FF), freeze drying under vacuum (FD), freezing at given temperature (F), cooling (C) and air drying at given temperature (AD), and the study tested their effect on estimation of leaf isoprenoid content and dry mass (drying treatments).

Figure 2. Leaf total isoprenoid content per dry mass (mg g^{-1}) (average $\pm$ SE of three to five independent replicates) after applying different sample processing methods to two tree species leaves: *Abies balsamea* (a and b) and *Pinus mugo* (c and d). Treatments legends in (a) and (c): FD (freeze drying at -54°C and 1.2 mbar for 5 days), FF (flash freezing in liquid nitrogen), F-82 (freezing at -82°C for 48 h), F-20 (freezing at -20°C for 48 h), C (cooling at 4°C for 48 h), AD70 (air-drying at 70°C for 48 h). In (b) and (d), x-axis corresponds to air-drying temperature until constant weight was reached: normally 72 h at $30\text{--}40^\circ\text{C}$, 48 h at $60\text{--}110^\circ\text{C}$, and less than 12 h at higher temperatures. The data was fitted by a two-parameter exponential decay function. Letters in (a) and (c) denote statistical differences between groups after Holm-Sidak pairwise test at $P < 0.05$ significance level.

Figure 3. Relationship between ($\log_{10}$ -transformed) leaf isoprenoid content (mg g^{-1}) remaining after different sample treatments and leaf dry to fresh mass ratio (g g^{-1}). All data pooled of independent leaf samples undergoing different leaf storing and drying procedures (see materials and methods section). The data was fitted by a linear regression.

Figure 4. Leaf dry to fresh mass ratio (g g^{-1}) (average $\pm$ SE of five to eight independent replicates) after applying different sample processing methods to four tree species leaves: *Abies balsamea* (a and b), *Pinus mugo* (c and d), *Quercus ilex* (e and f), and *Betula pendula* (g and h). Treatments legends in the left panels: FD (freeze drying at -54°C and 1.2 mbar for 5 days), FF (flash freezing in liquid nitrogen), F-82 (freezing at -82°C for 48 h), F-20 (freezing at -20°C for 48 h), C (cooling at 4°C for 48 h), AD70 (air-drying at 70°C for 48 h). In the right panels, x-axis corresponds to air-drying temperature until constant weight was reached: normally 72 h at $30\text{--}40^\circ\text{C}$, 48 h at $60\text{--}110^\circ\text{C}$, and less than 12 h at higher temperatures. The data was fitted by

529 a linear regression. Letters in (a), (c), (e) and (g) denote statistical differences between groups
530 after Holm-Sidak pairwise test at $P < 0.05$ significance level.

Fig1

Fig 225

Fig3² $\log_{10} [\text{leaf terpene content (mg g}^{-1}\text{)}]$

$$y = 7.00x - 2.58$$
$$r^2 = 0.70 ; P < 0.001$$

(a)

$$y = 19.83x - 8.43$$
$$r^2 = 0.60 ; P < 0.001$$

(b)

0.35 0.40 0.45 0.50 0.55 0.60

Leaf dry to fresh mass ratio

Fig4

Treatment

Temperature (°C)