Review of cleaning techniques and their effects on the chemical composition of foliar samples

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Chemical foliar analysis is a tool widely used to study tree nutrition and to monitor the impact and extent of air pollutants. This paper reviews a number of cleaning methods, and the effects of cleaning on foliar chemistry. Cleaning may include mechanical techniques such as the use of dry or moistened tissues, shaking, blowing, and brushing, or use various washing techniques with water or other solvents. Owing to the diversity of plant species, tissue differences, etc., there is no standard procedure for all kinds of samples. Analysis of uncleaned leaves is considered a good method for assessing the degree of air contamination because it provides an estimate of the element content of the deposits on leaf surfaces or when the analysis is aimed at the investigation of transfer of elements along the food chain. Sample cleaning is recommended in order (1) to investigate the transfer rate of chemical elements from soil to plants, (2) to qualify the washoff of dry deposition from foliage and (3) to separate superficially absorbed and biomass-incorporated elements. Since there is not a standard cleaning procedure for all kinds of samples and aims, it is advised to conduct a pilot study in order to be able to establish a cleaning procedure to provide reliable foliar data.

Introduction

Chemical foliar analysis is a widely used diagnostic and monitoring method in forestry and environmental studies. It has been used to estimate nutrient deficiencies and toxicities (e.g. Morrison 1974, van den Driessche 1974), and to monitor the nutritional status of forest trees (e.g. Kaupenjohann *et al.* 1989, Cape *et al.* 1990, Linder 1995, Zwoliński *et al.* 1998), particularly with respect to maximising growth. Chemical foliar analysis has also been employed to study the impact and extent of air pollutants (e.g. Hüttl and Fink 1991, Ericsson *et al.* 1995, Djingova *et al.* 1999), and the pollutant accumulation capacity of different plants (Bicchiega *et al.* 1994, Šomšák *et al.* 2000).

It is well known that plants, especially trees with their large specific surface area, act as filters for aerosol particles (Mayer and Ulrich 1978, Jonas and Heinemann 1985) and effectively intercept pollutants (Badino *et al.* 1998). Some of these particles are washed off by rainfall, thereby increasing, together with the ions leached from the leaves and needles, element concentrations in throughfall (Hyvärinen 1990). Parts of the deposited particles are not removed by rainfall, and become irreversibly adsorbed or incorporated into the hydrophobic wax layer of the foliage.

Foliar analysis includes a number of different stages, i.e. the planning, sampling, physical and chemical sample preparation, instrumental measurements and data evaluation. Physical sample preparation consists of cleaning, drying and homogenization of the samples. Chemical foliar analysis has traditionally been carried out on uncleaned samples. In some cases, however, it has been strongly recommended to clean foliage samples. If the aim of the research is the assessment of accumulated elements and their ecotoxicogical effects on plants, the samples

Table 1. Summary of the best cleaning procedure for foliar samples of conifers species.

Species	Washing method and aim	Use	Reference
Pinus sylvestris	200 ml of chloroform for 30 s and then rinsed twice with deionized water to study the effect on S and metals for foliar analysis	Recommended to distinguish external from internal tissue composition	Zwoliński *et al.* 1998
Pinus sylvestris	20 ml of chloroform for 15 s in a bioindicator study to analyse the inside conc. in needles	Useful to distinguish external from internal tissue composition	Rautio 2000
Picea abies	200 ml of chloroform for 30 s and then rinsed twice with deionized water to study the effect on S and metals in foliar analysis and to assess the nutritional status of the plan	Recommended to distinguish external from internal tissue composition	Zwoliński *et al.* 1998, Simmleit *et al.* 1989
Picea abies	Shaking 5 ml of chloroform or carbon of the tetrachloride for 30 s to study the contribution surface deposits to the element conc.	Recommended to obtain an accurate needle analysis and the correct interpretation of atmospheric pollution	Krivan *et al.* 1987
Picea abies	Chloroform for 1 min and then rinsed twice with distilled water to determine chemical foliar composition	Useful to distinguish external from internal tissue composition. Only negligible because leaching of K	Wyttenbach *et al.* 1985, Markert 1995, 1996, Bargagli 1998, 1999
Pinus densiflora and *Criptomeria japonica*	Water with 0.2 M of HCl to remove the external deposits from internal one	Recommended to distinguish external from internal tissue composition. No effect on K, Ca, Mg and P	Salkl and Maeda 1982
Abies balsamea	Chloroform for 15 s to determine accumulation of metal deposits on needle surface by removing epicuticular wax layer from foliar surface	Recommended to study the accumulation of the metal deposits in and on epicuticular wax layer	Lin and Schuepp 1996
Pinus sylvestris	Distilled water for about 1 min to remove materials deposited on needle surfaces in a monitoring environmental pollution study	Recommended to compare the results of chemical analysis of collected in various locations	Dmuchowski and Bytnerowicz 1995
Conifer species	Chloroform for about 3 min in biomonitoring studies	Ensure removal of the waxy material which accumulates atmospheric deposition and can be analysed separately	Kovacheva *et al.* 2000

should be carefully cleaned during physical sample preparation. In addition, if the purpose of foliar analysis is to investigate the transfer rate of elements from the soil to the plants, then sample cleaning is necessary owing to the presence of surface contamination (Robert *et al*. 1996). Sample cleaning is absolutely essential if the purpose is to distinguish between pollutants deposited on the surface of leaves, e.g. soil dust, and the composition of the internal tissues (Little and Martin 1972, Little 1973, Wedding *et al*. 1977, McColl and Bush 1978, Lindberg and Harriss 1981, Höfken and Gravenhorst 1982, Lindberg and Lovett 1985, Potter and Ragsdale 1991, McCrimmon 1994, Alfani *et al*. 1996, 2000, Markert 1996). However, sample cleaning is not mandatory when investigating the effects of the total element input in the food chain on the health of animals (Markert 1996).

Particular attention should be paid to the cleaning procedure because, according to Markert (1996), an incorrect cleaning method can introduce an error of between 100% and 300% in the accuracy of the analysis.

The aim of this article is to review the different cleaning methods, and their effects on the chemical composition of foliar samples.

Cleaning techniques

Sample cleaning procedures can include mechanical techniques such as the use of dry or moistened tissues, wiping with a cloth, blowing with a jet of nitrogen, shaking, and brushing the samples, as well as washing techniques involving washing with water or other solvents (Markert 1996, Kovacheva *et al*. 2000). Tables 1 and 2

Table 2. Summary of the best cleaning procedure for foliar samples of broadleaf species.

Species	Washing method and aim	Use	Reference
Lolium multiflorum	Distilled water for 15 min in biomonitoring studies	Recommended to distinguish external from internal tissue composition	Steubing 1982
Ailanthus altissima	1% of Alconox™, followed by 0.01 M of EDTA to remove surface contamination in studies of particulate pollutants analysis	Recommended to distinguish external from internal tissue composition	Porter 1986
Populus nigra cv. *italica*	Deionized water with detergent to distinguish the lead adsorbed from that deposited	Recommended to distinguish external from internal Pb tissue composition	Capelli *et al.* 1989
Quercus myrsinaefolia	Water with 0.2 M of HCl to remove the external deposits from internal one	Recommended to distinguish external from internal tissue composition. No effect on K	Salkl and Maeda 1982
Empetrum nigrum	Distilled water for 1 min to minimize the effect of surface contamination in study of the chemical composition and the ecophysiological response	Recommended to distinguish external from internal heavy metals conc. but not micronutrients	Monni *et al.* 2000
Agrostis palustris	Deionized water for 30 s to assess the reliable nutrient status of the plant	Recommended to distinguish external from internal B, Al, Fe, Zn, composition	McCrimmon 1994
Quercus ilex	No washing if the aim is to assess the elemental accumulation in leaves	Useful to a reliable evaluation of pollutant deposition, since surface deposition on leaves in an urban area is due principally to airborne pollutants and soil dust component is insignificant	Alfani *et al.* 2000
Ornamental species	Distilled water for 10 washing process for a biomonitoring study	Recommended to distinguish internal from external heavy metals composition	La Malfa *et al.* 1996, Djingova *et al.* 1999

show the most used cleaning procedures for plan samples and their effect. A number of different washing techniques have been used to investigate the location of elements in foliage. The most common technique is to wash the samples by shaking the leaves or needles with washing agent(s) for a few minutes. Sometimes the samples are only immersed in the washing solution. Less common methods are mechanical cleanings that include the stripping (Rentschler 1982, Cercasov 1985, Krivan and Schaldach 1986, Krivan *et al*. 1987) and the rain drop simulator (Potter and Ragsdale 1991) techniques. A cleaning procedure involving ultrasonic treatment (Godzik *et al*. 1979) to detach the particles from the leaf surface has also been used (Buchauer 1973, Hall *et al*. 1975).

In the stripping technique, leaves or needles are dipped into a solution of resin dissolved in acetone (Krivan *et al*. 1987). The leaf is then suspended vertically and, after drying, the resin is stripped off with tweezers. The hardened resin contains all the particulate material attached to the leaf surface. According to scanning electron microscopy, this procedure does not damage the leaf surface (Rentschler 1982, Krivan and Schaldach 1986, Krivan *et al*. 1987). Polyviny butyrals such us Mowital (Hoechst AG), and different types of Pioloform (Wacker Chemie GmbH) have been used in a variety of solvents. Similarly, collodion dissolved in amylacetate has also been used to separate the epicuticular wax from the intracuticular wax (Haas and Rentschler 1984).

Potter and Ragsdale (1991) and Lin and Schuepp (1996) used the rain drop simulator technique to estimate the washoff of dry deposition from the leaves of forest tree leaves by simulated acidic rainfall. The rain drop simulator consisted of rain water in an automatic dispensing burette, and a three nipple manifold which delivered drops of simulated rain at a controlled size and rate. A vibrating glass arm distributed the drops at random over the leaf surface. Wyttenbach and Tobler (1988, 2000) washed needles of *Picea abies* with a mixture of toluene and tetrahydrofuran for one minute with mechanical vibration.

A number of studies have been carried out to develop a suitable cleaning method for plants

(Bewley 1979, Garber 1970, Iserman 1977, Kloke and Riebartsch 1964, Wallace *et al*. 1980, Thomas *et al*. 1984, Kowalenko 1984, Krivan and Schaldach 1986, Porter 1986, Lieth and Markert 1988, Wyttenbach *et al*. 1985, 1993). There is no ideal washing procedure or technique because e.g. the effects of washing also depend on the foliar matrix. Various washing agents and combinations of these have been used:

1. distilled water or tap water (Arkley *et al*. 1960, Fergusson *et al*. 1980, Capelli *et al*. 1989, Gay and Murphy 1989, Dmuchowski and Bytnerowicz 1995, Romano and Abate 1995, Alfani *et al*. 1996, Markert 1996, Monni *et al*. 2000), chloroform (Godzik *et al*. 1979, Wyttenbach *et al*. 1985, Keller *et al*. 1986, Krivan and Schaldach 1986, Van Praag and Weissen 1986, Pfirrmann *et al*. 1989, Zwoliński *et al*. 1998, Rautio 2000),
2. distilled water followed by chloroform or vice versa (Krivan and Schaldach 1986, Krivan *et al*. 1987, Simmleit *et al*. 1989), different detergent solutions (Nakos 1979, Salkl and Maeda 1982, Capelli *et al*. 1989, Moraghan, 1991, McCrimmon 1994),
3. toluene and tetrahydrofurane (Wyttenbach and Tobler 1988, Schleppi *et al*. 2000),
4. distilled water and acetone (Carten and Hanson 1990),
5. distilled water and a detergent solution and hydrochloric acid (Worley 1993),
6. weak acid solutions (Keller and Preis 1967, Little 1973, Lindberg and Harriss 1981, Rea *et al*. 2000),
7. EDTA solutions (Priebe *et al*. 1981, McCrimmon 1994, Markert 1996),
8. hydrochloric acid (Salkl and Maeda 1982),
9. ammonium acetate solutions (Martinez *et al*. 1971).

Salkl and Maeda (1982) tested three different washing techniques for leaves of *Quercus myrsinaefolia* Blume, and needles of *Cryptomeria japonica* (L. f.) D. Don and *Pinus densiflora* Siebold. and Zucc. They used distilled water, 2% domestic detergent (containing LAS and sodium pyrophosphate) or 0.2 M hydrochloric acid (HCl) solution for 4 minutes. The most effective method was washing with HCl since this reagent removed

a significant proportion of the Al, Cr, Fe, Pb and Ti on the needle surfaces. Rea *et al.* (2000) tested distilled water and a dilute nitric acid solution and reported that nitric acid was more effective than water. Although detergents and acids are the most effective washing agents (Little 1973, Salkl and Maeda 1982, Krivan *et al.* 1987), water is the medium that is most widely used on a routine basis for washing plant tissues prior to analysis. Distilled water is recommended as the most effective, safe and cost efficient agent in many cases (Bargagli 1998). However, it is not suitable for conifer needle samples (Capelli *et al.* 1989). Therefore the washing of conifer needles with chloroform for 1 to 3 minutes is recommended (Kovacheva *et al.* 2000). According to Markert (1996), chloroform agent removes 80% of the Al, As, Fe, Sb deposited on the surface wax, and results in a constant elementary composition for needles of different ages (Bargagli 1999). However many laboratories try to avoid the use of chloroform due to its occupational health hazard and polluting effect in the environment.

Nevertheless, the choice of washing agents depends on the plant species and on the application (Porter 1986), to the different surface properties of leaves and leaf age (Krivan *et al.* 1987, Lin and Schuepp 1996, Rea *et al.* 2000, Rossini Oliva 2000). The washing of leaves with a rough surface is more effective than that of leaves with a smooth surface. If the leaves have a wax layer, e.g. coniferous needles, then a stronger washing solvent is recommended. In addition, leaf age affects the choice of washing agents because young conifer needles have a thinner wax layer than older ones. The different accumulation capacity of the plant species may also affect the choice of washing agents (Little 1973). The washing procedure also depends on other factors, e.g. the aim of the experiment, washing agent, washing time and volume of washing agents, temperature, and physical and chemical properties of the accumulated elements (Dickinson 1971, Roberts 1972, Fortmann and Johnson 1984, Wyttenbach *et al.* 1985, Krivan and Schaldach 1986, Krivan *et al.* 1987, Pfirrmann *et al.* 1989, Simmleit *et al.* 1989, Zwoliński *et al.* 1998, Kovacheva *et al.* 2000).

The effect of washing time depends on e.g. the properties of the elements in question. A longer washing time is more effective than a shorter one. According to Steubing (1982), washing with distilled water for 1 minute removed only a minor proportion of the pollutants, but washing for 15 minutes considerably increased the yield. However, increasing the washing time to 30 minutes did not remove any more pollutants (Steubing 1982).

The washing time is also problematic due to the leaching effect. If the washing time is long then this may result in the leaching of individual elements or pollutants. The same washing time and temperature can also give different results for the same samples (Markert 1995, Rea *et al.* 2000). A heterogeneous particle size also makes it more difficult to find a comparable procedure (Markert 1995) because large particles (> 1 μm) are more easily removed by washing than small particles (< 1 μm) (Fortmann and Johnson 1984, Lin and Schuepp 1996, Bargagli 1998). This means that it is not possible to achieve a standard washing agent, time and temperature. However, a standard procedure has been recommended for some species. For instance, Wyttenbach *et al.* (1985), Markert (1996), Zwoliński *et al.* (1998) and Rautio (2000), have used the same washing procedure for pine needles: shaking the samples for 30 seconds with 20 ml of chloroform and rinsing the samples twice with deionized water. This procedure removed surface-deposited elements, but it did not extract biomass-incorporated elements (Wyttenbach *et al.* 1985). It has not been possible to adapt this procedure directly for other species (Little 1973, Salkl and Maeda 1982, Porter 1986, Wyttenbach and Tobler 1988, Koricheva and Haukioja 1995, Rea *et al.* 2000, Rossini Oliva 2000).

Effects of cleaning on foliar chemistry

Plant leaves, especially conifer needles, are effective filters of aerosol particles. A part of the dry deposition on needles is irreversibly adsorbed, and therefore superficial leaf contamination increases with leaf age. Gay and Murphy (1989) investigated the uptake rate and fate of SO_2 absorbed by the forest canopy using the radioactive tracer ^{35}S. About 50% of the ^{35}S absorbed

by the needles was associated with material that could be washed off the needle surface. Dollard (1980) reported similar or even higher surface absorption. For this reason, many researchers recommend the washing of samples prior to chemical analysis if the aim is to estimate the internal pool of essential elements.

No significant changes in the concentrations of most of the major elements (e.g. N, P, K, Ca, Mg, Cl, S) or micronutrients (B, Zn, Mn), have been reported after cleaning (Wyttenbach *et al.* 1985, Keller *et al.* 1986, Krivan and Schaldach 1986, Porter 1986, Krivan *et al.* 1987, Pfirrmann *et al.* 1989, Simmleit *et al.* 1989, Moraghan 1991, Worley 1993, Zwoliński *et al.* 1998, Alfani *et al.* 2000).

La Malfa *et al.* (1996) reported differences in the Pb concentrations of washed with distilled water and unwashed leaf samples (70% less in washed leaves) and Romano and Abate (1995) reported for lead a significant difference in washed leaves of *Lantana camara* L. (74% reduced) and *Pinus halepensis* L. (44.8% reduced). Zwoliński *et al.* (1998) found that washing the needles of Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.) with chloroform resulted in significant differences in the Fe (50%), Cu (30%) and Pb (3%) concentrations between washed and unwashed pine needles, and in the Fe (42%), Cu (59%), Pb (28%) and Cd (11%) concentrations correspondingly in spruce needles. No significant differences were found for Ca, K, Mg, Mn and Zn. Wyttenbach *et al.* (1985) also reported significant differences for Al, As, Fe, Sb, Sc and V in washed and unwashed Norway spruce needles.

The significant differences in the concentrations of some elements (As, At, Br, Cr, Fe, Cd, Ni, Pb, Sb, Ti, V) between cleaned and uncleaned samples indicate that foliage has considerable amounts of removable particulate material, and this may lead to overestimates of the internal pool of essential elements. This also means that cleaning in general, independently of the plant species, has a significant effect on removing the following pollutants: As, At, Br, Cr, Cd, Ni, Pb, Sb, Ti and V (Zech 1969, Little 1973, Nakos 1979, Kabata-Pendias and Pendias 1984, Wyttenbach *et al.* 1985, Keller *et al.* 1986, Van Praag and Weissen 1986, Krivan

and Schaldach 1986, Krivan *et al.* 1987, Capelli *et al.* 1989, Pfirrmann *et al.* 1989, Simmleit *et al.* 1989, McCrimmon 1994, Koricheva and Haukioja 1995, Romano and Abate 1995, Alfani *et al.* 1996, Lin and Schuepp 1996, Robert *et al.* 1996, Schleppi *et al.* 2000). Iron is one of the essential elements that is most affected by washing the needles. 60% to 80% of the Fe is removable and therefore assumed to be derived from surface contaminants, primarily soil dust (Wyttenbach *et al.* 1985, Keller *et al.* 1986, Pfirrmann *et al.* 1989, Porter 1986).

The proportion of pollutants removed by cleaning depends on many factors, e.g. the type of sample and the procedure used. For instance, Little (1973) reported that washing elm leaves with water removed 27% of the Zn, 8% of the Pb and 13% of the Cd. Rossini Oliva (2000) found that washing *Duranta repens* L. leaves with water removed 42% of the Fe, 51% of the Cr and 46% of the V, while for *Nerium oleander* L. washing removed only 32% of the Fe and 33% of the Cr.

Salkl and Maeda (1982) reported that the removal efficiency of 16 different elements from pine needles using water-washing procedures ranged from 0% for Zn to 48% for Ni. Washing with water was particularly ineffective for the removal of Cr, Cu, Mn, Pb and Zn from pine needles, but it was effective for the removal of Pb, Fe, Al, Cr and V from the leaves of ornamental species (Rossini Oliva 2000). Values ranging from 10% to 96% have been reported for the removal of Pb (Suchodoller 1967, Motto *et al.* 1970, Schuck and Locke 1970, Little 1973, Lerche and Breckle 1974, Ward *et al.* 1974, Ward *et al.* 1975a, b, Holl and Hamp 1975, Carlson *et al.* 1976, Nakos 1979, Rentsehler 1982, Keller *et al.* 1986, Krivan and Sehaldach 1986, Krivan *et al.* 1987). Most of the airborne particles containing Cd also remain on the surface of tree foliage, and partial penetration into the living tissues of plants is possible (Little and Martin 1972, Hagemayer *et al.* 1986, Krivan *et al.* 1987, Rossini Oliva 2000).

Quality assurance and quality control is a very important step in the chemical analysis. However, very few publications that include foliar analysis have reported the quality of the chemical analysis.

Conclusions

Chemical foliar analysis has traditionally been carried out on uncleaned samples. Cleaning plant samples is not recommended when monitoring the distribution of pollutants emitted e.g. by factories, mines or traffic. Analysis of uncleaned leaves is considered a good method for assessing the degree of air contamination because it provides an estimate of the element content of the deposits on leaf surfaces and when the analysis is aimed at the investigation of transfer of elements along the food chain. Sample cleaning is frequently not recommended because of its leaching effect, it is the opinion of the authors that leaching is not a problem if washing time is careful controlled.

However, in some cases, it is strongly recommended to clean foliage samples. The main aim of sample cleaning is to remove elements or particles deposited on plant surfaces. This allows e.g. distinguishing between atmospheric deposition and incorporation from the soil or the sea, and reduces the effect of precipitation. According to the literature, sample cleaning is recommended in order (1) to investigate the transfer rate of chemical elements from soil to plants, (2) to qualify the washoff of dry deposition from foliage and (3) to separate superficially absorbed and biomass-incorporated elements.

A number of different techniques have been used to clean plant samples. There are many washing agents available, and their effects depend on e.g. washing time, temperature, volume of the agent and structural properties of the sample. Distilled water is recommended in many cases, being effective, safe and cost efficient. The plant species, leaf age and the structure of the leaf surfaces have also an effect on the washing efficiency. The effect of washing time depends on the properties of the elements, but in general a longer time is more effective than a shorter one.

Cleaning has an effect on the foliar concentrations of Al, As, At, Br, Cd, Cr, Fe, Ni, Pb, V, T and other heavy metals. Thus the error for these elements can be very large if the samples are not washed. No significant changes in the concentrations of most of the major essential elements (N, P, K, Ca, Mg, S) and micronutrients (B, Mn and Zn) have been reported after washing.

The final conclusion is that there is no standard and general cleaning procedure. Therefore, if no information is reported in the literature for a particular plant species and for a particular aim of the investigation, it is advised to conduct a pilot study, to be able to establish a cleaning procedure to provide reliable foliar data.

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