Optimization of the sequential elution technique for geochemical background monitoring: the influence of bioindicator sample mass on the reproducibility of analysis

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Research Article

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Abstract

The study addresses the uneven distribution of chemical elements in bioindicator biomass (mosses and lichens) when using the sequential elution technique (SET) for geochemical monitoring. The aim was to improve analytical reproducibility by applying a larger sample volume (50 g wet mass). SET enables the quantitative determination of elements in different cellular compartments, which is important for assessing biogeochemical migration pathways, bioavailability, and potential ecosystem effects. However, its application to geochemical background studies is limited by low element concentrations and high variability in results obtained from small biomass samples. Experiments were carried out on the moss Hylocomium splendens and the lichen Cladonia stellaris collected in background areas of Altai Krai, Russia. The mosses and lichens were divided into upper, middle, and lower sections, after which the biomass was fractionated into four fractions. Results obtained using standard (10–20 g fresh weight) and large-volume (50 g fresh weight) biomass samples were compared. When the large-volume biomass sample was used, the relative standard deviation for most elements did not exceed 30%. Modification of the SET allows minimizing the influence of uneven atmospheric deposition of elements on bioindicators and ensures a more accurate assessment of the geochemical background and pollution levels.

Introduction

Geochemistry is the science that studies the Earth's chemical composition and the processes of element migration occurring in various geospheres. One of the key areas of geochemistry is the study of the geochemical background – the natural concentration of chemical elements in various natural environments. Knowledge of the geochemical background plays an important role in assessing the state of the environment, as it allows us to identify deviations caused by human activity, predict changes in ecosystems and assess potential risks (Kabata-Pendias, 2011). In recent decades, rapid industrial development and urbanisation have led to a significant increase in anthropogenic impact on natural systems. An increasing amount of pollutants is entering the environment, making the problem of environmental pollution particularly relevant. In this context, the geochemical background is used as a reference for the normal content of elements in soil, water and the atmosphere. Comparing actual concentrations of chemical elements with their background values allows the presence and extent of pollution to be determined.

Environmental monitoring systems are widely used to assess changes in the environment; these systems incorporate various methods for analysing the concentration of elements in the air, water and soil. One of the most common approaches is bioindication, which uses indicator organisms capable of accumulating chemical elements, reflecting pollution levels in both spatial and temporal terms (Bargagli, 1995; Markert et al., 1999). Bioindication methods allow not only the assessment of the total concentration of elements in the environment, but also the analysis of their forms, migration and bioavailability, which is particularly important for the study of heavy metals and other potentially toxic elements (Conti & Cecchetti, 2001; Nieboer et al., 1972). Bioindication is widely used in the fields of

pollution monitoring, soil assessment, the study of geochemical processes, climate change monitoring and water quality assessment.

Mosses and lichens have been used as biomonitors of atmospheric heavy metal pollution since the late 1960s (Brown & Buck, 1978; Chiarenzelli et al., 2001; Conti & Tudino, 2016; Kropacheva et al., 2017; Qarri et al., 2019; Riget et al., 2000; Thakur et al., 2024). Unlike higher plants, mosses and lichens have no roots, so their main source of nutrients is atmospheric precipitation. As these groups of organisms lack a protective cuticle, they can absorb ions from the air, rainwater or snow. Mosses and lichens grow slowly, which means they can accumulate pollutants very effectively (Bargagli, 2016). Their strong ability to retain particulate matter is also the reason why they can be used as good bioindicators (Varela et al., 2023).

The sequential elution technique (SET) of biomass, proposed by Brown and Buck (1978), has been successfully applied to determine the cellular distribution of elements in mosses and lichens. This approach is based on the sequential extraction of elements associated with different cellular structures. The method provides a quantitative determination of elements in extracellular and intracellular spaces, the determination of the total amount of elements in the fractions, and the subsequent comparison of their sum with the gross contents (Pérez-Llamazares et al., 2011b). The use of the SET offers significant advantages for geochemical monitoring, providing detailed information on the state of the environment. The presence of elements in different fractions reflects the varying strength of their binding to the biomass, which influences the elements' migration capabilities.

The extracellular fraction consists of elements bound to the outer surface of cell membranes, as well as elements dissolved in the intercellular space. The concentrations of elements in this fraction change rapidly and reflect the current state of the environment at the time of sampling, as well as short-term changes in environmental conditions. The intracellular fraction is most significant for assessing the physiological state of the bioindicator, as the elements accumulated within the cell are directly involved in cellular metabolism and can alter it. According to Costas and López (2001), the intracellular concentration of elements is resistant to short-term environmental changes, as changes in the content of elements within cells occur slowly. Thus, the intracellular fraction reflects the average environmental conditions of the bioindicator and, in essence, represents the local geochemical background. This fraction allows for the assessment of the degree of element bioconcentration and phytotoxicity, as well as the detailed calculation of environmental risks. The solid residue fraction comprises elements bound in the cell wall, as well as elements associated with crystalline deposits within the cells. This fraction reflects long-term geochemical conditions and is resistant to short-term environmental changes. This fraction most fully characterises global and stable geochemical conditions, allowing the contribution of elements to bioconcentration and their influence on the formation of the geochemical clark to be assessed. Thus, analysing the distribution of elements between biomass fractions is an effective tool for assessing both current and long-term geochemical conditions, and enables the identification of natural and anthropogenic sources of element input into the environment.

The effectiveness of SET for biomass in anthropogenic pollution studies is not in doubt (Fernández et al., 2004; Pérez-Llamazares et al., 2009; Vázquez et al., 1999). However, the use of SET to study background concentrations of elements is complicated due to the problem of detecting low concentrations of elements. Using small biomass samples results in a large standard deviation, which makes it difficult to compare the data obtained. Increasing the sample weight complicates sample preparation at all stages, from controlling the moisture content of the biomass and fractional separation to the chemical decomposition of the resulting solutions and solids. The aim of our study was to adapt the SET for geochemical background monitoring tasks. Such studies are characterised by a large number of samples. Our task was to address the problem of uneven distribution of elements across the area of geochemical background bioindicators by using larger-volume bioindicator samples, as well as to modify the sample preparation of fractions to speed up the entire analysis. The proposed adaptations will enhance the practical applicability of the SET for geochemical background monitoring and enable the collection of more accurate and reliable data for assessing the state of the environment and the risk of its contamination.

Materials and Methods

Sampling Site

In 2021 and 2022, moss (Hylocomium splendens (Hedw.) Schimp.) and lichen (Cladonia stellaris (Opiz) Pouzar & Vězda) samples were collected using the five-point sampling method (sampling area ~ 50 m²) at two points located 150 m apart in a ribbon forest 6.5 km north of the village of Pankrushikha (Altai Krai) (Fig. 1). All samples were collected in locations with similar environmental conditions. Each sample consisted of three pieces of lichen thallus or moss cover. The approximate dimensions were: lichen thallus – 20 × 30 × 6.5 cm, moss cover – 25 × 30 × 3.5 cm. Pieces of lichen thallus or moss covers were placed in clean containers in layers, so that the upper part of the bottom layer was in contact with the upper part of the second layer, and the basal part of the second layer was in contact with the basal part of the top layer. A sheet of filter paper moistened with bidistilled water was placed at the bottom of the container, and a second sheet of filter paper was placed on top to cover the layers. The container closed tightly. A sample of moss or lichen packaged in this way will remain fresh for up to two months.

Laboratory Sample Preparation

Under laboratory conditions at room temperature, the moss and lichen samples were divided into three parts: upper, middle, and lower. The top part represents the youngest portion of the sample, generally tender green with a crown; the middle part is mature portion of the sample; in this section the moss is pale yellow, while the lichen is light green; the bottom part represents the oldest portion of the sample, usually yellow-brown color. During the separation process, the total sample from each portion was averaged; debris and dead fractions were removed from the sample. The three sections obtained were placed in separate sealed boxes on moistened sheets of filter paper for 1–3 days to ensure that the

entire sample had the same moisture content. After achieving same moisture, in experiment 1 (Fig. 2) parallel subsamples (10 g wet mass) were taken from each part (n = 3) for biomass fractionation, along with bulk subsamples for mass balance calculations. In Experiment 2 (Fig. 2), a single large-volume sample with an optimal sample weight (50 g wet weight) was taken from each portion of biomass.

Sequential elution technique (SET)

Sequential elution technique was carried out according to the methodology presented by Pérez-Llamazares et al. (2011a) with the separation of four fractions: Fraction 0 includes elements sorbed on dust particles that have not yet bound to the biomass; fraction 1 includes elements from the intercellular space and those bound to the external surface of cell walls; fraction 2 includes elements bound to the inner surfaces of cytoplasmic membranes and/or dissolved inside the cell; fraction 3 includes elements strongly bound within the cell wall and associated structures, as well as the solid residue, which contains insoluble elements and crystalline deposits within the cell. Fraction 0 is considered not bound to the biomass and was referred to as the wash-off fraction. Fractions 1 and 2 are considered weakly bound to the biomass. Fraction 3 is considered strongly bound to the biomass (Table 1).

Table 1
Scheme of the SET

Fraction	Reagents	Time
0	H_2O (bidistilled), twice; biomass/solution = 1:10	30–60 s
1	0.1 M Na_2EDTA + H_2O (bidistilled); biomass/solution = 1:10	24 h
2	1 M HNO_3 + H_2O (bidistilled); biomass/solution = 1:10	20 min
3	drying to constant weight	min 24 h

Sample preparation

Experiment 1

The 0–2 fraction solutions were filtered through a filter (average pore size 8–12 µm) to separate the suspended solids. Then the solutions were evaporated to a volume of 10 mL and chemically digested with a mixture of acids in an open system (Table 2, Fig. 2).

Fraction 3, the bulk biomass, and the substance on the filters were dried to an air-dry state and ashed at 450°C with a gradual temperature increase of 50°C. The resulting ash was chemically decomposed in a mixture of acids in an open system (Table 2, Fig. 2).

Experiment 2

The 0–2 fraction solutions were evaporated to a volume of 10 mL without filtration and then chemically decomposed using a mixture of acids in an open system (Table 2, Fig. 2).

Fraction 3 and the bulk biomass were dried to air-dry condition and chemically decomposed in a mixture of acids and hydrogen peroxide in a closed system under pressure using a HotBlock (Table 2, Fig. 2).

Table 2
Scheme for decomposing material of fractions

	Experiment 1	Experiment 2
fraction 0	HNO_3	HNO_3 + HF + $HClO_4$ +HCl
fraction 1	HNO_3 + $HClO_4$ +HCl	open system
fraction 2	HNO_3	
fraction 3	ashing at 450°C with a gradual temperature increase of 50°C	HNO_3 + HF + H_2O_2
substance on the filters		closed system under pressure using a HotBlock
Bulk biomass		-

Measurement

The elemental concentrations in the solutions were determined by ICP-AES (iCAP XP Pro Duo, Thermo, USA) and AAS (iCE3500, Thermo Fisher Scientific, USA). The operating parameters of the instrument were set in accordance with the manufacturer's recommendations.

Statistical analysis and mathematical processing of results

The reproducibility of the method was assessed using the relative standard deviation (RSD). The RSD was calculated according to the following formula 1:

$$\mathrm{RSD} = \left(\mathrm{SD}/\mu\right) * 100\% \quad (1)$$

where SD is the standard deviation and μ is the arithmetic mean of the measurement results. The RSD values were calculated separately for each element and for each experimental group (mosses and lichens, different fractions).

Results and Discussion

Experiment 1

The application of SET to small biomass samples (10–20 g fresh weight) revealed significant variation in element concentrations across different fractions and parts of mosses and lichens (Fig. 3). Relative standard deviation (RSD) values in many cases exceeded generally accepted limits for analytical reproducibility (RSD > 30%), indicating problems with analytical accuracy when using SET in geochemical studies. Increased RSD values (> 30%), and in some cases extreme values reaching 50–120%, were observed for a significant number of elements. These values were not limited to specific fractions or biological samples under study, which indicates that they were caused by a combination of low absolute element concentrations rather than errors in the analytical procedure. In addition, the reproducibility of the results differed between the vertical parts of the samples. For both mosses and lichens, the lower parts of the samples generally exhibited higher RSD values compared with the upper and middle parts. Differences in reproducibility were also observed between fractions. Fractions 1 and 3 generally exhibited higher RSD values compared with fractions 0 and 2; however, this trend was not strictly systematic. For several biophilic elements characterized by high concentrations in biomass, such as K, Ca, Mg, and Zn, the RSD values also exceeded 30%. Thus, the impact of uneven atmospheric delivery of elements to the area of bioindicators complicates the use of SET in studies of background element concentrations.

It should be emphasized that Experiment 1 was conducted for the purpose of a diagnostic assessment of the baseline methodological reproducibility. The observed reproducibility is considered solely as a characteristic of the methodological limitations of the standard application of the SET and is not interpreted as reflecting the actual distribution of elements in biomass.

Possible solutions to the problem of uneven distribution of elements on bioindicators. Selection of a large-volume sample.

Experiment 1 demonstrated that the uneven distribution of atmospheric deposition on the surface of the bioindicator is a significant issue in geochemical research and can substantially influence the interpretation of results. Due to the specific nature of the SET method, the number of options for solving this problem is limited. Conventional sample averaging is only possible through quarting, since the method is applied to wet and structurally intact biomass, which eliminates the possibility of additional sample averaging through drying and grinding. The most practical way to reduce the impact of uneven atmospheric deposition is to increase the sample weight.

The consequences of changing the parameters of the original procedure must be taken into account: an increase in the mass of the fractionated sample leads to an increase in the volume of extractants at each stage of fractionation. It is important to find a balance that ensures the representativeness of the original sample without increasing the time required for subsequent sample preparation of the resulting fraction solutions. In some cases, it is possible to analyze not the entire volume of the fraction solutions, but aliquots from each solution; however, this requires additional studies in each individual case, which again prolongs the entire sample preparation and analysis procedure.

Experiment 2

Taking into account the limitations revealed in Experiment 1, Experiment 2 implemented revised parameters of the technique in an effort to improve reproducibility: the elimination of small parallel samples and the use of a single, larger biomass sample (50 g wet weight). In Experiment 2, reproducibility was assessed only for fraction 3 and the bulk sample, which could be averaged not only by quarting but also by grinding after drying. The solutions (fractions 0–2) were analyzed in their entirety, which did not allow for the calculation of RSD. (Fig. 4)

Within these SET parameters, the RSD values were significantly lower. For most elements, the RSD values were within the range of 0–30%, while for a considerable number of elements, especially in the upper and middle parts of the samples, the RSD did not exceed 10–20%. Cases where measurement results showed large dispersion were rare, and formally anomalous RSD values were practically absent.

The concentrations of elements in Experiment 2 were not directly compared with those in Experiment 1. The decrease in RSD values reflects improved reproducibility due to better spatial averaging of the biomass.

Conclusion

The present study aimed to evaluate and optimize the reproducibility of SET in geochemical background studies using bioindicators—mosses and lichens. The variation in results for a significant portion of the elements using small biomass samples exceeds acceptable limits, which significantly limits the reliability of results obtained using SET in such studies.

The use of a larger biomass sample (50 g fresh weight) led to a noticeable decrease in the relative standard deviation values for most elements in bulk samples and fractions 3 samples. Further increasing the sample weight becomes less practical, as it leads to a further increase in eluent volumes, which requires more time and effort for subsequent sample preparation.

The proposed optimization is not intended to alter the conceptual foundations of the SET, but rather to refine the conditions for its application in geochemical background monitoring. The conclusions drawn should be regarded as methodological recommendations designed to improve the reliability and reproducibility of analytical data when studying low concentrations of elements in bioindicators. Determining the practical sample mass limit expands the scope of application of the SET in studies of environmental background conditions and establishes a basis for its more accurate and reproducible use in analytical and geochemical research.

Declarations

All authors have read, understood, and have complied as applicable with the statement on “Ethical responsibilities of Authors” as found in the Instructions for Authors

Competing interests

The authors declare no competing interests.

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Author Contribution

Yanming Zhang: Writing – original draft, Visualization, Resources, Methodology, Validation, Formal analysis, Investigation. Marya Kropacheva: Writing – review & editing, Conceptualization, Methodology, Investigation, Supervision

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Figures

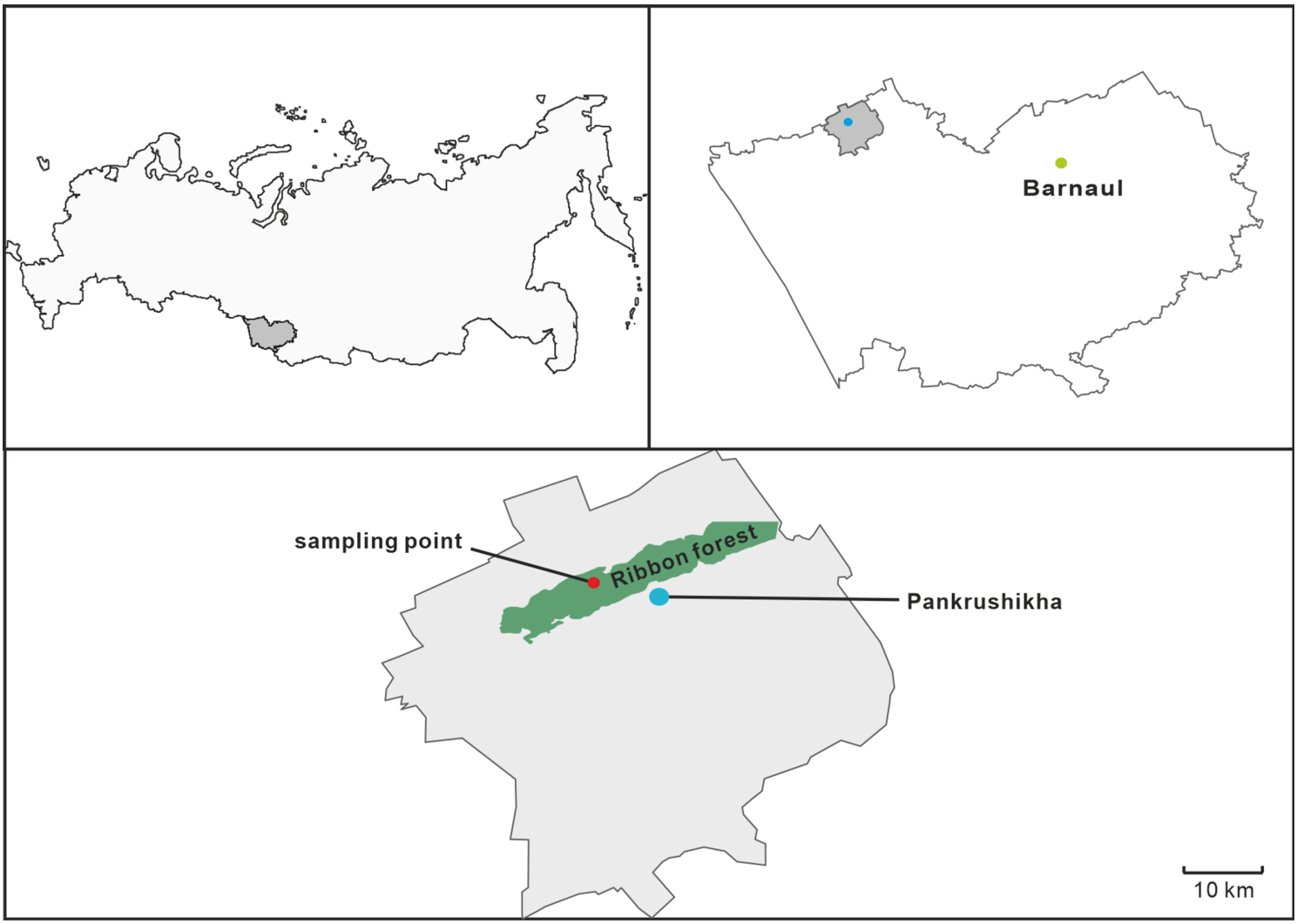

Figure 1

Location of the sampling site

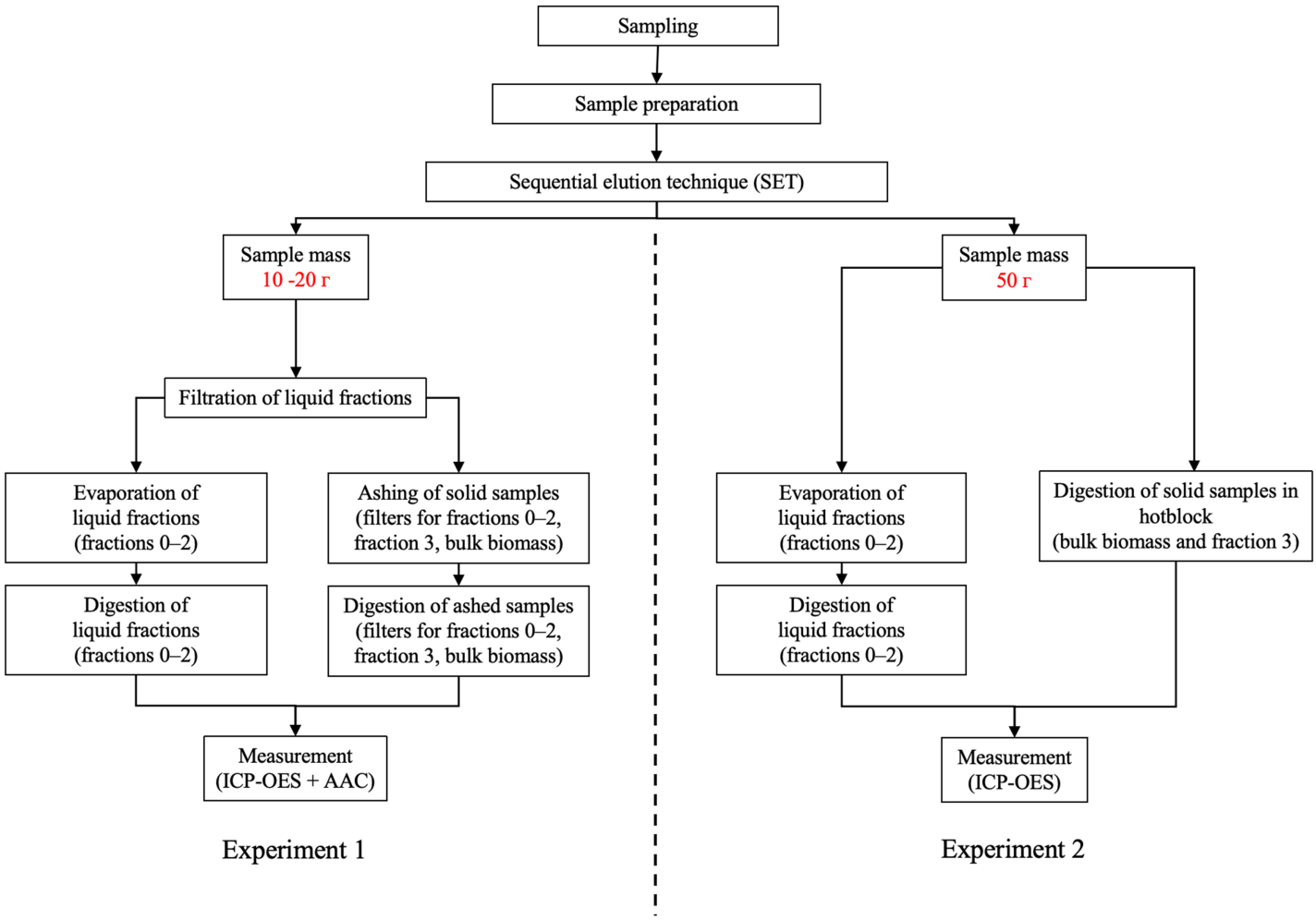

Figure 2

Scheme of the experiment

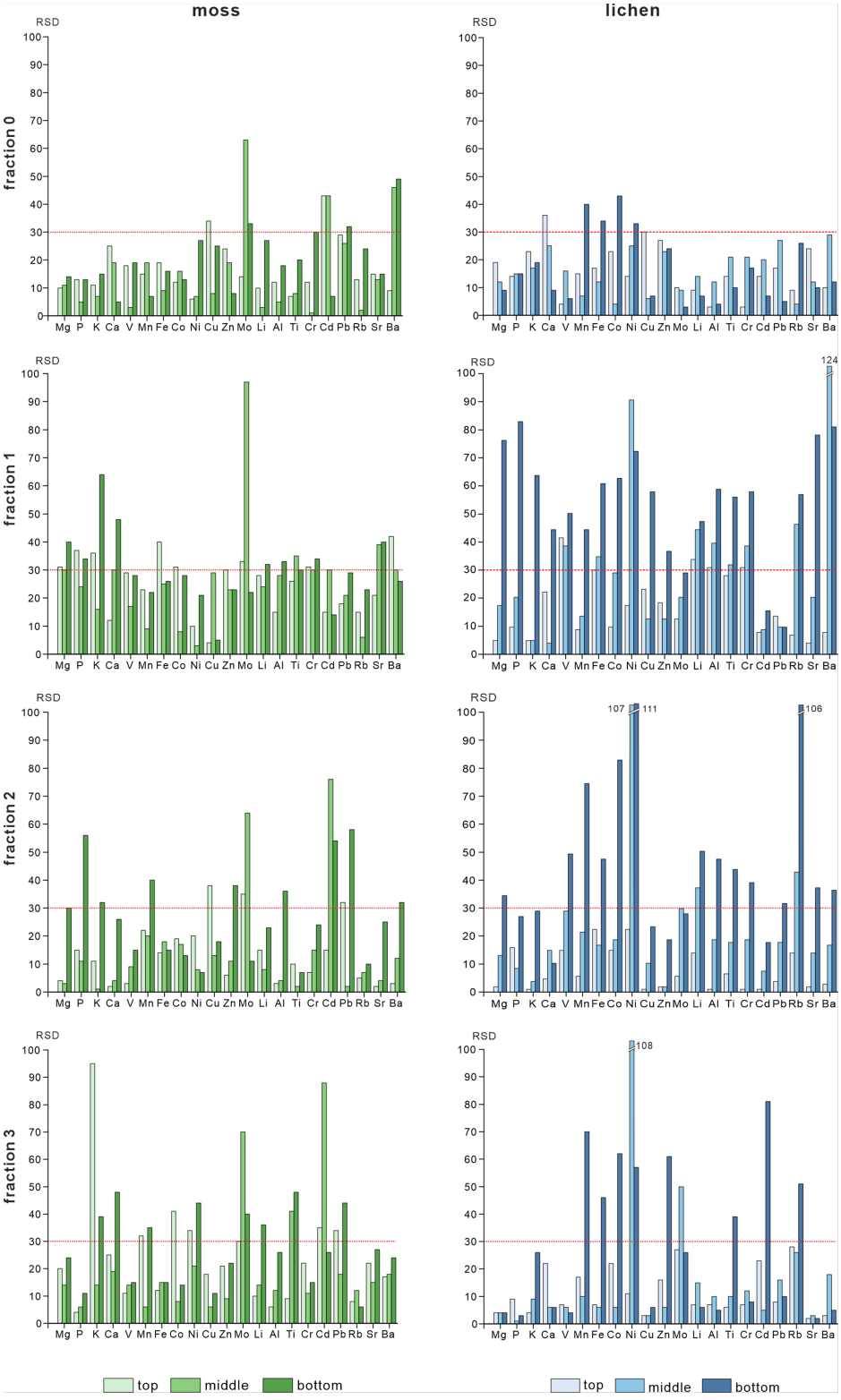

Figure 3

Relative standard deviation (RSD) of elements in fractions 0- 3 of mosses and lichens

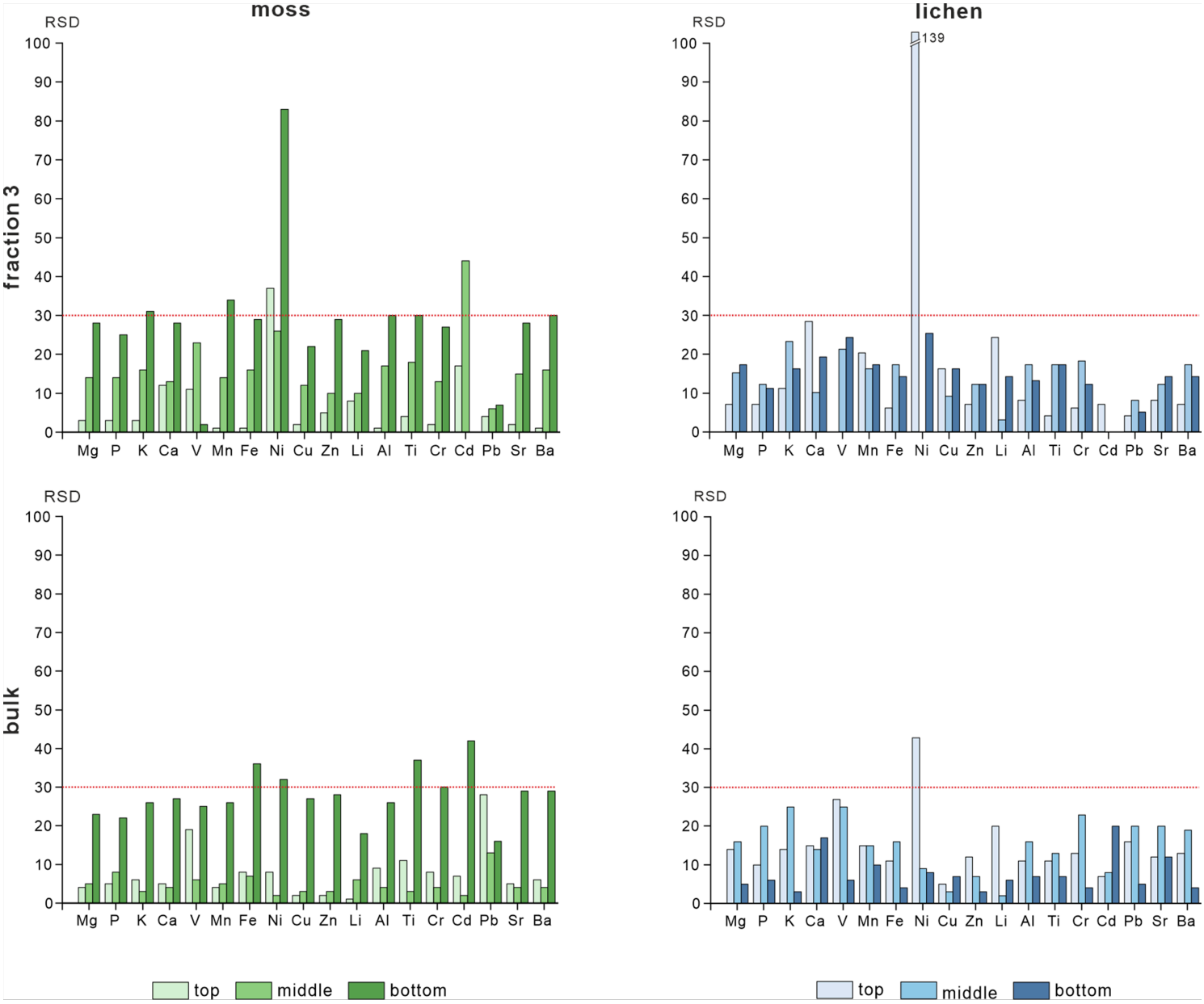

Figure 4

RSD of elements in fraction 3 and bulk of mosses and lichens when using a large-volume sample