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Total-reflection X-ray fluorescence analysis (TXRF) of plant's guttation fluids as a new, fast, and non-invasive strategy for the assessment of the bioavailability of Zn, Cd and Pb in contaminated soils

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ABSTRACT

The assessment of potentially toxic elements (PTE) concentration in biofluids is often used for the evaluation of their bioavailability in polluted environments. In the soil-plant system, the analysis of the composition of the xylem fluid can provide a real snapshot of the elements taken up from the soil by the plant. However, xylem collection is often difficult, and, for herbaceous plants, it requires cutting the plant. Alternatively, xylem can be collected through leaves as naturally exuded drops (i.e. guttation), thus in a fully non-destructive way. The guttation phenomenon is yet limited to micro-volumes, therefore the analysis with most techniques is challenging. For the first time, the capability and reliability of total-reflection X-ray fluorescence (TXRF) spectrometry for the quantification of PTE in plant's guttation fluids was tested in this work. In particular, the study was led on fluids sampled from *Lolium rigidum* plants grown in a soil contaminated with Zn, Cd, and Pb. Two different TXRF spectrometers were used and compared, equipped either with Mo- or W-anode-based X-ray sources; inductively coupled plasma optical emission spectroscopy (ICP-OES) and graphite furnace atomic absorption spectrometry GF-AAS were used as reference techniques. Differently from these latter, approximately 30 μL of sample were sufficient for the quantification of Zn, Cd, and Pb through TXRF (along with the determination of other essential plant nutrients). Furthermore, the comparison with ICP-OES and/or GF-AAS showed various improvements in using TXRF, including a fast sample preparation, a reduced use of chemicals, the multi-elemental capability. These results suggest that TXRF analysis of plant guttation fluids could represent a novel non-destructive, expeditious and "green" analytical approach for the study of Zn, Cd, Pb and other PTE availability in polluted soils.

1. Introduction

Biological fluids are widely used as a source of biomarkers, whose study allows evaluating the chemical exposure to pollutants and their effects on biota [1]. Further, the concentration of potentially toxic elements (PTE) in biofluids can be indicative of their bioavailability and bioaccessibility in polluted environments and therefore connected to the actual risk [2,3]. Although for various kinds of biofluids (e.g. human or mammals' blood, urine, breast milk, etc.) it is not that difficult to collect adequate amounts of sample for analysis, for smaller organisms and plants, the extrusion of fluids is often challenging and can difficultly exceed volumes of tens to hundreds of microliters. As such, the scanty amount of sample collectible makes the elemental analysis with traditional atomic spectroscopies, e.g. inductively coupled plasma atomic emission spectroscopy (ICP-AES), mass spectroscopy (ICP-MS) and atomic absorption spectroscopy (AAS) very tricky. Beside these techniques, total-reflection X-ray fluorescence spectrometry (TXRF) has gained over the last years increasing interest as a valuable and reliable multi-elemental micro-analytical technique [4], also for the analysis of biological fluids.

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For example, TXRF has been successfully applied for the determination of PTE such as Ni, Zn, As, Se, Cd, Pb, etc. in human and mammals' body fluids [5,6], coelomic fluids of soil invertebrates [3,7,8] and xylem sap [9,10]. This latter is basically a water-solution, streaming one-way from plant roots to shoots, which provides the supplying of mineral nutrients to the aerial parts of the plant. Hence, studying the composition of xylem saps can be used to assess the nutritional status of crops, but also to monitor the uptake and translocation of undesired substances from soil, such as PTE and/or organic contaminants. In the case of herbaceous plants, including those usually grown in laboratory experiments and often used in phytoremediation and biomonitoring, the xylem sap is usually collected by sucking the fluid that exudes after cutting the plant close to the root collar. However, the xylem sap collectible per plant is rather scarce, hardly exceeding the volume of 1 mL. This has led to the selection for such studies preferentially, if not exclusively, of plants with higher rates of xylem flux (e.g. cucumber) [9]. Moreover, the invasiveness of this kind of sampling procedure causes great damages to the plant and makes further sampling over time on the same individual impossible.

An alternative way of xylem sap sampling could be the collection of plant's guttation fluids [11], thus overcoming many of the above-mentioned limitations. In fact, guttation is the natural process of xylem sap exudation in the form of round-shaped droplets through foliar structures called hydathodes, which usually occurs in low transpiration and elevated root pressure conditions. This implies that, after guttation, such plant's saps can be collected directly from the leaves, thus avoiding any damage to the plant. Moreover, even if the volume of guttation saps collectible from an individual plant is extremely scarce (few microliters in most cases), it can be sufficient to investigate its elemental composition via TXRF. Indeed, to perform analyses under total reflection conditions, samples must be provided as thin films by depositing 5–20 μL of sample on a reflective carrier and subsequently drying the drop [12]. This means that, in principle, even a single guttation drop can be analysed. Therefore, TXRF appears to be a suitable analytical method for the analysis of plant guttation fluids, providing a completely non-destructive approach for the investigation of the composition of xylem fluids. According to the authors' knowledge, this application of TXRF spectrometry has never been explored before.

In this study, a new, fast and non-invasive method for the analysis of plant's guttation fluids with TXRF is proposed. For the purpose, guttation fluids exuded from *Lolium rigidum* plants grown in a multi-contaminated soil (Zn, Cd, and Pb) have been collected and analysed for the quantification of Zn, Cd, and Pb. The analytical performances of TXRF were compared with the ones of reference methods for elemental analysis such as ICP-AES and GF-AAS. Moreover, the capability of the method to appreciate variations in Zn, Cd, and Pb concentration in guttation fluids from plants grown in soils featuring different availability conditions of such PTE was evaluated.

This research represents the first example of PTE bioavailability assessment in contaminated soils using naturally exuded plant's biofluids in a non-destructive and expeditious way.

2. Materials and method

2.1. Plant guttation fluids collection

Annual ryegrass (*Lolium rigidum*) plants were grown in pots (18 cm diameter, 22 cm height) containing nearly 6 kg of a polluted soil from the former mining district of Montevecchio, Guspini, Sardinia, Italy. The total concentration of Pb, Cd and Zn was above the Italian national regulatory thresholds for agricultural sites [13], being on average $4.5{\cdot}10^3$ mg Pb kg^{-1}, 23 mg Cd kg^{-1}, $3.1{\cdot}10^3$ mg Zn kg^{-1} [14]. To study different bioavailability conditions, one pot was filled only with the polluted soil (Soil 1), another one with the same soil amended with a biochar, produced by pyrolysis of biomass of *Populus nigra* grown on a Pb contaminated soil [14], at 3 % *w*/w (Soil 2), one pot was filled with the soil inoculated with a suspension of a consortium of plant growth promoting rhizobacteria (PGPR) in sterile Ringer's (Soil 3), and a last one with the soil amended with biochar at 3 % *w*/w and inoculated with PGPR (Soil 4). Pots were planted with annual ryegrass seeds (6 g m^{-2}), to have an average of 15 ryegrass plants in each pot, and the plants were grown for 30 days in a greenhouse with natural light at an average temperature of 20–25 °C and 60–70 % relative humidity.

Guttation fluids were collected after 30 days from sowing at the beginning of the tillering phase, as it is one of the greatest vegetative development phases with great metabolic activity. To promote plant's guttation, pots were abundantly watered until reaching the 100 % of the soil water holding capacity the evening before saps collection to increase root pressure and were kept covered overnight with a plastic bag (Fig. 1). This latter procedure was adopted to increase the relative humidity and limit foliar transpiration, thus favouring guttation fluids exudation. Early in the morning, the plastic bags were removed, and the guttation droplets were collected in Eppendorf tubes by means of 200 μL micropipettes (Fig. 1d) and immediately stored at −20 °C. Each sample was composed of droplets collected from about 30 leaves per pot, representing a composite sample of all the plants grown in the pot. For each composite sample, the volume of fluid collected was of 50 to 100 μL per pot.

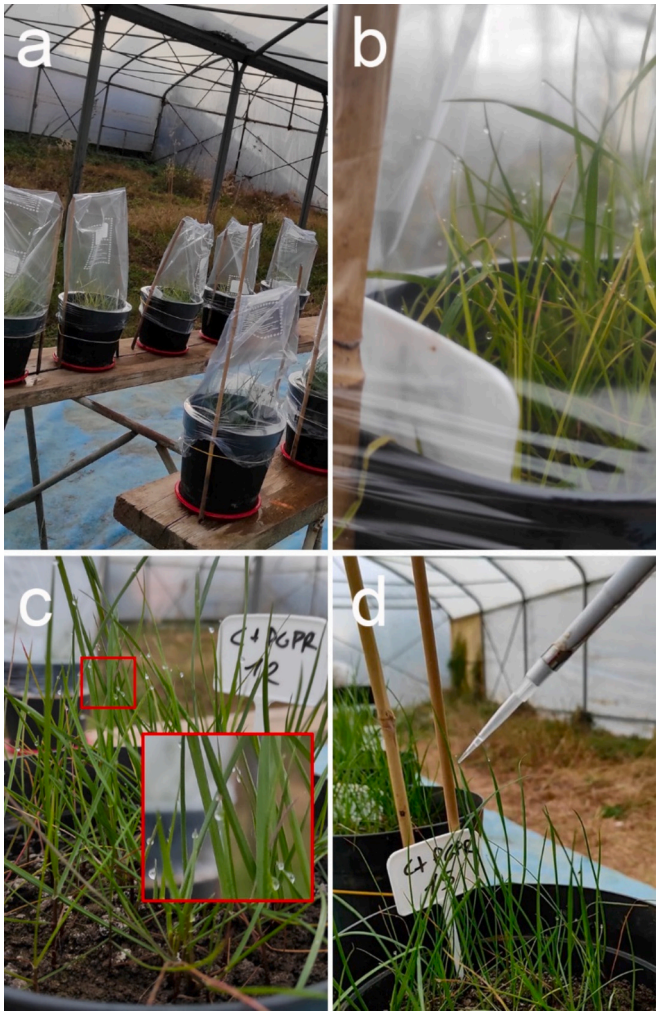

Fig. 1. Guttation fluid sampling procedure: a) after watering, plants are kept covered with plastic bags overnight to favour guttation; b) the cover has to be removed; c) guttation drops are visible at plants' tips and d) are collected with micropipettes. See also the supplementary video of guttation fluid collection (Video S1).

2.2. TXRF analysis

In TXRF analysis, as the sample constitutes a very thin layer, no matrix effects such as absorption and enhancement, common to conventional XRF spectrometry, occur. Therefore, it can be assumed that the intensities are proportional to the mass fractions of the elements of interest. For that reason, internal standardization is one of the most used strategies for quantification purposes in TXRF analysis. It is based on the addition of a defined amount of a liquid mono-element standard (internal standard, IS) to the target sample. Then the concentration of each analyte in the sample is calculated considering the signal and concentration of the internal standard, and the relative sensitivity of the analyte [15]. For the analysis of samples with minute amount availability, as in the case of this work, it is not possible to mix the IS with the sample before sample deposition on the reflective carrier. Indeed, it is a common practice to mix the sample with the internal standard solution directly over the reflector within the same drop [16,17]. However, it is here proposed and tested for the analysis of guttation fluids a separate deposition of sample and IS, which aims to allow the possible enrichment (therefore the determination) of low-concentrated analytes through multiple depositions of the sample For these reasons, and in order to verify whether the sample deposition strategy could affect the results, two different procedures for sample deposition on the TXRF reflector were tested,: i) deposition and drying of 10 μL of the sample (S) after internal standard deposition (10 μL) and drying (IS+S) and ii) deposition and drying of 10 μL of the IS after sample deposition (10 μL) and drying (S+IS). Yttrium (Y) (2 mg L^{-1} Y standard solution, Sigma-Aldrich) was chosen as IS; indeed, Y characteristic fluorescence lines (14.958 and 16.739 for Y-Kα and Y-Kβ, respectively) do not interfere or overlap with those of the elements of main interest for this work (Zn, Cd, and Pb).

Due to the lack of a specific commercial reference material, a 2 mg L^{-1} Certipur® ICP multi-element IV standard solution (Merck KGeA, 64,271 Darmstadt, Germany) was used for the validation.

Three replicates were prepared for each of the two deposition procedure (i.e., IS+S and S+IS) and analysed using two S2 Picofox TXRF spectrometers (Bruker Nano GmbH, Berlin, Germany), equipped with Mo- (Micro X-ray Lab, University of Bari, Italy) and a W-anode-based X-ray tubes (Department of Chemistry, University of Girona, Spain), respectively (hereafter, Mo-TXRF and W-TXRF). W-TXRF was used for the detection and quantification of high-Z elements, in particular for Cd.

The IS+S procedure was finally used, and all plant guttation fluids were prepared for TXRF analysis as follows: 10 μL of a 2 mg L^{-1} Y standard solution were pipetted over a clean siliconized quartz reflector and left, until complete dryness, on a heating plate at 50 °C under a laminar flow hood; then, 10 μL of sample was deposited onto the IS and dried. The final IS concentration, calculated for the sample volume, was 1 mg L^{-1}. Each of the four fluids collected from Soils 1–4 was analysed in triplicate.

The experimental parameters of the two spectrometers is reported in Table 1.

The limit of detection (LoD) was determined in the following way:

$$LoD = \frac{3C_e\sqrt{N_{BG}}}{N_e}$$

where C_e and N_e are the concentration and the net area of the element's fluorescence peak and N_{BG} is the background area under the same fluorescence peak [12].

2.3. GF-AAS analysis and ICP-OES analysis

Graphite Furnace Atomic Absorption Spectrometry (GF-AAS) was chosen as reference method for the quantification of Cd and Pb and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) for Zn.

Table 1
Experimental parameters of the two S2 Picofox TXRF spectrometers used in this work.

Instrumental features	Mo-TXRF	W-TXRF
Anode	Mo	W
X-ray tube	Air-cooled metal ceramic	Air-cooled metal ceramic
Maximum power	40 W	50 W
Optics	Multilayer monochromator (17.5 keV)	Multilayer monochromator (35 keV)
Silicon drift detector	Area: 30 mm^2	Area: 30 mm^2
	FWHM: 139 eV (Mn-Kα)	FWHM: 147 eV (Mn-Kα)
Filter	Mo 10.0 μm	Ni 50.0 μm
Atmosphere	Air	Air
Voltage	50 kV	50 kV
Current	750 μA	1000 μA
Measuring time	1000 s	1000 s
Analytical lines	Zn-Kα: 8.637 keV	Zn-Kα: 8.637 keV
	Pb-Lα: 10.551 keV	Pb-Lα: 10.551 keV
	Cd-Lα: 3.133 keV	Cd-Kα: 23.173 keV

For GF-AAS analysis, a PinAAcle AA 900 T2 (Perkin-Elmer) spectrometer was used, equipped with hollow cathode lamps (HCL) of λ = 228.8 nm and 283.2 nm for Cd and Pb, respectively. In particular, guttation fluid samples were diluted 1:20 with distilled water for the analysis of Cd and Pb. Besides, Zn was quantified using a Spectro Arcos FH3 ICP-OES analyzer (Ametek) after a 1:500 sample dilution with distilled water.

2.4. Statistics

Student's *t*-test ($p < 0.05$) was applied to compare Mo-TXRF with W-TXRF results and both of them with GF-AAS and/or ICP-OES results.

3. Results and discussion

3.1. Optimization of sample deposition

Table 2 compares TXRF results and recovery values for Zn, Pb and Cd as obtained testing the two alternative sample depositions. A good accuracy was achieved in all the cases, with recoveries in the range 91–100 % for the IS+S sequence and 91–99 % for the S+IS.

However, the IS+S sequence showed higher precision than the S+IS strategy, as can be inferred by the smaller RSD values. In addition, the limits of detection (LoD) for Zn and Pb were 30 % lower in the case of the IS+S deposition strategy. For Cd, analysed with the W-TXRF, an improvement of 10 % in LoD was obtained with the S+IS strategy.

After verifying that the deposition strategy did not affect the results, the best LoD and accuracy were obtained using the IS+S deposition, therefore all the guttation fluid samples were prepared accordingly. Similarly, a testing composite sample of guttation fluid, obtained randomly collecting guttation drops from plants from Soil 1–4, was analysed with both Mo- and W-TXRF spectrometers and, additionally, with GF-AAS and ICP-OES, respectively, for validation (Table 3). The concentrations determined for Zn and Pb through TXRF analysis, once corrected according to recovery values (Table 2), were not significantly different using either Mo- or W-TXRF for $p < 0.05$ (*t*-test). Likewise, also Pb and Zn concentrations quantified by using GF-AAS and ICP-OES were in accordance with those obtained by TXRF ($p < 0.05$, *t*-test). Cadmium was identified and quantified only using the W-TXRF spectrometer by detecting the characteristic fluorescent signal at 23.173 keV (Cd-Kα), and the concentration obtained was in accordance with GF-AAS results ($p < 0.05$, *t*-test). Conversely, the high concentration of K (Table S1), whose Kα line (3.314 keV) overlaps Cd K-lines (3.133–3.316 keV), did not allow the detection of Cd and the determination of its LoD with the Mo-TXRF.

A slight increase of the LoD for Zn was observed for the guttation

Table 2
Zn , Pb and Cd concentration (C), standard deviation (SD), relative SD (RSD), recovery values and limit of detection (LoD) obtained for the standard reference solution (S) analysed with Mo-excitation (for Zn and Pb) and W-excitation (for Cd) TXRF spectrometers adopting the two different sample deposition strategies: IS+S or S+IS (IS: Y standard solution).

	IS+S					S+IS				
	C	*SD*	*RSD*	Recovery	*LoD*	*C*	*SD*	*RSD*	Recovery	*LoD*
Element	(μg L^{-1})	(μg L^{-1})	(%)	(%)	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(%)	(%)	(μg L^{-1})
Zn	914	13.6	1.5	91	0.8	909	35.6	3.9	91	1.3
Pb	933	24.5	2.6	93	0.6	952	31.1	3.3	95	0.9
Cd	1003	34.2	3.4	100	20.6	987	54.7	5.5	99	18.5

Table 3
Zn, Pb and Cd concentration (C), standard deviation (SD), relative SD (RSD), limit of detection (LoD) and recovery values for the guttation fluid sample (GS) analysed with a Mo- (for Zn and Pb) and W-TXRF spectrometer (for Cd) adopting the IS+S sample deposition strategy (IS: Y standard solution). The results of GF-AAS (Pb and Cd) and ICP-OES (Zn), performed on the same sample, are reported for validation.

	Mo – TXRF			W – TXRF			GF-AAS (Pb - Cd)/ICP-OES (Zn)		
	C	*SD*	*LoD*	*C*	*SD*	*LoD*	*C*	*EU*	*LoD**
	(μg L^{-1})			(μg L^{-1})			(μg L^{-1})		
Zn	31,811	1,400,399.7	2.7	31,515	661.8	24.4	34,000	6800	1735
Pb	3921	1.5	0.6	395	24.1	18.7	460	92	11
Cd	n.a.		n.a.	65.7	10.4	16.2	63	13	5

n.a.: not available; EU: expanded uncertainty, k = 2; * limit of detection after sample dilution (1:20 v/v for GF-AAS and 1:500 v/v for ICP-OES analysis).

fluid sample (GS) compared to the reference standard (Table 1). This is mainly attributable to the little higher average *Z* of the GS respect to the multielement standard solution, although both solutions gave very similar dry depositions (fluorescence self-absorption effects related to "coffee-ring" formation can be excluded). In fact, compared to the multielement standard solution, GS also contained organic compounds [11] and Zn concentration (~30,000 μg L^{-1}) was much higher. This can lead to an increase in the background signal and therefore an increment of the LoD.

3.2. TXRF analysis of plants' guttation fluids

The elements identified in guttation samples through the Mo-TXRF spectrometer were P, S, Cl, K, Ca, Mn, Fe, Ni, Cu, Zn, Br, Rb, Sr and Pb (Fig. 2, Table 1S). In addition to these elements, the W-TXRF spectrometer allowed also the identification of Cd, by means of the detection of Cd-Kα fluorescence signals, corresponding to the peak at 23.173 keV (Fig. 2). Indeed, using the Mo-TXRF, the energy of the characteristic X-rays of the anode material (Mo-Kα = 17.480 keV) is not sufficient to produce electronic transitions in K electron shells of Cd atoms. In fact, the Mo source can excite the K-lines of all the elements from P to Y (also Mg in particular cases) and the L-lines of elements heavier than Mo. Therefore, with a Mo-TXRF, Cd might be detected only through Cd L-lines (3.133–3.316 keV). However, such signals are often covered by K Kα lines (3.314 keV), being K much highly concentrated in the target (approximately 150 mg L^{-1}, Table 1S).

For both Mo- and W-TXRF spectra (Fig. 2), the Si peak (1.740 keV) is not attributable to the sample, but rather to the excitation of the quartz

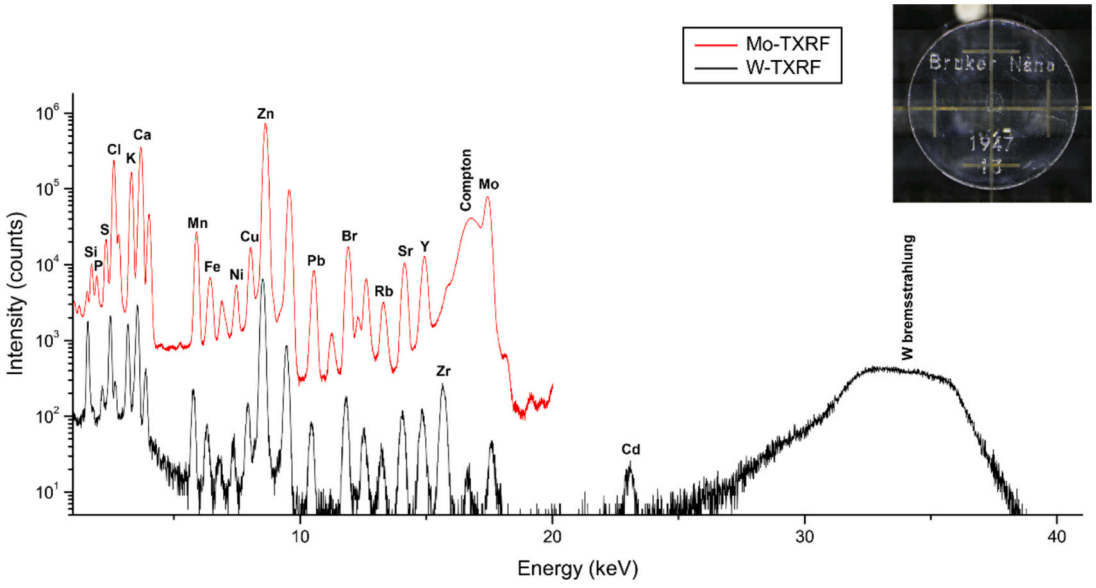

Fig. 2. Comparison of TXRF spectra of a guttation fluid sample (from plants grown in Soil 3) analysed using the Mo-TXRF spectrometer (red line) and the W-TXRF spectrometer (black line). A picture of the sample deposition is shown in the right-top corner of the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(SiO_2) sample carrier; similarly, the peak at 14.958 keV is attributable to X-ray emission occurring after Y Kα transitions, being Y here used as internal standard. Furthermore, in the W-TXRF spectrum the Zr peak (15.775 keV) is due to a detector artefact (the main component of the collimator in front of the detector window is Zr).

3.3. Analysis of Zn, Cd and Pb

In Table 4, the concentrations of Pb, Cd and Zn in guttation fluids sampled from annual ryegrass plants grown on the four soils and analysed with the two TXRF spectrometers and with GF-AAS/ICP-OES are shown.

Since the amount of sample was very low, not all the samples could be analysed for the three PTE of interest also with GF-AAS or ICP-OES. Therefore, guttation fluid from Soil 2 was tested for Pb, Cd and Zn while guttation fluids from Soil 3 and Soil 4 only for Cd and Zn. The guttation fluid collected from plants grown on Soil 1 was instead not enough to perform replicates with GF-AAS. This underlines the importance of having an analytical technique suitable for the analysis of microvolumes like TXRF for guttation fluid investigations.

The Pb concentration obtained analysing Soil 2 sample with GF-AAS is statistically equal to those obtained with both the TXRF spectrometers. In the case of Cd, W-TXRF and GF-AAS agreed for guttation samples from Soil 3 and 4, while for both techniques, Cd was below the limit of quantification for the fluid from Soil 2. As reported in the previous paragraph, elements whose Z > 42 (Mo), hence including Cd, are not detectable with Mo-TXRF through K-emission lines, but through L-lines. However, L-lines intensity is generally lower than K-lines; moreover, while for some “high-Z" elements (e.g. Pb) L-lines are efficiently excited by Mo source and do not overlap the fluorescence peaks of major elements of the sample, for elements such as Cd L-lines are most commonly hindered by more intense K-signals of elements typically more concentrated organic fluids, such as K and Ca. Thus, Cd is practically undetectable with Mo-TXRF spectrometers in plant saps using a Mo anode-based X-ray source. This is a crucial aspect since Cd is a rather diffused and mobile soil pollutant [18], and its detection in plant fluids is therefore of primary importance when studying the bioavailability of PTE in soil.

Zinc concentrations obtained with Mo- and W-TXRF spectrometers agree with ICP-OES results (where available), but they are significantly different one to the other ($p < 0.05$, *t*-test), in the case of fluids from Soil 2 and Soil 3. As visible in Fig. 2, for both spectrometers the TXRF spectra show an increase of the background in the Zn K-lines (8.639–9.572 keV) energy region due to the scattering caused by the high concentration of the Zn. Moreover, in the case of Mo-TXRF spectrometer, a strong tailing of the peak is observed causing a left asymmetry of the Zn peak. This variability in the background shape and in the profile of the Zn peaks can lead to a slight fluctuation of the quantification between Mo- and W-TXRF. This does not affect the agreement of the results with the reference methods (Table 3) but it seems to influence the agreement between the two TXRF spectrometers. In conclusion, TXRF analysis can provide the same results given by either GF-AAS or ICP-OES.

Table 4
Comparison between Pb, Cd and Zn concentration in plants' guttation fluid samples as obtained with Mo-TXRF and W-TXRF spectrometers. GF-AAS and ICP-OES results, where available, are reported, too.

		Mo -TXRF		W-TXRF		GF-AAS (Pb - Cd) / ICP-OES (Zn)	
		C	*SD*	*C*	*SD*	*C*	*EU*
		(μg L^{-1})		(μg L^{-1})		(μg L^{-1})	
Pb	Soil 1	248	13.3	213.	24.2	n.a.	
	Soil 2	138	14.9	146	13.3	148	30
	Soil 3	1506*	53.5	1705*	26.6	n.a.	
	Soil 4	334	2.5	296	28.9	n.a.	
Cd	Soil 1	n.a.		68	14.5	n.a.	
	Soil 2	n.a.		<LOD		<LOD	
	Soil 3	n.a.		300	14.5	248	50
	Soil 4	n.a.		101	32.5	76	15
Zn	Soil 1	57,462	3901.0	55,594	1664.7	n.a.	
	Soil 2	15138*	87.1	11530*	250.8	13,000	2600
	Soil 3	78401*	1901.6	84229*	2573.4	69,500	13,900
	Soil 4	25,842	2346.0	25,859	949.6	29,000	6000

n.a.: not available; EU: expanded uncertainty, k = 2; *significantly different for $p < 0.05$ (t-test).

The LoD for Zn with ICP-OES is 1735 μg L^{-1}, while the ones for Pb and Cd with GF-AAS are 11 and 5 μg L^{-1}, respectively. It should be noted that, as the samples had to be diluted (1:20 *v*/v for GF-AAS, 1:500 v/v for ICP-OES), the limit of detection increased noticeably for such techniques. Differently, using Mo-TXRF LoDs are greatly lower: LoD for Zn is more than six-hundred times lower than the ICP-OES one (i.e. 2.7 vs 1735 μg L^{-1}), while the LoD for Pb is about one eighteenth of the one obtained with GF-AAS (i.e. 0.6 vs 11 μg L^{-1}). Mo-TXRF LoDs for Zn and Pb are the lower than W-TXRF ones, which are approximately tenfold and thirty times higher, respectively. However, this difference in performances is well known in the literature [19–21]. In fact, the excitation energy of Mo (Kα = 17.479 keV) is closer to the energies of the fluorescence lines used for the detection and quantification of Zn and Pb (Zn-Kα = 8.639 keV; Pb-Lα = 10.552 keV), which results in a higher sensitivity than using W-bremsstrahlung excitation (35 keV). Contrariwise, the LoD for Cd was lower using W-TXRF, precisely because the higher energies of W-bremsstrahlung allowed to use Cd -Kα as analytical lines instead of Cd-Lα (used with Mo-TXRF with the drawbacks connected with the fluorescence emission of light elements described before). Even if the LoD obtained using W-TXRF for Cd (16.2 μg L^{-1}) is much higher than that of GF-AAS (5 μg L^{-1}), the techniques gave similar results in the quantification of this element in three guttation fluids. In the case of Soil 2 sample, for both techniques Cd concentration was below the LoD.

Based on the results described so far, and considering the overall procedure for the preparation and analysis of guttation fluids with TXRF spectrometry (both using Mo and W sources), the pros in using TXRF are quite evident in comparison to the other two reference methods adopted. By using only 10 μL of sample, TXRF provided a multi-elemental characterization of the guttation fluids (from S to Cd), as can be observed in the two spectra of Fig. 2. This implies that the overall amount of sample required, also considering replicates, remains altogether in the order of a few tens of microliters, which is a volume likely to be collected from herbaceous plants, also in the field. Differently, with GF-AAS, 10 μL are required for the analysis of each single element, thus at least 30 μL per element in the case of the direct analysis of three replicates of GS. Although the dilution of the sample would help in the increase of the sample volume, for less-concentrated analytes there would be the risk not to reach the detection limit of the technique. As a result, in the case of limited amounts of sample, this spectroscopic approach is not always suitable, especially when a several elements must be quantified (e.g. multiple PTE and mineral nutrient elements). For example, it was not possible to measure Cd in guttation fluids from Soil 1 with GF-AAS (as long as Pb in fluids sampled from plants grown on Soil 3 and Soil 4) right due to sample scantiness.

On the other hand, ICP-OES is a multi-elemental technique but requires larger volumes (generally ≥5 mL) of sample. For this reason, the dilution of the sample is often needed to reach adequate quantities, but this can compromise the analysis in case the concentrations of the analytes of interest are low. In other cases, multiple dilutions are required due to very-high concentrations of certain elements, as in this study for Zn. Conversely, TXRF simultaneously provided reliable results in a range of concentrations of various orders of magnitude (10^2 to 10^5 μg L^{-1}), with a single internal standard addition [12]. Also compared with ICP-OES, the advantages of TXRF for the analysis of such small sample volumes are quite evident and underline the validity of this technique for the analysis of guttation fluids. At last, ICP-MS analyses would be much more expensive in terms of equipment procurement and maintenance, as well as operator's expertise needed.

3.4. Other implications

The TXRF results obtained through this study denoted a certain variability in Zn, Cd and Pb concentration among guttation fluids collected from plants grown in differently treated soils. The lowest concentrations were observed in the fluids from Soil 2. Higher levels of these PTE were found from plants grown on Soil 3, even exceeding those found for Soil 1 for Pb and Cd (Table 4). In the case of Soil 2, this can be ascribed to the soil application of biochar, which has well-known properties of PTE adsorption, thus reducing pollutants mobility in soils and, hence, their availability for the plant [22]. In fact, biochar is often used for soil remediation purposes. Conversely, the treatment with PGPR of Soil 3 can explain the high values of the concentrations recorded, even higher than in the control soil for Cd and Pb, higher than in the biochar treated soil, but lower than the untreated soil in the case of Zn. Indeed, besides promoting the mobilization of beneficial plant nutrients from soil, PGPR can also increase the mobility and availability of undesired PTE [23–25]. Finally, in the guttation fluids of plants grown on Soil 4, a level of PTE intermediate between Soil 2 and 3 was found, which can be explained as a partial mitigation, provided by the biochar, of the increase of metal mobility triggered by PGPR. An in-depth investigation of the mechanisms acting in the plant-soil system after the application of different management strategies to the studied multi-contaminated soil is out of the aims of this work. Nonetheless, these findings underline the potential of such TXRF based-approach for the assessment of the actual PTE availability in polluted soils.

It is also noteworthy that, along with PTE, TXRF analysis revealed the presence in the guttation fluids of many mineral elements required for plant nutrition. Indeed, both macronutrients (P, S, K, Ca) and micronutrients were identified, including those potentially toxic at high concentrations (Mn, Fe, Cu, Zn, Ni), as well as other non-essential elements such as Br and Sr (Fig. 2, Table S1). This implies that the TXRF approach described so far is not only adequate for the assessment of inorganic contaminants in plant fluids but can provide at the same time useful information on the nutrients uptake from the soil and their translocation from roots to shoots.

4. Conclusions

This paper demonstrates the suitability and reliability of TXRF spectrometry for the determination of three prominent potentially toxic elements (i.e. Zn, Cd, and Pb) in plant's guttation fluids. The composition of the fluids, showing minimal dry residues, allowed the direct analysis of the fluids following a simple two-step sample deposition procedure (deposition of 10 μL of Y internal standard solution followed by the deposition of 10 μL of guttation fluid sample). The accuracy of the method was ascertained by analysing a multielement standard, and the comparison with the results from GF-AAS and ICP-OES techniques allowed method validation for the analysis of guttation fluids. TXRF showed significant advantages in the analysis of guttation fluids: i) differently from GF-AAS and ICP-OES, whose application requires the selection of the elements of interest, TXRF has simultaneous multi-elemental capability; ii) TXRF is a micro-analytical technique requiring a minimal sample preparation, similarly to GF-AAS. However, differently from this latter, the combined multi-elemental capability of TXRF allows the triplicate analysis of the fluids for several elements using only 30 μL sample. Nevertheless, the TXRF spectrometer configuration is an important aspect to consider, influencing the analytical performances. In fact, due to the high K concentration in guttation fluids, Cd detection and quantification was possible only by using the W-TXRF while the Mo-TXRF showed the best LoDs for Zn and Pb (more than ten times lower than W-TXRF). Nowadays, instruments equipped with both Mo- and W- anode-based X-ray tubes are commercially available.

Finally, the results of the present study suggested that PTE (e.g. Zn, Cd, and Pb) concentration in guttation fluids could be related to their bioavailability in soil. All these considerations support the hypothesis that TXRF analysis of plant guttation fluids can represent a first step for a novel non-destructive, simple, expeditious and "green" analytical approach for the assessment of the availability Zn, Cd and Pb and other PTE in polluted soils, also able to provide information about the nutritional status of plants and crops.

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CRediT authorship contribution statement

Carlo Porfido: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ignazio Allegretta:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation. **Eva Marguí:** Writing – review & editing, Investigation, Data curation. **Matteo Garau:** Writing – review & editing, Investigation. **Maria Vittoria Pinna:** Writing – review & editing, Investigation. **Concetta Eliana Gattullo:** Writing – review & editing. **Roberto Terzano:** Writing – review & editing, Supervision. **Matteo Spagnuolo:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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