VORTEX ASSISTED DISPERSIVE LIQUID-LIQUID MICROEXTRACTION FOR DETERMINATION OF MOLYBDENUM IN PLANTS BY INDUCTIVELY COUPLED PLASMA OPTICAL EMISSION SPECTROMETRY

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ABSTRACT

A new procedure for determining trace concentrations of Mo in plants combining dispersive liquid-liquid microextraction and inductively coupled plasma optical emission spectrometry is here proposed. An automated discrete sample introduction system using a Flow Blurring® multiple nebulizer (FBMN) and a solenoid valve were used to insert the organic rich phase into the plasma. The experimental conditions for the microextraction procedure were: 0.5% m v^{-1} of 8-hydroxyquinoline, pH 3.6 and 50 µL of 1-undecanol as extractant. A limit of detection of the instrument of 0.20 µg L^{-1}, a limit of detection of the procedure of 17 µg kg^{-1} and an enhancement factor of 246 were obtained employing the developed procedure. Three certified reference materials were used to check accuracy and no significant differences were found at a 95% confidence level between certified and determined values. The developed procedure was also successfully applied for determination of Mo in three different varieties of sugar cane leaves samples.

KEYWORDS: Foliar analysis; Micronutrient; Dispersive liquid-liquid microextraction; Green analytical chemistry; Flow injection analysis; *Flow Blurring*® multiple nebulizer.

1. Introduction

Molybdenum is an important micronutrient for plants acting on the fixation of atmospheric nitrogen by bacteria which promotes the synthesis of new proteins.[1,2] Determination of Mo in plants is of utmost importance because trace quantities of Mo are required to perform vital functions, however, concentrations greater than 5 μg g^{-1} can be potentially toxic and led to the death of the plant.[3]

Spectrometric techniques such as flame (FAAS) and electrothermal atomic absorption spectrometry (ETAAS), inductively coupled plasma optical emission spectrometry (ICP-OES) or mass spectrometry (ICP-MS) have been applied for a variety of samples for determination of Mo. Usually plant samples are acid digested prior to measurements.[4] Despite the fact that high sensitivity is achieved, some spectrometric techniques require an extraction step due to extremely low analyte concentrations and high matrix contents in the sample digests.[3,5,6]

In the past, liquid-liquid extraction (LLE) was extensively applied for separation and preconcentration of trace concentrations of metal ions, however, these procedures involved the use of high volumes of toxic and expensive organic solvents.[7] Nowadays, green procedures have gained attention in chemistry, and the miniaturization of the LLE has become a trend in modern analytical chemistry.[8] In this context, microextraction procedures are attractive due to its low consumption of organic solvents, which is an important aspect for green analytical chemistry procedures.

Dispersive liquid-liquid microextraction (DLLME) has been used for preconcentrating Mo in several samples.[5,9–11] The principle of the procedure is based on the extraction of the metal-complex into a small volume of an organic solvent. Complexing agent and buffer solution are added to an aliquot of the digested sample,

and complexation occurs at a fixed pH value. Conventionally, an appropriate mixture of extraction and dispersion solvents is rapidly injected into an aqueous solution, resulting in a cloudy emulsion consisting of tiny droplets of the extraction solvent dispersed in the aqueous sample. The large contact surface area between aqueous and organic phases leads to the establishment of a fast chemical equilibrium. As a result, analytes are transferred into the organic droplets. Afterwards, the cloudy emulsion is centrifuged to achieve phase separation. Finally, the analyte rich phase is removed and analyzed for determination of analytes by an appropriate instrumental technique.

In order to enhance the extractant phase dispersion, vortex-assisted DLLME has been introduced by Yiantzi *et al.*,[12] The use of vortex agitation to disrupt the extractant phase reduces the consumption of organic solvents, because the use of a third component (*i.e.*, disperser solvent) is not needed.

Several atomic spectrometric techniques have been combined with DLLME for Mo determination. Some techniques such as ETAAS[13] and LIBS[9] require a very low amount of sample for analysis.

Flame AAS is widely used as a simple and inexpensive technique.[5] A strategy based on the use of vortex assisted solidified organic drop (VA-SOFDME) microextraction combined with FAAS using discrete nebulization was proposed by Oviedo *et al*.[5] for the determination of Mo in corn roots and leaves. The use of discrete injection for Mo determinations in various samples was further investigated by Oviedo *et al*.,[14] and it was demonstrated that this procedure is sensitive and efficient for the determination of Mo in situations in which low sample volume is available and the sample consumption by the chosen determination technique is elevated.

In contrast, few papers proposed the combination of DLLME and ICP-OES, probably because of negative effects of organic matrices on argon plasma.[15] Moreover, after the DLLME procedure, the low quantity of organic extract is usually dissolved in another miscible organic solvent[5,16–18] because low sample volume is not compatible with conventional liquid sample introduction by pneumatic nebulization in ICP-OES. Depending on the added miscible organic solvent volume, this step might deteriorate the enhancement factor. In order to address these problems, a new multinebulizer based on *Flow Blurring*® technology[19] and a solenoid valve have been employed. The new multinebulizer has been adapted to analytical applications as a liquid sample introduction system (*i.e.*, *Flow Blurring*® multiple nebulizer (FBMN)-based system). The configuration of the multinebulizer allows the simultaneous introduction of the solutions (*i.e.*, sample, reagents, diluents, *etc*.) by distinct and independent channels and gas through a common orifice. This new and high efficiency nebulization system has been previously used for correction and compensation of matrix effects[20,21] and inorganic acid interferences.[22]

Another quite interesting feature of the FBMN-based system is the possibility of introducing samples with high organic contents into the plasma without using oxygen as auxiliary oxidant.[23] A more effective combustion of the organic samples was achieved when aqueous solutions were simultaneously introduced through a different nebulizer channel. Therefore, the amount of carbon residue deposited on the injector tip and torch was significantly reduced.[23]

On the other hand, the introduction of a low volume of analyte rich phase obtained in the DLLME can be achieved using discrete sample introduction with a solenoid valve. This strategy allows the introduction of smaller volumes of sample into the plasma without negatively affect the figures of merit.[5]

Considering the recent advances in the field of liquid-liquid microextraction and the capability of the multinebulizer system, we propose here the use of the DLLME combined with the innovative FBMN-based system for determination of Mo in plant samples using ICP-OES. In this work we evaluate the possibility of introducing the rich organic phase directly into the plasma without dilution, aiming at higher enhancement factor and lower limit of detection (LOD). We also combine the FBMN-based system with a solenoid valve to facilitate solution handling in an automated discrete sample introduction system.

2. Material and methods

2.1. Instrumentation

All measurements were performed with an Agilent 720-ES inductively coupled plasma optical emission spectrometer (Melbourne, Australia). A two liquid channels FBMN was operated in a commercial cyclonic-type spray chamber (Model Tracy, Glass Expansion Ptr. Ltd., Melbourne, Australia) having a 50 mL internal volume. This association is called FBMN-based system. The operational parameters of the ICP-OES are shown in Table 1.

Table 1. Operating conditions of the ICP-OES.

RF applied power (kW)	1.2
Argon gas flow rate (L min^{-1})	
Plasma gas	15
Auxiliary gas	1.5
Nebulizer gas	0.75
Organic extract uptake rate (µL min^{-1})	45
Nitric acid solution uptake rate (µL min^{-1})	190
Viewing mode	Axial
Analytical emission line (nm)	Mo I (281.615)

2.2. Reagents and analytical reference solutions

All reagents used were of analytical grade. Solutions were prepared using ultrapure water with resistivity of 18.2 MΩ cm from a Milli-Q purification system (Millipak-40 Filter Unit 0.22 µm NPT, Bedford, MA, USA). To minimize contaminations all laboratory glassware and polypropylene flasks were kept in 10% v v^{-1} nitric acid solution for 24 h and then washed with ultrapure water before use.

Concentrated high purity grade nitric acid was obtained from Merck (Darmstadt, Germany). Analytical reference solutions were prepared by appropriate dilutions of a stock solution of Mo(VI) 1000 mg L^{-1} (High Purity Standards, Charleston, SC, USA). The L(+)-ascorbic acid was purchased from Merck. Complexing agent (8-hydroxyquinoline (8-HQ), Sigma-Aldrich, Saint Louis, MO, USA) solution of 16.5 % m v^{-1} was prepared daily by dissolving the appropriate amount of reagent in ethanol 99.5% v v^{-1} (Merck) and stored in a brown glass bottle. Acetate buffer was prepared by

dissolving the appropriate amount of sodium acetate (Panreac Químicas S.A., Castellar del Vallès, Spain), and the pH was adjusted to 3.6 by adding aliquots of HNO_3 and/or NaOH (Scharlau, Barcelona, Spain) solutions. Extracting solvent (1-undecanol, 99% v v^{-1}) was purchased from Sigma-Aldrich. The accuracy of the developed procedure was evaluated with three certified reference materials: rice flour NIST 1568a, corn bran NIST-8433 and apple leaves NIST-1515 (National Institute of Standards and Technology, Gaithersburg, MD, USA). Three samples of sugar cane leaves were used to assess the applicability of the developed procedure. Forty sugar cane leaves were randomly collected from each sample. The central nervure of the leaves was removed and discarded, then the samples were washed with plenty deionized water, dried at 65 ºC for 72 h in a forced air oven. Samples were ground in a cutting mill equipped with a 20-mesh sieve and stored in polyethylene flasks.

2.3. Microwave-assisted sample digestion

Plant samples were microwave-assisted acid-digested using an Ethos 1 microwave oven (Milestone, Sorisole, Italy). Sample masses of 500 mg were microwave-assisted digested using 6 mL of HNO_3 solution 7.0 mol L^{-1} plus 2 mL of H_2O_2 30 % m m^{-1} (Panreac). The heating program was applied in two steps: (1) 15 min to reach 200 ºC and (2) 15 min at 200 ºC, and an additional 15 min cooling step. A maximum 1.5 kW of microwave power was applied. After completing the digestion and cooling down steps, the digests were transferred to 50 mL conical tubes and 5 mL of NaOH 3.5 mol L^{-1} along with 3 mL of acetate buffer were added before final dilution to 30.0 mL. The pH values of all digests were measured and they were around 3.6 ± 0.1.

2.4. Liquid-liquid microextraction procedure

A 15 mL aliquot of digested sample was added to a glass tube plus 0.0825 g of ascorbic acid, 1 mL of acetate buffer and 0.5 mL of 8-HQ solution aiming at a complexing agent final concentration of 0.5% m v^{-1}. Solutions were shaken manually and left at room temperature for 10 min, allowing the complex formation between Mo-8-HQ. Then, 50 µL of 1-undecanol was added to the mixture and shaken using vortex by 2 min. The solution was centrifuged at 4000 rpm for 8 min to separate the two phases, with the organic phase containing the analytes at the top. The organic extract was collected from the glass tube directly by the tube of the flow system. The microextraction procedures applied here were previously optimized by Jesus *et al.*,[9] However, the 8-HQ concentration was increased from 0.1 to 0.5 % m v^{-1} in order to guarantee an excess of complexing agent in digests of plant samples.

2.5. Experimental setup for extract injection

A solenoid valve controlled the injection of the extract (NResearch, 161T031, West Caldwell, NJ, USA). The solenoid valve control was implemented using a lab made interface programmable via USB. An ATMEGA P328 microcontroller was used to execute the program and ULN2803 integrated circuit to control the output ports. To control the solenoid valve a program was written using Arduino® language. The code for the developed software is displayed in the Supplementary Material (Table S1).

A representation of the experimental setup for introduction of the extract is shown in Figure 1. Two different types of propulsion tubes were used depending on the sample: (i) for organic extract (S), a propulsion tube compatible with most organic-based solvents (F-4040-A, id. 0.25 mm, Ismatec, Switzerland) was employed; and (ii)

for aqueous solution of nitric acid (1% v v^{-1}) (A), a Tygon® propulsion tube (R-3607, id. 0.51 mm, Ismatec, Switzerland) were used. A Teflon® tube (length 25 cm, i.d. 0.5 mm, UpChurch Scientific, Oak Harbor, WA, USA) was used for the analytical path (L).

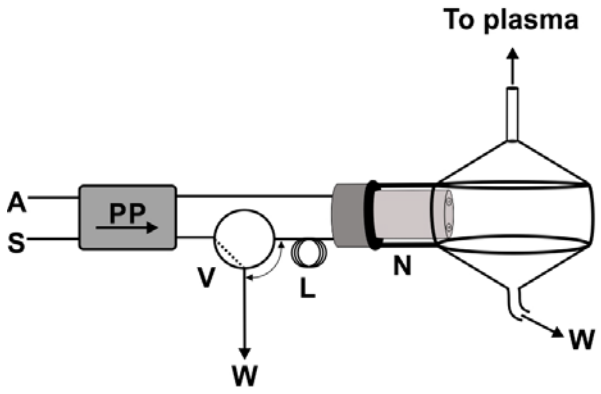

Figure 1 – Flow analysis module developed for the determination of Mo in plant samples. PP – Peristaltic pump; V – Solenoid valve; L – Analytical path; N – FBMN. A – Nitric acid aqueous solution (1 % v v^{-1}); S – Organic extract; W – Waste.

The program for controlling the solenoid valve was implemented in four steps. Initially the valve was switched to the sampling position, for a period of 110 s (Step 1) to load the organic extract into the analytical path (L). The organic extract introduction takes approximately 1 min and during the rest of the time (*i.e.*, 50 s) 1-undecanol was introduced as carrier solvent. Then, the valve was switched to the waste position for 10 s (Step 2). The discrete extract injection was executed 6 times (6 cycles). In each cycle, the solenoid valve was first switched to the sampling position, and the 1-undecanol carried the extract towards the spray chamber for a period of 10 s (Step 3), and between injections, a 20 s cleaning step was used (Step 4). It is also important to mention that during the whole discrete injection procedure the continuous nebulization

of the HNO_3 solution was necessary to clean the spray chamber, and also helped to prevent the deposition of carbon residues on the quartz torch.

All measurements were based on peak area. Figures 2-A and 2-B presented the transient signal obtained for injections of 30 µg L^{-1} preconcentrated standard and apple leaves digest (NIST-1515), respectively.

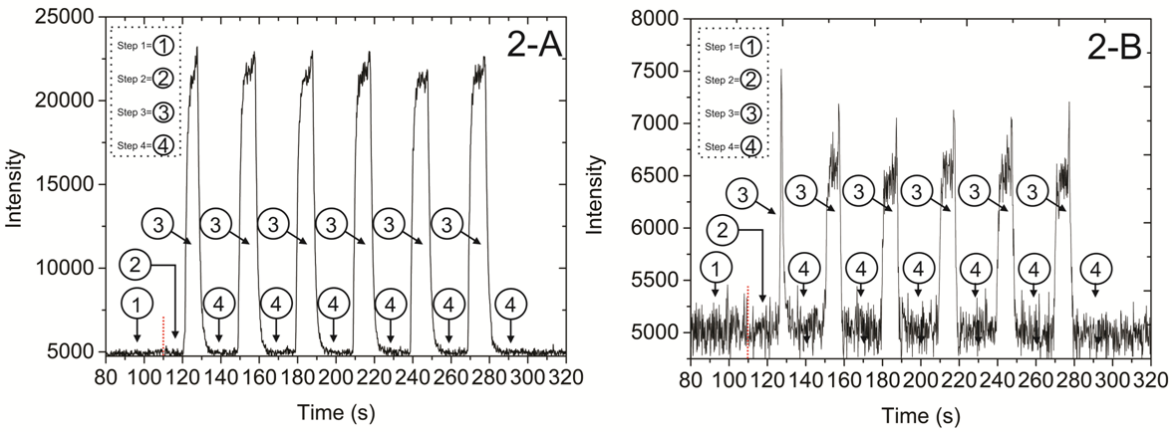

Figure 2 – Transient signals for injections of 30 µg L^{-1} preconcentrated standard (2-A) and apple leaves digest (NIST-1515) (2-B), illustrating the sequence of the solenoid valve control program.

As it can be seen in Figures 2-A and 2-B it is noticeable that the first peak from both signal registers have smaller half width than the subsequent peaks. This trend was observed in all experiments since the organic extract front is recessed from the exit of the analytical path. This makes that the first injection carries a slightly smaller volume of the extract compared to subsequent injections. This effect is more pronounced in Figure 2-B due to the low concentration of Mo in the apple leaves digest. Thus, the integrated area of the first peak was not considered for any calculation in this work, and all calculations were based on the 5 subsequent peaks (n = 5).

It is important to highlight some facts related to sample throughput. Considering the sample preparation procedure, the heating program took a total of 30 min with and additional cooling step of 15 min. The microextraction procedure required a total time of 20 min to obtain the organic extract and, finally, a total of 5 min was required to obtain transient signals. Hence, a total analysis time of 70 min is needed. Just for comparison purpose, the total analysis time is 50 min without using the DLLME step. Finally, it should be borne in mind that ten samples could be simultaneous digested and they could be extracted at the same time, therefore, a throughput of 5 samples per hour could be analyzed using DLLME-ICP-OES.

2.6. Addition of a reducing agent

During the microextraction procedure was observed the formation of a gelatinous reddish-brown precipitate after the addition of 8-HQ and buffer solutions to the sample digests. This behavior was observed with sugar cane leaves samples and with the apple leaves standard reference material (NIST-1515). Formation of precipitate made impossible complete separation of the organic droplet. Taking into account the color of the precipitate and the high concentration of Fe in samples and reference material, one hypothesis to explain this behavior is the formation of insoluble Fe hydroxides. Iron concentrations in sugar cane leaves samples were previously determined by ICP-OES and the found values were in the 115 - 190 mg kg^{-1} range. Due to the high stability constants, Fe(III) hydroxides are formed in higher concentrations than Fe(II) hydroxides and, furthermore, their dimers and trimmers are more stable.[24] In addition, co-precipitation of Al(III) hydroxides and other insoluble species could arise. Thus, the microextraction procedure here involved a reduction step before the microextraction in order to reduce Fe(III) to Fe(II). Ascorbic acid was chosen as

reducing agent and optimization was performed to determine the optimal concentration of reductant. Three concentrations of reducing agent were studied: 0.1; 0.5 and 1% m v^{-1}. After the addition of ascorbic acid, buffer and complexing agent solutions, a visual inspection of the mixture was done to evaluate if a precipitate would be formed. A 0.5% m v^{-1} ascorbic acid solution was efficient to prevent the formation of precipitate and this concentration was selected for further experiments.

3. Results and discussion

3.1. Figures of merit

The performance for the developed procedure was evaluated using Mo(VI) aqueous reference solutions. Table 2 shows the figures of merit obtained for the DLLME-ICP-OES developed procedure. For comparison purposes, figures of merit obtained when the automated flow analysis system was coupled to the ICP-OES and aqueous solutions were directly introduced, are also shown. The limits of detection and quantification were calculated by $3S_b/m$ and $10S_b/m$, respectively, where S_b is the standard deviation from 10 blank measurements and m is the slope of the calibration curve. The limit of detection of the procedure ($LOD_{procedure}$) was 17 µg kg^{-1} for 10 measurements of digestion blanks. The relative standard deviations were 4.3 and 2.7% (n = 5) for solutions containing 0.90 and 50 µg L^{-1} Mo, respectively. It is noticeable that the use of DLLME significantly improved the sensitivity. A 246-fold enhancement factor was calculated as the ratio of sensitivities obtained with and without DLLME procedure. The high enhancement factor achieved is due to the fact that the organic extract was introduced into the plasma without an additional dilution step as needed in several procedures previously described in the literature[5,10,16]. However, it is important

to highlight that the organic extract is diluted in the aerosol phase inside the spray chamber, since the HNO_3 solution is continuously introduced.

Table 2. Figures of merit of DLLME-ICP-OES and ICP-OES.

Parameter	DLLME-ICP-OES	ICP-OES
Linear working range (μg L^{-1})[a]	0.90-50	50-500
Correlation linear coefficient[a]	0.9978	0.9959
LOD (μg L^{-1})	0.20	8
LOQ (μg L^{-1})	0.65	26
Sensitivity (cps L μg^{-1})	5179 ± 98	21.0 ± 0.7
Relative sensitivity[b]	246	
Relative LOD[c]	40	

[a]Number of calibration points = 6.
[b]Sensitivity DLLME-ICP-OES/ Sensitivity ICP-OES.
[c]LOD ICP-OES/LOD DLLME-ICP-OES

As can be observed in Table 2, the relative LOD value obtained was not enhanced by the same extent to the corresponding enhancement factor (*i.e.*, relative sensitivity). Since LOD value depends on both sensitivity and standard deviation of the blank signal, the comparatively high LOD obtained in DLLME-ICP-OES can be mainly attributed to the increment of the standard deviation of the blank signal when the organic extract was introduced. In fact, the standard deviation of the blank signal for DLLME-ICP-OES was 6 times higher than that obtained for ICP-OES.

A comparison among the figures of merit obtained in this work and the previously reported procedures for the determination of Mo in plant samples is shown in Table 3. The limit of detection in our work is comparable to the one obtained by Belatto *et al.*[16], which determined Mo in plants using CPE and ICP-MS.

Using the automatic sample introduction system, the RSD values obtained for analytical solutions ranged from 1.4 to 4.7 %. A higher range of RSD values (6.0 to 14.5%) was obtained by Oviedo *et al.*,[5] which used manual discrete sample introduction. Thus, the use of an automatic sample introduction system improved the precision of the developed procedure.

The $LOD_{procedure}$ obtained in our work is comparable to the one obtained by Oliveira et al.[25], but it is important to mention that the extraction procedure carried out by these authors consumed large volumes of concentrated NH_4SCN and $SnCl_2$ solutions, and methyl isobutyl ketone which can be potentially toxic to the analyst with repetitive exposure. The main advantage of the microextraction procedure is to achieve similar performance consuming less organic solvents and hazardous substances.

The combination of SPE and ICP-OES proposed by Azeredo *et al.*[26] led to lower LOD (0.001µg L^{-1}) for determining Mo in plant samples. This may be related with the high sample consumption (*i.e.*, 1 L min^{-1}) of the liquid sample introduction system used (*i.e.*, ultrasonic nebulizer). However, the performance of the SPE procedure relies on the preparation of the absorbent, which might be a time consuming and laborious procedure.

The combination of LLE and LLME for determining Mo in plants using fiber optics-linear array detection spectrophotometry (FO-LADS) and UV-Vis spectrophotometry was also reported by Gharehbaghi and Shemirani[10] and Ghiasvand *et al.*[11], respectively. Even though these procedures do not require expensive instrumentation, they are prone to interferences from matrix components and have numerous steps. In addition, despite its low cost the combination of spectrophotometry and extraction/microextraction strategies did not provide higher sensitivity and enhancement factor for Mo determination in plants such as the procedure here proposed.

Table 3. Comparison of figures of merit for the determination of Mo in plants

Extraction procedure	Detection	LOD ($\mu g\ L^{-1}$)	$LOD_{procedure}$ ($\mu g\ kg^{-1}$)	Relative sensitivity	Reference
Dispersive liquid-liquid microextraction	ICP-OES	0.2	17	246[c]	This work
Vortex assisted solidified organic drop microextraction	FAAS	4.9	680	67[c]	5
Liquid–liquid extraction	HR-CS FAAS[a]	20	16	-	25
Cloud point extraction	ICP-MS	0.8[e]	-	70[c]	16
Solid phase extraction	ICP-OES	0.001	-	-	26
Dispersive liquid-liquid microextraction	FO-LADS[b]	1.43	-	72.6[c]	10
Homogeneous liquid-liquid extraction	UV-Vis spectrophotometry	-	-	125[d]	11

[a]High-resolution continuum source flame atomic absorption spectrometry.
[b]Fiber optics-linear array detection spectrophotometry.
[c]Obtained as the ratio of the sensitivities of the calibration curves.
[d]Obtained with the ratio of the analyte in the sedimented phase and in the initial sample solution.
[e]Limit of detection in $\mu g\ kg^{-1}$ based on blank uncertainties and calibration curves from both isotopes (^{98}Mo and ^{95}Mo).

3.2. Determination of Mo in plant materials

To assess the accuracy of the developed procedure, Mo was determined in three certified reference materials (Table 4). According to a t-test, the determined concentrations were in agreement with the certified values at a 95% confidence level. Recoveries ranged between 98 – 102 % and the confidence intervals found were in the range of 0.017 – 0.14 mg kg^{-1}. The procedure was applied for the determination of Mo in three samples of sugar cane leaves (Table 4). A range of concentrations of 0.12 – 0.41 mg kg^{-1} were found and the confidence intervals ranged from 0.08 – 0.09 mg kg^{-1}. Sugar cane leaves samples used in this work were part of an experiment aiming genetic improvement and development of new plant specimens.

Table 4. Determination of Mo in plant materials.

Sample	Found (mg kg^{-1})[a]	Certified (mg kg^{-1})[a]	Recovery (%)[b]
Corn bran NIST-8433	0.248 ± 0.021	0.252 ± 0.039	98 ± 17
Rice flour NIST-1568a	1.42 ± 0.14	1.45 ± 0.08	99 ± 11
Apple leaves NIST-1515	0.096 ± 0.017	0.094 ± 0.013	102 ± 24
Sugar cane leaves #1	0.12 ± 0.08	-	-
Sugar cane leaves #2	0.27 ± 0.08	-	-
Sugar cane leaves #3	0.41 ± 0.09	-	-

[a]Mean ± confidence interval at 95%.
[b]Recovery ± combined standard uncertainty.

4. Conclusions

As demonstrated here, DLLME proved to be a valuable tool for the separation and pre-concentration of Mo in plant samples. The use of the new automatic system combined with the FBMN-based system for insertion of extracts into the ICP-OES instrument proved to be efficient for the introduction of the organic analyte-rich phase without any additional dilution step, consuming low extract volume, improving precision and decreasing the amount of waste generated. The addition of ascorbic acid to the samples prior to the microextraction procedure was important for avoiding the formation of insoluble hydroxides. Finally, the developed procedure is applicable for accurate determination of trace concentrations of Mo in plant samples by ICP-OES.

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