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Short Report

# Ultrasound-assisted Extraction of Ca, K and Mg from *In Vitro* Citrus Culture

***Sandra C. C. Arruda[a], Adriana P. M. Rodriguez[a] and Marco A. Z. Arruda*[*,b]***

[a] *Laboratório de Biotecnologia Vegetal, Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, CP 96, 13400-970 Piracicaba - SP, Brazil*

[b] *Instituto de Química, Universidade Estadual de Campinas, CP 6154, 13084-971 Campinas - SP, Brazil*

Apresenta-se um procedimento para extração de Ca, K e Mg de amostras de cultivo *in vitro* de plantas, comparando-se culturas de calo com diferentes potenciais embriogênicos de C*itrus sinensis* e *Citrus limonia*, com o emprego de energia ultra-som. Foram investigados os parâmetros relacionados com a extração de metais, tais como tipo de amostragem do material, concentração do ácido e tempo de sonicação. Visando a comprovação da exatidão, o procedimento de extração ultra-sônica foi comparado com o de digestão ácida assistida por microondas, e não foram observadas diferenças estatísticas significativas em um nível de confiança de 95%. Com este procedimento de extração simples e preciso, foi possível estabelecer diferenças nas concentrações de Ca, K e Mg durante a formação/desenvolvimento dos embriões de *Citrus*, bem como entre as culturas (embriogênicas e não-embriogênicas). Finalmente, a extração ultra-sônica demonstrou-se uma excelente alternativa para menor manipulação das amostras e redução de custos operacionais.

An ultrasound extraction procedure for Ca, K and Mg from *in vitro* plant cultures is proposed, comparing cultures of different embryogenic levels of *Citrus sinensis* and *Citrus limonia,* employing ultrasound energy. Parameters related to metals extraction, such as plant material sampling, acid concentration and sonication time were investigated. For accuracy check, the proposed ultrasound extraction procedure was compared with a microwave-assisted digestion procedure and no differences in the results were verified at 95% of the confidence level. With this simple and accurate extraction procedure, it was possible to determine differences in Ca, K and Mg concentrations during *Citrus* embryo formation/development and between cultures (embryogenic and non-embryogenic). Finally, the ultrasound extraction method demonstrated to be an excellent alternative for handless sampling and operational costs.

**Keywords:** ultrasound extraction, FAAS, FAES, Ca, K and Mg determination

## Introduction

Classical sample preparation methods commonly used for biological studies are those based on dry or wet decomposition,[1,2] which, in general, require intensive sample handling and time/reagent consumption. In this context, the use of ultrasound can be an excellent alternative to minimize sample preparation[3-5] and to avoid solvent extraction,[6,7] because its energy facilitates and accelerates some analytical steps, permitting less time consumption and decreasing the cost per analysis. Besides these characteristics, accuracy, precision, and handless manipulation can be achieved when ultrasound-assisted extraction methods are used.[4]

As an example, the application of ultrasound as an auxiliary energy source for solid sample treatment was made by Luque de Castro and Silva.[8] The authors emphasized different applications of ultrasound mainly related to sample treatment of agricultural, biological and environmental samples. Carvalho *et al.*[9] pointed out the sonochemical degradation that occurs inside extracted solutions when using the ultrasonic bath. Chloride, sulfate, nitrate, acetate, formate and oxalate species were monitored by ion chromatography in both aqueous and acetone solutions. The authors present several unidentified peaks, which indicate the generation of new ionic species. These peaks were absent in the original samples (airborne particulate matter).

* e-mail: zezzi@iqm.unicamp.br

Mecozzi *et al.*[10] described an ultrasound-assisted procedure for the extraction of the bioavailable fraction of humic substances in marine sediments. The proposed method was based on consecutive sample extractions by 0.5 mol $L^{-1}$ NaOH and ultrasound. Only 30 min was necessary to complete the procedure, in contrast to another method (24 h shaking in 8 mol $L^{-1}$ HCl), which was also proposed by the same authors.

An ultrasound extraction method for elemental analysis by atomic spectrometry was recently proposed by Ashley *et al.*[11] Selected standard reference materials were subjected to ultrasound extraction in different acid solutions [25% v $v^{-1}$ $HNO_3$, 25% v $v^{-1}$ $HNO_3$/HCl or concentrated $HNO_3$/HCl (1:1)]. Good recoveries (>80%) were achieved when concentrated $HNO_3$/HCl was used for As, Cd, Cu, Mn, Pb and Zn. However, several elements (Ba, Co, Cr, Fe, Mg, Ni, V) yielded *ca.* 75% recoveries in this same condition.

Due to the importance of Ca, K and Mg in some biochemical processes,[12-14] their quantification is of utmost importance for the understanding of these processes. In this way, efficient methods of sample preparation, mainly those presenting accuracy and fastness, are necessary for appropriate metal quantification.

This work aims to evaluate the efficiency of ultrasound extraction of Ca, K and Mg from *in vitro* embryogenic and non-embryogenic *Citrus* sp cultures followed by determination of the metals concentrations by FAAS/FAES.

## Experimental

*Instruments and apparatus*

For ultrasound extraction procedure, a Neytech model 28H ultrasonic bath at a frequency of 47±3 kHz was used. The water volume inside the bath was 1 L.

For checking the accuracy of the results obtained with ultrasound extraction, embryogenic and non-embryogenic samples of *Citrus* were also prepared using a laboratory QW-3000 microwave oven (QCI), equipped with temperature and pressure sensors, Teflon® vessels and magnetron of 2450±13 MHz with nominal power of 1200 W.

A Perkin-Elmer model AAnalyst 300 Flame Atomic Absorption Spectrometer (FAAS), equipped with a deuterium lamp background correction system was used throughout this work for the analysis of *Citrus* after metal extraction as well as for digested samples. Hollow cathode lamps (Perkin-Elmer) of calcium and magnesium were used as primary radiation source. Potassium was determined by Flame Atomic Emission Spectrometry (FAES), employing the same equipment already described. Analytical measurements were based on time average absorbance and the main FAAS/FAES operating conditions were those recommended by the manufacturer.

*Reagents and solutions*

All solutions were prepared with analytical reagent-grade chemicals (Merck). It was used a high purity deionized water (18 MΩ cm), purified by a Milli-Q Water Purification System (Millipore).

Working standard solutions of calcium (1.0 - 5.0 mg $L^{-1}$), as $CaCO_3$, magnesium (0.1 - 0.5 mg $L^{-1}$), as $Mg(NO_3)_2$, and potassium (4.0 - 20.0 mg $L^{-1}$), as KCl, were prepared by sequential dilutions from stock solutions of 100 mg $L^{-1}$. For calcium and magnesium standard solutions, $La_2O_3$ was used as a concomitant suppresser, at a final concentration of 0.1% m $v^{-1}$ La. For potassium standard solutions the same lanthanum solution was used as the ionization buffer. Lanthanum solutions were prepared using 10 mL of conc. HCl.

*Storage and cleaning materials*

All solutions were stored in polypropylene bottles of high density (Nalgene). Plastic bottles and glassware materials were cleaned by soaking in 10% v $v^{-1}$ nitric acid for 48 h, followed by five rinses with Milli-Q water, and dried in a hood at room temperature.[15] When necessary, the extracted samples were stored at 4°C until the analysis was done.

*Plant material*

*In vitro* embryogenic (*Citrus sinensis* L. Osbeck) and non-embryogenic (*Citrus limonia* L. Osbeck) plant materials were obtained according to the protocol established by Benedito and co-workers.[16]

Twenty replications were prepared for each culture and for each sampling week (7 - 14 -21 - 28 days). Each replication consisted of one Petri dish. From these Petri dishes, three of them were randomly chosen to provide plant material samples for ultrasound-assisted extractions and microwave sample preparation. For FAAS and FAES analysis, each sonicated and digested extract was quantified in triplicate. Embryogenic and non-embryogenic plant material was sampled (n=3) weekly, during four weeks.

*Sampling method*

Since cultures were heterogeneous in terms of the type and size of cell clusters (callus), a preliminary test was

performed to define the sampling conditions. Two different ways of sampling were used, which were named simple *sampling* and sub-sample sampling. Metal extraction was done to compare both methods (n=15). In simple sampling, a random callus sample (150 mg) was collected from each Petri dish. In sub-sample sampling, the callus content in each Petri dish was sub-divided in four parts, following a collection of sub-samples with 37.5 mg from each part, totaling 150 mg. The best sampling method was used for the weekly sampling of plant material.

*Ultrasound-assisted extraction: effects of acid concentration and time*

After achieving a precise procedure for callus sampling, an experiment was performed with different acid concentrations (0.014, 1, 2, 3 and 4 mol $L^{-1}$), to establish the best conditions for metals extraction from this type of sample. As nitric acid is generally used for sample extraction/decomposition[17-19] and because it provides a good medium for working with FAAS technique,[20-22] it was chosen as the extracting medium for this purpose.

Since the sonication time plays an important role in metal extraction,[23-25] an experiment using 5, 10, 15 and 30 min of sonication time was performed in order to establish the best extraction time condition.

*Optimization of ultrasound-assisted extraction procedure*

In order to obtain an efficient ultrasound extraction, all conditions recommended by Nascentes *et al*.[26] were strictly followed when ultrasonic bath was used. In addition, after the optimization of the acid concentration and time, all conditions were applied for Ca, K and Mg extractions from plant material. Accurately weighed embryogenic and non-embryogenic *Citrus* callus (0.15g ± 0.1 mg) were transferred to each digestion tube for ultrasound extraction and 40 mL of the 0.014 mol $L^{-1}$ nitric acid were added to the samples. Each digestion tube was then placed in the Neytech ultrasonic bath and only one sample was sonicated every time. After the extraction, 0.5 mL of 10% (m $v^{-1}$) lanthanum solution was added to each sample and the volume was completed up to 50 mL with 0.014 mol $L^{-1}$ nitric acid.

Since very few solid particles remained in the solution after extraction, the analyses were performed by direct nebulization of the extracting solution. No nebulizer blockage of the flame atomizer was observed during the 8-h work period, confirming that even with the presence of small particles (probably smaller than 40 $\mu$m) in the liquid phase, they were nebulized and atomized.

*Accuracy check for ultrasound extraction*

In order to check the accuracy of the ultrasound extraction, the results obtained with this procedure were compared to those obtained using acid microwave-assisted digestion.[17] Embryogenic and non-embryogenic *Citrus* samples of 150 mg were weighted and transferred to a Teflon vessel, in which 6 mL of $HNO_3$ (concentrated ) + 1 mL of $H_2O_2$ (30% v $v^{-1}$) were added. The solution was heated by microwave action only after the initial reaction had subsisted. The microwave program used was the same already proposed.[17]

## Results and Discussion

*Sampling method*

Although the samples used seem to be composed by uniform masses of cells under the naked eye, different types of cell aggregates can be observed under the stereo-microscope. With the sampling procedure described in the "Plant Material" section, (the collection of *sub-sample sampling*), precise results (n=15) for metals quantification were obtained (*ca.* 1.5% RSD as repeatability) in comparison with the *simple sampling* procedure (> 15% RSD). These results confirm the importance of the sampling method for this type of material, since sonication effectiveness depends on both sample mass and particle sizes.[26]

*Ultrasound extraction: effects of acid concentration and time*

The concentration of nitric acid in the extraction solution and the sonication time also proved to be important parameters. According to the results obtained, it was noted that with the use of a less concentrated nitric acid solution (0.014 mol $L^{-1}$) for calcium extraction (n=3), higher values and more precise results (1656±7 mg $L^{-1}$) were achieved, when compared to higher nitric acid concentration (4 mol $L^{-1}$). In this situation the calcium concentration observed was 1176±11 mg $L^{-1}$. This can be explained by the viscosity/density of the extraction medium, since the propagation of ultrasonic waves is more effective in lower viscosity/density medium. At higher viscosity the cavitation process is more difficult to be induced and the number of cavitating bubbles per unit of volume is reduced.[24,25] It is interesting to emphasize that when only water was used as the extracting medium, lower calcium concentration was measured (*ca.* 1000 mg $L^{-1}$). In this way, 0.014 mol $L^{-1}$ $HNO_3$ was chosen as the extracting medium for future experiments.

According to the sonication time, the extraction process can be improved. Quantitative extraction was already obtained with 10 min of sonication, however, when 30 min was used, a lower extraction rate was obtained. This can be explained because different chemical species can be formed when longer sonication times are used due to the recombination of different structures in the liquid, inducing the formation of new species and reducing ions.[9]

Although slight differences were observed when 10 or 15 min of sonication was used for calcium extraction, 15 min produced more precise results (RSD *ca.* 1%), leading to the conclusion that the best extraction condition would be achieved with $HNO_3$ 0.014 mol $L^{-1}$ associated to 15 min for sonication time. Under these conditions an increase of *ca.* 2°C in the water bath occurred after each extraction cycle. In this way the water bath was exchanged every 5 extraction cycles.

*Accuracy check for ultrasound extraction and Ca, K and Mg determinations*

The accuracy of the ultrasound extraction procedure was checked using acid microwave-assisted digestion. Two different procedures were applied for all metals studied in this work and no difference at 95% confidence level was observed (Table 1). It is interesting to emphasize that only slight differences were observed when the two sets of results were compared, indicating the accuracy of the ultrasound extraction procedure.

After establishing the best conditions for metals extraction in an accurate way, Ca, K and Mg determinations from embryogenic and non-embryogenic cultures were performed (see Figure 1). According to the results obtained after both ultrasound extraction and metal determinations, all metals evaluated presented variations in theirs concentrations during embryo formation and development. Calcium, potassium and magnesium levels are different in both plant material analysed. The differential levels of Ca, K and Mg in embryogenic and non-embryogenic plant materials (see Figure 1) may be related to the differences in embryogenesis expression.

**Table 1.** Comparison of Ca, K and Mg concentration (mg $L^{-1}$) obtained from microwave decomposition (MW) and ultrasound extraction (US) procedures (n=3) using non-embryogenic (RL) and embryogenic (V63) samples

| Species | MW | | US | |
|---|---|---|---|---|
| | RL | V63 | RL | V63 |
| Ca | 1580±4 | 2300±6 | 1599±5 | 2249±7 |
| K | 2390±9 | 5039±12 | 2372±12 | 4992±10 |
| Mg | 170±1 | 230±2 | 179±1 | 235±1 |

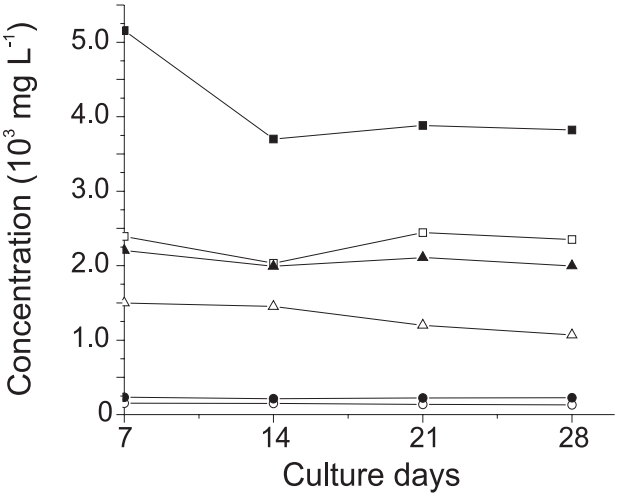


**Figure 1.** Variation on metal concentration (mg $L^{-1}$) in embryogenic cultures – V63 (K: −■−; Ca: −▲− and Mg: −●−) and non-embryogenic cultures - RL (K: −□−; Ca: −△− and Mg: −○−) during 7, 14, 21 and 28 days of culture. RSD for metal concentrations < 1 %.

## Conclusions

The extraction procedure proposed proved to be advantageous in accuracy, precision, and handless manipulation, when compared with classical sample preparation procedures.

The efficiency of the extraction procedure, based on the ultrasound energy was remarkable, allowing the substitution of hazardous and/or high handling methods by a clean chemistry–type procedure and giving accuracy to the experiment. These characteristics associated to the quickness of the process and the sample quantities required for the determination of metals, can be useful in analyses of plant tissue culture material. In these experiments, a simple, fast, precise and economic methodology is very advantageous, since a large number of samples is generally required for the analyses at different periods of time. The procedure presented here can be adapted, with minor adjustments, to other types of cultures, as well as other plant tissue culture samples.

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