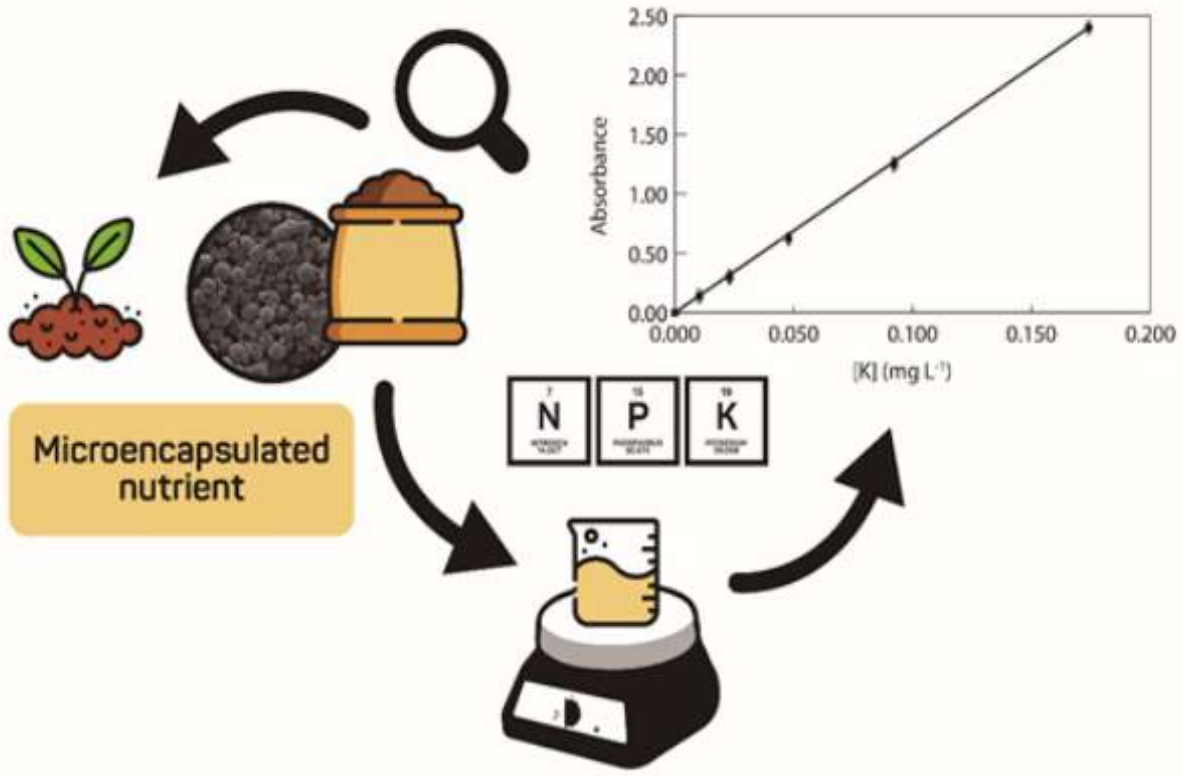
# Eclética Química Journal

Volume 46 • issue 2 • 2021


## NPK polymeric microparticles:
Application and validation of analytical methods for determination of a promising fertilizer

### Organic chemistry
Phytochemical and antibacterial investigation of *Moringa oleifera* seed: experimental and computational approaches

### Metal complex
Chemical, spectroscopic characterization, molecular modeling and antibacterial activity assays of a silver (I) complex with succinic acid

### Vegetable oils
Heterogeneous photodegradation of bisphenol A and ecotoxicological evaluation post treatment

### Emerging pollutant
Ultrasound-assisted extraction for the determination of α-linolenic and linoleic acid in vegetable oils by high performance liquid chromatography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Instituto de Química
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ISSN 1678-4618

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# Editorial

Dear authors and readers, the Editor of Eclética Química Journal, Editorial Board and Staff feel happy presenting this issue which contains valuable results of important investigations developed in a complicated scenery due to Covid-19, but that keep alive the flame of hope for better times. It is well known that antibiotic resistance and the growth of new strains of bacteria are of great concern to the human health. It requires the development of new drugs with better efficiency to treat the infections caused by these human pathogens. Particularly, the commonly used medicinal plants of Brazilian communities may help to diminish this problem, which is the subject of the first article. The *Moringa oleifera* seed extract showed antibacterial activity against both Gram-positive and Gram-negative bacteria and isolated compounds like kaempferol and quercetin were effective against infections caused by bacteria with the order of potency *Pseudomonas aeruginosa* > *Klebsiella pneumonia* > *Staphylococcus aureus* > *Escherichia coli* > *Streptococcus pneumonia*. Sequentially, a compound from succinic acid and $Ag^{+}$ ion was obtained and characterized as bidentate coordinated $Ag_2C_4H_4O_4$ complex. Density functional theoretical studies confirmed the coordination of each carboxylate group to one silver atom by the two oxygen and the bond lengths O···Ag theoretically determined range from 2.325 to 2.338 Å. This complex showed *in vitro* antibacterial activity against the bacterial strains of *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa* complex and to be active over *M. tuberculosis* $H_{37}$Rv strain. Following, microparticles (MP) containing nitrogen (N), phosphorus (P) and potassium (K) were synthesized with a mixture of polymers and a linear, precise and accurate methodology was developed to determine the NPK content. The MP can be applied as controlled release alternative to traditional fertilizers. Next, an emerging pollutant with endocrine disrupting properties, bisphenol A (BPA), present at trace levels in various aqueous medium, was removed by an advanced oxidation process, specifically heterogeneous photocatalysis using $TiO_2$. The methodology was efficient to completely remove the BPA even using solar radiation as UV source. Ecotoxicological and chronic toxicity evaluations indicated that the post-treatment aqueous samples showed better performance compared to the initial ones. Closes this issue a study that consists in developing a methodology for determining α-linolenic acid (ALA, ω-3) and linoleic acid (LA, ω-6) in vegetable oils with ultrasound-assisted extraction, which was validated by high-performance liquid chromatography (HPLC) for the quantification of polyunsaturated fatty acids. The proposed method is simple, fast, linear, precise and accurate for the quantitative determination of ALA and LA in vegetable oil.

Lastly, the Editor and members of Editorial Board of Eclética Química Journal pay a tribute to the effort developed by Authors and Reviewers during this confusing time by helping us to go ahead with mutual and essential collaboration.

Assis Vicente Benedetti
Editor-in-Chief of EQJ

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**2. Experimental**
***2.1 Surface characterization***
*2.1.1 Morphological analysis*

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## Bibliographic references

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Foster, J. C.; Varlas, S.; Couturaud, B.; Coe, J.; O'Reilly, R. K. Getting into Shape: Reflections on a New Generation of Cylindrical Nanostructures' Self-Assembly Using Polymer Building Block. *J. Am. Chem. Soc*. **2019**, *141* (7), 2742−2753. https://doi/10.1021/jacs.8b08648

**Book**
Hammond, C. *The Basics of Crystallography and Diffraction*, 4th ed.; International Union of Crystallography Texts on Crystallography, Vol. 21; Oxford University Press, 2015.

**Book chapter**
Hammond, C. Crystal Symmetry. In *The Basics of Crystallography and Diffraction*, 4th ed.; International Union of Crystallography Texts on Crystallography, Vol. 21; Oxford University Press, 2015; pp 99−134.

**Book with editors**
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**Website**
*ACS Publications Home Page*. https://pubs.acs.org/ (accessed 2019-02-21).

**Document from a website**
American Chemical Society, Committee on Chemical Safety, Task Force for Safety Education Guidelines. *Guidelines for Chemical Laboratory Safety in Academic Institutions*. American Chemical Society, 2016.

https://www.acs.org/content/dam/acsorg/about/governance/committees/chemicalsafety/publications/acs-safety-guidelines-academic.pdf (accessed 2019-02-21).

**Conference proceedings**
Nilsson, A.; Petersson, F.; Persson, H. W.; Jönsson, H. Manipulation of Suspended Particles in a Laminar Flow. In Micro Total Analysis Systems 2002, Proceedings of the μTAS 2002 Symposium, Nara, Japan, November 3−7, 2002; The Netherlands, 2002; pp 751−753. https://doi.org/10.1007/978-94-010-0504-3_50

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Department of Commerce, United States Patent and Trademark Office. Section 706.02 Rejection of Prior Art [R-07.2015]. *Manual of Patent Examining Procedure (MPEP)*, 9th ed., rev. 08.2017, last revised January 2018. https://www.uspto.gov/web/offices/pac/mpep/s706.html#d0e58220 (accessed 2019-03-20).

**Patent**
Lois-Caballe, C.; Baltimore, D.; Qin, X.-F. Method for Expression of Small RNA Molecules within a Cell. US 7 732 193 B2, 2010.

**Streaming data**
American Chemical Society. Game of Thrones Science: Sword Making and Valyrian Steel. *Reactions*. YouTube, April 15, 2015. https://www.youtube.com/watch?v=cHRcGoje4j4 (accessed 2019-02-28).

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Eclética Química Journal

Original article

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# Phytochemical and antibacterial investigation of *Moringa oleifera* seed: experimental and computational approaches

Sunday Adewale Akintelu[1,2], Aderonke Similoluwa Folorunso[3], Abel Kolawole Oyebamiji[2,4+]

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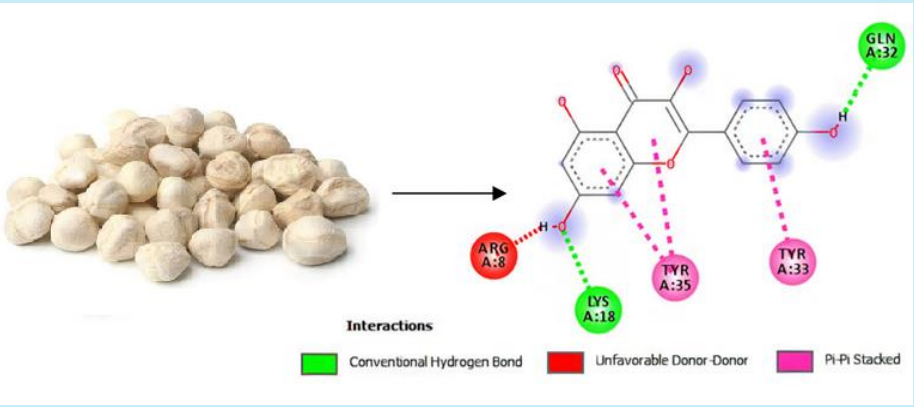
**ARTICLE INFO**

*Article history:*
**Received:** March 31, 2020
**Accepted:** December 12, 2020
**Published:** April 01, 2021

*Keywords*
1. *Moringa oleifera*
2. antibacterial
3. phytochemical and multidrug resistant
4. DFT
5. Docking

**ABSTRACT:** The advent of antibiotic resistance and the growth of new strains of bacteria are of great concern to the world health sectors. Effective treatment of the infections caused by these human pathogens call for designing and development of new drugs with better efficiency. This study aimed at investigating the antibacterial activity of *Moringa oleifera* seed extract against some bacteria strains.




The phytochemical and antibacterial activities of *Moringa oleifera* seed extract against both Gram-positive and Gram-negative bacteria were conducted using standard methods. The phytochemical analysis of the studied seed extract revealed the presence of alkaloids, tannins, saponins, phenols and flavonoids as secondary metabolites. The obtained result from the antibacterial study indicated that the extract exhibited high inhibition zones against all bacteria strain studied. Also, four molecular compounds from *Moringa oleifera* seed were selected based on their percentage yield and were optimized using density functional theory and they were used against five bacterial cell lines. It was observed that kaempferol and quercetin inhibited *Escherichia coli*. Kaempferol inhibited *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Streptococcus pneumonia* while Quercetin showed highest inhibition against *Staphylococcus aureus* when compared with other compounds. This showed the effectiveness of *Moringa oleifera* seed in eradicating some infections caused by bacteria.

## 1. Introduction

Infectious diseases are syndromes that emanate from an infected person or animal to a susceptible host[1]. Infectious diseases remain the major cause of premature death in the world and it has been reported to be responsible for the death of about 50,000 people on a daily basis. The increase in the rate of bacterial infections has become alarming in the past few decades, thereby resulting into a severe health problem[2]. *Staphylococcus aureus, Bacillus subtilis, Bacillus licheniformis* and *Esherichia coli* have been reported as bacterial species for pathogenic infections of skin, septic arthritis, food intoxication food-borne illness, diarrhea and wounds[3–5]. Antimicrobial agents are very crucial in reducing the world encumbrance of infectious diseases[6].

However, the multidrug resistance associated with pathogenic bacteria have led to serious health challenges[7,8]. Previous studies on rapid and extensive emergence of resistance to recent discovering of antimicrobial agents confirmed a short life expectancy of newly made antimicrobial agent[9,10]. The use of medicinal plants as source of drugs could be one of the most probable means of treating sickness and ailments in developing countries[11]. *Moringa oleifera*, popularly referred to as "horseradish tree", is a medicinal plant found in the tropical and subtropical regions of the world and has been reported to possess antioxidant, anti-bacteria, anti-inflammatory, anti-cancerous activities[12,13].

The discovery of some molecules used for protein–protein crossing point target have numerous challenges. Molecular docking revealed the connection between pharmacophore and receptor and it has the ability to provide assistance in detecting the appropriate binding site in the receptor. More so, calculated docking could be in the form of dock score, which is the arithmetic way of estimating the power of the relationship between docked compounds once the docking is accomplished[14–16].

Thus, the compounds (catechin, epicatechin, kaempferol, quercetin) that were isolated from *Moringa oleifera* seed by Onuah et al.[17], Sun et al.[18], Ablajan and Tuoheti[19], Saldanha et al.[20] were theoretically studied via density functional theory and docking methods. Thus, in respect to the confirmation of the rapid global blowout of resistance to existing antibiotics, the need to find new anti-bacteria agents to reduce or end the epidemic health implication associated with multidrug resistant is of paramount importance.

## 2. Experimental

### *2.1 Sources of materials*

Dry seed of *Moringa oleifera*, accession No. 266266, used in this study, were obtained from Obafemi Awolowo University Teaching and Research Farm (O.A.U.T. & R.F.), Ile-Ife, Nigeria, (7°33′N; 4°33′E).

The identification of the plant was done by a Plant Taxonomist in the Department of Botany, Obafemi Awolowo University. The pods enclosing the seed were removed. The seeds were air dried. Seeds of *Moringa oleifera* with good quality (Fig. 1) were selected and the seed coat and wings of the seeds were removed manually. The seeds were pulverized to fine powder, sieved and properly kept prior further analysis.

**Figure 1.** *Moringa oleifera* seeds.

### *2.2 Extract preparation*

About four hundred grams (400 g) of powdered seeds of *Moringa oleifera* were transferred into 1 L volumetric flask and 800 mL of methanol was added. The solution was allowed to stay for 72 h with vigorous agitation at 3 h interval. The extracting solvent was removed by decantation. The residue was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filtered and concentrated on a digital rotary evaporator (Heidolph laborata 4010) to obtain 9.20 g crude extracts of *Moringa oleifera*.

### *2.3 Qualitative phytochemical analysis*

The presence of alkaloids, reducing sugar, flavonoids, phenols, saponins and tannins in the seed extract of *Moringa oleifera* were qualitatively examined using standard methods[21], as described below.

#### *2.3.1 Determination of alkaloids*

Zero point five gram (0.5 g) of *Moringa oleifera* seed extract was stirred with 5 mL of 1% HCl on water bath, it was filtered and a few drops of Meyer's reagent was added to 1 mL of the filtrate. There was an occurrence of turbidity, which confirmed the present alkaloids.

#### *2.3.2 Determination of tannins*

Zero point three gram (0.3 g) of *Moringa oleifera* seed extract was dissolved in 10 mL of distilled water and filtered. Then freshly prepared $FeCl_3$ solution was added to 5 mL of the filtrate. There was an observation of greenish dark coloration, which indicated the presence of tannins.

#### *2.3.3 Determination of reducing sugar*

Two drops of Fehling's solution were added to a solution containing 0.5 g of *Moringa oleifera* seed extract in 5 mL distilled water and the solution was heated on water bath. The change in blue color of Fehling's solution, which gave a red precipitation, indicated the presence of reducing sugar.

#### *2.3.4 Determination of saponins*

The formation of bubbles, which persisted for 20 min when *Moringa oleifera* seed extract dissolved in distilled water was vigorously shake, confirms the presence of saponins.

#### *2.3.5 Determination of phenols*

The intense coloration observed when $FeCl_3$ solution was added to 0.4 g *Moringa oleifera* seed extract in distilled water confirmed the presence of phenols.

#### *2.3.6 Determination of flavonoids*

To the solution of *Moringa oleifera* seed extract, 5 mL of $AlCl_3$, 2 mL methanol, 2 mL of concentrated HCl, 2 mL of KOH solution and few drops of magnesium turning was added. A pink coloration was observed which confirmed the presence of flavonoids.

### *2.4 Bacterial analysis*

The antibacterial activity of the seed extract was examined using pure cultures of clinical isolates Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pneumonia*) alongside with Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*), obtained from the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria, were used for this study. The culturing and identification of the selected bacterial species was done using modified method given by Colle et al.,[22]. The antibacterial sensitivity of the seed extract of *Moringa oleifera* against selected bacteria were examined with agar well diffusion method. The culture media was sterilized for 15 min at 121 °C. 0.1 mL standard inoculum of the test bacteria previously inoculated with bacterial suspension (100 mL of medium/1 mL of $10^7$ CFU) to attain $10^5$ CFU/mL of medium was introduced into the sterilized media. The inoculum was spread through the media. A sterile cork borer was used to bore a 6 mm well into the agar medium. Zero point one milliliter (0.1 mL) of 2, 4 and 6 mg $mL^{-1}$ concentration obtained by re-dissolved 10 mg of *Moringa oleifera* seed extract in 5, 2.5 and 0.8 mL of methanol, respectively, was transferred into each of the well on the medium. The plates were allowed to stand for 1 h before incubation for another 24 h at 37 °C. The zones of inhibition on the plates were measured with a transparent ruler in millimeters. Filter paper discs loaded with 5 µg of


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ampicillin were used as control. The plates were kept in the fridge at 5 °C for 2 h to allow plant extracts diffusion then after incubation at 37 °C for 48 h, the zones of inhibition were measured. The effect of the seed extract of *Moringa oleifera* on bacteria strains was compared with standard control at concentrations of 2, 4 and 6 mg $mL^{-1}$. The percentages of zones of inhibition were calculated to determine the antibacterial activity of the seed extract of *Moringa oleifera*. The antibacterial investigation was on triplicate analysis. The adopted formula from the study[23] was used for calculating the percentage growth inhibition, as Eq. 1:

$$PGI\ \% = \frac{(BDC - BDT)}{BDC \times 100} \quad (1)$$

where PGI is percent growth inhibition, BDC is the bacteria colony diameter in control and BDT means bacteria colony diameter in treatment.

### *2.5 Theoretical calculation*

Four (4) selected molecular compounds[17–20] from molecules present in *Moringa oleifera* were used for this study. The selected compounds were chosen due to their highest percentage yield and the selected compounds were optimized using density functional theory at 6-31G* basis set and several molecular descriptors [$E_{HOMO}$, $E_{LUMO}$, band gap, dipole moment, hydrogen bond donor (HBD), hydrogen bond acceptor (HBA) log P, polarizability and Polar suface area (PSA)] which described anti-bacteria activity of the studied flavonoids were obtained using Spartan'14 software by wavefunction Inc. Also, the docking study were executed on the studied compounds and the studied enzymes (2J5O[24], 2RQX[25], 2W9T[26], 1GRX[27], 2M6U[28]) using Discovery studio, AutoDock Tool, AutoDock Vina and Pymol which serves as a post-dock software.

## 3. Results and discussion

### *3.1 Qualitative phytochemical analysis*

The qualitative phytochemical analysis of *Moringa oleifera* seed extract confirmed the presence of alkaloids, tannins, saponins, phenols, flavonoids and the absence of reducing sugar (Tab. 1). However, the absence of reducing sugar indicated that the anti-bacteria potency of plant extracts against Gram-negative bacteria was due to the presence of phenolic compounds[29]. Findings from literature revealed that medicinal plants containing alkaloids, phenols and flavonoids as bioactive metabolites have good antibacterial properties[30–33]. Therefore, the presence of these metabolites in the seed extract of *Moringa oleifera* could render it as a good antibacterial agent.

**Table 1.** Results of phytochemical analysis of the seed of *Moringa oleifera* seed extract.

| Phytochemicals | Inferences |
|---|---|
| Alkaloids | + |
| Tannins | – |
| Reducing sugar | – |
| Saponins | + |
| Phenols | + |
| Flavonoids | + |

+ represents presence of bioactive compounds, while; – represents absence of bioactive compounds.

### *3.2 Antibacterial activities of Moringa oleifera seed extract against test bacteria*

The percentage zones of inhibition exhibited by *Moringa oleifera* seed extract against selected bacterial strains are showed in Fig. 2. The antibacterial study revealed that *Moringa oleifera* seed extract showed some levels of inhibition against all the bacteria strain at various concentrations. The decreasing order of susceptibility of the bacteria strain to *Moringa oleifera* seed extract at 2 mg $mL^{-1}$ was *Staphylococcus aureus* > *Klebsiella pneumonia* > *Escherichia coli*, *Streptococcus pneumonia* > *Pseudomonas aeruginosa*. Furthermore, when the percentage zones of inhibition of *Moringa oleifera* seed extract at 2 mg $mL^{-1}$ was compared with the control, the percentage zones of inhibition observed was called "weak inhibition", because percentage zones of inhibition were below the average of that of the control.

At concentration of 4 mg $mL^{-1}$, an increase in percentage zones of inhibition was observed and was found higher than the average percentage zones of inhibition exhibited by the control when compared at


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same concentration. This was called "moderate inhibition". The increasing order of potency of the extract against the bacteria strain was *Klebsiella pneumonia* > *Staphylococcus aureus* > *Pseudomonas aeruginosa*, *Klebsiella pneumonia* > *Escherichia coli* > *Streptococcus pneumonia.*

The highest percentage zones of inhibition were detected at concentration of 6 mg $mL^{-1}$. The increasing order of effectiveness of *Moringa oleifera* seed extract against the test bacteria was given as *Pseudomonas aeruginosa* > *Klebsiella pneumonia* > *Staphylococcus aureus* > *Escherichia coli* > *Streptococcus pneumonia.* The zones of inhibition in this study were lower than that obtained from the study of Folorunso et al., on biosynthesis, characterization and antimicrobial activity of gold nanoparticles from leaf extracts of *Annona muricata*[23] and higher than obtained from the study of Sarital et al., on the in vitro antimicrobial activity of some medicinal plants against human pathogenic bacteria[34]. This is an indication that *Moringa oleifera* seed extract has good inhibitory efficiency against bacteria and could possibly be used as an antibiotic for the treatment of infected diseases caused by bacteria.

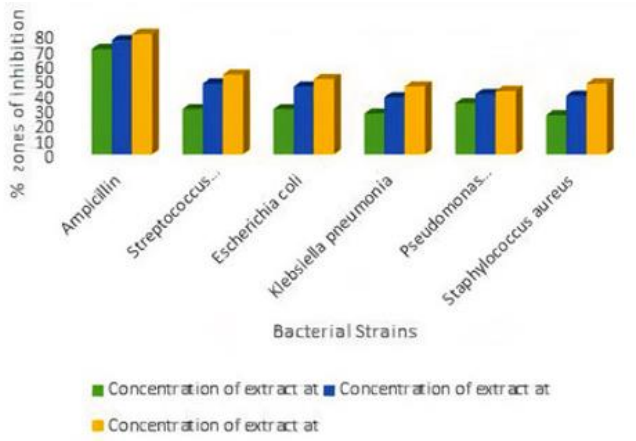


**Figure 2**. Antibacterial activities of *Moringa oleifera* seed extract against test bacteria.

### *3.3 Docking calculations*

Docking study was carried out on the flavonoids in *Moringa oleifera* seed and series of bacteria cell lines (2J5O, 2RQX, 2W9T, 1GRX, 2M6U). The obtained result, i.e., binding affinity between the studied compounds, were displayed in Tab. 2. As reported by Oyebamiji et al., molecules that have lower binding affinity show the molecule with tendency to inhibit well[35].

**Table 2.** Binding affinity for bacteria cell lines.

| Compound | *Escherichia coli* (IGRX)/kcal $mol^{-1}$ | *Klebsiella pneumonia* (2RQX)/kcal $mol^{-1}$ | *Pseudomonas aeruginosa* (2J5O)/kcal $mol^{-1}$ | *Staphylococcus aureus* (2W9T)/kcal $mol^{-1}$ | *Streptococcus pneumonia* (2M6U)/kcal $mol^{-1}$ |
|---|---|---|---|---|---|
| Catechin | -6.1 | -5.2 | -5.7 | -7.2 | -6.4 |
| Epicatechin | -6.2 | -5.6 | -5.5 | -6.4 | -6.9 |
| Kaempferol | -6.3 | -5.8 | -5.8 | -7.2 | -7.1 |
| Quercetin | -6.3 | -5.6 | -5.7 | -7.5 | -6.2 |

Thus, it was observed that kaempferol and quercetin are one of the vital flavonoids present in *Moringa oleifera* seed, as shown in Tab. 2. As displayed in Tab. 2, both kaempferol and quercetin inhibited *Escherichia coli* most. Kaempferol inhibited *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Streptococcus pneumonia* more than other studied compounds. Also, only quercetin possess the ability to inhibit *Staphylococcus aureus* more than other compounds used in this work. The residues involved in the interaction are shown in Figs. 3–8.

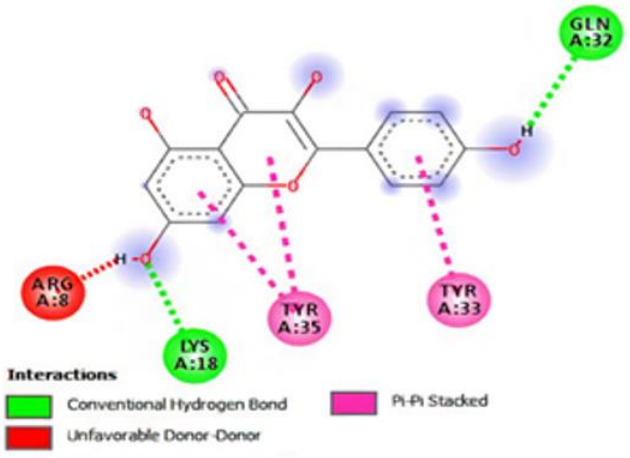


**Figure 3.** Molecular interactions of kaempferol with the residue in the gouge of *Escherichia coli* (IGRX).


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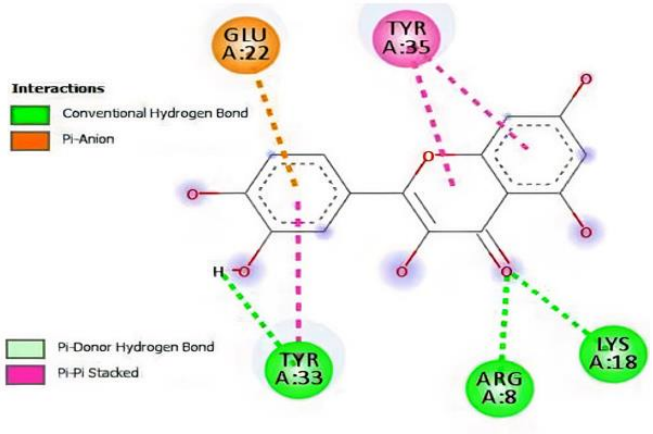


**Figure 4.** Molecular interactions of quercetin with the residue in the gouge of *Escherichia coli* (IGRX).

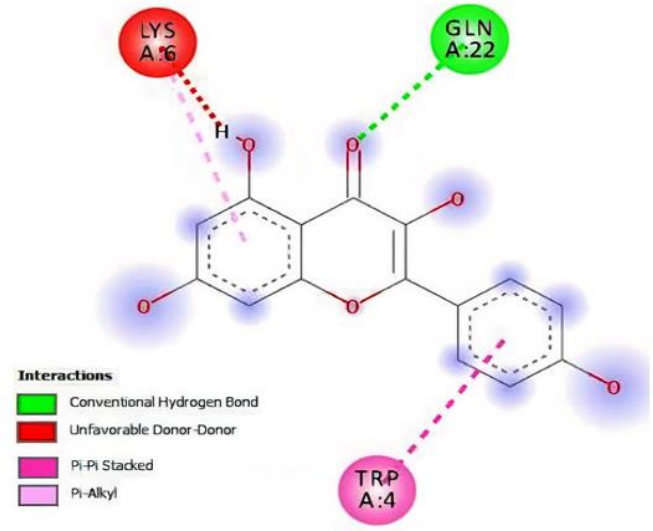


**Figure 5.** molecular interactions of kaempferol with the residue in the gouge of *Klebsiella pneumonia* (2RQX).


**Figure 6.** Molecular interactions of kaempferol with the residue in the gouge of *Pseudomonas aeruginosa* (2J5O).

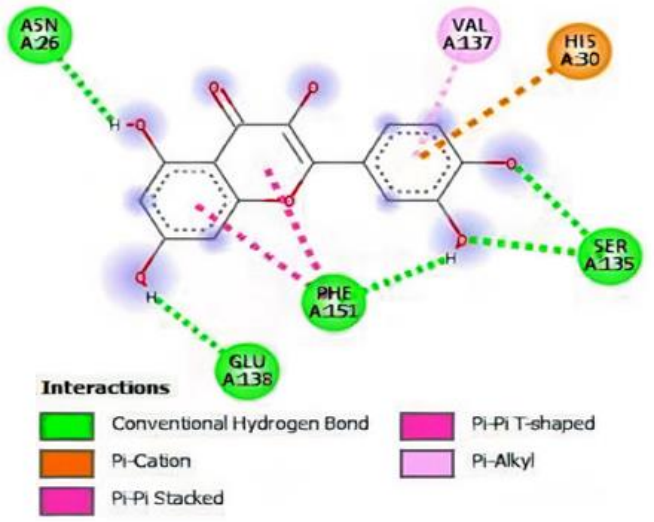


**Figure 7.** Molecular interactions of quercetin with the residue in the gouge of *Staphylococcus aureus* (2W9T).

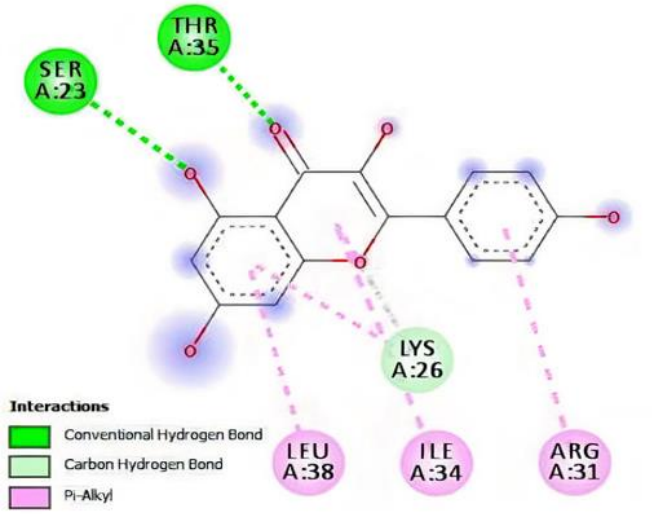


**Figure 8.** Molecular interactions of kaempferol with the residue in the gouge of *Streptococcus pneumonia* (2M6U).

Moreover, the molecular descriptors obtained for the studied compounds, as shown in Tab. 3, were subjected to Lipinski rule of five (Molecular Weight ≤ 500, Log P ≤ 5, HBD ≤ 5, and HBA ≤ 10)[36] and it was observed that the calculated molecular descriptors obtained from the studied compounds using density functional theory method obeyed the rule. This showed that all compounds used in this work have drug potential.


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**Table 3.** The obtained molecular descriptors.

| Compound | Molecular formula | Molecular weight/amu | Area/Å$^2$ | Vol/Å$^3$ | PSA | HBD | HBA | POL | Log P |
|---|---|---|---|---|---|---|---|---|---|
| Catechin | $C_{15}H_{14}O_6$ | 290.271 | 284.90 | 269.39 | 100.612 | 5 | 6 | 61.88 | 1.50 |
| Epicatechin | $C_{15}H_{14}O_6$ | 290.271 | 282.56 | 269.14 | 101.091 | 5 | 6 | 61.87 | 1.50 |
| Kaempferol | $C_{15}H_{10}O_6$ | 286.239 | 276.40 | 260.69 | 95.604 | 4 | 6 | 61.52 | 0.32 |
| Quercetin | $C_{15}H_{10}O_7$ | 302.238 | 283.80 | 267.49 | 113.340 | 5 | 7 | 62.10 | -0.07 |

## 4. Conclusions

*Moringa oleifera* seed extract could be modified as antibiotic ingredient to combat both Gram-positive and Gram-negative bacterial infections, which could help in some way to reduce or overcome the health challenges caused by bacterial resistance to many locally or commercially available antibiotics. Therefore, isolation of active compounds from *Moringa oleifera* seed is highly recommended for the discoveries of proficient antibacterial agent that can serve as conventional antibiotics that can constrain or cure the infectious diseases caused by pathogenic bacterial strains. In this work, kaempferol and quercetin inhibited *Escherichia coli* more than other studied compounds. Also, kaempferol possess the ability to inhibit *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Streptococcus pneumonia* more than other studied compounds, as well as Quercetin possess the capability to inhibit *Staphylococcus aureus* more than other compounds. The molecular descriptors were also in line with Lipinski rule.

## Acknowledgments

We are grateful to Computational Chemistry Research Laboratory, Department of Pure and Applied Chemistry, Ladoke Akintola University of Technology for the computational resources and Mrs E.T. Oyebamiji as well as Miss Priscilla F. Oyebamiji for the assistance in the course of this study.

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Eclética Química Journal, vol. 46, n. 2, 2021, 17-25
ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p17-25

Eclética Química Journal

Original article

iq.unesp.br/ecletica

| Vol. 46 | n. 2 | 2021 |

# Chemical, spectroscopic characterization, molecular modeling and antibacterial activity assays of a silver (I) complex with succinic acid

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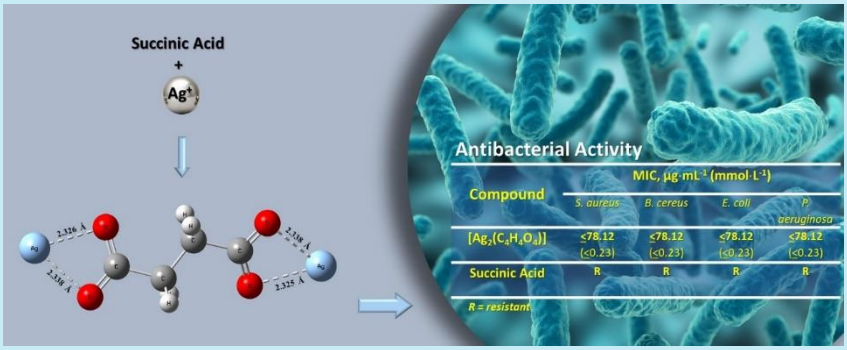
**ARTICLE INFO**

*Article history:*
**Received:** August 05, 2020
**Accepted:** December 24, 2020
**Published:** April 01, 2021

*Keywords*
1. *Silver complex*
2. Succinic acid
3. DFT
4. Antibacterial activity
5. *Mycobacterium tuberculosis*

**ABSTRACT:** A silver(I) complex with succinic acid in the form of succinate is presented. Chemical characterization confirms the molecular composition $Ag_2C_4H_4O_4$ for the complex. Infrared spectra suggest a bidentate coordination of both carboxylate groups of succinates to the two Ag(I) ions. Density functional theory (DFT) studies were used in the structures of succinic acid and Ag(I) succinate complex with coordination formula $[Ag_2(C_4H_4O_4)]$ in order to optimize them to their minimum energy. The studies confirmed that each carboxylate group of the succinate anion is coordinated to one silver atom by the two oxygen in a bidentate mode and the bond lengths O···Ag theoretically determined range from 2.325 to 2.338 Å. The complex $[Ag_2(C_4H_4O_4)]$ showed *in vitro* antibacterial activity against the bacterial strains of *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Pseudomonas aeruginosa* complex. Anti-*Mycobacterium tuberculosis* analyses were also performed and the $[Ag_2(C_4H_4O_4)]$ complex was shown to be active over *M. tuberculosis* $H_{37}Rv$ strain with $MIC_{90}$ of 23.94 μg $mL^{-1}$ while succinic acid itself showed a value higher than 25.00 μg $mL^{-1}$.





ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p26-35

## 1. Introduction

According to recent reviews[1,2], the bacterial antibiotic resistance is increasing at an alarming rate. The indiscriminate use of antibiotics is largely responsible for the occurrence of resistant bacteria.

One of the most common bacterial strains is *Escherichia coli*, which is usually found in intestines of warm-blooded organisms. Most strains of *E. coli* are harmless, but some can cause severe food poisoning. It is transmitted to humans mostly by the ingestion of contaminated foods, such as raw meat, milk and vegetables. *Escherichia coli* can cause neurological complications (seizures, stroke and coma) in about 25% of patients with hemolytic uremic syndrome and chronic kidney sequelae in about 50% of survivors. The *E. coli* resistance to the standard antibiotic treatment with fluoroquinolones is now widespread. In some countries, this treatment has shown to be ineffective in more than 50% of patients[3].

There has been a great increase in resistance to first-line drugs to treat infections caused by *Staphylococcus aureus*. People infected with *S. aureus* resistant to methicillin are estimated to be 64% more likely to die than people infected with a nonresistant strain of the same bacteria[3].

Tuberculosis (TB) is a disease responsible for more than 1.6 million deaths annually and remains a remarkable public health case of concern worldwide. The number of infections is growing up in developed countries, especially for immunosuppressed patients (such as people with diabetes and HIV/AIDS), individuals receiving antitumor therapy and diabetic individuals[4]. Despite the improvement of TB treatment, it is greatly affected by growth of resistant strains of *Mycobacterium tuberculosis*[5]. In 2017, a range of 483,000–639,000 people worldwide developed TB resistant to rifampicin, the most effective first-line drug, according to the World Health Organization Tuberculosis Report[4]. More alarming is that, within this group, 82% developed multidrug-resistant TB[6]. Therefore, the challenge is the search for new substances with antimicrobial activities with a remarkable effectiveness when tested against bacteria (mainly the resistant strains), compared to those drugs used nowadays.

A well-known strategy to obtain active compounds against bacteria includes preparation and uses of silver(I) complexes and Ag nanoparticles. Silver(I) is known for its antimicrobial activities for a long time in medicine and materials sciences[7–12]. The Ag(I) sulfadiazine complex, for example, has been clinically used as antibacterial and antifungal drug for more than 50 years. This complex is an insoluble compound which slowly liberates Ag(I) ions when used as a cream to treat bacterial infections in severe burns[8].

Silver(I) ions interact with DNA[13] or with S-donor ligands in vital enzymes and inactivate them[14,15]. Consequently, there is an increase of pyrimidine dimerization by a photodynamic process and interruption of DNA replication. So, Ag(I) complexes with N- and O-donor ligands have increased ability to replace such molecules by the S-donor ligands of target bacterial proteins[10,16,17].

Succinic acid (Fig. 1) is a nonhygroscopic acidulant of relatively low acid strength largely used in food and beverage industries. It is also a precursor to produce some polyesters and a component of alkyd resins heavily used in automotive and electronics industries[18]. The ligand is very well studied and has no toxicity or mutagenicity. So, if the complex is to be applied *in vivo* in the future, safety on the ligand is ensured. Furthermore, succinic acid has a chemical structure with two carboxylate groups, which are particularly good coordination points to prepare metal complexes. Succinic acid is an intermediate metabolic of the Krebs cycle. So, it could be expected that this characteristic promotes a higher intracellular absorption of the complex when compared to $AgNO_3$ and Ag(I) complexed to other ligands reported in the literature.

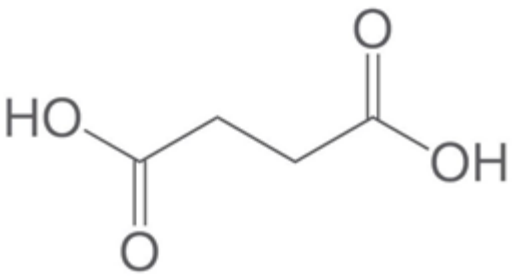



**Figure 1.** Chemical structure of succinic acid.


Eclética Química Journal, vol. 46, n. 2, 2021, 26-35
ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p26-35

Succinic acid complexes have already been described in the literature, such as the interaction of neodymium(III) and iron(III) with it and some of its derivatives[19]. Sladkov *et al.*[20] studied complexation of uranyl ($UO_2$) and plutonyl ($PuO_2$) with succinic acid in aqueous acid solutions.

In the present work, synthesis and characterization combining experimental and density functional theory (DFT) studies of an Ag(I) complex with succinic acid in the form of succinate are described. Antibacterial assays of the complex against the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 14579 and the Gram-negative *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were also performed and are described in this manuscript.

## 2. Experimental

### *2.1 Materials*

Succinic acid and silver nitrate were analytical grade chemical products from Sigma/Aldrich laboratories. All other chemicals were purchased from different sources. The reagents were used as received.

### *2.2 Synthesis*

An aqueous solution (20 mL) with 3.0 mmol of succinic acid was adjusted to pH 7.0 with KOH; 20 mL of another aqueous solution containing 6.0 mmol of Ag(I) nitrate was added under stirring to the succinic solution in a dark room. A white precipitate was immediately formed. The solid was collected by filtration and left to dry in a desiccator with $P_4O_{10}$ protected from light. *Anal. Calc. for* $[Ag_2(C_4H_4O_4)]$ *(%) C 14.5, H 1.2. Found (%) C 14.6, H 1.0. Yield: 75%.*

### *2.3 Instrumental methods*

Carbon, hydrogen and nitrogen (CHN) elemental analyses were performed using a CHNS-O 2400 series II (Perkin Elmer) analyzer. Infrared (IR) spectra were obtained on a FTIR Cary 630 Agilent spectrophotometer, equipped with attenuated total reflectance (ATR) sampling apparatus. The resolution was set at 4 $cm^{-1}$. Thermal analyses were performed on a thermoanalyzer TG/DTA simultaneous SDT Q-600 (TA Instruments) under the following conditions: aluminum crucible, synthetic air (100 mL $min^{-1}$), heating rate of 10 °C per min and temperature range from 30 to 1000 °C.

### *2.4 Computational simulations*

All the methods described in this section were based in previous work[21]. The chemical structures of succinic acid and the Ag(I) succinate complex $[Ag_2(C_4H_4O_4)]$ were optimized to the minimum of energy by the application of DFT with B3LYP[22–24]. The basis sets 6-31+G(d,p)[25–27] were applied for carbon (C), hydrogen (H) and oxygen (O) atoms. The LANL2DZ[28] effective core potential basis set was used for Ag atoms. Frequency calculations were employed to confirm that the optimized structures were at their minimum energy and no imaginary frequencies were found. Gaussian program 09 was used to perform all the calculations[29]. Gauss View 5.09 program was used to generate some structures[30].

### *2.5 Determination of the minimal inhibitory concentration (MIC)*

Determination of the minimum inhibitory concentration (MIC) of the free ligand and $[Ag_2(C_4H_4O_4)]$ was performed using different reference bacterial strains, *S. aureus* ATCC 25923, *B. cereus* ATCC 14579, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, as described in CLSI 2016[31,32]. The bacterial strains were inoculated in tubes containing 10.0 mL of brain heart infusion (BHI KASVI) and incubated for 18 h at 35–37 °C. Sufficient inoculums of each bacterial suspension were added in new tubes of sterile BHI medium until reaching 1.0 turbidity of the McFarland nephelometric scale (~ $3.0 \times 10^8$ CFU $mL^{-1}$). A volume of 50 µL of the stock solution (20 mg $mL^{-1}$) of the free ligand and $[Ag_2(C_4H_4O_4)]$ suspended in 20% dimethyl sulfoxide (DMSO) aqueous solution were added from the second well (B) of the 96-well microplate plus 50 µL of sterile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DOI: 10.26850/1678-4618eqj.v46.2.2021.p26-35

BHI medium, followed by serial dilutions (5.0 to 0.078 mg $mL^{-1}$) and 100 µL of the microorganism suspensions, on the McFarland 1.0 scale, were added to each well, reaching turbidity 0.5 McFarland (~ $1.5 \times 10^8$ CFU $mL^{-1}$) in a final volume of 200 µL $well^{-1}$. In the first well of the microplate, used as a growth control, 50 µL of sterile BHI medium, 50 µL of 20% aqueous DMSO solution and 100 µL of bacterial suspensions were added, on scale 1.0 McFarland. The microplates were incubated for 18 h at 35–37 °C in a humid chamber under agitation at 150 rpm. After the incubation period, 15 µL of 0.02% resazurin in sterile aqueous solution was added to each hole in the plates. After 3 h of reincubation, the reading was performed. When active (or able to replicate), the bacterial cells convert the "blue" resazurin to "pink" resorufin. The lower concentration that resulted in the inhibition of bacterial growth (or blocked the conversion of resazurin to resorufin) was considered the MIC value. The tests were performed in triplicate.

### *2.6 Anti-Mycobacterium tuberculosis analyses*

The $MIC_{90}$ of compounds against the standard *M. tuberculosis* $H_{37}Rv$ strain was determined with resazurin microtiter assay (REMA). Briefly, the compounds were dissolved in Middlebrook 7H9 broth, oleic albumin dextrose catalase (OADC) and glycerol 0.5%. The solutions of the compounds, in a range concentration of 0.09 to 25 mg $mL^{-1}$, were placed in a microplate (96-well) containing the bacterial inoculum, adjusted to $10^5$ CFU $mL^{-1}$. The plates were incubated for seven days at 37 °C, 5.0% $CO_2$ atmosphere. An aqueous solution of resazurin (0.01%) was added and the fluorescence was read at 530/590 nm after incubation of 24 h. The $MIC_{90}$ is the lowest concentration of the compound, which inhibits 90% of bacterial growth[33]. Three replicates were performed.

## 3. Results and discussion

### *3.1 IR spectra*

Some bands of the IR spectrum of the complex change significantly when compared to the succinic acid and its potassium salt indicating coordination of succinate to the metal by the carboxylate group. Figure 2 shows the IR spectra of succinic acid, $K^+$-succinate (the anionic form of succinic acid) and the $[Ag_2(C_4H_4O_4)]$ complex. The spectrum of succinic acid shows a very large stretching O–H band in the range 3300–2500 $cm^{-1}$. It also shows a combined absorption pattern in the range 3300–2500 $cm^{-1}$, with a broad O–H band superimposed on the sharp stretching bands of C–H. The reason for a so broad O–H stretching band of succinic acid is certainly because carboxylic acids usually exist as hydrogen-bonded dimers. This band disappears in the spectra of both potassium salt and complex. The second change is in the stretching and bending vibrations C=O, C–O and O–H in the region 1300–1700 $cm^{-1}$. The C=O stretching mode of succinic acid occurs at 1675 $cm^{-1}$, the O–H bend at 1408 and 890 $cm^{-1}$ and the C–O stretch at 1304 $cm^{-1}$. For $K^+$-succinate, the $COO^-$ ions generate a strong asymmetric stretching vibration ($\nu COO^-_{as}$) at 1559 $cm^{-1}$ and a weak symmetric stretching vibration ($\nu COO^-_{sym}$) at 1392 $cm^{-1}$. These bands are located at 1507 $cm^{-1}$ ($\nu COO^-_{as}$) and at 1385 $cm^{-1}$ ($\nu COO^-_{sym}$) in the silver-succinate complex. The difference between $\nu COO^-_{as}$ and $\nu COO^-_{sym}$ ($\Delta\nu$) depends on the type of coordination between the metal and the carboxylate. The carboxylate group keeps the $C_2$ symmetry when coordinating as a bridging or a bidentate group. The metal atom is equally associated with the two oxygen atoms in the succinate salt. The difference $\Delta\nu$ between the $COO^-$ asymmetric and symmetric stretching frequencies in the carboxylate salt and in the complex is 167 and 122 $cm^{-1}$, respectively, suggesting a bidentate coordination of each carboxylate group to the two Ag(I) ions[34,35].

The structure of succinate molecule is highly symmetric. So, the IR bands of the two carboxylate groups would have the same frequencies and such bands are superimposed. Consequently, $\Delta\nu$ should be the same for both carboxylate groups. In summary, each carboxylate group is coordinated to one Ag(I) ion in a bidentate mode.


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DOI: 10.26850/1678-4618eqj.v46.2.2021.p26-35

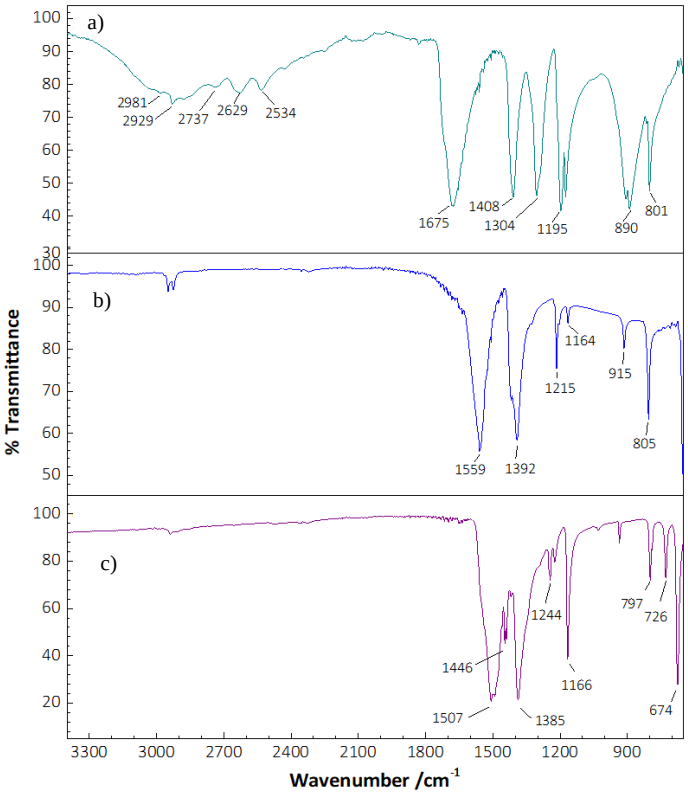


**Figure 2.** Infrared spectra of succinic acid (a), potassium succinate (b) and [$Ag_2(C_4H_4O_4)$] (c).

### *3.2 Thermogravimetric measurements*

Figure 3 shows the thermogravimetric curve for the [$Ag_2(C_4H_4O_4)$] complex, with only one well-defined mass loss. The loss of about 34% occurred in the range 260–310 °C and corresponds to the net mass loss. The final residue percentage is in accordance with the formation of metallic silver. Differential scanning calorimetry (DSC) analysis of [$Ag_2(C_4H_4O_4)$] (Fig. 4) shows the occurrence of only one exothermic event at 304 °C, which corresponds to oxidation of the ligand leading to the formation of the residue of $Ag^0$.

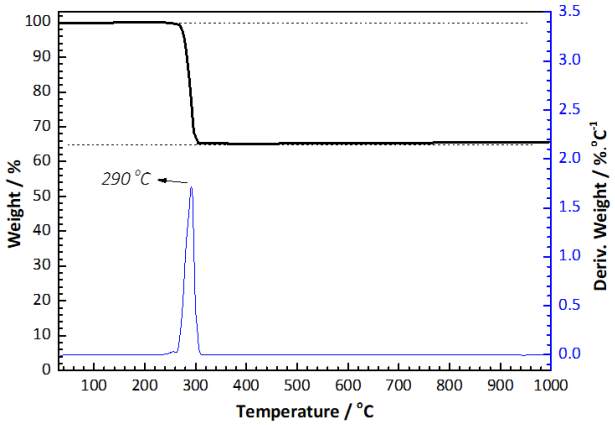


**Figure 3.** Thermogravimetric curves (TG and DTG) for [$Ag_2(C_4H_4O_4)$].

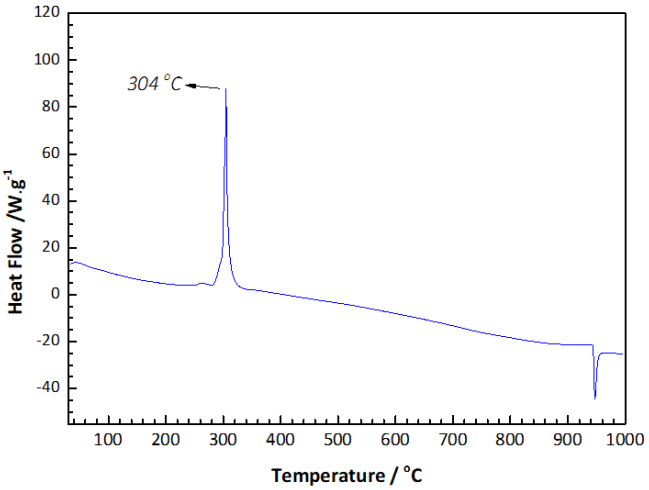


**Figure 4.** Differential scanning calorimetry (DSC) for [$Ag_2(C_4H_4O_4)$].

### *3.3 Electronic and structural properties by density functional theory (DFT)*

Succinic acid and [$Ag_2(C_4H_4O_4)$] were theoretically studied and their structural parameters after complexation and their frontier molecular orbitals (FMO) were determined. The structural parameters calculated were the bond lengths for the metal and oxygen (O···Ag) (Fig. 5).


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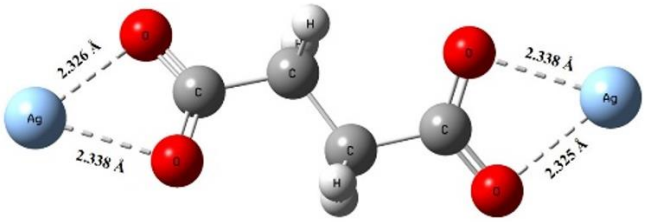


**Figure 5**. Structural formula for $[Ag_2(C_4H_4O_4)]$ and the bond length values for the O···Ag bonds.

By analyzing the results, it is possible to observe that each carboxylate group of the succinate coordinates to one Ag atom by the two O atoms in a bidentate mode and the theoretically determined bond lengths O···Ag were in the range from 2.325 to 2.338 Å. It is important to note that, for optimization, the Ag atom was placed close to the O atom and no imaginary frequencies were identified after optimization, showing that the structure determined in Fig. 6 was at minimum energy. The theoretically determined values for the O···Ag bond are similar to the crystal data found in the literature for a silver complex with mixed ligands 2-aminobenzonitrile and 4-methylbenzoic acid, where the carboxylate group is coordinated to silver in a bidentate chelate mode[36]. The frontier molecular orbitals highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO) and the energy gap for succinic acid and the $[Ag_2(C_4H_4O_4)]$ complex were evaluated and are also shown in Fig. 6.

The results indicate that the HOMO and LUMO orbitals of succinic acid, as well as the HOMO of $[Ag_2(C_4H_4O_4)]$, are located over all molecular structures. The exception was the LUMO of the complex which is most located over the Ag atoms. The results for the determined energy gap were $\Delta E$ = 7.21 eV for the ligand and $\Delta E$ =3.32 eV for the complex. The values of gap show that there is a significant decrease in the energy gap (≅ 3.89 eV) after complexation, which indicates that the complex is more reactive than the ligand.

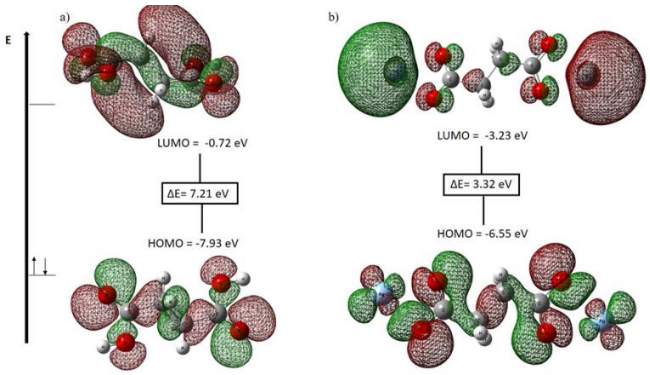


**Figure 6.** Frontier molecular orbitals and energy gap for: a) succinic acid and b) $[Ag_2(C_4H_4O_4)]$ complex.

### *3.4 Minimum inhibitory concentration (MIC)*

The $[Ag_2(succ)]$ shows its inhibitory activity against Gram-positive and Gram-negative bacteria used in the assay. The free ligand succ and DMSO aqueous solution did not show antibacterial activities. The results are summarized in Table 1.

The results suggest that antibacterial activity of $[Ag_2(C_4H_4O_4)]$ is due to the release of $Ag^+$ ions in the same way it was described for Ag-sulfadiazine and for other Ag complexes. Inhibition (in $mmol \cdot L^{-1}$) provided by $[Ag_2(C_4H_4O_4)]$ was higher than that observed by the starting salt $AgNO_3$. It is already described in the literature that $Ag^+$ can link to the cell membrane and consequently inhibits cell divisions. Silver ions also binds to bacterial DNA and RNA and inhibits bacterial replication[37].

**Table 1.** Minimum inhibitory concentration (MIC) values of $AgNO_3$, succinic acid and $[Ag_2(succ)]$.

| | *S. aureus* ATCC 25923 | | *B. cereus* ATCC 14579 | | *E. coli* ATCC 25922 | | *P. aeruginosa* ATCC 27853 | |
|---|---|---|---|---|---|---|---|---|
| | MIC $\mu g \cdot mL^{-1}$ | MIC $mmol \cdot L^{-1}$ | MIC $\mu g \cdot mL^{-1}$ | MIC $mmol \cdot L^{-1}$ | MIC $\mu g \cdot mL^{-1}$ | MIC $mmol \cdot L^{-1}$ | MIC $\mu g \cdot mL^{-1}$ | MIC $mmol \cdot L^{-1}$ |
| $[Ag_2(succ)]$ | ≤ 78.12 | ≤ 0.23 | ≤ 78.12 | ≤ 0.23 | ≤ 78.12 | ≤ 0.23 | ≤ 78.12 | ≤ 0.23 |
| Succinic acid | R | | R | | R | | R | |
| $AgNO_3$ | ≤ 78.12 | ≤ 0.45 | ≤ 78.12 | ≤ 0.45 | ≤ 78.12 | ≤ 0.45 | ≤ 78.12 | ≤ 0.45 |

R = Resistant. Student t-test was used to determine the statistical significance for *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa* replicates. Results were expressed as the mean (***p < 0.005).


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ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p26-35

So, the use of silver complex promotes a slower release of the silver ions when compared to silver nitrate. This characteristic increases the antibacterial effect of the compound. For example, the role of silver and sulfadiazine in the mechanism of action of silver sulfadiazine on burn wound infections was investigated[38]. The efficacy of silver sulfadiazine is thought to result from its slow and steady reactions with serum and other sodium chloride-containing body fluids, which permits the slow and sustained delivery of silver ions into the wound environment. In this circumstance, a relatively minimum amount of sulfadiazine appears to be active.

Furthermore, the ligand succinic acid is an intermediate metabolic of the Krebs cycle. So, it is suggested that this characteristic promotes a higher intracellular absorption of the complex when compared to $AgNO_3$ and Ag(I) complexed to other ligands reported in the literature.

The results obtained in this work demonstrated that the $[Ag_2(C_4H_4O_4)]$ complex showed significant growth inhibition activity of the tested bacterial species, when compared with other studies that report the inhibitory activity of bacterial growth by Ag(I) complexes with several ligands, as Ag(I) with furosemide (MIC = 0.39 mmol $L^{-1}$ for gram-positive bacteria strains)[32], sulfathiazole (MIC 3.45 mmol $L^{-1}$ for gram-negative bacteria and 6.90 mmol $L^{-1}$ for Gram-positive bacteria strains) and sulfamethoxazole (MIC = 1.74 mmol $L^{-1}$ for gram-negative bacteria and 13.9 mmol $L^{-1}$ for gram-positive bacteria)[39]. The results demonstrate the potential for using the $[Ag_2(C_4H_4O_4)]$ complex as an antibacterial drug in the future.

### *3.5 Anti*-Mycobacterium tuberculosis *activity*

The $MIC_{90}$ values for $[Ag_2(C_4H_4O_4)]$, succinic acid and rifampicin were obtained through REMA. Rifampicin, the most effective first-line drug, was used as a control and showed a $MIC_{90}$ of 0.08 μg $mL^{-1}$. The silver complex with succinate was shown to be active with a $MIC_{90}$ of 23.94 μg $mL^{-1}$ while succinic acid itself presented a value higher than 25.00 μg $mL^{-1}$. In spite of being active, the $[Ag_2(C_4H_4O_4)]$ complex has a MIC value lower than that of other antimycobacterial agents used in TB clinical treatments as ethambutol ($MIC_{90}$ = 5.62 μg $mL^{-1}$) and *p*-aminosalicylic ($MIC_{90}$ = 1.25μg $mL^{-1}$).

## 4. Conclusions

A new silver complex with succinic acid was obtained and characterized. On the basis of elemental and thermogravimetric analyses, the complex was formulated as $[Ag_2(C_4H_4O_4)]$. Infrared spectra showed that the value for Δν decreases for both carboxylate groups because of the high symmetry of succinate anion compared to the free succinic acid molecule. The reduction of the Δν value indicates coordination of each carboxylate group to each Ag(I) in a bidentate mode. The theoretical structures for succinic acid and the $[Ag_2(C_4H_4O_4)]$ complex were optimized to the minimum of energy using DFT. The studies confirm that each carboxylate group of the succinate coordinates to one Ag atom by the two O atoms in a bidentate mode as suggested by the IR data and the bond lengths O···Ag theoretically range from 2.325 to 2.338 Å.

The complex shows inhibitory activity against the considered strains used in the assay with MIC values ≤ 0.23 mmol $L^{-1}$. Succinic acid, $K^+$-succinate and DMSO aqueous solution were inactive. Anti-*Mycobacterium tuberculosis* analyses were also performed with $[Ag_2(C_4H_4O_4)]$ and succinic acid. The $[Ag_2(C_4H_4O_4)]$ complex was shown to be active presenting a $MIC_{90}$ of 23.94 μg $mL^{-1}$ while succinic acid itself presented a value higher than 25.00 μg $mL^{-1}$. The results obtained indicate the significant activity *in vitro* of the silver complex with succinic acid and warrants for additional studies in the search of safer silver-based antimicrobial drugs.

## Acknowledgments

This study was supported by grants from the Brazilian Agencies FAPESP (São Paulo State Research Council, Grants #2018/12590-0, #2017/11570-3 and 2018/12062-4), CNPq (National Council of Scientific and Technological Development, Grant #300968/2016-7) and FUNADESP (National Foundation for the Development of Private Higher Education, Grant #2700375). Douglas H. Pereira also acknowledges the

Center for Computational Engineering and Sciences (Financial support from FAPESP, Grants #2013/08293-7 and #2017/11485-6) and the National Center for High Performance Processing (CENAPAD) in São Paulo for the computational resources. This study was partially financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001.

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Eclética Química Journal, vol. 46, n. 2, 2021, 26-35
ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p26-35

Eclética Química Journal

Original article

iq.unesp.br/ecletica

| Vol. 46 | n. 2 | 2021 |

# Nitrogen, phosphorous and potassium polymeric microparticles: application and validation of analytical methods for determination of a promising fertilizer

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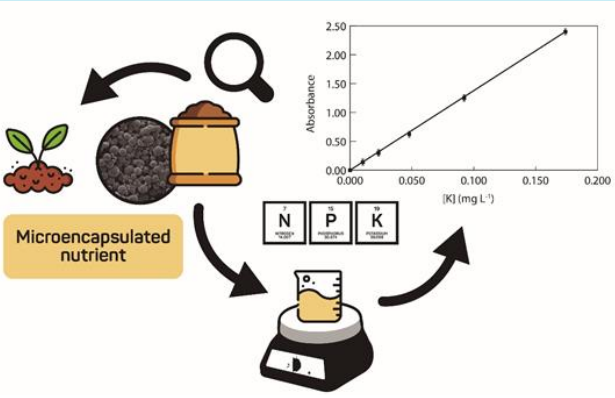
**ARTICLE INFO**

*Article history:*
**Received:** June 19, 2020
**Accepted:** September 10, 2020
**Published:** April 01, 2021

*Keywords*
1. polymeric microparticles
2. encapsulated fertilizers
3. Kjeldahl method
4. stannous chloride method
5. flame atomic absorption spectroscopy

**ABSTRACT:** Traditional analytical methods were applied to microparticles synthesized using a mixture of polymers. The microparticles containing nitrogen, phosphorus and potassium (NPK) were developed for application as a controlled release alternative to traditional fertilizers. Thus, analytical methods for the evaluation of nitrogen, phosphorus and potassium (essential nutrients for plants) in polymeric microparticles were applied and the results validated for this kind of sample. The linearity of the methods was established in the range of 10 to 20 mg $L^{-1}$ for N, 0 to 2 mg $L^{-1}$ for P and 0 to 2.4 mg $L^{-1}$ for K. The relative standard deviation (RSD) of intermediate precision of the method was < 1% and recoveries from 99 to 108% for all nutrients were reached. The method proved to be selective, linear, precise and accurate for quantifying NPK in polymeric microparticles. In this way, it was possible to evaluate the correct nutrient encapsulation capacity in the synthetized polymeric microparticles, which is of fundamental importance since this will directly interfere in the success of the intended fertilization process.




Methods that allow to evaluate the correct encapsulation capacity of nitrogen, phosphorus, and potassium in microparticles with fertilization purposes are of great importance, since these controlled release systems promise to be more efficient and minimize the environmental impacts compared to conventional fertilization.

## 1. Introduction

Controlled release polymeric microstructured systems can be defined as solid colloidal particles with a maximum size of 1000 µm, which carry an active substance for subsequent slow and controlled release in the medium for which they have been developed[1]. Due to this property, these systems have some advantages, including reduction in the number of chemical substances needed in their applications, propensity to reduce the risk of contamination, ability to reduce the amount of energy spent since the number of necessary applications is reduced and to maintain the concentration of the substance for a greater period of time[2–5].

In this way, this technology allows a change in the physicochemical properties of the active principles, making it an interesting alternative for the controlled/proper release of nitrogen, phosphorus and potassium (NPK), since traditional fertilizers may lose part of their nutrients when applied to the environment, leading to unwanted environmental contamination[6–9]. Therefore, the controlled/modified release technology becomes an important alternative that minimizes the problems caused by traditional fertilizers.

There are several determining factors in the choice of the preparation method, namely: the type of polymer(s) to be used, the application site of the particles and the desired size, among others[10,11]. In view of this, the emulsification solvent diffusion method is attractive, given that it is easy to apply and it is highly reproducible and compatible with biodegradable polymers. The emulsification solvent diffusion method was first described by Fessi (1988)[12]. It consists of the interfacial polymer deposition after the displacement of a water-miscible semipolar solvent from a lipophilic solution[13,14]. This method has been extensively reported in the literature and applied to a wide range of active principles[15–17].

There are some studies in the literature that have used poly(ε-caprolactone) (PCL) for the synthesis of microparticles carrying several micro-active substances of interest, such as pharmaceuticals and herbicides, via the emulsification solvent diffusion method, among others[18–23]. Poly(ε-caprolactone) is an aliphatic biodegradable polyester, insoluble in water, that has slow degradation in aqueous medium and poses no environmental harm. As a result, it is chosen for the synthesis of carrier systems.

In addition to aliphatic polyesters, alginate polymers can be used for the synthesis of controlled release systems[24,25]. The properties of polyglycerol (PG) are like those of alginate polymers and is obtained by glycerine polymerisation, which is abundantly generated during biodiesel production. For this reason, the development of processes that fosterers the use of this residual glycerine is of great importance, because it exhibits an interesting environmental aspect. One of them[26] is using this residual glycerine via PG to synthesise controlled release systems, providing an alternative use/reuse of this large-scale produced industrial waste with no relevant applications.

In view of the characteristics, the microparticulate system synthesized with PCL and PG developed in the present study was used to encapsulate three essential nutrients for plants, nitrogen, phosphorus and potassium. These nutrients are classified as macronutrients because they are necessary at higher concentrations (mmol $kg^{-1}$ – dry matter), compared with other essential nutrients used for plants[27].

Nitrogen is essential because it promotes vigorous plant growth and is one of the responsible substances for protein development. Phosphorus promotes cell division and the formation of the cell structures of the plants. It stimulates healthy root growth, is essential for seed germination and assists in the conversion of solar energy into chemical energy for the photosynthesis process. Potassium promotes the formation of fruits and confers resistance to diseases and high temperatures. It is essential for photosynthesis and is responsible for maintaining water levels in the plants[27–29].

There are few reports in the literature[30,31] concerning the quantification of NPK in microstructured release polymeric systems. Therefore, the application and validation of analytical methods for the quantification of these chemicals in such systems is important, since these particles can provide a new direction for fertilization procedures. Consequently, the goal of the present study was to apply and validate analytical methods for determining NPK in polymeric


Eclética Química Journal, vol. 46, n. 2, 2021, 36-47
ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p36-47

microparticles obtained by the emulsification solvent diffusion method, with fertilization purposes.

## 2. Experimental

### *2.1 Reagents and solutions*

The following reagents were used for the synthesis of the microparticles: boric acid (Sigma Aldrich); nitric acid (Sigma Aldrich); sulphuric acid (Synth); methyl orange (Sigma Aldrich); polyvinyl alcohol (PVA) (Sigma Aldrich); methylene blue (Sigma Aldrich); sodium carbonate (Synth); stannous chloride (Sigma Aldrich); chloroform (Sigma Aldrich); ethanol (Sigma Aldrich); phenolphthalein (Synth); glycerol (Synth); sodium hydroxide (Sigma Aldrich); ammonium molybdate (Sigma Aldrich); phosphorus, nitrogen, and potassium (999 ± 4 mg $L^{-1}$) (Sigma Aldrich); poly (ε-caprolactone) (Sigma Aldrich); polyglycerol (70wt.%) (Verti Ecotechnologies UFMG); copper sulphate (Sigma Aldrich) and methyl red (Sigma Aldrich).

All solutions were prepared using analytical-grade reagents and the final volumes were adjusted with deionized water (Milli-Q system - Millipore Corporation). The glassware was previously washed in baths containing 10%v/v nitric acid ($HNO_3$) (Synth) for 24 h. All solutions used were stored and kept at 8 °C for preservation.

### *2.2 Instrumentation*

The instruments used in this study were: magnetic stirrer (Hot Lab II Nalgon), hot plate (Hidrosan; 50 to 320 °C), macro-Kjeldahl distillation apparatus (Quimis), oven (Ethik Technology, Nova Ética - 404/D; 50 to 200 °C), atomic absorption spectrophotometer (SpectrAA 50 B - Varian) and spectrophotometer (UV-Vis 220-2000UV - Biospectro).

### *2.3 Preparation of microparticles*

The polymeric microparticles were prepared according to the emulsification solvent diffusion method described by Chagas *et al.*[30] and involves the interfacing deposition of the polymer after the displacement of a semipolar solvent, miscible in water, from a lipophilic solution. For this study, the organic phase was prepared with PCL and PG polymers. The aqueous phase was composed of PVA, deionized water and an aliquot of NPK standard solution. After the dissolution of the components of both phases, the organic phase was added to the aqueous one.

### *2.4 Kjeldahl method for quantification of nitrogen in polymeric microparticles*

The quantification of nitrogen was carried out according to the Kjeldahl method. It consisted of digesting 100 mL of the sample with 50 mL of digesting solution (134 g of potassium sulphate and 7.3 g of copper sulphate, weighted in an analytical balance, and 134 mL of sulfuric acid for 1 L of solution) in a hot plate at 320 °C. Then, 300 mL of distilled water were added along with 50 mL of sodium hydroxide (50% w $v^{-1}$)/sodium thiosulphate (2.5% m $v^{-1}$) solution to the solution resulting from the digestion. This mixture was made in a Kjeldahl flask using a measuring cup. Subsequently, the sample was submitted to distillation in a traditional Kjeldahl distiller. The product of this reaction was collected in 50 mL boric acid solution presented in an Erlenmeyer flask. This acid solution was produced with 0.0200 g of methyl red, 10 mg of methylene blue and 20 g of boric acid, weighed in analytical balance, in ethanoic acid medium. Then, the titration of the distilled solution with sulphuric acid (0.01 mol $L^{-1}$) was performed[32]. The analytical curve was constructed with serial dilutions of a standard nitrogen solution (1000 mg $L^{-1}$).

### *2.5 Stannous chloride method for the quantification of phosphorus in polymeric microparticles*

The quantification of phosphorus was made according to the stannous chloride method[32]. It was performed a 100-times dilution of the particulate suspension, in which the diluted samples were digested with 1 mL of concentrated sulphuric acid and 5 mL of concentrated nitric acid in a hot plate at 250 °C. Later,


Eclética Química Journal, vol. 46, n. 2, 2021, 36-47
ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p36-47

20 mL of distilled water and 0.05 mL of phenolphthalein were added to the filtrate solution. This solution was neutralised with sodium hydroxide solution (NaOH) (6.00 mol $L^{-1}$). Subsequently, this solution was titrated with a solution obtained by mixing concentrated sulfuric and nitric acids. Finally, 4 mL of ammonium molybdate solution (25 g of ammonium molybdate, weighted in analytical balance, and 280 mL of sulphuric acid for one liter of solution) and 0.50 mL of the stannous chloride solution (2.5 g of stannous chloride, weighted in analytical balance, for 100 mL of glycerol) were added. Then, the solution was analyzed in a UV-Vis spectrophotometer at 690 nm with a 10 mL glass cuvette. The analytical curve was constructed with serial dilutions of a standard phosphorus solution (1000 mg $L^{-1}$).

### *2.6 Development of the potassium quantification method in polymeric microparticles*

The quantification of potassium was carried out by flame atomic absorption spectrophotometry, in which the conditions were: 5 mA for the lamp current, air/acetylene flame and air support, flame stoichiometry adjusted to obtain an oxidizing environment, wavelength of 766.55 nm and slot width of 1.0 nm. For the preparation of the samples, a multivariate optimization study was conducted to adequate the ideal conditions, previously studied and reported by the research group involved in this work[32]. The most efficient digestion occurred with 5 mL of the filtrate solution and 10 mL of concentrated nitric acid at 60 °C under magnetic stirring for 90 min. The solutions resulting from the digestion were measured in a 100 mL volumetric flask and later analyzed for the quantification of potassium. The analytical curve was constructed with serial dilutions of a standard potassium solution (100 mg $L^{-1}$).

### *2.7 Methods validation*

The quantification methods were validated according to a guideline document (DOQ-CGCRE-008: 3 Revision - Feb/2010) of the National Institute of Metrology, Standardization and Industrial Quality (INMETRO)[33].

### *2.8 Specificity*

To confirm the specificity of the methods, the absence of interference caused by substances that are part of the microparticles (PCL, PG and PVA) was checked. In this way, the microparticles were prepared without the nutrients (NPK) and analyzed under the same experimental conditions of samples with the presence of nutrients.

### *2.9 Linearity, limit of quantification and limit of detection*

The linearity of the methods was determined using three analytical curves carried out in three different days, generating a total of nine curves with several concentrations diluted in water. Three analyses of each solution were performed to check the repeatability of the methods responses in each concentration. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the slope and the standard deviation of the mean intercept in three calibration curves of each method.

### *2.10 Accuracy*

The accuracy of the methods was assessed by the standard addition of solutions with known concentrations of each nutrient to the samples of microparticle suspensions (without nutrients). The recovery was determined as the percentage difference between the average experimental concentration and the theoretical concentration at each level.

### *2.11 Precision*

The precision was determined by means of repeatability (intraday) and intermediate precision (interday). The repeatability was assessed analyzing samples of microparticles (without NPK) with standard addition of each nutrient solution on the same day and under the same experimental conditions. For the intermediate precision, the analyses were performed on three different days.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### *2.12 Compatibility of the samples*

The matrix can have a considerable effect on the way the chemical analysis is conducted and in the quality of the results. Such effects are called matrix effects. The most common approach for the compatibility of matrix effects is the construction of a calibration curve with standard samples of known analyte concentrations, which attempts to approximate the matrix of the sample as much as possible[34].

Thus, samples of 5 mL aliquots of previously digested polymeric microparticles were transferred into 100 mL volumetric flasks. It was added N, P and K standard solutions into these flasks to obtain the analytical curves for every chemical element. The analyses were performed in triplicate for each used method. An acid solution previously prepared according to each method was used as a solution with no matrix effects.

### *2.13 Tendency/recovery*

When applied to a series of test results, tendency implies a combination of random and systematic error components. The latter cannot be admitted. Tendency can be expressed as analytical recovery defined by the Eq. 1[33].

$$\text{Recovery} = \frac{\text{observed value}}{\text{expected value}} \times 100\% \quad (1)$$

### *2.14 Determination of encapsulation efficiency*

The percentage rate of nutrients combination (NPK) [encapsulation efficiency (EE)] in the microparticles is of great importance, because it is the condition that will indicate the concentration of the active principle that the polymeric particles are able to blend for the subsequent controlled release[35,36].

The percentage of NPK blend with the microparticles was determined by specific methods for each chemical element. Samples of 100 mL microparticles suspension containing NPK were filtered in quantitative filter paper (slow filtration - blue ribbon 3552, Nalgon). Then, the EE was determined by the difference between the quantification of nutrients concentrations in the filtrate solution and their total concentration (100%) present in the microparticles suspension (Eq. 2)[35,36].

$$\text{EE (\%)} = \frac{(X_o - X_f)}{X_o} \times 100\% \quad (2)$$

where $X_o$ is the nutrient concentration quantified in the solution of microparticles and $X_f$ is the nutrient concentration quantified in the filtered solution of microparticles.

### *2.15 Morphological analysis of the particles*

The particle synthesis procedure reported in the Chagas *et al.*[30], was performed. In this way, the suspension obtained was filtered and the retained material was dried in a desiccator. After complete drying, 10 mg of the sample were placed on a carbon ribbon. The samples were taken to a vacuum metallizer for the deposition of gold (100 to 200 nm). Then, the morphology of the samples of metallized particles using a scanning electron microscope (SEM) were analyzed. Size distributions were measured and expressed as means of three determinations.

## 3. Results and discussion

### *3.1 Morphological analysis of the particles*

The SEM analysis of the particles indicated that the formulation used for the synthesis was efficient (Fig. 1). Spherical particles without aggregates were observed. The average size ranged from 5 to 60 µm and the particles were classified as microparticles. To be considered nanoparticles, the average diameter should be less than 1000 nm[30,31, 34–36], as presented in a previous work[30].


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ISSN: 1678-4618
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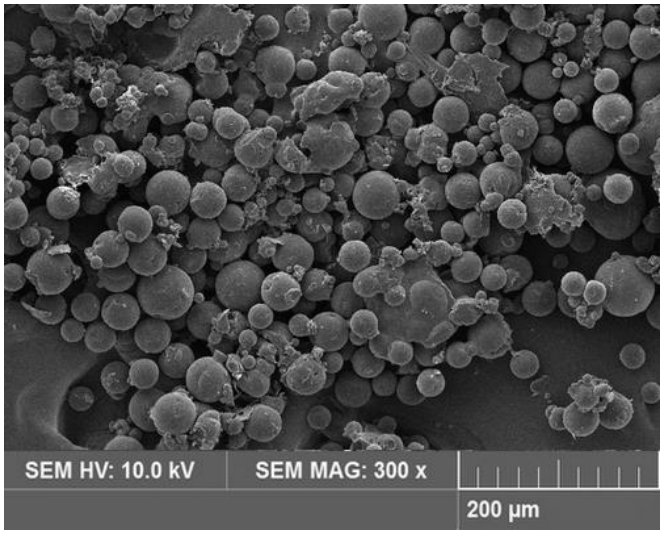


**Figure 1.** Scanning electron microscope images of the polymeric microparticles containing the nutrients NPK at 300 × magnification. SEM HV = scanning electron microscope high vacuum; MAG = magnification.

### *3.2 Validation of the proposed analytical methods*

The linearity of the analytical methods was assessed using linear regressions of nine analytical curves for each nutrient and each point on the curve was analyzed three times (intraday-interday). The correlation coefficients (r) of the mean analytical curves (n = 9) and the respective parameters (A and B) are shown in Tab. 1. The validity of the regression is observed when the slope of the curve is significantly different from zero, which is represented by values of $F_{cal}$ (calculated F) less than that of $F_{tab}$ (tabulated F). The calculated values for F of the three nutrients (NPK) are shown in Tab. 1, they are smaller than the $F_{tab}$ value, thus it is assumed that the slope of the line is not zero and the linear curve fit is accepted for both nutrients with $p < 0.0001$. In this way, it can be affirmed with 95% confidence that the model is linear and well-adjusted in the concentration range studied[37]. The correlation coefficient is a parameter that allows to estimate the quality of the obtained curve because the closer to 1, the smaller the dispersion of the experimental points. The National Health Surveillance Agency (ANVISA/Brazil) and the National Institute of Metrology, Standardization and Industrial Quality (INMETRO/Brazil) recommend a correlation coefficient greater than or equal to 0.99[33]. All linear correlation values shown in Tab. 1 for R were above 0.99, therefore, each method was considered linear[33,34]. The results indicate that the sampling conditions proposed in this study are adequate for the analysis of NPK in the investigated levels, 10.00 to 20.00 mg $L^{-1}$ for N; 0 to 2.40 mg $L^{-1}$ for P and 0 to 2.00 mg $L^{-1}$ for K[30].

**Table 1.** Parameters obtained with the linear regressions of the analytical curves for NPK nutrients.

| Nutrient | Interval | Parameters | Results | Statistic α < 0.05 |
|---|---|---|---|---|
| Nitrogen | 10.00 to 20.00 mg $L^{-1}$ | Slope | 0.3 ± 0.1 | $F_{cal}$ = 3.9253<br>$F_{tab}$ = 4.62 |
| | | Intercept | 0.2 ± 0.3 | $F_{cal}$ = 1.0692<br>$F_{tab}$ = 4.62 |
| | | R | 0.999 ± 0.001 | |
| Phosphorus | 0.00 to 2.00 mg $L^{-1}$ | Slope | 1.859 ± 0.004 | $F_{cal}$ = 1.4100<br>$F_{tab}$ = 4.62 |
| | | Intercept | 0.015 ± 0.007 | $F_{cal}$ = 1.3856<br>$F_{tab}$ = 4.62 |
| | | R | 0.9992 ± 0.0003 | |
| Potassium | 0.00 to 2.40 mg $L^{-1}$ | Slope | 0.072 ± 0.008 | $F_{cal}$ = 8.4279<br>$F_{tab}$ = 39.45 |
| | | Intercept | 0.0026 ± 0.0007 | $F_{cal}$ = 1.3074<br>$F_{tab}$ = 4.62 |
| | | R | 0.9993 ± 0.0007 | |

NPK = nitrogen, phosphorus, and potassium; R = correlation coefficient; $F_{cal}$ = calculated F; $F_{tab}$ = tabulated F.


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Repeatability, or intraday precision, was evaluated by the determination of the intraday RSD and the intermediate precision, or interday precision, was determined by the interday RSD. The RSD values are shown in Tab. 2.

**Table 2.** Values of the relative standard deviation (RSD) of NPK concentrations (C) for assessing the methods precision.

| Nutrient | C / mg $L^{-1}$ | RSD - day 1 / % | RSD - day 2 / % | Interday RSD / % |
|---|---|---|---|---|
| **N** | 5.00 | 0.72 | 0.81 | 0.72 |
| | 10.00 | 2.02 | 2.14 | 2.52 |
| | 15.00 | 2.95 | 4.30 | 1.73 |
| **P** | 0.45 | 2.18 | 3.45 | 2.67 |
| | 0.75 | 0.78 | 1.32 | 3.52 |
| | 1.15 | 1.77 | 2.28 | 1.77 |
| **K** | 0.04 | 1.50 | 1.50 | 1.44 |
| | 0.60 | 4.03 | 1.61 | 1.53 |
| | 1.20 | 1.74 | 3.85 | 4.94 |

C = concentration; RSD = relative standard deviation.

The intra and interday RSD values determined in Tab. 2 were all below the limit established by ANVISA and the Institute of Human Sciences (IHC), which is a maximum of 5%[33,34]. Therefore, the methods could be considered accurate. The accuracy was determined by experimentally determined concentration values, compared with the theoretical concentrations, as shown in Tab. 3.

**Table 3.** Nitrogen, phosphorus, and potassium concentration values determined for assessing the methods accuracy.

| Nutrient | Theoretical concentration / mg $L^{-1}$ | Empirical concentrations / mg $L^{-1}$ | Recovery / % |
|---|---|---|---|
| **N** | 5.00 | 5.39 | 107.80% |
| | 5.00 | 5.37 | 107.40% |
| | 5.00 | 5.45 | 109.00% |
| | 10.00 | 10.48 | 104.80% |
| | 10.00 | 10.34 | 103.40% |
| | 10.00 | 10.21 | 102.10% |
| | 15.00 | 15.20 | 101.30% |
| | 15.00 | 15.00 | 100.00% |
| | 15.00 | 14.80 | 98.60% |
| **P** | 0.25 | 0.24 | 96.00% |
| | 0.25 | 0.26 | 108.00% |
| | 0.25 | 0.24 | 96.00% |
| | 0.55 | 0.53 | 96.00% |
| | 0.55 | 0.53 | 96.00% |
| | 0.55 | 0.54 | 98.18% |
| | 1.15 | 1.20 | 104.34% |
| | 1.15 | 1.19 | 103.48% |
| | 1.15 | 1.14 | 99.14% |
| **K** | 0.06 | 0.06 | 101.67% |
| | 0.06 | 0.05 | 98.33% |
| | 0.06 | 0.05 | 98.33% |
| | 0.30 | 0.32 | 106.60% |
| | 0.30 | 0.32 | 108.00% |
| | 0.30 | 0.31 | 103.30% |
| | 1.20 | 1.16 | 97.25% |
| | 1.20 | 1.18 | 98.33% |
| | 1.20 | 1.14 | 95.50% |

N = nitrogen; P = phosphorus; K = potassium.


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Accuracy is the relationship between experimentally determined mean concentrations and the corresponding theoretical ones. The recovery percentage varied from 95.50 to 108%. In this way, all results achieved the established acceptance criteria for concentrations (from 95 to 110%) and the methods were considered accurate[33,34]. The LOD and LOQ using the data obtained with the linear regressions were also determined (Tab. 4).

**Table 4.** Limit of detection (LOD) and limit of quantification (LOQ) values for the developed methods to analyse NPK in polymeric microparticles.

| Nutrient | LOD / mg $L^{-1}$ | LOQ / mg $L^{-1}$ |
|---|---|---|
| N | 5.04 | 5.32 |
| P | 0.046 | 0.110 |
| K | 0.042 | 0.051 |

The compatibility of the samples was assessed through the experimentally determined concentration values in solutions prepared with solvents, compared with solutions prepared without NPK (Figs. 2, 3 and 4).

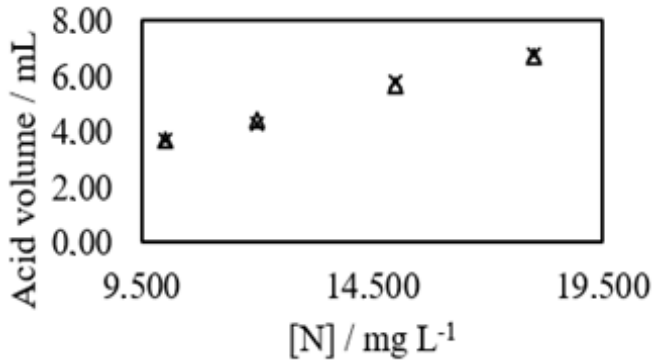


**Figure 2.** Curves for the assessment of compatibility of samples in the analyses of N from polymeric microparticles using the Kjeldahl method (n = 3).
Triangle = curve prepared with solutions in solvent. X = curve prepared with polymeric microparticles.

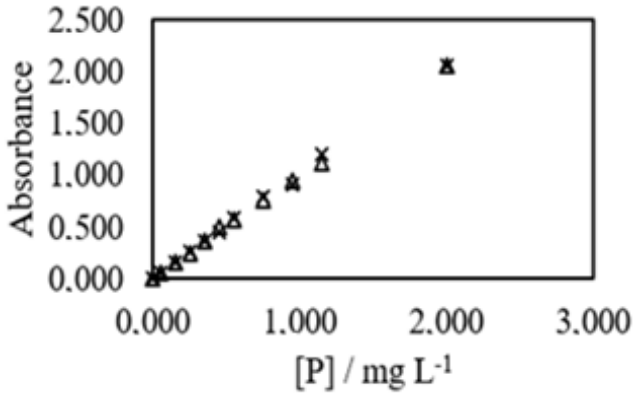


**Figure 3.** Curves for the assessment of compatibility of samples in the analyses of P from polymeric microparticles using the stannous chloride method (n = 3).
Triangle = curve prepared with solutions in solvent. X = curve prepared with polymeric microparticles.

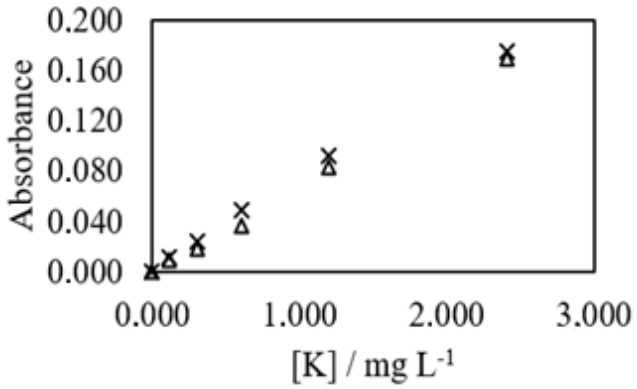


**Figure 4.** Curves for the assessment of compatibility of samples in the analyses of K from polymeric microparticles using flame atomic absorption spectrophotometry (n = 3).
Triangle = curve prepared with solutions in solvent. X = curve prepared with polymeric microparticles.

The correlation coefficients of the analytical curves of solutions in solvent medium and microparticle medium (n = 3) with their respective parameters (A and B) of the regression equations presented in Figs. 2, 3 and 4 are shown in Tab. 5.


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**Table 5.** Parameters obtained with the linear regressions of the analytical curves of solutions in solvent medium and microparticle medium for the NPK nutrients.

| Nutrient | Solutions in solvent medium | | | Solutions in microparticle medium | | |
|---|---|---|---|---|---|---|
| | A | B | R | A | B | R |
| N | 0.3906 | 0.2468 | 0.9987 | 0.4044 | 0.4262 | 0.9972 |
| P | 1.0261 | 0.0040 | 0.9987 | 1.0117 | 0.0025 | 0.9984 |
| K | 0.0586 | 0.0028 | 0.9990 | 0.0577 | 0.0018 | 0.9993 |

A = slope; B = intercept; R = correlation coefficient.

The results obtained, showed in Tab. 5, indicate that the correlation and the angular and linear coefficients of the curves had no significant difference. Therefore, the methods used did not require compatibility of samples and the analytical curves prepared in solvents could be used.

All validation parameters assessed for the analytical methods proposed were considered satisfactory since they met the specifications established by ANVISA and INMETRO[33,37]. Therefore, the methods could be considered s pecific, linear, precise and accurate. As a result, they are applicable in analysis for determining NPK in polymeric microparticles.

### *3.3 Application of the proposed methods to determine the encapsulation efficiency and NPK content*

The microparticles obtained were analyzed using each specific method previously validated in this work to determine the efficiency of NPK encapsulation. The encapsulation values were then determined by the difference between the total concentration (100%) of the nutrients present in the microparticle suspension and the quantified ones in the filtered solution of microparticles[30]. The results are shown in Tab. 6.

The values of EE percentage shown in Tab. 6 were satisfactory, since there are few studies in the literature assessing the EE percentage for the active substances proposed in the present study with the use of polyglycerol. In addition, these values were within the averages shown in the literature for other active principles[18–23,38–41]. The differences in the values for the encapsulation showed in Tab. 6 are due to the results related to the multivariate experimental designs employed, in which each assay promotes different levels of the investigated parameters and, as a result, a different response can be generated. This study was previously discussed and published by this group[30].

**Table 6.** Results of encapsulation efficiency (EE) through the application of the developed analytical methods for the quantification of NPK in polymeric microparticles.

| Sample | EE per nutrient / % | | |
|---|---|---|---|
| | Nitrogen | Phosphorus | Potassium |
| **01** | 96.27 | 96.58 | 59.62 |
| **02** | 83.80 | 77.80 | 48.60 |
| **03** | 93.68 | 77.60 | 27.80 |
| **04** | 87.64 | 99.20 | 43.00 |
| **05** | 70.08 | 80.60 | 29.40 |
| **06** | 47.58 | 91.60 | 45.20 |
| **07** | 61.85 | 81.00 | 34.60 |
| **08** | 78.31 | 95.60 | 33.00 |
| **09** | 78.31 | 96.20 | 36.20 |

The highest values of the nitrogen and phosphorus encapsulations in polymeric microparticles were because these nutrients feature high aqueous solubility, which promotes high interaction with PG[4]. The lowest rate of potassium encapsulation, in comparison with the other nutrients, can be explained by the interaction competition between N and P. The developed and validated analytical methods are applicable to samples of polymeric microparticles.

Although the analytical methods applied here are well established in the literature, from the best of the author's knowledge, their application to the types of samples described in this research were not found, except to those previously published by our research group[30].

## 4. Conclusions

The proposed analytical methods for detection and quantification of NPK in microparticles, i.e., Kjeldahl,


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ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p36-47

stannous chloride and flame atomic absorption spectrophotometry, respectively, proved to be specific, linear, accurate and precise in the concentration ranges assessed. They are suitable for determining encapsulation efficiency and NPK contents in poly(ε-caprolactone) and polyglycerol microparticles.

## Acknowledgments

The authors are thankful to: Chemical Network of Minas Gerais [Rede Mineira de Química (RQ-MG) (FAPEMIG CEX - RED-00010-14)]; National Council for Scientific and Technological Development – CNPq [Conselho Nacional de Desenvolvimento Científico e Tecnológico (Project No. 479273/2013-8)]; Foundation for Research Support of the State of Minas Gerais [Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG)] and the Research and Postgraduate Department [Pró-Reitoria de Pesquisa e Pós-Graduação (PROPP)] of the Federal University of Ouro Preto (UFOP) for their financial support and scholarships granted. The authors are especially thankful to the Laboratory of Environmental Sanitation under the coordination of Professor Aníbal Santiago, PhD, and Professor Hubert Mathias Roeser, PhD; and to Verti EcoTecnologias Ltd.

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Eclética Química Journal, vol. 46, n. 2, 2021, 36-47
ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p36-47

Eclética Química Journal

Original article

iq.unesp.br/ecletica

| Vol. 46 | n. 2 | 2021 |

# Heterogeneous photodegradation of bisphenol A and ecotoxicological evaluation post treatment

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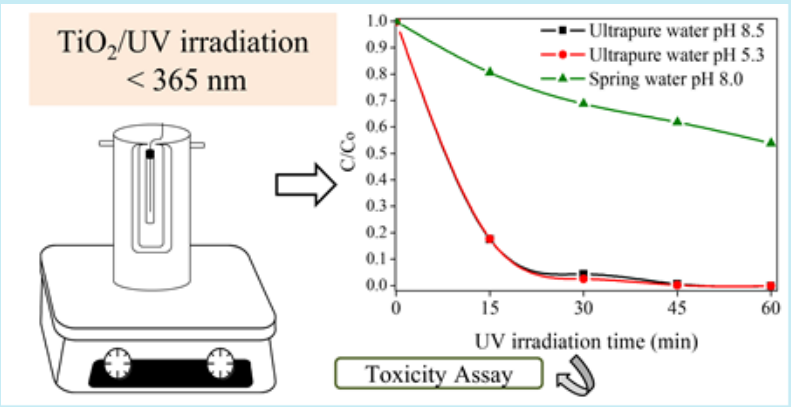
**ARTICLE INFO**

*Article history:*
**Received:** May 12, 2020
**Accepted:** September 08, 2020
**Published:** April 01, 2021

*Keywords*
1. emerging contaminants
2. titanium dioxide
3. ecotoxicological assays
4. *Daphnia similis*
5. *Raphidocelis subcapitata*

**ABSTRACT:** Bisphenol A (BPA) is an emerging pollutant with endocrine disrupting properties that can be found at trace levels in various aqueous environments. Conventional water and wastewater treatments are not designed to efficiently remove these substances. Therefore, this work investigates the removal of BPA by an Advanced Oxidation Process (AOP), specifically heterogeneous photocatalysis using $TiO_2$. The influences of the $TiO_2$ concentration (1.0–10.0 mg $L^{-1}$), pH (5.3 and 8.5) and effects matrix composition were studied for the removal of BPA at a concentration of 0.8 mg $L^{-1}$. The results indicated that BPA was completely removed after 45 min of treatment using 7.5 and 10 mg $L^{-1}$ of $TiO_2$, under constant aeration and artificial UV irradiation, at the different pH values. The use of solar radiation as an UV source was also effective, removing BPA after 60 min of irradiation at pH without adjustment, as well as at pH 8.5. Ecotoxicological evaluation indicated that the post-treatment samples did not present acute effects towards *Daphnia similis*. Evaluation of chronic toxicity with *Raphidocelis subcapitata* showed that there was a reduction in the negative effect of BPA under the growth rate of algae biomass after 60 min of treatment, compared to the initial sample.





ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p48-56

## 1. Introduction

The endocrine disruptors (EDs) are a class of substances that can interfere in the natural functioning of the endocrine system, resulting in health problems in animals and humans[1]. Several studies have associated exposure to EDs with disturbance of the human reproduction system, with effects including infertility, endometrioses, breast cancer, prostate cancer and decreased sperm production[2]. Substances such as natural and synthetic hormones, as well as some drugs, are included in this class of substances[2,3]. Endocrine disruptors have been found at trace levels (from μg $L^{-1}$ to ng $L^{-1}$) in various aqueous environments, including superficial water, groundwater, domestic wastewater, and even potable water[4].

Among the synthetic compounds that are considered EDs, one that is of increasing concern is bisphenol A [BPA, 2,2-bis(4-hydroxyphenyl) propanol]. The worldwide production of BPA in 2015 exceeded 5.4 million tons. The BPA monomer is used in the production of polycarbonate plastics, epoxy resins, stickers, pipes, fire retardants and thermal papers. The main route of human exposure to BPA is by food and potable water consumption[5–9].

Conventional water and wastewater treatments present limitations in terms of the ability to efficiently remove EDs[10]. Therefore, several alternative treatments are being investigated in order to improve their removal. Among these, the advanced oxidation processes (AOPs) have shown high efficiency in the removal of organic contaminants, including BPA, from aqueous systems[9,11,12].

During AOPs, highly reactive radicals, especially the hydroxyl radical (•OH, with E° = 2.8 V), are generated, which can mineralize organic compounds to $CO_2$, $H_2O$ and inorganic ions[13]. Heterogeneous photocatalysis using $TiO_2$/ultraviolet (UV) is an example of an AOP. Under ultraviolet irradiation (UV) with energy higher than the bandgap of the $TiO_2$, electrons from the valence band (VB) are excited to the conduction band (CB), resulting in an electron/hole pair ($e^-/h^+$) that can interact with adsorbed water or oxygen molecules, generating oxidant radicals[14–16].

Organic contaminants and their degradation products can present potential risks to the environment[17], with intermediate compounds sometimes being less biodegradable or more toxic than the parent compounds. The use of ecotoxicology bioassays represents a useful tool for evaluation of exposure to these substances[18].

The literature shows a great number of studies[8,9,11] using AOP for the removal of BPA from aqueous matrixes; however, there are few works of the ecotoxicological behavior of the samples after AOP. The main objectives of the present work were to evaluate the efficiency of heterogeneous photocatalysis using $TiO_2$/UV for the removal of BPA (at a concentration of 0.8 mg $L^{-1}$), as well as to investigate the possible toxic effects of the treated samples towards two trophic levels of aquatic organisms, namely the microalga *Raphidocelis subcapitata* and the microcrustacean *Daphnia similis*.

## 2. Experimental

### *2.1 Chemicals*

Bisphenol A ($C_{15}H_{16}O_2$, purity ≥ 99.0%) was purchased from Sigma-Aldrich (Brazil). Acetonitrile [high performance liquid chromatography (HPLC) grade] was obtained from Dinamica (Brazil). The $TiO_2$ (P25) was from Evonik (Brazil). Stock solutions of 1000 mg $L^{-1}$ BPA were prepared in acetonitrile:water (60:40 v/v) and were stored at 8 °C, protected from light. High-purity water was produced using a Milli-Q (Millipore) purification system.

### *2.2 Photodegradation study*

A double-jacketed borosilicate glass reactor, as described by Kondo and Jardim[15], was used for the BPA photodegradation assays. A mercury vapor lamp (125 W, $\lambda_{max}$ of 365 nm), delivering total accumulated radiation of 55.2 J $cm^{-2}$ after 60 min, was installed in the reactor. The sample solution consisted of a 1.0 L volume containing 0.8 mg $L^{-1}$ BPA. The lamp was turned on 10 min before initiation of the degradation process. Assays were performed without pH adjustment (pH 5.3) and with adjustment to pH 8.5 using 1.0 mol $L^{-1}$ solutions of NaOH or HCl. The $TiO_2$ was added (1.0–10 mg $L^{-1}$) and the suspension was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ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p48-56

irradiated for 1 h at room temperature (23 °C) under constant aeration at a flow rate of 60 mL $min^{-1}$. Samples were withdrawn at 0, 15, 30, 45 and 60 min, filtered through an ester membrane (0.45 μm pore size) and stored prior to subsequent analysis by HPLC. The natural degradation of BPA was evaluated using a solution containing 0.8 mg $L^{-1}$ BPA, which was kept in the dark for 10 weeks. Control assays were also performed using $TiO_2$, UV irradiation and aeration separately.

Solar radiation was also used as a source of UV. Suspensions (1 L volume) containing the same initial concentration (0.8 mg $L^{-1}$) of BPA and 10 mg $L^{-1}$ of $TiO_2$ were placed in a glass container (30 × 23 cm, 2.8 cm high) and kept under solar irradiation for 1 h (average total radiation of 11.3 J $cm^{-2}$). These assays were performed in the city of Itajubá, in the state of Minas Gerais, Brazil (latitude 22°24'45'', longitude 45°26'58'', 850 m above sea level), during July 2017.

### *2.3 Chromatographic analysis*

The BPA concentration was monitored by HPLC using an Agilent 1260 Infinity Series chromatograph equipped with a fluorescence detector and a ZORBAX SB-C8 Rapid Resolution HT column (1.8 μm × 3.0 mm × 150 mm). The mobile phase was a mixture of acetonitrile and ultrapure water (60:40 v/v), under isocratic conditions for 10 min, at a flow rate of 0.20 mL $min^{-1}$. The column temperature was 45 °C. The sample volume was 10 μL and BPA was detected using excitation and emission wavelengths of 275 ($\lambda_{excitation}$) and 300 nm ($\lambda_{emission}$). Validation of the method was carried out according to the guidelines provided by ANVISA (Brazilian National Health Surveillance Agency), considering the following parameters: selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and robustness[19].

### *2.4 Acute toxicity test with Daphnia* similis

The acute toxicity assay was performed according to Brazilian Association of Technical Standards (NBR 12713/2016)[20]. Samples of natural spring water were spiked with 0.8 mg $L^{-1}$ of BPA, the pH was adjusted to 8.0 and the photocatalytic process was conducted using 10 mg $L^{-1}$ of $TiO_2$ under artificial UV irradiation. Samples were removed at different oxidation times (0, 15, 30, 45 and 60 min), followed by filtration (10 mL) and introduction of five neonates of *D. similis* (aged between 6 and 24 h). The samples were incubated at 22 °C in a static system with a 12 h light/dark photoperiod. The immobilized organisms were quantified after exposure times of 24 and 48 h. Determinations were made of the initial and final values of conductivity, dissolved oxygen, pH and temperature. All experiments were performed with the samples in quadruplicates.

Determination of the median effective concentration ($EC_{50}$) was performed using different concentrations of BPA (3.0, 5.0, 8.0, 10.0 and 12.0 mg $L^{-1}$), with the calculations employing Spear software[21]. The $EC_{50}$ was used in order to establish whether the degradation samples caused any acute effects in the organisms.

### *2.5 Chronic toxicity test with R. subcapitata*

Chronic toxicity bioassays using the microalgae *R. subcapitata* were performed according to NBR 12648/2011[22]. The inocula were incubated in autoclaved LC Oligo culture medium. Algae aged between 5 and 7 days (exponential phase of growth) were inoculated in 125 mL Erlenmeyer flasks containing 100 mL of test solution (94.0 mL of LC Oligo culture medium and 6.0 mL of treated sample), in order to obtain $10^5$ cells $mL^{-1}$. A control sample was prepared using 100.0 mL of LC Oligo medium containing $10^5$ cells $mL^{-1}$. The inoculated samples were maintained for 72 h at 23 ± 2 °C, under 4500 μmol photons $m^{-2}$ $s^{-1}$, with shaking at 120 rpm. The Erlenmeyer flasks containing the test media were arranged randomly on the table of the orbital shaker (Model MA140, Marconi), with their positions being changed daily. After 72 h, 5.0 mL aliquots of the samples were removed and fixed using 5% lugol in order to quantify the algal biomass. All the experiments were performed using the samples in triplicate.

The final algal growth was analyzed by cell quantification using a Neubauer chamber and an optical microscope. The results were calculated as the


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final algal biomass and the growth inhibition percentage (%I), according to Eq. 1:

$$\%I = \frac{Mc-Ma}{Mc}\ x\ 100 \quad (1)$$

where $M_c$ is the average for the control cells and $M_a$ is the average for the cells in the test solution.

## 3. Results and discussion

### *3.1 Chromatographic conditions and method validation*

The quantification results showed a linear regression coefficient of 0.9995. The LOD and LOQ values were 5.0 and 10.0 µg $L^{-1}$, respectively. The retention time was 5.6 min and the method was selective and robust (± 5.0%). The accuracy was quantified using ultrapure water spiked with 0.3, 0.5 and 0.8 mg $L^{-1}$ of BPA, with average recoveries between 95 and 102%. The precision of the method was determined considering the repeatability and the intermediate precision.

### *3.2 Stability of BPA in aqueous solution*

The weekly evaluation of solutions of BPA at 0.8 mg $L^{-1}$ showed an average recovery between 88 and 101% after 10 weeks. According to Corrales *et al.*[23], the half-life time of BPA in the environment is 38 days. Under natural conditions, BPA can be removed by direct photolysis and biological degradation. In the present work, the natural degradation of BPA was not observed since the stability assays were performed in a closed and controlled environment.

### *3.3 Control assays*

The experiments using $TiO_2$, UV irradiation and aeration separately did not show any BPA removal after 1 h, indicating that BPA was not adsorbed onto the $TiO_2$ surface and that aeration was ineffective in transferring BPA from the aqueous solution to the gas phase. In both cases, the BPA decrease was less than 1% of the original concentration. Direct photolysis at pH 8.5 resulted in a 3% decrease of BPA after 1 h of irradiation, while no concentration change was observed using pH without adjustment.

These results are similar to those obtained by Silva *et al.*[24], who used 120 mg $L^{-1}$ $TiO_2$ (Sigma–Aldrich), without irradiation, and observed no removal of 5.0 mg $L^{-1}$ BPA after 2 h. Repousi *et al.*[25] reported that 260 µg $L^{-1}$ BPA in 20 mg $L^{-1}$ humic acid solution did not undergo direct photolysis after 45 min of irradiation using a 100 W $Xe/O_3$ lamp.

### *3.4 Bisphenol A photodegradation study*

#### *3.4.1 Influence of $TiO_2$ concentration on BPA degradation*

Considering the range of $TiO_2$ concentrations tested (from 1.0 to 10.0 mg $L^{-1}$), the best efficiencies were obtained using 7.5 and 10.0 mg $L^{-1}$, with removal of > 99% BPA after 45 min of irradiation, at both pH values employed (Fig. 1). When 5.0 mg $L^{-1}$ of the catalyst was used, BPA removal exceeded 98% after 1 h of reaction. Use of $TiO_2$ at a concentration of 2.5 mg $L^{-1}$ resulted in BPA removals of up to 80 and 88% after 1 h of irradiation at pH 5.3 and 8.5, respectively. At a $TiO_2$ concentration of 1.0 mg $L^{-1}$, 40% BPA removal was obtained at both pH values.

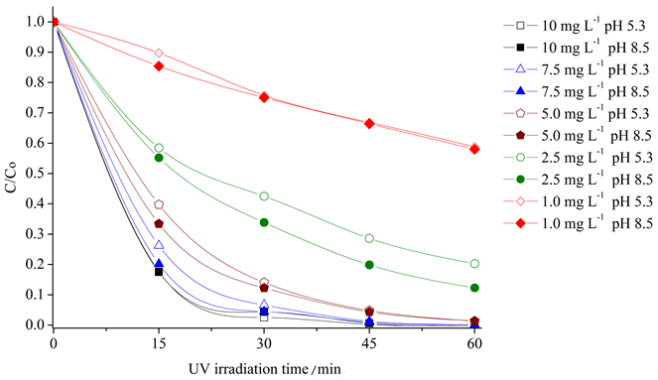


**Figure 1.** Comparison of the removal of 0.8 mg $L^{-1}$ BPA from ultrapure water using artificial UV, $TiO_2$ and aeration.

As can be seen in Fig. 1, there were no significant differences between the BPA removals achieved at the different pH values. The effect of pH on the BPA


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ISSN: 1678-4618
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removal efficiency was due to the ionization of the molecule at basic pH, along with different surface charges of the catalyst[26]. $TiO_2$ semiconductor particles present a zero point of charge (ZPC) pH of 6.0, so the particles are negatively charged at pH lower than 6.0[27]. Since the photocatalytic process occurs at the interface, lower efficiency can be expected at pH 8.5, due to electrostatic repulsion and, consequently, reduced adsorption of BPA molecules onto the $TiO_2$ surface[28]. Nevertheless, more hydroxyl radicals are generated at alkaline pH, due to the higher concentration of hydroxyl ions in the solution, which probably overcame the decreased BPA adsorption, resulting in efficient removal of the compound[27].

Use of a higher $TiO_2$ concentration increased the surface area and the quantity of active sites available, resulting in increased BPA removal efficiency[26,27].

Tsai *et al.*[28] reported that removal of BPA at 10.0 mg $L^{-1}$ reached > 99% after 1 h of irradiation using $TiO_2$/UV, with an initial catalyst concentration of 0.5 g $L^{-1}$. Abo *et al.*[11] studied the removal of 25.0 mg $L^{-1}$ BPA at pH 5.8, using different semiconductors. Removal of up to 98% BPA was observed after 1 h of irradiation, using 0.1% (w/w) of $TiO_2$ and 0.5 mmol $L^{-1}$ of sodium hypochlorite.

### *3.4.2 Solar radiation*

No removal of BPA was observed after 1 h of direct photolysis under solar irradiation. The accumulated UV radiation doses were 10.6 and 12.3 J $cm^{-2}$ for the assays using solutions without pH adjustment and at pH 8.5, respectively. However, combining the optimized $TiO_2$ concentration of 10 mg $L^{-1}$ and solar radiation resulted in complete depletion of the initial BPA concentration after 1 h, at both pH values (5.3 and 8.5). The accumulated UV radiation doses were 11.4 and 9.45 J $cm^{-2}$ for pH 5.3 and 8.5, respectively.

### *3.4.3 Influence of the matrix on BPA degradation*

Evaluation of BPA removal using spring water, instead of ultrapure water, was necessary for the subsequent ecotoxicological investigations. Under these conditions, BPA removal reached 46% after 1 h of reaction at unadjusted pH 8.0, under artificial UV irradiation (Fig. 2). The lower BPA removal efficiency, compared to the value obtained using ultrapure water (> 99% removal in 45 min of irradiation) could be attributed to the presence of ions (85.4 µS $cm^{-1}$) and organic compounds in the spring water, which were not present in the ultrapure water. Teixeira and Jardim[26] reported that anions, such as $Cl^-$, $SO_4^{2-}$ and $PO_4^{3-}$ could reduce the photomineralization ratio in a range from 20 to 70%, due to adsorption of the anions onto the oxidant sites of the catalyst. Furthermore, the presence of species, such as $HCO_3^-$ and $CO_3^{2-}$, could hinder the photocatalytic process, since these anions could react with the hydroxyl radicals, hence competing with the target compound[29].

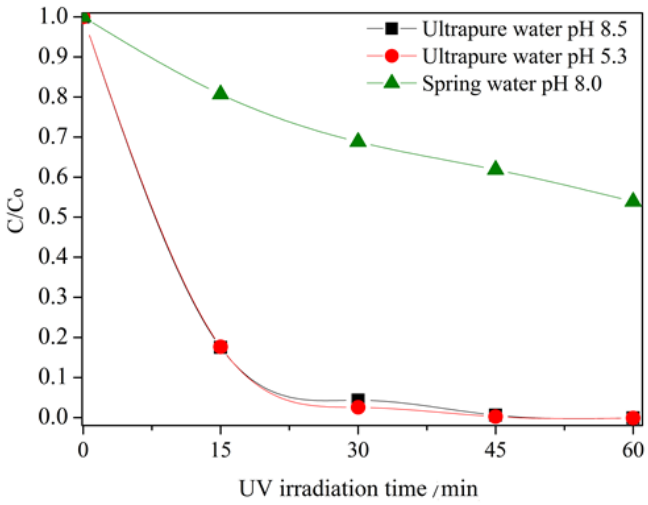


**Figure 2.** Comparison of the efficiencies of BPA removal from ultrapure water and natural spring water using the heterogeneous photocatalyst system ($TiO_2/O_2$/artificial UV). [$TiO_2$] = 10.0 mg $L^{-1}$.

The chromatograms acquired during the BPA removal study using spring water showed signals at retention times different to that for BPA, which was indicative of the generation of BPA degradation intermediates.

## *3.5 Toxicity assays*

The results of the toxicity assays indicated that the samples obtained during BPA removal using the photocatalytic degradation process did not present acute effects towards *D. similis*. This absence of any


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acute effects towards the microcrustacean could be explained by the fact that all the solutions used contained BPA at concentrations lower than the $EC_{50}$ value of 8.97 mg $L^{-1}$ (Fig. 3). The results of this bioassay also showed that there was no toxicity associated with any possible intermediates generated.

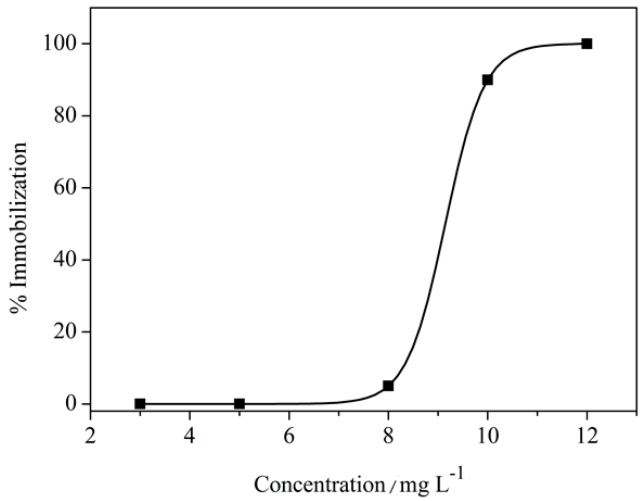


**Figure 3.** Dose-response curve after 48 h exposure of *D. similis* to BPA.

In a similar study with *Daphnia magna*, Erjavec *et al.*[30] investigated the acute effects of BPA degradation by heterogeneous photodegradation using $TiO_2$ supported on glass fiber filters. The immobilization rate of the organism exceeded 30% after 24 h of exposure to the original 10 mg $L^{-1}$ solution of BPA. It was also observed that the samples removed during the AOP process presented lower toxicity compared to the original solution, which was suggested to be due to the decrease of the BPA concentration during the heterogeneous photocatalysis process.

Initial samples containing 0.8 mg $L^{-1}$ BPA caused 25% growth inhibition of *R. subcapitata*, compared to the control. Samples removed after 15 and 30 min of irradiation in the presence of $TiO_2$ caused 18 and 15% enhancements of algal biomass growth, respectively. However, samples removed after 45 and 60 min of irradiation once again inhibited algal growth, with 10 and 7% decreases, respectively (Fig. 4). Statistical analysis (ANOVA) indicated that there were significant differences among the algal biomasses obtained for the control and the samples collected after different oxidation times (95% confidence, $p$ = 0.0007), even after 72 h of exposure. Statistical analysis showed that in the absence of BPA, the heterogeneous photocatalysis process did not affect the algal biomass, with no significant difference relative to the control (ANOVA, 95% confidence, $p$ = 0.745).

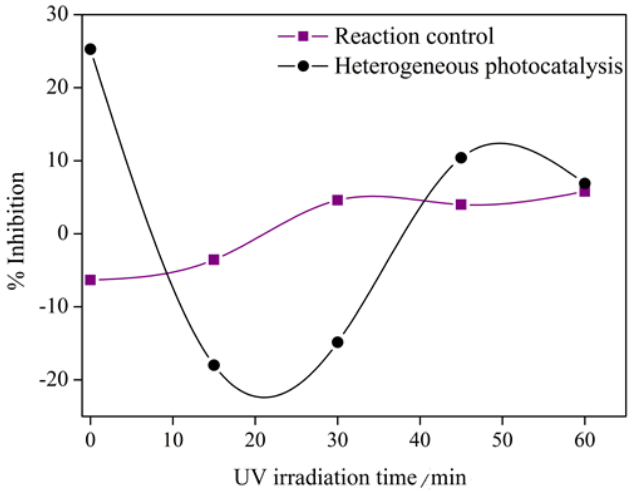


**Figure 4.** Percentage inhibition of *R. subcapitata* in filtered samples collected after different times of oxidation of 0.8 mg $L^{-1}$ BPA and the reaction control, using natural spring water containing 10.0 g $L^{-1}$ $TiO_2$ suspension, with irradiation using a 125 W medium-pressure Hg vapor lamp.

Similar results were obtained by Candido *et al.*[31] during evaluation of the chronic toxicity towards *R. subcapitata* of ibuprofen solutions after heterogeneous photocatalysis using $TiO_2/O_2$/UV. It was observed that the initial samples containing 1.0 mg $L^{-1}$ ibuprofen caused 20% inhibition of algal growth, while samples removed after 5 and 10 min of reaction induced 10% increases of the algal biomass. However, statistical analysis showed no significant differences among the Chl-a values after exposure for 96 h.

The abiotic parameters that could interfere in the ecotoxicological evaluation were monitored at the beginning and the end of each experiment. The data in Tab. 1 show the physicochemical parameters such as conductivity, pH, temperature and dissolved oxygen (DO) concentration during the *D. similis* bioassays. As the initial and the final values of those parameters do not show significant difference, the abiotic interference was not observed.


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ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p48-56

**Table 1.** Physicochemical variables of spring water samples during the acute ecotoxicity assay with *D. similis*, in the determination of $EC_{50}$ and in the times $t_0$ and $t_{60}$ of the heterogeneous photocatalytic process in the presence and absence of BPA.

| Samples | | pH | Conductivity / µS $cm^{-1}$ | DO / mg $L^{-1}$ | Temperature / °C |
|---|---|---|---|---|---|
| Spring water | Initial | 8.21 | 75.8 | 7.53 | 23.0 |
| | Final | 8.86 | 88.2 | 7.59 | 23.6 |
| Reaction control ($t_0$) | Initial | 7.94 | 67.7 | 9.05 | 22.4 |
| | Final | 8.64 | 82.1 | 7.64 | 22.9 |
| Reaction control ($t_{60}$) | Initial | 7.34 | 73.3 | 8.69 | 22.6 |
| | Final | 8.47 | 85.8 | 7.65 | 23.3 |
| Heterogeneous photocatalysis ($t_0$) | Initial | 7.58 | 73.8 | 8.51 | 22.9 |
| | Final | 8.53 | 84.8 | 7.70 | 22.9 |
| Heterogeneous photocatalysis ($t_{60}$) | Initial | 7.50 | 73.6 | 8.56 | 22.8 |
| | Final | 8.44 | 83.6 | 7.66 | 22.9 |
| $EC_{50}$ 3.0 mg $L^{-1}$ | Initial | 7.25 | 73.9 | 7.72 | 23.1 |
| | Final | 8.31 | 87.6 | 7.60 | 23.3 |
| $EC_{50}$ 8.0 mg $L^{-1}$ | Initial | 7.36 | 71.6 | 7.76 | 23.0 |
| | Final | 8.33 | 83.4 | 7.57 | 23.3 |
| $EC_{50}$ 12.0 mg $L^{-1}$ | Initial | 7.57 | 72.3 | 7.77 | 23.0 |
| | Final | 8.22 | 87.1 | 7.53 | 23.2 |

$EC_{50}$: effective concentration 50; DO: dissolved oxygen.

The tendency of the obtained results for the chronical assay indicated that the treated samples showed interference to the algal biomass growth of the *R. subcapitata*. However, the extrapolation of obtained results in laboratorial conditions are limited, since in the natural environment synergic effects of different exogenous substances may occur. Therefore, more detailed studies are necessary in order to predict the harmful effects due to the presence of BPA in the environment.

## 4. Conclusions

The $TiO_2/O_2$/artificial UV process was effective in degrading BPA present in aqueous solution with total removal (BPA concentration lower than the LOQ) within 45 min of reaction, under the pH conditions tested using 10 mg $L^{-1}$ $TiO_2$. When solar UV irradiation was used, total removal of BPA was observed after 60 min, under the same conditions employed in the assays with artificial UV.

The BPA $EC_{50}$ value for *D. similis* was 8.97 mg $L^{-1}$. Acute toxicity assays performed using samples after AOP treatment indicated that there were no negative effects towards the microorganisms after 48 h of exposure. In the chronic assays using the alga *R. subcapitata*, a 25% inhibition of algal biomass growth was observed using the initial BPA solution of 0.8 mg $L^{-1}$, while samples collected after 15 and 30 min of irradiation in the presence of $TiO_2$ caused an increase of 18 and 15% in algal biomass growth, respectively.

## Acknowledgments

The authors would like to acknowledge financial support from the following Brazilian Research agencies: CAPES, CNPq and FAPEMIG.

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Eclética Química Journal

Original article

iq.unesp.br/ecletica

| Vol. 46 | n. 2 | 2021 |

# Ultrasound-assisted extraction for the determination of α-linolenic and linoleic acid in vegetable oils by high performance liquid chromatography

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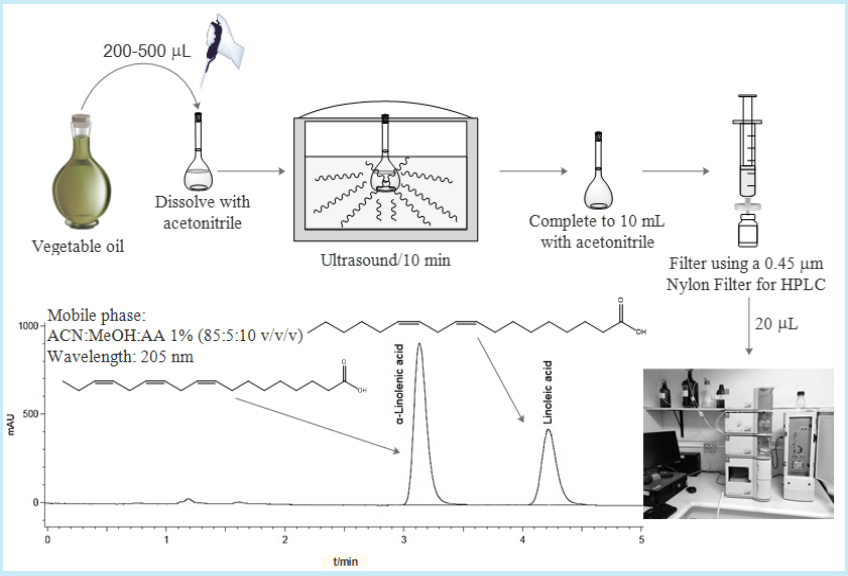
**ARTICLE INFO**

*Article history:*
**Received:** March 26, 2020
**Accepted:** December 03, 2020
**Published:** April 01, 2021

*Keywords*
1. α-linolenic acid
2. linoleic acid
3. vegetal oils
4. HPLC

**ABSTRACT:** The present research consisted in determining α-linolenic acid (ALA, ω-3) and linoleic acid (LA, ω-6) in vegetable oils with ultrasound-assisted extraction. The analytical method was validated by high performance liquid chromatography (HPLC) for the quantification of both polyunsaturated fatty acids (PUFA). The determination was made at a wavelength of 205 nm, mobile phase composed of acetonitrile: methanol: acetic acid 1% (85:5:10), with a flow of 1 mL $min^{-1}$ and during 5 min. The calibration graph data (50 to 300 mg $L^{-1}$) adjusted to the linear regression model with a coefficient of determination ($R^2$) of 0.99914 and 0.99986 for ALA and LA, respectively. The repeatability, reproducibility, accuracy and percentage of recovery complied with international guidelines for analytical methods.




The proposed method is simple, fast, linear, precise and accurate for the quantitative determination of ALA and LA in vegetable oils. The previous ultrasound application during 10 min achieves significantly higher concentrations ($p < 0.05$) when ultrasound is not applied. In conclusion, the present method can serve as a useful alternative for routine determinations of ALA and LA in vegetable oils, as it proved to be an easy, fast and reliable method.




ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p57-66

## 1. Introduction

Fatty acids (FA) are carboxylic acids with a hydrocarbon chain that can vary from $C_6$ to $C_{24}$ and can be present in nature as pure free fatty acids (FFA), FA salts or as part of more complex molecules known as lipids. Fatty acids can be unsaturated and sub-classified as monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA)[1]. In general, MUFA and long-chain PUFA are involved in neuronal processes: their deficiency and other nutrients between the last trimester of pregnancy and the first two years of childhood could damage brain function[2]. On the other hand, they are important for the treatment of eye, skin, central nervous system and viral diseases[3], prevention and management of violence in schizophrenia[4]. In addition, mitochondrial FA (β oxidation) are crucial for the maintenance of thermogenesis[5].

Cis, cis-9, 12-octadecadienoic acid or linoleic acid (LA) and cis, cis, cis-cis, 9, 12, 15-octadecatrienoic acid or α-linolenic acid (ALA) are among the best-known PUFAs present in plants, nuts, seeds and vegetable oils[6,7]. These PUFAs are considered essential FAs: they are not synthesized by humans, hence it is necessary to include them in the diet[2,8,9]. Other FA present in vegetable oils are palmitic acid, stearic acid, palmitoleic acid, oleic acid, arachidic acid, behenic acid, lignoceric acid, etc[10]. On the other hand, a group of PUFA is known as Omega 3, which includes ALA, stearidonic acid, eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid present in various sources, such as plants, fish, fish oils, seaweed[11] and as omega 6 to LA[12]. Also, vegetable oils can be composed of phenolic acids, flavonoids, vitamins A and E[13].

Concerning LA (Omega 6, ω-6) benefits, it was found to effectively reversed the inflammatory responses induced by palmitic acid treatment in microglial cells[14]. Likewise, it has been reported that this acid constitutes a safe, easy to apply, well tolerated and effective anti-inflammatory prophylactic treatment of chronic migraine[15]. Furthermore, the intake of LA is associated with a lower risk of suffering from diabetes mellitus type 2 (DM2), as well as a better glycemic control and/or insulin sensitivity[16].

On the other hand, ALA (Omega 3, ω-3), a known precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is considered as a nutraceutical/pharmaceutical candidate due to its safe use as a food ingredient. Regarding the benefits of ALA, cardioprotective, anticancer, neuroprotective, anti-osteoporotic, anti-inflammatory and antioxidant effects have been reported[17], as well as bone protector[18] and coronary heart disease prevention capacities[19]. Alfa-linolenic acid could be involved in the preventive effect on cognitive impairment and dementia[20] and other researches consider it as a nutraceutical to prevent devastating damages caused by cerebral ischemia[21,22].

Hence, fatty acids such as ALA and LA are plant-origin dietary components of great nutritional interest, which justifies the necessity of research concerning their concentration in vegetable oils. Developing faster techniques for ALA and LA quantification is of crucial importance. Currently, emerging (non-thermal) technologies, such as high-pressure processing (HPP), ultrasound treatment (US), ionizing radiation (IR), pulsed electric field (PEF) and cold plasma, are applied for the preservation of food components[23]. Moreover, studies report that ultrasound-assisted oil extraction increases yield and reduces extraction time without affecting the quality of oil, compared to other extraction methods[24,25]. In addition, it was shown that the sample exposure time to ultrasound gives more optimal results when analyzing short-chain fatty acids (SCAF) compared to shaking the sample in a solvent[26], this is because ultrasound in the immiscible liquid-liquid-solid system produces high-speed impacts between solid particles and liquid phases and therefore promotes mass transfer between phases[27,28]. To date there are various methods for determining FFA, such as thin layer chromatography (TLC)[29], volumetric methods, gas chromatography (GC)[30] and particularly high performance liquid chromatography (HPLC)[31], which would provide various advantages over the official GC method, especially in terms of simplicity, separation times, sensitivity, speed[32] and its importance in many different fields[33,34].

Therefore, the present research proposes an easy methodology for the determination of ALA and LA in vegetable oils by HPLC with diode array detector


Eclética Química Journal, vol. 46, n. 2, 2021, 57-66
ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p57-66

(HPLC-DAD) with ultrasound-assisted determination (UAD). The use of ultrasound as a method for the preparation of samples for solid-liquid extraction is widespread among laboratories and can be considered fast and effective. This opens a gateway to new perspectives, mainly in relation to analytes that are strongly bound to the matrix[35]. Therefore, the goal of this research was to compare concentrations of ALA and LA with and without UAD in soybean, Sacha Inchi, linseed oils that are highly commercialized and Loche pumpkin and Macre pumpkin oils that present scarce studies in relation to the composition of ALA and LA.

## 2. Materials and methods

### *2.1 Chemical reagents and samples*

Analytical standards of ALA and LA were obtained from Sigma-Aldrich. Likewise, acetonitrile (ACN) and methanol (MeOH) HPLC grade and acetic acid (AA) 99.8%, were obtained from Merck. Ultrapure water (18.2 MΩ cm) was produced by a Thermo Scientific Barnstead Easypure II - RF purifier. The samples of vegetable oils (soybean, Sacha Inchi, linseed, Loche pumpkin and Macre pumpkin) were provided by the Quality Control Laboratory of the Universidad Católica de Santa María.

### *2.2 Chromatographic conditions*

A HPLC Hitachi LaChrom Elite with diode array detector (DAD), Merck LiChroCART 250-4 RP-18e column (5 µm), ACN mobile phase:MeOH:AA 1% v/v/v (85:5:10) was used. The system was kept in isocratic conditions exposed to a 205 nm wavelength and with a flow of 1 mL $min^{-1}$ during a period of 5 min.

#### *2.2.1 Evaluation of the linearity of the ALA and LA quantification method by HPLC*

To prepare the calibration graph, a standard stock solution was prepared weighing 10.0 mg of the ALA and LA standards in a 10.0 mL flask, achieving a concentration of 1000 mg $L^{-1}$ in ACN. Six calibration solutions were prepared from the stock solution: 50, 100, 150, 200, 250 and 300 mg $L^{-1}$. Finally, 20 µL were injected into the HPLC column under the established chromatographic conditions. This procedure was performed three times. Linear regression was performed using the method of the least squares in order to find the equation of the straight line.

$$y = a + bx \quad (1)$$

Where, $y$ is the absorbance, $x$ is the concentration, $a$ is the intercept and $b$ is the slope.

Then, the coefficient of determination $R^2$, which should be greater than 0.99, was calculated[36].

#### *2.2.2 Determination of the detection and quantification limits*

The limit of detection (LOD) that corresponds to the minimum amount of analyte that the method can determine, but not quantify, was calculated using Eq. 2[36].

$$LOD = \frac{Y_{bl}+3(Sbl)}{b}\frac{1}{\sqrt{n}} \quad (2)$$

On the other hand, the limit of quantification (LOQ) that corresponds to the minimum amount of analyte that the method can quantify with precision and accuracy was calculated with Eq. 3[36]:

$$LOQ = \frac{Y_{bl}+10(S_{bl})}{b}\frac{1}{\sqrt{n}} \quad (3)$$

Where, $Y_{bl}$ (blank response) corresponds to the intercept that relates the concentration and the average response (area) and $S_{bl}$ (standard deviation of the blank) to the intercept corresponding to the calculated line of the concentration versus the standard deviation[36].

#### *2.2.3 Precision*

Six samples of vegetable oil were prepared and quantified (ALA and LA) through HPLC. To evaluate repeatability, the six analysis were performed by the same analyst; for reproducibility, another six analysis were performed by two analysts. Accuracy was


Eclética Química Journal, vol. 46, n. 2, 2021, 57-66
ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p57-66

expressed as the relative standard deviation (RSD) using Eq. 4[36]:

$$RSD = \frac{s}{\bar{x}} \times 100 \quad (4)$$

Where $\bar{x}$ is the average of the six measurements and *s* corresponds to the standard deviation of those measurements.

### *2.2.4 Evaluation of accuracy*

Accuracy was calculated using the percentage recovery method *(%R*). To do this, the concentration of ALA and LA in a sample of vegetable oil was quantified and then, the sample was enriched with 100 mg $L^{-1}$ of ALA and 150 mg $L^{-1}$ of LA. Once analyzed by HPLC, the *%R* was calculated using Eq. 5:

$$\%R = \frac{C_e - C}{C_{st}} \times 100 \quad (5)$$

where, *C* is the concentration of ALA and LA in the vegetable oil, $C_e$ is the concentration after enrichment with ALA and LA standards in vegetable oil and $C_{st}$ is the concentration of ALA and LA standards with which the vegetable oils were enriched.

## *2.3 Samples preparation*

For the analysis of the vegetable oils, 200 µL of each of soy, Sacha Inchi and linseed oils and 500 µL of each pumpkin oil, Loche and Macre, were poured in 10 mL volumetric flasks. Then, 5 mL of ACN were added to the samples, which were covered with aluminum foil and exposed to ultrasound during 10 min in the Branson 2510 ultrasound. Finally, the flasks were made up to with ACN, filtered using 0.45 µm nylon membranes and 20 µL of the solution was injected to HPLC. Also, analyses of the same oils were performed without the application of ultrasound. The volume of oil analysis was higher for pumpkin oils because it gave results below the LOQ.

### *2.3.1 Concentration*

Alfa-linolenic acid and LA concentrations were expressed in g $L^{-1}$ using Eq. 6:

$$Concentration(g/L) = \frac{Concentration(mg/L)}{1000} \times \frac{10}{v} \quad (6)$$

where, concentration *(*mg $L^{-1}$*)* is obtained by replacing the area in the equation of the line, which is divided by 1000 to transform mg to g, (*10*) corresponds to the volume of the flask where the vegetable oil is dissolved, and (v) corresponds to the volume of vegetable oil in milliliters.

## *2.4 Statistics*

The data obtained were evaluated using descriptive statistics [mean, standard deviation (SD) and relative standard deviation (RSD)]. On the other hand, the calibration equation was established by applying a linear regression model that relates the analyte concentrations to the signals (area)[37]. The concentrations of ALA and LA were expressed in g $L^{-1}$ ± SD. For the comparison of the results, a two-way analysis of variance (ANOVA) with a significance of 95% was used and then Tukey's test post-hoc method was used. All results were obtained using GraphPad Prism 6.0 and OrginPro 9.0 software.

# 3. Results

## *3.1 The ALA and LA determination method by HPLC*

The chromatogram (Fig. 1) shows the retention times for ALA and LA, which are 3.2 and 4.3 min, respectively, at the established chromatographic conditions.

To evaluate the linearity of the method, the areas of the ALA and LA calibration solutions were measured from 50 to 300 mg $L^{-1}$. Concerning ALA, it resulted with a coefficient of determination $R^2$ of 0.99914, a slope "*b*" of 228877 and an intercept "*a*" of 1850196. Knowing that the y axis corresponds to the area in mAU and the x axis to the concentration of ALA in mg $L^{-1}$, the Eq. 7 is obtained.

$$Area = 228877[Concentration] + 1850196 \quad (7)$$

Likewise, concerning LA, it resulted with coefficient of determination $R^2$ of 0.99986, a slope "*b*" of 111061 and an intercept "*a*" of 430963. Knowing


Eclética Química Journal, vol. 46, n. 2, 2021, 57-66
ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p57-66

that the y axis is the area in mAU (milli-Absorbance Units) and the x axis is the concentration of LA in mg $L^{-1}$, Eq. 8 is obtained:

$$Area = 111061[Concentration] + 430963 \quad (8)$$

Concerning the results of the precision evaluation, the repeatability tests resulted in an RSD of 0.77 and 0.72% for ALA and LA, respectively. For reproducibility, the RSD was 1.27 and 1.34% for ALA and LA, respectively.

Regarding the sensitivity of the method, the LOD for ALA and LA were 6.14 and 3.16 mg $L^{-1}$, respectively, and the LOQ for ALA and LA were 12.77 and 6.84 mg $L^{-1}$, respectively.

The accuracy assessed by the percent recovery method (*%R*) resulted in values of 100.50 and 100.19% for ALA and LA, respectively.

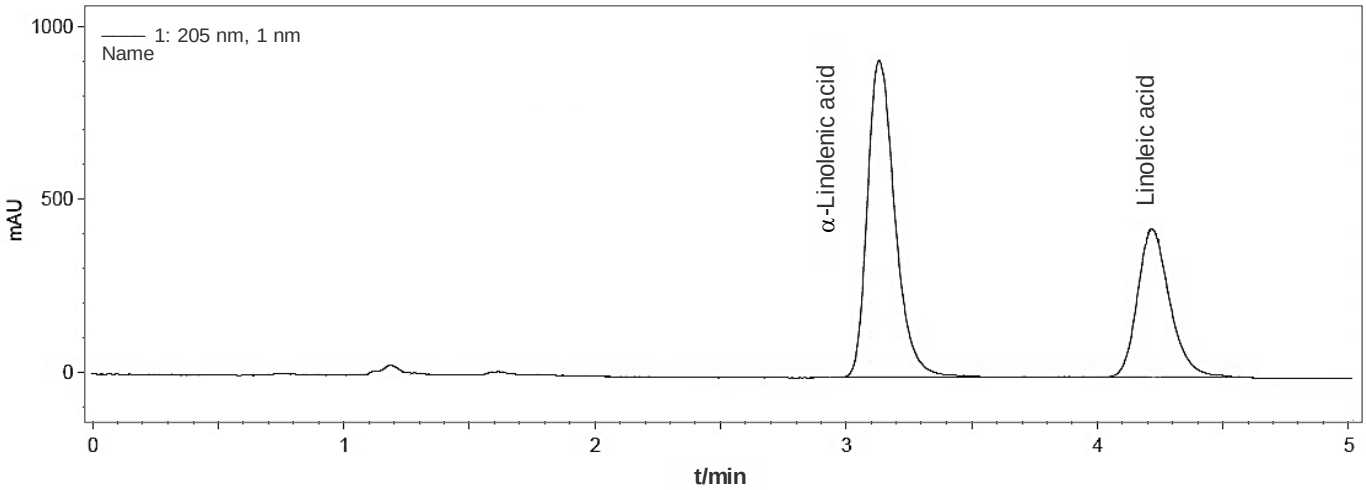



**Figure 1.** The ALA and LA chromatogram at 205 nm.

### *3.2 Quantification of ALA and LA in vegetable oils with ultrasound-assisted determination*

In the present investigation, ultrasound was used to accelerate the preparation and the determination of fatty acids from samples of vegetable oils. The areas determined in the quantification of ALA and LA in the studied vegetable oils are shown in Tabs. 1 and 2, which also show the concentration in mg $L^{-1}$ calculated with Eqs. 7 and 8, the average concentrations ± SD and the concentrations in g $L^{-1}$ ± SD of both acids under study, which was calculated with Eq. 6. Likewise, the same table shows the results with and without UAD.

**Table 1.** Concentration (g $L^{-1}$) of free α-linolenic acid (ALA) in vegetable oils.

| Vegetable oil | With UAD | | Without UAD | |
|---|---|---|---|---|
| | Average concentration mg $L^{-1}$ ± SD | Concentration g $L^{-1}$ ± SD | Average concentration mg $L^{-1}$ ± SD | Concentration g $L^{-1}$ ± SD |
| Soybean oil | 16.2 ± 0.2 | 0.81 ± 0.01[a] | 13.2 ± 0.4 | 0.66 ± 0.02[a] |
| Sacha Inchi oil | 44.5 ± 0.1 | 2.23 ± 0.01[b] | 38.1 ± 0.5 | 1.91 ± 0.03[b] |
| Linseed oil | 54.8 ± 0.7 | 2.74 ± 0.03[c] | 50.1 ± 0.7 | 2.51 ± 0.03[c] |
| Loche pumpkin oil | 17.9 ± 0.4 | 0.36 ± 0.01[d] | 15.7 ± 0.6 | 0.31 ± 0.01[d] |
| Macre pumpkin oil | 15.1 ± 0.5 | 0.30 ± 0.01[e] | 13.5 ± 0.4 | 0.27 ± 0.01[e] |

*__UAD:__ ultrasound-assisted determination, __different letters:__ significant difference.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ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p57-66

**Table 2.** Concentration (g $L^{-1}$) of free linoleic acid (LA) in vegetable oils.

| Vegetable oil | With UAD | | Without UAD | |
|---|---|---|---|---|
| | Average concentration mg $L^{-1}$ ± SD | Concentration g $L^{-1}$ ± SD | Average concentration mg $L^{-1}$ ± SD | Concentration g $L^{-1}$ ± SD |
| Soybean oil | 47.1 ± 0.5 | 2.36 ± 0.02[a] | 40.0 ± 0.6 | 2.00 ± 0.03[a] |
| Sacha Inchi oil | 16.0 ± 0.4 | 0.80 ± 0.02[b] | 13.0 ± 0.6 | 0.65 ± 0.03[b] |
| Linseed oil | 16.8 ± 0.1 | 0.84 ± 0.01[b] | 13.2 ± 0.2 | 0.66 ± 0.01[b] |
| Loche pumpkin oil | 45.9 ± 1.0 | 0.92 ± 0.02[c] | 41.7 ± 1.0 | 0.83 ± 0.02[c] |
| Macre pumpkin oil | 55.0 ± 1.0 | 1.10 ± 0.02[d] | 50.7 ± 0.6 | 1.01 ± 0.01[d] |

***UAD:** ultrasound-assisted determination, **different letters:** significant difference.

The ANOVA performed on the data in Tabs. 1 and 2 showed that the concentration of ALA and LA differ in at least one sample of vegetable oil ($p < 0.05$). On the other hand, statistical analysis also indicates that the application of ultrasound reports significantly higher results ($p < 0.05$) than when no ultrasound was applied.

In relation to the results obtained by UAD, the Tukey's test showed that the concentration of ALA in all the studied oils was different from 95% confidence. The concentration of ALA in oils in descending order is: linseed (2.74 ± 0.03 g $L^{-1}$) > Sacha Inchi (2.23 ± 0.01 g $L^{-1}$) > soybean (0.81 ± 0.01 g $L^{-1}$) > Loche pumpkin (0.36 ± 0.01 g $L^{-1}$) > Macre pumpkin (0.30 ± 0.01 g $L^{-1}$). On the other hand, Tukey's test showed that there is no significant difference between the concentration of LA in linseed oil and Sacha Inchi oil at 95% confidence. These concentrations differ from the ones of the other oils studied. The order of concentration of LA in the studied oils is the following: soybean (2.36 ± 0.02 g $L^{-1}$) > Macre pumpkin (1.10 ± 0.02 g $L^{-1}$) > Loche pumpkin (0.92 ± 0.02 g $L^{-1}$) > linseed (0.84 ± 0.01 g $L^{-1}$) = Sacha Inchi (0.80 ± 0.02 g $L^{-1}$).

## 4. Discussion

The International Conference on Harmonization (ICH) standards establish that, for the validation of the method, linearity, precision, accuracy, LOQ and LOD must be determined[38,39]. A method is considered linear if the coefficient of determination $R^2$ is greater than 0.99, precise when the RSD is less than 2% and accurate as long as the recovery percentage (*%R*) is close to 100% (90–110%)[36]. The method turned out to be linear with an $R^2$ = 0.99914 for ALA and 0.99986 for LA; precise with an RSD of 1.27% for ALA and 1.34% for LA; and in reproducibility and repeatability tests, the RSDs for ALA and LA were 0.77 and 0.72%, respectively. The LODs were 6.14 for ALA and 3.16 mg $L^{-1}$ for LA, and LOQs were 12.77 and 6.84 for ALA and LA, respectively.

Nowadays, ultrasound is used for the isolation of organic compounds present in solid samples, such as soils, animal tissues, plants, etc., since they are comparable to methods that involve more intensive treatments and with various solvents[35,40,41]. In addition, the application of ultrasound showed that in terms of yield and extraction time, it does not affect the quality of the oil[25]. Several investigations were developed with different methods of extraction and determination of fatty acids. The ALA was found in linseed oil[17,42], in linseeds[18] and perilla, canola and soybean oil[20]. It is also reported that LA was not found in soybeans oil[43], however Sacha Inchi did show the presence of ALA and LA[44,45]. In the present investigation, ALA (± SD) and LA (± SD) were found in Sacha Inchi oils (2.74 ± 0.03 g $L^{-1}$; 0.80 ± 0.02 g $L^{-1}$), linseed oil (2.74 ± 0.03 g $L^{-1}$; 0.84 ± 0.01 g $L^{-1}$), Loche pumpkin oil (0.36 ± 0.01 g $L^{-1}$; 0.92 ± 0.02 g $L^{-1}$), Macre pumpkin oil (0.30 ± 0.01 g $L^{-1}$; 1.10 ± 0.02 g $L^{-1}$) and soybean oil (0.81 ± 0.01 g $L^{-1}$; 2.36 ± 0.02 g $L^{-1}$).

In relation to HPLC methods, some use direct derivatization for the determination of both FFA with and without saponification of the samples, showing recoveries in the range 98.30 – 103.40% and the coefficients of variation were in the range 0.70 – 3.10%[31]. In the present investigation, it also turns out to be a simple, fast and accurate method that resulted in recoveries of 100.19 and 100.50% for LA and ALA, respectively, with a determination coefficient of less


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ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p57-66

than 1.50 % as opposed to the derivation method that gave a maximum result of 3.10%.

In another study, a method was developed and validated by HPLC-DAD for the determination of short chain fatty acids such as: acetic, propionic, butyric, valeric and isovaleric acids in newborn meconium. The method presented a high precision (RSD ≤ 2.5%), high coefficients of determination of the calibration curves ($R^2$ > 0.997). Assay recovery ranged from 90 ± 2 and 106 ± 2%. The validation parameters obtained in said study showed that the HPLC-DAD method is a reliable and useful tool for the determination of short chain fatty acids in meconium samples, where the samples were exposed to ultrasound energy at 40 °C for 80 min and subsequent shaking for 60 min[46]. This study showed that the application of ultrasound for only 10 min optimizes the determination process of FFA from vegetable oils giving results significantly higher than when no ultrasound is applied, in addition, the method by HPLC-DAD developed also presents high accuracy, with coefficients greater than 0.997.

Another method by HPLC with conductivity detector for the separation and simultaneous determination of myristic, palmitic, stearic, oleic and linoleic acids presented calibration curves of the five well correlated FA ($R^2$ > 0.999)[32]. In addition, 2- (7H-dibenzo [a, g] carbazol-7-il) ethyl 4-methylbenzenesulfonate (DBCETS) was proposed as a new fluorescent labeling reagent to detect FFA by HPLC in serum sample analysis resulting in low detection limits of 0.28–0.57 ng $mL^{-1}$ and guaranteed high accuracy and reliability of results[47]. Unlike the present investigation where the detection limits for ALA and LA were 6.14 and 3.16 mg $L^{-1}$, respectively. Clearly the method developed by HPLC-DAD offers high detection limits compared to the method by HPLC with fluorescence detector; however, both analyses are oriented to different matrices and the concentration of FA in oils is relatively higher than in serum, demonstrating that the methodology developed provides reliable results in the analysis of ALA and LA in vegetable oils.

## 5. Conclusions

A method for the determination of ALA and LA by HPLC at 205 nm in vegetable oils has been developed. For this method, the sample preparation uses between 200 to 500 µL of oil, a 10-min ultrasound application during a short run time (5 min), at a flow of 1 mL $min^{-1}$, obtaining retention times of 3.2 and 4.3 min for ALA and LA, respectively. The method for determining ALA and LA was found to be linear ($R^2$ > 0.99), precise (RSD < 2.0%) and accurate (90% < *%R* <110%). The validated method allowed the determination of ALA and LA concentrations in samples of vegetable oils (Sacha Inchi, linseed, Loche pumpkin, Macre pumpkin and soybean). Therefore, the present method can serve as a useful tool for the routine determinations of ALA and LA in vegetable oils since it turned out to be an easy, fast and reliable method.

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ISSN: 1678-4618
DOI: 10.26850/1678-4618eqj.v46.2.2021.p57-66