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DESENVOLVIMENTO E VALIDAÇÃO DE MÉTODO PARA QUANTIFICAÇÃO DE INIBIDORES DA COLINESTERASE EM SORO EM CASOS DE SUSPEITA DE INTOXICAÇÃO AGUDA

Florianópolis, 2023.

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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota."
Madre Teresa de Calcuta

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DEVELOPMENT AND VALIDATION OF A METHOD FOR QUANTIFICATION OF CHOLINESTERASE INHIBITORS IN SERUM IN CASES OF ACUTE POISONING

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This article presents the validation of a method by Gas Chromatography system coupled to a Mass Spectrometer (GC-MS) for the determination and quantification of Aldicarb, Propamocarb, Fenitrothion and Chlorpyrifos in serum samples, to aid diagnosis of patients with acute intoxication by pesticides. Sample is prepared by Solid Phase Extraction and method validation followed Brazilian Health Regulatory Agency parameters. The main results of the method were linearity between 2-30 ug/mL for Propamocarb, Fenitrothion and Clorpyrifos and 5-30 ug/mL for Aldicarb, with a Limit of Quantitation of 2 ug/mL and 5 ug/mL respectively, and Limit of Detection of 0.89 ug/mL for Aldicarb, 0.26 ug/mL for Propamocarb, 0.14 ug/mL for Fenitrothion and 0.38 ug/mL for Chlorpyrifos. Precision and accuracy presented most results according to legislation and matrix effect were not observed. The identification and determination of the concentration of pesticides helps in elucidation of cases with suspected intoxication, in the diagnosis and treatment of patients in an emergency state of acute intoxication, and it also generates a greater amount of data and information, contributing to a greater spectrum of awareness and prevention policies for the population.

Keywords: Pesticides. Intoxication. GC-MS. Serum. Validation.

INTRODUCTION

Pesticides are products used in agriculture and livestock to control insects, pests and fungi, and they are products with a high level of toxicity, they can endanger the health of workers and the general population (Cancer National Institute, 2022). Exposure to pesticides can cause chronic or acute poisoning, which may be through direct contact (inhaled, oral or topical) or indirect (consumption of contaminated food or water) and may occur due to intentional or accidental contact in toxic and even lethal doses (Ministry of Health, 2006).

There are several pesticides available in Brazil, many of which are banned in Europe and the United States and allowed in Brazil (DE MORAES, 2019). In addition, Brazil is the world's largest consumer of pesticides (SOUSA et al, 2022) and the list of active ingredients present in pesticides authorized in the country includes some known toxicity to human health and the environment (FRIEDRICH *et al*, 2021). Furthermore, on Brazil in the year of 2017, 62 deaths and 3370 cases of pesticides exposure was registered, knowing that most of the intoxication cases are by accident or attempted of suicide, being that national statistics shows that children are a predominance for the accidental domestic intoxication, and adolescents for the attempted of suicide (SINITOX, 2017).

Among the classifications of pesticides, there are the organophosphates (OPs) and the carbamates (CMs), also called cholinesterase inhibitors, because that can bind, or inhibit, cholinesterase, making it unable to breakdown acetylcholine (EXTOXNET, n.d.). This end up causing an accumulation of acetylcholine, which acts on the Central and Peripheral Nervous System, causing cholinergic hyperstimulation and leading to an acute cholinergic syndrome,

with the appearance of muscarinic, nicotinic and Central Nervous System signs and symptoms (RAMESH, 2023). Organophosphates can bind irreversibly to cholinesterase, and some examples of it include malathion, chlorpyrifos, fenthion and parathion (O'Malley, 2022). Carbamates bind cholinesterase in a reversible way, and among the main carbamates are propamocarb, methomyl and aldicarb, the latter being most found together with other compounds in "Chumbinho", a rodenticide still used illegally (ANVISA, 2020).

According to Caldas, (2000) frequent cases of acute poisoning by carbamate and organophosphate insecticides are found in the hospital emergency room, whether accidental or due to a suicide attempt . These acute poisonings are the result of multiple or single contact with one (or more) pesticide(s) within a 24-hour period, with the onset of symptoms immediately or within two weeks, which may occur mildly, moderately or severely (Paraná State Department of Health, 2018).

In a descriptive study shown in an article on Hospital Admissions due to Poisoning, it is reported that most intensive care unit (ICU) admissions were caused by pesticide poisoning, around 9.4%, and of these, 10.4% were children (DOS REIS , 2013). In this same article, the hospitalization period of patients accidentally or voluntarily exposed to cholinesterase inhibitors is observed, which varies from 1 to 40 days, and the longer the patient remains hospitalized, the greater the use of hospital resources, antidote and professionals of health.

Data from the 2021 Annual Report of the Center for Information and Toxicological Assistance of Santa Catarina (CIATox-SC), point to the severity of cases of pesticide poisoning, and show that on 2021, eight deaths happened because of the exposition to pesticides, and also point to the lethality of pesticides, that was observed in 16,8 in each 1000 cases of medical care of patients exposed to pesticides (CIATox, 2021).

There is a difficulty on differentiating the class of the etiological agent, since organophosphate intoxication is usually suspected when the patient presents a clinical condition of involvement of the Central Neural System, when the patient works or resides in rural area or when even receiving atropine, the patient does not present improvement of muscarinic conditions. However, clinically there may be no differences between carbamates and organophosphate intoxications, as in the case of Aldicarb which can cause severe symptoms (CALDAS, 2000).

With a laboratory method that allows differentiating the class of the etiological agent, the evaluation of the patient's condition would not have to be only clinical. Based on the data presented, the severity of the symptoms also exemplified, and the scarcity of methods for this context, it is understood the importance and the need to be able to identify and/or quantify the pesticide in the biological samples, in order to direct the clinical conduct in cases of suspected acute intoxication. Therefore, the objective of this work was to develop and validate an analytical method using Gas Chromatography system coupled to Mass Spectrometry, aiming to identify and quantify cholinesterase inhibitors in the serum of intoxicated patients.

MATERIALS AND METHOD

Materials

The matrix used was serum, and for the development and validation of the method, a pool of serum was created from samples that would be discarded. From this pool of sera, the

points of the calibration curve and other concentrations of the validation of the method were made, adding the pesticide standards equivalent to the desired concentration.

The reagents used were methanol, acetonitrile and ethyl acetate (Synth®). Methanol was used to clean the chromatographic system, acetonitrile for the protein precipitation step, for sample preparation, and ethyl acetate for resuspension, after sample concentration. Initially, the pesticide standards were the following traceable standards (Sigma-Aldrich®): Methomyl, Aldicarb, Propamocarb, Forate, Terbufos, Fenitrothion, Methyl Parathion, Malathion and Chlorpyrifos, standards available for use and the most found in intoxications. As the results were obtained, the number of pesticides was reduced, leaving Aldicarb, Propamocarb, Fenitrothion and Chlorpyrifos, which presented better results.

The equipment used is the Gas Chromatograph system coupled to the Mass Spectrometer (GC-MS) (Shimadzu®), equipped with a capillary column with diphenyl/dimethyl-polysiloxane phase, 5:95, measuring 30 x 0.25mm x 0.25um. with helium gas mobile phase.

Method

First, the test spots were prepared, and the serum sample was purified and concentrated. The serum needs to be centrifuged, for the protein precipitation, removing coarse impurities present in the sample, and were tested on this stage the best solvent and proportion of volume to be used on the final methodology. Subsequently, for sample purification, Solid Phase Extraction (SPE) was performed, which was evaluated and developed according to some variables such as: volume, type of extractor column, solvents and pH, so that the best results could be achieved. The columns/cartridges used in the SPE were 3mL Strata-X. All these results can be seen on Results and Discussion. Then, the extracts were concentrated in a sample concentrator equipment until dryness, being resuspended in ethyl acetate after the end of this step and filtered with 13MM syringe filters, being, finally, able to be injected in the Gas Chromatograph system. The final methodology of these steps is described in the flowchart in Figure 1.

For the development of the chromatographic method, the Real Time Analysis software was used, where variables such as temperature and time were selected, and the selection of ions monitored in the GC-MS was performed for the chromatographic analytical run step.

The monitored ions are arranged in letter A), the oven temperature ramp can be observed in letter B) and the flowchart of the method can be seen in letter C).

Figure 1 - Monitored ions (A), oven temperature ramp (B) and methodology for sample preparation (C) used for Aldicarb, Propamocarb, Fenitrothion and Chlorpyrifos analysis.

A)

GC MS

GCMS-QP2010

Ion Source Temp. : 250 °C

Interface Temp. : 300 °C Detector Voltage : Relative to the Tuning Result Absolute

Solvent Cut Time : 7 min 0 kV

Micro Scan Width : 0 u Threshold : 0

Use MS Program : Set... GC Program Time : 38.43 min

Group#1 - Event#1

	Start Time (min)	End Time (min)	Acq. Mode	Event Time(sec)	Scan Speed	Start m/z	End m/z	Ch1 m/z	Ch2 m/z	Ch3 m/z
1	7.80	12.50	SIM	0.30				41.00	87.00	71.00
2	12.50	16.14	SIM	0.30				58.00	42.00	59.00
3	16.14	17.72	SIM	0.30				136.00	42.00	94.00
4	17.72	20.37	SIM	0.30				75.00	121.00	97.00
5	20.37	21.29	SIM	0.30				57.00	231.00	103.00
6	21.29	22.55	SIM	0.30				109.00	125.00	263.00
7	22.55	22.80	SIM	0.30				125.00	109.00	277.00
8	22.80	22.97	SIM	0.30				127.00	125.00	173.00
9	22.97	23.71	SIM	0.30				97.00	199.00	197.00
10	23.71	35.01	SIM	0.30				466.00	203.00	125.00
11	0.00	0.00	Scan	0.00	0	0.00	0.00			

B)

Rate	Final temperature	Hold time
	60	5
15	107	2
10	260	0
20	300	0
10	310	10

C)

Source: The author (2023)

Validation

Once the sample preparation methodology were defined, calibration curves were developed containing the chosen pesticides and the results were analyzed, seeking the lowest possible lower limit of detection values, as well as the best selectivity and specificity. Analyzes corresponding to the acceptance criteria for validation of the method according to RDC N° 166 of July 24, 2017 and N° 27 of May 17, 2012 from the Brazilian Health Regulatory Agency, ANVISA's, which consist of: Selectivity, Residual Effect, Matrix Effect, Linearity, Intraday Precision, Interday Precision Accuracy, Sensitivity and Stability, in addition to Lower Limit of Detection and Quantitation. Each parameter has acceptance criteria that must be met.

RESULTS AND DISCUSSION

The sample preparation methodology was analyzed by the comparison of areas and data of the chromatography method were analyzed according to validation parameters consisting of: Linearity, Selectivity, Residual Effect, Sensitivity, Stability, Precision, Accuracy and Matrix Effect.

Development of the methodology for sample preparation

The tests of sample preparation methodology were divided in two phases, the proteins precipitation and the Solid Phase Extration (SPE). For protein precipitation were tested the solvent and the proportion of sample/solvent volume. For the SPE, were tested the following variables: type of sortive phase, solvent, volume of solvent elution and washing step. All testes were made with nine pesticides (Metomil, Aldicarb, Propamocarb, Phorate, Terbufos, Methyl parathion, Fenitrothion, Malathion and Chlorpyrifos) using a triplicate of 3 concentrations (LLOQ, CQM and ULOQ).

Solvent (protein precipitation)

The following solvents were evaluated: acetonitrile, ethyl acetate and methanol, with a triplicate of each one. Using acetonitrile, the largest areas of the analytes tested were obtained, which is, therefore, the chosen solvent for protein precipitation.

Sample/solvent volume proportion (protein precipitation)

After choosing the solvent, the proportions of volume 1:2 and 1:1 of sample/solvent were tested. The areas obtained were larger for the 1:1 sample/solvent volume proportion, producing a better result and being chosen for the final methodology.

Type of sortive phase (SPE)

The extractor columns tested were C18 and Strata-X sortive phases. The results were favorable for the Strata-X column.

Solvent (SPE)

After choosing the sortive phase, were tested the best solvent between methanol and ethyl acetate. Best results were found using methanol.

Volume of solvent elution (SPE)

Then, on the elution part, the results of extractions using 3 different volumes of solvent were analyzed: 1mL, 2mL and 3mL. The largest areas were obtained using 2mL of solvent.

Washing step (SPE)

Samples were tested with and without the washing step, and there was no considerable change in the results, leading to exclusion of the washing step.

Validation of chromatographic method

Linearity

Five to six points of each analyte were prepared in the following concentrations: 5, 10, 15, 20, 25 and 30 ug/mL for Aldicarb, 2, 6, 10, 15, 20 and 30 ug/mL for Propamocarb, Fenitrothion and Chlorpyrifos, these points being respectively LLOQ (Lower Limit of Quantitation), LQC (Lower Quality Control), CQM 1 and 2 (Middle Quality Control), CQA (Higher Quality Control) and ULOQ (Upper Limit of Quantitation). Therefore, the LQ (limit of quantitation) was 5 ug/mL for Aldicarb and 2 ug/mL for Propamocarb, Fenitrothion and Chlorpyrifos, and LD (limit of detection) was 0.89 ug/mL for Aldicarb, 0.26 ug/mL for Propamocarb, 0.14 ug/mL for Fenitrothion and 0.38 ug/mL for Chlorpyrifos, calculated based on RDC 166, 2017.

For all the four pesticides, the correlation coefficient (r) was above 0.99, where it can be seen in graphs on letter C of figure 2, along with Linearities, and the peaks integration of the medium point of calibration curve on letter A and the six points of the linearity on letter B.

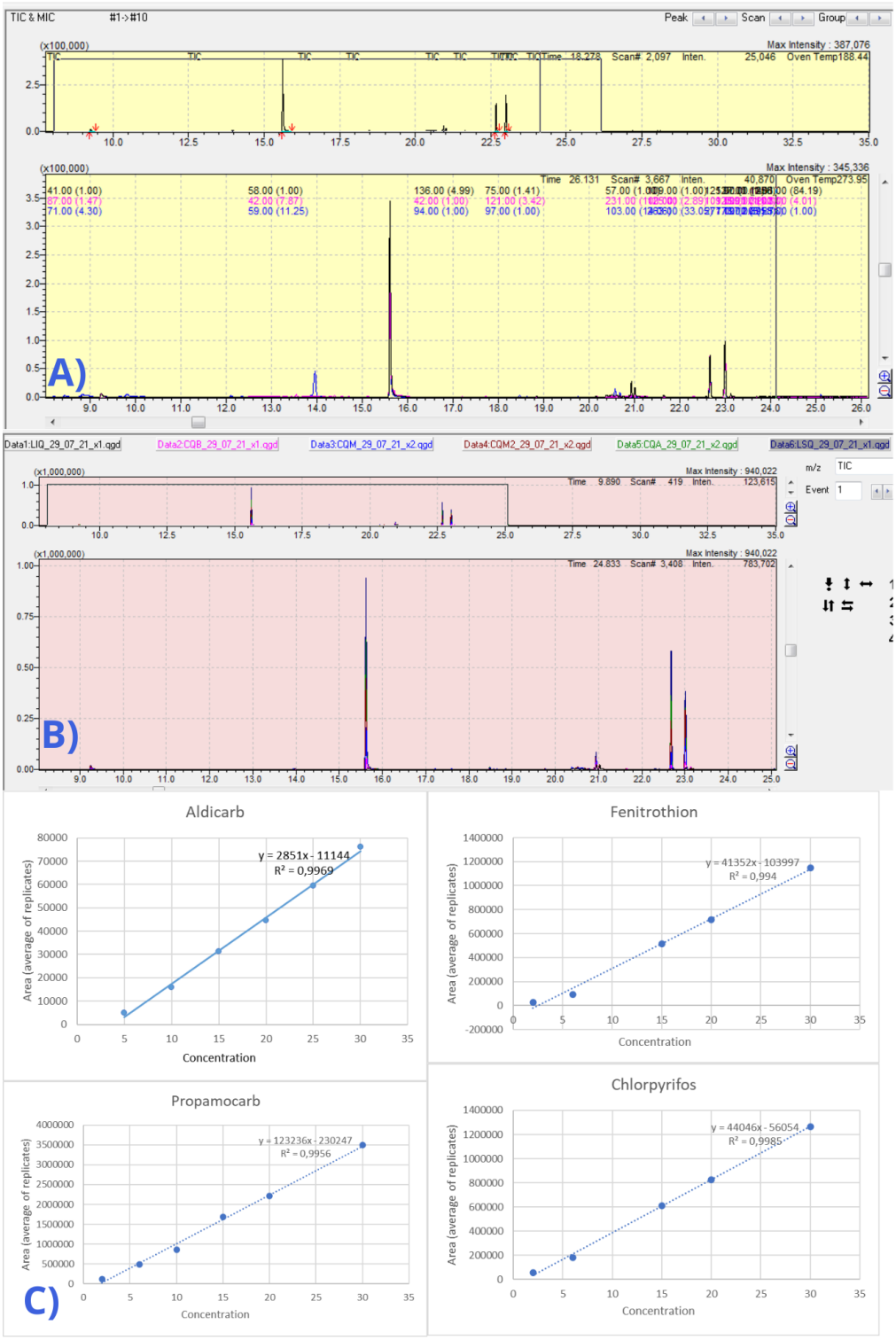
All linearity results were considered within the parameters established by RDC N° 166, 2017.

Precision and Accuracy

Precision and accuracy were determined on the same day (intraday) and in three different runs on different days (interday). Precision is measured by coefficient of variation and accuracy by the error between the measured value and the actual value.

In Table I is presented the results of the precision of each analyte and each day, and each day has from three to five replicates and at least 5 points. Some precision and accuracy results, in some concentrations, slightly exceed the established limits, being a parameter that can be reviewed and repeated in the future. However, in the case of Fenitrothion, regarding the accuracy parameter and the lower limit of quantification concentration, the results extrapolated the limit proposed in the legislation, and for this analyte it's recommended repeat the accuracy test or maybe increase the LLOQ on the future.

Figure 2 – Chromatograms and graphs of Linearity of Aldicarb, Propamocarb, Fenitrothion and Chlorpyrifos.



Source: The author (2023)

Table I – Results of precision, accuracy, selectivity, residual effect a matrix effect for Aldicarb, Propamocarb, Fenitrothion and Chlorpyrifos

Pesticides	Concentration (ug/mL)	Interim Precision (%)	Intraday Precision (%)	Interim Accuracy (%)	Intraday Accuracy (%)	Selectivity (%)	Residual Effect (%)	Matrix Effect (p)
Aldicarb	5	17,5	15,2	13,13	13,65	zero	zero	0,98435
	10	10,3	7,2	9,51	3,75	3		
	15	18,5	11,6	11,81	-	zero	zero	
	20	20,2	15,8	14,56	8,69	zero		
	25	19,7	10,1	15,57	7,91	zero	zero	
	30	29	20,1	12,6	11,02	zero		
Propamocarb	2	32,3	9,1	31,29	37,75	0,66	2,189	0,40358
	6	12,5	10,9	12,45	6,68	1,86		
	10	13,9	5	12,6	11,6	1,14	2,250	
	15	11,1	9,1	21,85	7,57	1,89		
	20	10,3	13,2	20,42	8,87	0,65	1,844	
	30	14,2	6,3	23,95	4,98	1,2		
Fenitrothion	2	31,2	27,1	116,08	37,28	3,34	8,652	0,9748
	6	16,5	9	6,14	7,17	5,13		
	10	8,5	5,3	16,42	7,29	6,22	8,164	
	15	17,1	-	10,02	-	zero		
	20	17,3	4,4	12,35	3,54	zero	8,930	
	30	8,5	8	7,41	6,03	zero		
Clorpyrifos	2	19	9,4	25,19	25,34	10,01	9,143	0,61668
	6	14,1	5,4	22,89	11,35	1,36		
	10	13,7	-	11,05	-	5,09	9,623	
	15	19,6	14,1	16,88	11,21	7,37		
	20	15,4	13,2	12,22	10,06	7,43	10,531	
	30	23,1	3,2	18,08	2,1	5,27		

Source: The Author (2023)

Selectivity and Residual Effect

Selectivity was analyzed with 10 samples of the biological matrix, in comparison with LLOQ results, and selected 6 (six) results, as can be seen in Table I. In this method, a pool of serum was performed using more than 20 different sources of serum samples, such as hemolyzed, lipemic and normal samples. All results were less than 20% (twenty percent) of the analyte response in the LLOQ samples, as expected, being able to conclude that there are no interfering peaks close to the analyte retention time.

As for the Residual Effect, which can also be seen in Table 1, a blank sample injection was performed before and two after each ULOQ, and the results were all less than 20% comparing them with the LLOQ, may concluding that there are no residue of the higher concentrations on the others concentrations, especially on the LLOQ.

Matrix Effect and Stability

The matrix effect was evaluated by applying the Student's T Test in order to verify the significant difference between the values and identify if there is interference in the results when performing an analysis in the matrix (serum) in relation to water. The results can be seen in Table 1, and, as they are all above 0.05, there is no statistically significant difference, and no matrix effect was observed for any of the analytes. Therefore, it’s able to conclude that there is no interference of the matrix (serum) on the results and on the method.

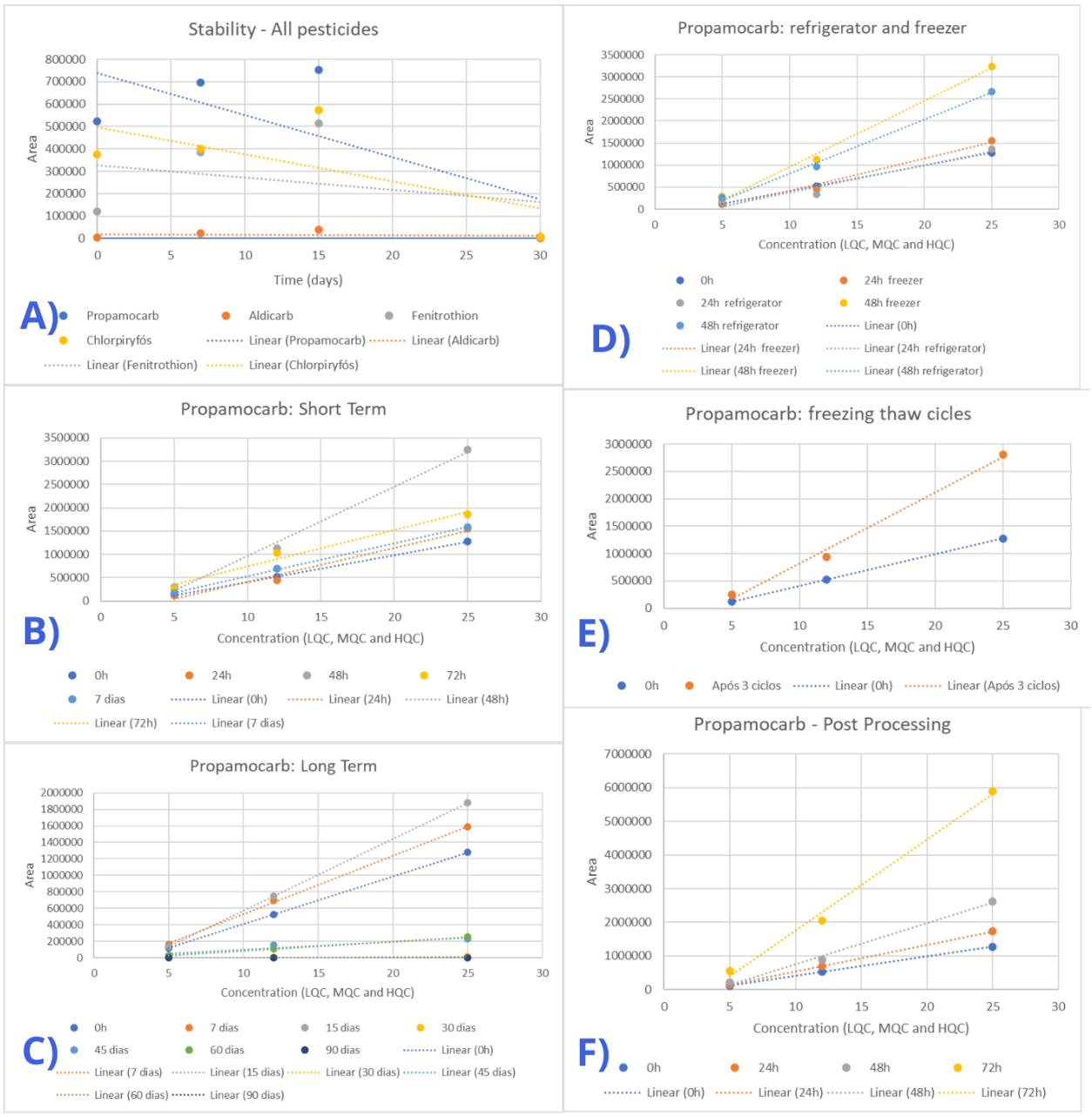
As for stability, the following conditions were evaluated: short term (24, 48, 72h and 7 days), long term (15, 30, 45, 60 and 90 days), post processing (24, 48 and 72h), cycles of thawing (3 cycles), all of these being kept in the freezer, and in the times 24h and 48h, points stored in the refrigerator and in the freezer were evaluated.

The results can be analyzed in Figure 2, where the behavior of the pesticides analyzed in terms of area X time can be seen in graph A, and in graphs B to F shows Propamocarb behavior, was chosen between the four pesticides just for example of each of the parameters of stability analyzed. In graphs B and C, the stability of Propamocarb for short and long duration is observed, remaining stable at 15 days, with a drop in area values over time after 30 days. Therefore, it is concluded that Propamocarb in whey is stable for up to 15 days in the refrigerator. The same analysis was performed for the other pesticides, as can be seen in graph A of Figure 2, reaching the conclusion that Aldicarb, Fenitrothion and Chlorpyrifos are stable for 7, 15 and 15 days, respectively.

Graph D shows the comparison between samples stored in the refrigerator and samples stored in the freezer. Both in the 24h and 48h time, there was an increase in the area of the samples stored in the freezer, with an average difference between the points of 15.75% in the 24h time and 20.74% in the 48h time. This occurred in all the pesticides analyzed. With this, it can be concluded that the way the samples are packaged influences their stability, with greater preservation and stability of frozen samples than those kept only under refrigeration.

Finally, in graphs E and F, the behavior of the samples after cycles of thawing and after post-processing is observed, noting an increase in the areas, which can be caused by evaporation of solvent and concentration of the samples, when they are at environment temperature. As a conclusion, it isn't recommended thawing the samples more than once and post-processing them, because it can cause a not trustable result.

Figure 2 – Stability Results for Aldicarb, Propamocarb, Fenitrothion and Chlorpyrifos



Source: The author (2023).
*LQC = Lower quality control; MQC= Middle quality control; HQC = Higher quantitative control

CONCLUSION

Based on the results presented and discussed, the method meets most of the validation parameters and has the potential to be used in the routine for diagnosing intoxications caused by cholinesterase inhibitor pesticides. In the future, it is intended to apply this method to biological samples of patients suspected of being poisoned by pesticides, and to implement the

method in the routine of the Clinical Analysis sector of the University Hospital of the Santa Catarina Federal University (UFSC), thus helping in the greater efficiency in the diagnosis and treatment of patients. In addition, it can generate data and information that can contribute to a greater spectrum of awareness and prevention policies for the population.

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ATTACHMENT 1: Instructions to authors (BJPS)

Brazilian Journal of
Pharmaceutical Sciences

Brazilian Journal of
Pharmaceutical Sciences

SCOPE AND POLICY

The Brazilian Journal of Pharmaceutical Sciences (BJPS) is a peer-reviewed electronic journal published continually by the School of Pharmaceutical Sciences of the University of São Paulo. The purpose of the Brazilian Journal of Pharmaceutical Sciences is to publish manuscripts that significantly contribute to knowledge in all areas of Pharmaceutical Sciences, including:

1. Medicinal Chemistry & Pharmacognosy
2. Pharmaceutical Technology, Drug development, Pharmaceutics & Biopharmaceutics, Drug Delivery
3. Clinical Pharmacy and Pharmacokinetics, Pharmaceutical Care, Pharmaceutical Services
4. Clinical-Bioanalytical Chemistry and Pharmaceutical Analysis
5. Food Science and Nutrition, including Food Analysis and Technology, Nutrigenomics, Immunonutrition, Functional foods and supplements, Food bioactive compounds, the interaction between drugs and foods
6. Vaccines and Biologicals
7. Cosmetology
8. Toxicology

Journal's policy, which are used as guidelines for the review of manuscripts:

1. Out of scope

The paper must report on recent advances in pharmaceutical technology, biopharmaceutics, pharmaceutical biotechnology, medication use, and pharmacist services of major importance.

Immediate rejection criteria:

a. Description of analytical methods with no relation to pharmaceutics, biopharmaceutics, or pharmaceutical biotechnology.

b. Mere description of compound synthesis, processes, validation, etc. without any pharmaceutical application.

c. Inappropriate promotion of a trademark or a product.

d. Regulatory issues with no relation to pharmaceutics, biopharmaceutics, or pharmaceutical biotechnology.

2. Too preliminary

A paper must be based on a thorough and extensive study, using established or well-described methods and including proper controls. Research must be hypothesis-driven and conclusions must be supported by the data presented.

Immediate rejection criteria:

a. No proof of concept in either an in vivo or an in vitro evaluation.

b. No clear indication of the use of the product or formulation.

c. No clear description of the materials & methods used in the pharmaceutical field. d. Use of inadequate or insufficient methods.

e. Inappropriate statistical analysis.

f. Lack of proper controls.

g. Lack of coherent discussion of the results.

3. Lack of novelty:

The study described in the manuscript must represent a novel approach.

Immediate rejection criteria

a. Repetition of previously published data, either partially or entirely.

b. Simple variation of parameters of a formulation, processes, synthesis, etc.

c. Modification of well-known delivery systems with no novelty and/or benefit.

d. Plagiarism (intentional and unacknowledged copying of other's work) and self-plagiarism (re-use of parts of an author's previously published work without proper attribution).

Before entering the review process, all manuscripts will go through a similarity check using the plagiarism detection software iThenticate. If the editors agree that there is a high percentage of similarity to other texts, the manuscript will be rejected immediately.

The following papers **will not be accepted** for publication:

a. Studies on human subjects not approved by an accredited Ethics Committee or without written informed consent from the subject or legal guardian.

b. Studies on animals not approved by an accredited Ethics and Animal Care Committee.

c. Manuscripts describing plant extract activity that do not Identify quali and quantitative chemical markers of the extract.

PREPARATION OF THE MANUSCRIPT

a. Manuscripts must be submitted in English.

b. Submission of a manuscript to BJPS implies that the data have not been published previously and will not be submitted for publication elsewhere while the manuscript is under review.

c. Co-authors should be individuals who have contributed substantially to the content of the paper.

Manuscripts in accordance to the “Preparing your manuscript section” will be submitted for peer review to at least two independent, anonymous referees indicated by the Associated Editors. Based on peer review, the Associate Editors will suggest manuscript acceptance or not to the Editor-in-Chief, who is responsible for the final decision.

In the case revision is suggested, the authors are asked to resubmit the manuscript incorporating the suggestions and recommendations of the referees within 15 calendar days. If the revised version is not received within the time specified from the date of the notice, the manuscript process will be canceled. All revisions must be accompanied by a letter detailing the changes made to the original document and answering all the reviewer comments, on a point-by-point basis. All alterations must be identified in the revised manuscript.

Manuscripts must have their copyright enclosed as a file to the BJPS. The manuscript will not be sent to reviewers if the signed copyright was not included in the submission package. This document must be hand- signed by all authors, no exception. Later, if the manuscript was accepted, an English certificate will be requested for the last version of the manuscript.

The dates of receipt and acceptance will be published for each article. Authors are expected to return reviewed manuscripts to the Journal within 15 calendar days and to return galley proofs of accepted manuscripts within 72 hours. The total number of “late” days will be added to the submission date at the time of publication.

Authors are required to suggest 4 potential reviewers with information on institutional and e-mail addresses. At least two of these potential reviewers must be from countries other than the corresponding author. The Editors reserve the right to nominate these or other reviewers for manuscript evaluation.

Manuscripts that do not agree to the Instructions will be refused prior to peer review.

Disclosure instructions

Authors must disclose the use of generative AI and AI-assisted technologies in the writing process by adding a statement at the end of their manuscript in the core manuscript file, before the References list. The statement should be placed in a new section entitled ‘Declaration of Generative AI and Aiassisted technologies in the writing process’.

This declaration does not apply to the use of basic tools for checking grammar, spelling, references etc. If there is nothing to disclose, there is no need to add a statement.

Statement model:

During the preparation of this work the author(s) used [NAME TOOL / SERVICE] in order to [REASON]. After using this tool/service, the author(s) reviewed and edited the content as needed

MANUSCRIPT CATEGORIES

The authors should state in the cover letter that the manuscript is intended to be Full-length Original Paper, Short Communication, Review Article, Mini-review article, Concepts and Comments and Book Reviews.
The Journal will also publish Thematic or Congress Abstracts Supplements under invitation by the Editors or previous approval by the Editorial Board.

BJPS will publish the following type of articles:

Full-length Original Paper
Each manuscript should clearly state its objective or hypothesis; the experimental design and methods used; the essential features of any interventions; the main outcome measures; the main results of the study; and a discussion placing the results in the context of published literature.

The manuscript should contain:
a. abstract of no more than 200 words
b. no more than 6 keywords
c. a running title to be used as a page heading, which should not exceed 60 letters and spaces
d. manuscript's main body is divided into separate sections (Introduction, Material and Methods, Results and Discussion).
e. no more than 40 references (without exceptions)
f. Supplementary data can be submitted as a *Supplementary information* session.

Short Communication
Short communication is **a report on a single subject**, which should be concise but definitive. The scope of this section is intended to be wide and encompass methodology and experimental data on subjects of interest to the readers of the Journal.
The manuscript should contain:
a. abstract of no more than 200 words
b. no more than 6 keywords
c. a running title to be used as a page heading, which should not exceed 60 letters and spaces
d. manuscript's main body is divided into separate sections (Introduction, Material and Methods,
Results and Discussion), without a separate section for conclusions
e. no more than 20 references (without exceptions)
f. no more than three illustrations (figures and/or tables)

Review Article

A review article should provide a synthetic and critical analysis of a relevant area and should not be merely a chronological description of the literature. A review article by investigators who have made substantial contributions to a specific area of Pharmaceutical Sciences will be published by invitation of the Editors. However, an outline of a review article may be submitted to the Editors without prior consultation. If it is judged appropriate for the Journal, the author(s) will be invited to prepare the article for peer review.

The manuscript should contain:

a. abstract of no more than 250 words

b. no more than 6 keywords

c. a running title to be used as a page heading, which should not exceed 60 letters and spaces

manuscript main body divided into sections with appropriate

titles and subtitles

d. no more than 90 references (without exceptions)

Mini-review Article

A mini-review is focused on a restricted part of a subject normally covered in a review article. The structure of the mini-review follows the same rules as the review.

Concepts and Comments

The Concepts and Comments section provides a platform for readers to present ideas, theories and views.

The manuscript should contain:

a. abstract of no more than 250 words

b. no more than 6 keywords

c. a running title to be used as a page heading, which should not exceed 60 letters and spaces

d. manuscript's main body is divided into sections with appropriate titles and subtitles

e. no more than 40 references (without exceptions)

Book Reviews

Written by experts nominated by the Editors or written by the Authors.

PREPARING YOUR MANUSCRIPT

Cover Letter

It is important that you include a cover letter with your manuscript. Take the time to consider why this manuscript is suitable for publication in the *Brazilian Journal of Pharmaceutical Sciences*. Why will your paper inspire other members of your field, and how will it drive research forward. Please explain these points in your cover letter.

The cover letter should also contain the following information:

a. Title of article.

b. Name(s), filiation and ORCID number of all author(s).

c. Information of Corresponding Author: name and e-mail (full address and telephone number are optional informations).

Authorship requirements

Only people who directly contributed to the intellectual content of the paper should be listed as authors. Manuscripts must be submitted electronically only. Confirmation of submission will be sent by email to all authors, for their agreement.

Authors should meet all of the following criteria, thereby taking public responsibility for the content of the paper:

a. Conceived, planned, and carried out the experiments presented in the manuscript or interpreted the data, or both.

b. Wrote the paper, or reviewed successive versions.

c. Approved the final version.

d. Holding positions of administrative leadership, contributing to patients, and collecting and assembling data, however important to the research, are not by themselves criteria for authorship. Any person who has made a substantial, direct contribution to the work but cannot be considered an author should be cited in the Acknowledgment section, with permission and include a description of his/her specific contribution to the research.

Text format

a. The text of a manuscript can only be accepted as a Microsoft Word file created with MS Word as a "doc", "docx" or "RTF" document.

b. Manuscripts should be sent in 30-36 lines, 1,5 spaced,

c. Each page should contain the page number in the upper right-hand corner starting with the title page as page 1.

d. Report all measurements in Système International, SI (http://physics.nist.gov/cuu/Units) and standard units where applicable

e. Names of plants, animals and chemicals should be mentioned according to International Rules available.

f. Names of drugs can follow the International rules (DCI) or current Brazilian rules (DCB)

g. Trademarks may be mentioned only once in the text (between parenthesis and initial in capital letter)

h. Do not use abbreviations in the title and limit their use in the abstract and text.

i. The length of the manuscript and the number of tables and figures must be kept to a minimum.

j. Ensure that all references are cited in the text.

k. Generic names must be used for all drugs. Instruments may be referred to by proprietary name; the name and country of the manufacturer should be given in parenthesis.

ORGANIZATION OF THE MANUSCRIPT

Most articles published in BJPS will be organized into the following sections:
Title
Running Title Authors (full names)
Corresponding author information (Abbreviated name, institutional address, phone, e-mail, ORCID link)
Abstract, Keywords **INTRODUCTION MATERIAL AND METHODS**
First Subtitle (if there is any) *Second subtitle* (if there is any) **RESULTS**
DISCUSSION ACKNOWLEDGMENTS REFERENCES
Tables with a descriptive title and footnote legends
Figures with a descriptive legend and uniformity in format.

Continuous page numbers are required for all pages including figures. There are no specific length restrictions for the overall manuscript or individual sections. However, we request authors to present and discuss their findings concisely. We recognize that some articles will not be best presented in our research article format. If you have a manuscript that would benefit from a different format, please contact the editors for further discussion.

TITLE PAGE

Title
The title should be as short and informative as possible, should not contain non-standard acronyms or abbreviations, and should not exceed two printed lines. The title should be centered and written in bold as the example below:

FREEZE-DRYING OF AMPICILLIN SOLID LIPID NANOPARTICLES USING MANNITOL AS CRYOPROTECTANT

Running title
This short title, to be used as a page heading, should not exceed 60 letters and spaces.

Authors and Affiliations
Full name (matched with superscript numbers identifying affiliation) must be written in bold and centered. Institution(s) (Department, Faculty, University, City, State, Country) of each author (in English must be centered and written in italic.

Example:

Hongmei Xia [1*] , Yongfeng Cheng [2] , Yinxiang Xu [3] , Zhiqing Cheng [1]

[1] [2]College of Pharmacy, Anhui University of Chinese Medicine, Hefei, People's Republic of China, School [3] of Life Science, University of Science and Technology of China, Hefei, People's Republic of China, Zhaoke (Hefei) Pharmaceutical Co. Ltd., Hefei, People's Republic of China

Corresponding author

One of the authors should be designated as the corresponding author. It is the corresponding author´s responsibility to ensure that the author list is accurate and complete. If the article has been submitted on behalf of a consortium, all consortium members and affiliations should be listed in the Acknowledgments section. Provide the name, email address, and ORCID number of the author to whom correspondence should be sent identified with an asterisk.

Abstract

Since abstracts are published separately by Information Services, they should contain sufficient hard data to be appreciated by the reader. The abstract should not exceed 200 words and should be prepared in a single paragraph without topics and no margins. The abstract should briefly and clearly present the objective, experimental approach, new results as quantitative data if possible, and conclusions. It should mention the techniques used without going into methodological detail and mention the most important results. Abbreviations should be kept to a minimum and should be defined in both the Abstract and text. Please do not include any reference citations in the abstract. If the use of a reference is unavoidable, the full citation should be given within the abstract.

Keywords:

A list of keywords or indexing terms (no more than 6) should be included avoiding generic terms. Keywords must be separated by dots with only the first letter of the first word in upper case.

Example:

Apoptosis pharmacokinetics. Toxicology.

INTRODUCTION

The Introduction should put the focus of the manuscript into a broader context and reflects the present state-of-art of the subject. This should state briefly and clearly the objectives of the investigation with reference to previous works. The introduction should justify the hypothesis of the study. An extensive review of the literature should be avoided and when possible replaced by recent reviews of the subject.

MATERIAL AND METHODS

These should be described in sufficient detail that the work can be reproduced. Well-established procedures and techniques require only a citation of the original source, except when they are substantially modified. Reports of experimental studies on humans and animals must certify (including the number of protocols) that the research received prior approval by the appropriate institutional review Ethics Committee.

RESULTS

Results must be presented clearly and concisely and in a logical order. This section should provide the results of all of the experiments required to support the conclusions of the paper. When possible, use figures or tables to present data rather than text. Large datasets, including raw data, should be submitted as supplementary files; these are published online and linked to the article.

DISCUSSION

Discussion should interpret the results and assess their significance in relation to existing knowledge. Speculation not warranted by actual data should be avoided. The Discussion should spell out the major conclusions and interpretations of the work including some explanation of the significance of these conclusions.

ACKNOWLEDGMENTS

When appropriate, briefly acknowledge technical assistance, advice, and contributions from colleagues.

People who contributed to the work but do not fit the criteria for authors should be listed in the Acknowledgments section, along with their contributions. Donations of animals, cells, or reagents should also be acknowledged. You must also ensure that anyone named in the Acknowledgments agrees to being so named. Financial support for the research and fellowships should be acknowledged in this section (agency and grant number).

Figures

Figures must be submitted in high-resolution version (300 dpi). They must be submitted separately from the text, in the file upload section of the submission platform.

Preparing figure files for submission

The use of figures is mandatory for original articles since it increases the clarity of data. The use of color figures in articles is free of charge. The following guidelines must be observed when preparing figures. Failure to do so is likely to delay the acceptance and publication of the article.

a. Each figure of a manuscript should be submitted as a single file.

b. Figures should be numbered in the order they are first mentioned in the text, and uploaded in this

order.

c. Figure titles and legends should be provided in the main manuscript as a List of Figures, not in the graphic file.

d. The aim of the figure legend should be to describe the key messages of the figure, but the figure should also be discussed in the text.

e. An enlarged version of the figure and its full legend will often be viewed in a separate window online, and it should be possible for a reader to understand the figure without moving back and forth between this window and the relevant parts of the text.

f. The legend itself should be succinct, while still explaining all symbols and abbreviations. Avoid lengthy descriptions of methods. Statistical information should be given as well as the statistical tests used.

g. Arrows or letters should be used in the figure and explained in the legend to identify important structures.

h. Figures with multiple panels should use capital letters A, B, C, etc. to identify the panels.

i. Each figure should be closely cropped to minimize the amount of white space surrounding the illustration. Cropping figures improves accuracy when placing the figure in combination with other elements when the accepted manuscript is prepared for publication.

j. Individual figure files should not exceed 5 MB. If a suitable format is chosen, this file size is adequate for extremely high-quality figures.

Please note that it is the responsibility of the author(s) to obtain permission from the copyright holder to reproduce figures (or tables) that have previously been published elsewhere. In order for all figures to be open-access, authors must have permission from the rights-holder if they wish to include images that have been published elsewhere in non-open-access journals. Permission should be indicated in the figure legend, and the original source included in the reference list.

Supported file type

The following file format can be accepted: TIFF (suitable for images) or JPEG with 300 dpi, and Word file for the manuscript.

Tables

a. Tables must be submitted in Word (.doc) or Excel (.xls), not as an image.

b. Tables must be numbered consecutively with Roman numerals in the text.

c. Tables must have a concise and descriptive title.

d. All explanatory information should be given in a footnote below the table. Footnotes should be used to explain abbreviations and provide statistical information, including statistical tests used.

e. All abbreviations must be defined in this footnote, even if they are explained in the text.

f. Tables must be understandable without referring to the text.

g. Tables occupying more than one printed page should be avoided, if possible.
h. Vertical and diagonal lines should not be used in tables; instead, indentation and vertical or horizontal space should be used to group data.

Citations

References should be prepared and listed according to Vancouver's standard reference style. Entries should be arranged in alphabetical order by the author at the end of the paper. All authors' names should be given. The accuracy and completeness of reference data is the responsibility of the authors.
Only published references should be included in the reference list. Meeting abstracts, conference talks, or papers that have been submitted but not yet accepted should not be cited. Limited citation of unpublished work should be included in the body of the text only. All personal communications should be supported by a letter from the relevant authors.
References should be cited in the text by the authors' names, with only the first letter in capital letter followed by the year of publication. For more than three authors, the first has to be cited followed by the expression *et al.* (in italic). Small letters close to the year must differentiate references of the same authors and year of publication

Examples:
(Zhang, 2017)
(Ima, Souza, 2015)
(Fujisawa, Atsumi, Kadoma, 1989) (Aviral *et al*., 2009)
(Liu *et al.,* 2011a) (Liu *et al.,* 2011b)

References

Published Papers: Write all author's names up to 6 authors and followed by et al. (in case there are more than 6), Title (Only the first letter in upper case). Journal abbreviation without dots. Year;Volume(issue number):first page-last page.

Abe T, Fukushima N, Brune K, Boehm C, Sato N, Matsubayashi H, et al. Genome-Wide allelotypes of familial pancreatic adenocarcinomas and familial and sporadic intraductal papillary mucinous neoplasms. Clin Cancer Res. 2007;13(20):6019-25.

Ali A, Iqbal F, Taj A, Iqbal Z, Amin MJ, Iqbal QZ. Prevalence of microvascular complications in newly diagnosed patients with Type 2 diabetes. Pak J Med Sci. 2013,29(4): 899-902.

Calvo A, Gimenez MJ. Ex Vivo Serum Activity (Killing Rates) After Gemifloxacin 320 mg Versus Trovafloxacin 200 mg Single Doses Against Ciprofloxacin-Susceptible and -Resistant Streptococcus pneumonia. Int J Antimicr Ag. 2007;20:144-6.

Article accepted for publication but not yet published: First 6 authors followed by et al. Title. Journal (abbreviation in normal font), Year of expected publication (in press) at the end of the citation.

Janiszewski M, Lopes LR, Carmo AO, Pedro MA, Brandes RP, Santos CXC, et al. Regulation of NAD(P)H oxidase by associated protein disulfide isomerase in vascular smooth muscle cells. J Biol Chem. 2005 (in press).

Internet Communication: Ensure that URLs are active and available. Provide DOI, if available.

Brasil. Ministério da Saúde, Secretaria de Vigilância em Saúde. Leishmaniose visceral grave: normas e condutas [Internet]. Brasília (DF): Ministério da Saúde, 2006. [citado 2008 Jan 7]. 60 p. (Série A. Normas e Manuais Técnicos). Disponível em: http://dtr2001.saude.gov.br/editora/produtos/livros/ pdf/06_0072_M.pdf

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Developmental toxicology. [cited 2015 Apr 10]. Available from: http://www.devtox.org/nomenclature/organ.php.

Whole Book: Authors, Book title, Edition, City, Publisher, Year.

Hewitt W. Microbiological assay for pharmaceutical analysis: a rational approach. Boca Raton: CRC Press; 2003.
Jenkins PF. Making sense of the chest x-ray: a hands-on guide. New York: Oxford University Press; 2005. 194 p.

Laws:

Agência Nacional de Vigilância Sanitária (Brasil). Resolução nº. 259, de 20 de setembro de 2002. Regulamento Técnico para Rotulagem de Alimentos Embalados. Diário Oficial da União 23 set 2002; Seção 1.

Milech A, et al., Oliveira JEP, Vencio S, organizadores. Diretrizes da Sociedade Brasileira de Diabetes. São Paulo: A.C. Farmacêutica;2016.

Book Chapter: Authors, Chapter Title, Editors, Book title, Edition, City, Publisher, Year, Pages of citation.

Conference or Symposium Proceedings: Cite papers only from published proceedings.

Hejzlar RM, Diogo PA. The use of water quality modelling for optimizing operation of a drinking water reservoir. In: Proceedings of the International Conference Fluid Mechanics and Hydrology. 1999 Jun 23-26; Prague. Prague: Institute of Hydrodynamics AS CR; 1999. p 475-482.

Report

Rojko JL, Hardy WD Jr. Feline leukemia virus and other retroviruses. In: Sherding RG, editor. The cat: diseases and clinical management. New York: Churchill Livingstone; 1989. p. 229-332.

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Proceedings of the 10th annual meeting of the Canadian Society for Pharmaceutical Sciences. *J Pharm Pharm Sci*. 2007 Dec 3;10(4):1s-186s.

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Physician's Desk Reference (PDR). Release 2003.1AX. [CD- ROM]. Montvale: Thomson PDR; 2003.

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Dean AG, Dean JA, Coulombier D, Brendel KA, Smith DC, Burton AH, et al. *Epi info, version 6.04: a word processing database and statistics program for public health on IBM- compatible microcomputers.* [Computer program]. Atlanta: Centers of Disease Control and Prevention;1998. World Health Organization. WHO. Working to overcome the global impact of neglected tropical diseases, First WHO report on neglected tropical diseases. Geneva, Switzerland: WHO Press; 2010.

Patent

Larsen CE, Trip R, Johnson CR. Methods for procedures related to the electrophysiology of the heart. Patent No.5.529.067. Novoste Corporation; 1995.

Thesis and Dissertations

Joselevitch C. Visão no ultravioleta em Carassius auratus (Ostariophysi, Cypriformes, Cyprinidae):

estudo eletrofisiológico do sistema cone - células horizontais. [Master's dissertation]. São Paulo: Instituto de Psicologia, USP; 1999.

Marcolongo R. Dissolução de medicamentos: fundamentos, aplicações, aspectos regulatórios e perspectivas na área farmacêutica. [dissertação]. São Paulo: Universidade de São Paulo, Faculdade de Ciências Farmacêuticas; 2003.

Unpublished results and Personal communication: Reference should appear in the text with the individual name(s) and initials and not in the reference list.
(Santos CS, da-Silva GB, Martins LT, unpublished results).

It is assumed that the author has obtained permission from the source when personal communication is cited.

HOW TO SUBMIT THE MANUSCRIPT

Submission should be sent electronically through the ScholarOne system (https://mc04.manuscriptcentral.com/bjps-scielo).

If you need further assistance, please contact the Journal staff (bjps@usp.br).