*2021 International Nuclear Atlantic Conference – INAC 2021*
*Virtual meeting, Brazil, November 29 – December 2, 2021*

# Limitations of Thin Layer Chromatography (TLC) in the evaluation of radiolabeling of new peptide compound

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## 1. Introduction

One of the focuses of Nuclear Medicine is the search for compounds and techniques for diagnosing and treating diseases, including malignant tumors [1]. Usually, the treatment of these tumors requires chemotherapy, radiotherapy, and surgery sessions. However, these processes have side effects and efficiency limits. Thus, the search for new, more effective, and safer drugs is essential [2].
One class of drugs used in Cancerology is as theranostic agents, which aims, with a single compound, to diagnose and treat tumors. Depending on the purpose, high-energy radioisotopes are coupled to a therapeutic carrier molecule to damage cancer cells, signalizing them, or even the carrier itself will present an antitumor effect. For those purposes, common carrier molecules are peptides, including lectins from vegetal species [3, 4].
The peptidic lectin SteLL, extracted from a folk medicine plant known as aroeira-da-praia (*Schinus therebinthifolia*), presents antitumoral activity [5]. In this way, we can assume that the radiolabeling of SteLL with a gamma or positron emitter could lead to a theranostic agent.
Radiolabeling efficacy, shown as a percentage, should be determined on all radiopharmaceutical products prior to use. For this purpose, thin layer chromatography is one of the most used techniques. Thin-layer chromatography (TLC) is an analytical methodology used to obtain quality control data from different radiopharmaceuticals and assess radiolabelling and purity coefficients. A reproducible TLC method can quantify free pertechnetate ($Na^{99m}TcO_{4-}$) and hydrolyzed technetium ($^{99m}TcO_2$), the most common impurities in technetium-99-metastable radiopharmaceuticals [6, 7].
Despite its widespread use, it is noteworthy that TLC has some limitations, such as the deposition of more than one molecule in the same retention factor (Rf), inability to identify the compound of interest and impurities [8, 9].
In this work, we reproduced TLC methods reported by scientific papers. Those methods are cited as

reliable for the estimation of radiolabeling percentage using different peptidic compounds. Our objective was to verify if those methods could also be reliable to estimate the radiolabeling percentage of $^{99m}$Tc-SteLL and impurities.

## 2. Methodology

**Materials:** stannous chloride ($SnCl_2$ $H_2O$) solution in HCl 0.1 N at 1 mg/ml, isolated SteLL solution at 1 mg/ml, obtained according Ramos *et al.* [5]; silica gel TLC F254 100 aluminum plates (10x2 cm diameter), Whatman® qualitative paper grade (10x2 cm diameter), cellulose chromatography papers 3MM (10x2cm diameter), NaCl 0.9%, acetone, 0.22 µm syringe filters and pH measuring stripes. A miniGITA TLC scanner (Raytest, Germany) was used for plate counting and Rf estimation.

To obtain a $^{99m}$Tc-SteLL, we synthesized samples with 100 µL of isolated SteLL and 100 µL of stannous chloride solution, stirred and preserved at room temperature, protected from light, for 20 minutes. Then one mCi of sodium pertechnetate eluate was added to each sample, stirred, and held for 10 minutes until pH correction to 7, using 1 M NaOH and 0.01 N HCl. This radiolabeling method was adapted from Patricio *et al*. [10] and Koch *et al.* [11].

The total volume of samples was adjusted to 2 mL with NaCl 0.9%. Aliquots of 10 µL of each sample were submitted to different chromatographic methods (Table I). Stationary phases were dried and counted by the TLC scanner.

Table I: literature-cited methods applied on $^{99m}$Tc-SteLL radiolabeling efficacy.

| METHOD | STATIONARY PHASE | MOBILE PHASE | PRODUCTS RF |
|---|---|---|---|
| Monteiro *et al.,* 2010 [9] | Silica gel | 0.9% NaCl | $Na^{99m}TcO_{4-}$ = 1.0. |
| | Whatman 1M paper | ethyl acetate:methanol (8:2) | $Na^{99m}TcO_{4-}$ and $^{99m}TcO_2$ = 0. |
| Patrício *et al.,* 2011 [10] | Whatman No. 1 paper | Acetone | $Na^{99m}TcO_{4-}$= 1.0. $^{99m}$Tc-Lectin = 0.0. |
| Dias *et al.,* 2005 [12] | Whatman 3MM paper | Saline, acetone | $^{99m}TcO_2$ and $^{99m}$Tc-MDP= 1.0. |

We also modified the radiolabeling method, employing different pH and temperature conditions to the samples, to verify if better radiolabeling efficacy could be obtained.

An additional batch of pertechnetate and stannous chloride 1:1 was synthesized to verify if it would be possible to remove colloidal impurities, mainly $^{99m}TcO_2$, employing filtration, as reported by Diniz *et a*l. (2005) [13]. For this, we used 0.22 µm syringe filters.

## 3. Results and Discussion

Studies cite that, due to its higher molecular weight, the $^{99m}$Tc-SteLL compound will remain at the point of application (Rf 0). Other analytes, if present, should remain at Rf 0 or 1, depending on the chromatographic method. These results are shown for radiopharmaceuticals such as $^{99m}$Tc-MIBI [9], $^{99m}$Tc-Cramoll [10], and $^{99m}$Tc-cefuroxime [14].

Under all conditions, our results showed that $Na^{99m}TcO_4$ remained at Rf ± 1, separated from the other compounds. Otherwise, $^{99m}TcO_2$ remained at the point of application (Rf ± 0), regardless of the

chromatographic method, which is a problem since $^{99m}$Tc-SteLL should have an Rf close or equal to 0. The results are in Table II.

Table II: Retention factors (Rf) for hydrolyzed technetium and free pertechnetate under different chromatographic conditions.

| PRODUCT | STATIONARY PHASE | MOBILE PHASE | RF |
|---|---|---|---|
| Hydrolyzed Technetium ($^{99m}TcO_2$) | W1[a] | Acetone | 0.033 |
| | | NaCl 0.9% | 0.041 |
| | 3MM[b] | Acetone | 0.041 |
| | | NaCl 0.9% | 0.033 |
| | SG[c] | Acetone | 0.041 |
| | | NaCl 0.9% | 0.133 |

a: qualitative Whatman® grade 1 paper; b: 3MM cellulose chromatography paper; c: silica gel TLC F254 100.

The same results were observed in the system using acetate:methanol (8:2) mobile phase. These findings did not agree with published studies, in which it is possible to keep the separation of impurities from the radiolabeled molecule of interest.

In none of the batches it was possible to quantify if there was radiolabeling of SteLL, or only formation of impurities since it was only possible to separate the free pertechnetate.

Submitting samples through 0.22 µm filtration also was not useful. In these cases, the attempt to pass only $^{99m}$Tc-SteLL was unsuccessful, as the mixture did not pass through the filter, possibly due to the molecular size being greater than the supported limit.

Our results suggest that there may be flaws in the use of TLC to quantify the radiolabeling efficiency and purity of new compounds, especially when dealing with $^{99m}$Tc-labeled peptides. Since it was impossible to identify different Rfs for the radiolabeled peptide and impurities, many of the reported studies may overestimate the radiolabeling efficiency, ignoring the presence of impurities critical to the quality of the product. Many articles reporting radiolabeling with $^{99m}$Tc do not cite methods for quantifying impurities.

## 4. Conclusions

We conclude that the use of thin layer chromatography to assess radiolabeling of new molecules may be limited. Our results suggest the lack of reproducibility of methodologies and difficulty identifying and quantifying synthesis impurities, leading to false-positive results. New methods and protocols are necessary for evaluating radiolabeled peptidic agents.

## Acknowledgments

The authors thanks to the Comissão Nacional de Energia Nuclear (CNEN) for financial support and the Universidade Federal de Pernambuco for scientific support.

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