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# Validation of the chromatographic method and implantation of the skin absorption assay (OECD TG 428)

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**Abstract:** Alternative methods may be defined as any method that can be used to replace, reduce or refine the use of animals of experimentation in biomedical research, testing or teaching. Thus, the development and implantation of alternative methods to animal experimental and applied biometrology are extremely relevant nowadays to demonstrate reliability in the results obtained with the requisite quality. The present work aims to present the validation of the chromatographic method (HPLC-DAD), developed by our group at INMETRO**,** using the quantification of caffeine for the implantation of the porcine skin absorption assay (OECD TG 428).

**Keywords**: Alternative methods; validation; bioassays; skin absorption

## 1. INTRODUCTION

The application of metrology in biological assay is hampered by the high complexity of the samples analyzed, once the conditions of storage, manipulation, improper analytical and environmental can derail the entire experiment because of the sensitivity of these samples. Allied to this, intensive research and laboratory analysis are performed for using animals and *in vivo* tests are replaced by *in vitro* models. Alternative methods may be defined as any method that can be used to replace, reduce or refine the use of animals of experimentation in biomedical research, testing or teaching.

The "cosmeceuticals", cosmetics with a therapeutic purpose, are dermatologically tested by means of *in vitro* bioassays to ensure that it does not cause adverse effects that may cause damage to health. On the other hand, for medicinal products being released to the market, clinical trials are required. These ensure appropriate dosage, safe dose, release parameters, among other pharmacological action that classifies the formulation/ingredient as dermal or transdermal, with local or systemic action, respectively[1]. Thus, the development and implantation of alternative methods to animal experimental that can ensure the reliability of results is extremely relevant nowadays.

Laboratories should demonstrate such reliability, that are able to provide data with the requisite quality, using some tools: the use of validated analytical methods, the use of internal quality control procedures, participation in proficiency testing, accreditation and the establishment of metrological traceability of the results of the measurements. In the present work were used polysulfone synthetic membranes and biological samples porcine skin of animals slaughtered for food consumption. Synthetic membranes are used in the release assay and porcine samples to assess absorption or permeation, both assays used caffeine as reference substance, as recommended by the Organization for Economic Co-operation and Development (OECD), by the *in vitro* method of skin absorption (OECD Guideline For The Testing Of Chemicals - Skin Absorption: *In Vitro* Method N° 428). The quantification of caffeine is carried out using the analytical method by High Performance Liquid Chromatography coupled to the Diodes Array Detector (HPLC-DAD)[3]. Thus, the present study has the objective of presenting the development and validation of the chromatographic method used in the implantation of the cutaneous absorption assay at National Institute of Metrology, Quality and Technology (Instituto Nacional de Metrologia, Qualidade e Tecnologia - INMETRO).

## 2. METHODOLOGY

### *2.1. Permeation and skin release tests*

For the release test of the *in vitro* method presented, polysulfone synthetic membranes were used in an automatic system with Franz vertical diffusion cell with constant agitation of 300 rpm and temperature at 37 °C. The volume used was 7 ml for the receptor fluid (0.01 M phosphate buffer, pH 7.4) and the area available for diffusion was 1.77 $cm^2$. A solution of caffeine (Sigma Aldrich® - 99.7%) of 4000 µg/mL concentration in ethanol: water (50:50 v/v) was used as standard reference substance. Different times were established throughout the assay to (0, 30, 60, 120, 240, 360, 480, 600, 720, 840, 960, 1080, 1200, 1320 and 1440 minutes), to collect 1.0 mL of the receptor fluid. After that, of the same amount of the solution returned to the system. For the skin permeation assay the same conditions were used, however the membranes used were prepared from porcine skin with a thickness of approximately 800 µm, composed of stratum corneum, epidermis and dermis. The thickness of each skin sample was measured by a calibrated micrometer (figure 1) so that the measurement between the samples is homogeneous and traceable. For the quantification of the caffeine obtained in the release assay, only the aliquots withdrawn from the receptor fluid were injected. For the permeation assay, in addition to the aliquots of the receptor fluid, were analyzed extracts obtained from the removed stratum corneum by adhesive tapes, surface of the skin, skin (epidermis and dermis) and adjacent skin to the diffusion area. All samples were injected in duplicate and caffeine quantified by the analytical method developed by our group at INMETRO, described in Table 1, by HPLC-DAD.

**Figure 1.** Measurement of porcine skin sample thickness by micrometer.

### *2.2. Analytical method*

From the stock solution of 4000 µg/mL the analytical curve was prepared with the following

concentrations 0.04, 0.08, 0.15, 0.25, 0.50, 1, 2.5, 5, 10, 20, 40, 60, 80, 100 µg/mL, for the quantification of caffeine with the analytical method (table 1).

**Table 1**. Chromatographic conditions used for the analysis of caffeine.

| | |
|---|---|
| Mobile phase | methanol: water (25:75) |
| Injection volume | 2 µL |
| Flow rate | 0.3 mL/min |
| Column | Pursuit 5 C18 (150 x 2 mm; 5 µm) - Varian |
| Temperature | 30 ºC |
| Wave-length | 273 nm |
| total run time | 5.0 min |

### *2.3. Validation*

Validation studies were carried out in the release and permeation method, following the procedures described in DOQ-Cgcre-008[2], evaluating the selectivity and matrix effect, linear range, detection limit, quantification limit, accuracy (recovery) and precision (repeatability and intermediate precision).

## 3. RESULTS AND DISCUSSION

Figure 2 shows the chromatographic profile obtained by the release assay.

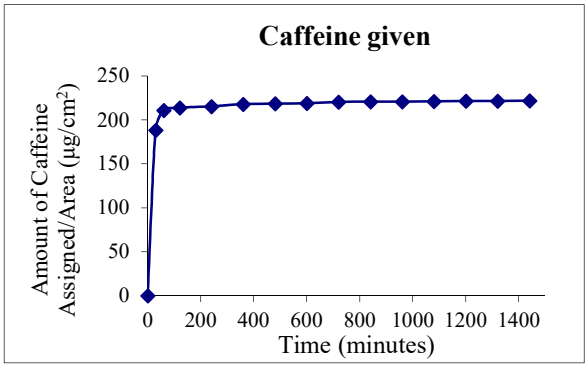


**Figure 2.** Caffeine release profile assigned to the receptor fluid.

The absorption profile of caffeine in the receptor fluid using porcine biomembrane is shown in figure 3, as well as the analytical curve used for the quantification of the same substance, figure 4.

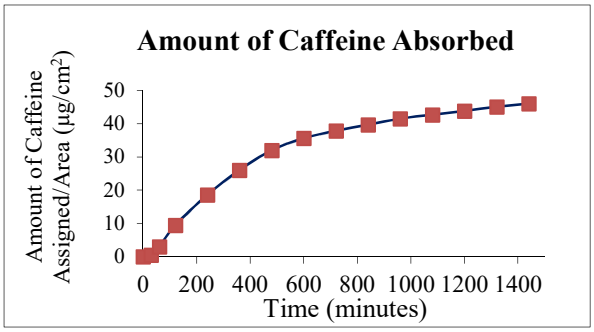


**Figure 3.** Absorption profile of caffeine in the receptor fluid.

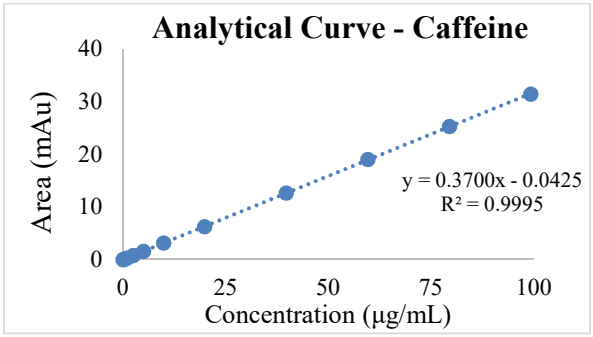


**Figure 4.** Caffeine characteristic analytical curve (0.04 to 100 µg/mL).

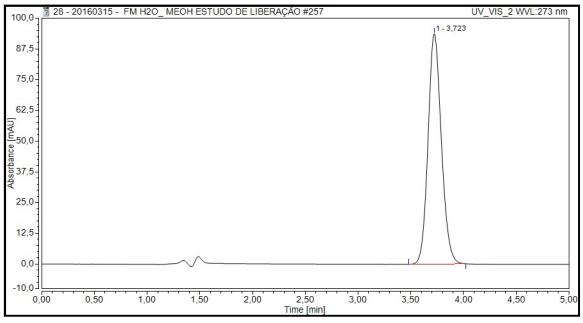

**Figure 5.** Chromatographic profile of the standard 0.5 µg/mL in ethanol:water (50:50 v/v).

The validation results are summarized in Table 2.

**Table 2.** Performance parameters of the release and permeation method in porcine skin ($R^2$ - coefficient of determination; LOD - limit of detection; LOQ - limit of quantitation; RSD - relative standard deviation).

| **Parameter** | **Release** | **Permeation** |
|---|---|---|
| Selectivity | selective | Discrete matrix effect |
| Linear Band | 0.04 - 100 µg/mL; $R^2 > 0.999$ | |
| LOD / LOQ | 0.01 / 0.04 µg/mL | |
| Recovery | > 85% | > 75% |
| Repeatability | RSD < 4% | |
| Accuracy intermediate (animals and different days) | RSD < 6% | |

## 4. CONCLUSION

The implantation of the proposed method was performed, since the caffeine (reference substance) could be quantified in the receptor fluid, as well as in the other components of the cutaneous permeation assay. The analytical curve used to determine the concentration of caffeine presents linearity within its range of application, obtaining $R^2 > 0.999$. For the release assay it was possible to recover > 85% and for the permeation > 75%. This allowed quantification of the released and permeated caffeine. The OECD TG 428 test is suitable for evaluating the skin permeation of substances in dermal products. It also highlights the importance of alternative methods, allowing the substitution of *in vivo* methods by *in vitro*.

## 5. REFERENCES

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