Department of Chemistry and Drug Technologies

PhD in Pharmaceutical Sciences
XXXVII Cycle

Production and quality control according to current Good Manufacturing Practice of the radiopharmaceutical 18F-Fluoromethylcholine

Doctoral Thesis
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1. INTRODUCTION

Prostate cancer is the most common malignant tumor in men and represents the third leading cause of cancer mortality in men [(1)].

Most cases involve males over 70 years old; however, despite the high incidence, the risk of the disease having an unfortunate outcome is low, especially if action is taken in time. Interestingly, compared to 2015, a reduction in mortality rates of 15.6% was estimated in 2020 [(2)]. Today, the survival of patients with prostate cancer is 92%, 5 years after the first diagnosis. This value is in constant and significant growth thanks to increasingly early diagnoses due to the progressive diffusion of large-scale screening. Recent advances have greatly improved the ability to detect and evaluate the development of prostate cancer and its metastatic spread, especially bone metastasis (BM).

An early diagnosis can significantly improve the patient's prognosis, and this is possible through various clinical tests, including:

- Digital rectal exam. First diagnostic approach to appreciate the shape, consistency and approximate volume of the gland, as well as the presence of suspicious nodules.
- Biopsy under ultrasound guidance. The prostate ultrasound has the task of highlighting sonographically dubious areas and improving the accuracy of the prostate biopsy, which is the clinical examination that allows the neoplasm to be diagnosed with greater certainty.
- Prostate specific antigen (PSA) measurement is a protein produced by normal and malignant cells of the prostate gland. The PSA test measures the serum PSA level in the blood.

Currently, PSA is the most used biomarker for PCa (prostate cancer) screening, with which early prostate tumors can be identified even years before they are noticeable to digital exposure or to the association of symptoms.

On the other hand, the PSA test may not be entirely reliable because high PSA levels also occur in benign pathologies such as prostatic hypertrophy or prostatitis or after the performance of a prostate biopsy.

On the contrary, PSA levels may decrease in circulation following the use of 5-alpha-reductase inhibitors (finasteride, dutasteride) used in the treatment of benign prostatic hypertrophy or in drug-prevention programs for prostate cancer [(3)].

Although in the initial screening phase the test can generate false positives, today it appears to be a very reliable indicator of disease recurrence after initial treatment.

Despite several advances, one of the main obstacles to clinical care remains the recurrence of tumors after prostatectomy, radiotherapy or other local intervention techniques.

PSA levels, along with other parameters, can help determine the risk of having bone metastases. A classification report is used to classify patients into three risk levels:

- low risk (PSA < 10 ng/mL, clinical stage T1-T2a, Gleason score ≤ 6);
- intermediate risk (PSA 10.1-20 ng/mL, clinical stage T2b-T2c, Gleason score = 7);
- high risk (PSA > 20 ng/mL, clinical stage T3a or higher, or Gleason score ≥ 8) [(4)].

Numerous techniques can be used to pinpoint the locations of biochemical recurrences and detect prostate cancer.

Diagnostic imaging techniques play a fundamental role in the diagnosis and staging of the disease: computed tomography (CT), magnetic resonance imaging (MRI), bone scan are used to detect primary and metastatic prostate cancer, for staging and for risk stratification. Together with positron emission tomography (PET/CT), it is possible to obtain: i) differential diagnoses between benign and malignant lesions; ii) staging; iii) restaging at the end of treatment; iv) monitoring the effectiveness of therapy; v) identification of primary tumor of unknown location; vi) biological characterization of the neoplastic tissue; vii) localization of the most appropriate site within a mass to be subjected to biopsy.

The use of radiotracers for PET analysis enables to identify the tumor mass and determine its staging. The PET technique, combined with MRI or CT, is indicated in the identification of prostate cancer for subjects whose PSA values are very high >10 ng/mL and with a Gleason score >7 and for subjects who present levels of PSA < 2ng/mL but with biochemical relapse.

There are currently several radiotracers used in PET analyses. These differ from each other for the carrier portion necessary to reach the organ or system of interest, or for the portion of the radioactive nuclide which allows the distribution of the radiopharmaceutical in the body to be followed through diagnostic instrumentation. The choice of the radiotracer is therefore fundamental and varies based on the type of tumor or the half-life of the radioactive nuclide.

18F-Fluorodeoxyglucose (18F-FDG), a marker of glucose metabolism, has a limited role in PCa due to the relatively low metabolic rate of well-differentiated prostate tumors [(5)].

The limited usefulness of the 18F-FDG has led to the development of other radiotracers for PCa. Choline derivatives are a class of PET tracers that have been extensively evaluated during the last decade [(6)].

Choline is a substrate for the synthesis of phosphatidylcholine and an important phospholipid in cell membranes. Cell membrane biosynthesis is rapid in tumor tissues, and positive regulation of malignancy-induced choline kinase activity results in increased uptake of choline by tumor cells. Radiolabeled choline PET-CT imaging has emerged as a promising method for disease localization in PCa patients with biochemical recurrence after primary treatment. The greatest advantage of this method is its ability to evaluate also disease recurrence at multiple anatomical sites with greater accuracy than conventional imaging and 18F-FDG PET-CT imaging. The short half-life of 11C ($t_{1/2}$ = 20 min) generally prevents late imaging where the tumor-to-blood ratio is considered optimal for detecting recurrence. Due to the multifaceted chemistry and short half-life of the 11C, the use of the

11C-Choline (11C-CCH) is limited. On the other hand, the 18F-Fluoromethylcholine (FCholine; 18F-FCH) is more widely available with stable biodistribution and a longer half-life ($t_{1/2}$ = 110 min). Additionally, delayed imaging can be performed to better differentiate physiologic urinary radiotracer excretion from recurrence. *In vitro* absorption and phosphorylation of 18F-FCH are similar to those of 11C-CCH and superior to those of other radiopharmaceuticals. Although new 68Ga-based radiopharmaceuticals including 68Ga PSMA-11, 68Ga- Bombesin, and 68Ga- RM2 have also been used for PCa imaging, PET-CT 18F-FCH is still considered an important diagnostic modality according to Clinical Practice Guidelines in Oncology (NCCN) guidelines due to its longer half-life [(5)].

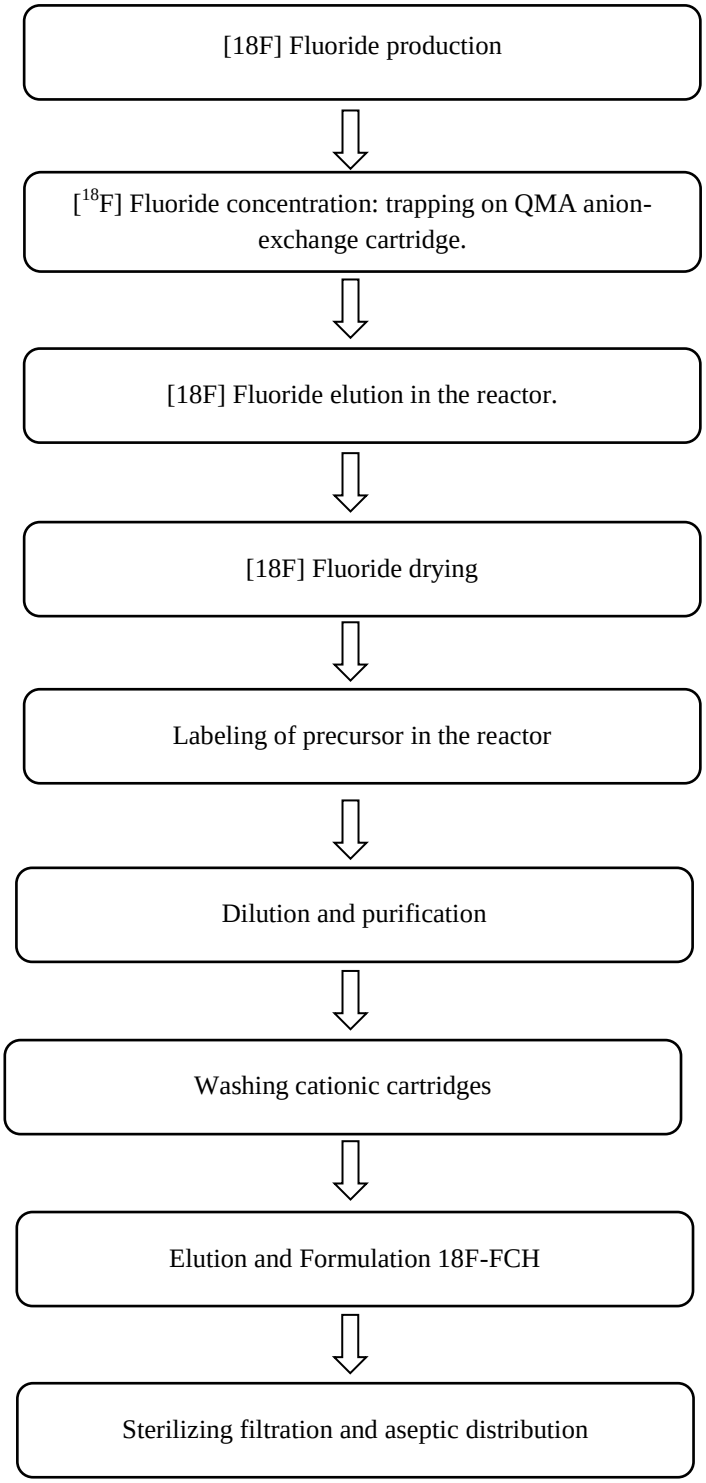
The use of 18F-FCH has limited value for imaging and diagnosis of primary malignant neoplasms of the prostate. It may be useful in patients with elevated PSA levels and negative repeat biopsies to guide repeat biopsy. The positive predictive value of PET/CT 18F-FCH for the detection of lymph node involvement and the sensitivity and specificity of PET/CT 18F-FCH for the evaluation of bone metastases is relatively high. PET/CT 18F-FCH is useful for distinguishing between locoregional recurrence and distant metastasis in cases of biochemical recurrence, particularly in interAveragete-to-high risk patients with certain criteria (e.g. high trigger PSA values and/or a short PSA doubling time and/or Gleason score > 7). PET/CT 18F-FCH may also play a role in radiation therapy dose escalation or salvage therapy [(6)].

The increased use of 18F-FCH has led to commercial availability of this tracer. For satisfying the demand of 18F-FCH, a high-yield production process is required. Using the new synthesis process compared to the one currently in use, the amount of 18F-FCH available for PET analysis is significantly increased.

To guarantee the quality of 18F-FCH, GMP (Good Manufacturing practice) standards must be applied starting from the design and implementation of the production and quality control activities. Equipment and quality control methods must be validated to ensure the quality and safety of 18F-FCH and of any other pharmaceuticals.

The primary objective of the radiopharmaceutical manufacturing process is to consistently produce a product of expected quality that adheres to predetermined specifications. Reliability and reproducibility of the manufacturing process are essential characteristics of a radiopharmaceutical manufacturing process. The robustness of routine processes is guaranteed by the design and validation of the entire production process, including analytical quality control methods. The validation and qualification activities are aimed at demonstrating that the critical aspects of the production process are under control. In particular, the validation of quality control (QC) analytical methods is fundamental to demonstrate the reliability of QC activities, which detect non-conformities or out of specification. Consequently, the manufacturing process must be designed to produce radiopharmaceuticals with uniform quality, purity and potency.

The following diagram outlines the main steps of the 18F-FCH manufacturing process.



2. DESCRIPTION OF THE PRODUCTION PROCESS

The production of 18F-Fluoromethylcholine (18F-FCH) consists of the following steps:

- production of the radionuclide anion $^{18}F^-$;
- production of 18F-Fluoromethylcholine and purification (using the Tracerlab module or the Trasis Allinone module);
- transfer of the solution to the dispensing system;
- dilution;
- final sterilizing filtration and distribution of the product in the primary container;
- secondary packaging (shielded container).

2.1 Description of the manufacturing process

2.1.1 Production of radionuclides

Radionuclide is a radioactive isotope that can be included for medical purposes in any medicine. Radioisotopes used for diagnostic purposes have the following characteristics:

- an adequate decay time, compatible with the patient's exposure time;
- a high-performance production method in order to meet market needs;
- the decay of the radionuclide must produce positrons or gamma rays [(7)].

2.1.2 Fluoride production 18

The production of 18Fluoride occurs using a ccyclotron (Figure 1), by proton irradiation of water enriched with oxygen-18 (target material). A cyclotron is a device that accelerates charged atomic or subatomic particles in a constant magnetic field. A cyclotron consists of two hollow semicircular electrodes, called dees, mounted back-to-back, separated by a narrow gap, in an evacuated chamber between the poles of a magnet. An electric field, alternating in polarity, is created in space by a radio frequency oscillator [(7)].

The beam is pulled out of the main chamber. It is collimated on the target, which is filled with different materials. Based on the loaded material, different radionuclides can be produced:

- ^{18}O water for the production of ^{18}F;
- ^{14}N gas for the production of ^{11}C;
- ^{16}O liquid for the production of ^{13}N;
- ^{15}N gas for the production of ^{15}O;
- ^{14}N gas for the production of ^{15}O;
- ^{20}Ne gas for the production of $^{18}F2$;
- $^{18}O2$ gas for the production of $^{18}F2$;
- ^{124}Xe Gas for the production of ^{123}I;
- ^{82}Kr gas for the production of ^{81}Rb.

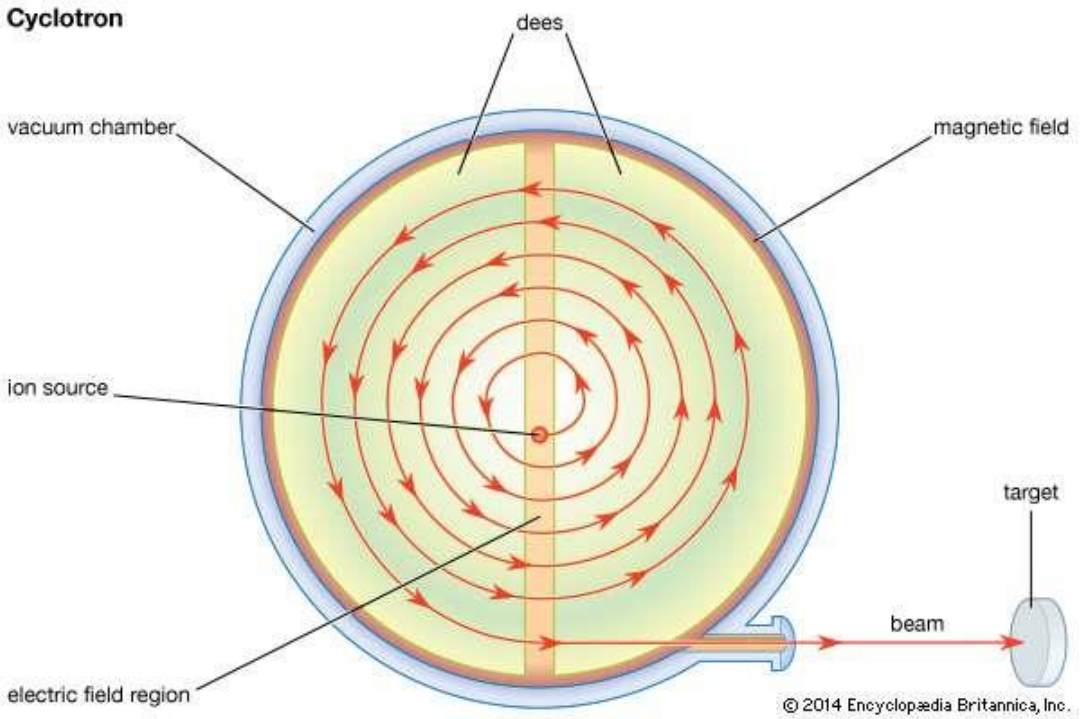


Figure 1. Scheme of a cyclotron accelerator.

The production of the radioisotope (Figure 2) starts from the production of hydrogen, that is conveyed inside the cyclotron where a source of ions carries out the heterolytic breaking of the bond; the hydride ions are accelerated by the cyclotron and pass through a carbon foil which allows the conversion of the hydride ions into protons by stripping the 2 electrons, this channeled into the target full of enriched oxygen-18 will allow the nuclear reaction to produce $18F^-$.

Nuclear Report: ^{18}O (p,n) → $^{18}F^-$

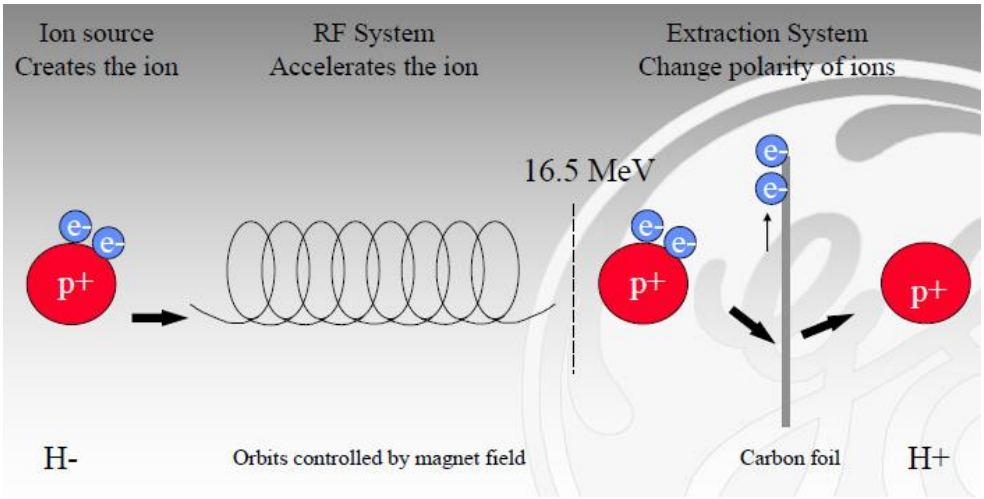


Figure 2. Radioisotope production in a cyclotron.

In ITEL Telecommunicazioni two GE PET trace 860 cyclotrons are installed, both equipped with two liquid targets for the production of $18F^-$. The volume by H_2O-18 for the production of $18F^-$ is 2.5 mL or 5 mL based on the activity to be produced with the bombardment, of one (single beam) or two targets (dual beam) respectively. The bombardment occurs with a proton beam with an intensity of 60 µA for a single target or 100 µA for a dual target, the irradiation time depends on the final activity required for the production of the product. The aqueous solution of the produced radionuclide is delivered through shielded transfer lines (1/16" OD, ETFE tubes) directly to the synthesis module, in which the synthesis process takes place (Figure 3 shows the automated synthesizer AllInOne and the control PC).

For radiation protection the synthesis takes place inside a shielded hot cell. For the synthesis of 18F-FCH, disposable cassettes are used, specifically developed for each synthesis module.

Figure 3. AllInOne synthesizer.

2.2 Tracerlab synthesis process description

The synthesis used in this process follows the process developed by David Kryza on the TracerLab MX FDG synthesis module [8].

2.2.1 Elution of [^{18}F]Fluoride ions

The [18F]-fluoride ions resulting from the bombardment of H_2O-18 are conveyed into the synthesis module and are trapped on the pre-conditioned QMA anion exchange cartridge present on the synthesis cassette. The QMA cartridge has the task of blocking the [^{18}F]-fluoride ions and allowing the elution of the enriched water.

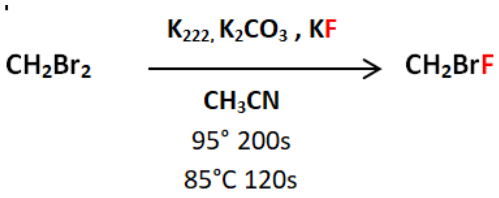
The [^{18}F]-fluoride ions are eluted from the cartridge to the reactor using 0.6 mL of an eluent containing potassium carbonate and kryptofix (K_{222}) in a 50/50 acetonitrile/water mixture (QMA eluent), (Scheme 1).

2.2.2 Evaporation of solvents

After transferring the eluent containing the $^{18}F^-$ activity in the reactor, the solvents are evaporated to dryness at 95°C. During the drying process, small amounts of acetonitrile are added to the reaction vessel 3 times (80 µl each time). Evaporation occurs by heating the reactor under a nitrogen flow and under vacuum.

2.2.3 Precursor labeling

The solution containing the dibromomethane dissolved in approximately 3 mL of acetonitrile, is transferred automatically from its vial in the reactor. A nucleophilic substitution reaction occurs at 95°C for 3.7 minutes in which a bromide from dibromomethane is replaced by [18F$^-$] ion obtaining the Bromo[18F]fluoromethane (Scheme 1). The labeling reaction is the most critical step in the synthesis of 18F-FCH. Any trace of impurities in the input activity, reagent, eluent mixture, nitrogen, QMA cartridge or collector could result in a decrease in labeling yield.



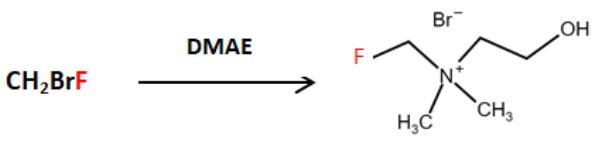
Scheme 1. Labeling reaction.

2.2.4 Bromo[18F]fluoromethane purification

The gaseous Bromo[18F]fluoromethane is passed through 3 polar absorption silica cartridges, to separate any remaining unreacted dibromomethane from the [18F]bromofluoromethane, this process is developed as the remaining portion of remaining unreacted dibromomethane would react with the 2-(dimethylamino)ethanol (DMAE) in the next step obtaining unwanted by-products.

2.2.5 Alkylation reaction

The gaseous Bromo[18F]fluoromethane passes, then, through two polymer-based reversed phase cartridges (Oasis HLB), the first loaded before the synthesis with 2-(dimethylamino)ethanol (DMAE), here the alkylation reaction takes place, obtaining the Dimethyl(fluoromethyl)(2-hydroxyethyl)ammonium bromide (F-18-Fluorocholine bromide) (Scheme 2).



Scheme 2. Alkylation reaction.

2.2.6 F-18-Fluorocholine bromide purification

Once the F-18-Fluorocholine bromide on solid phase is obtained, the Oasis HLB cartridges are rinsed with water for injections (30 mL), ethanol (10 mL) and again water for injections (30 mL). The rinse fluids are passed through two silica-based cation exchange cartridges (CM cartridges) where the 18F-FCH is retained.

2.2.7 Elution of 18F-FCH

18F-FCH is eluted and simultaneously formulated by 13 mL of 9 mg/mL NaCl solution. The product is pushed by nitrogen gas from the synthesis cell to the dispensing insulator (Class A) *via* a disposable capillary, the produced stock solution undergoes a first sterilizing filtration using a 0.22 µm sterilizing filter and elutes into a sterile vial. The volume of the 18F-FCH stock solution is ≥ 11 mL (Scheme 3).

Scheme 3. 18F-FCH formulation.

2.3 Description of Trasis Allinone synthesis process

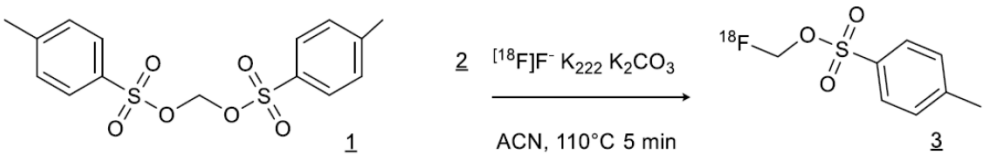
2.3.1 Elution of [18F]Fluoride ions

The ions [18F]-fluoride resulting from the bombardment of H_2O-18 and conveyed into the synthesis module are trapped on the pre-conditioned QMA anion exchange cartridge present on the synthesis cassette. The QMA cartridge blocks the [18F]-fluoride ions and allow the elution of the enriched water.

The QMA cartridge is washed with 400 µl of acetonitrile to remove remaining traces of water, followed by [18F]-fluoride ions are eluted from the cartridge to the reaction reactor using 0.75 mL of an eluent containing potassium carbonate and kryptofix (K_{222}) in a 25/75 water/acetonitrile mixture (QMA eluent, intermediate 2 Scheme 4)[15].

2.3.2 Labeling of the precursor with 18F in the reactor

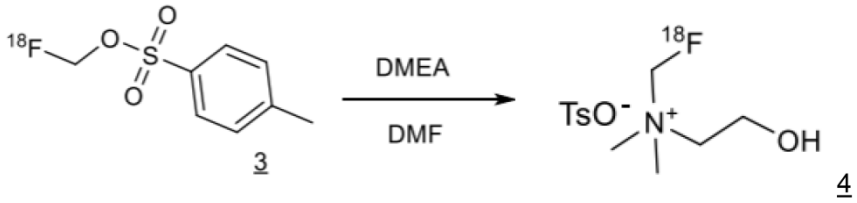
The methylene bis(toluene-4-sulfonate) (reagent 1, Scheme 4) solution is added inside the reactor in acetonitrile; the fluoride anion displaces one of the tosyl groups forming the intermediate [18F]Fluoromethyl 4-methylbenzenesulfonate (intermediate 3, Scheme 4).



Scheme 4. Intermediate reaction formation.

2.3.3 Quaternization of the amin

Upon completion of the previous reaction, a mixture of 2-(dimethylamino)ethanol (DMAE) and *N*,*N*-dimethylformamide (DMF) (1:1 v:v) is added to the reactor, which is heated to achieve quaternization of the amine which leads to the formation of *N*-([18F]fluoromethyl)-2-hydroxy-*N*,*N*-dimethylethane-1-amino 4-methylbenzenesulfonate (Scheme 5).



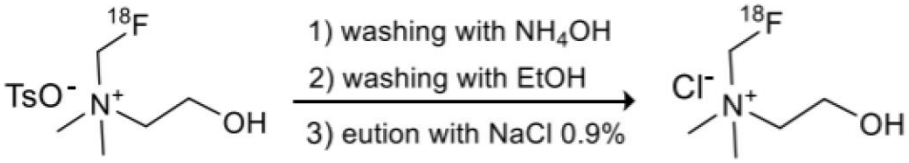
Scheme 5. Amine quaternization.

2.3.4 Dilution and purification

The compound is cooled and diluted in water, then purified through silica-based reversed phase cartridge (Sep-Pak tC18 cartridges), the tC18 allows to block unreacted reagents, for example methylene bis(toluene-4-sulfonate and secondary products), Oasis HLB, and finally trapped on the polymer-based cation exchange cartridge (WCX cartridge) [(16)].

2.3.5 Washing cationic cartridges

The WCX cartridge is washed with diluted ammonia to remove the unreacted DMAE from the product (DMAE not having affinity to the cartridge is easily eluted), washing with ethanol is used to eliminate the DMF present in the product and finally a final wash with water (Scheme 6).



Scheme 6. Elimination of impurities and product formulation.

Washing the WCX cartridge to remove unreacted DMAE is necessary, so that DMAE residues are as low as possible in the final product. DMAE is a competitive inhibitor of cell transport 18F-FCH[9]. By decreasing the final concentration of DMAE, and the one of ^{18}F-Fluoromethylcholine on tumor sites, furthermore a reduced concentration of DMAE allows to reduce interference in molecular imaging 18F-FCH.

2.3.6 [18F]-FCM elution

18F-FCH, the active ingredient, is eluted from the cationic cartridges using 15 mL of a 0.9% NaCl solution and then purified on an additional non-polar HLB cartridge prior be to collected in the final vial. This step ensures the removal of any non-polar impurities. The product is pushed by nitrogen gas from the synthesis cell to the dispensing insulator (Class A) via a disposable capillary, the produced stock solution undergoes a first sterilizing filtration using a 0.22 µm sterilizing filter and elutes into a sterile vial. The volume of 18F-FCH stock solution is ≥ 11 mL.

2.4 Formulation and terminal sterilization

The stock solution is diluted with a 9 mg/mL sodium chloride solution, obtaining a solution of 18F-FCH with a contraction of 222 MBq/mL. The drug solution comes dispensed with a semi-automated dispensing system and is filtered through a terminal sterilizing filter before being dispensed into each individual sterile vial.

2.5 Secondary packaging

After dispensing, the vials are placed in the safety shielded containers and removed from the dispensing isolator.

3. COMPARISON OF SYNTHESIS ROUTES

3.1 18F-FCH production with Tracerlab module

The production of 18F-FCH with dibromethane precursor produced using the process developed by David Kryza on the TracerLab MX FDG synthesis module [8], with some small modifications obtained following subsequent studies to improve the final purification process, one of which consists in the replacement of a C18 cartridge with a second CM cartridge.

This production process, well consolidated over the years, uses a disposable FDG synthesis cassette (Figure 4), adequately modified for the synthesis of 18F-FCH (Figure 5), for the synthesis of the radio tracer.

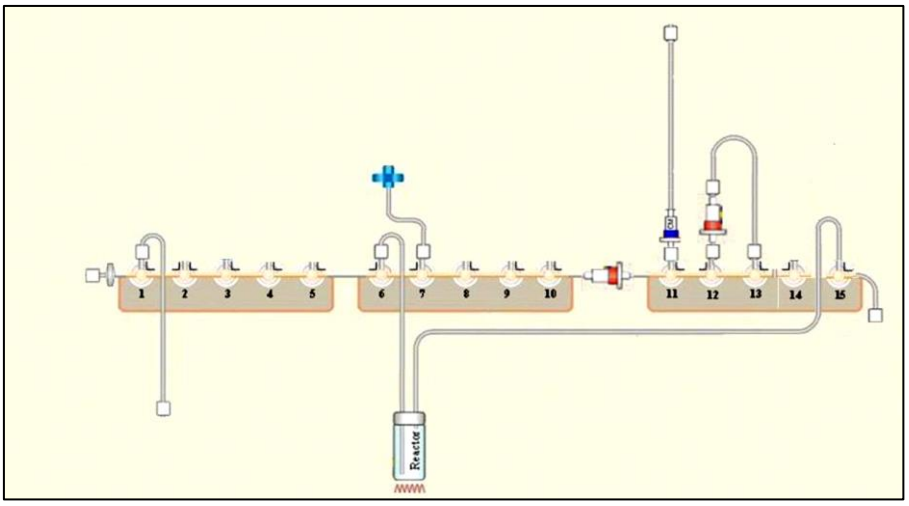


Figure 4. FDG production cassette.

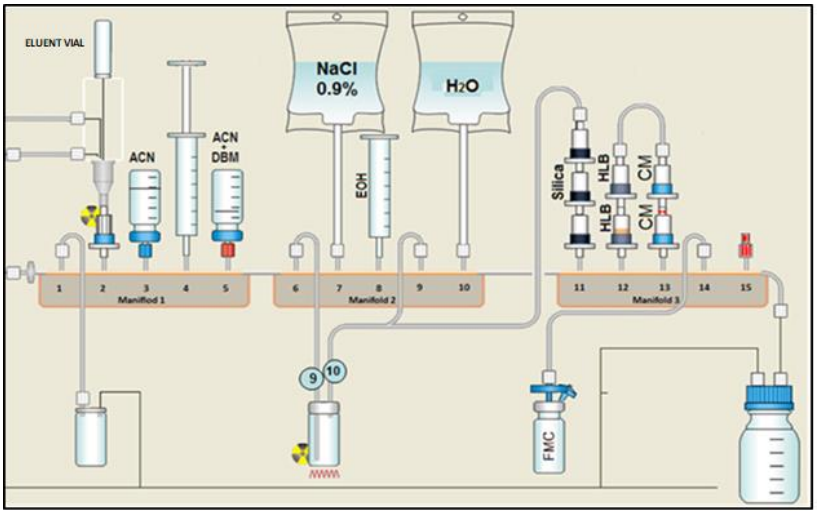


Figure 5. Modified cassette for 18F-FCH production.

By using this synthetic route, human error is introduced into the synthesis process due to the various modifications between capillaries and purification cartridges to be inserted/replaced in the synthesis cassette and also to the handling of the raw materials involved in the synthesis which must be loaded manually by the production operator. Among these steps, the fundamental ones are the following:

- DMAE loading on HLB cartridge;
- Dibromomethane dissolving in Acetonitrile;
- Adhesion of the bottle containing QMA eluent on the synthesis module.

3.2 18F-FCH production with Trasis AllinOne

The cassette developed by Trasis (Figure 6) for production of 18-FCH is a single-use cassette, used together with the Trasis AllInOne synthesizer for the automated synthesis of 18F-FCH. The cassettes include all the components necessary for the production of FCH, from receiving the activity from the cyclotron to transferring the final product.

Figure 6. Trasis module with 18F-FCH production cassette.

The raw materials involved in the reaction, if not present on the cassette, are prepared in vials. In this case, the manual operation consists in inserting the bottle onto the spike having the same color as thring on the vials. Allowing to significantly reduce human errors.

3.3 18F-FCH production comparison

The use of 18F-FDG cassettes on the Tracerlab synthesis module adapted for the production of 18F-FCH is now dated. Unfortunately, the latter are modified/assembled by the production operator before carrying out the synthesis, furthermore in the different productions they are manipulated by different operators which leads to a process that is not entirely uniform.

The production process with trasis module instead involves a series of advantageous improvements, as listed below:

- The new synthesis route does not generate radioactive volatile gases, as no gaseous reactions are part of the process, unlike the Tracerlab method where gaseous dibromomethane is produced.
- Bromo[18F]fluoromethane is a volatile intermediate produced by the older synthesis process (dibromomethane) and the discharge of the radioactive gas is a potential risk. With the

synthesis on the Trasis Allinone module, no radioactive atmospheric release is expected, which brings a great advantage and an improvement in operator safety.

- Residual DMAE, a toxic solvent, is reduced approximately 50-fold below the European Pharmacopoeia limit.
- Better yield: Although the old method provides a reproducible and reliable synthesis, the new synthesis module offers a more robust process and higher yield.
- The Plug and play system of the Trasis cassette eliminates the laboratory preparation of the synthesis cassette. Reducing pre-synthesis manipulations by operators results in fewer potential errors and a more reliable process. Furthermore, the use of a disposable reagent kit avoids the risk of cross-contamination.

Production capacity and efficiency (reliability) are directly affected. A greater synthesis yield is observed: the average yield of 18F-FCH with the Tracerlab process is around 7-9 %, while the average yield with the Trasis Allinone process is around 35 %.

4. QUALIFICATION AND VALIDATION

Good Manufacturing Practice (GMP) regulations require manufacturers to manage critical aspects of their operations by implementing qualification and validation procedures throughout the entire product and process lifecycle [10]. The validation process is essential to verify that a given procedure, process, equipment, material, activity or system will produce the expected results, with the ultimate goal of ensuring the quality of (radio)pharmaceuticals [10]. This ensures that the manufacturing process is well controlled and consistent, with the end result of providing safe and effective products to the market. The qualification process involves checking several aspects, including manufacturing, quality control systems and equipment, computer systems and personnel. These steps are essential to guarantee the proper functioning and reliability of the systems and maintain the quality of the overall result.

4.1 Qualification of the synthesis module (AllInOne Trasis)

The qualification of the synthesis module (AllInOne Trasis) is developed through three different sequential phases:

- installation qualification (IQ);
- operational qualification (OQ);
- performance qualification (PQ):

These phases are generally performed separately, each is documented through a specific qualification protocol. The objective of the qualification is to provide documented evidence that the systems or equipment are installed in accordance with the reference specifications, operate within the specified operating ranges and achieve results that meet user-defined requirements.

The purpose of the qualification is to provide documentary evidence that the systems or equipment are installed and operate in compliance with the reference specifications, defined in the User Requirements Specification. Qualification activities should consider all phases, from initial development of user requirements specifications until the end of use of the equipment, facility, utility or system. Below the main stages and some suggested criteria (although this depends on the circumstances of the individual project and may be different) [10] are described.

4.2 Qualification of the installation

Installation Qualification (IQ) requires verification of all major components of equipment, systems and services against the manufacturer's and/or supplier's technical specifications and documentation.

The main objectives of IQ are to highlight and document:

- installation regarding detailed specifications and manufacturer recommendations;
- the installation status of the utilities;
- all critical measuring instruments of the equipment;

- verification of the existence, at least in draft form, of adequate operating procedures for the use, maintenance and cleaning of the equipment;
- availability of manuals for using the equipment;
- availability of a list of critical spare parts of the equipment;
- connection to utilities;
- the correct environmental conditions for installation.

4.3 Operational qualification

Operational Qualification (OQ) is a fundamental procedure used to verify that equipment operates correctly according to functional specifications within the approved range for the equipment.

The testing procedure involves a detailed evaluation of the functionality and performance of the equipment in line with pre-determined specifications. The purpose of this test is to ensure that the equipment operates efficiently and effectively while meeting the required quality standards. As a result, OQ test results are vital to the overall performance and maintenance of the equipment, providing a basis for the continued use and maintenance of the equipment.

The objectives of the Operational Qualification (OQ) process are multiple and listed below:

- verify the existence of appropriate operating procedures for the use, maintenance and cleaning of the equipment, at least in draft form;
- verify the existence of a suitable staff training procedure
- check the calibration status of the "critical" instrumentation;
- check the calibration status of any measuring devices not installed on the equipment, but used to collect the Qualification data;
- check the operating status of the utilities for the specified parameters (temperature, pressure, power supply, etc.);
- verify (where applicable) that functions, alarms and local control panels operate according to manufacturer specifications;
- control critical operating parameters in specified workflows;
- validate the user's access rights for the software used; during which the functionality of the automated module must be verified without reagents or chemical components;
- a verification of the user access policy to the software, with reference to the different possible levels of privileges (for example, admins have the right to modify any parameters, sequences, methods, etc., while operators only have the possibility to execute the dispensing programs);
- a verification of the software sequences, if applicable;
- a check of the possible effects of a general power failure (for example to verify the presence and/or need for a UPS);
- a verification of data backup and restoration.

4.4 Performance qualification

Performance Qualification (PQ) is a critical step in ensuring that equipment consistently produces finished products or conditions that meet User Requirements Specifications (URS) while operating within the normal range of control parameters. PQ must be performed for each product manufactured using the qualified equipment or system. Therefore, the PQ must be performed for each product that is manufactured using the equipment/system being qualified.

The objectives of the PQ are the following:

- verify the existence of adequate approved operating procedures for the use, maintenance and cleaning of the equipment in question;
- verify the training status of the staff regarding the use and management of the equipment in question;
- verify the "critical" calibration status of the instrumentation;
- check the calibration status of any measuring devices not installed on the equipment, but used to collect the Qualification data;
- verify the efficiency attributes required for the equipment.

The production equipment used for the synthesis and distribution of 18F-Fluorocholine has been validated according to a risk assessment approach. Each test was chosen to demonstrate the reliability and reproducibility of each task.

The individual tests performed, with the acceptance criteria and the related results constitute company know-how. All phases for the validation of the production equipment have been carried out, including the Averagefill for the operators.

By way of example, the tests performed on the AllInOne synthesis module are shown below:

- installation qualification:
  - the list of equipment components;
  - the visual inspection of equipment;
  - the environmental condition;
    - inside the hot cell;
    - Hot cell environment prerequisites;
  - the installation:
    - connections inside the hot cell;
    - external connections to the hot cell;
- Operational qualification;
  - Operating mode
    - testing of linear actuators (activity piston and syringe drives);
    - testing of rotary actuators;

        - reaction oven test;
        - vacuum pump test;
        - circuit leak test;
        - reproducibility of the pressure circuit and leak test;
        - test of the cassette fixing mechanism: visual inspection;
    - the radioactivity detector response;
- performance qualification;
    - the stages of the process.

The results obtained during the validation process confirmed the reliability of the synthesis method.

5. VALIDATION OF THE METHODS

The quality of the 18F-FCH, as final product, must be verified before administration to patients, to ensure that its characteristics (i.e. identity and purity) are suitable for the intended use.

To obtain accurate results, it is necessary to validate the analytical methods used for radiopharmaceutical analyses. Analytical method validation requires testing of several parameters, as outlined in the guidance document published by the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH). This document has been adopted by the European Medicines Agency (CPMP/ICH/381/95 2014) and is therefore applicable in all Member States of the European Union (EU).

Using these guidelines, all analytical methods used in chemical quality control procedures are validated.

The validation steps must be documented. Prior to the validation study, a validation protocol must be generated. The protocol must contain information on the intended purpose of the analytical procedure, the performance characteristics to be validated and the associated acceptance criteria. The results of the validation study must be summarized in a validation report [(11)].

Below some of the typical validation features that need to be taken into consideration are listed:

- specificity: ability to measure the desired analyte in a complex mixture; ·
- precision: agreement between measured value and real value;
- linearity: proportionality of the measured value with respect to the concentration;
- detection limit: minimum quantity of detectable analyte;
- limit of quantification: minimum quantity of measurable analyte.

The following Table 1 summarizes the chemical and microbiological analytical methods used to evaluate the quality of 18F-FCH, as well as the equipments involved.

Table 1. Methods to be validated.

METHOD	EQUIPMENT	TYPE OF METHOD
Evaluation of residual solvents	GC	Impurity test: limit
Chemical purity	HPLC	Impurity tests: quantitative
Chemical purity	TLC	Impurity test: limit
Radiochemical purity	TLC	Impurity test: limit
Radiochemical purity	Radio-HPLC	Impurity tests: quantitative
Bioburden	Filter pump	Impurity test: limit
Sterility	Insulator	Impurity test: limit

The following paragraphs describe in detail the various analytical techniques used, the description of the validation, the equipment, the reagent and the solutions to be prepared, the analysis conditions and the tests.

Following the correct execution of the validation protocols, after their approval, the analytical procedures can be considered validated and used for the analysis of the final product.

The execution of some analyzes performed on the final product are performed according to scientifically validated methods described in the European Pharmacopoeia, therefore unless otherwise indicated, it is not necessary for the analyst to validate the test methods. Refer to the Table 2 below for a summary of chemical analytical procedures that do not require validation.

Table 2. Methods for which the corresponding validation is not required.

METHOD	EQUIPMENT
Wait	Visual inspection
Identification A	Gamma ray spectrometer
Identification B	Dose calibrator
pH	pH meter
Radionuclide purity	Gamma spectrometer
Radioactive concentration	Dose calibrator
Filter integrity test	Bubble point test

5.1 Radiochemical purity

By measuring the radiochemical purity, the content of impurities labeled with [18F]Fluoride, due to reaction by-products is established.

The formation of by-products can be due to:

- a malfunction of the purification in the product labeling phase;

- the action of solvents;

- a change in temperature;

- a change in pH;

- the presence of oxidizing or reducing agents;

- incomplete reaction and/or radiolysis.

Impurities can reduce the quality of PET images, causing unnecessary exposure in non-target organs and poor diagnostic quality.

5.1.1 Radiochemical purity – (Radio TLC)

Using the Radio-TLC methodology it is possible to detect all the radioactivity applied without recovery problems. The determination of radiochemical purity occurs through the ratio between the area of the peak of the desired radiopharmaceutical and the overall area of all the peaks of the detected impurities.

The analysis is carried out with thin layer chromatography by applying a very small portion of the solution containing the radiotracer, the prepared plate is immersed in the solvent contained in the development chamber. The slide is immersed so that the drop of radiotracer is above the solvent.

During the chromatographic process, the sample components are distributed between the adsorbent (silica gel) and the solvent, depending on their distribution coefficients. Each component of a given sample is characterized by an Rf value (Retardation factor), which is defined as the ratio of the distance traveled by the component to the distance traveled by the solvent front from the original application point of the sample. Rf values are mainly used for the identification of different components.

Radioactivity is measured through a radiochromatographic scanner, through which a chromatogram is obtained with the radioactive peaks of the radiotracer and the various impurities. The radiochemical impurity is calculated as the percentage ratio of the radioactivity of the unwanted component to the total radiochemical activity.

5.1.2 Description of the analytical method

- ✓ The analyzes are performed by depositing approximately 5µL of the 18F-FCH solution to be analyzed on a TLC slide 1 cm from the bottom of the slide, to be subjected to development in an appropriate chamber where there is a mobile phase with a volume of 10 mL, paying attention to do not wet the seed line. Following the chromatographic run, the plate is dried in a stream of air at room temperature and subjected to analysis by the radiometric detector. The analysis condition is summarized below:
  - stationary phase: TLC Silica gel 60F254 on glass support, TLC plates dimensions 200mmx50mm ;
  - mobile phase: 9 mg/mL NaCl solution in ultrapure water/acetonitrile (50:50 v/v);
  - solvent front: 8 cm;
  - application: approximately 5 µl.

Method validation

- ✓ The tools used for validation are:
  - scanner TLC;
  - micropipettes P100;
  - micropipettes P10;
  - development chamber;
  - graduated cylinder 25 mL class A;
- ✓ The materials used for validation are:
  - TLC Silica gel 60F254 a glass support, TLC plates dimensions 200mmx50mm;
- ✓ The reagents used for validation are:
  - acetonitrile;
  - 9mg/mL NaCl solution in ultrapure water.
- ✓ The solutions used for validation are:
  - Mobile phase: 9mg/mL NaCl solution in ultrapure water and acetonitrile 50:50 (v/v);
  - [18F]-F^- aqueous solution;
  - solutions of 18F-FCH at different concentrations.

5.2 Chemical purity: evaluation of residual solvents

Radiopharmaceuticals may contain small amounts of organic solvents as impurities, a result of the solvents used during the reaction synthesis. Gas chromatography (GC) is used to analyze these impurities and these methods must be validated according to ICH general guidelines.

5.2.1 Description of the analytical method

The test is performed using a GC. The analytical conditions are summarized below:

- carrier gas flow: 1.5 mL/minute;
- synthetic airflow: 300 mL/minute;
- hydrogen flow: 30 mL/minute;
- initial oven temperature: 60°C;
- ramp at temperature: +25°C/min
- final oven temperature: 160°C;
- injector temperature: 250°C;
- detector temperature: 275°C;
- injection volume: 1 µl;
- Split ratio: 10
- course duration: 13 minutes;
- column: Restek DB-624.

Before analyzing the sample, it is necessary to verify the adequacy of the system. A reference solution (MIXTURE C5) containing is prepared Acetonitrile 408.7 mg/mL; Ethanol 4970.7 mg/mL; Isopropanol 4951.8 mg/mL; Dimethylaminoethanol: 97.45 mg/mL; Dibromethane: 9.91 mg/mL.

The test is judged compliant if the concentrations obtained fall within the range of ± 20% compared to the theoretical concentrations and the resolution between Ethanol and Acetonitrile must be ≥ 2. All individual solvent peaks can be identified by comparing the retention time values for each raw material with the retention time values of the reference solution:

- the average retention time of ethanol is 2.7 minutes;
- the average retention time of isopropanol is 2.9 minutes;
- the average retention time of acetonitrile is 3.1 minutes;
- the average retention time of dibromomethane is 4.6 minutes;
- the average retention time of dimethylaminoethanol is 4.9 minutes;

5.2.2 Method validation

✓ The tools used for validation are:

- GC Agilent 8890;
- Column Restek DB-624, poly(cyanopropyl)(phenyl)(dimethyl)siloxane (1.8 µm,30mx320 µm);
- micropipettes P1000;
- micropipettes P100;
- 10mL and 25mL class A volumetric flasks.
- Polyethylene stoppers for flasks.

✓ The reagents used for validation are:

- ultrapure water;
- Ethanol standard solution
- Isopropanol standard solution
- Acetonitrile standard solution
- Dibromethane standard solution
- Dimethylaminoethanol standard solution

The stock and reference solutions used for validation are:

Mother Solutions

MOTHER SOLUTION ETHANOL (SM1)

MOTHER SOLUTION ISOPROPANOL (SM2)

MOTHER SOLUTION ACETONITRILE (SM3)

MOTHER SOLUTION DMAE (SM4)

MOTHER SOLUTION DBM (SM5)

Reference solutions

Mixture C1

Withdraw 630 mL of SM1 (Ethanol), 630 mL of SM2 (Isopropanol), 52 mL of SM3 (Acetonitrile), 100 mL of SM4 (Dimethylaminoethanol, DMAE), 200 mL of SM5 (Dibromethane, DBM) and place them in a 10 mL flask. Bring to volume with double-distilled water

Concentration of the C1 mixture:

- Acetonitrile : 81.74 mg/mL
- Ethanol: 994.14 mg/mL
- Isopropanol: 990.36 mg/mL
- DMAE: 19.49 mg/mL

- DBM: 1.98 mg/mL

Mixture C2

Withdraw 1260 mL of SM1 (Ethanol), 1260 mL of SM2 (Isopropanol) and 105 mL of SM3 (Acetonitrile), 200 mL of SM4 (Dimethylaminoethanol, DMAE), 400 mL of SM5 (Dibromethane, DBM) and place them in a 10 mL flask. Bring to volume with double-distilled water.

Concentration of the C2 mixture:

- Acetonitrile: 163.5 mg/mL
- Ethanol: 1988.28mg/mL
- Isopropanol: 1980.72mg/mL
- DMAE: 38.98 mg/mL
- DBM: 3.96 mg/mL

MIXTURE C3

Withdraw 1890 mL of SM1 (Ethanol), 1890 mL of SM2 (Isopropanol) and 157.2 mL of SM3 (Acetonitrile), 300 mL of SM4 (Dimethylaminoethanol, DMAE), 600 mL of SM5 (Dibromethane, DBM) and place them in a 10 mL flask. Bring to volume with double-distilled water.

Concentration of the C3 mixture:

- Acetonitrile: 245.22 mg/mL
- Ethanol: 2982.4 mg/mL
- Isopropanol: 2971.08 mg/mL
- DMAE: 58.47 mg/mL
- DBM: 5.94 mg/mL

MIXTURE C4

Withdraw 2520 mL of SM1 (Ethanol), 2520 mL of SM2 (Isopropanol) and 208 mL of SM3 (Acetonitrile), 400 mL of SM4 (Dimethylaminoethanol, DMAE), 800 mL of SM5 (Dibromethane, DBM) and place them in a 10 mL flask. Bring to volume with double-distilled water.

Concentration of the C4 mixture:

- Acetonitrile: 326.96 mg/mL
- Ethanol: 3976.56 mg/mL
- Isopropanol: 3961.44 mg/mL
- DMAE: 77.96 mg/mL
- DBM: 7.93 mg/mL

MIXTURE C5

Withdraw 3150 mL of SM1 (Ethanol), 3150 mL of SM2 (Isopropanol) and 262 mL of SM3 (Acetonitrile), 500 mL of SM4 (Dimethylaminoethanol, DMAE), 1000 mL of SM5 (Dibromethane, DBM) and place them in a 10 mL flask. Bring to volume with double-distilled water.

Concentration of the C5 mixture:

- Acetonitrile: 408.7 mg/mL
- Ethanol: 4970.7 mg/mL
- Isopropanol: 4951.8 mg/mL
- DMAE: 97.45 mg/mL
- DBM: 9.91 mg/mL

MIXTURE C6

Withdraw 3780 mL of SM1 (Ethanol), 3780 mL of SM2 (Isopropanol) and 314 mL of SM3 (Acetonitrile), 600 mL of SM4 (Dimethylaminoethanol, DMAE), 1200 mL of SM5 (Dibromethane) and place them in a 10 mL flask. Bring to volume with double-distilled water.

Concentration of the C6 mixture:

- Acetonitrile: 490,44 mg/mL
- Ethanol: 5964.84 mg/mL
- Isopropanol: 5942.16 mg/mL
- DMAE: 116.94 mg/mL
- DBM: 11.88 mg/mL

Preparation of reference solutions for verifying the specificity of the Method: Stock solutions

ETHANOL MOTHER SOLUTION (SM1a)

40 µL of standard ethanol in 2 mL flask and make up to the mark with double-distilled water.

ISOPROPANOL MOTHER SOLUTION (SM2a)

40 µL of standard isopropanol in 2 mL flask and make up to the mark with double-distilled water.

ACETONITRILE MOTHER SOLUTION (SM3a)

40 µL of standard acetonitrile in 2 mL flask and make up to the mark with double-distilled water.

DMAE MOTHER SOLUTION (SM4a)

20 µL of standard dimethylaminoethanol in 2 mL flask and make up to the mark with double-distilled water.

DBM MOTHER SOLUTION (SM5a)

2 µL of standard dibromethane in 2 mL flask and make up to the mark with double-distilled water.

Reference solutions

MIXTURE S1

Withdraw 315 mL of SM1a (Ethanol), 315 mL of SM2a (Isopropanol) and 26 mL of SM3a (Acetonitrile), 11 mL of SM4a (Dimethylaminoethanol, DMAE), 4 mL of SM5a (Dibromethane, DBM) and place them in a 1 mL flask. Bring to volume with double-distilled water.

Concentration of the S1 mixture:

- Acetonitrile: 408.7 mg/mL
- Ethanol: 4970.7 mg/mL
- Isopropanol: 4951.8 mg/mL
- DMAE: 97.45 mg/mL
- DBM: 9.91 mg/mL

5.3 Chemical purity: evaluation of DMF content

Dimethylformamide is one of the solvents present in the 18F-FCH production kit, therefore it is necessary to determine its concentration in the finished product.

Description of the analytical method

The test is performed using a GC. The analytical conditions are summarized below:

- Detector type: FID
- Carrier gas flow (Nitrogen): 3 mL/min
- Makeup Azoto: 30 mL/min
- Split ratio: 5
- Synthetic air flow: 350 mL/min
- Hydrogen Flow: 35 mL/min
- Initial temperature (for 4 min, from 0 to 4 min) 40 °C
- Temperature ramp (from 4 to 7 min) 60 °C/min
- Final temperature (for 1.5 minutes from 7 to 8.5 min) 220 °C
- Injector temperature: 250 °C
- Temperature detector: 320 °C
- Chromatographic column: Agilent capillary col. (DB-624 830m 0.320 mmx 1.80µm)
- Sample: 0.2 µl

Method validation

The tools used for validation are:

- GC Agilent 8890;
- Col. capillary Agilent DB-624 830m 0.320 mmx 1.80µm);
- micropipettes P1000;
- micropipettes P100;
- 10 mL and 25 mL class A volumetric flasks;
- Polyethylene stoppers for flasks.

The reagents used for validation are:

- ultrapure water;
- DMF.

Preparation of stock solutions

Mother solution A

Take 185 µL of Dimethylformamide and place them in a 100 mL flask. Bring to volume with double-distilled water.

Concentration of Ref. sol. A: DMF: 1760 mg/mL

Mother solution B

Take 371 µL of Dimethylformamide and place them in a 100 mL flask. Bring to volume with double-distilled water.

Concentration of Ref. sol. B: DMF: 3520 mg/mL; DMF retention time ≈ 6.42 min

5.4 Chemical and radiochemical purity

High performance liquid chromatography (HPLC) is an important technique also for the analysis of the radiotracer 18F-FCH, as it provides separation of the various components with high resolution. The instrument used to determine radiochemical purity with HPLC is the radiometric detector. The measurement is performed using the HPLC to determine of 18F-FCH and ^{18}F total impurities, using the chemical detector (UV detector) connected in series to the radio detector. In particular, 18F-FCH is detected at 278 nm. The analysis is performed by injecting 20 µL of the final product. The injection is the same for the determination of radiochemical and chemical purity. Chemical purity is calculated by comparing peak areas to the validated calibration curve of 18F-FCH.

Operating conditions

- the Column — Cation-exchange resin R2 (5 µm)
  - length: 250 mm
  - internal diameter: 4.6 mm
  - particle diameter: 5 µm
  - stationary phase: cation exchange resin R2
- Mobile phase: Phosphate buffer brought to pH 2.5 with pyridine
- Flow: 1 mL/min
- the Injection volume: 20 µl (for HPLC ICS-5000)
- the Column temperature: 20-30°C
- the Chromatography run duration: 20 minutes
- the Spectrophotometer wavelength: 270 nm

System description

The ICS-5000 HPLC consists of the following components:

- Dual pump;
- Detector compartment:
  - the Column, size: l = 0.25 m; Ø = 4.6 mm, Stationary phase: Cation-exchange resin R2 (5 µm) e preColumn;
  - the Column thermostating system;
  - the UV spectrophotometer, 270 nm;
- PC
  - the Application software for machine management;
- Detector radiometrico

5.4.1 Tools, Materials and Reagents

The following materials will be used for the development activities:

Instruments:

- HPLC ICS-5000
- Chromatographic column
- Micropipettes P1000
- Micropipettes P10
- pH-metro Mettler Toledo Seven Multi S40
- Mettler XS105DU analytical balance

Materials:

- Volumetric flasks 2 mL, 10 mL class A
- Polyethylene stoppers for flasks

Reagents:

- HPLC grade water
- Sodium dihydrogen phosphate monohydrate
- Anhydrous pyridine
- Phosphoric acid
- Standard di FMC (Fluoromethylcholine chloride)
- Standard di FEC (Fluoroethylcholine chloride)
- Sample of [18F]-Fluorocholine chloride
- Sample [18F]-Fluoride

Preparation of reference solutions for HPLC:

REFERENCE SOLUTION A

Dissolve 5 mg of standard Fluoromethylcholine (FMC)in HPLC-grade water and dilute to a volume of 20 mL using the same solvent.

Solution concentration: FMC: 250 µg/mL

REFERENCE SOLUTION B

Dissolve 5 mg of standard Fluoroethylcholine (FEC) in HPLC-grade water and dilute to a volume of 20 mL using the same solvent.

Solution concentration: FEC: 250 µg/mL

REFERENCE SOLUTION C

Mix 1 mL of Ref. sol. A with 1 mL of Ref. sol. B.

Solution concentration: FMC: 125 µg/mL FEC: 125 ug/mL

REFERENCE SOLUTION D

Mix 1 mL of ref. sol. A with 1 mL of test solution 18F-FCH 1010 MBq/mL a t_0 Sample preparation:

18F-FCH SOLUTION

Radioactive sample of 18F-FCH al tempo t_0

Active Concentration: 18F-FCH: 1010 MBq/mL.

18F-FCH-5% SOLUTION

Take 10 µL of radioactive sample of [^{18}F]- FCH and dilute it with 190 µL of chromatographic grade water until an active concentration equal to 5% of the initial concentration is obtained.

Active Concentration: 18F-FCH: 50.5 MBq/mL.

18F-FCH -EXPIRATION TIME SOLUTION

Take 10 µL of radioactive sample of [^{18}F]- FCH and dilute it with 430 µL of chromatographic grade water until the concentration of [^{18}F]- FCH equal to that present at the expiry time.

Active concentration: 18F-FCH: 23 MBq/mL.

18F- FCH -5%- EXPIRATION TIME SOLUTION

Take 10 µL of 18F-FCH -expire time solution and dilute it with 190 µL of chromatographic grade water until obtaining an active concentration equal to 5% of the concentration at the expiration time.

Active concentration: 18F-FCH: 1.15MBq/mL.

CALCULATIONS

For the calculation of [^{19}F]-FCH and concentrations of chemical impurities, the following formula is used: $y= mx + q$

Where:

y = value of the area of the analyte identified in the sample with a UV spectrophotometer, expressed in mAU; x = analyte concentration expressed in µg/mL

q = calibration line intercepts, processed annually;

m = calibration line slope, processed annually.

5.5 BIOBURDEN

The evaluation of the microbial load (bioburden) in the radiotracer final product is carried out in order to determine the possible presence of microorganisms in the 18F-FCH final product before terminal sterilization. The test is performed once the product has completely decayed, at least 48 hours after the production time.

The test involves the filtration of the product to be analyzed with the aid of a filter pump on sterile and disposable filter membranes with porosity of ≤ 0.45 μm.

The membrane acts as a barrier and captures microorganisms larger than the pores.

After filtration, the membrane is transferred to a culture medium and placed in an incubator for at least 5 days at 32.5°C for the detection of bacteria and at 22.5°C for the detection of fungi. Once the incubation time has elapsed, microbial contamination is read.

Regarding the maximum charge allowed on the unfiltered bulk sample, adopt the NMT limit of 10 CFU/mL. This limit is used instead of the general NMT 10 CFU/100 mL limit defined for products sterilized by filtration (EMA Human and Veterinary Notes for Guidance on Manufacture of the Finished Dosage Form - CPMP/QWP/486/95), since the volume to be analyze of the product is equal to 1mL.

Validation takes place using all the microbial strains indicated in the European Pharmacopoeia (paragraph 2.6.1) and on S. epidermidis (company environmental isolate strain).

Equipment used:

- Incubators 32.5°C
- Incubators 22.5°C
- Laminar flow hood
- Micropipettes P100
- Micropipettes P1000
- Testing station
- Membrane filtration pump
- Filter funnels
- Sterile 15mL test tubes

Culture media and reagents

- Counting plates containing Tryptic Soy agar (TSA) medium
- Counting plates containing Sabouraud Dextrose agar (SDA) medium
- Plates containing TSA for membrane filter
- Plates containing SDA for membrane filter
- Bottles containing NaCl Peptone Buffer pH = 7.0
- Bottles containing peptone water

Table 3. Microorganisms to test.

Microorganism	**Name**	**ATCC code**
Bacteria	*Staphylococcus aureus*	6538
	Bacillus spizizenii	6633
	Pseudomonas paraeruginosa	9027
	Staphylococcus epidermidis (endogenous isolate)	
Fungi and yeasts	*Candida albicans*	10231
	Aspergillus brasiliensis	16404

For each microorganism, a microbial suspension (also called "test suspension") is prepared to be used for validation activities.

Before carrying out the validation activities, the following control is required for each microbial test suspension prepared:

- *Title control (VT):* allows you to check the titer of the prepared suspension by inoculating onto two counting plates.

The validation activity of the membrane filtration method involves carrying out the following checks:

- *Positive Product Control (PPC):* represents the actual test to verify the fungistasis and/or bacteriostasis of the product in the analytical conditions and with the materials used. Each microbial suspension is filtered on a membrane in the presence of the bulk solution (previously diluted 1:10 and filtered at 0.22 µm), and then incubated on the solid culture medium(s) (SDA and/or TSA depending on of the microorganism);
- *Positive Control without Product (PCWP):* allows you to verify, in the analytical conditions subject to validation, the growth of each microorganism in the absence of product. Each microbial suspension is filtered on a membrane and incubated on solid culture medium(s) (SDA and/or TSA depending on the microorganism);
- *Negative Control without Product (NCWP):* consists of verifying the sterility of soils and materials and correct manual skills during the execution of the tests. The diluent used is membrane filtered and incubated on both solid culture Average (SDA and TSA). Only one negative control is needed per day.

- *Negative Control with Filtered Product (NCPF):* consists of verifying the sterility of soils, materials, effectiveness of product filtration at 0.22 µm and correct manual skills during the execution of the tests. This control therefore involves membrane filtration and incubation on both solid culture Average (SDA and TSA) of the bulk product (previously diluted 1:10 and filtered at 0.22µm).
- *Negative Control with Unfiltered Product (NCPNF):* it is the actual bioburden test; The unfiltered bulk product at 0.22µm diluted 1:10 is membrane filtered and incubated on both solid culture Average (SDA and TSA).

5.6 STERILITY TEST

The sterility test is a microbiological test required by GMP, necessary to confirm that the 18F-FCH product is sterile and does not contain viable microorganisms. The sterility test can be performed using two methods:

- **Direct inoculation** where a small quantity of product is inoculated directly into the culture medium, this method, although simple, has limitations as if the sample has an initial turbidity, it may be difficult to detect the turbidity due to microbial growth at the end of the incubation period.
- **Membrane filtration**, this is the method of choice for verifying the sterility of filterable pharmaceutical products as reported in the European Pharmacopoeia (2.6.1). With this method, the samples are filtered through a 0.45 µm membrane and collected in a canister to which the culture media are added. These canisters are subsequently incubated for 14 days. This method offers greater sensitivity than direct inoculation, as the entire product sample to be analyzed can be filtered. Furthermore, filtration allows the filtered sample to be washed before adding the culture Average, so as to eliminate components that may generate turbidity or inhibit growth in the medium.

Validation takes place using all the microbial strains indicated in the European Pharmacopoeia (paragraph 2.6.1) and on S. epidermidis (company environmental isolate strain).

Equipment used:

- Incubators 32.5°C
- Incubators 22.5°C
- Laminar flow hood
- Micropipettes P100
- Micropipettes P1000
- Testing station
- Refrigerator 4°C
- Isolator Theme
- Pump
- Filtering devices
- Particle counter

Culture media and reagents

- Counting plates containing Tryptic Soy agar (TSA) medium
- Counting plates containing Sabouraud Dextrose agar (SDA) medium
- Bottles containing Tryptic Soy Broth (TSB) culture medium

- Bottles containing Fluid Thioglycolate Medium (FTM) culture medium
- Plates containing TSA medium with neutralizers for passive sampling
- Plates containing TSA medium with neutralizers for surface sampling
- Bottles containing peptone water

Table 4: Microorganisms to test.

Microorganism	**Name**	**ATCC code**
Bacteria	*Staphylococcus aureus*	6538
	Bacillus spizizenii	6633
	Staphylococcus epidermidis	N/A (environmental isolated)
	Pseudomonas Paraeruginosa	9027
	Clostridium sporogenes	19404
Fungi and yeasts	*Candida albicans*	10231
	Aspergillus brasiliensis	16404

The validation process is divided into two phases:

- the first is performed under a laminar flow hoodwhere the manipulation of the microbiological strains and the preparation of the samples to be analyzed takes place.

- the second takes place inside a Tema isolator (Class A) in which the material to be used undergoes a biocontamination cycle with 35% H_2O_2. Biocontamination allows to eliminate any contamination present on the devices or bottles to be used in the test (all microbiological components and soils do not come into contact with the peroxide as they are contained in closed glass bottles).

The 18F-FCH product samples used are dispensed into primary containers (glass bottles), after sterilizing filtration at 0.22 µm.

Validation tests:

Proceed with the validation of the membrane filtration method, carrying out the following checks:

a. *Positive Product Control (PPC):* represents the actual test to verify the fungistasis and/or bacteriostasis of the finished product in the analytical conditions and with the materials used. Each microbial suspension, previously inoculated into a bottle of peptone water, is filtered on a membrane in the presence of the finished product, and then incubated in one of the two liquid culture media

(TSB or FTM depending on the microorganism used);

b. Positive Control Without Product (PCWP): allows to verify, in the analytical conditions subject to validation, the growth of each microorganism in the absence of product. Each microbial suspension, previously inoculated into a bottle of peptone water, is filtered through a membrane and incubated in one of the two liquid culture Average (TSB or FTM depending on the microorganism used);

c. Negative Control (CN): consists of verifying the sterility of soils, materials and correct manual skills during the execution of the tests. Instead of the product, an identical quantity of previously sterilized water for injectionswill be tested: everything is tested by membrane filtration and incubated in the two liquid culture media (TSB and FTM).

d. Product control (PC): consists of checking the finished product during the execution of tests. Each batch of finished product is tested by membrane filtration and incubated in the two liquid culture media (TSB and FTM).

Microbial suspension controls: carry out the following checks for each microbial suspension ("test suspension") used:

Title Verification (VT): allows you to check the titer of the microbial suspension by inoculating 2 counting plates (TSA for bacteria, SDA for fungi and yeasts).

6. PROCESS VALIDATION

Process validation is a key element in ensuring that a manufacturing process is designed and controlled to consistently and reliably meet predetermined requirements. With process validation, the data of the production process is provided, which must respect the desired quality characteristics, as well as confirming that the process and the final product have quality and repeatability characteristics.

The concept of validation has changed over the years, and today it brings together several activities: analytical methods, instrument calibration, process utilities, raw materials and packaging materials, equipment, facilities, production and packaging process, cleaning process, operators and software.

In the EMA Guidelines-Annex 15, three different approaches to process validation are mentioned:

1. traditional validation;
2. continuous validation of the process;
3. hybrid approaches.

The approach used for process validation is the traditional one.

Traditional process validation is performed once pharmaceutical development and process validation are completed, but before the finished product is commercialized. The number of batches selected for validation should be based on the variability and complexity of the process. In the validation of the radiotracer process it is necessary to satisfy a series of parameters, in progressive order:

1. activity produced by the cyclotron
2. activity produced after synthesis
3. radiotracer volume obtained
4. aseptic dispensing: class A conditions
5. final radiotracer concentration
6. minimum/maximum volume of customer vials
7. pre-release Quality Control Analysis
8. terminal sterilizing filter integrity
9. post-release Quality Control Analysis

At the end of production, the actual number of vials used must be known considering production risks (for example: additional vials requested during production or vials not produced due to low activity). Customer's number of vials must be ≤ 20 vials per lot. In addition to customer vials, each lot contains quality control (QC) vials. All quality specification criteria at the time of release are met.

With the validation of the production process, it was demonstrated that the process is valid by providing reproducible results, respecting the defined specificity criteria. 3 batches of process validation were carried out to perform and satisfy the specifications in all phases and in-process controls.

7. RESULTS

The following paragraphs summarize the data obtained during the validation activities. Some raw data are reported as examples.

7.1 Radiochemical purity

7.1.1 Identification

- **Objective**

The purpose of this test is to evaluate the ability of the analytical method to measure a specific analyte in the presence of other components.

In Radio-TLC analysis, the identification test is achieved when the stain due to the substances of interest is well separated from the stains due to other substances in the finished product.

- **Method of verification**

Fill the TLC development chamber with minimum 10 mL of mobile phase, the chamber is closed for at least 30 minutes. Apply 5 µL of the solution to be tested at 1 cm from the lower edge of the TLC plate. Then, the plate is placed in the development chamber until the solvent front reaches 2/3 of the plate. The plate is removed from the developing chamber and allowed to dry at room temperature. The plate is scanned by a radio-TLC detector.

- **Criteria for acceptance**

Resolution times of [18F]-Fluoride should be about 0.0 min that of 18F-FCHabout 0.5 min.

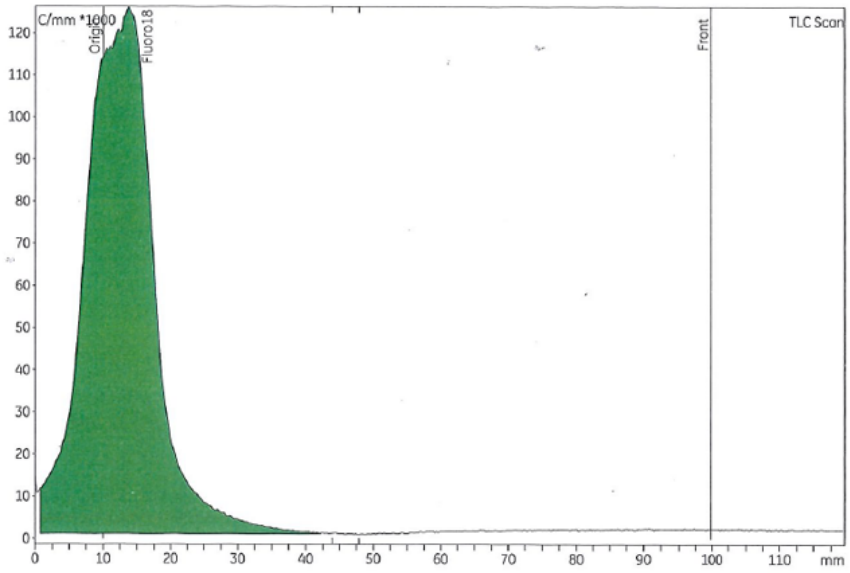
- **Results**

The results of the identification test are shown in Table 5, the chromatograms of the identification test in Figures 7 and 8.

Table 5: identification test results.

Sample	RF (mm)	Acceptance criterion (mm)
[18F]-Fluoride	0.044	≈ 0.0
18F-FCH	0.533	≈ 0.5

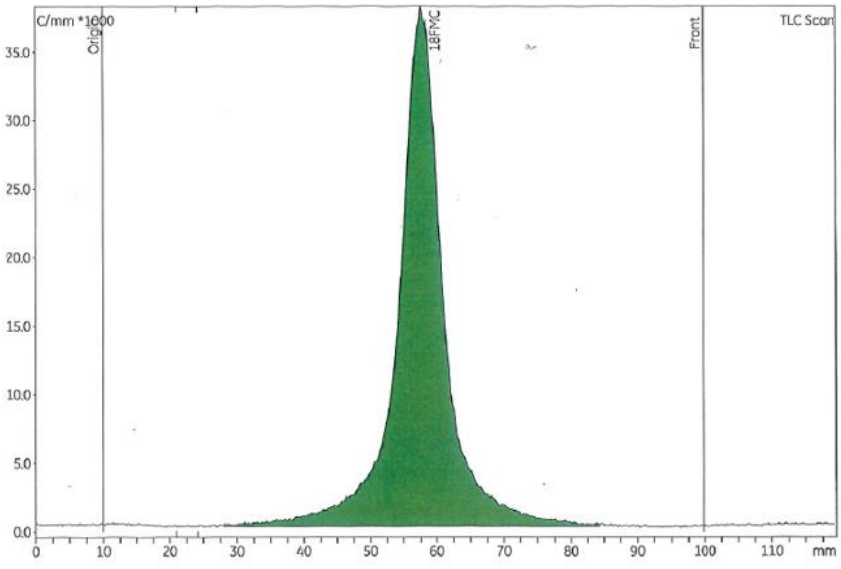
Figure 7. Identification test of [18F]-Fluoride.



Integration TLC Scan

Substance	R/F	%Total %	Type	Area Counts	%Area %
Fluoro18	0.044	90.63	DD(M)	1483439	100.00
Sum in ROI	-	-	-	1483439	100.00
Total area	-	-	-	1636754	-
Area (total) RF	-	-	-	1090846	-
BKG1 (CPS)	-	-	-	1151.3	-
Remainder (Tot)	-	-	-	153315.19	9.37

Figure 8. Identification test of 18F-FCH.



Integration TLC Scan

Substance	R/F	%Total %	Type	Area Counts	%Area %
18FMC	0.533	87.97	DD(M)	302763.1	100.00
Sum in ROI	-	-	-	302763.1	100.00
Total area	-	-	-	344157.4	-
Area (total) RF	-	-	-	303777.5	-
BKG1 (CPS)	-	-	-	385.54	-
Remainder (Tot)	-	-	-	41394.31	12.03

A sample of the drug product was analyzed according to the analytical procedure. The resolution between the two spots generated by [18F]-Fluoride and 18F-FCHwas calculated using the following formula:

$$R_S = \frac{1{,}18 * (Rif_B - Rif_{UN})}{W_{UN} + IN_B}$$

where:

Rif_{UN} = distance of the [18F]-Fluoride spot from the deposition point

Rif_B = distance of the 18F-FCH from the point of deposition

W_{UN}= width of the peak at half the maximum height of the substance a

IN_B= width of the peak at half of the maximum height of the substance b

7.1.2 Resolution, LOD, Composition

- **Objective**

The resolution between the peaks of [18F]-Fluoride and 18F-FCH in solutions of 18F-FCH-95% and 18F-FCH -95% expire time solution?

The composition of the analyzed solutions by calculating the percentage of [18F]-Fluoride and 18F-FCH peaks, relative to the total activity.

Check the LOQ of [18F]-Fluoride, which is the limit of detection (LOD) of the analytical method. The LOD is defined as the minimum detectable amount of analyte 18F-FCH and [18F]-Fluoride) in a test sample, expressed in count area.

- **Method of verification**

Samples are analyzed according to the identification method. Reference solutions are obtained by mixing the finished 18F-FCH and [18F]-Fluoride product at the time of calibration and expiration.

- solution of 18F-FCH -95% (obtained by mixing 475µl of 18F-FCH and 25µl of [18F]-Fluoride)
- solution of 18F-FCH -95% exp. (obtained by mixing 475µl of 18F-FCH and 25µl of [18F]-Fluoride at expire time.)

The signal-to-noise ratio is calculated by the following formula:

$$S/N = \frac{2H}{h}$$

Where:

H = height of the analyte peak, measured from the peak maximum to the extrapolated baseline, where the signal baseline is observed over a distance equal to at least five times the width at half peak height;

h = range of noise in the chromatogram obtained after injection of a blank, observed over a distance equal to at least five times the width at half peak height.

- **Criteria for acceptance**

The resolution (Rs) between spots ([18F-]Fluoride versus 18F-FCH) must be ≥ to 1.50. Composition: the % of [18F]-Fluoride in the analyzed samples must be less than 5% of the total radioactivity. LOQ: The signal-to-noise ratio for the peak of [18F]-Fluoride must be ≥10.

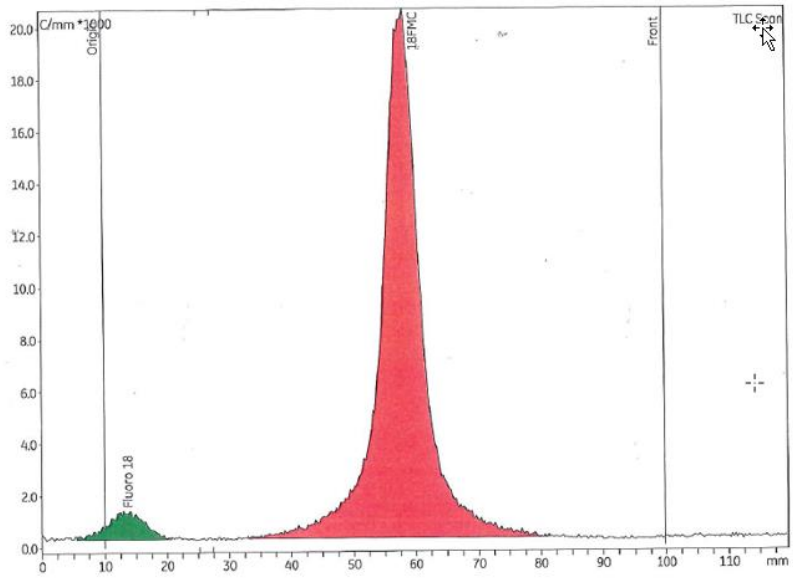
- **Results**

The results of the resolution, LOQ and composition tests are reported in Table 6, the resolution test chromatograms in Figures 9 and 10.

Table 6: Test Results: Resolution; LOQ, Composition.

<table>
<tr><th>Solution Test</th><th colspan="2">Resolution</th><th>Criteria for acceptance</th></tr>
<tr><td>18F-FCH-95%</td><td colspan="2">3.94</td><td>Rs tra [18F]-Fluoride - [18F] 18F-FCH ≥ 1,5</td></tr>
<tr><td>18F-FCH -95% exp</td><td colspan="2">3.86</td><td>Rs tra [18F]-Fluoride - [18F] 18F-FCH ≥ 1,5</td></tr>
<tr><td></td><td colspan="2">Composition of areas (%)</td><td></td></tr>
<tr><td rowspan="2">18F-FCH -95%</td><td>[18F]-Fluoride</td><td>4,57</td><td rowspan="4">[18F]-Fluoride ≤ 5 % of radioactivity total</td></tr>
<tr><td>18F-FCH</td><td>95,43</td></tr>
<tr><td rowspan="2">18F-FCH -95%</td><td>[18F]Fluoride</td><td>4,78</td></tr>
<tr><td>18F-FCH</td><td>95,22</td></tr>
<tr><td></td><td colspan="2">LOQ calculated</td><td></td></tr>
<tr><td>[18F]Fluoride-95%</td><td colspan="2">46.6</td><td rowspan="2">LOQ ≥10</td></tr>
<tr><td>18F-FCH -95% exp</td><td colspan="2">39.5</td></tr>
</table>

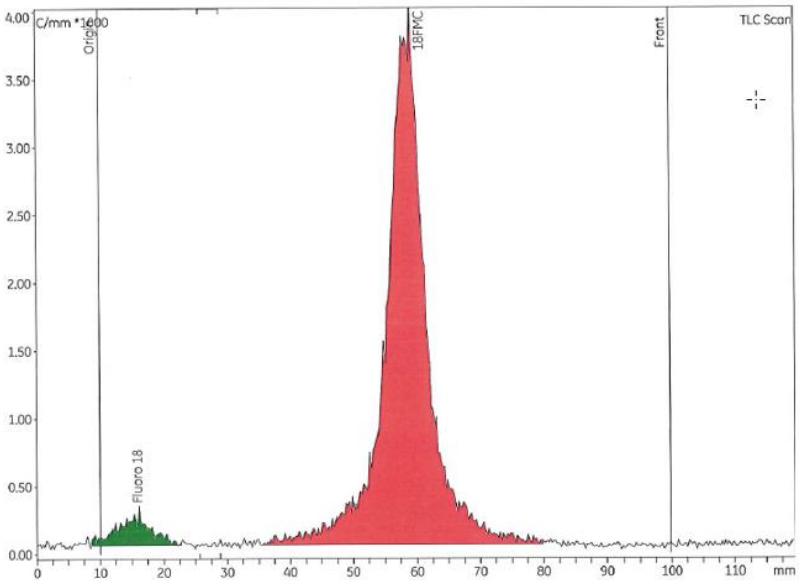
Figure 9. 95% 18-FCH resolution at time t0. t0: end of synthesis time.



Integration TLC Scan

Substance	R/F	%Total %	Type	Area Counts	%Area %	FWH mm	S/N ratio	R/T mm	Signal to nois
Fluoro 18	0.039	3.87	DD(M)	7445.8	4.57	6.90	12.2	13.47	46.6
18FMC	0.536	80.92	DD(M)	155496.6	95.43	6.46	219.1	58.13	68.0
Sum in ROI	-	-	-	162942.3	100.00	-	-	-	-
Total area	-	-	-	192164.7	-	-	-	-	-
Area (total) RF	-	-	-	161207.0	-	-	-	-	-
BKG1 (CPS)	-	-	-	319.56	-	-	-	-	-
Remainder (Tot)	-	-	-	29222.33	15.21	-	-	-	-

Figure 10. FMC 95% resolution at Texp. Texp: Product expiration time (FCH).



Integration TLC Scan

Substance	R/F	%Total %	Type	Area Counts	%Area %	FWH mm	S/N ratio	R/T mm	Signal to nois
Fluoro 18	0.064	3.98	DD(M)	1418.38	4.78	6.93	4.1	15.72	39.5
18FMC	0.542	79.35	DD(M)	28247.62	95.22	6.20	93.2	58.65	60.5
Sum in ROI	-	-	-	29666.00	100.00	-	-	-	-
Total area	-	-	-	35598.23	-	-	-	-	-
Area (total) RF	-	-	-	29547.62	-	-	-	-	-
BKG1 (CPS)	-	-	-	64.615	-	-	-	-	-
Remainder (Tot)	-	-	-	5932.23	16.66	-	-	-	-

7.2 Chemical purity: evaluation of residual solvents

7.2.1 Specificity

Assess the ability of the analytical method to specifically measure the analyte in the presence of other components, which may be present in the matrix of the sample to be analysed and which may possibly influence the analysis itself. Specificity is achieved when the peak corresponding to a specific substance is well separated from the peaks of the other substances potentially present in the sample.

- **Method of verification**

Prepare the mixtures below and analyze them as prescribed by the analytical method:

a. inject the blank solution by injection of 0.9 mg/mL NaCl diluent solution

b. injection standard solution of solvents under test: Mixture SM1a

c. injection standard solution of test solvents: Mixture SM2a

d. standard solution injection of test solvents: Mixture SM3a

e. standard solution injection of solvents under consideration: Mixture SM4a

f. standard solution injection of solvents under consideration: Mixture SM5a

g. standard solution injection of solvents such as Ethanol, Isopropanol, Acetonitrile, Dibromethane, Dimethylaminoethanol at specification levels: Mixture S1.

Then, visually compare the chromatograms obtained and verify the absence of unidentifiable and interfering peaks in the chromatogram obtained from the analysis of Mixture S1. If interfering peaks are present, calculate the resolution of the Acetonitrile, Isopropanol and Ethanol, Dibromomethane and Dimethylaminoethanol peaks relative to any peaks produced by the interfering compounds. Also calculate the resolution between the peaks of the same residual solvents using the following formula:

$$R_s = \frac{1.18 * (Tr_b - Tr_a)}{W_a + W_b}$$

where:

Tra = retention time of substance a

Trb = retention time of substance b

Wa = peak width of substance a at mid-height

Wb = width of the peak of substance b at mid-height

- **Acceptance Criteria**

Chromatograms should be visually compared, and no peaks related to the excipients, active ingredient (18F-FCH) and impurities should interfere with the peaks of the solvents under test. If peaks attributable to the active ingredient or excipients/impurities are present, their resolution with respect to the peaks of the test solvents should be ≥ 1.5. In addition, the resolution between the solvent ethanol and acetonitrile should be ≥ 2, while their resolution of the other peaks of the test solvents should be ≥ 1.5. The retention time of each solvent-related peak within the S1 mixture shall not differ by more than 5% from the retention time of the peak of the same solvent within the reference solution containing the single solvent.

- **Results**

Results for the evaluation of the specificity of the GC method are reported in Table 7. Chromatograms of the tested solutions in the Figure 11-15. Solution retention time and resolution results in Table 8. Chromatogram of the product solution in Figure 16.

Table 7. Evaluation of the specificity of the GC method.

Injection	Analytes	Retention time (min)	Retention times ± 5 % (min)
White	Front	NA	NA
Solution SM1a	Ethanol	2,82	2.68 ÷ 2.96
Solution SM2a	Isopropanol	3,07	3.07 ÷ 3.22
Solution SM3a	Acetonitrile	3,23	3.23 ÷ 3,39
Solution SM4a	DMAE	4,73	5.02 ÷ 5.27
Solution SM5a	DMF	5,02	4.49 ÷ 4.97

Figure 11. White: ultrapure water injection.

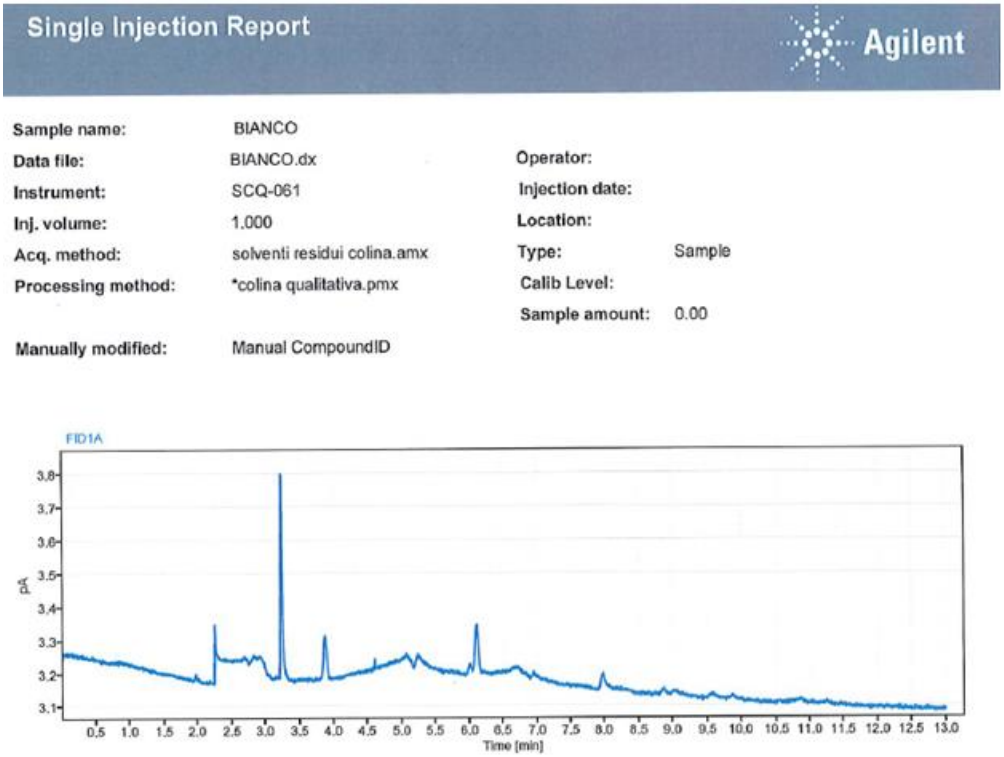


Figure 12. Specificity: Ethanol solution injection; SM1a.

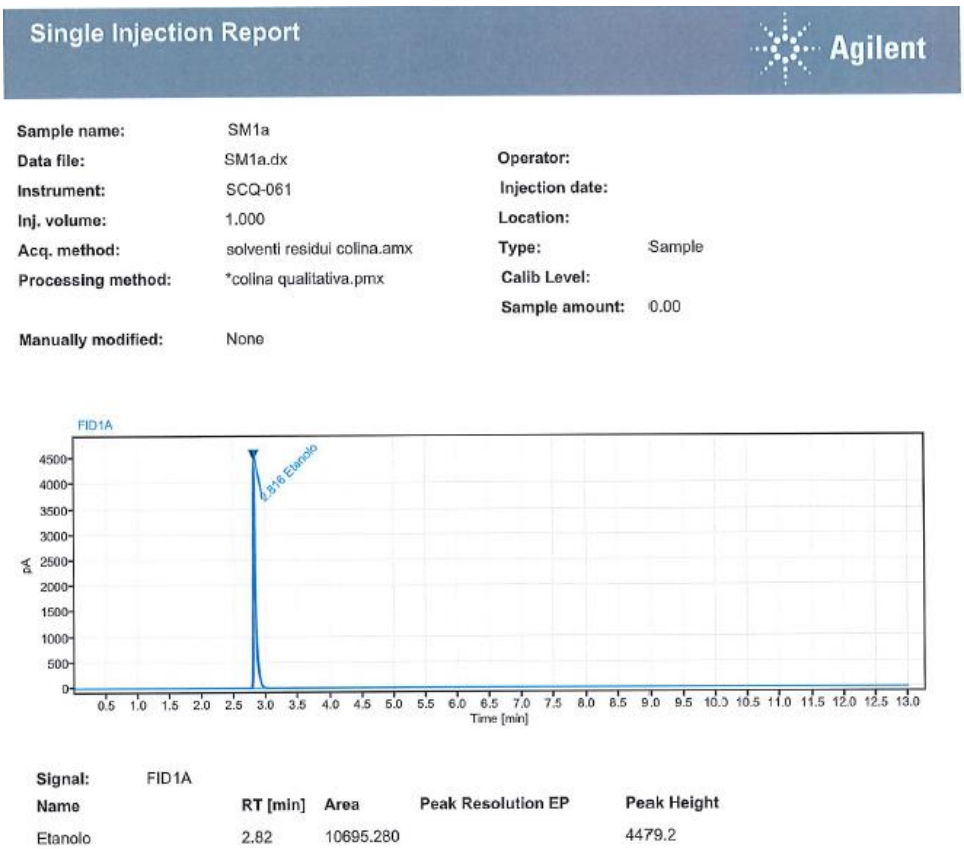


Figure 13. Specificity: Acetonitrile solution injection; SM3a.

Figure 14. Specificity: DMAE solution injection; SM4a.

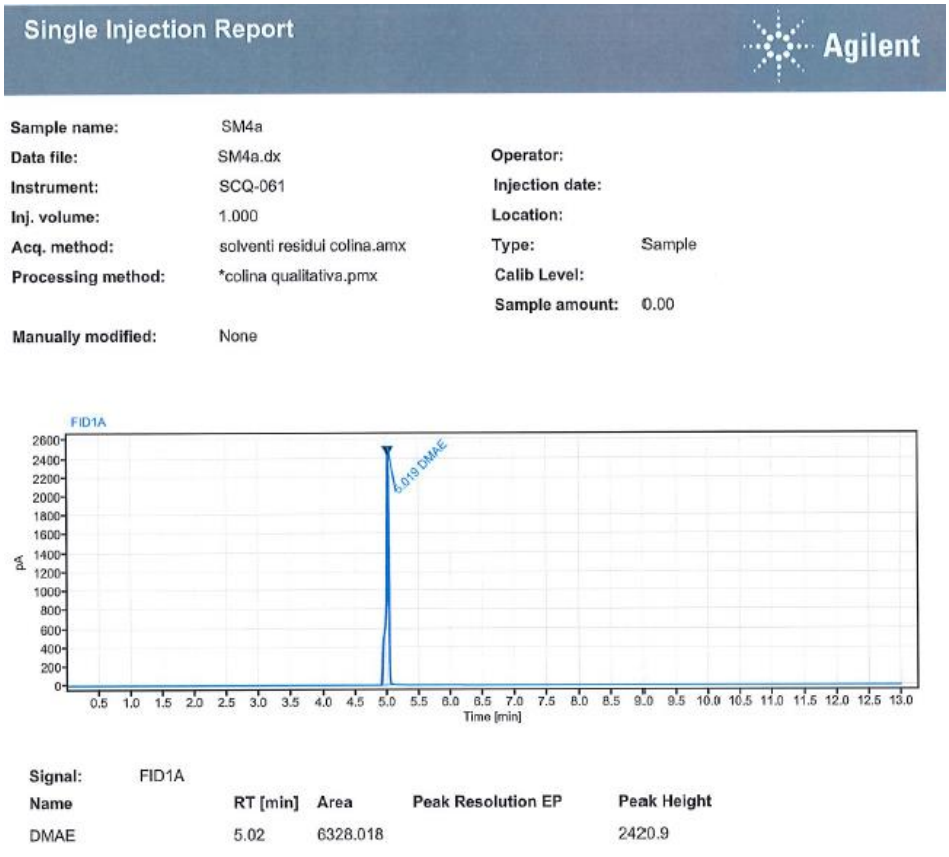


Figure 15. Specificity: DBM solution injection; SM5a.

Table 8: Solution retention time and resolution.

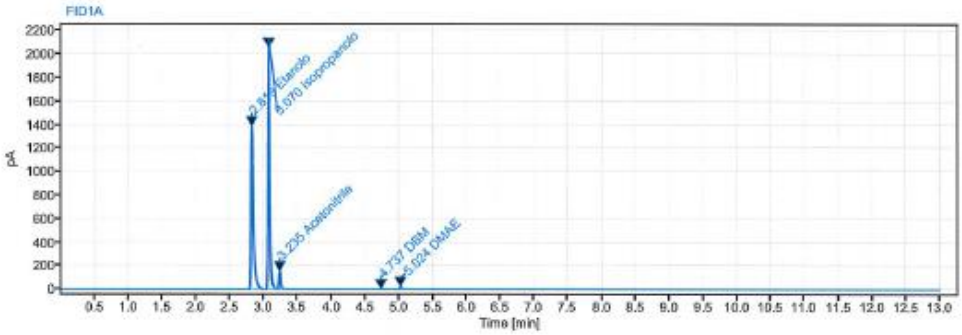
Analytes retention time		**Resolution (RS)**
Analyte name	**Analyte name**	
Ethanol	2.82	NA
Isopropanol	3.07	5.36
Acetonitrile	3.23	4.00
DBM	4.74	40.22
DMAE	5.04	7.53

Figure 16. Sample Resolution.

Single Injection Report

Agilent

Sample name:	S1		
Data file:	S1.dx	**Operator:**	
Instrument:	SCQ-061	**Injection date:**	
Inj. volume:	1.000	**Location:**	
Acq. method:	solventi residui colina.amx	**Type:**	Sample
Processing method:	*colina qualitativa.pmx	**Calib Level:**	
		Sample amount:	0.00
Manually modified:	None		



Signal: FID1A

Name	RT [min]	Area	Peak Resolution EP	Peak Height
Etanolo	2.82	3189.852		1385.0
Isopropanolo	3.07	3567.389	5.36	2049.1
Acetonitrile	3.23	252.883	4.00	151.5
DBM	4.74	0.481	40.22	0.4
DMAE	5.02	38.991	7.53	19.5

7.2.1 Linearity

- **Objective**

Check whether the method can produce analytical results proportional to the amount of analyte present in the test samples, within a given concentration range.

The linearity of a method is expressed through the correlation coefficient (r) calculated through the linear regression obtained from the analytical results as the analyte concentration present in the analysis samples changes.

The correlation coefficient, the slope values of the straight line, the intercept with the y-axis and the sum of the residual squares will be given for each analyte.

- **Criteria for acceptance**

The correlation coefficient (r), calculated from the linear regression obtained by interpolation between the peak area values obtained and the corresponding concentrations of Acetonitrile, Ethanol and Isopropanol, Dibromomethane, Dimethylaminoethanol must be ≥ 0.99.

- **Method of verification**

Prepare the mixtures of the various solvents named C1, C2, C3, C4, C5, C6, inject each mixtures 3 times. Define the average areas of the 6 reference mixtures.

Calculate the linear regression between the peak area values obtained and the concentrations of each analyte in the analyzed mixtures. The linear regression function returns a matrix showing the values reported in Table 9:

Table 9: Linear regression parameters.

m (slope)	**q (intercept)**
Standard deviation (m)	Standard deviation (q)
r^2	Standard deviation (y)
F di Fisher	Degrees of freedom
SSreg (sum of squares regression)	SSres (residual sum of squares)

- **Results**

Results summarized in tables for each individual solvent; chromatograms of some analytical runs are represented in a representative manner.

Table 10:Ethanol linearity.

Ethanol linearity				
	Conc. (µg/mL)	**Average Area peak (pA*min)**	**Validation Parameters**	
Sol. C1	994,4	656,304		
Sol. C2	1988,28	1332,441	Slope (m)	0,67
Sol. C3	2982,4	2019,671	Intercept (q)	3,2699
Sol. C4	3976,56	2681,370	Coeff.Correlation (r)	1
Sol. C5	4970,70	3329,281	Som. Quad.Res (SSres)	845
Sol. C6	5964,84	3988,526		

Table 11: Isopropanol linearity.

Isopropanol linearity				
	Conc. (µg/mL)	**Average Arae peak (pA*min)**	**Validation Parameters**	
Sol. C1	990.36	746.405		
Sol. C2	1980.28	1498.165	Slope (m)	0.73
Sol. C3	2971.08	2220.225	Intercept (q)	37.668
Sol. C4	3961.44	2972.107	Coeff.Correlation (r)	1
Sol. C5	4951.8	3641.669	Som. Quad.Res (SSres)	2003
Sol. C6	5942.16	4391.625		

Table 12. Acetonitrile linearity.

Acetonitrile linearity				
	Conc. (µg/mL)	**Average Arae peak (pA*min)**	**Validation Parameters**	
Sol. C1	81.74	53.277		
Sol. C2	163.5	108.366	Slope (m)	0.659
Sol. C3	245.22	161.296	Intercept (q)	0.9965
Sol. C4	326,96	223.841	Coeff.Correlation (r)	0.999
Sol. C5	408,72	269.040	Som. Quad.Res (SSres)	68
Sol. C6	490,64	321.453		

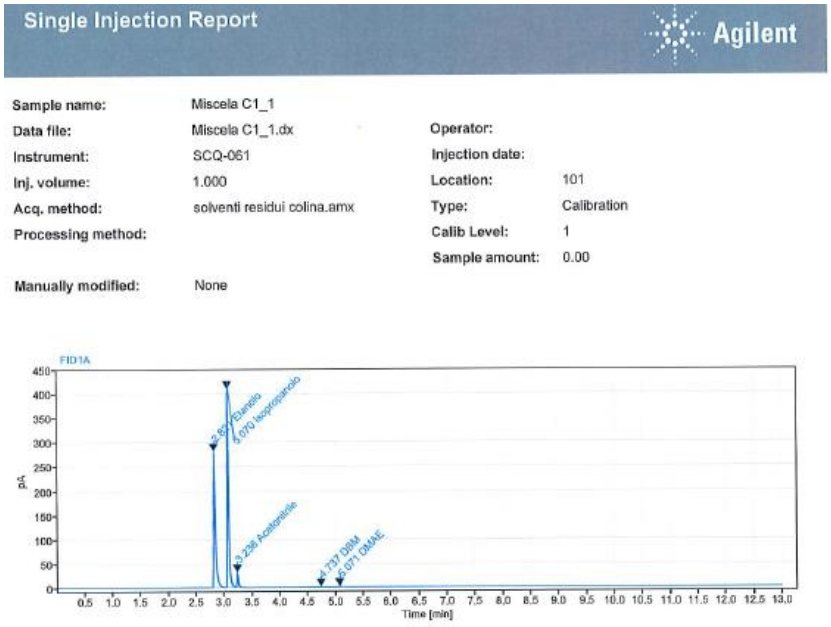
Table 13: DMAE linearity.

DMAE linearity				
	Conc. (µg/mL)	**Average Arae peak (pA*min)**	**Validation Parameters**	
Sol. C1	19.49	2.110		
Sol. C2	38.98	11.130	Slope (m)	0,59
Sol. C3	58.47	21.706	Intercept (q)	-11.221
Sol. C4	77.96	34.059	Coeff.Correlation (r)	0.997
Sol. C5	97.45	49.236	Som. Quad.Res (SSres)	15
Sol. C6	116.94	57.947		

Table 14: DBM linearity.

DBM linearity				
	Conc. (µg/mL)	**Average Arae peak (pA*min)**	**Validation Parameters**	
Sol. C1	1.98	0.206		
Sol. C2	3.96	0.348	Slope (m)	0,06
Sol. C3	5.94	0.500	Intercept (q)	0,0873
Sol. C4	7.93	0.644	Coeff.Correlation (r)	0,998
Sol. C5	9.91	0.753	Som. Quad.Res (SSres)	0,001
Sol. C6	11.88	0.865		

Figure 17: Linearity test, Mixture C1 injection. First concentration to be tested.



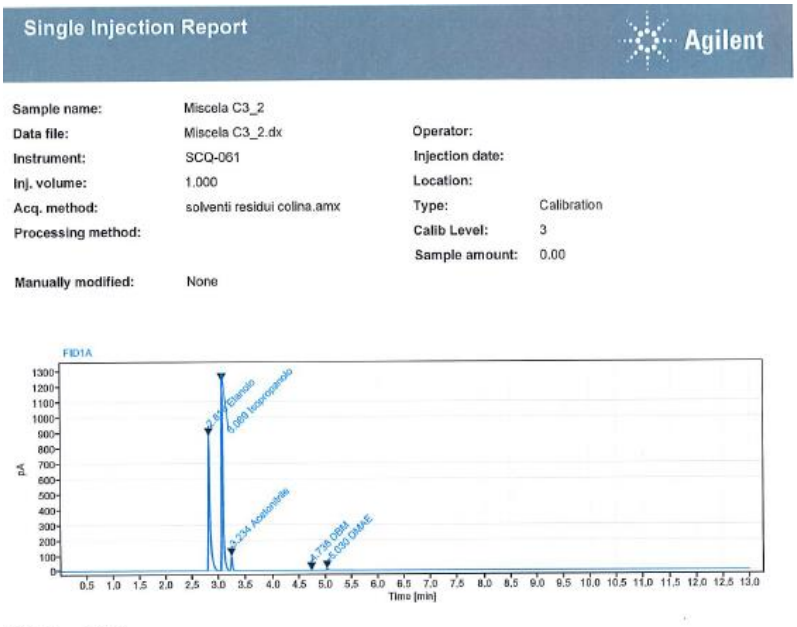
Single Injection Report — Agilent

Sample name:	Miscela C1_1		
Data file:	Miscela C1_1.dx	Operator:	
Instrument:	SCQ-061	Injection date:	
Inj. volume:	1.000	Location:	101
Acq. method:	solventi residui colina.amx	Type:	Calibration
Processing method:		Calib Level:	1
		Sample amount:	0.00
Manually modified:	None		

Signal: FID1A

Name	RT [min]	Area	Concentration []	Peak Resolution EP
Etanolo	2.82	647.853	962.0	
Isopropanolo	3.07	738.354	956.1	5.26
Acetonitrile	3.24	52.793	78.9	3.95
DBM	4.74	0.213	1.9	39.32
DMAE	5.07	2.642	23.3	4.19

Figure 18: Linearity test, Mixture C3 injection. Third concentration to be tested



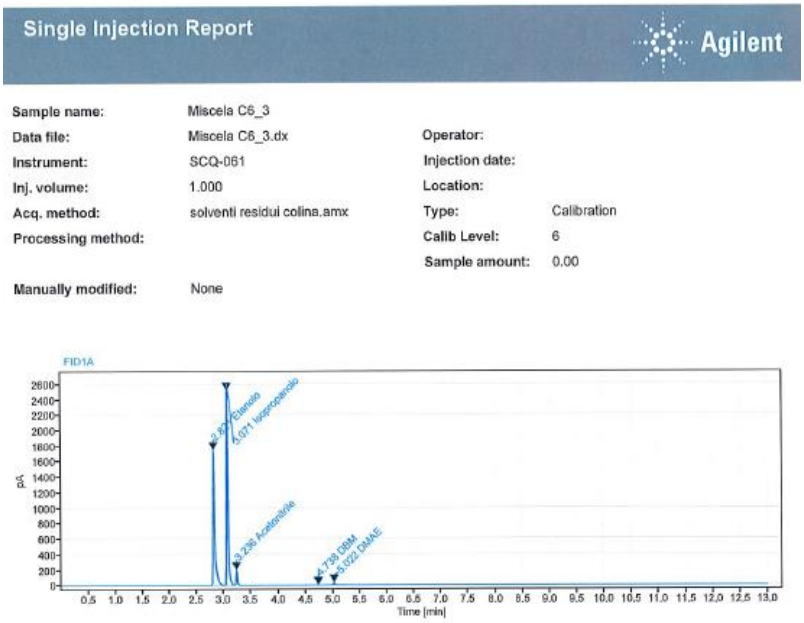
Single Injection Report — Agilent

Sample name:	Miscela C3_2		
Data file:	Miscela C3_2.dx	Operator:	
Instrument:	SCQ-061	Injection date:	
Inj. volume:	1.000	Location:	
Acq. method:	solventi residui colina.amx	Type:	Calibration
Processing method:		Calib Level:	3
		Sample amount:	0.00
Manually modified:	None		

Signal: FID1A

Name	RT [min]	Area	Concentration []	Peak Resolution EP
Etanolo	2.82	2011.061	2996.6	
Isopropanolo	3.07	2209.913	2963.6	5.53
Acetonitrile	3.23	160.561	242.6	4.10
DBM	4.74	0.477	5.8	40.96
DMAE	5.03	21.869	55.6	7.88

Figure 19: Linearity test, Mixture C6 injection. Sixth concentration to be tested.



Single Injection Report — Agilent

Sample name:	Miscela C6_3		
Data file:	Miscela C6_3.dx	Operator:	
Instrument:	SCQ-061	Injection date:	
Inj. volume:	1.000	Location:	
Acq. method:	solventi residui colina.amx	Type:	Calibration
Processing method:		Calib Level:	6
		Sample amount:	0.00
Manually modified:	None		

Signal: FID1A

Name	RT [min]	Area	Concentration []	Peak Resolution EP
Etanolo	2.82	3935.006	5868.1	
Isopropanolo	3.07	4336.670	5865.0	5.49
Acetonitrile	3.24	316.742	479.8	4.09
DBM	4.74	0.829	11.0	40.74
DMAE	5.02	57.169	114.9	7.46

7.2.2 Limit of Quantification (LOQ) and Limit of Detectability (LOD) Assessment.

- **Objective**

  Verify the minimum amount of analyte that can be detected and quantified with precision and accuracy (LOQ).

  Verify the minimum amount of analyte that can be detected but not necessarily quantified as an exact value (LOD).

- **Criteria for acceptance**

  LOQ must be less than the specification limit.

  LOD must be less than the LOQ and the specification limit.

- **Method of verification**

  The LOQ is calculated with the linearity test data using the following equation:

$$LOQ = \frac{10 * \sigma}{b}$$

  where: σ is the standard deviation of the response; b is the slope of the calibration curve.

The LOD (Limit of detection) is calculated by extrapolation of the calibration curve using the following equation:

$$LOD = \frac{3{,}3 * \sigma}{b}$$

where: σ is the standard deviation of the response; b is the slope of the calibration curve.

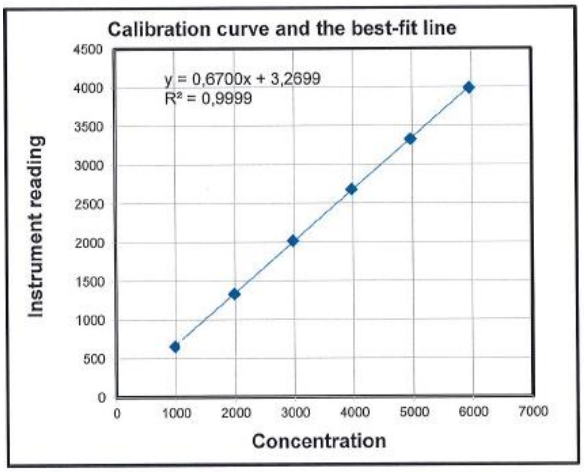
- **Results**

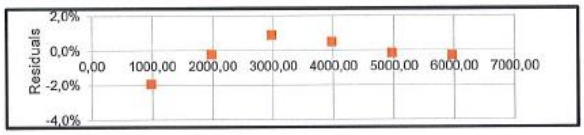
Test results for the evaluation of the limit of quantification and detection are reported in Table 15. Solvent calibration curves; Figure 20 Ethanol; Figure 21 Acetonitrile; Figure 22 Isopropanol; Figure 23; DMAE, Figure 24 DBM.

Table 15: Test data limit quantification and detection of solvents: ethanol, acetonitrile, isopropanol, DMAE, DBM.

	Ethanol	**Acetonitrile**	**Isopropanol**	**DBM**	**DMAE**
Standard deviation σ	13.5274	3.8376	20.8300	0.0175	1.7848
Slope (b)	0.670	0.659	0.7330	0.0671	0.5950
LOD (µg/mL)	66.63	19.22	93.78	0.86	9.90
LOQ (µg/mL)	201.90	58.25	284.18	2.60	30.00
Limit of Specification	5000 (µg/mL)	410 (µg/mL)	5000 (µg/mL)	10 (µg/mL)	100 (µg/mL)

EtOH





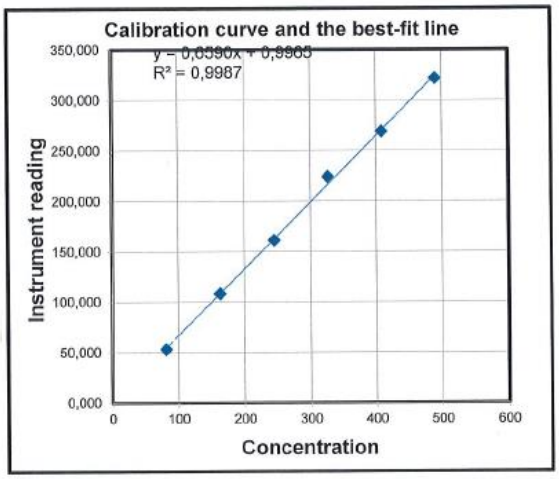
Standard deviation of the residuals = 1,0%

	value	Error (σ)	% Error
Slope	0,6700	0,0035	0,52%
Intercept	3,2699	13,5274	413,69%

LOQ	LOD
201,90	66,63

Figure 20. Curve calibration Ethanol.

ACN



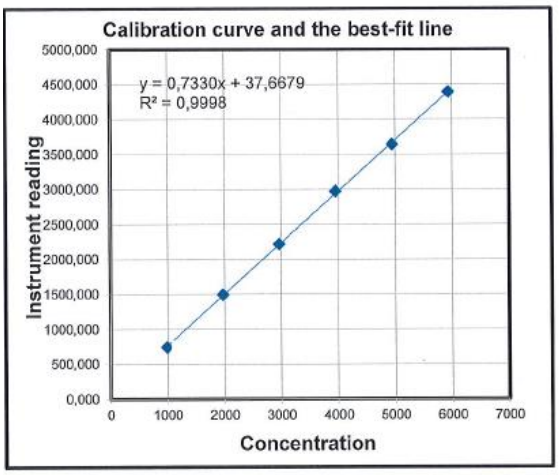
Standard deviation of the residuals = 2,1%

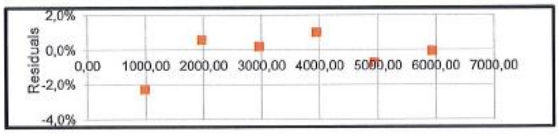
	value	Error (σ)	% Error
Slope	0,6590	0,0121	1,83%
Intercept	0,9965	3,8386	385,22%

LOQ	LOD
58,25	19,22

Figure 21. Curve calibration acetonitrile.

IPA





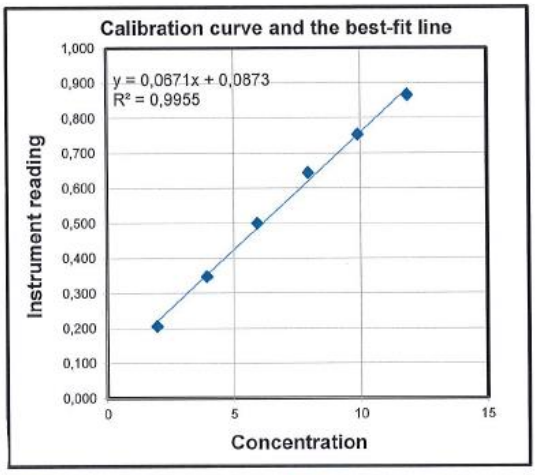
Standard deviation of the residuals = 1,2%

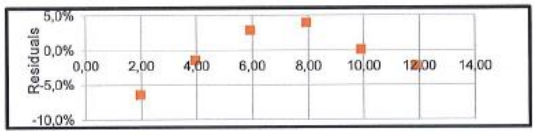
	value	Error (σ)	% Error
Slope	0,7330	0,0054	0,74%
Intercept	37,6679	20,8300	55,30%

LOQ	LOD
284,18	93,78

Figure 22. Ccurve calibration isopropanol.

DBM





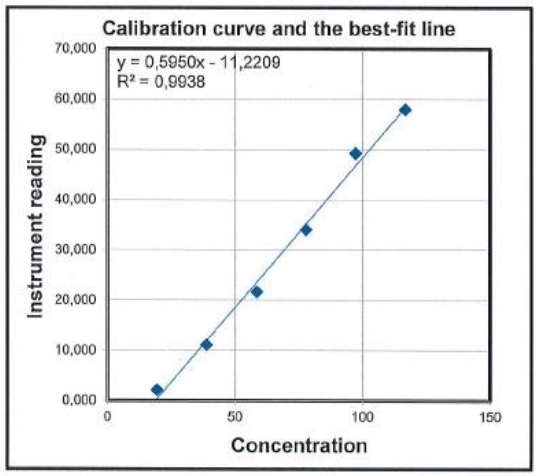
Standard deviation of the residuals = 3,8%

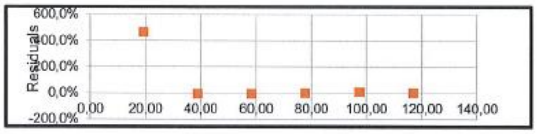
	value	Error (σ)	% Error
Slope	0,0671	0,0023	3,37%
Intercept	0,0873	0,0175	20,01%

LOQ	LOD
2,60	0,86

Figure 23. C curve calibration DBM.

DMAE





Standard deviation of the residuals = 190,1%

	value	Error (σ)	% Error
Slope	0,5950	0,0235	3,95%
Intercept	-11,2209	1,7848	-15,91%

LOQ	LOD
30,00	9,90

Figure 24: curve calibration DMAE.

7.2.3 Verifying the Accuracy of the Analytical Method

- **Objective**

  Evaluation of the accuracy of the analytical method, i.e. verifying the extent to which it is capable of obtaining results close to the expected value for analysis mixtures containing Ethanol, Isopropanol, Acetonitrile, Dibromethane and Dimethylaminoethanol.

- **Criteria for acceptance**

  The overall Recovery value (%) of the analysed reference mixtures must be between 90 % and 110 % of the corresponding theoretical expected values. The Global Recovery (%) is calculated as the average of the three global media recoveries (media of the Recovery (%) of the three replicates for each mixture).

- **Method of verification**

  Accuracy is assessed by analysing each injection of the six solutions used to calculate the calibration curve,

  Calculate the Recovery value (%) of each analyte in each sample mixture using the following formula:

$$\text{Recovery}\% = \frac{A_i}{C_{rif_i}} * 100$$

where:

Ai = calculated concentration value for an analyte in the sample mixture.

Crif i = theoretical concentration value of the analyte under test in the sample mixture.

Compare the calculated recovery value with the expected acceptance criterion.

Print out the reports showing the areas of the peaks detected and attach them to the protocol.

- **Results**

The data for this test can be retrieved from the linearity test of the individual solutions. Table 16 shows the results expressed in %.

Table 16: global recovery of tested solvents.

	Ethanol	**Isopropanol**	**Acetonitrile**	**DMAE**	**DMF**
Global recovery %	99.80	99.80	99.87	99.68	101.21

7.2.4 Checking the repeatability of the analytical method

- **Objective**

Assessing the repeatability of the method by calculating the relative standard deviation (RSD %).

- **Criteria for acceptance**

The RSD % of the concentrations and retention times obtained for each analyte must be ≤ 5%.

- **Method of verification**

The reference solution C5, used for the linearity test, is injected six times. Calculate the recovery % value of each analyte in each mixture and then calculate the RSD% as follows:

Standard Deviation: $$S = \sqrt{\sum_{i=1}^{n} \frac{\left(x_i - \bar{x}\right)^2}{\left(n-1\right)}}$$

Relative Standard Deviation: $$RSD\% = \frac{S}{\bar{x}} * 100 = \frac{\sqrt{\sum_{i=1}^{n} \frac{\left(x_i - \bar{x}\right)^2}{\left(n-1\right)}}}{\frac{\sum_{i=1}^{n} x_i}{n}} * 100$$

where:

S: Standard deviation of individual recoveries %

$\bar{x}$: Average of individual recoveries %

n: number of retrievals

t: t of student at 95% probability

- **Results**

Table 17 shows the values of the relative standard deviation of the solvents.

Table 17: relative standard deviation of the tested solvents.

	Ethanol	**Isopropanol**	**Acetonitrile**	**DMAE**	**DMF**
RSD%	2.18	1.92	2.08	4.23	4.84

7.3 Chemical purity: DMF solvent evaluation

7.3.1 Linearity assessment

- **Objective**

To verify that the method is capable of obtaining analytical results proportional to the amount of analyte present in the test samples, within a given concentration range.

- **Criteria for acceptance**

The correlation coefficient (r), calculated from the linear regression obtained by interpolation between the values of the areas of the peaks obtained and the corresponding DMF concentrations must be ≥ 0.997.

- **Method of verification**

Prepare DMF mixtures named C1, C2, C3, C4, C5 and C6. Each solution is injected 3 times.

Verify Linearity by averaging the areas of the 6 reference mixtures (C1, C2, C3, C4, C5, C6).

Preparation of reference solutions for linearity determination.

The following Table 18 shows the preparation of reference solutions for the linearity test. The solutions are diluted with double-distilled water.

Table 18: DMF solutions, linearity test execution.

	C1	**C2**	**C3**	**C4**	**C5**	**C6**
	20%	**40%**	**60%**	**80%**	**100%**	**120%**
Ref. Sol. A (μL)	1000		3000		5000	
Ref. Sol. B (μL)		1000		2000		3000
Final Volume	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL
DMF Concentration (μg/mL)	176	352	528	704	880	1056

- **Results**

Table 19 shows the values of the analytical runs of the solutions with different DMF concentrations.

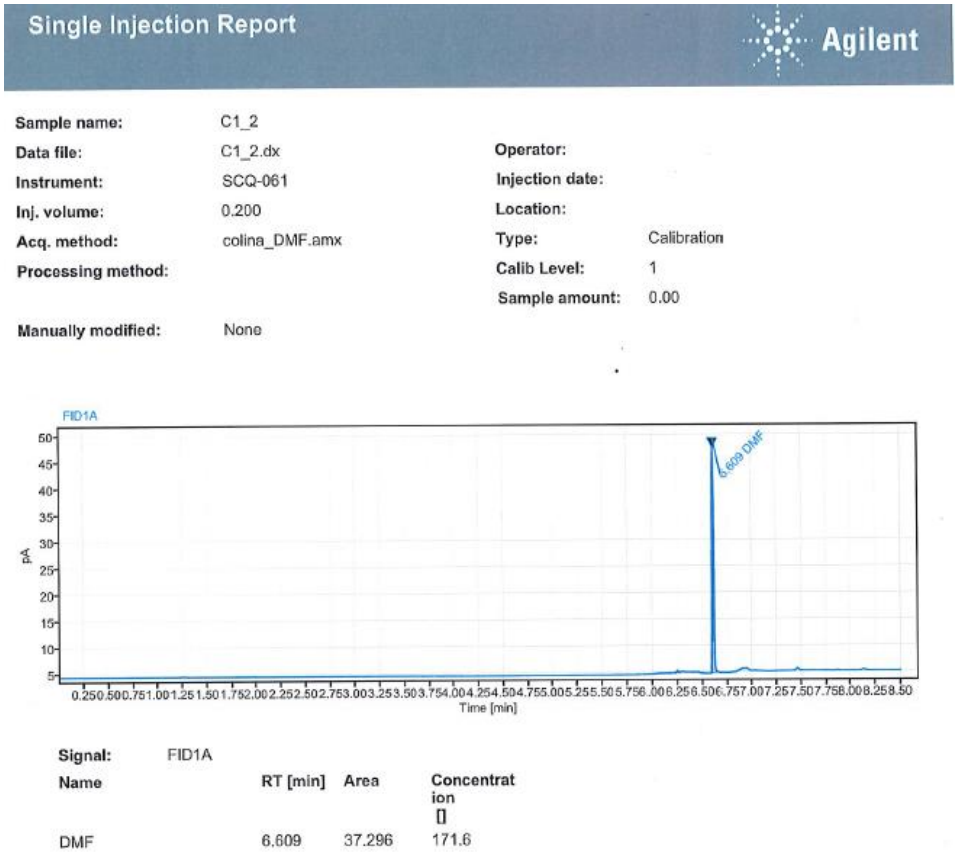
Le 3 prove analitiche per la linearità della soluzione C1 sono illustrate a titolo di esempio nelle figure 25,26 e 27.

Table 19: Linearity tests of DMF solutions at different concentrations.

DMF linearity				
	Conc. (µg/mL)	**Average peak area (pA*min)**	**Validation Parameters**	
Sol. C1	176	38.59		
Sol. C2	352	79.805		
Sol. C3	528	121.507	Slope (m)	0.2188
Sol. C4	704	158.419	Intercept (q)	2.8785
Sol. C5	880	197.755	Correlation coefficient (r)	0.999
Sol. C6	1056	230.059		

Figure 25. Linearity test, injection of mixture C1. First replicate to be tested.

Figure 26. Linearity test, injection of mixture C1. Second replicate to be tested.



Single Injection Report

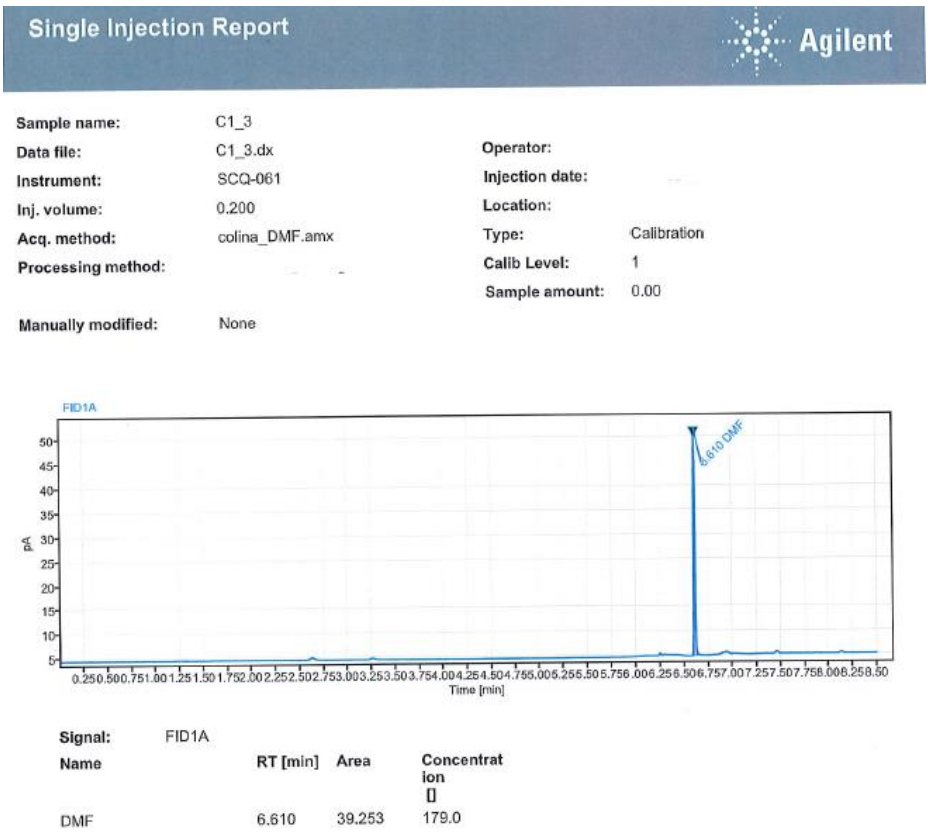
Agilent

Sample name:	C1_2		
Data file:	C1_2.dx	**Operator:**	
Instrument:	SCQ-061	**Injection date:**	
Inj. volume:	0.200	**Location:**	
Acq. method:	colina_DMF.amx	**Type:**	Calibration
Processing method:		**Calib Level:**	1
		Sample amount:	0.00
Manually modified:	None		

Signal: FID1A

Name	RT [min]	Area	Concentration []
DMF	6.609	37.296	171.6

Figure27. Linearity test, injection of mixture C1. Third replicate to be tested



Single Injection Report

Agilent

Sample name:	C1_3		
Data file:	C1_3.dx	**Operator:**	
Instrument:	SCQ-061	**Injection date:**	
Inj. volume:	0.200	**Location:**	
Acq. method:	colina_DMF.amx	**Type:**	Calibration
Processing method:		**Calib Level:**	1
		Sample amount:	0.00
Manually modified:	None		

Signal: FID1A

Name	RT [min]	Area	Concentration []
DMF	6.610	39.253	179.0

7.3.2 Evaluation of Detection and Quantification Limits

- **Objective**

  - Verify the minimum amount of analyte that can be detected and quantified precisely and accurately.

  - Verify the minimum amount of analyte that can be detected but not necessarily quantified as an exact value.

- **Criteria for acceptance**

  LOQ must be less than the specification limit.

  LOD must be lower than the LOQ and the specification limit.

- **Method of verification**

  LOD and LOQ are calculated using the formulae

$$LOQ = \frac{10 \times \sigma}{b} \qquad LOD = \frac{3{,}3 \times \sigma}{b}$$

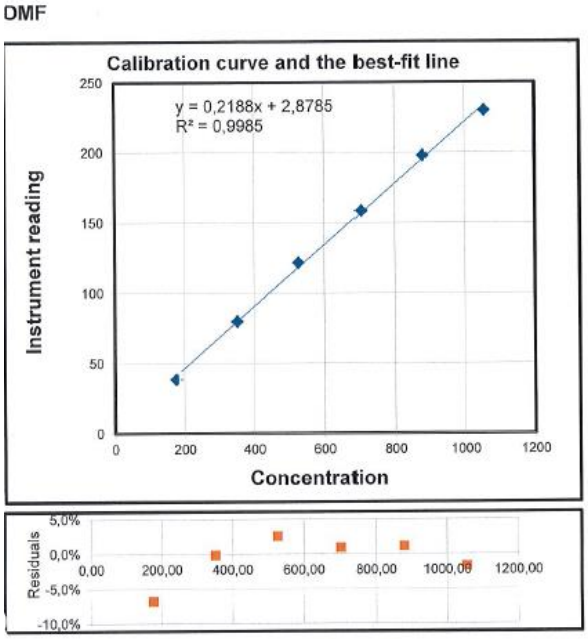
- **Results**

  Test results for the evaluation of the limit of quantification, the limit of detection and standard deviation are reported in table 20. DMF calibration curves Figure 28.

Table 20: DMF test data, limit of quantification, limit of detection and standard deviation.

	Standard deviation (σ)	**Slope (b)**	**LOD (g/mL)**	**LOQ (g/mL)**	**Specification limit**
DMF	3.3	0.2188	44.47	134,77	880

Figure 28. DMF calibration curve.



Standard deviation of the residuals = 3,3%

	value	Error (σ)	% Error
Slope	0,2188	0,0043	1,97%
Intercept	2,8785	2,9495	102,47%

LOQ	LOD
134,77	44,47

7.3.3 Verification of method accuracy

- **Objective**

  To assess the accuracy of the analytical method, i.e. to verify the extent to which it is capable of obtaining results close to the expected value for analysis mixtures containing Dimethylformamide.

- **Criteria for acceptance and veridication method**

  See paragraph 7.2.4.

- **Results**

  Overall recovery for DMF = 99.85 %.

7.3.4 Verification of the repeatability of the analytical method

- **Objective**

  To assess the repeatability of the method by calculating the relative standard deviation % (RSD %)

- **Criteria for acceptance and veridication method**

  See paragraph 7.2.5.

- **Results**

  RSD % for DMF = 3.73 %.

7.4 Chemical Purity

7.4.1 Identification and System suitability test

- **Scope**

The purpose of this test is to assess the ability of the analytical method to measure the analyte in the presence of other components, which may be present in the sample to be analysed and could interfere with the analysis.

In HPLC analysis, specificity is achieved when the peak corresponding to the analyte being tested is separated from the peaks of other substances that may be present in the solution. This is demonstrated by injecting each substance and checking for the absence of overlapping signals from other impurities. If interfering peaks are present, the chromatograms are analysed and the resolution to the peaks is calculated.

- **Objective**

The objective of the test is to evaluate the retention times of the individual analytes (reference a and b) and to verify the resolution of the Fluoromethicoline (FMC) and Fluoroethylcholine (FEC) peaks in reference solution C.

- **Method of verification**

Prepare as indicated in paragraph 5.4 the mixtures indicated below and analyse them as prescribed by the analytical method:

- Injection Reference Solution A
- Injection Reference Solution B
- Injection Reference Solution C

The resolution (Rs) between two adjacent peaks is expressed by the ratio of their distance to the semi-sum of their respective widths at the base.

Calculate the resolution of peaks FMC and FEC in reference solution c using the following formula:

$$R_s = \frac{1.18 * (Tr_b - Tr_a)}{W_a + W_b}$$

where:

Tra = retention time of substance a

Trb = retention time of substance b

Wa = peak width of substance a at mid-height

Wb = peak width of substance b at mid-height

- **Criteria for acceptance**

Verify that:

- The retention time of the Fluoromethylcholine (FMC) is approximately 8 min.

- The relative retention time of the Fluoroethylcholine (FEC) is about 1.1 relative to the FMC.

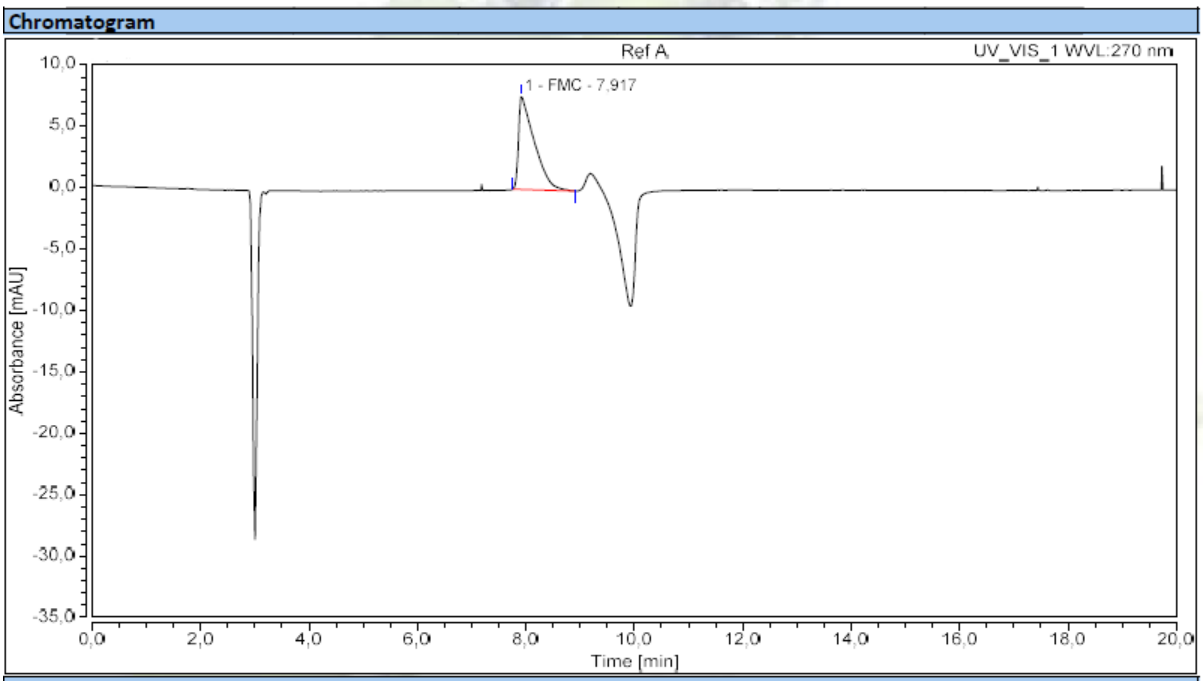
- The resolution between the FMC peak and FEC must be ≥ of 1.5.

- **Results**

The results of the injections of the solutions of Fluoromethylcholine (FMC); Fluoroethylcholine (FEC); and of the FMC/FEC 50/50 v/v solution, are reported in Table 21. The chromatograms of the solutions are shown in the figures: 29, 30 e 31.

Table 21: retention time solution FMC; FET; FMC/FET.

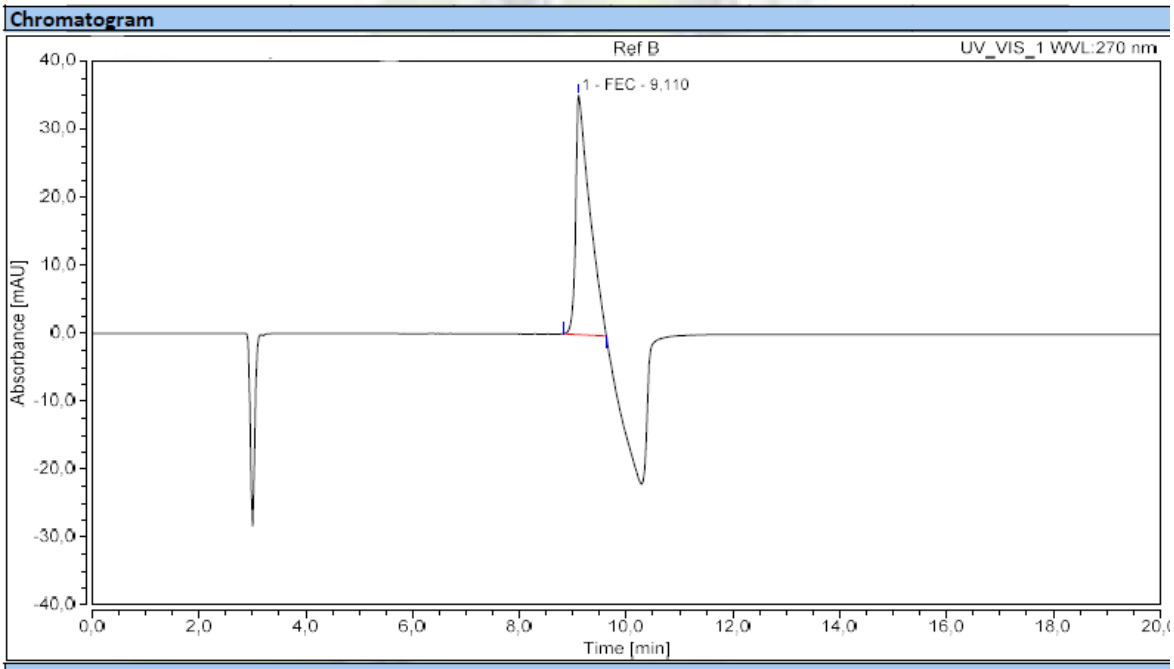
Injected solutions	Retention time min
Solution A (FMC)	7.917
Solution B (FET)	9.110
Solution C (FMC/FET)	8.030/ 9.347
Resolution	3.17



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Rel.Area %
1	FMC	7,917	2,552	7,600	100,00
n.a.	FEC	n.a.	n.a.	n.a.	n.a.
Total:			2,552	7,600	100,00

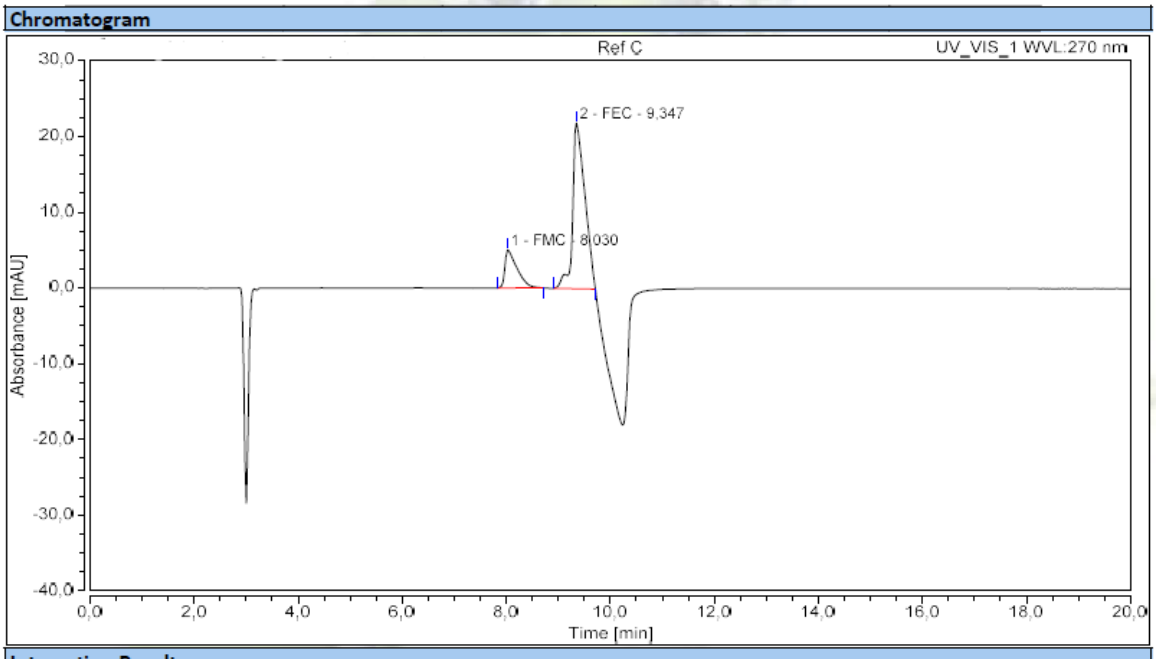
Figure 29. FMC identification.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Rel.Area %
n.a.	FMC	n.a.	n.a.	n.a.	n.a.
1	FEC	9,110	10,576	35,238	100,00
Total:			**10,576**	**35,238**	**100,00**

Figure 30. FEC identification.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Rel.Area %
1	FMC	8,030	1,350	5,040	18,52
2	FEC	9,347	5,940	21,871	81,48
Total:			**7,290**	**26,911**	**100,00**

Figure 31. FMC and FEC solution identification.

7.4.2Verification of identification test and Loq

- **Objective**
  - To verify the retention times of the analyte FMC in the reference solution a and in a radioactive sample of 18F-FCH;
  - to verify the amount of analyte, 18F-FCH that produces an area whose signal-to-noise ratio is ≥10 on the radiometric channel This concentration is assumed to be the lower limit of quantification (LOQ), i.e. the lowest quantitatively detectable analyte concentration in an analysis sample.

- **Method of verification**

  - Reference Solution D;

  - [18F]-FCH solution;

  - [18F]-FCH-5% solution;

  - [18F]-FCH- expire time solution;

  - [18F]-FCH-5%- expire time solution.

  Inject 25 ul of each test solution.

  Identify and integrate peaks on the radiometric channel: the 18F-FCH peak and other 18F impurities.

  The signal-to-noise ratio is calculated using the following formula: $S/N = \frac{2H}{h}$

  where:

  H = height of the analyte peak, measured from the peak maximum to the extrapolated baseline, where the signal baseline is observed over a distance of at least five times the peak width at half height;

  h = noise interval in the chromatogram obtained after injection of a blank, observed over a distance of at least five times the peak width at half height.

- **Acceptance Criteria**

  - Identify the 18F-FCH peak by evaluating the retention time of the [18F]-FCH on the radiometric channel, which must be similar to the retention time of the [19F]-FCH analyte on the UV channel.

  - The signal-to-noise ratio for the peak of [18F]-FCH must be ≥ 10.

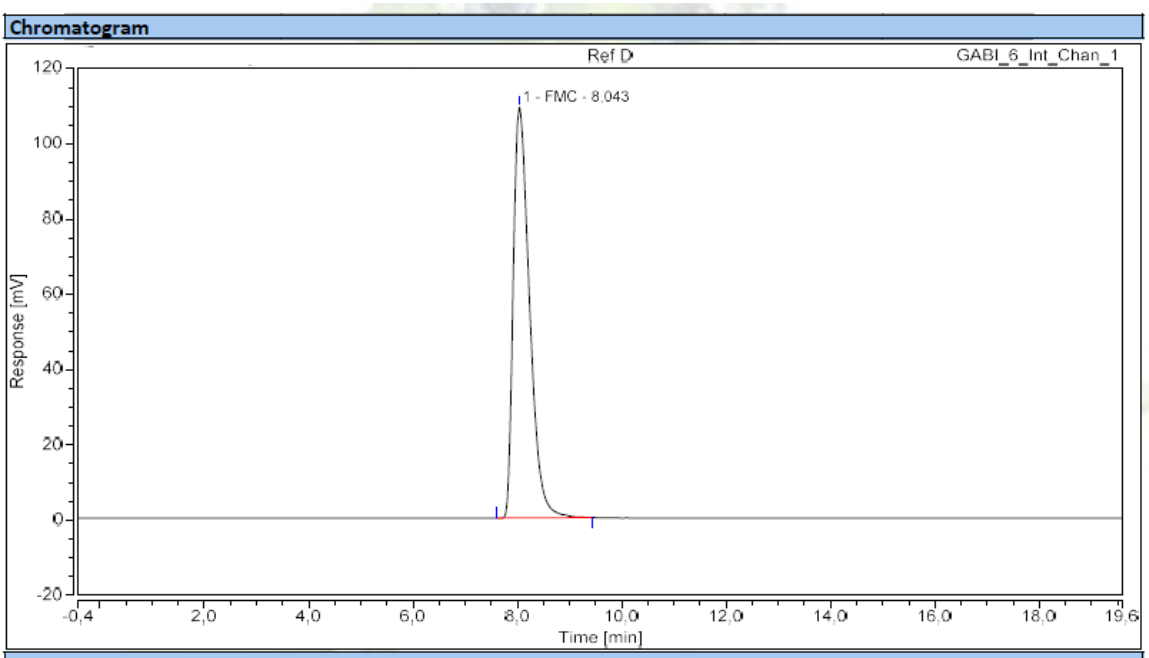
- **Results**

  The results of the 18F-FCH solution runs are reported in Table 22. 18F-FCH (18F-FCH solution at a concentration of 222Mbq/mL at the end of synthesis); [18F]-FCH -5% (18F-FCH solution diluted to 5%, at the end of synthesis); 18F-FCH-expire time (FMC solution at a concentration of 222Mbq/mL at the expiration time); 18F-FCH-5%- expire time (FMC solution diluted to 5% at the expiration time).

The chromatograms of some analytical runs are shown representatively, Figure 32 Solution Ref D radiometric channel, Figure 33 Solution Ref D UV channel, Figure 34 Solution 18F-FCH.

Table 22: FMC retention times on UV channels and radiometric channel.

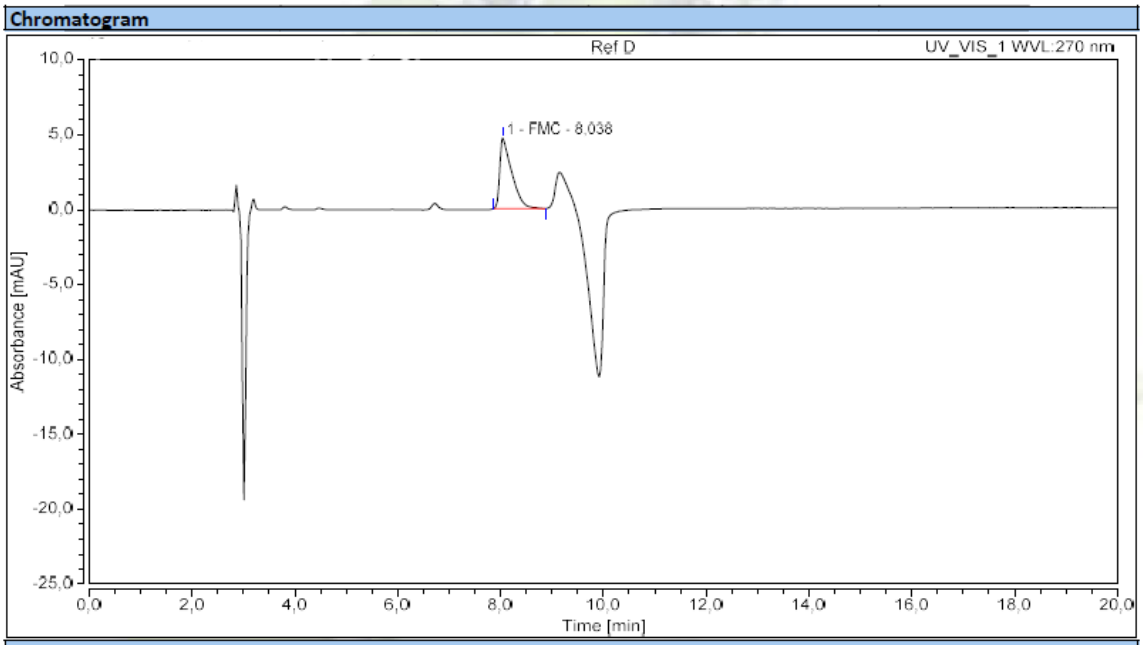
Sample	Retention time FMC UV channel	Retention time FMC radiometric channel	S/N
Reference Solution D	8.038	8,043	36382
Sample	**Maximum height**	**Retention time FMC radiometric channel**	**S/N**
18F-FCH solution	225.683	8.255	75227.6
18F-FCH -5% solution	9.751	8.265	3250.3
18F-FCH- expire time solution	3.408	8.258	1136
18F-FCH-5%- expire time solution	0.041	8.291	13.66
White	Observed noise	0.006	



Integration Results

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Rel.Area %
1	FMC	8,043	39,446	109,146	100,00
n.a.	FEC	n.a.	n.a.	n.a.	n.a.
Total:			39,446	109,146	100,00

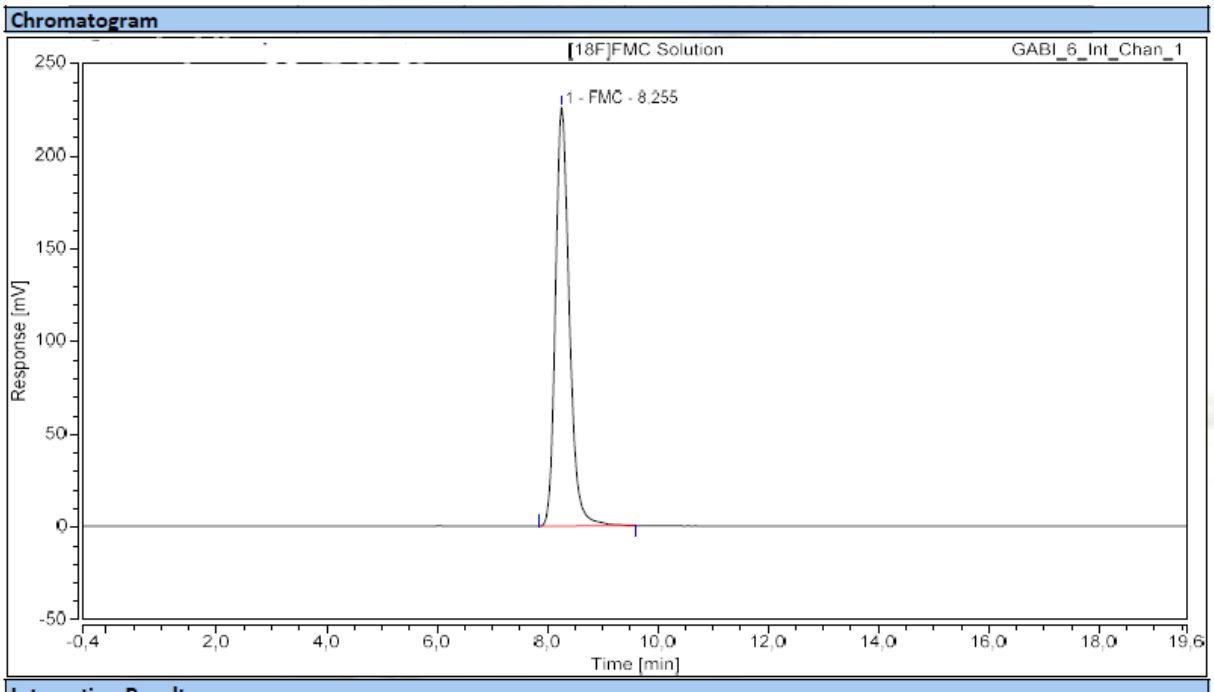
Figure 32. Radiometric channel Ref D analysis



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Rel.Area %
1	FMC	8,038	1,273	4,710	100,00
n.a.	FEC	n.a.	n.a.	n.a.	n.a.
Total:			**1,273**	**4,710**	**100,00**

Figure 33. Ref D channel UV analysis



Integration Results

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Rel.Area %
1	FMC	8,255	65,791	225,683	100,00
n.a.	FEC	n.a.	n.a.	n.a.	n.a.
Total:			**65,791**	**225,683**	**100,00**

Figure 34. Analyses [^{18}F]-FMC solution

7.5 Bioburden

- **Objective**

  To verify that the membrane filtration analytical method is applicable to the bulk solution of 18F-FCH not subjected to sterilising filtration, confirming the absence of bacteriostasis and/or fungistasis phenomena of the product on the ATCC microorganisms and S. epidermidis (ITELPHARMA environmental isolate strain) tested.

- **Acceptance Criteria**
  - With reference to the titre control (IT), performed on the microbial test suspension prepared for each microorganism tested:

    - the growth of each inoculated microorganism must take place within the incubation times indicated in the Table 23;

    - the inoculated count plates for the control must show a CFU/100 µl number < 100.

  - With reference to the PPC and PCWP samples from the validation activities

    - the growth of each ATCC microorganism tested on the different culture mediamust take place within the incubation times indicated in Table 24;

    - for each micro-organism in the PCWP samples, the resulting colony count (CFU) must be between 50 and 200 % of the mean value resulting from the relevant titre control (IT);

    - for each micro-organism of the PPC samples, the resulting number of colonies (CFU) must be between 50 and 200 % compared to the colony count (CFU) value resulting from the respective PCWP sample (positive control).

  - With reference to the NC samples (NCWP and NCPF) in the validation activities

  - no growth must be detected after the incubation period foreseen in Table 24.

  - With reference to the NC samples (NCPNF) foreseen by the validation activities

    - after the incubation period foreseen in Table 24, < 10 CFU values must be found per plate.

Table 23. Incubation times culture Average

Culture medium	Incubation temperature	Incubation time		
		PPC	PCWP	NCWP/NCPF/NCPNF
Plates SDA	20-25°C	≤ 5 gg for fungi and yeasts	≤ 5 gg for fungi and yeasts	5 gg
Plates TSA	30-35°C	≤ 3 gg for bacteria ≤ 5 gg for fungi and yeasts	≤ 3 gg for bacteria ≤ 5 gg for fungi and yeasts	5 gg

Table 24: Incubation times and temperatures of microbial suspension controls.

Culture medium	Incubation temperature	Incubation time		
		PPC e PCWP	PC	CN
TSB	20-25°C	Visible turbidity≤5gg	14 gg	14 gg
FTM	30-35°C	Visible turbidity≤5gg	14 gg	14 gg

- **Results**

The results of the control of the titre of the individual microbiological strains, and of the various tests carried out on the 3 batches are reported in table 25.

Table 25: Bioburden test results.

	Coltur ground	Title control IT (Average CFU)	PCWP (CFU)	Batch Product	PPC (CFU)	NCPF (CFU)	NCPNF (CFU)	NCWP (CFU)
S.aureus ATCC 6538	TSA	29	22	Lot#1	20	TSA Lot#10	TSA Lot#10	TSA 0 CFU
				Lot#2	24			
				Lot#3	18			
P.aeruginosa ATCC 9027	TSA	25	26	Lot#1	26			
				Lot#2	28	TSA Lot#20	TSA Lot#20	
				Lot#3	24			
B.subtilis ATCC 6633	TSA	31	36	Lot#1	36			
				Lot#2	40			
				Lot#3	38	TSA Lot#30	TSA Lot#30	
C.albicans ATCC10231	TSA	26	19	Lot#1	23			
				Lot#2	20			
				Lot#3	22			
C.albicans ATCC10231	SDA	26	20	Lot#1	21	SDA Lot#10	SDA Lot#10	SDA 0 CFU
				Lot#2	18			
				Lot#3	17			
A.brasiliensis ATCC16404	TSA	36	31	Lot#1	33			
				Lot#2	32	SDA Lot#20	SDA Lot#20	
				Lot#3	30			
A.brasiliensis ATCC16404	SDA	36	29	Lot#1	31			
				Lot#2	32			
				Lot#3	38	SDA Lot#30	SDA Lot#30	
S. Epidermidis (environmental isolate)	TSA	45	45	Lot#1	45			
				Lot#2	48			
				Lot#3	43			

7.6 Sterility

- **Objective**

Verify that the membrane filtration analytical method for determining the sterility for injectable solutions of 18F-FCHconfirms the absence of bacterial or fungal growth, and also evaluate the growth of microorganisms within the product by inoculation with ATCC microorganisms and on *S. epidermidis* (ITELPHARMA environmental isolate strain).

- **Acceptance Criteria**
  - With reference to the microbial suspensions ('test suspensions') performed for the validation tests:

  - the growth of each inoculated and plated microorganism must occur within the incubation times indicated in Table 26;

  - the inoculated count plates for titre control (VT) must show a number of CFU ≤100 CFU per 100 µl;

  - With reference to the PPC and PCWP samples from the validation activities:

  - the growth of each micro-organism tested must take place within the incubation times indicated in Table 27 ;

  - for FTM medium bottles, the pink upper zone must not extend more than half of the total height of the medium;

  - the growth of each micro-organism in the PPC samples must be visually comparable to that observed in the corresponding PCWP sample (positive control without product), and in any case within 5 days;

  - With reference to the PC and CN samples from the validation activities

  - no growth must be observed after the incubation period foreseen in Table 27.

Table 26.: Incubation times and temperatures of microbial suspension controls.

Culture medium	Incubation temperature	Incubation time
Plates di TSA	30-35°C	≤3gg
Plates di SDA	20-25°C	≤5gg

Table 27: Sample incubation times and temperatures.

Culture medium	Incubation temperature	Incubation time		
		PPC e PCWP	PC	CN
TSB	20-25°C	Visible turbidity ≤ 5gg	14 gg	14 gg
FTM	30-35°C	Visible turbidity ≤ 5gg	14 gg	14 gg

- **Results**

The following tables 28; 29 and 30 show the results of microbiological growth (in days) and sterility of the batches analyzed.

Table 28: Sterility and microbial growth test batch 1.

Product batch/ Run #	Microorganism/ ATCC	VT	Average							
			FTM				TSB			
			PPC Growth after (days):	PCWP Growth after (days):	PC	CN	PPC Growth after (days):	PCWP Growth after (days):	PC	CN
RUN#1	*C.albicans ATCC10231*	Conform			Sterile	Sterile	3	3	Sterile	Sterile
	B.Spizizenii ATCC 6633	Conform					3	3		
	A.brasiliensis ATCC16404	Conform					3	3		
	S. epidermidis (endogenous)	Conform					4	4		
	S.aureus ATCC 6538	Conform	3	3						
	C.sporogenes ATCC11437	Conform	3	3						
	P.Paraeruginosa ATCC 9027	Conform	3	3						

Table 29: Sterility and microbial growth test batch 2.

Product batch/ Run #	Microorganism/ATCC	VT	Average							
			FTM				TSB			
			PPC Growth after (days):	PCWP Growth after (days):	PC	CN	PPC Growth after (days):	PCWP Growth after (days):	PC	CN
RUN#2	*C.albicans ATCC10231*	Conform			Sterile	Sterile	3	3	Sterile	Sterile
	B. spizizenii ATCC 6633	Conform					3	3		
	A.brasiliensis ATCC16404	Conform					3	3		
	S. epidermidis (endogeno)	Conform					4	4		
	S.aureus ATCC 6538	Conform	3	3						
	C.sporogenes ATCC11437	Conform	3	3						
	P. araeruginosa ATCC 9027	Conform	3	3						

Table 30: Sterility and microbial growth test batch 3.

Product batch/ Run #	Microorganism/ATCC	VT	Average							
			FTM				TSB			
			PPC Growth after (days):	PCWP Growth after (days):):	PC	CN	PPC Growth after (days):	PCWP Growth after (days):	PC	CN
RUN#3	*C.albicans ATCC10231*	Conform			Sterile	Sterile	3	3	Sterile	Sterile
	B.Spizizenii ATCC 6633	Conform					3	3		
	A.brasiliensis ATCC16404	Conform					3	3		
	S. epidermidis (endogeno)	Conform					3	3		
	S.aureus ATCC 6538	Conform	3	3						
	C.sporogenes ATCC11437	Conform	3	3						
	P.Paraeruginosa ATCC 9027	Conform	3	3						

8. STABILITY

A stability study of the pharmaceutical product 18F-FCH was performed to confirm that the quality of the product complies throughout the shelf life, and within the use-by date. The purpose of stability tests is to provide evidence of how the quality of the pharmaceutical product changes over time under the influence of a variety of environmental factors such as temperature, humidity and light, and to establish the shelf life of the pharmaceutical product and storage conditions.

Drug substance stress tests can help identify probable degradation products, which in turn can help establish the degradation pathways and intrinsic stability of the finished products.

Stability study data should be provided on at least three primary batches of the finished product. The batches were produced considering a minimum, interAveragete and maximum batch size, following the same synthetic route as the production batches and using production methods and procedures that simulate the final process for the production batches. The overall quality of the pharmaceutical batches used in the formal stability studies must be representative of the quality of the material to be produced on a production scale.

The tests covered physical, chemical and microbiological attributes.

In general, the drug product should be evaluated under storage conditions (with appropriate tolerances) that test its thermal stability and, where appropriate, moisture sensitivity. The storage conditions and duration of the selected studies should be sufficient to cover storage, shipping and subsequent use. Stability studies were conducted on the finished product packaged in a container closure system equal to that proposed for storage and distribution.

The study was performed on samples produced using the manufacturing method described in the previous section, with the same formulation and stored in the same container with the proposed closure [(12)].

Three batches of 18F-FCH (the same as used for process validation) were stored for stability testing at room temperature (25 ± 2°C) and tested a:

- after manufacture (End of Synthesis);

- calibration time (End of Synthesis + 4 hours);

- Expiry time (End of Synthesis + 10 hours).

As the radiopharmaceutical is intended to be supplied in a multi-dose vial, stability during the proposed shelf life after removal of subsequent doses was studied, thus simulating actual use of the product. For this purpose, each vial was punctured 10 times after the control point at the time of release and stored under the conditions described below.

The stability of the product under abnormal conditions of light and humidity was not assessed: the product is packaged in a sealed glass vial (which provides a permanent barrier to the passage of

moisture), which in turn is contained in a shielding container [13].

Tests performed at t_0 were performed in accordance with the specifications and methods described in the previous sections. The tests performed at t_0 at the end of synthesis include all tests performed during a commercial release.

Tests for sterility and radionuclidic purity (Test B) were performed after an appropriate period of product decay, and then directly after the proposed shelf life.

Stability tests (appearance, Test A, Test B, identification C, radiochemical purity, chemical purity, residual solvent, pH) were performed at 4 and 10 hours after production. These tests are summarised in Table 31.

The stability data show that the drug product is stable in the proposed formulation in the dispensing vials, and therefore suitable for its intended use up to 10 hours after the end of synthesis. A shelf life of up to 10 hours after production is proposed for the drug product when stored at an ambient temperature of 25 °C.

The stability study was conducted as part of the production process validation. The analytical methods applied during the stability study are the same as those proposed for release and previously validated[13].

Table 31: Test and specification analysis.

Test analysis	**Methods**	**Specification**
Appearance	Visual inspection	Clear, colourless solution, free of visible particles
pH	Potentiometric	4.5 - 8.5
Identification test C:	HPLC	Similar retention time than standard FMC in UV chromatogram
Radiochemical identification	HPLC	18F-FCH 95% of area due to total radioactivity
Radiochemical purity (Test A)	TLC	≤ 5 % of total radioactivity
Radiochemical purity (Test B)	Spot test	Kryptofix ≤ 220 µg/mL (= 2.2 mg/ Vmax)
Chemical purity	GC	DMAE ≤ 0.10 g/l DBM ≤ 0.01 g/l ETHANOL ≤ 5 g/L (= 50 mg/Vmax) ISOPROANOL ≤ 5 g/L (= 50 mg/Vmax) ACETONITRILE ≤ 0.41 g/L(=4.1mg/Vmax) DMF ≤ 0.088 g/L (= 0.88 mg/Vmax)

The results obtained from one of the three initial test and stability studies carried out at ITEL Telecomunicazioni Srl are summarised in Table 32.

Table 32: Stability results.

Criteria	**Specification**	**Time points**		
		t_0	t_0 + 4h	t_0 + 10h
Visual test	Clear, colorless, free from visible particles	C	C	C
pH	4.5 – 8.5	5.54	6.66	6.54
Radiochemical purity (HPLC + γ detector)				
Identification C [^{18}F]-Fluorocholine chloride ([18F]-FCH) – Identity	Similar retention time than FMC standard in UV chromatogram	C	C	C
[^{18}F]-Fluoromethylcholine chloride ([18F]-FCH) (%)	≥ 95 % of total radioactivity	100 %	100 %	100 %
Radiochemical purity (TLC + γ detector)				
[18F] Fluoride (%)	≤ 5 % of total radioactivity	n.d.	n.d.	n.d.
Radionuclidic Purity				
Gamma Ray – (keV)	511 keV ± 5 % (485 - 537 keV)	508 Kev	-	-
Half-life Deviation (%)	109.8 ± 4 % (105 – 115 min)	109.8	-	-
Gamma-ray spectrometry Radionuclidic impurities (%)	Total radioactivity due to impurities (>t_0 + 24h) ≤ 0.1 % of total radioactivity	**(> t_0 + 24h)** ≤ 0.1 % of total radioactivity		
Chemical Purity (spot test)				
Kryptofix	≤ 220 µg/mL (= 2.2 mg/ Vmax)	≤ 220 µg/mL	≤ 220 µg/mL	≤ 220 µg/mL
Chemical Purity (GC + FID detector)				
Dimethylaminoethanol (DMAE) (g/l)	≤ 0.10 g/l	0.0215 g/L	0.0204 g/L	0.0197 g/L
Dibromomethane (g/l)	≤ 0.01 g/l	n.d.	n.d.	n.d.
Residual solvents: Ethanol (g/l)	≤ 5 g/L (= 50 mg/Vmax)	0.3510 g/L	0.2649 g/L	0.2410 g/L .
Residual solvents: Acetonitrile (g/l)	≤ 0.41 g/L (= 4.1 mg/Vmax)	n.d.	n.d.	n.d.
Residual solvents: Isopropanol (g/l)	≤ 5 g/L (= 50 mg/Vmax)	n.d.	n.d.	n.d.
Residual solvents: Dimethylformamide (g/l)	≤ 0.088 g/L (= 0.88 mg/Vmax)	n.d.	n.d.	n.d.
Activity concentration				
[^{18}F]-Fluoromethylcholine chloride (MBq/mL)	200-244 MBq/mL (= 222 MBq/mL ± 10 %)	229	-	-
Filter Integrity				

Criteria	Specification	Time points		
		t_0	t_0 + 4h	t_0 + 10h
Pre-filter (bar)	According to filter supplier Specification Millipore: ≥ 3.45 bar (or 50 psi)	4.12	-	-
Final filter (bar)		4.17	-	-
Sterility				
Sterility (Vials without punctures)	Sterile Complies with Eur. Ph.	Sterile		
Sterility (Vials with punctures)	Sterile Complies with Eur. Ph.	Sterile		
Bacterial Endotoxins				
Bacterial Endotoxins – Vials without punctures (U.I./mL)	< 17.5 U.I/mL	< 17.5 U.I/mL		
Bacterial Endotoxins – Vials with punctures (U.I./mL)	< 17.5 U.I/mL	< 17.5 U.I/mL		
Compatibility tests				
Container-content interactions	Percentage of error < 10 %	*	0.50%	1.62%

n.d.; No detectable – n.q.: No quantifiable; c: Compliance; *: t_0 is the reference point for the container-content interaction test.

Compatibility study

Evaluation of the interaction between solution and container. The vial containing the product is pierced with a needle (on the elastomeric cap) and turned upside down. The weight of an empty syringe is measured, a sample of solution is taken with it (about 500 µl) at t_0, t_0 +4h (t_c), t_0 + 10h (t_{exp}), subsequently the weight of the full syringe and the activity contained in it are measured. The radioactive concentration is reported (calculated) at the calibration time and compared as a function of weight. The results are reported in Table 33.

Table 33. Compatibility study.

	Time (h:m)	Weight in (g)	Activity (MBq)	Activity (MBq) / Weight (g)	Calculated activity in MBq/g at t_c	% of Dev
Vial t_0 (reference)	10:25	0.50245	324	644.84	220.27	0.00
Vial (t_0+4h)	13:21	0.49919	106.4	213.14	221.38	0.50
Vial (t_0+10h)	19 :39	0.49203	9.733	19.78	226.70	2.92

9. SPECIFICATION

A specification is a list of tests referring to validated or European Pharmacopoeia analytical methods, and appropriate acceptance criteria, i.e., numerical limits, ranges or other criteria for the described tests.

It establishes the set of criteria that the new drug producted must meet in order to be considered acceptable for its intended use. "Compliance with specifications" means that the drug producted, when tested according to the listed analytical procedures, meets the listed acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as a condition of approval.

Specifications are based, where it is possible, on the European Pharmacopoeia General Chapter, "Radiopharmaceuticals Preparations" (Ph. Eur.0125), Parenteral preparations

(Ph. Eur. 0520) and [18F]-Fluoride base monograph. ICH reference guideline:

- ICH Q3A(R2) – "Impurities in drug substances", Step 4, 25 Oct, 2006;
- ICH Q3B(R2) – "Impurities in new drug products", Step 4, 25 Oct, 2006;
- ICH M3(R2) – "Guidance on nonclinical safety studies for the conduct of human

  clinical trials and marketing authorization for pharmaceuticals", Step 4, 11 Jun, 2009;
- ICH Q6A – "Specifications: test procedures and acceptance criteria for new drug substances and new drug products: chemical substances", Step 5, May 2000.

Test results from stability and scale-up / validation batches, with emphasis on the primary stability batches, have been used to establish and justify specifications [(14)].

In developing the specifications, considerations were given to those characteristics that may affect the efficacy and safety of the products, namely:

- sterility and bacterial endotoxins;
- radioactive strength;
- radiochemical purity;
- specific activity, if applicable;
- appearance of the finished pharmaceutical product;
- radionuclidic purity, if applicable;
- chemical purity.

The data collected from stability studies and validation protocols led to the definition of the specification limits for the radiotracer 18F-FCH: the data are collected and shown in Table 34.

Table 34: Specification limits 18F-FCH.

ANALYTICAL PARAMETER	STRUMENT	SPECIFICATION LIMITS
Appearance	Screened syringe with white background	Clear solution, free of visible particles
pH	pH-meter	4.5 – 8.5
Identification A: Radionuclidic purity	Gamma spectrometer	485 – 537 keV (511 keV ± 5 %)
Radionuclide purity (Test A) Half-life	Dose calibrator	Half-life time 105 - 115 min (109.8 min ± 4 %)
Identification C: Radionuclidic purity	HPLC	The main peak in the radiochromatogram obtained with the test solution is similar in retention time to the main peak obtained with the reference solution of [^{19}F]-FMC
Radionuclide purity (Test A)	HPLC	[18F]-Fluorocholine chloride (18F-FCH) ≥ 95% of the area due to total radioactivity
Radionuclide purity (Test B)	TLC	[18F]Fluoride and other impurities ≤ 5% of the area due to total radioactivity
Radiochemical purity	Colour Spot Test	Kryptofix ≤ 220 µg/mL
Chemical purity	GC	Ethanol residuo ≤ 5 g/L
Chemical purity	GC	Isopropanol residuo ≤ 5 g/L
Chemical purity	GC	Acetonitrile residuo ≤ 0.41 g/L
Residual solvents	GC	DBM ≤ 0.01 g/L
Residual solvents	GC	DMAE ≤ 0.10 g/L
Chemical purity	GC	DMF ≤ 0.88 g/L
Radionuclidic purity (Test A)	Gamma Mucha Spectrometer	Total radioactivity due to impurities (other than 511 KeV or 1,022 KeV) must be ≤ 0.1 % of the total radioactivity. Fluorine 18 must be ≥ 99.9 % of the total radioactivity
Radioactive concentration	Dose Calibrator	200-244 MBq/mL (222 MBq/mL±10%)
Sterility (1)	Isolator and incubators	Sterile
Bacterial endotoxins	Endosafe-PTS	≤ 17.5 EU/mL
Radioactive concentration (1)	Ortec Gamma Spectrometer	Total radioactivity due to impurities must be ≤ 0.1%, [18F]-Fluoride ≥ 99.9% of total radioactivity
Terminal Filter Integrity	Bubble point test	> 3.45 bar (or 50 psi)
Pre-Filter Integrity		

[1]Tests performed after decay (at least 48 hours after the production time).

10. Conclusions

The main objective of the present industrial doctorate was to develop a production-scale manufacturing process for 18F-FCH according to European Good Manufacturing Practices.

The first step was the design of the production process. We started from the evaluation of the new synthesis route. This new route guarantees a robust process with higher final yields than the synthesis route used in the company. The comparison between the two different synthesis processes was carried out by analyzing the data in the literature, the economic characteristics and the safety aspects.

The synthesis route, which uses gaseous Bromo[18F]fluoromethane as precursor to produce 18F-FCH, has numerous disadvantages:

• Gaseous Bromo[18F]fluoromethane, is a highly volatile gas, if the containment cells to produce the radiotracer were to present problems, the gas would leak, causing damage to the operators.

• The synthesis is developed on cassettes (physical skeletons on which the chemical synthesis is performed), not specific radiotracer, as cassettes to produce 18F-FDG modified for the production of 18F-FCH are used.

• Very low synthesis yields are usyally obtained, compared to the starting activity.

The synthesis route that uses methylene-bis(toluene-4-sulfonate) as precursor bypasses all the disadvantages of the previous synthesis. Infact:

• No radiative gas is produced during the synthesis, improving operator safety.

• The synthesis is developed on specific plug-and-play cassettes for this synthesis (reduction of human errors).

• The synthesis yields are significantly higher, allowing the synthesis of the radiotracer to start with a lower initial starting activity (economic advantages).

• Quantity of DMAE is significantly lower than the limits defined by the European Pharmacopoeia (reduction of DMAE competition for the transport of 18F-FCH).

• the number of commercial lots is reduced, managing to guarantee with a single lot more doses of the radiotracer (economic and technical advantage).

The second phase of the study consisted of the implementation of the production process of 18F-FCH wich uses methylene-bis(toluene-4-sulfonate) as a precursor, according to GMP guidelines. In particular, the following activities were carried out:

– qualification of the synthesis module (AllInOne Trasis);

– verification of the performance qualification of the synthesis module;

– development of the chemical quality control equipment (HPLC, GC, TLC);

– validation of the chemical analytical method (HPLC, GC, TLC);

– validation of the microbiological analytical method (Bioburden, sterility);

– validation of the process;

– execution of the stability study.

The qualification of the synthesis module included installation and operational qualification. During the installation qualification (IQ), all major components of the equipment, systems and services were verified against the technical specifications and documentation of the manufacturer and/or supplier. The installation of the equipment, its connection to utilities, the operating procedures for use, maintenance and cleaning of the equipment, the availability of technical documentation, including the list of critical spare parts, were validated.

The operational qualification (OQ) demonstrated that the equipment operates in accordance with the functional specifications within the approved range. The OQ tests were successfully completed.

The product formulation was the focus of the performance qualification of the synthesis and dispensing system. Through the data obtained from the tests and subsequently from the qualification, an improved production range was obtained, obtaining a higher number of doses in a single production process.

In the third phase, radioactive samples were generated for the validation of the chemical analytical methods. For the validation of an analytical method, the draft of the method to be validated should be available, including the analytical conditions and instrument setup. Radiochemical purity, chemical purity and chemical purity for residual solvents were validated using radio-TLC, HPLC and GC respectively. Tests were performed to validate the analytical method to demonstrate that the methods are reliable and can be used for the analysis of 18F-FCH. Different solutions were tested on the equipment. The validation activities demonstrated that the quality control activities are reliable and allow the detection of non-compliance or out-of-specification.

In the fourth step, radioactive samples were generated for the validation of the microbiological analytical methods. After their decay, the generated samples were used to validate the Bioburden of the unfiltered product and the sterility of the product following terminal sterilizing filtration. To validate the analytical method, microbiological tests were performed by testing ATCC microorganisms and *S. epidermidis* (ITELPHARMA environmental isolate strain). The validation activities demonstrated that quality control activities are reliable, and the final product does not present bacteriostasis and/or fungistasis phenomena.

The final stage was to perform process validation, stability study and definition of the specifications of the final product. Reliability and reproducibility of the manufacturing process are essential characteristics of a pharmaceutical manufacturing process. Three batches of 18F-FCH were produced

and analyzed to demonstrate that the entire manufacturing process is robust and reliable. The data confirmed the reliability and reproducibility of the manufacturing process.

In conclusion, the data obtained during the qualification of the production equipment installation confirmed the correct installation of all components, the calibration of the instrumentation and the quality of the raw materials. The operating limits and the applicability of the operating and cleaning procedures were demonstrated during the operational qualification phase. The performance qualification was carried out after the completion of the installation and operational qualification. It certified the operation of the equipment in the operating range of the manufacturing process.

The chemical analytical test methods were validated with appropriate limits of detection and quantification. The data confirmed the reliability of the HPLC, GC and TLC scanners.

Microbiological testing (bioburden and sterility) of the product was performed after method validation. Validation confirmed that the product does not interfere with the recovery of microorganisms.

The manufacturing process was validated to demonstrate its robustness. Validation ensures consistent product quality before a product is released to the market. The manufacturing process was validated by testing three complete batches.

Validation activities confirmed that all manufacturing processes are clearly defined, capable of producing a finished product that is consistent and compliant with specifications. The data were submitted to AIFA for assessment before the start of production of batches to be administered to patients.

AIFA reviewed the equipment qualification, method and process validation data.

ITEL Telecomunicazioni obtained a new authorization from AIFA (reported below) after the positive assessment of the data relating to the 18F-FCH process.

Figure 35. ITEL Telecomunicazioni authorization for 18F-FCH.

AGENZIA ITALIANA DEL FARMACO

ALLEGATO 1

SCOPO DELL'AUTORIZZAZIONE

Denominazione ed indirizzo del sito: ITEL TELECOMUNICAZIONI S.R.L. - VIA ANTONIO LABRIOLA ZONA INDUSTRIALE SNC , 70037 RUVO DI PUGLIA(BA)

Prodotti Medicinali Umani

Attività Autorizzate

Attivita' di Produzione (Parte 1)

Parte 1 - ATTIVITA' DI PRODUZIONE

1.1	**Prodotti sterili**		
	1.1.1	*Preparati in asepsi*	
		1.1.1.4	Liquidi di piccolo volume Requisiti speciali: Radiofarmaci
	1.1.2	*Sterilizzati terminalmente*	
		1.1.2.3	Liquidi di piccolo volume Requisiti speciali: Radiofarmaci
	1.1.3	*Certificazione del lotto*	
1.5	**Confezionamento**		
	1.5.2	*Confezionamento secondario*	
1.6	**Test per il controllo di qualità**		
	1.6.1	*Microbiologici: sterilità*	
	1.6.3	*Chimico/Fisici*	
	1.6.4	*Biologici*	

Restrizioni o chiarimenti inerenti le operazioni di produzione

1.1.1.4 Liquidi di piccolo volume: 18F-Fluorodesossiglucosio; 18F-Flutemetamolo; 18F-Fluorocolina, 18F-Fluorodopa, 18F-Florbetaben, 18F-PSMA-1007, 18F-JK-PSMA-7;
1.1.2.3 Liquidi di piccolo volume: 18F-Fluorodesossiglucosio; 18F-Sodio fluoruro;
1.1.3 Certificazione del lotto: solo radiofarmaci;
1.5.2 Confezionamento secondario: solo radiofarmaci;
1.6.1 Microbiologici: sterilità : solo radiofarmaci;
1.6.3 Chimico/Fisici: anche purezza radionuclidica; solo radiofarmaci;
1.6.4 Biologici: test endotossine; solo radiofarmaci;

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