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CICLO XXIV

COORDINATORE Prof. FRANCESCO BERNARDI

SYNTHESIS AND QUALITY ASSESSMENT OF THE POSITRON EMITTER RADIOTRACER 2-[^{18}F]-FLUOROETHYLCHOLINE, A [^{18}F]-LABELLED CHOLINE ANALOGUE FOR TUMOURS IMAGING.

Settore Scientifico Disciplinare CHIM/06

Dottorando
Dott. ASTI MATTIA

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(firma)

Tutore
Prof. UCCELLI LICIA

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(firma)

Anni 2008/2011

… Mientras haya unos ojos que reflejen
los ojos que los miran,
mientras responda el labio suspirando
al labio que suspira,
mientras sentirse puedan en un beso
dos almas confundidas,
mientras exista una mujer hermosa,
habrá poesía !

Gustavo Adolfo Becquer

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INTRODUCTION:

1.1. CHOLINE METABOLIC PATHWAYS:

In 1998, choline was identified as an essential nutrient for humans by the National Academy of Sciences, United States (1). Choline (trimethyl-β-hydroxy-ethylammonium) is a quaternary amine linked to a two carbon atoms backbone with an alcoholic moiety (2) and its concentration in plasma has a physiological range of 10–50 μM (3). Choline is predominantly utilized for the synthesis of essential lipid components of the cell membranes, phosphatidylcholine (PCh) and sphingomyelin, and for the production of potent lipid mediators such as platelet-activating factor and lysophosphatidylcholine (4). Quantitatively, PCh is the most important metabolite of choline and accounts for approximately one-half of the total membrane lipid content (5). Similarly to folate, choline is a source of labile methyl groups (6). In the liver and kidney mitochondria, choline is oxidized to betaine (trimethylglycine), which then enters the one-carbon cycle and serves as a methyl donor in the re-methylation of homocysteine to methionine, to ultimately generate the methylation agent S-adenosylmethionine (7). Finally, choline is best known for its key role in neurons as the precursor of the neurotransmitter acetylcholine (8, 9). Acetylcholine could also be synthesized and released from non-neuronal cells; however, its role outside of neurons is not clearly defined (10). The choline metabolic pathways are resumen in figure 1, while, in figure 2 an overview with molecular structures is shown.

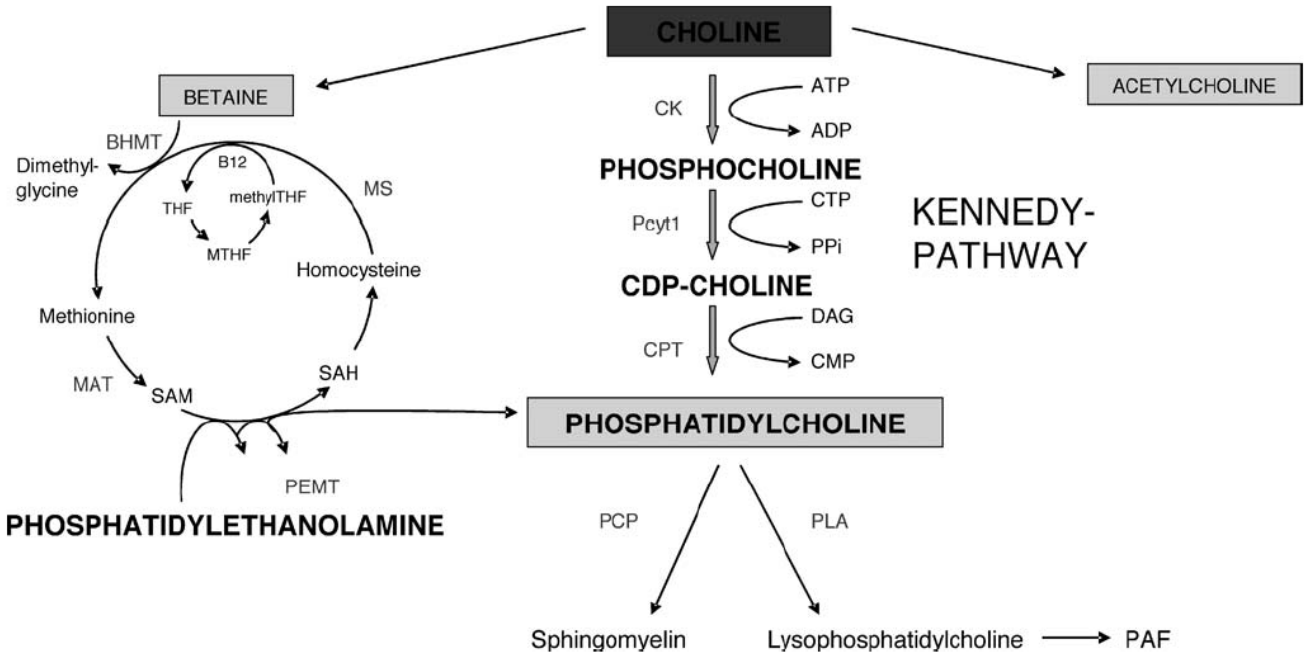


Figure 1: Choline metabolic pathways. ADP, adenosine diphosphate; ATP, adenosine triphosphate; BHMT, betaine:homocysteine methyltransferase; B12, vitamin B12; CDP, cytidine diphosphate; CK, choline kinase; CPT, CDP-choline:DAG cholinephosphotransferase; CMP, cytidine monophosphate; CTP, cytidine triphosphate; DAG, diacylglycerol; MAT, methionine adenosyltransferase; methylTHF, 5 methyltetrahydrofolate; MS, methionine synthase; MTHF, 5,10-methylene-tetrahydrofolate; PAF, platelet activating factor; PCP, phosphatidylcholine: ceramidecholine phosphotransferase; Pcyt1, CTP:phosphocholine cytidyltransferase; PEMT, phosphatidylethanolamine N-methyltransferase; PLA, phospholipase A2; PPi, pyrophosphate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate. The figure is printed from (11).

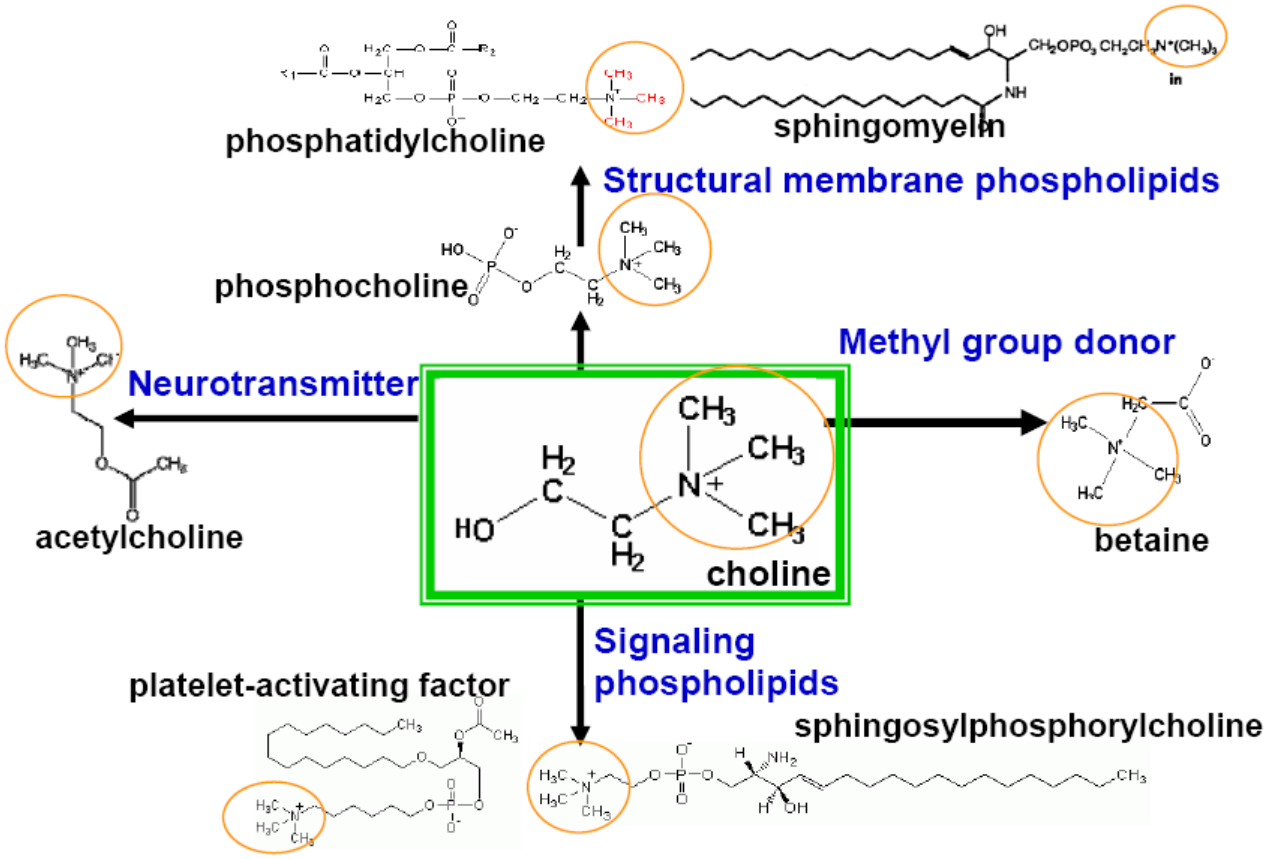


Figure 2 Metabolic fate of choline.

The majority of cellular choline is phosphorylated by choline kinase (ChK) to phosphocholine, then CTP can then be added to phosphocholine by Pcyt1 to yield CDP-choline. The formation of PCh results from the reaction of CDP-choline with diacylglycerol (DAG), catalyzed by the enzyme CDP-choline DAG cholinephosphotransferase (CPT, Fig. 1). This pathway is known as the Kennedy (CDP-choline) pathway for de novo synthesis of PCh and is essential for the formation of membrane PCh in all nucleated cells. Since its discovery in the 1950s by Eugene Kennedy, this pathway has been extensively studied, and most aspects of its regulation are well established. In recent years, focus is more on cloning and characterization of genes participating in the pathway (12–16), with emphasis on the rate-regulatory gene Pcyt1 (17-19); the Kennedy pathway is not considered to be regulated by other steps in the pathway, nor by the rate of choline transport.

During evolution, the liver has retained a backup pathway for choline production from the second most abundant membrane phospholipids, phosphatidylethanolamine (PE, Fig. 1), to provide this essential metabolite when dietary choline is limited, as, for example, during starvation, embryonic development, pregnancy, or lactation. PE is transformed to PCh in a three-step methylation by SAM, which is catalyzed by phosphatidylethanolamine-N-methyl-transferase (PEMT). The PE methylation pathway has been extensively studied by Dr. Dennis Vance and his collaborators (20, 21). More recently, his laboratory developed a PEMT knockout mouse model, providing valuable data on the role of this pathway in the liver, PCh synthesis, lipoprotein secretion, and bile and homocysteine production (15, 22).

1.2. CHOLINE TRANSPORT:

Choline is a positively charged quaternary amine and requires a protein-mediated mechanism to effectively pass the membrane lipid barrier. The mechanisms underlying choline transport have not been completely elucidated; however, three protein-mediated and thus saturable uptake systems, following Michaelis-Menten kinetics, are well documented (23–28).

The first mechanism is the facilitated diffusion that has an apparent K_m of 10 μM of choline, driven by a choline concentration gradient as described in red blood cells (24).

The second is a high-affinity, Na^+- and energy-dependent transport system with a K_m of 0.5–3 μM that is sensitive to inhibition by low concentration of hemicholinium-3 (HC-3, K_i of 1–3 μM; Ref. 25). This ‘‘active transport’’ system is coupled to the biosynthesis of acetylcholine and is primary to neuronal tissues (25).

The third, also ‘‘active transport,’’ system, which is somewhat lower in affinity for choline (K_m ca. 20–200 μM), is ubiquitously distributed, and less effectively inhibited by HC-3 (K_i ca. 20–200 μM) than the neuronal system (26–28). This system operates in most cells as a means of choline uptake for the purpose of phospholipid synthesis.

1.3. CHOLINE METABOLISM AND MALIGNANT TRANSFORMATION:

Choline metabolism and choline-derived metabolites can undergo extensive alterations as a result of a malignant transformation. The level of phospocholine in human breast cancer cells was found to be markedly higher than in normal human mammary epithelial cells (29-31). Both choline transport and phosphorylation were found to be augmented in human breast cancer cells relative to their normal counterpart. The level of phosphocholine correlated with the maximal rate of choline transport in the normal and cancerous cells, but it did not correlate with the tumorgenecity and invasiveness of the cancer cells. Progression of human mammary epithelial cells from a normal to a malignant phenotype was shown to be associated with a reversion in the balance, as well as an overall increase in the content of phosphocholine and GPCho (31). A similar trend was also exhibited by human prostatic epithelial cells (32). High levels of phosphomonoesters, including phosphocholine, were detected in human breast cancer biopsies and patients (33-35). Ras transformed cells, and multi oncogenic transformed cells have also exhibited an increase in phosphocholine content (36-38). Choline-kinase activation was shown to be critical for the proliferation of primary human mammary epithelial cells and breast tumors progression (39). Indeed, cessation of phosphocholine synthesis by novel choline kinase inhibitors exhibited antitumor activity (40-42).

Choline is a precursor of choline derived phospholipids, but can be also recovered as a product of their hydrolysis. The synthesis and degradation of phospholipids may be induced by growth factors that play a major role in malignant transformations (43, 44). Several studies have demonstrated regulation of PCh metabolism by a receptor tyrosine kinase cascade, downstream of the ras/raf interaction (45, 46). In turn, molecules derived by the breakdown of choline-containing-phospholipids, such as diacylglycerol, ceramide,

and phosphocholine can act as second messengers in mitogenic signal transduction pathways (47-50).

1.4. POSITRON EMISSION TOMOGRAPHY TUMOURS IMAGING BY MEANS OF CHOLINE BASED TRACERS:

Tumor imaging with choline-based tracers was introduced by Hara and coworkers using carbon-11 (^{11}C) choline PET to successfully visualize brain tumors and prostate cancer (51,52). As a true tracer, ^{11}C choline is biochemically indistinguishable from natural choline. This compound has shown particular promise for imaging tumours of the genitourinary tract because of its limited urinary clearance and avidity for bladder and prostate cancers (53-58). However, the short decay half-life of the carbon-11 (20 minutes) has limited its use to centers equipped with an on-site cyclotron.

Conversely, ^{18}F is a radionuclide with optimal decay characteristics both for PET applications and for a widespread utilization. For these reasons, [^{18}F]-labeled analogues have been developed and tested as an alternative choline derivatives for PET imaging of prostate and brain tumours (59-61). Among the analogues the most used are also in clinical practice are [^{18}F]fluoromethyl-dimethyl-2-hydroxy-ethylammonium (^{18}FCH) and 2-[^{18}F]-fluoroethylcholine (^{18}FECH). In figure 3 are shown some choline analogues labelled with 18-Fluoro and ^{11}C-Choline for comparison. Figure 3 is derived from (64).

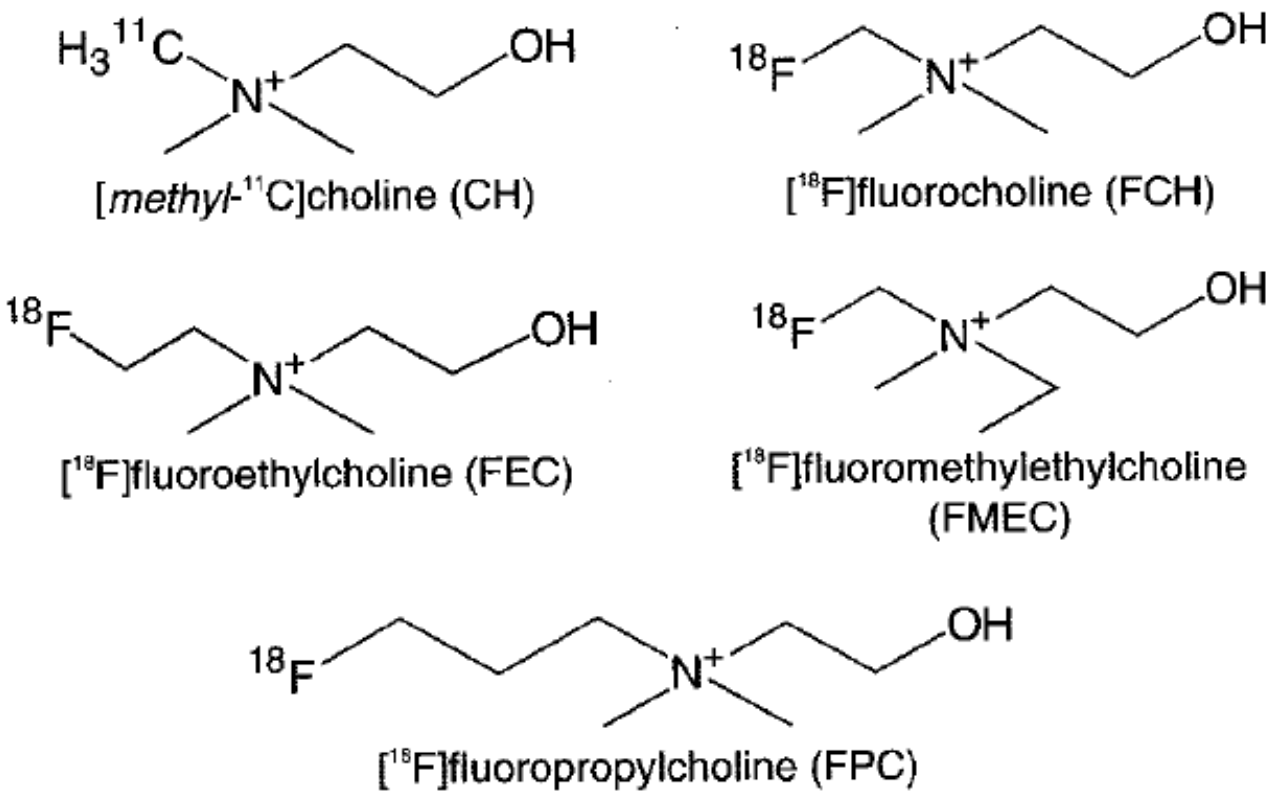
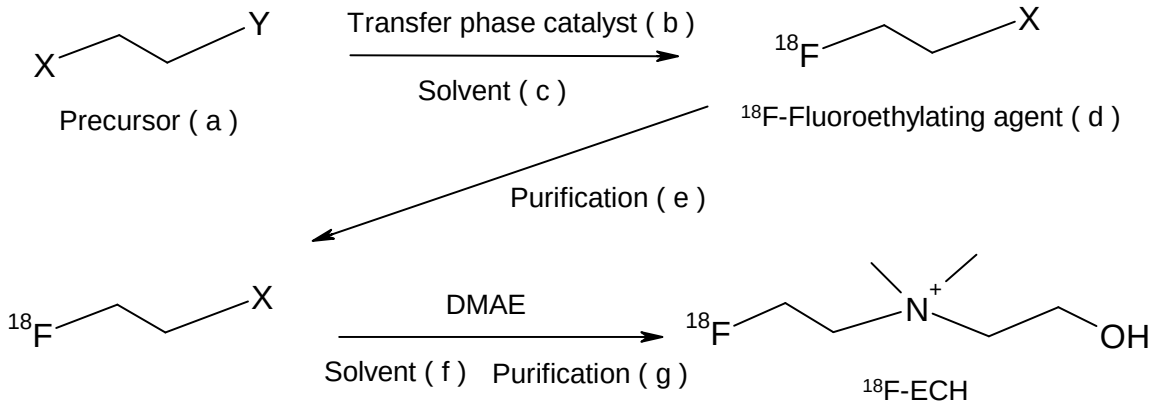


Figure 3. Chemical structure of positrons emitters – labelled choline analogues.

The sterical hindrance and structural features imparted by the introduction of a fluorine atom in the choline backbone have been thoroughly investigated both in vitro and in vivo (62–65). In these studies, it was reported that two methyl groups on the quaternary amine are essential for maintaining the affinity of the molecule for the choline transport system and for the specificity of choline kinase. The remaining third methyl group can be replaced by a longer alkyl chain where an ^{18}F atom is linked.

At the present time, it is not known which choline derivative is most advantageous for clinical use. There have been no direct in vivo comparisons between individual compounds, and previous in vitro comparisons have not controlled for potentially confounding factors, including the presence of synthetic contaminants such as dimethylethanolamine, which may modulate the phosphorylation and trans- port of these compounds in vitro.

As a result, the syntheses of ^{18}FCH and ^{18}FECH were developed in two-step reactions. Both the procedures resulted rather complicate to be carried out in an hospital environment and required time consuming purifications by means of gas chromatography (GC) or high-performance liquid chromatography (HPLC) (64,65). In figure 4 are shown the different precursors and purification methods that have been reported in literature.



(a, d) X = Br, OTs, OTf Y = Br, OTs (b) TBAHCO3 or Kry 2.2.2 (c) ACN, o-DCB, DMSO

(e) nothing, GC, distillation, SPE (f) nothing, ACO, ACN, DMSO, DMF (g) SPE, HPLC, SPE + HPLC

Figure 4 : ^{18}FECH syntheses using different reagents, solvents and purification methods

The choline metabolite peak on magnetic resonance spectroscopy (MRS) also has been proposed as an indicator of malignancy or proliferation (66). However, correlations between ^{18}F-FCH or ^{11}C-choline uptake on PET and choline metabolite concentrations on MRS have not always been observed, alluding to the possibility that increased choline spectral peaks on MRS may not specifically reflect free choline or the active accumulation of choline metabolites by cells (67,68). For example, in the case of a tumefactive demyelinating lesion, where increases in choline metabolite concentrations are frequently observed with MRS (presumably due to demyelination), there may not be corresponding increases in the uptake of ^{11}C choline or ^{18}F-FCH (68,69). In malignant glial tumours,

where mitogenic activity would be expected to result in the active utilization of choline, a direct regional correlation has been observed between ^{18}F-FCH uptake and choline metabolite peaks on spectroscopy (68). Without a better understanding of the biochemical basis of what is measured by both MRS and choline-based PET imaging, it will be difficult to integrate this information for clinical purposes (65). Because there is occasional discordance, there will most likely be complementary value to both measures.

2. AIM:

The first purpose of this dissertation was to develop a reliable synthesis of ^{18}FECH by using commercial synthesizers. Different synthetic approaches, reaction parameters, and purification methods were compared and optimized in order to achieve the highest radiochemical yield and purity. Aiming at a user- friendly and widely applicable procedure, the use of commercial reagents and the feasibility of a one-pot reactions and SPE purification were assessed.

Although ^{18}F-FECH is a radiopharmaceutical widely used in the clinical practice for the detection of prostate cancer recurrence and brain tumours by PET (11, 70), it is still subjected to experimental protocols because no Monographs is present on the European Pharmacopeia. For this reason the second purpose of this dissertation is to establish a what techniques and instruments are necessary for assessing the suitability of the ^{18}FECH preparations in humans. quality controls. For this reason, with no regard for the synthetic procedure, the final ^{18}FECH solution was submitted to an in-depth set of quality controls for attesting the reliability of the synthesis and for evaluating a safe employment of the radiotracer for clinical trials.

3. EXPERIMENTAL:

3.1. 2-[^{18}F]-FLUOROETHYLCHOLINE SYNTHESIS:

In recent years, the synthesis of ^{18}FECH has been the most investigated of the [18F]-radiolabelled choline derivatives likely because its synthesis was supposed to allow for easier automation. In the original method (65), [^{18}F]fluoride was reacted with a 1,2-bis (tosyloxy)ethane (diOTsEt) solution to give [^{18}F]fluoro-ethyltosylate (^{18}FEtOTs). Subsequently, a large excess of pure N,N-dimethylaminoethanol (DMAE) was added directly to the mixture in order to obtain ^{18}FECH. The reaction mixture was purified by evaporation, HPLC and then solid-phase extraction (SPE) obtaining the product with a 27% radiochemical yield (RCY), not decay corrected. This approach was recently improved with a fully automated synthesis and a yield of 36% was achieved (71). In other studies, different [^{18}F]-fluoroethylating agents (^{18}FEtX) were obtained by nucleophilic substitution on various precursors and reacted with DMAE solutions (72,73). However, in those studies, at least one SPE purification after the nucleophilic substitution and an HPLC purification after the alkylation reaction were performed. In spite of this and with some minor changes in the first described approach (65), Zuhayra et al. were able to synthesize ^{18}FECH with a 47% RCY (not decay corrected) (72). However, the use of a non-commercial precursor and of toxic solvent such as 1,2-dichlorobenzene is a strong drawback for applying this method in a clinical setting. Recently, it was reported that a drastic increase in [^{18}F] fluoroethylating agents yield could be obtained by adding alkali iodide to the reaction mixture (73). However, when ^{18}FECH synthesis was fully performed using that approach, only a 30% of RCY was obtained (74). Schmaljohann J et al. reported a ^{18}FECH synthesis in two steps and high yield by starting from BrEtONos precursor but the assessment of the chemical purity of the preparation was not completely fulfilled as only an UV detector was used in the HPLC analysis for quality control (75).

3.2. MATERIALS AND METHODS:

3.2.1 Reagents:

diOTsEt, DMAE, dibromoethane, ethanol and dry acetonitrile (ACN) were purchased from Sigma-Aldrich (Milan, Italy). Tetra-butylammonium bicarbonate (TBA) and Kryptofix 2.2.2/ K_2CO_3 solutions as well as 1,2-bis(nosyloxy)ethane (diONsEt), 2-Bromoethyltosylate BrEtOTs were obtained by ABX (Radeberg, Germany). Fluoroethylcholine chloride (FECH) and N,N-dimethylmorpholinium chloride (diMMCl), used as reference standards for the quality controls, were purchased from ABX and Labotest OHG (Dresden, Germany), respectively. All the reagents were used without further purification. Unless otherwise specified, the water used in any operation was distilled by using a MilliQ Simplicity 185 (Millipore, Milan, Italy). Sep-Pak light QMA (preconditioned CO_3^{2-} form), Sep-pak plus QMA, plus C-18 and CM cartridges were obtained by Waters (Milan, Italy). Cathivex- GS 0.22-µm filters were purchased from Millipore.

3.2.2. Instrumentations:

No-carrier-added [^{18}F]fluoride was produced by the ^{18}O (p,n)18F nuclear reaction on a MINItrace cyclotron (GE Medical system, Uppsala Sweden) using a 9.6 MeV proton beam. Enriched (> 98 %) [^{18}O]water was purchased from SRICI (Shanghai, China).

3.2.2.1. Automatic synthesizers:

An automated synthesis/dispense module is defined as an electromechanical device controlled by software to automatically perform a sequence of operations needed to synthesise and/or formulate and/or dispense a radiopharmaceutical. It usually consists of a combination of power supplies, actuators, pumps, heaters and sensors that are used in combination with an interconnected network of containers, reactors, tubing, syringes, solid

phase cartridges and/or preparative HPLC systems. The automated synthesis/dispense module can be a commercial piece of equipment or can be custom made. It is common for different radiopharmaceuticals to be made on the same automated synthesis/dispense module. Within the synthesis process, the automated synthesis/dispense module controls the mixing of starting materials and reaction parameters in such a way that a bulk solution of a radiopharmaceutical is produced. The containers and purification system used with the automated synthesis/dispense module can be single-use ('radiopharmaceutical cassette') or used in multiple production runs. It must be shown by appropriate cleaning protocols that the quality of the produced radiopharmaceutical is not negatively affected when used in multiple production runs. Cross contamination must be prevented. The containers and purification systems (i.e. the column of a preparative HPLC system) are considered part of the synthesiser. The electronic components of the synthesiser are usually resistant to high radiation. Components that come into contact with the starting materials, solvents and/or the radiopharmaceutical are chemically inert. Automated dispensing modules control formulation and dispensing of the radiopharmaceutical. This is usually done by using volume- or weight-measuring devices and radioactivity detectors in order to measure and dispense the correct quantities. For dispensing, single-use tubing systems should be used unless a validated cleaning protocol is performed. The measuring systems are calibrated. The synthesis process on the synthesiser is usually controlled by software containing specific time lists for each process. Automated systems may involve the use of radiopharmaceutical cassettes. A radiopharmaceutical cassette is defined as a system of single-use production hardware (such as tubes, valves and filters). It is used with a set of starting materials (such as precursors, solvents, catalysts, etc.) which may be contained in the cassette or provided separately (prefilled vs. empty cassette). In order to maintain a low bacterial endotoxin level and achieve a high sterility assurance level for the

radiopharmaceutical prepared with the use of a cassette, the cassette have a low bio-burden (76).

In this dissertation, synthesis of ^{18}FECH was accomplished by using two different automatic synthesizers. The first one, was an FX FN Tracer lab (GE Medical System, Uppsala, Sweden) without any modification from the original set up. F-X FN Tracer Lab is a multiple production synthesizer and an appropriate sequence of washing was adopted after every production.

The second synthesizer was a Mx Tacerlab (GE Medical System, Uppsala, Sweden), namely a cassettes based synthesizer. The advantages and drawbacks of both synthesizer in the preparation of ^{18}FECH will be described in the discussion session.

3.2.2.2. Quality controls instruments:

Radioactivity measurements were performed in a calibrated ion chamber (Aktivimeter ISOMED 2000, MED Nuklear- Medizintechnik, Dresden, Germany). The radiochemical and chemical purity of the ^{18}FECH solutions were assessed by means of: (1) ion chromatography using an isocratic pump (Gilson, Milan, Italy) equipped with a Waters 432 conductimeter (Waters, Milan, Italy) in series with a β+- flow count detector (Bioscan, Washington, DC) and with a 152 UV/VIS detector (Gilson, Milan, Italy); (2) Acquity UHPLC system with a binary solvent and auto-sampler manager modules (Waters, Milan, Italy). The instrument was equipped with an Acquity TUV detector (Waters) in series with a Herm LB 500 radiochemical detector (Berthold Technologies, Milan, Italy) and a Waters 432 conductimeter (Waters, Milan, Italy). The HPLC system was replaced with the UHPC system and the analysis method was further improved in order to obtain a complete assessment of the chemical and radiochemical composition of the final preparations:

Thin-layer chromatography (TLC) using an AR 2000 Imaging Scanner (Bioscan, Washington, DC, USA) and silica gel plates 60 F254 (Merck, Milan, Italy) was also performed.

Residual solvents amounts and chemical purity were also measured by gas chromatography on a FOCUS GC system (Thermo Scientific, Waltham, MA, USA) equipped with a flame ionization detector (FID) and an AI/AS 3000 series auto-sampler. The absence of bacterial endotoxin in the final radiopharmaceutical preparations was checked with a chromogenic limulus amebocyte lisate test performed with an Endosafe-PTS instrument (Charles Rives Laboratories, Charleston, SC, USA).

Nuclear Magnetic Resonance spectrometer Bruker Avance 300 (Bruker, Milan, Italy) was used for acquiring 1H NMR spectra in order to assess the stability of the reagents.

3.2.3. Radiopharmaceutical synthesis:

3.2.3.1. TRACERlab Fx F-N:

TRACERlab™ FX F-N (figure 5) is an automated versatile synthesizer for easy and efficient production of [^{18}F] tracers via nucleophilic substitution with [^{18}F] F-Fluoride trapped from [^{18}O] water. Purification of the products can be achieved by an integrated, preparative HPLC system. The system is based on a number of glassy reservoir and vessel connected by silicon tubing through two or three-ways valves. The flow of the liquid inside the synthesizer is regulated by an helium stream or by a mechanical vacuum pump. Reactions can be performed in a fixed

glassy carbon reactor where the temperature can range between 200 to RT ℃. A rapid tempering of the liquids in the reactor is also allowed by a helium flow directed on the reactor body. Solid phase extraction cartridges can be put on line in varying positions. Through application software, all process steps are easily programmed to produce the required tracers. After every synthesis, Fx F-N have to be cleaned by automatic rinsing protocols.

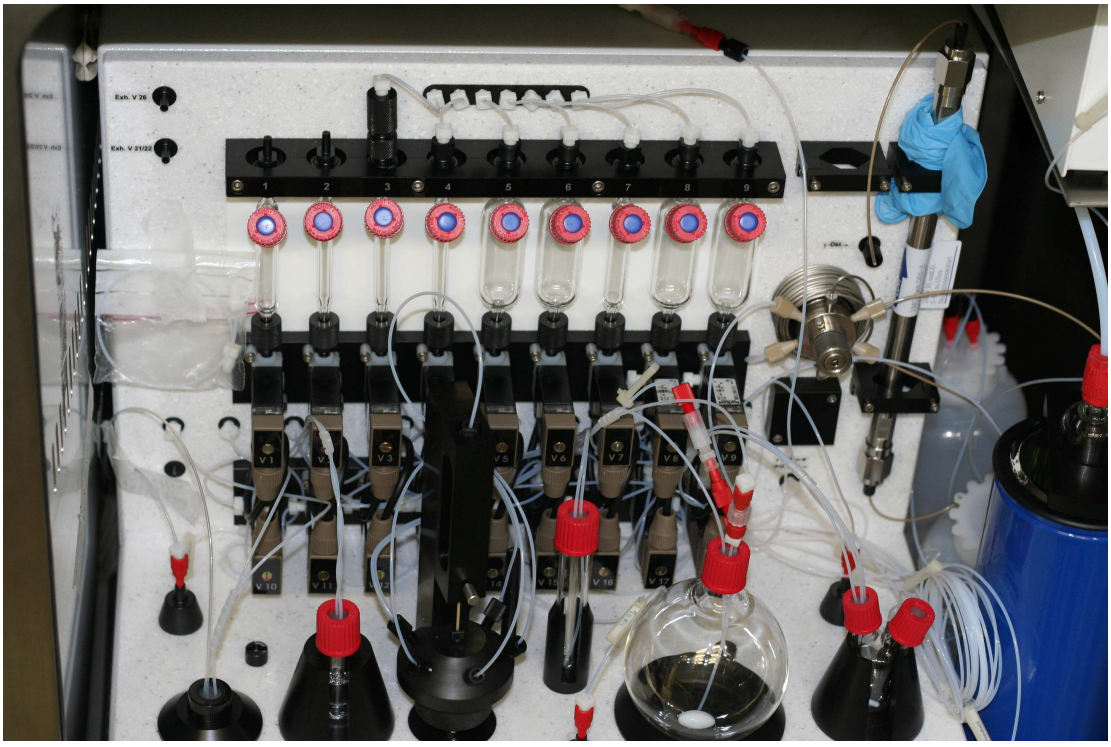


Figure 5: Hardware of an Fx-F-N TRACERlab (G.E.m.s)

In this study, ^{18}FECH was synthesized by two different approaches, following the paths shown in Figure 6. The first method is the two-step reaction already reported in literature (65). The second method is an innovative approach which allows the two reactions to be carried out simultaneously and that was developed by the author of this dissertation (77). Moreover, two different methods for purifying the crude ^{18}FECH solution (preparative HPLC and SPE) were used and compared.

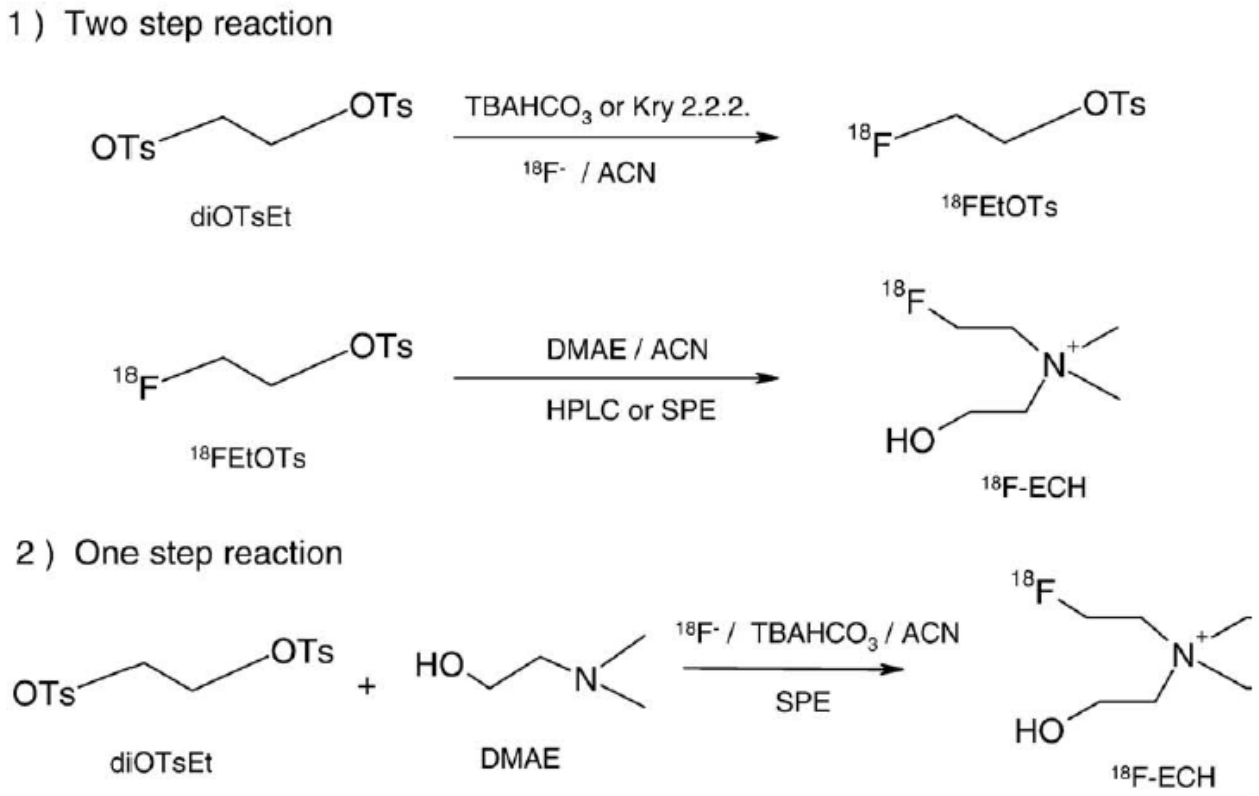


Figure 6: the two approaches for the 2-[^{18}F]-fluoroethycholine synthesis developed on the Fx F-N

3.2.3.1.1 [^{18}F]Fluoride production

Batches of 2035 MBq [18F]fluoride were produced by irradiating [^{18}O]water with a 25 µA current for 5 min. At the end of bombardment, the activity was transferred to the synthesizer and trapped into a light QMA cartridge. [^{18}F] Fluoride was then desorbed from the cartridge by using alternatively a 0.075 M TBA bicarbonate solution (600 µl) or a Kry 2.2.2 (22 mg)/K2CO3 (7 mg) ACN/water solution (50/ 50, v/v, 600 µl) as a phase transfer catalyst and directed into the reactor vessel. The reaction mixture was heated to dryness at 60 ℃ for 7 min and then at 120℃ for 5 min.

3.2.3.1.2. Two-step reaction

To obtain the ^{18}FEtOTs intermediate, a diOTsEt solution in ACN was added to the reaction vessel and heated for 5 min at 80℃. The temperatur e was cooled to 55℃ and ^{18}FEtOTs

was subsequently reacted with a DMAE solution in ACN for 10 min at 100℃. The reaction was performed in the same vessel, without carrying out any purification between the two steps. The effects on the yield of different phase transfer catalysts (TBA bicarbonate or Kry 2.2.2) and different amounts of reagents were assessed. Moreover, the influence of the ACN volume in both steps was considered.

3.2.3.1.3. One-step reaction

The [^{18}F]-fluoride production were carried out as described above, but only the TBA was used as a phase transfer catalyst. The nucleophilic substitution and the alkylation reactions were performed simultaneously by adding a solution of diOTsEt and DMAE dissolved in ACN to the [^{18}F]-fluoride anhydrous residue. The reactor was heated at 95 ℃ for 10 min and then cooled to 50℃. As before, the effects of the reagents amounts and total ACN volume on the synthesis yield were assessed. In fact, when an unique heating step occurred, the reaction volume was directly given by the amount of ACN used for dissolving both the reagent.

3.2.3.1.4. Purification of ^{18}FECH

After the synthesis, the reaction mixture was cooled to 50 ℃ and purified by following two different procedures:

- In the first one, reported by Hara et al. [65], the reactor vessel was rinsed with 4.5 ml water for dissolving the reaction products. The solution was then injected into a semi-preparative HPLC (column: YMC–Pac ODS–A, flow rate: 5 ml/min and eluent: $5 \cdot 10^{-2}$ M H_3PO_4, $1 \cdot 10^{-3}$ M 2-napthalenesulfonic acid). The separation was monitored by a β^+-flow count detector and an ultraviolet (UV) detector in series. A three-way valve was switched in order to collect the selected 2 ml containing the purified fraction into a bulb vessel.

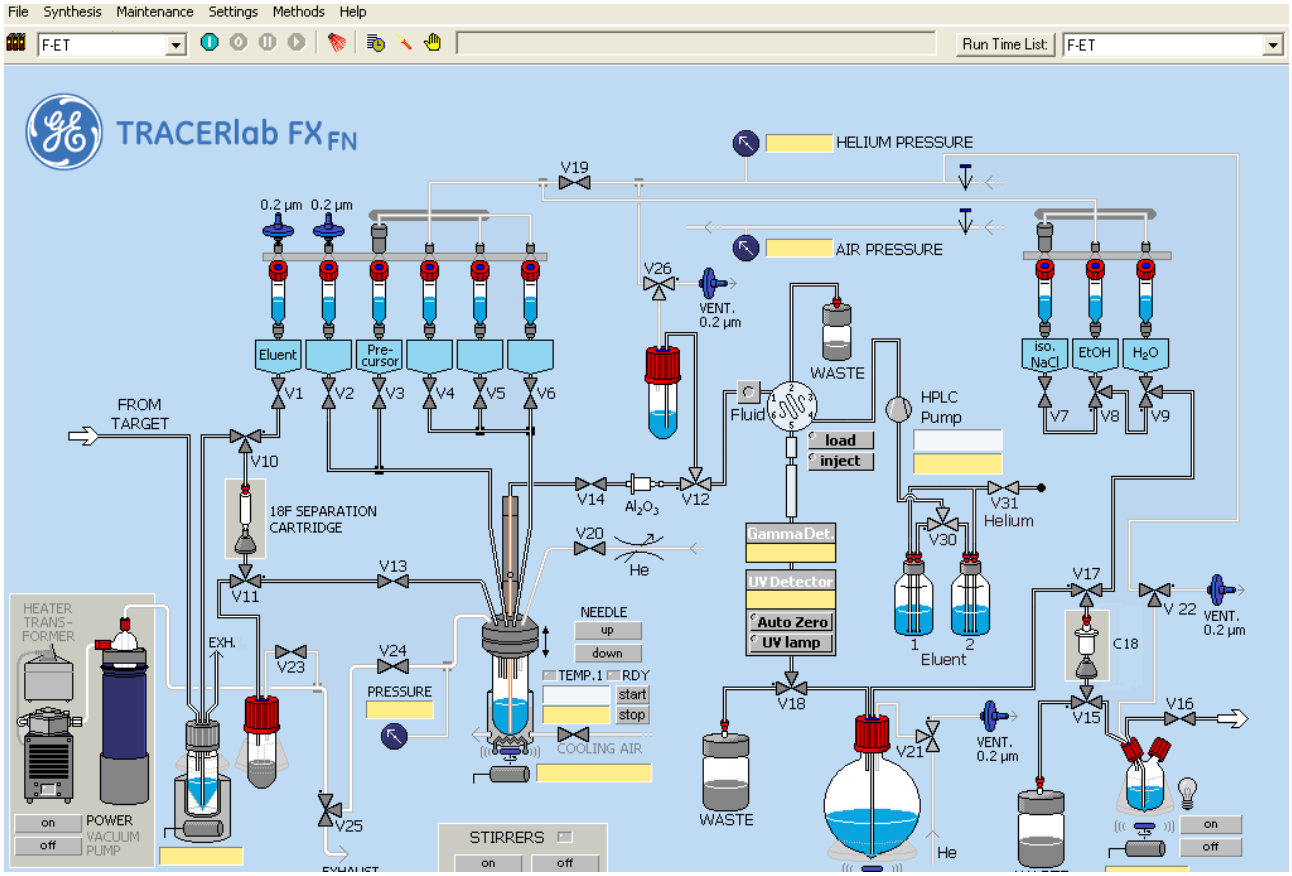


Figure 7: Synoptic of the Fx F-N assembly when a preparative HPLC purification is performed.

- In the second approach, the reaction vessel was rinsed with 10 ml water. The washing solution, containing ^{18}FECH, was filtered through two plus C-18 cartridges connected with two plus QMA cartridges and finally collected into a bulb vessel.

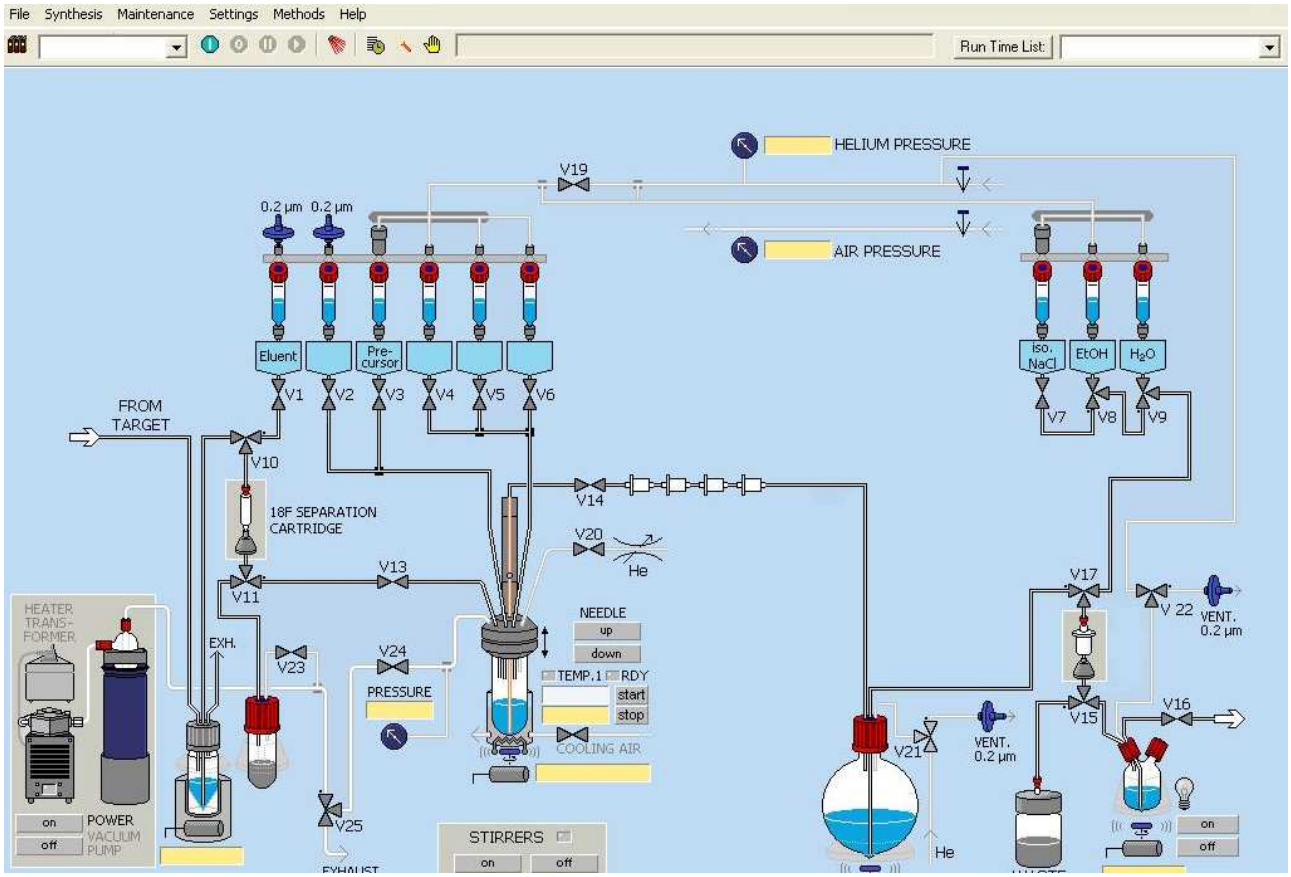


Figure 8: Synoptic of the Fx F-N assembly when a SPE purification is performed.

In both cases, the purified ^{18}FECH was first transferred into a cation exchange cartridge (plus CM), then, the cartridge was washed out with 10 ml water and 5 ml ethanol. Subsequently the ^{18}FECH was eluted with 3 ml 0.9% NaCl water solution and collected in the product vial. Before the final releasing, the preparation was filtered through a Cathivex-GS 0.22-µm sterile filter and diluted with 12 ml 0.9 % NaCl water solution.

Every synthesis and purification were carried out at least in triplicate. Either HPLC and SPE purification were carried out during the two step approach while only the SPE purification was performed in the one-step reaction.

3.2.3.2. Mx TRACERlab:

TRACERlab MX FDG was designed to use a disposable cartridge in the synthesis process, allowing multiple back-to-back production runs for ^{18}F-FDG, the most widely utilized PET tracer. The result is yield around 65 % (n.d.c.) with little maintenance, which is perfect for high-output FDG production as well as in-house production laboratories. Integrated diagnostics allow you to measure and record minute variables, not only ensuring consistent tracer production, but making GMP guidelines easy to meet. The TRACERlab MX FDG module features a Programmable Logic Controller (PLC) with an operation program that facilitates user interaction. The production process itself is designed to optimize production parameters.

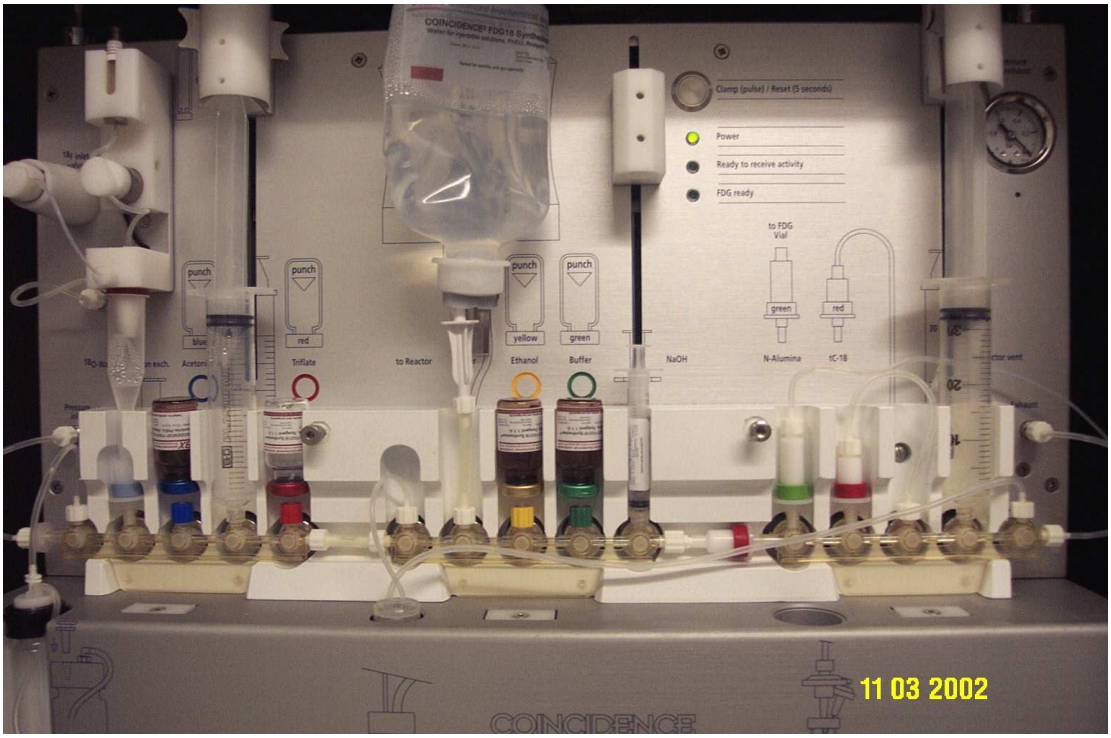


Figure 9: Hardware of an Mx TRACERlab with a disposable ^{18}F-FDG cassettes and reagents assembled (G.E.m.s)

The standard disposable cassette, assembled for synthesizing ^{18}F-FDG (fig 10) was adapted for fully automated ^{18}FECH synthesis. All the cartridges were removed and the orizontal tC-18 cartridge between block 2 and 3 was replaced by a silicon tubing. The 3 ml NaOH solution syringe in position 10 was replaced with a plastic plug as this manifold is not necessary for this sequence. A receiving cone and a QMA cartridge, for recovery and [$^{18}F^-$] elution, respectively, were placed in position 2 and a vial containing 3 ml acetonitrile for azeotropic distillation was put in position 3. The precursors, diOTsEt and DMAE, were both dissolved in 1 ml acetonitrile, 5 minutes before the synthesis start, and the vial was positioned in 5. Two vials containing 5 ml ethanol and 5 ml NaCl 0.9% were placed in Positions 8 and 9, respectively. Plus-CM (one or two) cartridge/s was/were tightly inserted in position 11 and the silicon tubing coming out from the cartridge/s was connected to the final vial. The position 12 was occupied by one plus QMA and one plus C-18 cartridge and was connected to a 25 ml vented collector vials with a short needle (in-let of the solutions), position 13 was connected to the 25 ml vial to through a silicon tubing and a long needle (out-let of the solutions). Other position were connected like as the standard FDG synthesis. All connection were screwed tightly. A visual comparison of the two set-up is shown in figure 10 and 11. The changes between the standard ^{18}F-FDG cassettes and the ^{18}FECH cassettes are resumed in table 1.

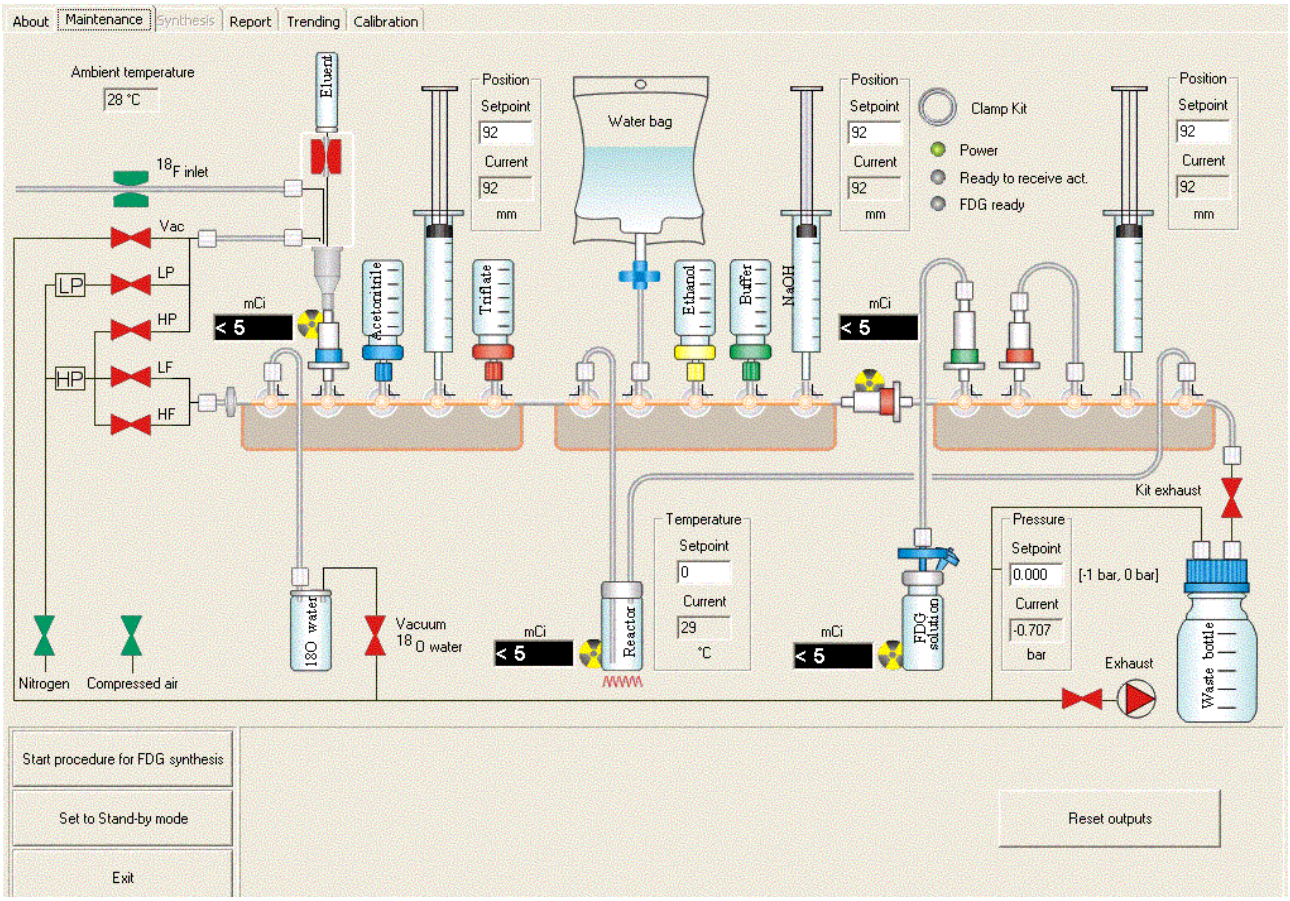


Figure 10: Synoptic of a traditional Mx TRACERlab assembly when ^{18}F-FDG is produced.

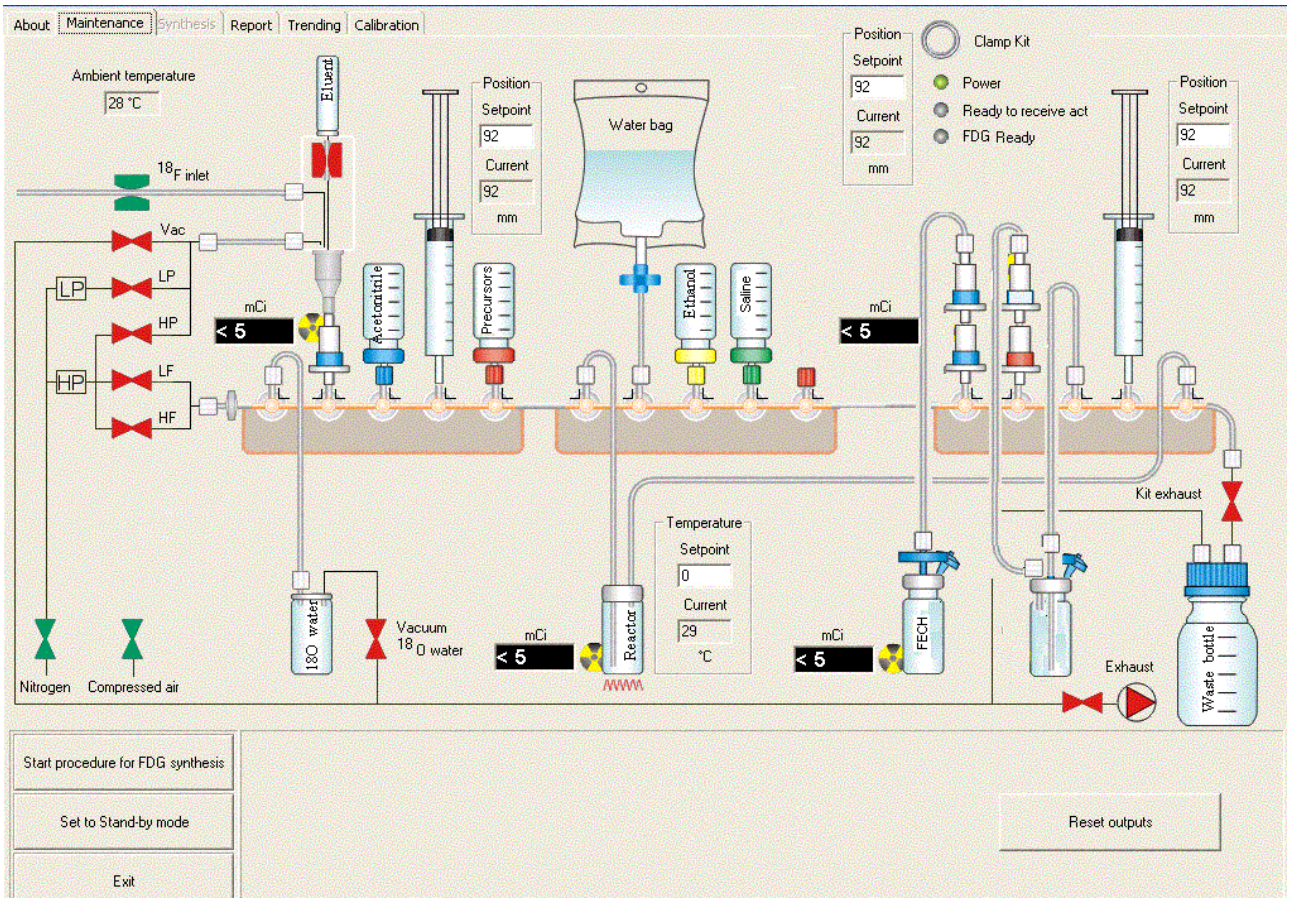


Figure 11: Synoptic of the Mx TRACERlab assembly when ^{18}FECH is produced.

Position	^{18}F-FDG	^{18}FECH
3	ACN (7 ml)	ACN (3 ml)
5	Mannose Triflate	diOTsEt + DMAE in ACN
8	EtOH (5 ml)	EtOH (5 ml)
9	Buffer solution	0.9 % NaCl solution
10	2 N NaOH Syringe	Capped
11	Sep-Pak plus N Allumina	1 or 2 Sep-Pak plus CM
12	Sep-Pak plus tC18	Sep-Paks plus C18 and QMA
13	Connected to plus tC18	Connected to a collect vessel

Table 1: Modification to the standard ^{18}F-FDG cassettes

The standard ^{18}FDG synthesis sequence was modified and fitted for synthesizing ^{18}FECH. In particular, the sequence was adapted for reproducing the one-step method developed for the FX F-N Tracer Lab and described in the precedent paragraph. The traditional two-step method would be difficult, if not impossible, to be transferred to the Mx TRACERlab disposable cassettes due to the limited number of position where the reagents vials can be placed and to the one-way direction of the manifolds.

The first steps of the program were similar to those used for ^{18}F-FDG synthesis but differently from FDG preparation, the step in which the precursors was dissolved in ACN was deleted because they were dissolved by the operator manually before starting the sequence.

The [^{18}F]-fluoride production were carried out as described above and the ^{18}F-Fluoride solution was transferred to the Mx TRACERlab through a PEEF tubing. Then, it was trapped

on a light-QMA cartridge and eluted to the reactor by a mixture of K_2CO_3/ K2.2.2 in a 1:1 solution of acetonitrile/water. The [^{18}F]-fluoride was dried by azeotropic distillation at 95℃ using three successive additions of acetonitrile (0.7 ml). A solution of di(OTs)Et (25 mg, 70 nmol) and 100 µl of DMAE (1.0 mmol) in dry acetonitrile (1 ml) was added to the dried residue. The reactor was then heated at 120℃ for 10 minutes and then tempered to around 50℃ in order to contemporary perform both the addition and N-alkylation reactions. The reaction mixture was purified by passing through a plus C18 cartridge and plus-QMA cartridge in position 12 and collected into the 25 ml vial. The reactor and the cartridges was rinsed with 15 ml of water and the washing was collected into the same 25 ml vial. After the first purification the ^{18}FECH solution was withdrawn with the 30 ml right syringe in position 14 and passed through one or two plus-CM cartridges (position 11). ^{18}FECH was retained in the cartridges while the crude solution was collected in a waste bottle positioned inside the manipulation hood. The collect vial and the cartridges was washed with 5 ml of EtOH and 20 ml of water and ^{18}FECH was then eluted with 5 ml of 0.9% NaCl solution. The ^{18}FECH solution was gathered in the final vessel by manually switching the waste bottle with a sterile vial. The preparation was diluted with 11 ml water. Total synthesis time was about 35 min.

As many different precursors were used in precedent studies (72-75, 78-80) and encouraging results were obtained, the feasibility of this labelling reaction with the Mx TRACERlab using BrOTsEt, diBrEt and diONosEt, was also investigated and whose RCY was compared with RCY using diOTsEt.

Step	Action
A	^{18}F-Fluoride trapping in the QMA cartridge
B	^{18}F-Fluoride elution to the reactor by Kry 2.2.2 mixture
C	Azeotropic evaporation
D	Addition of the precursors solution in the reactor vial
E	Template reaction of the precursors and ^{18}F-Fluoride
F	Cooling of the reaction
G	Purification between sep-pak plus C-18 and QMA
H	Washing the sep-paks and the reactor
I	Trapping the ^{18}FECH in 1 ore 2 Sep-Pak plus CM
J	Washing the CM and the collect vessel with 5 ml EtOH + 10 ml water
K	Elution of the ^{18}FECH with 5 ml 0.9 % NaCl
L	Dilution of the final solution in water

Table 2: ^{18}FECH synthesis sequence

3.2.3.3. Cartridges activation:

All the sep-pak described in any approach of this study were treated as follow: plus QMA cartridges were activated with 10 ml 0.05 M NaOH solution and subsequently rinsed with 10 ml water. Plus C-18 cartridges were washed with 10 ml of ethanol and 10 ml of water. The CM cartridges were activated by using 5 ml 0.9 % NaCl water solution and rinsed with 20 ml water.

The 0.9% NaCl solution was used for eliminating the K^+ counterions from the solid phase of the cartridges. In fact, the presence of K^+ cations in the final solution interferes with the DMAE detection by HPLC (vide infra). In the UHPLC methods this problem was avoided

but the activation was performed in same way to minimize the presence of K^+ in the preparations.

3.2.4. Quality controls.

3.2.4.1. Radiochemical and Chemical Purity:

An HPLC or UHPLC systems were equipped with an Ion Pac CS 14 column (Dionex, Milan, Italy) or a IC-PakTM Cation M/D column (Waters, Milan Italy), respectively, and three different detectors in series were used to assess the chemical as well as the radiochemical purity of the preparations (radiochemical, electrochemical and UV detectors). The analyses were performed with an aqueous solution containing 2 mM H_2SO_4, 2 mM CH_3HSO_3 and 12.5 % (v/v) acetonitrile as eluent, at the flow rate of 1 ml/min for both HPLC or UHPLC system.

The systems were calibrated by injecting not radioactive standard solutions of the most probable by-product and reagent (DMAE, diMMCl, FECH, K^+, o-Tosilate) at varying concentrations (10, 50, 100, 200 ppm) in order to identify the peaks produced by the detectors (Electrochemical and UV). The identification of the ^{18}FECH peak produced by the radio-detector was obtained by co-injecting a not radioactive FECH standard 100 ppm.

As expected the UHPLC system show best separation properties probably due to the best mechanical features.

Radiochemical purity (RCP) was also assessed by TLC using a MeOH/ammonium acetate 1 M solution 50/50 (v/v) as eluent and silica gel plates 60 F254 as stationary phase.

3.2.4.2. Solvents:

The concentration of volatile products such as EtOH and ACN or TBA (when used) and DMAE in the final solution was assessed by GC with an RTX-1301 column and a FID detector.

3.2.4.3. Others:

The sterility and the presence of bacterial endotoxin were tested on different preparations by following the European Pharmacopeia guidelines.

The presence of Kryptofix. 222, when used, was assessed by methods described in the European Pharmacopeia for FDG preparations. As a negative control for the assay, a ^{18}FECH solution prepared by using TBA as phase transfer catalyst was used.

3.2.4.4. Stability of the reagents solution:

When the synthesis was performed with the Mx TRACERlab, the sequence needs the contemporary pre-dissolution of diOtsEt and DMAE in acetonitrile, thus an additional analysis was performed on the reagent mixture in order to check the stability of the solution of the reagent over time. 20 mg of diOTsEt and 0.1 ml of DMAE were dissolved in 1 ml of d-ACN. The solution was stored overnight at RT and then an 1H NMR analysis at 25℃ was performed. Subsequently, the NMR tubing was heated to 80℃ directly inside the NMR magnet chamber and a further analysis was performed. ^{1}H NMR analyses of a 0.1 ml DMAE in 1ml d-ACN solution and of a 20 mg diOTsEt in 1 ml d-ACN solution were also carried out for comparison.

4. RESULTS AND DISCUSSION:

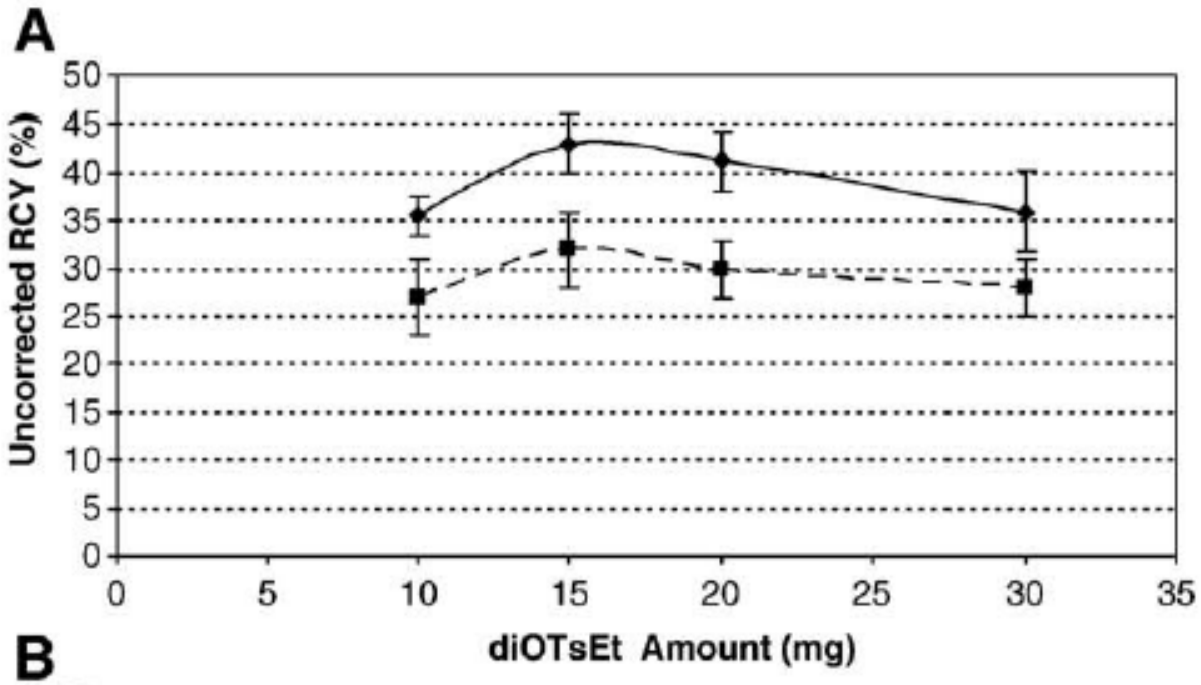
4.1. RADIOPHARMACEUTICAL SYNTHESIS:

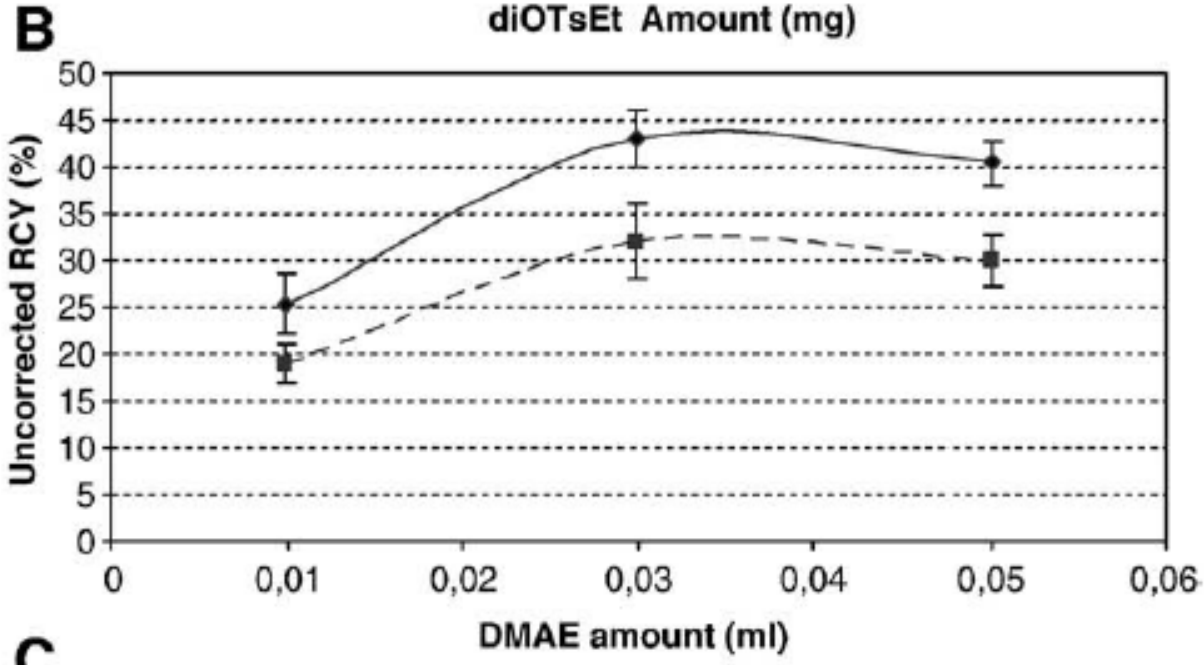
In this dissertation, synthesis of ^{18}F-FECH was carried out by means of different synthesizers and by using varying approaches. In the following sessions, the results of the trials will be summarized and discussed trying to underline the feasibility and reliability of the different approach in term of radiochemical yield but also of radiochemical purity. Final aim of this dissertation is to ensure a ^{18}FECH supply for clinical trials.

4.1.1. Automated synthesis (two step reaction) using FX F-N synthesizer:

When the synthesis was performed by using SPE as purification method, ^{18}FECH was synthesized in the best conditions in 55 min with a RCY of 43±3% or 32±4% (not corrected for decay) depending on if TBA or Kry 2.2.2 were used, respectively. Conversely, when the purification was performed by using a semipreparative HPLC, the synthesis time was 65 min and the yields dropped to 31±3% and 20 ±2%, respectively, in the best conditions.

As shown in Fig. 12, the RCY of the reaction was strictly dependent on the reagents amounts, and the best results were obtained when 15 mg diOTsEt and 0.03 ml DMEA were reacted.


A
Uncorrected RCY (%)
50
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
diOTsEt Amount (mg)


B
Uncorrected RCY (%)
50
45
40
35
30
25
20
15
10
5
0
0
0,01
0,02
0,03
0,04
0,05
0,06
DMAE amount (ml)

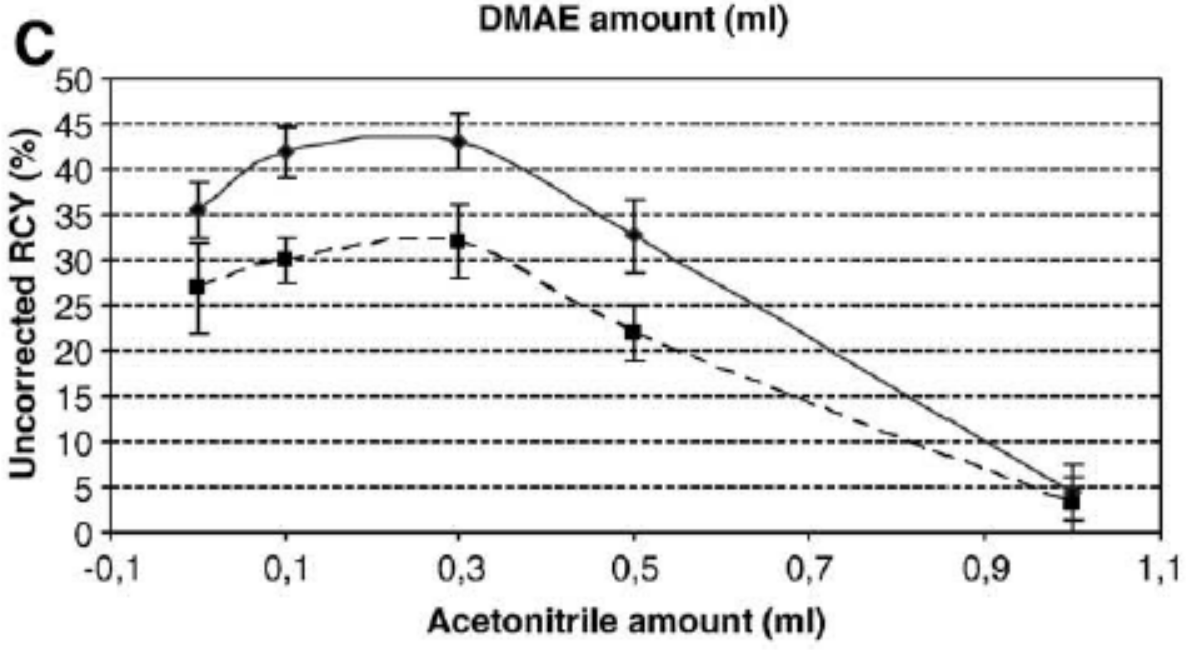

C
Uncorrected RCY (%)
50
45
40
35
30
25
20
15
10
5
0
-0,1
0,1
0,3
0,5
0,7
0,9
1,1
Acetonitrile amount (ml)

Figure 12: (A) Dependence of the ^{18}FECH radiochemical yield on the diOTsEt amount in the two-step reaction (n=3). (B) Dependence of the ^{18}FECH radiochemical yield on the DMAE amount in the two-step reaction (n=3). (C) Dependence of the ^{18}FECH radiochemical yield on the ACN volume dissolving DMAE in the two-step reaction (n=3). (---) Kry 2.2.2 or (—) TBA

While comparable results were achieved when diOTsEt was dissolved in an ACN volume ranging between 0.5 and 1 ml, thus indicating a low dependence of the nucleophilic substitution step on the solvent volume, ACN amount strongly influenced the alkylation step. In fact, as shown in Fig. 12, the RCY dropped drastically when more than 0.4 ml ACN were used to dissolve DMAE, while best conditions were obtained with 0.3 ml. A possible explanation of this findings is that the alkylation step was obtained at higher temperature than the ACN boiling point, so it is necessary to evaporate all the ACN amounts to start the reaction between the labelled precursor (^{18}FOTsEt) previously formed and DMAE. In such case DMAE would be both reagent and solvent of the reaction at the same time. This thesis is supported also to the fact that in other studies (71) the alkylation reaction was performed in neat DMAE without the presence of solvent, in this dissertation the use of ACN was preferred for transferring the DMAE in the synthesizer reactor due to the really low amount of DMAE used.

Also the use of the phase transfer catalysts and different purification methods influenced the RCY remarkably. When Kry 2.2.2 was employed, less than 10% RCY respect to TBA was obtained regardless of the purification method. The possible competition of the amino-polyether ternary amine with the DMAE in the alkylation reaction can reasonable explain the lower RCY.

4.1.2. Automated synthesis (one-step reaction) using FX F-N synthesizer.

When the one-step reaction was adopted, as both the reagent were mixed in the same vial and solved with ACN, the solvent volume greatly influenced the RCY. Namely, a

considerable decrease was observed when the total reaction volume was more than 0.5 ml (Fig. 13) while the best result was obtained when the total reaction volume was 0.4 ml.

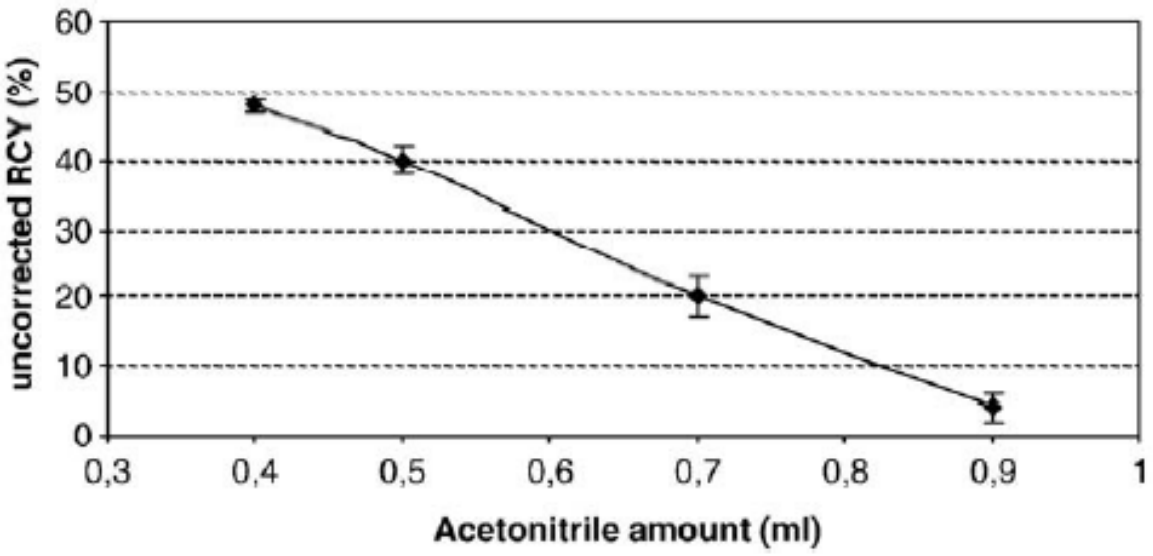


Figure 13. Dependence of the ^{18}FECH radiochemical yield on the total ACN volume in the one-step reaction (n=3).

With respect to the dependence of the reagents amounts on the RCY instead, the one-step reaction showed a similar trend to the two step one (data not shown) but best result was achieved with 12 mg diOTsEt and 0.03 ml DMAE, respectively.

^{18}FECH synthesis was carried out using only TBA as activator and SPE as purification method, as this condition showed the better results in the two-step method trial. A 48±1% RCY in 43 min was obtained (not corrected for decay) demonstrating the reliability and feasibility of the approach.

In fact, despite the presence of a large molar excess of the DMAE in the reaction mixture compared to [^{18}F]-fluoride, the^{18}FEtOTs formation did not seem to be affected by the competition between [^{18}F]-fluoride and DMAE in the nucleophilic substitution. This result leads to the reasonable conclusion that the formation of ^{18}FEtOTs intermediate is

kinetically favoured with respect to other by-products such as diMM^{+} that are derived from a direct reaction between DMAE and di(oTs)Et. In the present study, the simultaneous presence of the reagents in the reaction mixture brought to ^{18}FECH formation anyway.

This findings are in agreement with the results published in (78) were, at these reaction conditions, was found that the displacement of the –OSulfonate (-OTf, -OTs) group by the ^{18}F-fluoride take place also in presence of competing amines.

As summarized in Table 3 where all the approaches described so far are shown; the one-step method showed superior features with respect to the two-step approach in terms of RCY, synthesis time and feasibility, although both of them were comparable in term of chemical and radiochemical purity.

	Approach	Purification by	Duration	Catalyst	RCY % (d.c. / n.d.c.)	
	2 steps	HPLC	65 min.	TBA	47	31
	2 steps	HPLC	65 min.	Kry 2.2.2	30	20
→	2 steps	SPE	55 min.	TBA	60	43
	2 steps	SPE	55 min.	Kry 2.2.2	45	32
→	1 step	SPE	43 min.	TBA	62	48

Table 3: Summary of the approaches performed with the FX F-N Tracer Lab in the best conditions of reagents amounts (15 mg of diOTsEt and 0.03 ml of DMAE)

Moreover, the proof of concept of feasibility of the one-step approach can support the application of this method also to the preparation of other radiopharmaceuticals, such as ^{18}F-Fluoroethyltyrosine (^{18}FET), whose synthesis involves firstly the traditional preparation of the ^{18}F-Fluoro alkylating agent and then, the reaction with a second substrate.

Secondly, this method can more easily be subjected to automation and can more easily be transferred to other synthesizers with minor arrangement, as described hereafter for the Mx TRACERlab. Differently from reported in (80) both the approaches described herein, suggested that the purification of ^{18}FEtOTs intermediate is not mandatory in order to achieve high RCY and RCP, if a sufficient amount of di(OTs)Et was used in the reaction.

4.1.3. Automated synthesis (one-step reaction) with Mx TRACERlab:

It is worth to notice that the Mx TRACERlab platform is not able to support a traditional two-steps approach. This is manly due to the synthesizer and hardware cassettes body where only one position in the first cassette block is available for the insertion of a reagent vial. In the same way, in a traditional Mx TRACERlab is not possible to perform a preparative HPLC purifications of the products. In opposition, the one-step method can be easily performed by applying some modification to the standard ^{18}FDG cassettes and the SPE purification can be used. Thus, the procedure was studied in order to reply the steps followed by the FX F-N Tracer Lab and for this reason an additional 25 ml collect vessel had to be added to the assembly.

Moreover, the Mx TRACERlab approach was tested by using different symmetric or asymmetric O-sulfonates precursors trying to improve the RCY respect to the diOTsEt used in the FX F-N approaches. The results obtained are resumed in table 4.

Precursor	nmol/mg	Solvent	# CM	RCY (n.d.c)
diOTsEt	0.07 / 25	ACN	1	10 +/- 1 %
diOTsEt	0.07 / 25	ACN	2	17 +/- 2 %
diONosEt	0.07 / 30	ACO	2	0 % *
BrOTsEt	0.07 / 20	ACN	2	11 +/- 1 %
diBrEt	0.07 / 13	ACN	2	0 % **

Table 4: Summary of the results obtained using different precursors with the Mx TRACERlab.

* syntheses failed because hot ACO dissolve the cassette body.

** Synthesis failed because all the activity evaporate from the reactor.

As reported, when this approach was applied using the Mx TRACERlab, best results were obtained by using diOtsEt, with a RCY of 17±2 % and 10±1 (not decay corrected) in 35 min, if two or one plus CM were used, respectively. The lower yield compared to the same approach using the FX F-N Tracer Lab can be addressed to the following reasons: i) FX F-N use a fixed glassy carbon reactor with a conical bottom and with a deep assessment of temperature and pressure. On the other hand, the Mx TRACERlab owns a disposable glass reactor with a flat bottom and without the possibility to low the temperature of the reaction mixture if not by switching of the heater and waiting for some minutes ii) liquid movement through the Mx TRACERlab cassettes is dependent by the direction of the manifolds and all the manifold (excepted position 2) are two-way. This imply that some transferring of the liquid (reagent or product) have to follow longer and more complicated path. iii) in some steps, the liquid flow through the cassettes, has to be regulated by two 30 ml syringes withdrawn or pushing. In this cases the flow is quite high a this can cause some product loss during the SPE purification steps with CM cartridge/s. Anyway, the RCY

obtained using two plus-CM cartridge is comparable to that obtained in the preparation of ^{18}FMCH as reported in (81) with the same synthesizer.

Regarding the other O-Sulfonates precursors, a lower RCY (11 %) was obtained by using BrOTsEt instead of the symmetrical diOTsEt while complete failure was obtained with diNosEt and diBrEt. For BrOTsEt, we suppose that the reaction temperature should be firstly decreased in order to allow the substitution of the –OTs group with formation of ^{18}FBrEt but avoiding the evaporation of the intermediate, and then increased for allowing the substitution of the –Br atom.

Concerning diONsEt, we found that it is not soluble in ACN so the reaction has to be performed in ACO. However hot ACO is not suitable for the cassettes body and it was not possible to extract the reaction mixture from the reactor.

When diBrEt was used, we notice a complete loss of the radioactivity during the reaction probably due to the high volatility of the precursor and of the ^{18}F-Fluorinated intermediate. Also in this case, improved reaction conditions should be found and set.

It is worth to say that, using the Mx TRACERlab, the choice of the correct amounts of reagents (O-Sulfonate precursors, DMAE and ACN) is critical, above all for avoiding the clogging of the cassettes tubing and cartridge due to the precipitation of un-reacted O-sulfonates when the reaction is diluted with water and the reactor is rinsed.

4.2. QUALITY CONTROLS:

The synthesis of ^{18}FECH is a reaction involving complex interaction between three precursors such as [^{18}F]-Fluoride, diOTsEt and DMAE, as well as ancillary substances such as solvent (ACN) and phase transfer catalysts (TBA and Kry 2.2.2).

It is worth to notice that DMAE has two possible groups that can act as nucleophilic agents in the reaction with diOtsEt precursor or with ^{18}FOTsEt intermediate synthon (namely the amino and the hydroxyl group), this fact may potentially bring to the formation of different

and unexpected by-products both radioactive and un-radioactive. In addition, the presence of un-reacted ^{18}F-Fluoride and of ^{18}FOtsEt has to be assessed as radioactive contaminants. In figure 14 are resumed the radioactive products that may form during this reaction.

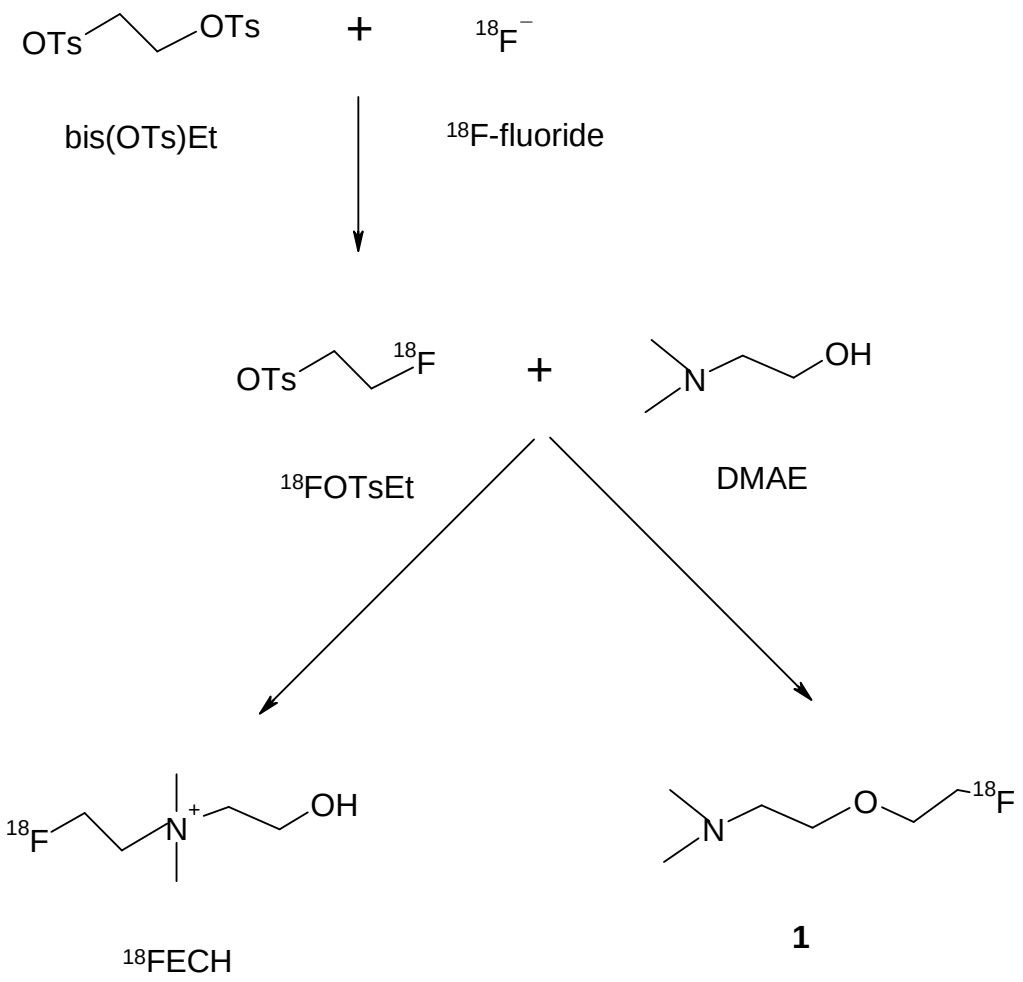


Figure 14: Potential ^{18}F-labelled products formed by reacting diOTSEt, ^{18}F-fluoride and DMAE.

Furthermore, due to the high excess of cold precursors compared to ^{18}F-Fluoride, double reactions between the precursors themselves have also to be taken in account and these kind of reactions would bring to not radioactive by-products. All these considerations may result in the formation of varying by-products and also to the presence of partially reacted precursors, such as O-Tosylated products. In figure 15 all the possible theoretical

reactions between precursors are resumed and the possible not radioactive products are shown.

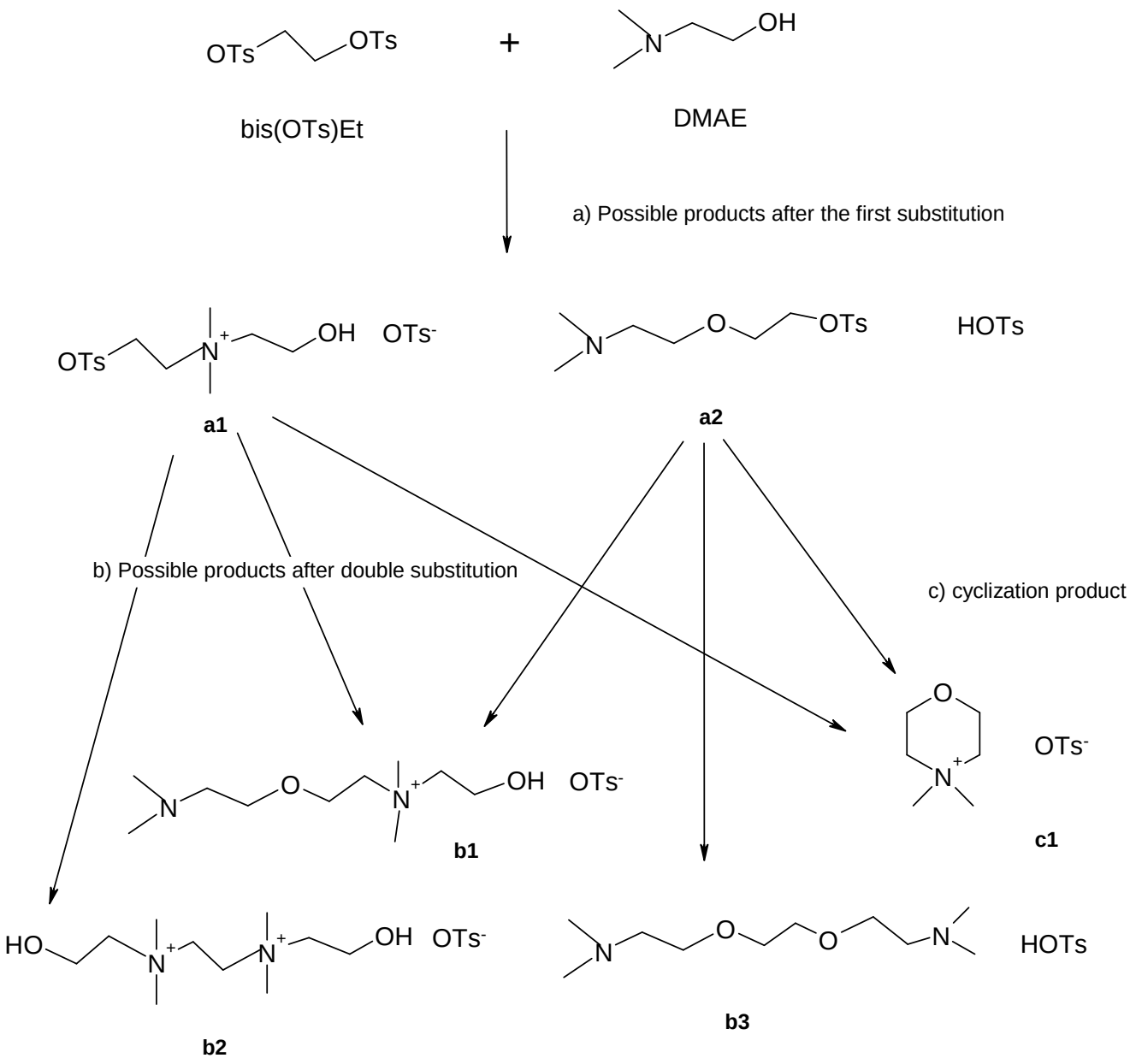


Figure 15: Potential not radioactive by-product formed by reacting diOTsEt and DMAE

For this reasons and in spite of the purification methods of choice, reliable quality controls must be designed and accomplished in order to assess the presence of all the possible by-products. In this study, three different chromatographic systems were adopted in order to

analyze the reaction mixtures and the final product. Due to the nature of the varying substances detected, both the HPLC and UHPLC systems are equipped with three detector in series.

4.2.1. Comparison between the synthesis and purification methods*:*

In table 5 are resumed the results obtained in the quality controls of the preparations performed with the two different approaches (one or two-step), the two purification methods (HPLC and SPE) and the two synthesizers (FX F-N and Mx).

Approach / Synthesizer	Purification	Conductometer				UV		Radio
		K^+	DMAE	FECH	DMM^+	UV by-prod	OTs^-	RCP %
2 steps / FX F-N	HPLC	10	ND	38	106	ND	ND	99
2 steps / FX F-N	SPE	11	ND	38	107	ND	ND	99
1 steps / FX F-N	SPE	10	ND	37	95	ND	ND	99
1 steps / Mx	SPE	280	230	54	70	present	ND	99

Table 5: Summary of the quality controls performed by UPLC on the preparation carried out with the two synthesizers, the two approaches and the two purification methods.

ND: under limit of detection

4.2.2. Radiochemical purity:

[^{18}F]-Fluoride, ^{18}FEtOTs and ^{18}FECH were evaluated by means of radiometric detection and, in the HPLC system, have a retention time of 2.5, 5.6 and 8.0 min, respectively.

In UHPLC system, a rapid and better separation could be obtained and the following retention times were found: [^{18}F]-Fluoride = 2.0, ^{18}FEtOTs = 3.2 and ^{18}FECH = 4.05.

The identity of ^{18}FECH was verified on conductimetric detector by injecting the cold FECH standard as shown in figure 16.

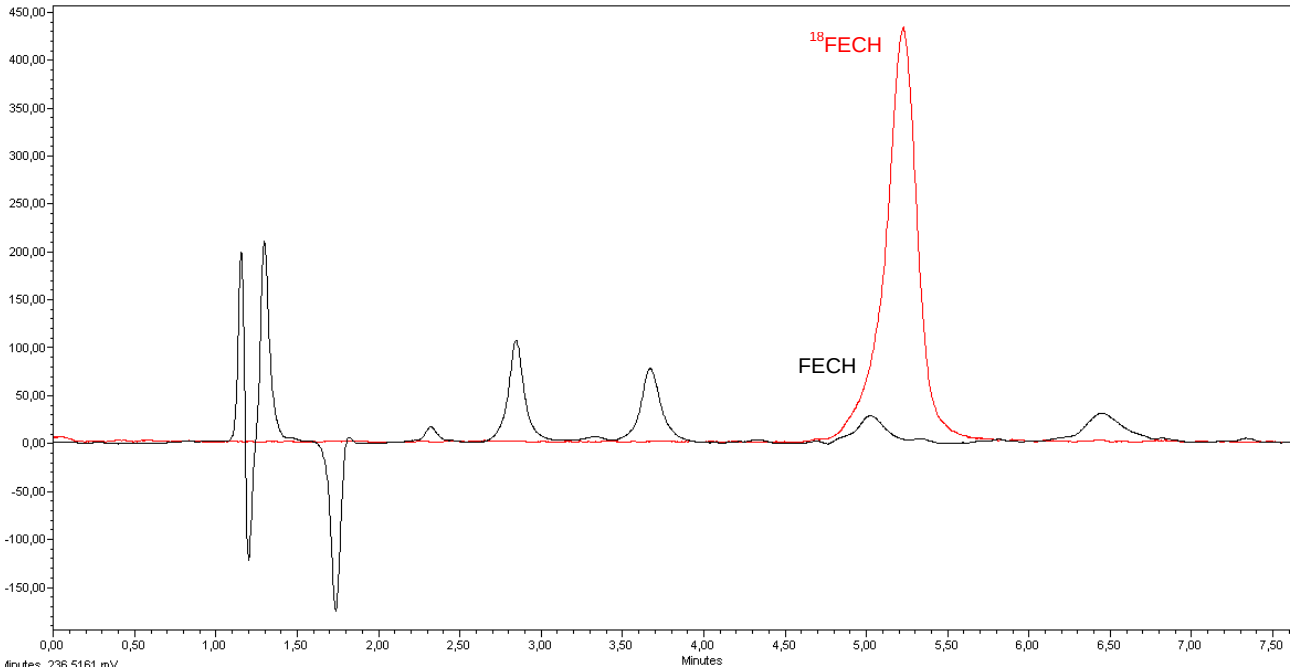


Figure 16: UPLC Chromatograms of a ^{18}FECH preparation on the radiometric detector (red) and a 100 ppm standard of cold-FECH on conductometer detector (black).

In figure 17 is shown a typical chromatogram obtained by injecting a bulk solution just after the SPE purification through the C-18 and QMA cartridge or a preparative HPLC purification, in spite of the approach used (one-step or two-steps) and in spite of the synthesizer used (FX F-N or Mx TRACERlab). In all the case the radiochemical purity is comparable and it is evident that it is not sufficient for an injectable product.

On other hands, as shown by the comparison between figure 17 and figure 18, the last filtration through the CM cartridge/s have a strong influence on the radiochemical purity and can be considered the most important step of the purification.

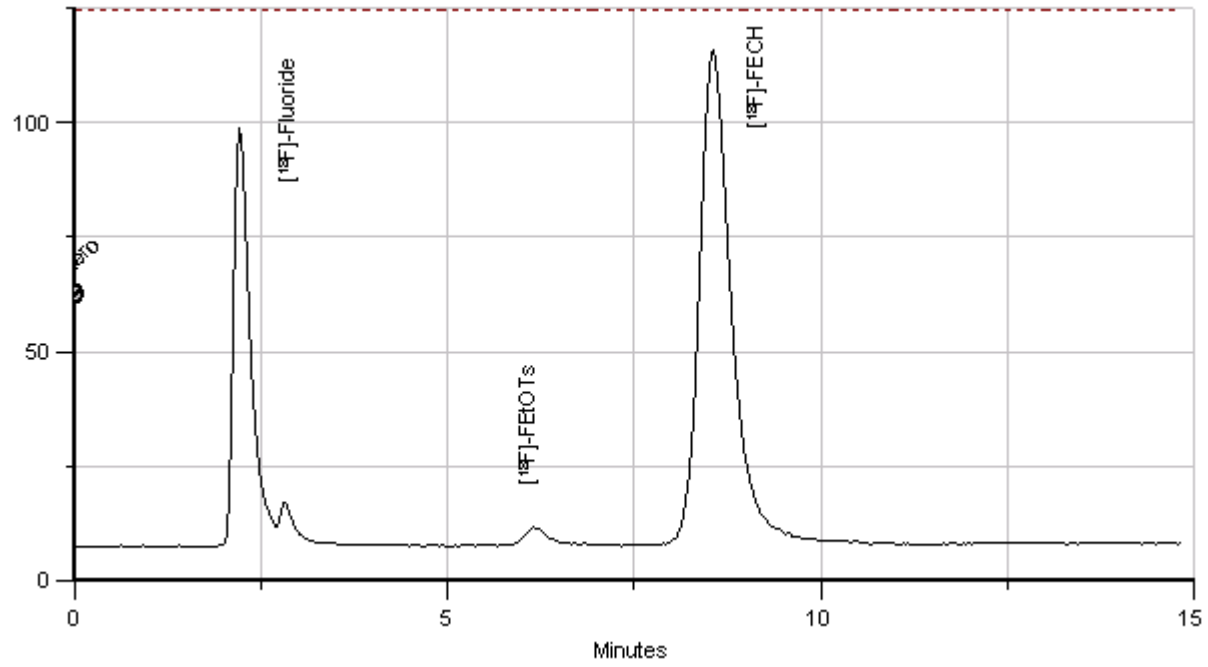


Figure 17 : Typical chromatogram (radiodetector) of the crude mixture before the CM purification in spite of the approach or of the synthesizer used.

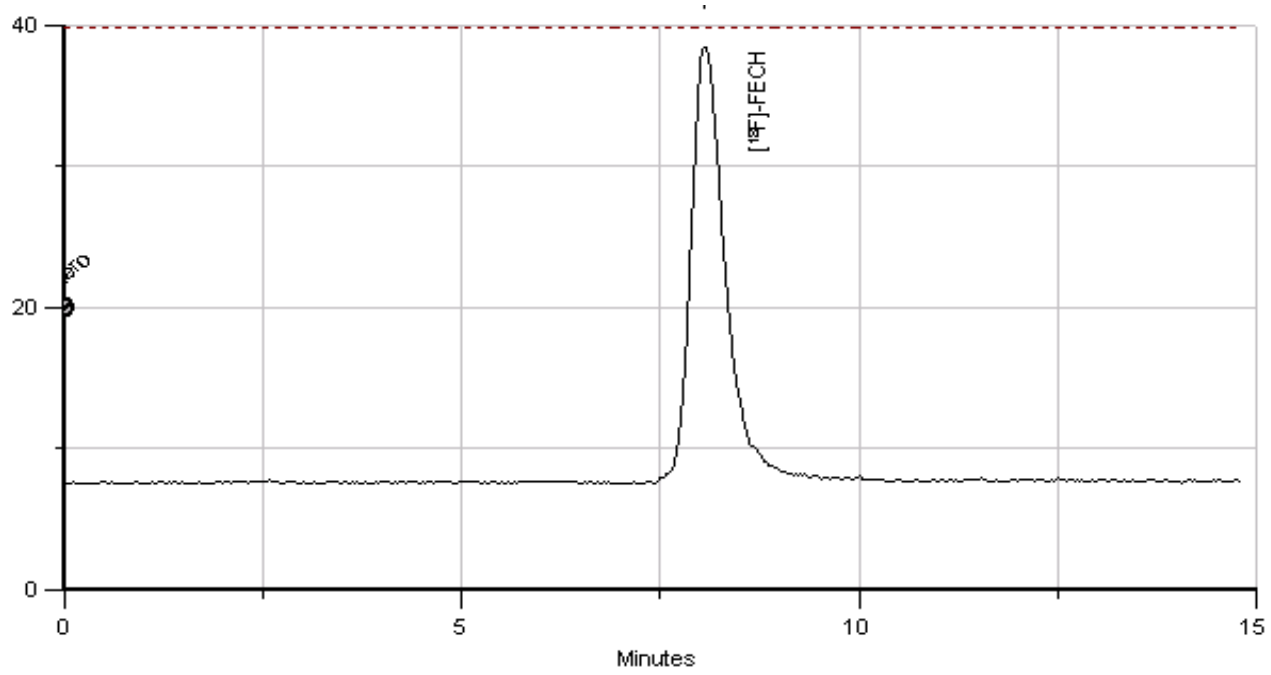


Figure 18 : Typical chromatogram (radiodetector) of the final solutions after the CM purification in spite of the approach or of the synthesizer used.

Regardless of the different synthetic approaches, the radiochemical purities of the ^{18}FECH solutions at the end of synthesis were comparable and were greater than 99 %. As an example, the comparison between the chromatograms obtained by a two steps/HPLC purification/FX F-N and a one step/SPE/Mx TRACERlab approach can be observed in figure 19.

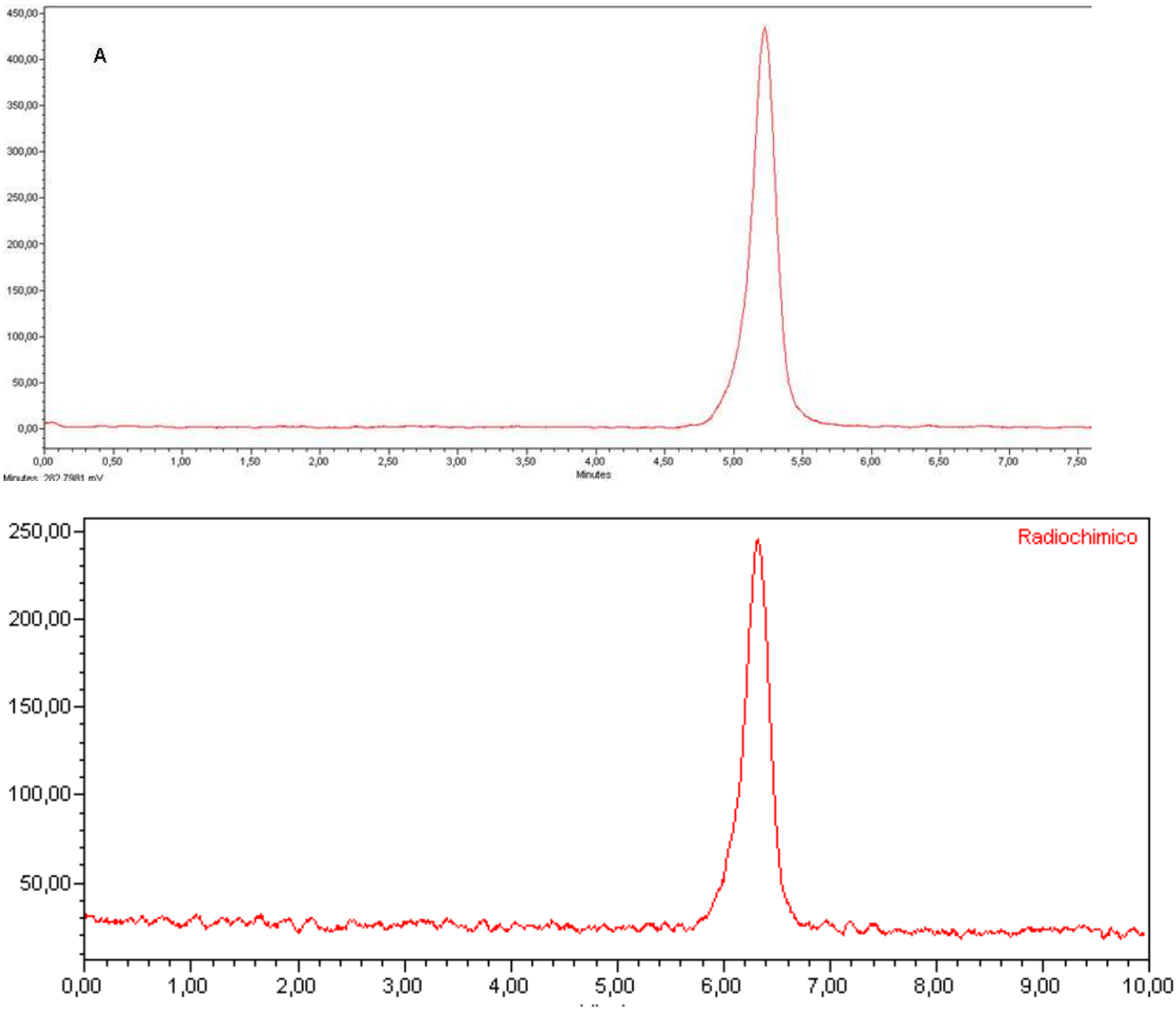


Figure 19: Comparison between two typical chromatograms (radio-detector) obtained from a ^{18}FECH preparation by a two steps/HPLC purification/FX F-N approach (A) and a one step/SPE/Mx TRACERlab approach (B).

Radiochemical purity (RCP) was also assessed by radio-TLC and the following retention times were obtained: [^{18}F]-fluoride: Rf=0.1 ^{18}FECH Rf=0.8, respectively.

4.2.3. Chemical purity:

In spite of the theoretic possibility to form the by-product illustrated in figure 15, the setting of the HPLC and UPLC analyses and the identification of the chromatographic peak are dependent to the commercial availability of the standards. Products **a1, a2, b1, b2, b3** in figure 15 are not commercially available so it was not be possible to determine their presence in the final solution. On the other hand, a calibration curve was fitted for the following standards: K^+, DMAE, FECH and dMM^+ (product **c1** in figure 15) by using the conductimetric detector.

HPLC system is able to separate Na^+, DMAE and $diMM^+$ but K^+ co-elutes with DMAE and the relative quantification of these products is difficult. DMAE (and K^+) and $diMM^+$ have a retention times (Rt) of 6.1 and 10.2 min, respectively.

In the UHPLC methods all the product are well separated and quantified. In the UHPLC system the following Rt were found: K^+ = 2.5 min. DMAE = 3.1 min. cold-FECH = 4.2 min. $diMM^+$ = 5.4 min. The chromatogram of the standard and the calibration curves are shown in figure 20 and 21, respectively.

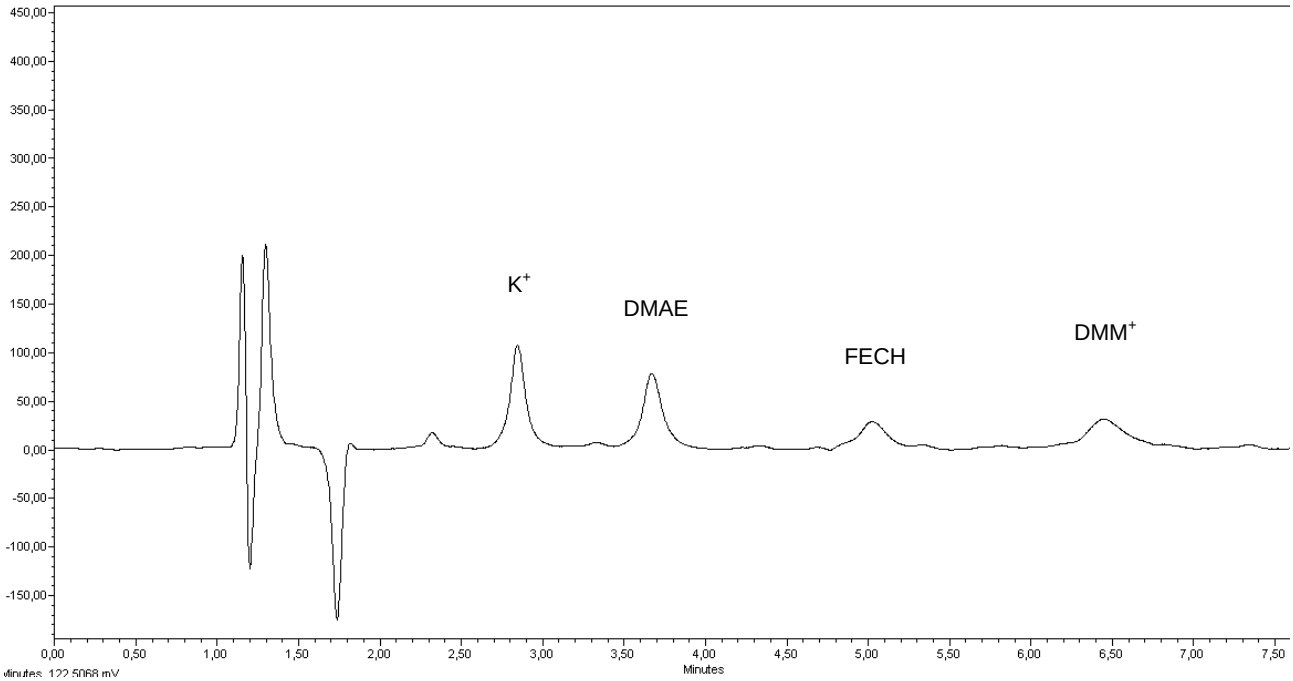


Figure 20: UPLC Chromatogram on the conductometer detector of the 100 ppm standard solutions

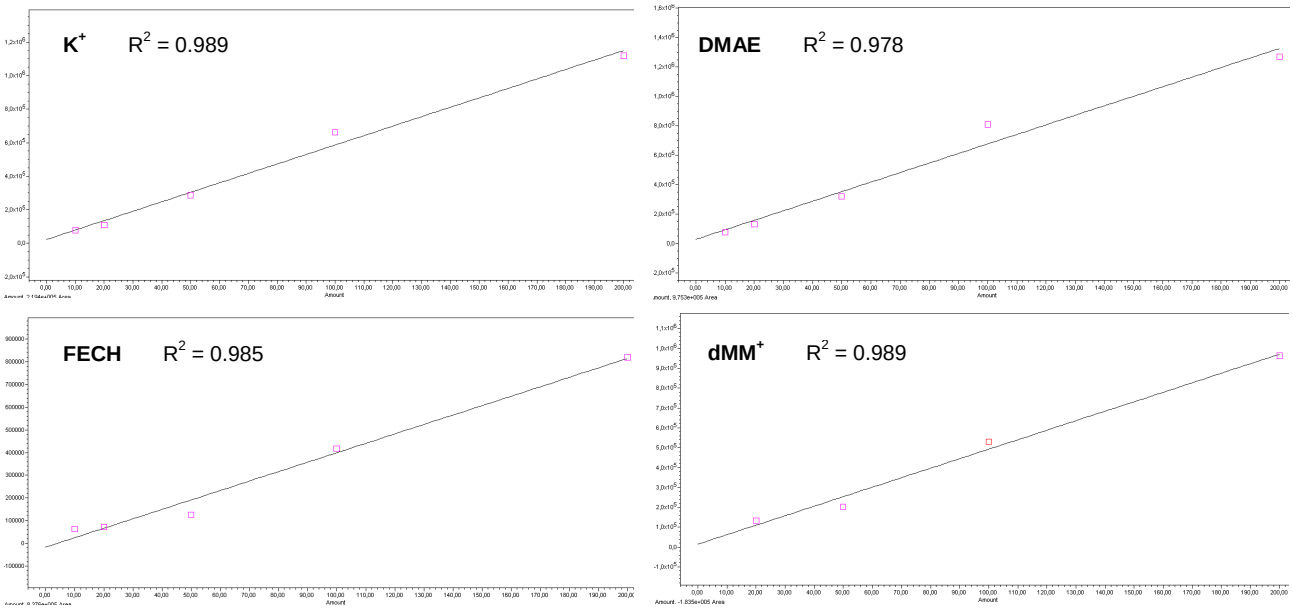


Figure 21: Calibration curves and correlation coefficients for K^+, DMAE, FECH and dMM^+ standard soltutions.

As reported in table 5, the chemical purity of the final solution is quite influenced by the approach used. In spite of the synthetic approach (one-step or two-steps) or of the synthesizer used for the synthesis, the formation of $diMM^+$, a cationic by-product formed

by the direct reaction of diOTsEt with DMAE followed by an intra-molecular cyclization, was found. The proposed mechanism of dMM^+ formation is showed in figure 22.

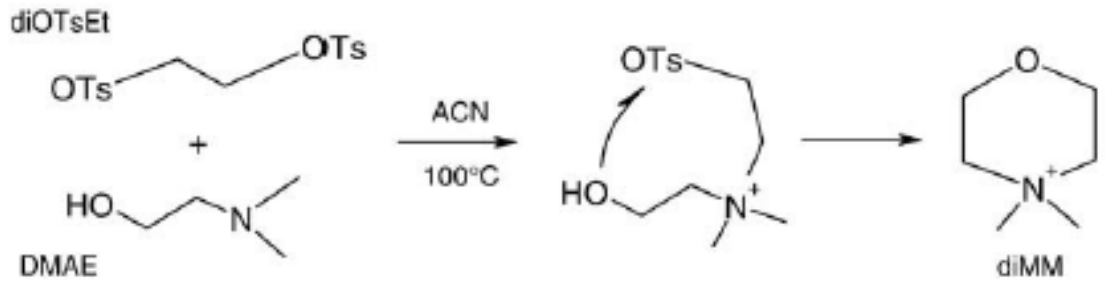


Figure 22. Proposed mechanism of dMM^+ formation.

To the best of our knowledge, the presence of $diMM^+$ in ^{18}FECH solutions was not described so far and its identity was confirmed by comparison with the commercial standard in the same HPLC or UHPLC conditions. As far as we know, toxicological data about $diMM^+$ in human beings were not reported so far, since the only studies published are related to plants growth. In the set of reactions performed in this dissertation, $diMM^+$ was detected in a concentration ranging between 41 and 179 mg/l regardless of the purification method. As reported in table 5 the mean concentration found when the Mx TRACERlab was used is lower than the one found in the other approaches. It is difficult to give a reason of this behaviour but it is supposed that the presence of two CM cartridge positively influence the retention of this compound, blocking the dMM^+ in the CM and thus limiting its amount in the final solution.

Differently from dMM^+, the amount of DMAE and K^+ is really low (under limit of detection and ca. 10 ppm, respectively) when the syntheses are performed with the FX F-N tracer lab, in spite of the purification method and approach. Indeed, when the Mx TRACERlab is used, the DMAE and K^+ amount rise to 230 and 280 ppm, respectively. These results may

indicate that the presence of the two CM cartridge has a negative impact on the elimination of this two substances from the final solution.

The toxicity of DMAE is not especially concerning since its use is documented as an active substance in dermal creams and treatment of mental impairment (71). However, increasing amounts of DMAE were showed to reduce the uptake of ^{18}FECH in prostate cancer cells in vitro (82,83), even if its inhibitory effect has not been yet clarified (60). Therefore, in order to avoid any physiological competition, the DMAE concentration in the final ^{18}FECH radiopharmaceutical solution should be limited.

As reported before, if K^+ cation is present in the final solution, the presence of DMAE was not appraisable in the HPLC method, as DMAE co-elutes with K^+ cations. The source of K^+ is normally the Kry 2.2.2./K_2CO_3 solution when it is used as transfer phase catalyst but it was also found the presence of a large amount of K^+ in the un-treated CM cartridges. The issue was solved by varying approach that allow the perfect separation and determination of DMAE amount:

i) differently from reported in other study (81), plus CM cartridges were activated with 0.9% NaCl water solution. We experienced that the use of NaCl both decrease the presence of K^+ inside the filters due to the K^+ substitution with Na^+ and positively acts on the ^{18}FECH retention in the CM cartridge, increasing the RCY during the synthesis.

ii) The UHPLC methods reported before, was developed. It is able to perfectly separate and give a quantification of DMAE and K^+

iii) DMAE amount was also checked with GC analysis (vide infra)

In our opinion, the possible co-elution of this two substances could explain the high variability in the DMAE concentrations in the synthesis of [^{18}F]-fluorinated choline

analogues reported so far (65, 71, 72, 81, 84). In all this cases DMAE amount was assessed by HPLC analysis only.

A comparison between the chromatograms obtained by a two steps/HPLC purification/FX F-N and a one step/SPE/Mx TRACERlab approach can be observed in figure 23.

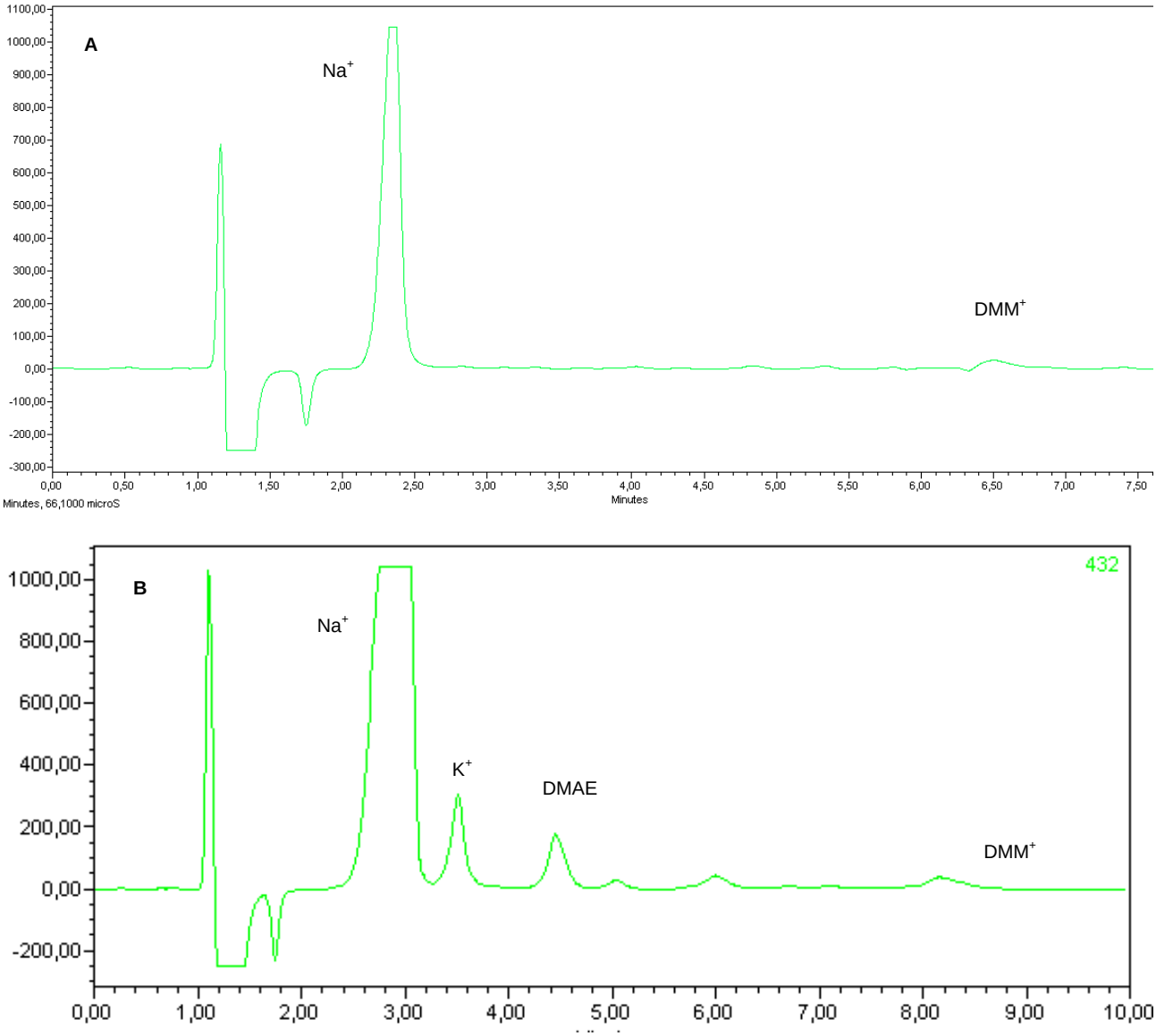


Figure 23: Comparison between two typical chromatograms (conductimeter-detector) obtained from a ^{18}FECH preparation by a two steps/HPLC purification/FX F-N approach (A) and a one step/SPE/Mx TRACERlab approach (B).

The presence of any UV-absorbing impurities, such as free OTs^- anions and tosylated by-products, was proved by UV detection at 206 nm. With the exception of the synthesis

performed with Mx TRACERlab where the presence of tosylated product were detected, no UV-absorbing products were found. A comparison between the chromatograms obtained by a two steps/HPLC purification/FX F-N and a one step/SPE/Mx TRACERlab approach can be observed in figure 24.

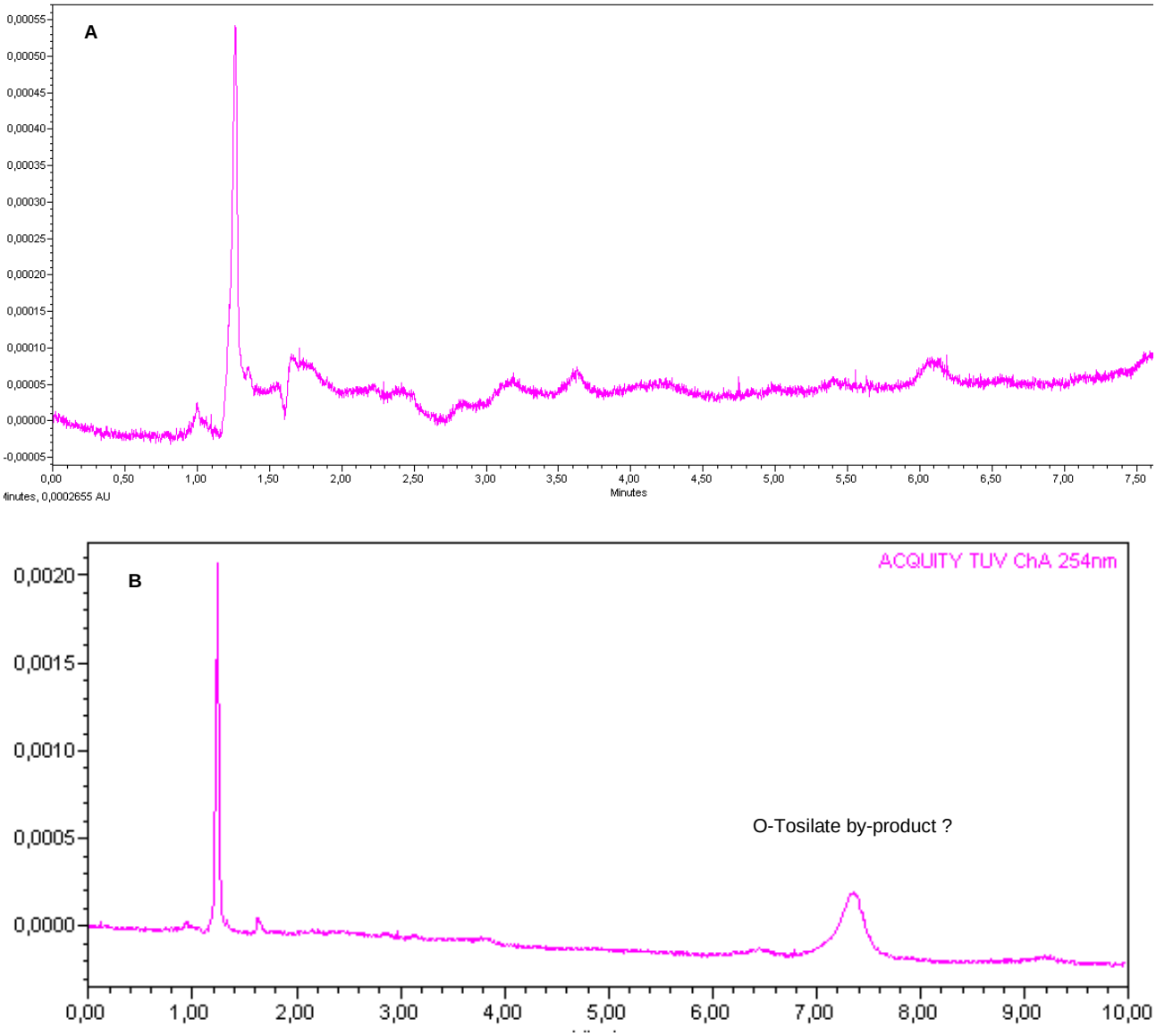


Figure 24: Comparison between two typical chromatograms (UV detector) obtained from a ^{18}FECH preparation by a two steps/HPLC purification/FX F-N approach (A) and a one step/SPE/Mx TRACERlab approach (B).

4.2.4. Solvents:

During the experiments, DMAE amount was also evaluated by means of GC analysis and the experimental setup for detecting DMAE together with TBA, ACN and EtOH in an unique run was developed. The mean concentrations of the latter three reagents were 115 mg/L, 192 mg/L and 117 mg/l, respectively; while the concentration of DMAE was around 0.5 mg/l. In general, a good agreement between the results obtained by UHPLC and GC analyses was accomplished. EtOH, ACN, TBA (when used) and DMAE in the final solutions exhibit the following retention times: Rt=2.6, 3.1, 4.5 and 6.1 min, respectively.

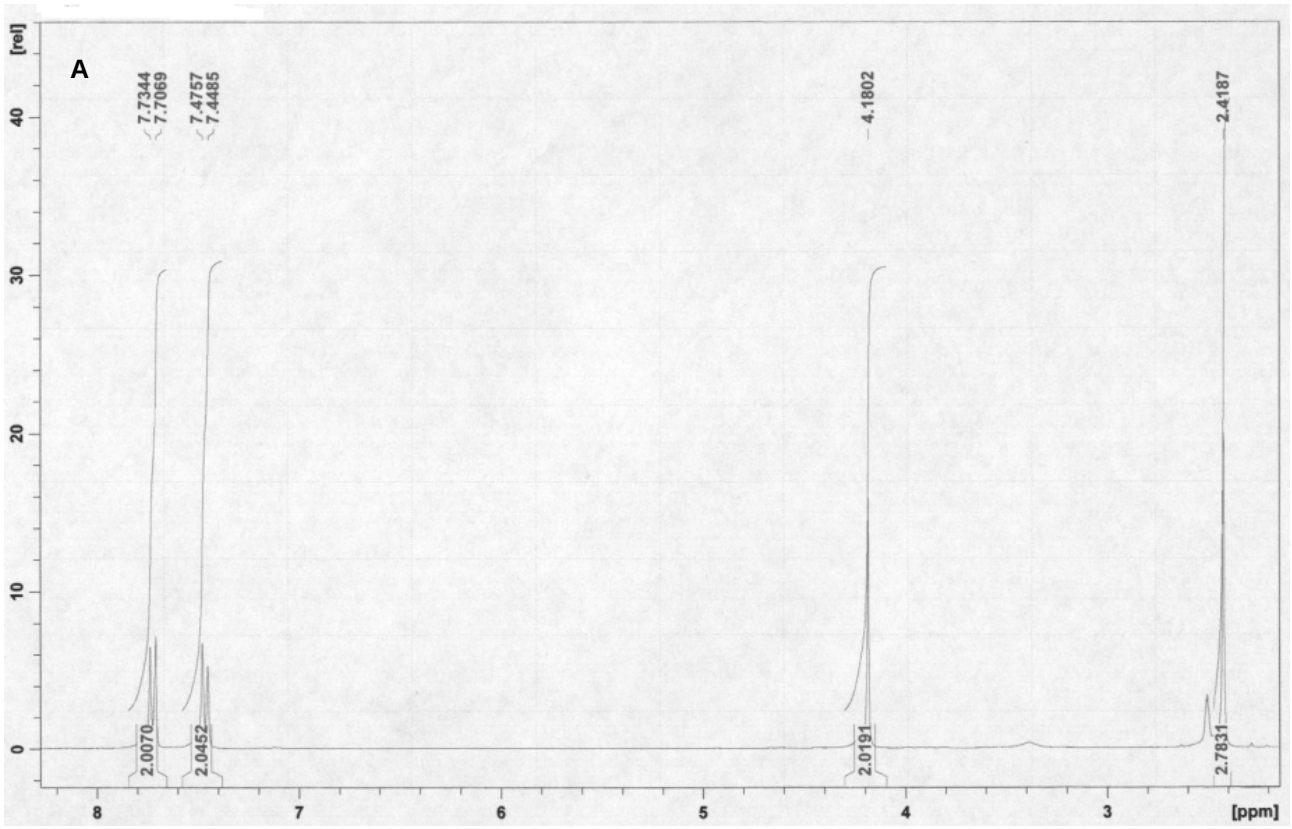
4.2.5. Others:

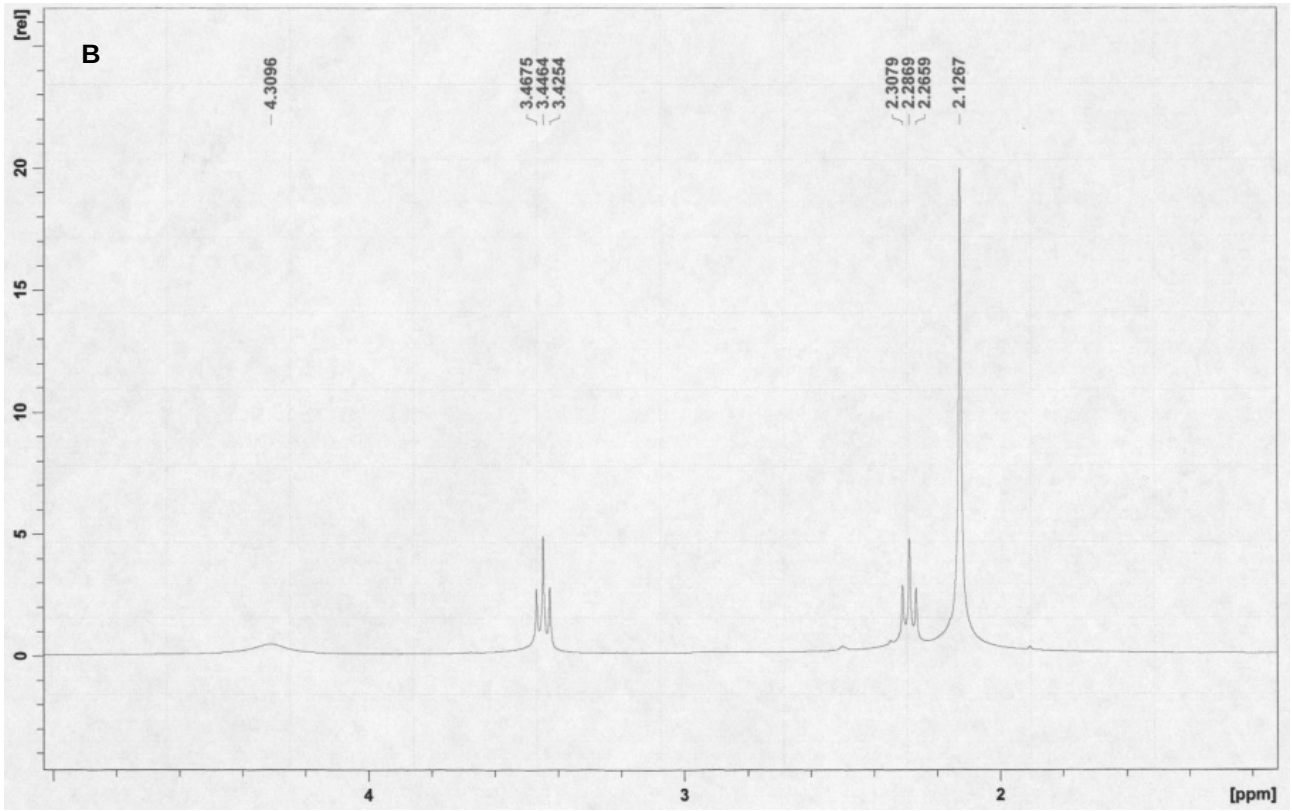
Both the ^{18}FECH preparation synthesized by using TBA or Kry 2.2.2. showed a visible spot when tested with the colorimetric method described in (85) and reported in Pharmacopeia for ^{18}F-FDG preparation. Probably, due to the presence of DMAE as an impurity in the final solutions, it was not possible to evaluate the concentration of Kry 2.2.2 by means of the colorimetric spot test reported in (85). This method appears not specific for Kry 2.2.2 but it is sensitive to any tertiary amine present in solution and gives false positive results if used for testing ^{18}FECH preparations.

All the tested samples were sterile and bacterial endotoxin free (< 0.5 EU/ml).

4.2.6. Stability of the reagents solution:

The feasibility of the ^{18}FECH synthesis with Mx Tacer Lab is dependent on the stability of the reagent precursors in a ACN solution as diOTsEt and DMAE were pre-dissolved into the vial in position 5 of the cassette. The stability of a ACN solution containing both the diOTsEt and DMAE reagents was assessed by ^{1}H NMR at two different temperature (25℃ and 80℃). The spectra of the mixture were compared with the single spectra of the two precursors. All ^{1}H NMR spectra are gathered in figure 25.


A
[rel]
7.7344
7.7069
7.4757
7.4485
4.1802
2.4187
2.0070
2.0452
2.0191
2.7831
[ppm]


B
[rel]
4.3096
3.4675
3.4464
3.4254
2.3079
2.2869
2.2659
2.1267
[ppm]

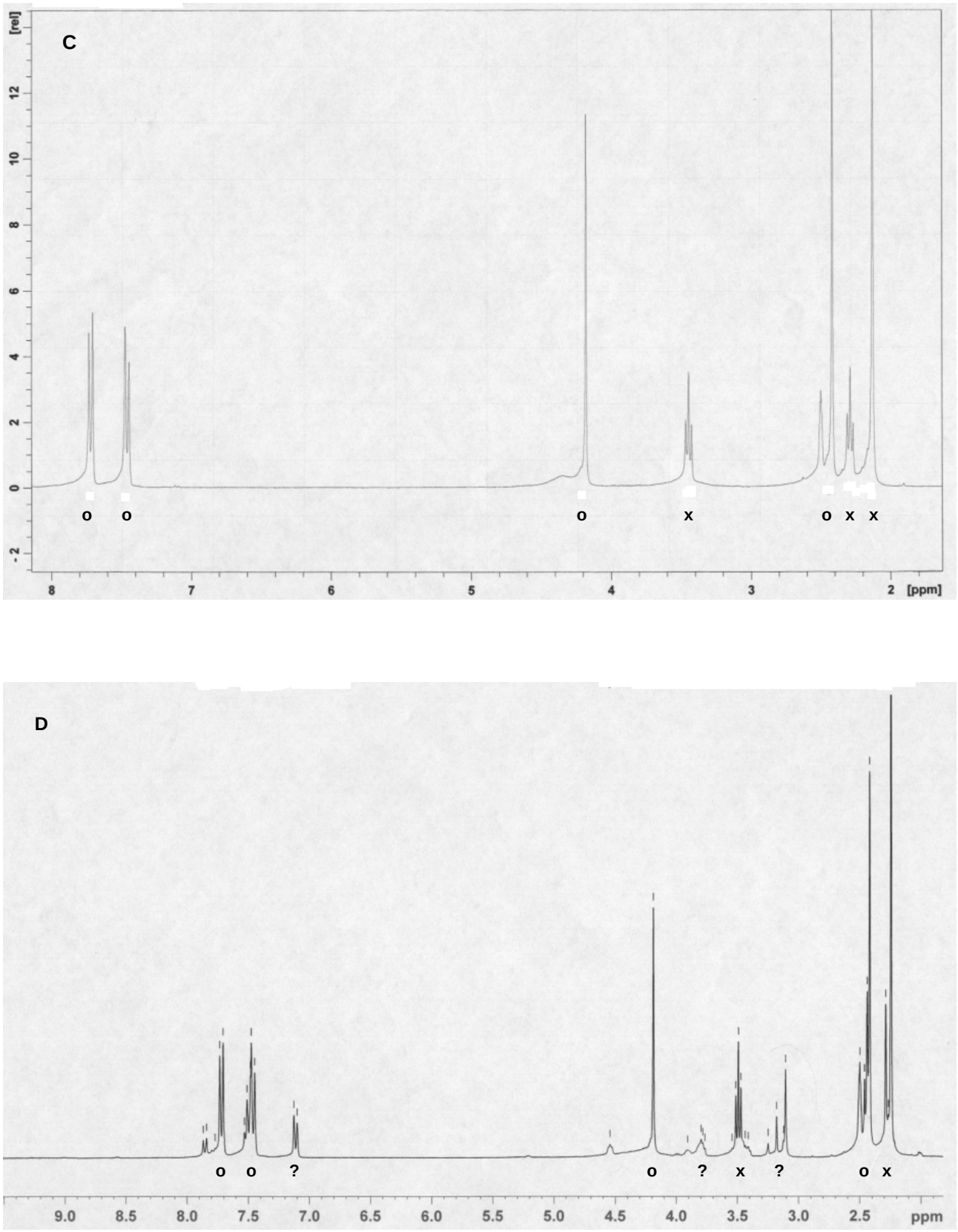


Figure 25: Comparison between the NMR spectra obtained by: 20 mg of diOTsEt dissolved in 1 ml of ACN (**A**) 0.1 ml of DMAE dissolved in 1 ml of ACN (**B**) a solution obtained by dissolving 20 mg of diOtsEt and 0.1

ml of DMAE in 1 ml of ACN at RT (**C**) a solution obtained by dissolving 20 mg of diOtsEt and 0.1 ml of DMAE in 1 ml of ACN heated at 80℃ (**D**)

o = signal assigned to diOTsEt **x** = signals assigned to DMAE **?** = new unknown signals (new products)

diOTsEt (Fig.25 **A**) ^{1}H NMR (d-ACN, 300 MHz) δ_H ppm: 2.42 (s, 6H, -CH_3), 4.20 (s, 4H, -CH_2), 7.45 (d, 4H, -CH), 7.72 (d, 4H, -CH).

DMAE (Fig.25 **B**) ^{1}H NMR (d-ACN, 300 MHz) δ_H ppm: 2.13 (s, 6H, -CH_3), 2.29 (t, 2H, -CH_2), 3.45 (t, 2H, -CH_2), 4.31 (s, 1H, -OH).

As showed in the panels of figure 25, the ^{1}H NMR of the mixture, acquired at 25℃, is equivalent at the sum of the two spectra of the single reagents. This means that no reaction between the reagents has happened after 24 h at RT. Conversely, in the ^{1}H NMR of the mixture acquired at 80℃, it is possible to notice the presence of new peaks indicating the starting of a reaction between DMAE and diOTsEt with subsequent formation of new products. In conclusion, the reagents mixture appears to be stable at RT and thus the reagent can be pre-solved and mixed up to 24 hours before starting the synthesis without influencing the yield of the reaction.

5. CONCLUSIONS:

The use of ^{18}FECH radiopharmaceutical in Nuclear Medicine departments is dramatically increasing due the possibility to give diagnostic information in fields where the traditional PET/CT imaging with ^{18}FDG is not useful, such as, mainly in prostate and brain cancer. The metabolic pathway followed by ^{18}F-labelled choline analogue allows to detect cancer cells showing uncontrolled growth and enhanced synthesis of phospholipid membranes.

In this dissertation a deep examination of the possible ways to produce ^{18}FECH were accomplished and the use of two different commercial automatic synthesizers, purification and synthetic approaches were evaluated. As the final aim of this discussion is to obtain a reliable synthetic way of a radiopharmaceutical for human use, particular attention was paid to develop a sure set of quality controls.

^{18}FECH synthesis was carried out with a FX F-N Tracer Lab obtaining medium or high RCY and high purity in every conditions. However, the needing of compliance to the European Pharmacopeia, strongly suggests the choice of an automatic synthesizers able to operate by using disposable device. For this reason, the one-step synthetic path was transferred to Mx TRACERlab.

Synthesis of ^{18}FECH with the Mx TRACERlab is also possible by using diOtEt as a precursor, but with a lower RCY. Anyway, the RCY is comparable to that obtained for commercial ^{18}FMCH with the same synthesizer, as already reported in literature and, if produced in a large scale, appears to be sufficient for a ^{18}FECH supply for a daily activity of a Nuclear Medicine facility. Concerning the use of other precursors, further studies are necessary, probably changing the reaction parameters such as reaction temperature and time.

The greatest concern by using the Mx TRACERlab synthesizer is about the chemical purity of the product that was little lower than the radiotracer produced by the FX F-N

Tracer Lab since still traces of DMAE, K^+ and O-Tosylate by-products were found in the ^{18}FECH preparations. On this topic too, further studies are ongoing.

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… Lo sposo leva lo sguardo sull'accompagnatore che a braccia incrociate ha assistito alla scena e ora dice piano: «Per lo meno vi siete incontrati. L'avete già fatto più volte e lo farete sempre di nuovo. Questo non capita a tutti».

Quindi segue la ragazza che a lunghi balzi s'inoltra nel deserto, in direzione dell'altra porta che si leva, enorme, sull'orizzonte settentrionale. Le due figure si fanno sempre più piccole in mezzo alle dune e presto di loro non resta che una traccia tortuosa di minuscoli crateri di sabbia. Lo sposo la segue con gli occhi lattiginosi, mentre con le dita palpa il boccio della rosa. «Com'è bella!» sussurra, «mio Dio, com'è bella!»

E, lasciandosi ricadere sulla sabbia, mormora ancora: «Mi troverà laggiù, dietro quella porta?»

Michael Ende "Lo specchio nello specchio"

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