An HPLC and UHPLC-HRMS Approach to Study PSMA-11 Instability in Aqueous Solution

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An HPLC and UHPLC-HRMS approach to study PSMA-11 instability in aqueous solution

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ABSTRACT

Background. Stability of precursors and reagents are of utmost importance for developing a suitable, fast and routinely reproducible analytical method for the quality control of radiopharmaceuticals, for the validation of the analytical method itself, as well as for radiolabeling procedure.

During the validation of the analytical method used for the determination of chemical and radiochemical purity of an injectable solution of 68Ga-PSMA-11, a trend to instability of the PSMA-11 standard, the same used as a precursor in the radiosynthesis of 68Ga-PSMA11, has been found. This instability led to the formation of a secondary compound in a time-dependent manner. The formation of this compound made difficult the validation of the analytical method and influenced the radiolabeling yield, by increasing free 68Ga which, obviously, decreased the final yield.

Results. The nature of this compound was investigated by adding chelators, such as EDTA, to PSMA-11 solutions and by using the combination of UHPLC-HRMS. The results led to the definition of the secondary compound structure, as ^{nat}Fe-PSMA-11, from the combination of the high affinity chelator HBED-CC, present in the molecule of PSMA-11, and environmental Fe(III).

Conclusions. Strategies to reduce the risk of low radiolabeling yields and to increase the stability of the standards were also discussed.

Keywords: Radiopharmaceuticals, Validation, Radioanalytical methods, ^{68}Ga-PET imaging, PSMA-11, HBED-CC, Prostate cancer, Radiopharmaceutical production, Quality Control, Chemical instability

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BACKGROUND

The radiopharmaceutical preparations or radiopharmaceuticals (RPs) are medicinal products which, when ready for use, contain one or more radionuclides included for a medical purpose *(1)*.

Since radiopharmaceuticals are intended for human administration, they must undergo strict quality control tests before release for clinical use. Basically, quality control involves specific tests which ensure the purity, potency, product identity, biologic safety, and efficacy of radiopharmaceuticals *(2)*.

The quality control (QC) of radiopharmaceuticals is described in the monographs of the European Pharmacopoeia (Eu. Pharm.), including the related method of analysis with the experimental details and the acceptance criteria.

Eu. Pharm. analytical methods do not need extensive validation, but they must be verified in each individual laboratory, to make sure that the method has been implemented properly (i.e., system suitability test, detector linearity, and limit of quantification).

On the other hand, if a monograph for a radiopharmaceutical has not been published, as for the Investigational Medicinal Products (IMPs), the analytical methods must be fully validated before use in routine quality control procedures. The validation aims to prove that the methods are suitable for their intended purpose *(1)*.

The Glu-urea-Lys(Ahx)-[(HBED-CC)] (PSMA-11) labelled with ^{68}Gallium, ^{68}Ga-PSMA-11, is the investigational radiotracer of choice for imaging of Prostate Cancer (PCa) *(3)*.

While performing the validation of the analytical method recommended in the Pharmeuropa draft monograph "Gallium (^{68}Ga) PSMA-11 injection" for the determination of chemical purity (CP) % and radiochemical purity (RCP) % of an injectable solution of ^{68}Ga-PSMA-11 *(4)*, we found out that the PSMA-11 standard was unstable in the acidic aqueous solution and a secondary compound was formed in solution, over time.

This instability has a major impact on the validation of the analytical method, which is reflected in the difficulty to evaluate the reproducibility and the intermediate precision; moreover, the decrease in the PSMA-11 concentration could lead to a lower ^{68}GaPSMA-11 radiolabeling yield.

Therefore, the aims of this paper were to investigate the causes of PSMA-11 instability in acidic aqueous solution by identification of the secondary compound, in order to improve the stability of PSMA-11 in solution, which is an essential step for the analytical method validation, and to evaluate possible strategies to limit the risk of a decrease in the radiolabeling yield.

MATERIALS AND METHODS

Chemicals and reagents

PSMA-11 standard, the same used as a precursor in the radiosynthesis of ^{68}Ga-PSMA11, was purchased from Advanced Biochemical Compounds, ABX (Radeberg, Germany). The lyophilized product was provided at - 20 ± 5°C.

Ethylenediaminetetraacetic Acid (EDTA) and Acetonitrile (ACN) were purchased from Carlo Erba Reagents S.r.l. (Cornaredo, Milan, Italy); Trifluoroacetic Acid (TFA), Formic Acid (FA) and metal-free water (Fluka Water TraceSelect® for trace Analysis) were purchased from Merck Life Science

S.r.l. (Milan, Italy); Ultrapure water (Milli-Q, 18.2 MΩ) was obtained from a Milli-Q® IQ Element purification (Merck KGaA, Darmstadt, Germany).

Radionuclide ^{68}Gallium (^{68}Ga) was routinely obtained as Gallium chloride ($^{68}GaCl_3$) solution by elution of a commercial ^{68}Ge/^{68}Ga generator, 1.11 GBq (68Germanio cloruro (68Ge)/68Gallio cloruro (68Ga) GalliaPharm®, Eckert&Ziegler Radiopharma GmbH, Berlin, Germany).

Hydrochloric Acid (HCl) 0.1 M for elution of ^{68}Ge/^{68}Ga generator was purchased from Eckert & Ziegler Radiopharma GmbH (Berlin, Germany).

All chemicals were of analytical grade and were used without further purification. HPLC eluents (Milli-Q water, FA, ACN and TFA) were of high-grade purity.

Analytical method validation

Since the Pharmeuropa draft monograph "Gallium (^{68}Ga) PSMA-11 injection" has not yet released for publication, the extensive validation of the analytical method, described in the draft for the determination of CP % and RCP % of an injectable solution of ^{68}Ga-PSMA-11, was carried out according to International Conference of Harmonisation (ICH) Q2 (R1) guidelines *(4-5)*.

The parameters assessed were: accuracy, reproducibility, intermediate precision, specificity, detection limit (LOD), quantification limit (LOQ), linearity, and range.

Briefly, to evaluate the linearity, the necessary experimental data were obtained by sequential injections of three replicates of each standard solution; whereas, to evaluate the reproducibility and the intermediate precision, three replicates of three concentrations of standard solutions were injected in a short period and three different days.

The LOQ and LOD were calculated by the standard deviation of the response and the slope obtained from the linearity plot. The accuracy was determined using the same set of data obtained following the linearity test.

Standard solutions preparation

To determine the CP %, the Pharmeuropa draft monograph "Gallium (^{68}Ga) PSMA-11 injection" requires to use a PSMA-11 reference solution corresponding to 30 µg of anhydrous and trifluoroacetic acid-free PSMA-11 dissolved in a solvent mixture of TFA and metal-free water (0.1% V/V) and diluted to V with the same solvent mixture (V being the maximum recommended dose in millilitres, which is 10 mL in our case) *(4)*. PSMA-11 standard solutions with a final concentration of 0.75, 1.5, 3, 6, and 12 µg/mL were prepared.

During tests, all PSMA-11 standard solutions were stored at –25°C.

All the equipment for the PSMA-11 standard solutions preparation was free from trace metals contamination.

HPLC Analysis

The PSMA-11 standard solutions were injected into a Thermo Scientific Dionex Ultimate 3000 HPLC system (Thermo Scientific, Bremen, Germany) equipped with LPG-3400SD pump, TCC-3000 column oven, UV VWD-3100 detector and radiometric detector at NaI (Gabi Star, Elysia-Raytest, Germany) connected in series.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC; ACE 3 µm C18, l = 0.6 m, Ø = 7 mm; Thermo Scientific, Bremen, Germany) with a linear A–B gradient (0-0.5 min 5% B, 0.5–10 min 5% B to 40% B, 10–11 min 40% B to 5% B, 11–16 min 5% B) at a flow rate of 0.6 mL/min and a total run of 16 min was used. Solvent A consisted of 0.1% TFA in Milli-Q water and solvent B of 0.1% TFA in ACN. UV absorbance was measured at 280 nm. The column temperature was kept at 24°C. The injection volume was 20 µl.

The Chromeleon data system software (Version 7.2.8) was used for data acquisition and mathematical

calculations.

Analysis of PSMA-11 instability

As the PSMA-11 is characterized by a highly efficient acyclic chelator with an EDTA-like structure and two additional phenol coordinating covalent bonds, the N,N'-bis [2-hydroxy-5-(carboxyethyl)benzyl] ethylenediamine-N, N'-diacetic acid (hereinafter HBED-CC, see figure 1), which demands rather low energy for metal ions complexing *(6-7)*, a series of analyses were performed to investigate if the secondary compound, formed during the storage of PSMA-11 standard solutions, was due to an impurity, an acidic degradation or thermal decomposition product, or to the HBED-CC tendency of chelating metal ions already at room temperature.

Figure 1. Chemical structure of PSMA-11 and EDTA.

Initially, the PSMA-11 standard solutions were prepared only in metal-free water and analyzed. Since the obtained chromatograms clearly showed the presence of the secondary compound in these solutions injected at 3 days from the preparation, in order to investigate the role of HBED-CC chelator in the formation of the secondary compound, a large excess of EDTA, an acyclic chelator available in our laboratory, was added only to two PSMA-11 standard solutions more than 3 days old, previously prepared in the solvent mixture of TFA and metal-free water (0.1% V/V), both with and without heating at 50°C of the solutions after adding of EDTA.

The addition of EDTA highlighted the role of likely metallic contaminants in the formation of secondary compound. PSMA-11 standard solutions were prepared from a stock solution (200 mg/mL)

obtained by adding 100 mg of EDTA to 10 mL of a solvent mixture of TFA and metal-free water (0.1% V/V); then, anhydrous and trifluoroacetic acid-free PSMA-11 was dissolved in this solvent mixture, that was previously incubated for one night at 50°C in order to remove the metallic contaminants from environment. The obtained solution was stored at room temperature and analyzed 1 hour after the addition of PSMA-11.

All PSMA-11 solutions were analyzed under the chromatographic conditions described before.

UHPLC-HRMS analysis

To identify the chemical structure of the secondary compound, a mass spectrometric study using Ultra High Performance Liquid Chromatography–High Resolution Mass Spectrometry (UHPLC–HRMS) of a 50 µg/mL PSMA-11 standard solution, showing the contaminant compound, was performed.

Briefly, 5 µl of the 50 µg/mL PSMA-11 standard solution were injected into a Thermo Scientific Dionex Ultimate 3000 UHPLC coupled to a Thermo high-resolution Q Exactive mass spectrometer (Thermo Scientific, Bremen, Germany). The column (Zorbax SB-C18 RRHT, 2.1x50 mm, 1.8 µ particle size, Agilent Technologies), thermostated at 30°C, was equilibrated with 0.3 mL/min of water 0.1% FA (A) with 5% ACN (B); after sample injection, B% was kept constant at 5% for 0.5', then linearly increased from 5 to 40% in 9.5 minutes; B% was then brought to 5% in 1 minute and kept at 5% B for 5 minutes for the reconditioning step. Each sample required a total run time of 16 minutes. Centroided MS and MS^2 spectra were recorded in both positive and negative polarities from 300 to 1800 and 200 to 2000 m/z in Full MS/dd-MS^2 (TOP2) mode, at a resolution of 70000 and 17500, respectively. The two most intense ions were selected for MS^2 nitrogen-promoted collision-induced dissociation (NCE = 30). Precursor dynamic exclusion (15 seconds) and apex triggering (1 to 6s) were set; peptide-like isotope pattern ions were preferred. The mass spectrometer was calibrated before the start of the analyses.

^{68}Ga-PSMA-11 synthesis

Synthesis of ^{68}Ga-PSMA-11 was carried out according to a well-established method *(7-9)*.

^{68}Ga-PSMA-11 was prepared using 10 µg of PSMA-11 precursor (10 µl of a 1 µg/µL aqueous solution), loaded in an automated radiosynthesis module (GAIA V2™, Elysia-Raytest, Germany), by using disposable reagents assembled in a disposable sterile cassette (SCX fluidic kit for the [^{68}Ga]-labeling of peptides, ABX, Germany).

The radionuclide ^{68}Gallium (^{68}Ga) was obtained as described in Chemicals and Reagents.

A series of modifications to the original synthesis procedure was performed to investigate the possibility to reduce the risk of low radiolabeling yields due to the formation of the secondary compound in the aqueous solutions of PSMA-11 synthesis precursor:

1. Using only freshly prepared aqueous solutions of PSMA-11 synthesis precursor (max. 3 days from the preparation);
2. Replacing the cation exchange (SCX) cartridge, commonly in use for the trapping and the purification of $^{68}GaCl_3$ eluted, with an SCX cartridge which shows greater affinity for metal impurities, in order to minimize the presence in the reactor of metal ions from the $^{68}GaCl_3$ eluate;
3. Increasing the amount of PSMA-11 precursor from 10 µg aliquots to 30 µg aliquots according to Pharmeuropa draft monograph "Gallium (^{68}Ga) PSMA-11 injection" *(4)*.

RESULTS

PSMA-11 instability in acidic aqueous solutions

Figure 2 shows a typical chromatogram of a 3 µg/mL PSMA-11 standard solution freshly prepared.

The average Retention Time (RT) of the peak of PSMA-11 (hereinafter principal peak) is 8.5 min.

This chromatogram was obtained during evaluation of the linearity of the analytical method.

Figure 2. Typical chromatogram of a 3 µg/mL PSMA-11 standard solution freshly prepared in a TFA and metal-free water mixture (0.1% V/V).

Figure 3 shows the chromatograms obtained from the 3 µg/mL PSMA-11 standard solution, injected at 3, 5, and 8 days from the preparation, and stored at −25°C during tests.

Figure 3. Chromatograms of a 3 µg/mL PSMA-11 standard solution injected at 3 (A), 5 (B), and 8 (C) days from preparation.

The chromatograms clearly show that PSMA-11 is not stable in acidic aqueous solutions.

When the solutions were injected at 3 days from the preparation, a secondary peak with an average RT of 7.4 min was clearly identified in addition to the principal peak related to PSMA-11; its area decreased over time in favour of the secondary peak area which became predominant.

This secondary peak was clearly identified in chromatograms obtained from all PSMA-11 standard solutions injected for evaluating the intermediate precision of the analytical method (0.75, 3, and 12 µg/mL), but it was more evident at low PSMA-11 concentration (3 µg/mL) compared to high concentration (12 µg/mL) (see figure 4).

To confirm this assumption, in the chromatograms of the PSMA-11 standard solutions with a final concentration ≥ 50 µg/mL, this peak resulted clearly identifiable only 6 months after the solution

preparation (see figure 4).

Figure 4. Chromatograms of 3 (A), 12 (B), and 50 (C) µg/mL PSMA-11 standard solutions injected at 3 days from preparation and chromatogram of a 50 µg/mL PSMA-11 standard solution injected 6 months after the preparation (D).

Moreover, in the chromatograms of the PSMA-11 standard solutions stored at room temperature, the secondary peak was clearly identifiable already when injecting the solutions at 6 hours from the preparation, suggesting a combined time/temperature effect (data not shown).

The RT of this secondary peak was similar to the RT of PSMA-11 ^{68}Gallium labelled peak (7.9 min RT, see figures 10, 11 and 12).

Analysis of PSMA-11 instability

The chromatograms of PSMA-11 standard solutions prepared only in metal-free water also clearly showed the secondary peak at 7.4 min RT, when the solutions were injected at 3 days from the preparation.

Interestingly, after adding of EDTA to PSMA-11 standard solutions 8 days old, with and without heating at 50°C of the solutions, the secondary peak area decreased over time in favour of the PSMA-11 peak area.

The complete transchelation effect of EDTA required 48 hours without heating and 5 hours when heating the PSMA-11 solutions to 50°C after adding of EDTA (see figures 5 and 6).

Figure 5. Chromatograms of a 12 µg/mL PSMA-11 standard solution 8 days old, injected at 0.5 (A), 5 (B), 24 (C), and 48 (D) hours from adding EDTA without heating.

Figure 6. Chromatograms of a 3 µg/mL PSMA-11 standard solution 8 days old, injected at 0.5 (A), 2 (B), and 5 (C) hours from adding EDTA and heated at 50°C.

The PSMA-11 solutions with EDTA was stable for 1 month at –25°C.

The formation of highly stable EDTA metal chelates in acidic aqueous solution allowed us to prepare PSMA-11 stable standard solutions for the validation of the analytical method. These solutions were prepared by adding of EDTA (10 mg/mL) to the solvent mixture of TFA and metal-free water (0.1% V/V) used for dissolving the PSMA-11.

In chromatograms obtained from these solutions, stored at room temperature, the secondary peak at 7.4 min RT was not clearly identifiable and the area of the peak due to PSMA-11 was stable at least for 12 months after the solution preparations (see figure 7).

Figure 7. Chromatograms of a 3 µg/mL PSMA-11 standard solutions obtained by dissolving the PSMA-11 in an EDTA, TFA and metal-free water mixture and performing the analysis immediately (A) and at 12 months from solution preparation (B).

UHPLC-HRMS analysis

The ESI MS negative base peak chromatogram of PSMA-11 showed two main peaks; the extracted ion chromatogram of the most intense ion of the peak at 8.9 min RT (998.3214 m/z) shows 2 not resolved peaks which can be attributed to the $[C_{44}H_{58}FeN_6O_{17}]^-$ ion, which corresponds both in terms of mass and isotopes patterns/relative intensity (bottom right frame of figure 8) to a complex of PSMA-11 with Fe(III) (structure reported in figure 9). The not resolved peak at 8.9 could be reasonably attributed to the two different diasteromeric forms of the complex. The extracted ion chromatogram of the most intense ion of the peak at 9.14 min RT (945.4099 m/z) shows a peak which can be attributed to the [M-H]- ion of HBED-CC; as shown in the bottom left frame of figure 8, the experimental (top) and predicted (bottom) spectra correspond both in terms of mass and isotopes patterns/relative intensity.

Figure 8. UHPLC-HRMS analysis of a 50 µg/mL PSMA-11 standard solution.

Figure 9. Chemical structure of Glu-urea-Lys(Ahx)-[Fe(HBED-CC)]$^-$

Analysis of ^{68}Ga-PSMA-11 formulation

We observed that the formation of a secondary compound in the aqueous solutions of PSMA-11 synthesis precursor can cause an increase in the percentage of ^{68}Gallium in ionic form in the final product (i.e., 10% vs 0.4%) and, consequently, a decrease in the percentage of the radiolabeling yield

non-decay corrected (n.d.c.) at end of synthesis (EOS) (i.e., 43% vs 91%).

In radiochromatograms obtained by analysis of a ^{68}Ga-PSMA-11 solution obtained with 10 µl of a PSMA-11 synthesis precursor solution (1 µg/µL) 8 days old (see figure 10, B), the area of the peak due to ^{68}Gallium in ionic form was greater (up to 10 times) than in those obtained by using the same aliquot of a freshly prepared (max. 3 days old) PSMA-11 synthesis precursor solution (see figure 10, A).

Figure 10. Radiochromatograms of ^{68}Ga-PSMA-11 solutions prepared by ^{68} Gallium radiolabeling a freshly prepared PSMA-11 aqueous solution (A) and a PSMA-11 aqueous solution 8 days old (B).

Conversely, in radiochromatograms obtained by performing the synthesis with 30 µl of a PSMA-11 synthesis precursor solution (1 µg/µL) 15 days old (see figures 11) as well as using an SCX cartridge more suitable to retain metal impurities and 10 µl of a not freshly prepared (≥ 3 days old) PSMA-11 synthesis precursor solution (1 µg/µL) (see figures 12), the area of the peak due to ^{68}Gallium in ionic form was more comparable to the one obtained by performing the synthesis with 10 µl of a freshly prepared PSMA-11 synthesis precursor solution (see figure 10, A).

Figure 11. Radiochromatogram of a ^{68}Ga-PSMA-11 solution prepared from 30 µg of PSMA-11 precursor.

Figure 12. Radiochromatogram of a ^{68}Ga-PSMA-11 solution prepared with a SCX cartridge more suitable to retain metal impurities.

DISCUSSION

During the validation of the analytical method recommended in the Pharmeuropa draft monograph "Gallium (^{68}Ga) PSMA-11 injection" for the determination of CP % and RCP % of an injectable solution of ^{68}Ga-PSMA-11 *(4)*, we had difficulties in evaluating the reproducibility and the intermediate precision, because of PSMA-11 instability in acidic aqueous solutions, leading to formation of a secondary compound, with a peak at 7.4 min RT, when the PSMA-11 solutions were stored at -25°C already after 3 days from preparation.

This appears clearer when working at lower concentrations (see figure 4).

As the PSMA-11 consists of HBED-CC (see figure 1), a highly efficient acyclic chelator which demands rather low energy for metal ions complexing *(6-7)*, we performed a series of analysis to investigate if the secondary compound formation was due to an impurity, an acidic degradation or thermal decomposition product, or to the HBED-CC tendency to coordinate the metal ions present in the environment, because of its fast coordination kinetics *(10)*.

The obtained results highlighted that the secondary compound was the result of the time concentration dependent interaction, already at room temperature, between HBED-CC and likely metal ions present in the PSMA-11 aqueous solutions in both with and without TFA.

The experiments carried out with EDTA supported this hypothesis; so, in order to perform the validation of the analytical method, PSMA-11 stable standard solutions were prepared by adding of EDTA (10 mg/mL) to the solvent mixture of TFA and metal-free water (0.1% V/V) recommended in

337 the draft for dissolving the PSMA-11.

338 However, to obtain PSMA-11 stable solutions, we found out that it was necessary to incubate the

339 solution containing EDTA for 1 hour at room temperature after the adding of PSMA-11.

340 Therefore, we supposed that the secondary compound was the result of the interaction between

341 HBED-*CC* and an environmental metal ion, coming, possibly, from the equipment used for the PSMA-11

342 standard synthesis.

343 To identify the metal ion responsible for the formation of the secondary compound, the UHPLC-

344 HRMS of a 50 µg/mL PSMA-11 standard, showing the contaminant compound, was performed.

345 The UHPLC–HRMS showed that the secondary compound is likely a complex between HBED-*CC* and

346 Fe(III) with molecular formula: $[C_{44}H_{58}FeN_6O_{17}]^-$ and structure reported in figure 9 *(11)*.

347 This observation is reasonable because Fe(III) is really an ubiquitous metal *(12)*.

348 Moreover, it could justify the large excess of EDTA required for obtaining the transchelation effect

349 of EDTA on HBED-*CC* metal ions complex, because the EDTA binds Fe(III) with a lower affinity (log

350 $K_{EDTA\text{-}Fe}$ = 25.1) than the HBED-*CC* (log $K_{HBED\text{-}CC\text{-}Fe}$ = 36.74) *(13-15)*.

351 The very high affinity between HBED-*CC* and Fe(III) would also justify the presence in the

352 chromatograms of the secondary peak already at 6 hours from the preparation of solutions when

353 they are stored at room temperature, as well as the relatively slow formation of the secondary

354 compound (within 3 days from the preparation of solutions) in PSMA-11 standard solutions stored at -

355 25°C, temperature at which the kinetic of coordination is negligible. This behaviour would be less

356 probable in presence of a metal ion with a lower affinity for the HBED-*CC* as Cu(II) (log $K_{HBED\text{-}CC\text{-}Cu}$ =

357 23.40) *(16-17)*.

358 Finally, the formation of the complex between HBED-*CC* and Fe(III) in the aqueous solutions of

359 PSMA-11 synthesis precursor could also justify the decrease in the radiolabeling yield by using not

360 freshly prepared solutions or solutions more than 3 days old, because the affinity of HBED-*CC* for

both Ga(III) and Fe(III) is similarly high (log K_{Ga} = 37.73, log K_{Fe} = 36.74) *(15; 18),* and higher than the affinity between HBED-CC and other commonly ubiquitous metal ions Cu(II) or Zn(II) or Co(II) or Ni(II) *(16; 18).*

Therefore, considering that Fe(III) can compete with ^{68}Ga(III) for binding the HBED-CC and that the concentration of $^{68}GaCl_3$ in generator eluates is very low *(15),* in order to reduce the risk of low radiolabeling yields we both increased the amount of PSMA-11 synthesis precursor from 10 μg to 30 μg, and used an SCX cartridge which shows greater affinity for metal impurities in order to minimize the presence of metal ions from the $^{68}GaCl_3$ eluted in the reactor.

These results on PSMA-11 instability are not in agreement with data previously reported by Migliari et Al. *(19).* The difference may be due to the lack of evaluation of intermediate precision; this test, being performed on different days, highlights the instability of the PSMA.

We observed also that performing the analysis of the PSMA-11 standard solutions at UV absorbance (λ) of 220 nm, according to reported in the paper of Migliari et al. *(19)*, as well as in the Chemistry, Manufacturing, and Controls (CMC) of the PSMA-11 supplied by ABX, the secondary peak is much less detectable than performing the analysis at 280 nm.

Moreover, a common practice, consisting in the analysis of a PSMA-11 and ^{nat}Ga-PSMA-11 mixture to perform the System Suitability Test of chromatographic system before QC starting, doesn't allow the detection of the secondary peak because of its co-elution with the ^{nat}Ga-PSMA-11 peak.

CONCLUSIONS

The stability of the PSMA-11 in (acidic or not acidic) aqueous solution is poor and, over time, a secondary compound is formed (RT ~ 7.4 min. See figure 3), which is more evident at low concentrations.

We have demonstrated that it's due to the interaction between HBED-CC and Fe(III), an ubiquitous

385 metal ion, possibly also in the environment of the PSMA-11 standard synthesis.

386 Since, the HBED-CC binds both Ga(III) and Fe(III) with a very high affinity, the formation of the
387 complex between HBED-CC and Fe(III) in PSMA-11 aqueous solutions has an important impact not
388 only for the validation of the analytical method, with the difficulty in evaluating the reproducibility
389 and the intermediate precision, but also for the synthesis of the ^{68}Ga-PSMA-11, because it can
390 increase ^{68}Gallium in ionic form in the final product, and, consequently, decrease the radiolabeling
391 yield.

392 This paper describes a possible way of obtaining PSMA-11 stable standard solutions, a mandatory
393 requirement for the validation of the analytical method before its use in routine QC procedures.

394 Moreover, in this paper are presented possible strategies to reduce the risk of low radiolabeling
395 yields.

396 Finally, this study highlights how the availability of a chelator with a preference for the Ga(III) over
397 other likely metal contaminants in eluates or used equipment would be very important not only to
398 obtain radiotracers with higher molar activity but also for developing a suitable, fast and routinely
399 reproducible analytical method for the QC.

400

401 **List of abbreviations**

402 ACN: acetonitrile; CP: Chemical Purity; EDTA: EthyleneDiamine Tetraacetic Acid; EOS: End Of Synthesis; ESI:
403 ElectroSpray Ionization; EU: European Union; HCl: Hydrochloric acid; HPLC: High-Performance Liquid
404 Chromatography; ICH: International Conference on Harmonization of Technical Requirements for Registration
405 of Pharmaceuticals for Human Use; IMPs: Investigational Medicinal Products; LOD: Limit Of Detection; LOQ:
406 Limit Of Quantification; MS: Mass Spectrometry; NDC: Non Decay Corrected; Eu. Pharm: European
407 Pharmacopoeia; PCa: Prostate Cancer; PSMA: Prostate Specific Membrane Antigen; QC: Quality Control; RCP:
408 RadioChemical Purity; RP-HPLC: Reversed-Phase High Performance Liquid Chromatography; RPs:
409 Radiopharmaceutical preparations Preparations or Radiopharmaceuticals; RT: Retention Time; SCX: Cation

Exchange Cartridge; TFA: Trifluoroacetic Acid; UHPLC–HRMS: Ultra High Performance Liquid Chromatography–High Resolution Mass Spectrometry; V: Volume.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article. More data are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AI contributed to the design of study as well as in writing of the manuscript and in data interpretation and analysis, in carried out particularly the synthesis of 68Ga-PSMA-11, the analysis of stability of PSMA-11 solutions and the validation of quality control procedures.

FG contributed in writing of the manuscript as wall as to designing, performing and evaluating the UHPLC–HRMS analyses.

VDI contributed to carried out the synthesis of 68Ga-PSMA-11.

GC contributed in the management of the 68Ge/68Ga generator.

SB contributed to the design of study as well as in writing of the manuscript and in data interpretation and analysis.

All authors have read and approved the final manuscript.

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Figures

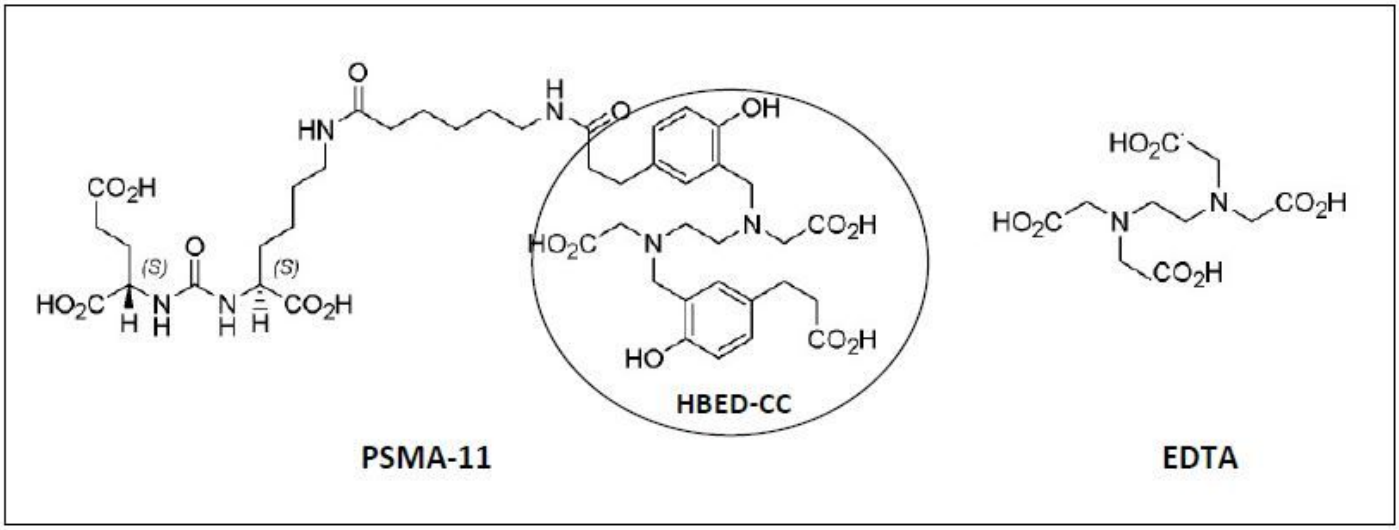

Figure 1

Chemical structure of PSMA-11 and EDTA.

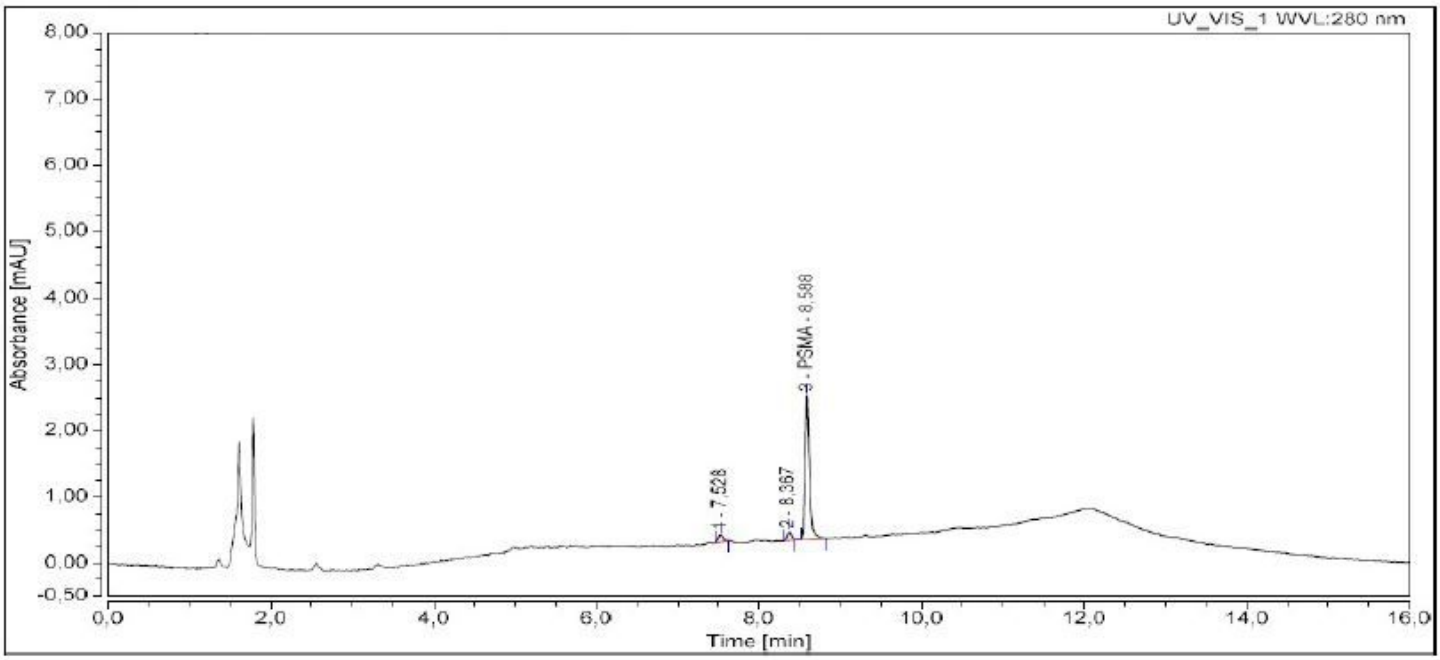

Figure 2

Typical chromatogram of a 3 µg/mL PSMA-11 standard solution freshly prepared in a TFA and metal-free water mixture (0.1% V/V).

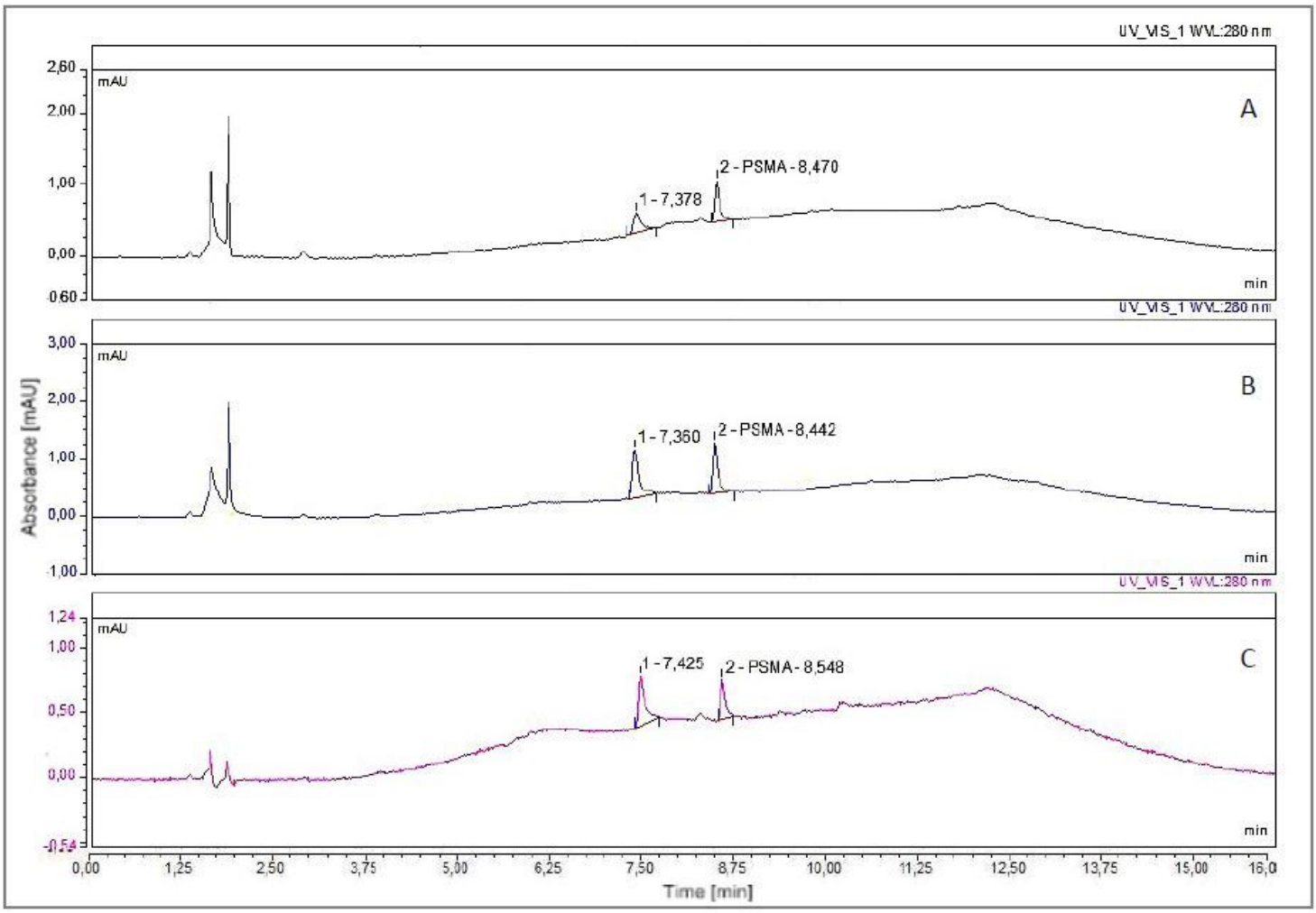

Figure 3

Chromatograms of a 3 µg/mL PSMA-11 standard solution injected at 3 (A), 5 (B), and 8 (C) days from preparation.

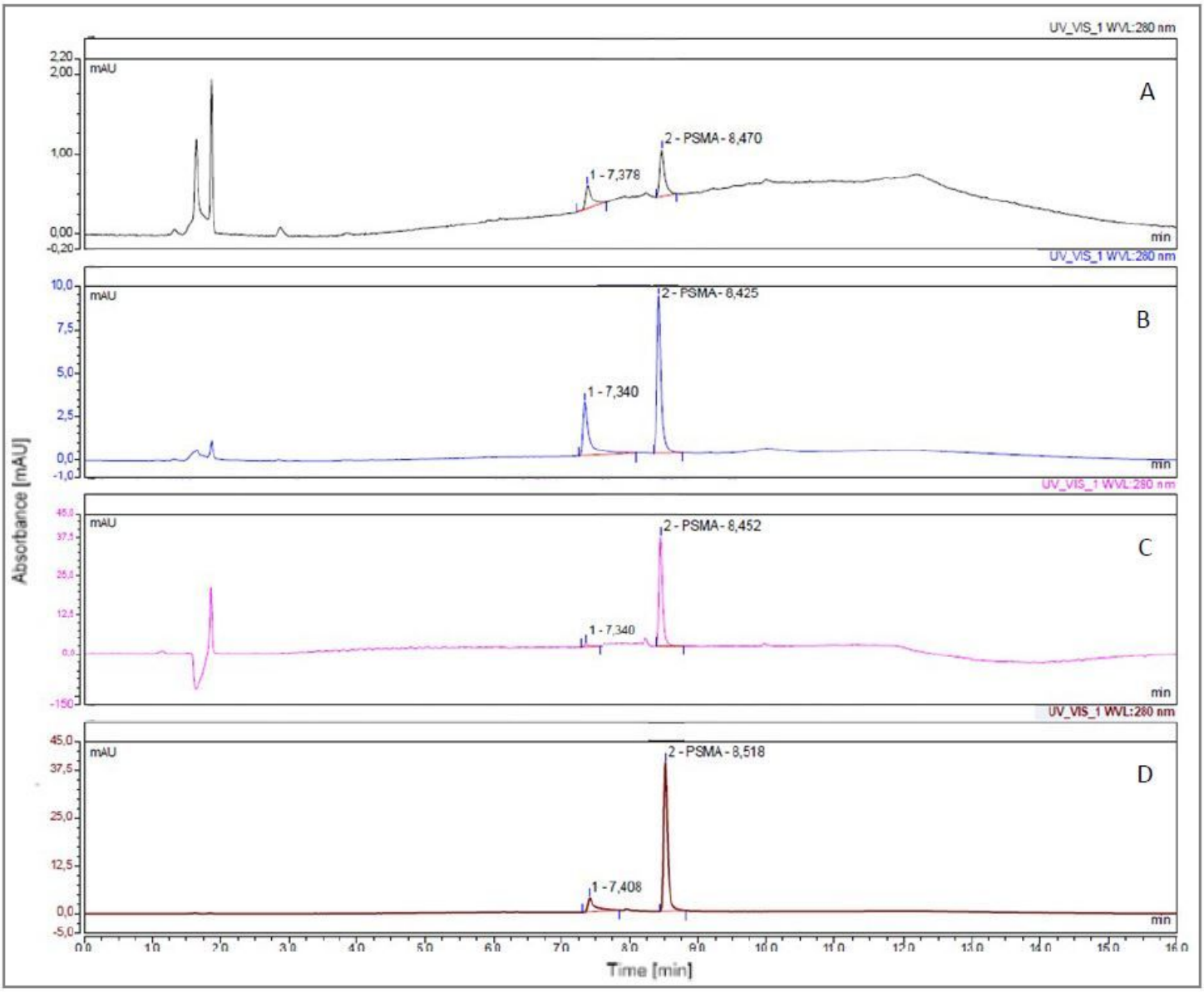

Figure 4

Chromatograms of 3 (A), 12 (B), and 50 (C) µg/mL PSMA-11 standard solutions injected at 3 days from preparation and chromatogram of a 50 µg/mL PSMA-11 standard solution injected 6 months after the preparation (D).

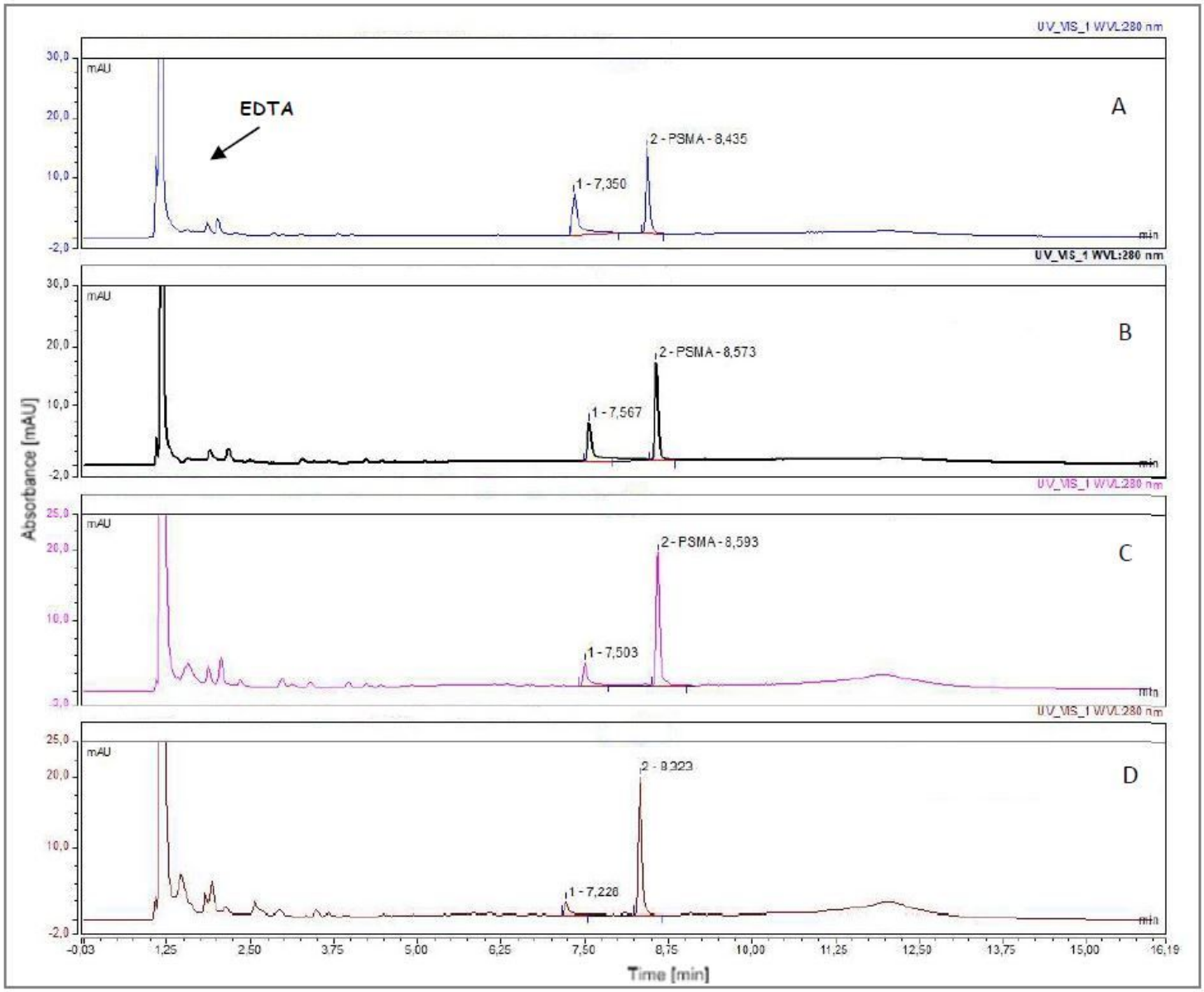

Figure 5

Chromatograms of a 12 µg/mL PSMA-11 standard solution 8 days old, injected at 0.5 (A), 5 (B), 24 (C), and 48 (D) hours from adding EDTA without heating.

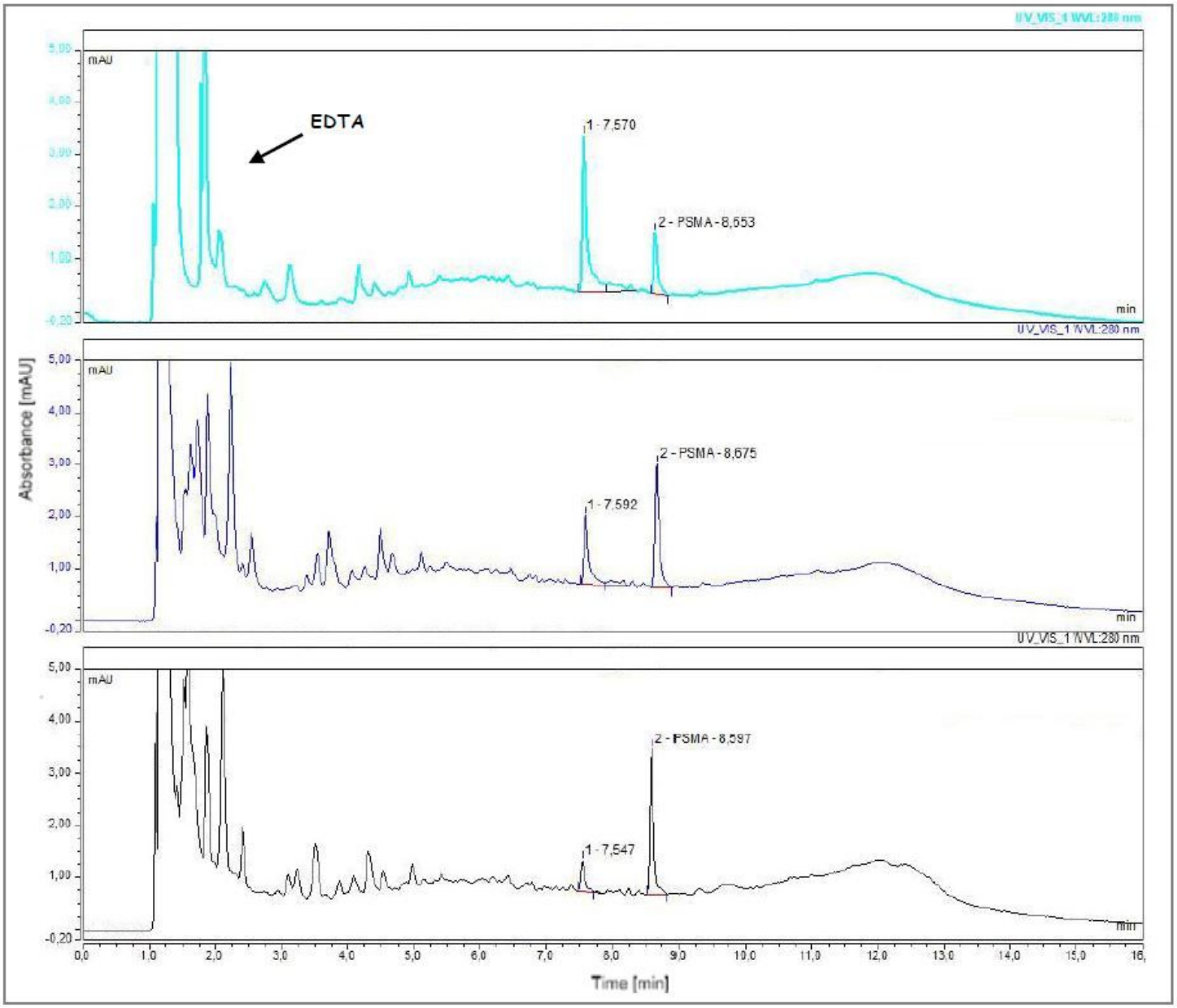

Figure 6

Chromatograms of a 3 µg/mL PSMA-11 standard solution 8 days old, injected at 0.5 (A), 2 (B), and 5 (C) hours from adding EDTA and heated at 50°C.

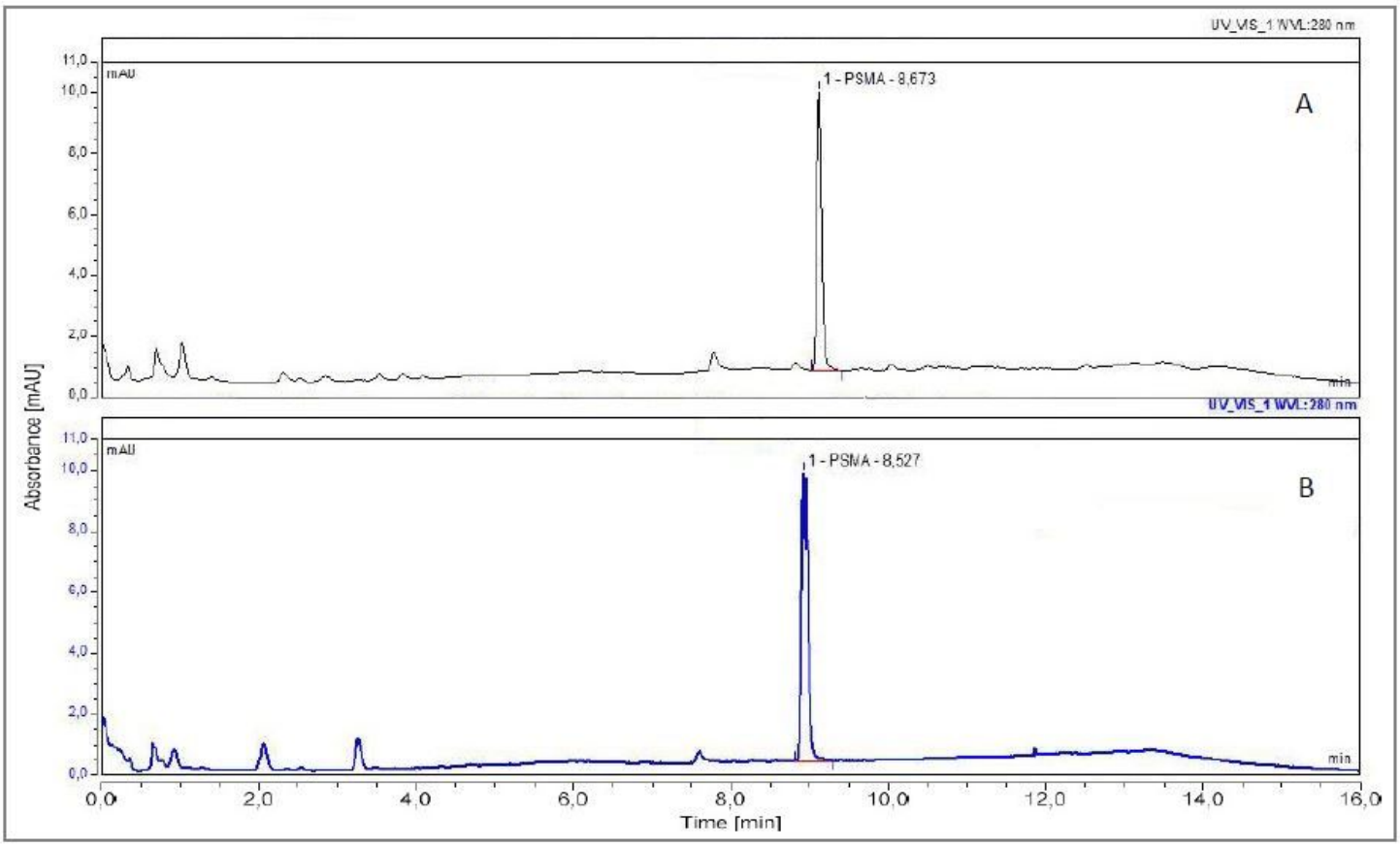

Figure 7

Chromatograms of a 3 µg/mL PSMA-11 standard solutions obtained by dissolving the PSMA-11 in an EDTA, TFA and metal-free water mixture and performing the analysis immediately (A) and at 12 months from solution preparation (B).

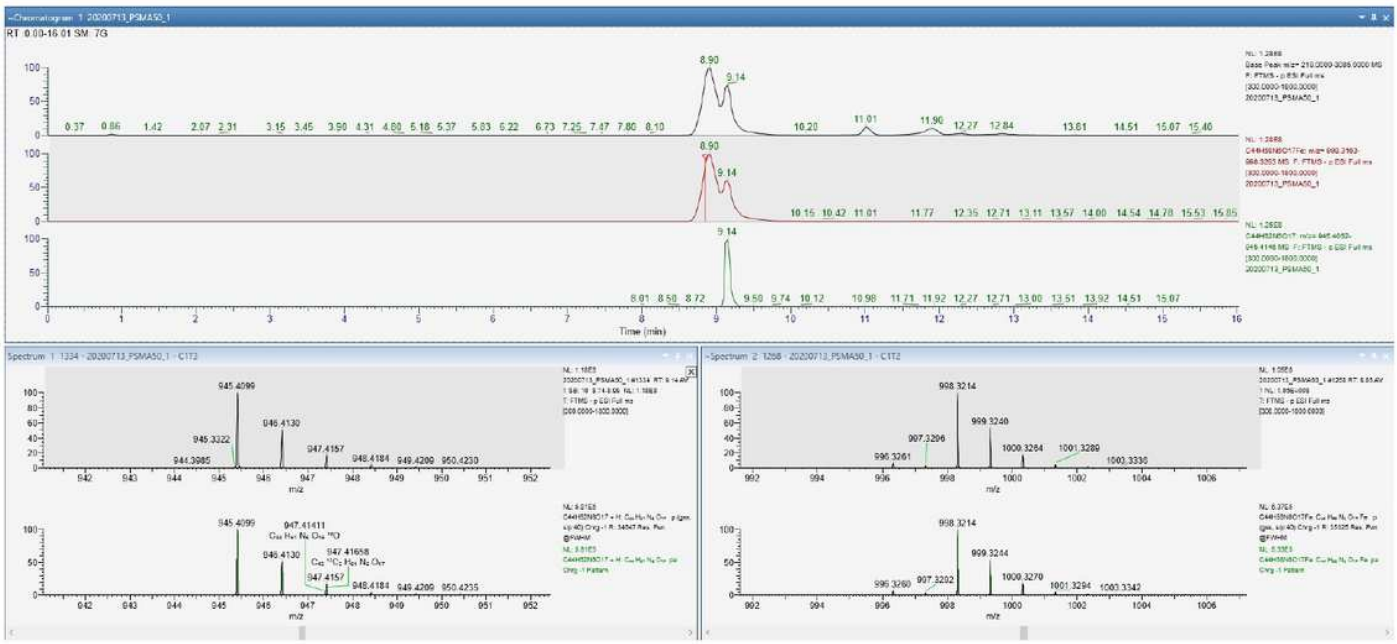

Figure 8

UHPLC-HRMS analysis of a 50 µg/mL PSMA-11 standard solution.

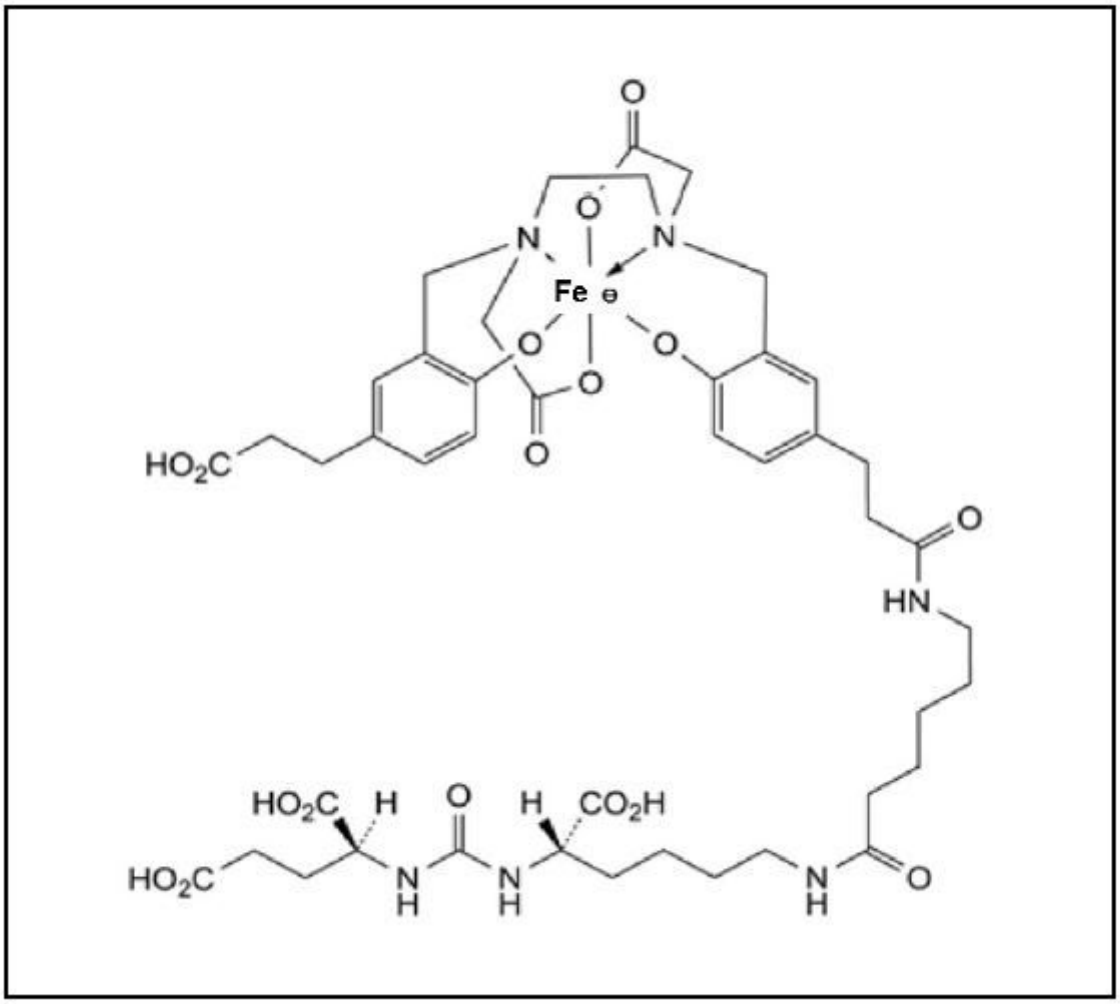

Figure 9

Chemical structure of Glu-urea-Lys(Ahx)-[Fe(HBED-CC)]-

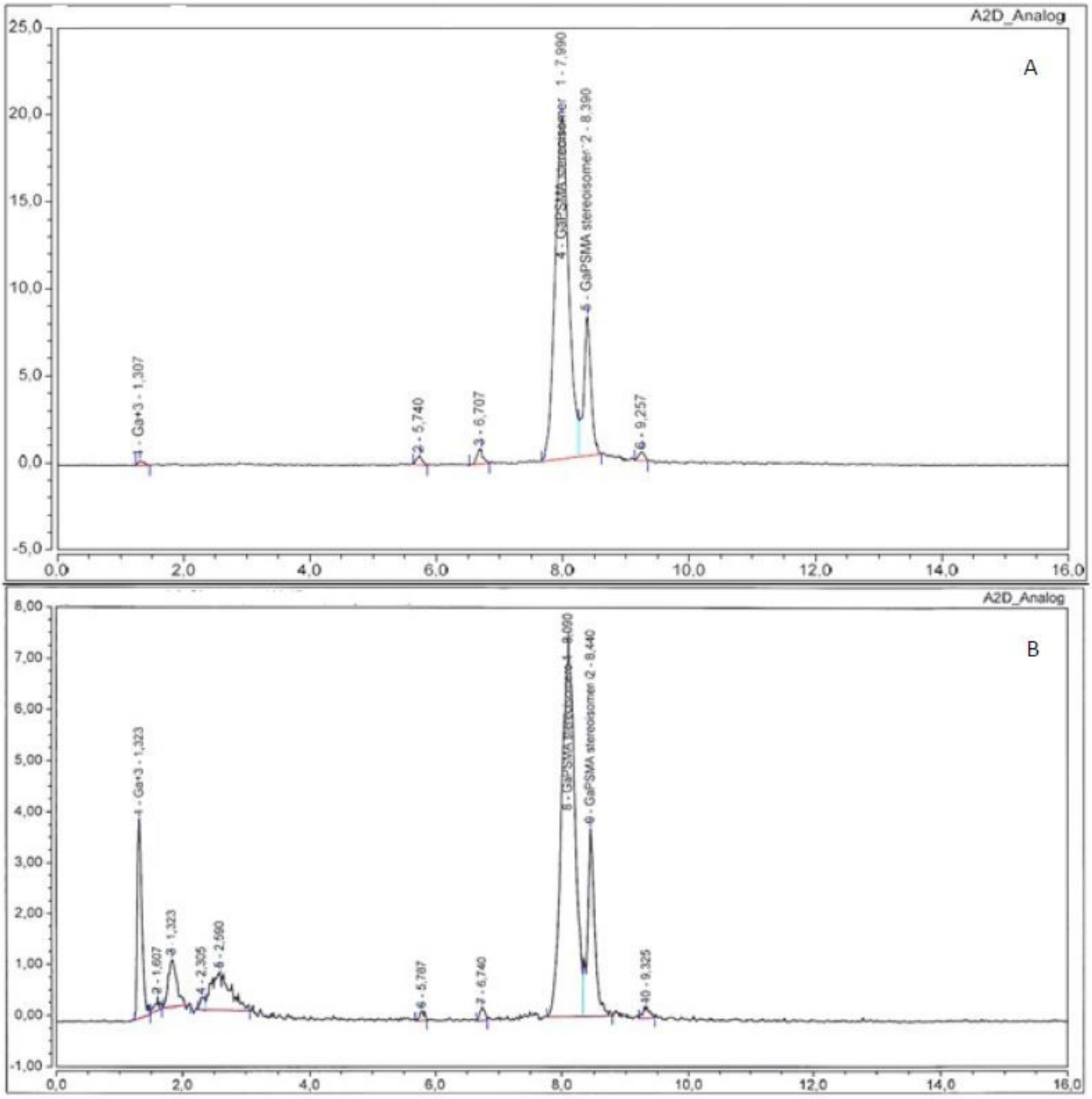

Figure 10

Radiochromatograms of 68Ga-PSMA-11 solutions prepared by 68 Gallium radiolabeling a freshly prepared PSMA-11 aqueous solution (A) and a PSMA-11 aqueous solution 8 days old (B).

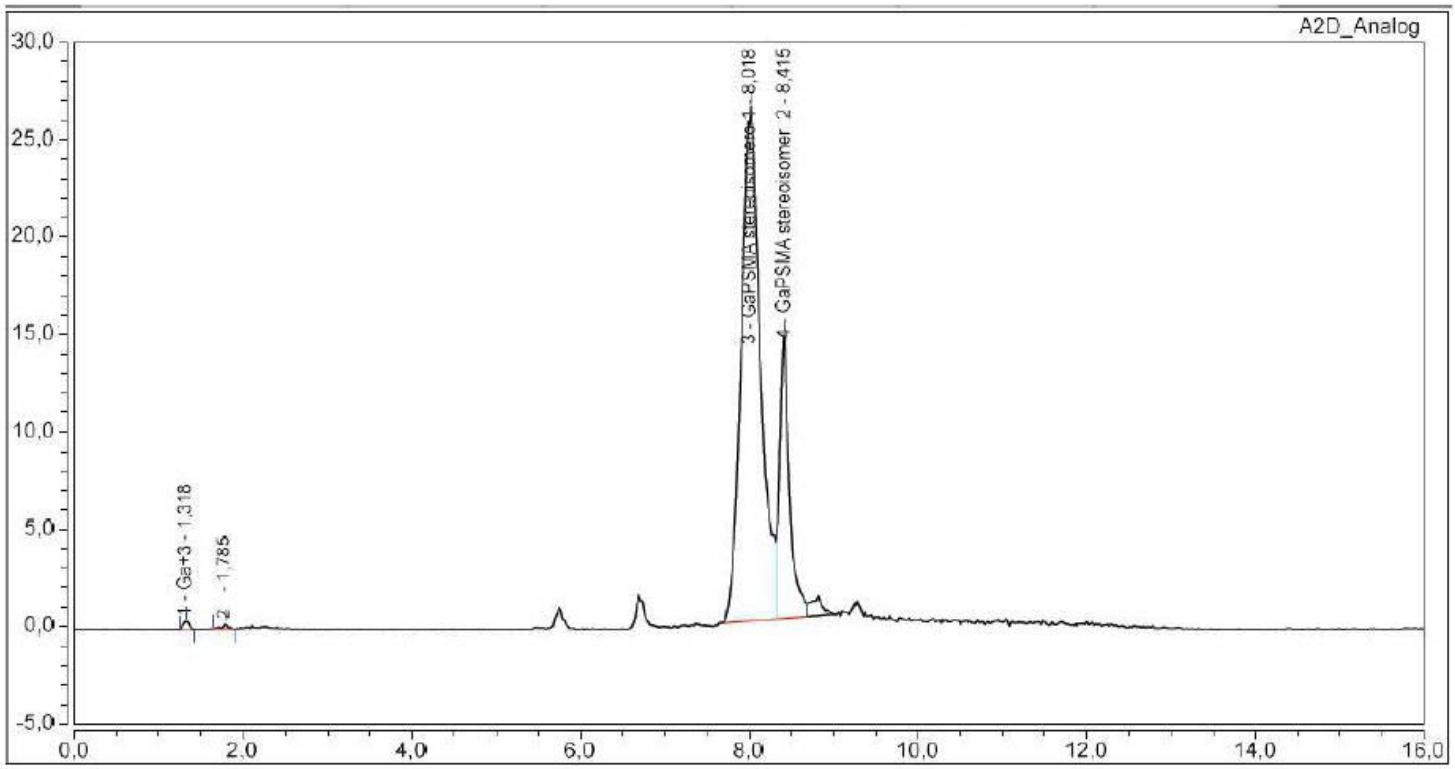

Figure 11

Radiochromatogram of a 68Ga-PSMA-11 solution prepared from 30 µg of PSMA-11 precursor.

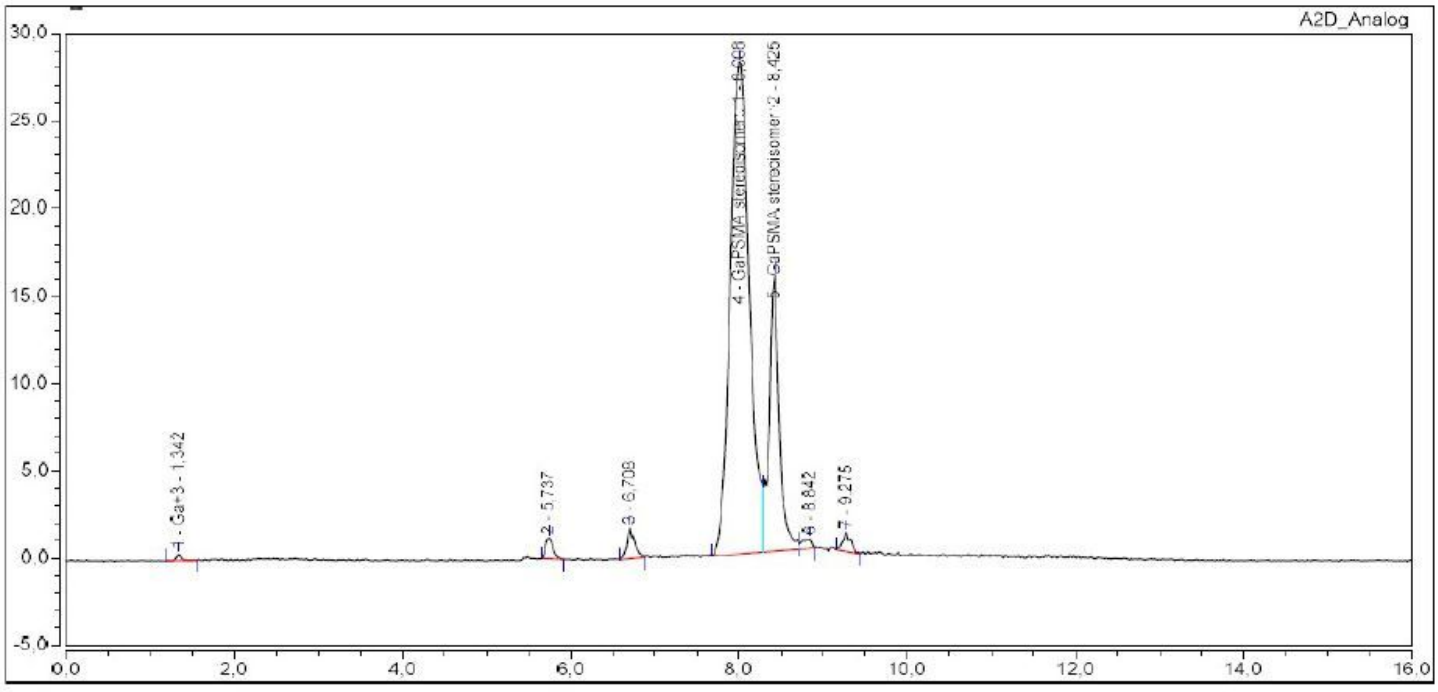

Figure 12

Radiochromatogram of a 68Ga-PSMA-11 solution prepared with a SCX cartridge more suitable to retain metal impurities.