NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

GONÇALO LEITE SANTOS
BSc in Chemical and Biochemical Engineering

DEVELOPMENT AND VALIDATION OF AN HPLC METHOD FOR THE DETERMINATION OF EIGHT CEPHALOSPORINS

MASTER IN CHEMICAL AND BIOCHEMICAL ENGINEERING
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Ad amicos meos et familiam

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"Eles não sabem, nem sonham, que o sonho comanda a vida. Que sempre que um homem sonha o mundo pula e avança como bola colorida entre a mãos de uma criança."

(António Gedeão).

ABSTRACT

Cephalosporins are a class of antibiotics effective against a wide range of bacterial infections, making them widely used in the pharmaceutical industry. Hikma, specifically Hikma Sintra facilities, is a company involved in the production of these antibiotics. The objective of this work was to develop and validate an analytical method capable of detecting all Cephalosporins produced at Hikma. This method is intended to monitor and verify the effectiveness of cross-contamination prevention measures within the facilities. The current work developed an HPLC (High Pressure Liquid Chromatography) method, through several iterations, that successfully detects all eight Cephalosporins. The main variable while developing the method was the percentage of organic, ACN (acetonitrile) or MeOH (methanol), in the different mobile phases attempted: 100 mM ammonium acetate buffer, 40 mM phosphate buffer and 10 mM sodium acetate buffer. The final method uses a XBridge BEH C18 5µm column at 32ºC, with a mobile phase consisting of 40 mM phosphate buffer, MeOH and ACN (82:13:5), at a flow rate of 1.2 mL/min and a UV detector set to 240 nm. The method demonstrated linearity with a correlation factor above 0.98 for all Cephalosporins, and precision, as indicated by a %RSD below 10% for all Cephalosporins, across six injections. The LOQ (Limit of Quantitation) and LOD (Limit of Detection) were determined to be 0.08 µg/mL and 0.02 µg/mL, respectively. The validated range of the method spans from 0.08 µg/mL to 0.5 µg/mL. Additionally, the swab recovery test confirmed the method's accuracy, with recovery percentages exceeding 75% for all Cephalosporins across the tested concentration range.

Keywords: HPLC, Contaminant, Cephalosporins, Validation, Development

RESUMO

As cefalosporinas são uma classe de antibióticos eficazes contra uma ampla gama de infecções bacterianas, tornando-as amplamente utilizadas na indústria farmacêutica. A Hikma, mais concretamente as instalações da Hikma Sintra, é uma empresa envolvida na produção destes antibióticos. O objetivo deste trabalho foi desenvolver e validar um método analítico capaz de detectar todos as Cefalosporinas produzidas na Hikma. Este método tem como objetivo monitorizar e verificar a eficácia das medidas de prevenção de contaminação cruzada dentro das instalações. O presente trabalho desenvolveu um método de HPLC (High Pressure Liquid Chromatography), através de várias iterações, que detecta com sucesso todas as oito Cefalosporinas. A principal variável durante o desenvolvimento do método foi a percentagem de orgânico, ACN (acetonitrilo) ou MeOH (metanol), nas diferentes fases móveis tentadas: 100 mM tampão acetato de amônio,40 mM tampão fosfato e 10 mM tampão acetato de sódio. O método final utiliza coluna XBridge BEH C18 5 µm a 32ºC, com fase móvel composta por tampão fosfato 40 mM, MeOH e ACN (82:13:5), com um fluxo de 1.2 mL/min e detector UV ajustado para 240 nm. O método demonstrou linearidade com fator de correlação acima de 0.98 para todas as cefalosporinas, e precisão, conforme indicado por um %RSD abaixo de 10% para todas as cefalosporinas, em seis injeções diferentes. O LOQ (Limite de Quantificação) e o LOD (Limite de Deteção) foram determinados em 0,08 µg/mL e 0,02 µg/mL, respectivamente. O intervalo do metodo varia de 0,08 µg/mL a 0,5 µg/mL. Além disso, o teste de recuperação do esfregaço confirmou a precisão do método, com percentagens de recuperação superiores a 75% para todas as cefalosporinas em toda a gama de concentrações testadas.

Palavras chave: HPLC, Resíduos, Cefalosporinas, Validação, Desenvolvimento

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ACRONYMS

US	United States.
FDA	Food and Drug Administration.
MEMA	Middle East, Mediterranean, African
QC	Quality Control
QA	Quality Assurance
GMPs	Good Manufacturing Practices
WHO	World Health Organization
SOP	Standard Operation Procedure
HPLC	High-Pressure Liquid Chromatography
UPLC	Ultra-Pressure Liquid Chromatography
GC	Gas Chromatography
LC	Liquid Chromatography
SFC	Supercritical Fluid Chromatography
ICH	International Council of Harmonization
DIW	Deionized Water
MeOH	Methanol
ACN	Acetonitrile
NIOH	Nacional Institute of Occupational Safety and Health

LOD	Limit of Detection
LOQ	Limit of Quantitation
CZ	Cefazolin
CP	Cefepime
CZI	Ceftazidime
CFT	Ceftriaxone
ZOX	Ceftizoxime
TAX	Cefotaxime
CFR	Cefuroxime
CX	Cefoxitin
UV	Ultraviolet
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene Difluoride
THPAB	Tetraheptylammonium bromide

SYMBOLS

t_r	Retention time, min
h	Hight of a chromatographic peak, µV
w_b	Width of a chromatographic peak, µV
t_0	Void time, min
k'	Retention factor
v_0	Void Volume, mL
F	Flow rate, mL/min
r	Column inner radius, mm
L	Column length, mm
α	Selectivity
k_j	Retention time of the peak j, min
k_i	Retention time of the peak i, min
x	Sample
n	Number of analyses
σ	Standard deviation
N	Number of plates
R_S	Resolution
T_f	Tailing factor

S/N	Signal to noise
A_S	Asymmetry factor
$\overline{x}$	Mean
RSD	Relative Standard Deviation, %
$[STD]$	Concentration of Cephalosporin in the standard, ppm
$[SMP]$	Concentration of Cephalosporin in the recovery sample, ppm
PA_{SMP}	Peak area of Cephalosporin in the recovery sample, µV.sec
PA_{STD}	Peak area of Cephalosporin in the standard, µV.sec

1

INTRODUCTION

1.1 Framework and motivations

1.1.1 Hikma

Hikma Pharmaceuticals was founded in 1978 by Samih Darwazah in Amman, Jordan. What drove Samih to start producing pharmaceutical products was the fact that many people in Jordan couldn't have access to medicines, so he wanted a way for the drugs to reach out to everybody. It was the first Arab company to export pharmaceutical products to the United States. Nowadays Hikma produces more than 760 generical products, injectables, branded generics, and specialty products, and in 2024 has more than 9000 collaborators. The company has 29 manufacturing factories, 13 US (United States) FDA (Food and Drug Administration)-inspected plants, 12 EMA-inspected plants, and 8 R&D hubs [1].

Currently, it ranks as the third largest supplier of generic injectables in the US, having many manufacturing facilities in the US, Europe, and MEMA (Middle East, Mediterranean, Africa). Hikma has three distinct businesses that make high quality medicines. The three businesses are injectables, generics and branded products. In 2022 the injectables were the biggest source of income with about 1 100 million US dollars of revenue, both branded and generic products rounded the 700 million US dollars of revenue [2].

1.1.2 Hikma Sintra

The work discussed in this thesis was conducted in the manufacturing facility of Sintra, Portugal. This facility is composed of three different factories specialized in injectables products: one for cephalosporins, one for liquid and lyophilization products, and another for oncology

products. All these products are injectable. In Hikma 1 is where are manufactured the liquids and lyophilization products and is also where the administration department resides. Hikma 4 produces high containment products, and they are liquids and lyophilization products same as Hikma 1. It's the most recent building in the facilities and it also has QC (Quality Control) laboratories.

At last, there is Hikma 2 where the content of this paper was conducted. In this building are produced cephalosporins in which, contrary to the other two, the final product is a solid (powder). Here the production process consists of the bottle filling, the raw product is the same as the final product. Hikma 2 as different sections:

- Production department, with three different lines of bottle filling and packaging of the final product.
- Warehouse for the feedstock (which also includes the materials used in the bottling process) and the packaging materials.
- Maintenance (where the water treatment is made).
- Quality department composed of QC and QA (Quality Assurance).

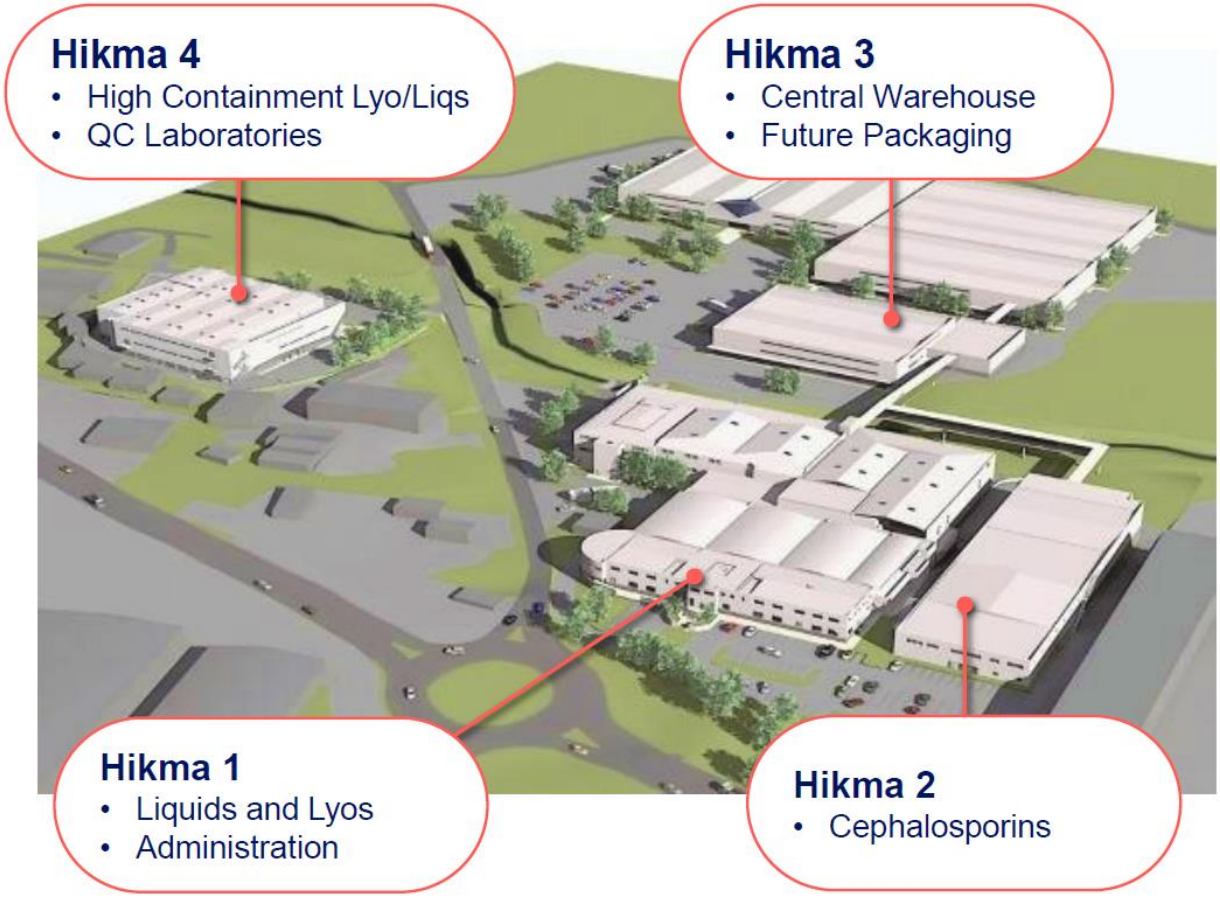


Figure 1.1 - Hikma Sintra plant layout [3].

Figure 1.1 illustrates the current plant layout of Hikma Sintra facilities. There are plans for future expansion of the factory that will almost double the area of production, implementing three more buildings.

1.1.2.1 Productive Process

As Figure 1.2 demonstrates, there are multiple phases that the product must go through, from the reception of the feedstock to the shipping of the final product.

Figure 1.2 - Productive process.

First the sterile feedstock arrives at the warehouse. To start the productive process, it is checked that the production lines meet all the conditions required, as such they are cleaned and tested. Gathered all the conditions required, the process can begin and the bottle filling starts. Previously, quality tests needed to be made on all the feedstock and materials used in the bottling filling. Before the product can be shipped and enters the market there is also the need to verify the quality of the product.

1.1.2.2 Products

Hikma 2 produces injectable solid cephalosporins (powder), mainly third generation cephalosporins, as shown in Table 1.1:

Table 1.1 - Products of Hikma 2

1st generation	**3rd generation**
Cefazolin Sodium	Cefotaxime Sodium
2nd generation	Ceftazidime Pentahydrate
Cefoxitin Sodium	Ceftizoxime Sodium
Cefuroxime Sodium	Ceftriaxone Sodium
4th generation	
Cefepime Hydrochloride	

1.1.2.3 Quality control

To ensure the quality of the product the company works with GMPs (Good Manufacturing Practices). This is a system that ensures the consistency of the products and the control

according to quality standards. GMPs are established by WHO (World Health Organization), and they should be present in the entire process of production and development of the medicine. As such, all the collaborators should be properly certificated, and all the results and documents should be properly stored and tracked, there are high standards regarding hygiene, and audits are made of all intellectual property of the company and all the facilities, regularly. The good practices established by WHO are supposed to be guidelines (strong recommendations), that must be adapted and changed accordingly to the specific needs in each different situation, and they must be validated. These guidelines don't cover personal safety, this is an aspect that must be analyzed by each government and applied accordingly [4].

Working with GMPs is needed because there are multiple legal entities responsible for the regulation of pharmaceutical drugs, and there is a need to harmonize the collaboration and to share the work between all the entities. These reglementary organizations publish books, called pharmacopoeias, that aim to implement legal guidelines and standards to the pharmacology industry.

Currently, there are a total of 56 national pharmacopoeias, 3 regional and subregional pharmacopoeias and 1 international pharmacopoeia that was published by the WHO (see appendix A) [5].

For every procedure Hikma has a SOP (Standard Operation Procedure). This is an internal document that is written according to the GMPs and provides indications for everything that is made in the company.

1.2 Case Study

Although cephalosporins aren't classified as hazardous drugs, a criterion established by NIOSH (National Institute of Occupational Safety and Health), when manufacturing these compounds is required attention to prevent cross-contamination [6]. Cephalosporins, like all non-penicillin beta-lactam antibiotics, have the potential to sensitize individuals, and therefore, the exposure to cephalosporins may cause allergic reactions to some patients. The cross-contamination between different beta-lactam classes and within classes may lead to life-threatening situations. FDA wrote a document with guidelines and GMPs to prevent the cross-contamination of non-penicillin beta-lactam antibiotics. One of the considerations made by the FDA, is that the separation of the production facilities is a GMP [7, 8].

The layout of Hikma Sintra facilities does not provide full separation between Hikma 1, Hikma 2, Hikma 3 and Hikma 4. While Hikma 2 operates mostly independently from the other

buildings, there are occasions when workers or specific equipment from the rest of the factory must enter Hikma 2. This flow cannot be completely halted, as doing so would require the facilities to be entirely independent from one another, which is not feasible under the current setup.

In Hikma 2, the product is a solid powder with a very small particle size, increasing the risk of cross-contamination, as these particles can easily adhere to clothing, worker´s hands, and objects. To mitigate this risk, there are numerous SOPs in place, designed to prevent cross-contamination. Strict rules must be followed to ensure the maintenance of quality standards across all products.

One of the key SOPs in place is SOP GEN020 [9], which regulates the flow of people and objects within the Hikma facilities. While there are no movement restrictions between Hikma 1, Hikma 3, and Hikma 4, strict protocols apply when entering Hikma 2, as demonstrated in Figure 1.3. Once someone or something enters Hikma 2, reentry to the other buildings is prohibited unless a cleaning process is completed. For example, if a worker enters Hikma 2 and needs to return to Hikma 1, they must go home to shower and change clothes before being allowed back into Hikma 1. Similarly, objects and equipment typically remain in Hikma 2 after entering. If relocating to other buildings is necessary, they must undergo a thorough cleaning and disinfection process prior to transfer.

To ensure that the cleaning of the facilities and equipment is being done correctly, as is the hygiene of the personnel, tests must be done regularly. Hikma has a procedure that is made once a month by the quality control department in Hikma 2, the containment, that describes the steps that must be followed to detect the presence or absence of cephalosporins in surfaces on the non-cephalosporins departments and on the hands of the workers of the cephalosporins area of production and quality control department when leaving the facilities [10].

As described in SOPSNT18144, an area of 100 cm^2 must be swabbed in every location in analysis and the swabbing of the hands must cover every bit of the hand, or as much as possible. After collecting all the samples, they must be prepared into a solution to be used in HPLC (High-Pressure Liquid Chromatography) equipment. The samples are prepared outside of Hikma 2 to prevent contamination, and they only enter Hikma 2 facilities when they are in the vials that are used in the HPLC analysis. This procedure is made by the Hikma 2 QC department.

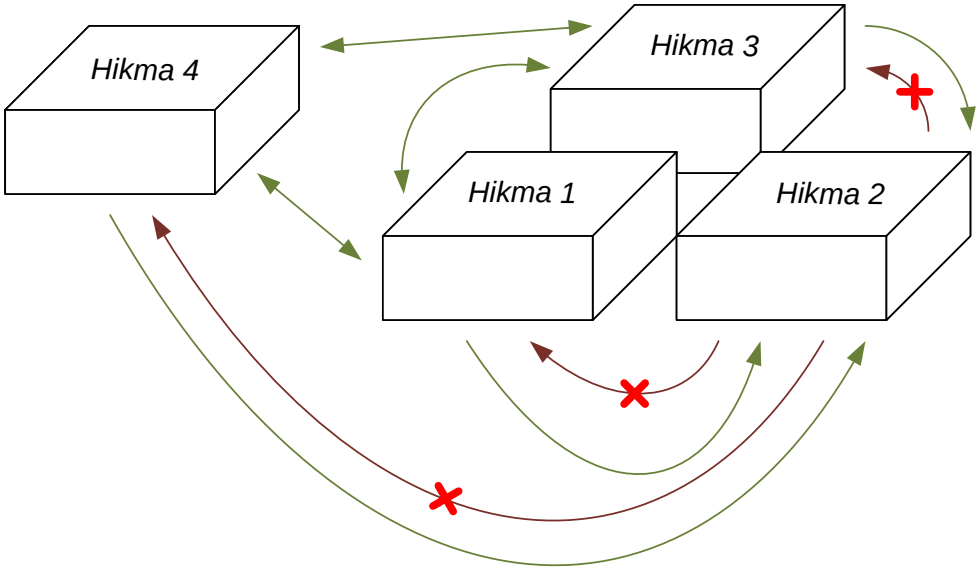


Figure 1.3 - People and object flow in Hikma Sintra facilities

The main goal of this procedure is to verify that there are not any of the cephalosporins produced in Hikma 2 in the rest of the facilities and personnel, and for that is used an analytical method of HPLC to analyze the samples. First, it is made a compost of all the eight cephalosporins produced in Hikma 2, to be able to compare the results obtained in the analysis of the samples. The results of the HPLC run of the compost should show eight different peaks that represent all the cephalosporins. Having this chromatogram, it is possible to compare the results of the samples and see if there is any peak in the same position as the peaks shown in the compost analyze. If the results don't show any evidence of the presence of cephalosporins, it means that all the procedures taken to prevent cross-contamination are being well performed.

1.3 Objectives

The current method for detection of cephalosporins in surfaces and personnel, only detects six of the cephalosporins produced in the factory: cefazolin, cefuroxime, ceftriaxone, ceftizoxime, cefoxitin and cefotaxime. Cefepime and ceftazidime cannot be detected using the current method.

This work aims to develop and validate a method of detecting all the eight cephalosporins produced in Hikma 2, including cefepime and ceftazidime.

It is possible to divide the objectives in two:

- Development of the method – study of the conditions that affect the chromatographic results and construction and optimization of an analytical method able to include the eight cephalosporins in the results.
- Validation of the method – confirm the reproducibility of the results obtained with the new method by doing different trials and statistical studies of the results.

1.4 Dissertation Structure

The structure of this work is divided into five main chapters:

- Introduction - This chapter presents the background and context that led to the objective of this work, along with the objective itself.
- State of the Art - A review of current knowledge on Cephalosporins, method development, validation, and the existing techniques used for detecting Cephalosporins.
- Materials and Methods - This chapter details the experimental procedures, describing all materials used and the specific methods followed.
- Results and discussion - The outcomes from the experimental procedures are presented and analyzed in this chapter.
- Conclusion - The final chapter summarizes the key results, their significance, and suggests potential future research directions.

2

STATE OF THE ART

2.1 Cephalosporins in Pharmaceutical Industry

Over the years, medical advancements have increasingly focused on addressing the growing issue of antimicrobial resistance among pathogenic bacteria [11]. Antibiotics are a unique class of medicine due to the rapid emergence of antibiotic-resistant bacteria, making the development of new antibiotic drugs critically important [12]. Table 2.1 shows some examples that support this evolving resistance of bacteria.

Table 2.1 - Evolving resistance of some antibiotics (adapted from literature) [12].

Antibiotic	Year deployed	Clinical resistance observed
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	Late 1960s
Nalidixic acid	1962	1962
Fluoroquinolones	1980s	1980s
Fidaxomicin	2011	2011

Cephalosporins are gram positive antibiotics that are structurally related to penicillin, they were originally derived from the fungus *Cephalosporum acremonium*. They are structurally like penicillin and have a beta-lactam ring and thiazolidine [13].

Penincilin Cephalosporin

Figure 2.1 - Penicillin and Cephalosporin core structure [14].

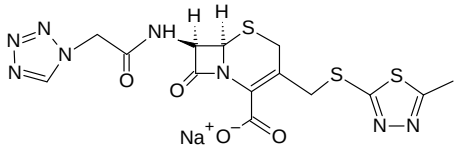
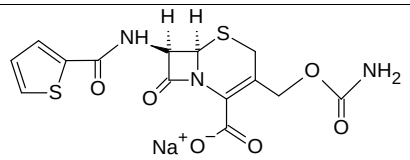
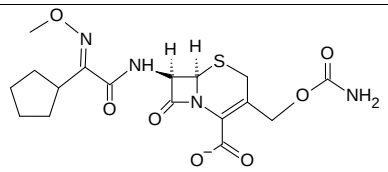
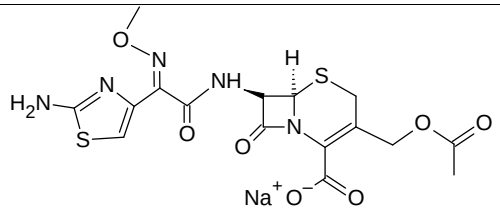
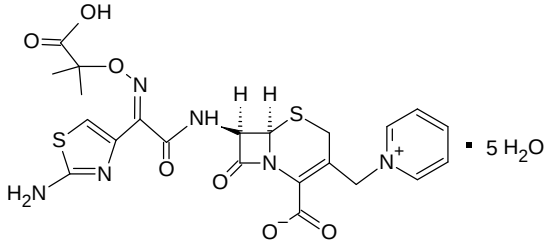
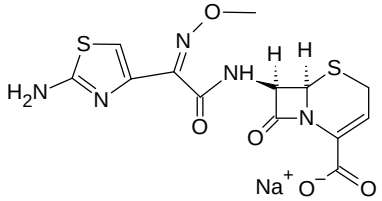
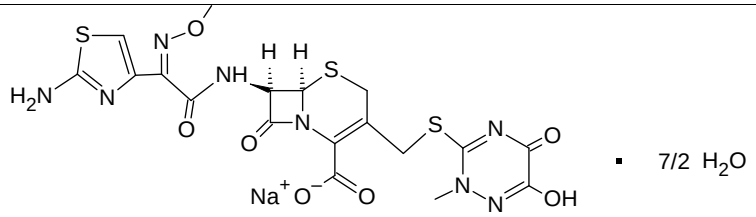
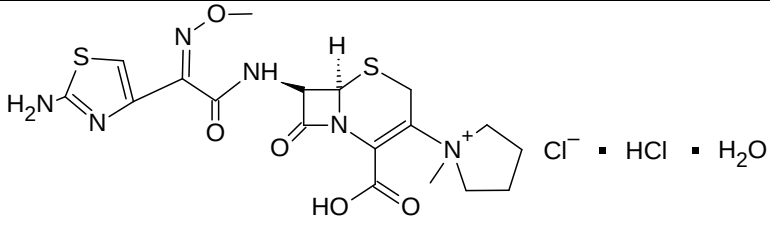
The way Cephalosporins work consists of inhibiting the synthesis of bacterial wall by binding to penicillin-binding proteins on bacteria. This leads to cell lysis, particularly in rapidly growing organisms. They differ from Penicillin activity in the ability to inhibit a broader range of proteins and can present sensitive to some extent to beta-lactamase [13].

To date, five generations of cephalosporins have been developed with different antibacterial abilities:

- First generation: active against many gram-positive bacteria.
- Second generation: broader antibacterial activity than first generation cephalosporins.
- Third: less active against gram-positive bacteria but more active against gram-negative bacteria and have greater stability against beta-lactamases
- Fourth generation: active against many gram-positive and gram-negative organisms.
- Fifth generation: active against a wider range of both gram-negative and gram-positive bacteria.

All cephalosporins of all generations present a core structure as shown in Figure 2.1. The entire structure of the eight cephalosporins produced by Hikma 2 is presented in the table 2.2.

Table 2.2 - Structure of the eight cephalosporins produced in Hikma 2

Cefazolin Sodium	
Cefoxitin Sodium	
Cefuroxime Sodium	
Cefotaxime Sodium	
Ceftazidime Pentahydrate	
Ceftizoxime Sodium	
Ceftriaxone Sodium	
Cefepime Hydrochloride	

Cephalosporins are commonly used to treat a wide range of infections, including respiratory tract infections, urinary tract infections, skin infections, peritonitis, and septicemia. The choice of cephalosporin depends on the specific characteristics of the bacteria that need to be targeted. For instance, if the infection is caused by anaerobic bacteria, second-generation cephalosporins like cefoxitin are more effective [15, 16].

Some bacteria that are inhibited by second-generation cephalosporins can develop resistance over time, necessitating the use of third-generation cephalosporins. For example, certain strains of *Pseudomonas* have shown greater susceptibility to ceftazidime, which offers more effective inhibition than earlier generations [17].

2.2 HPLC

Chromatography is a technique that allows the separation of components of complex mixtures. A chromatography system has three main components to work: a pump, a column and a detector. The column is composed of a mobile and a stationary phase and the sample is dissolved in the mobile phase which can be a gas, a liquid or a supercritical fluid. The choice of the phases is based on the distribution that the analyte can do throw both. The existence of a stationary phase, which can be a liquid or a solid, is important because some components are more strongly held in this phase than others, and consequently they move slowly with the mobile phase. These conditions allow the separation of the components into discrete bands that, using a detector, can be quantified and analyzed qualitatively [18, 19].

There are three main classifications for the chromatographic methods, GC (gas chromatography), LC (liquid chromatography) and SFC (supercritical fluid chromatography). LC can be carried out in columns or in plane surfaces unlike GC and SFC that are restricted specifically to columns [19].

Figure 2.2 shows how chromatography works in a column, having 3 components (Z, Y and X) that need to be separated, identified and quantified. The result of this chromatography is represented in Figure 2.3 that shows the peaks related to the different components and the time that they are retained in the column.

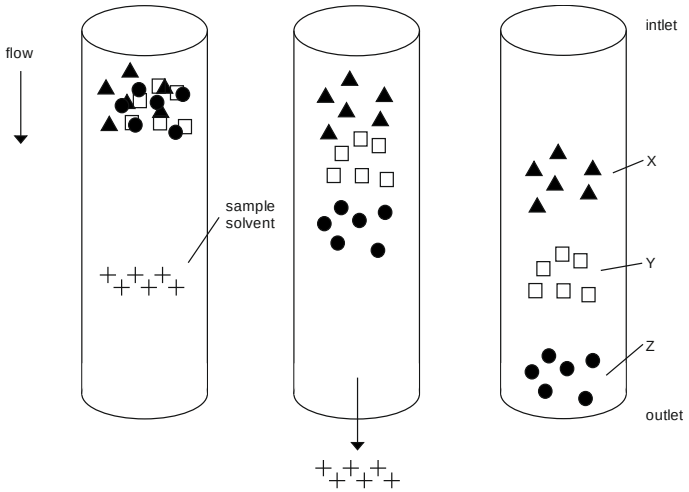


Figure 2.2 - Separation in a chromatography column of three different analytes (X,Y,Z) [18].

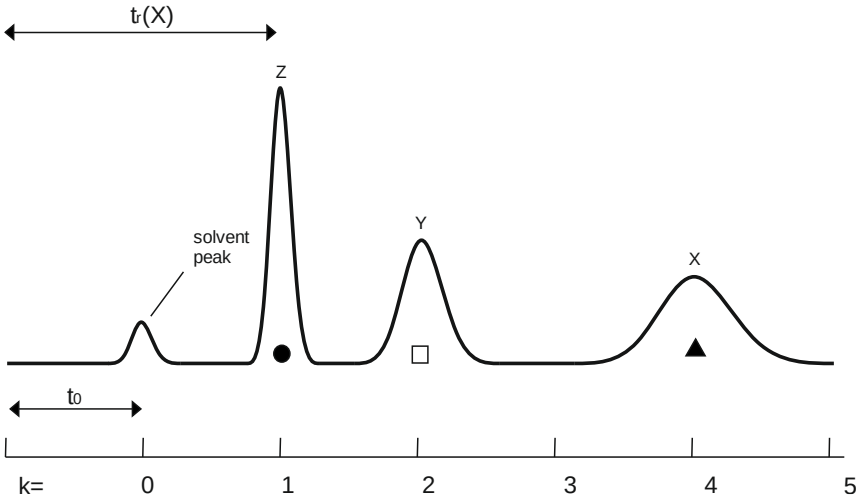


Figure 2.3 - Typical chromatogram of a separation of three different analytes (Z,Y,X) [18].

The peak is smaller if the component is more strongly held in the stationary phase because it represents the rate that the analyte moves thought the column, as such, if the strength that the stationary phase has to retain the component is bigger, the rate of motion through the column will be decreased. Movement along the column provides distance between the peaks of different components, if the detection was in the middle of the column the distance between the peaks would be very little compared to detecting in the end [19].

2.2.1 Fundamental concepts in HPLC

There are a few basics when working with HPLC. The goal is to separate the analytes to be able to quantify the different components and for that to happen the sample must be soluble. HPLC

uses a mobile phase to work and if the analyte is not soluble will be impossible for the analyte to move down the column with the mobile phase. Another important factor that needs attention is the fact that the analyte must interact with the stationary phase to obtain separation of the different components, and their retention ability must differ. With that in mind, the mobile phase is the one that really provides the separation of the different components, while the stationary phase retains the analytes, the mobile phase makes the different components travel down the column at different rates. The solution of analyte to be injected in the column must be dissolved in the mobile phase or in a weaker solvent before entering the system to reduce the anomalies in the results such as splitting peaks and fronting peaks.

2.2.1.1 Retention

A graphical representation of the results of a chromatography column is called a chromatogram. Figure 2.4 represents an example of a common chromatogram, it shows the retention time (t_r, min) of the component in the column, both height (h, µV) and width (w_b, µV) of the peak, and the void time (t_0, min) [20].

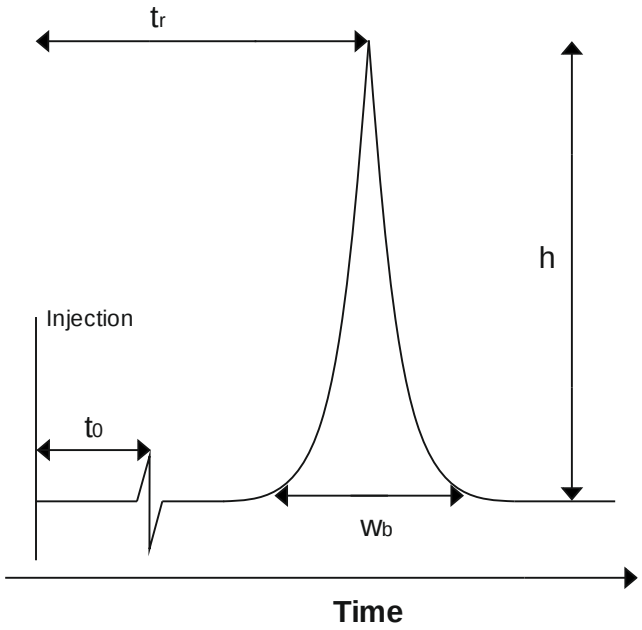


Figure 2.4 - Chromatogram that shows the void time (t_0), the retention time (t_r), the peak base width (w_b) and the peak height (h).

Retention time is the time between the sample injection and the point of the analyte detection and is identified in the maximum height of the peak. The measurement of the with was made in the base line and that is why it is used the subscript "b" when referring to the with. The amount of the component in the sample is proportional to the area and to the height of the peak, being the first more accurate [20].

Being the retention time used to identify the peak, there is a more fundamental concept that refers to the capacity of retention of the analyte in each phase, the retention factor or capacity factor [21], that relates retention and void times as shown in equation 1 and equation 2.

$$k' = \frac{t_r - t_0}{t_0} = \frac{V_r - V_0}{V_0} \quad \text{(eq.1)}$$

$$t_r = t_0(k' + 1) \quad \text{(eq.2)}$$

A retention factor of 0 indicates that there is no interaction with the stationary phase and the component elutes with the solvent. The higher the retention factor is, the longer the interaction with the stationary phase is and therefore the value of the retention time increases. When the retention time is higher than 20 the elution time becomes very long and consequently the sensitivity of the elution decreases drastically [21, 22, 23].

2.2.1.2 Void volume

Understanding the meaning of void volume (V_0, mL) is important since this concept is directly related to the void time that is presented in a chromatogram. The void volume represents the volume that isn't occupied with the stationary phase, and therefore represents the free volume available for the mobile phase [21]. Equation 3 demonstrates that it can be calculated by multiplying the flow rate (F, mL/min) with the void time.

$$V_0 = t_0 F \quad \text{(eq.3)}$$

Typically, the stationary phase occupies 30-40% of the empty column, as such, the void volume is normally 70-60% of the volume of the column, as shown in equation 4.

$$V_0 = 0.65\pi r^2 L \quad \text{(eq.4)}$$

, where r and L are the column inner radius and length.

Since the void volume depends directly on the dimensions of the column, the type of column used in the chromatography will seriously affect the results in the analyses, a bigger void volume will lead to bigger peak volumes and therefore lower analyte concentration. The diameter of the column affects the sensitivity of the analysis, a smaller diameter increases the sensitivity.

2.2.1.3 Selectivity

One of the aims in the development of a chromatographic method is to obtain a chromatogram with distanced peaks, for that the selectivity must be high. The lower the selectivity, the lower the distance between peaks. This parameter, calculated as in equation5, is the ratio between the retention factor of two peaks and it must be higher than 1 to occur peak separation [20].

$$\alpha = \frac{k_j}{k_i} \qquad \text{(eq.5)}$$

Here, the values of k_i and k_j represent the retention factor of the peaks i and j, being that the first peak to appear is i [18].

2.2.1.4 Efficiency

What defines the column efficiency is the narrowness of the peaks that is measured with the number of theorical plates. This is a way of relating the retention time with the standard deviation width of the peak (σ). Since the standard deviation width of the peak is 4 times the width of the peak the calculation of the number of theorical plates is given by equation 6.

$$N = \left(\frac{t_r}{\sigma}\right)^2 = 16\left(\frac{t_r}{w_b}\right)^2 \qquad \text{(eq.6)}$$

A high number of plates gives a higher efficiency to the chromatography that is represented by narrower peaks in the chromatogram.

There are some factors that can be changed to increase or decrease the number of theorical plates. Table 2.3 shows some conditions that can be changed and their effect on the theorical plates.

Table 2.3 - Effects on theorical plates when changing specific conditions [18].

Condition	Effect on N
Column Length (L)	Increases proportionately
Column Diameter (d_c)	None
Column Particle Size (d_p)	Decreases
Mobile Phase Flow Rate (F)	Decreases
Mobile Phase Viscosity (η)	Decreases
Temperature (T)	Increases
Sample Molecular Weight (M)	Decreases

2.2.1.5 Resolution

The separation of the peaks is dependent on both kinetic and thermodynamic factors. To calculate resolution, the thermodynamic factors are divided by the kinetic factors, having a ratio between the peak separation and the average peak width, as equation 7 demonstrates [21].

$$R_s = \frac{2(t_{r_1} - t_{r_2})}{w_1 + w_2} \qquad \text{(eq.7)}$$

Resolution is very important to define the peaks but in practice rarely is calculated and it's the chromatographer that looks directly to the chromatogram and defines the resolution. Of course, the concentration of the components usually isn't the same, so the peaks shape differs [21].

2.2.1.6 Tailing factor

In ideal conditions a chromatography peak shows a perfect symmetry being representative of a Gaussian peak. In practice this doesn't occur due to possible chemical reactions, isomerization, column overload and absorption or other strong reactions between the analyte and the stationary phase. Figure 2.5 demonstrates that the peak can be slightly tailing or fronting, meaning that can have a little distortion to the left or right, and equation 8 shows how to calculate it.

$$T_f = \frac{W_{0.05}}{2f} = \frac{AC}{2AB} \qquad \text{(eq.8)}$$

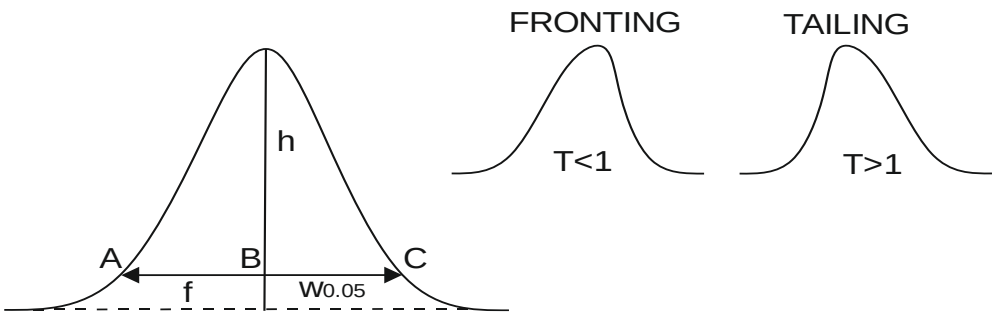


Figure 2.5 - Peak tailing and fronting [20].

The tailing factor normally should be between 0.9 and 1.4, being that the value of 1 represents a perfect symmetry.

2.2.1.7 Resolution equation

When developing a method is convenient to calculate the resolution merging three factors: Retention, Selectivity and Efficiency, as shown in equation 9.

$$R_s = \left(\frac{k'}{k'+1}\right)\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{\sqrt{N}}{4}\right) \qquad \text{(eq.9)}$$

Retention Selectivity Efficiency

Observing the resolution equation, it is possible to indicate that the three factors are relatively independent and to obtain a good resolution, changes in these parameters must be made.

2.2.1.8 Signal to noise (S/N)

When working with low concentrations of analyte the noise produced by the baseline can affect the analyses of the chromatogram, since that at a low scale, the baseline isn´t a straight line (it as ups and downs) and the detection of a certain peak may be very difficult to do. The signal to noise ratio is a very important factor to determine the performance of a certain peak, and to determine if that peak is easily detected. If the maximum height of a peak as similar values to the baseline noise, it means that both can´t be discriminated and the detection of the analyte in this concentration is not possible. Usually the minimum value of S/N accepted for the lower LOD (limit of detection) is 3 [18]. Figure 2.6 is representative of the measurement of s/n.

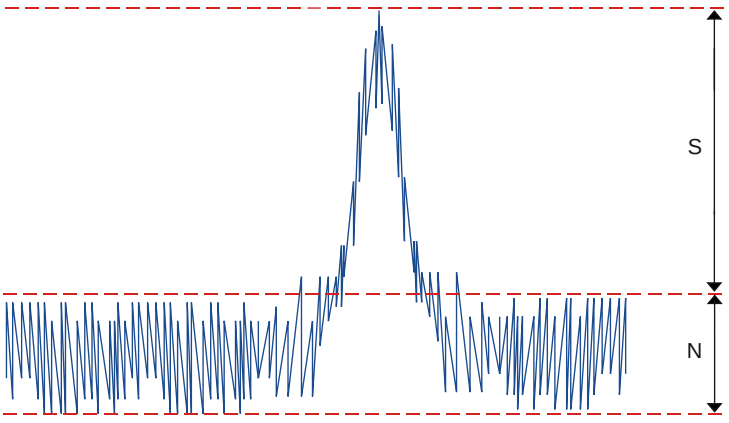


Figure 2.6 – Chromatographic noise (N) and signal (S) [22].

2.2.2 Columns

An HPLC system has some components that have influence on the performance of the chromatography, and one with the most importance is the type of column used.

Parameters such as the size and the composition of the column filling significantly influence the results obtained. Since the separation of different components in a chromatography occurs in the column, his chemistry is of great importance, and it varies according to the type of analyte being separated. The length of the column manly interferes with the retention time of the analyte in the column.

2.2.2.1 Particle Type

Many configurations for the type and size of the particle are currently available for use, as presented in Figure 2.7. Totally porous particles (a) and superficially porous particles (b) are the two types most used.

Totally porous particles have many advantages such as being able to allow larger injection volumes due to its larger column capacity and has more variety of options in the stationary phase, column, particle and pore size [18].

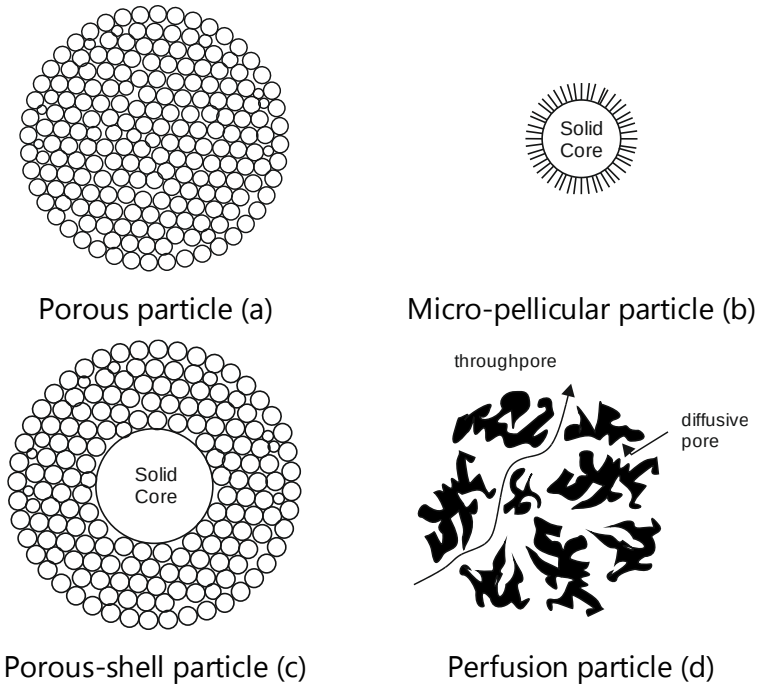


Figure 2.7 - Column particle types. [18]

Superficially porous particles are a very good option to increase the number of plates compared to the totally porous particles. As shown in Figure 2.7 it is a solid core surrounded by a porous shell.

Pellicular particles have a thin layer of stationary phase that surrounds a solid particle. It can only be used to inject small weight samples because they present a very low surface area and

as such their retention is reduced. This is an old technology, and the particle size is much bigger than the other, in the range of 30-80 μm [23].
Perfusion particles combine two sets of pores: Throughpores and diffusive pores. This allows a combination of diffusive and convective flow enabling better access of macromolecules to the inner of the particle [24].

2.2.2.2 Column chemistry

When separating different analytes, the packing of the column used is of the most importance and has a big influence in the separation results. There are different types of HPLC that can be chosen, and the choice of the type of column must be accordingly [25].
Partition chromatography is widely used and it´s a recurring choice for HPLC methods. It consists of the separation of solutes based on their partitioning between a stationary phase coated on a solid support and a liquid mobile phase. There are also other types of HPLC such as adsorption chromatography, ion exchange chromatography, size exclusion and affinity chromatography [26].
There are two main types of partition chromatography that are based on the polarity of the stationary phase: normal phase and reversed phase chromatography [27, 28].
In normal phase chromatography the stationary phase is polar and as such it shows greater retention for polar compounds. Reversed phase chromatography uses nonpolar stationary phase and uses weak polar mobile phases such as water, justifying the popularity of this type of HPLC [26].
RP-HPLC (reversed phase high pressure liquid chromatography) is commonly used because of its reliability, viability, reproducibility and practicality of use [25].
There are many different types of stationary phases used in HPLC, as described in Table 2.4, and they can be divided into three different groups: inorganic, polymeric and hybrid materials. Silica is the inorganic packing that has the most characteristics for an ideal support, thus being the predominant choice [29].
Figure 2.8 shows the molecular structure of bare silica that is the base for many stationary phases, changing the functional group that is bonded to silica. The most popular are the ODS (octadecyl silica columns), known as C18 columns, since the most used HPLC chromatography methods are reversed phase, and ODS are the best for that end [29].
In the unbonded region of the surface of the silica there are multiple silanols that react with molecules to form the stationary phase. Usually are used alkyl bonded ligands like C2, C8 and C18 to prepare packings for RP-HPLC [30].

Figure 2.9 shows examples of the molecular structure of some packings normally used.

Table 2.4 - Types of stationary phase used in HPLC [31].

Stationary Phase
Silica
Styrene-Divinylbenzene
Alumina
Magnesium Silicate
Controlled-Pore Glass
Hydroxyalkylmethacrylate Gel
Hydroxylapatite
Agarose
Porous Graphitic Carbon
Titania
Zirconia
Restricted Surface Access Phases

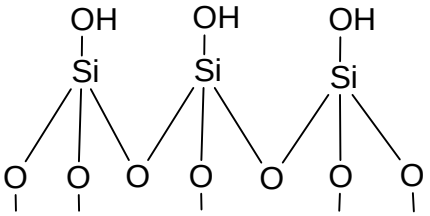


Figure 2.8 - Bare silica particle surface [32].

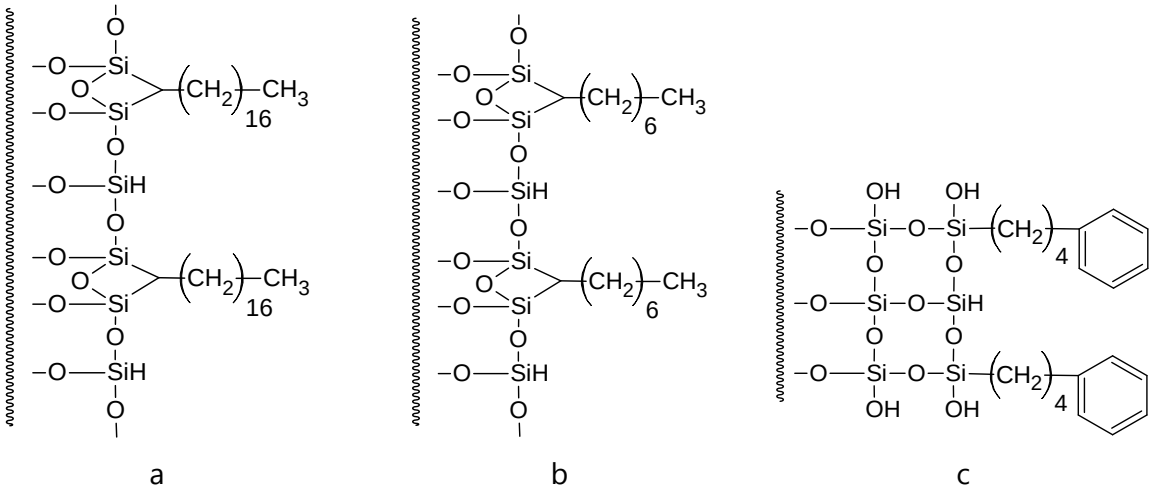


Figure 2.9 - (a) C18, (b) C8 and (c) phenyl bonded to silica hydride [33].

ODS columns are commonly used, they present a large alkyl chain and therefore the mobile phase has much more surface to cover, meaning that the separation could be much more efficient. Another advantage of using C18 columns is the low pressure in the system when the flow is increased, allowing to increase the flow in order to reduce the time of the analyses [34]. Figure 2.10 represents a comparison of multiple options of columns available on the market, showing the asymmetry factor (A_s) of the different columns. A high A_s means that the peaks will present fronting or tailing, needing for them to have a much more bigger gap between their retention times to have a good resolution.

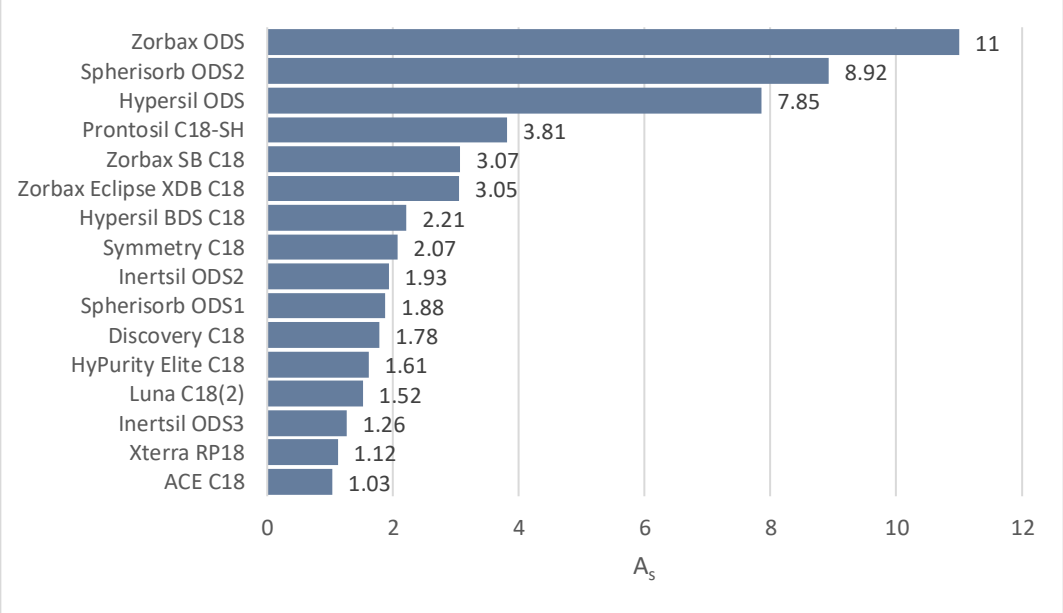


Figure 2.10 - Comparison of different C18 column brands [35].

2.3 Method Development

Liquid chromatography methods are extensively utilized in the pharmaceutical industry, in this case in the production of cephalosporins. Ensuring the purity of these products and detecting potential contaminants is crucial. As a result, various chromatographic techniques have been developed and tailored to the specific characteristics of the product or its contaminants, allowing for precise identification and quality control.

HPLC is the most used technology for analyzing cephalosporins. However, UPLC (Ultra-Performance Liquid Chromatography) is also widely employed due to its ability to provide shorter analysis times and greater efficiency. UPLC systems offer improved resolution and sensitivity

compared to HPLC, making them highly effective for detecting and quantifying cephalosporins in a more time-efficient manner. The main difference between these two is the particle size of the column, and there is the possibility to transition from HPLC to UPLC and vice versa.

When developing an analytical method in the pharmaceutical industry, there is a minimal approach to be taken that consists in four main objectives [36]:

- Identification of the attributes and characteristics of the drug that need to be tested
- Choice of the appropriate equipment and technology in which the characteristics in study will be detected and evaluated.
- Conduction of experiments having in mind characteristics of the method itself, such as accuracy, repeatability, specificity, range and robustness.
- Definition of a procedure strategy and analytical procedure description.

The first approach to be taken is evaluating the conditions that will affect the results pretended with the method, and for that the 6 M's analyses can be used, to divide and identify the major variability in the specific case in hands. Figure 2.11 represents the 6 M's analyses using an Ishikawa diagram for a better understanding of the characteristic of the method that will have more impact, and as such, lead the development of the method.

After assessing the key variables to manipulate, it is essential to review existing methods for detecting cephalosporins and gather detailed information on these variables. Since the primary goal is to detect Cefepime and Ceftazidime, the focus should be on methods specifically tailored to these cephalosporins.

The two main categories that influence the method are the Methods and the Machines. It is easier to optimize conditions such as the mobile phase composition, buffer type, pH of the mobile phase, injection volume, and column selection to achieve the desired outcomes.

Table 2.5 and Table 2.6 resume some of the HPLC methods developed that detect cephalosporins, specifically Cefepime and Ceftazidime. Some of them detect multiple Cephalosporins but don't detect simultaneously all the eight cephalosporins produced in Hikma 2, Cefazolin (CZ), Cefepime (CP), Ceftazidime (CZI), Ceftriaxone (CFT), Ceftizoxime (ZOX), Cefuroxime (CFR), Cefoxitin (CX) and Cefotaxime (TAX).

According to the literature, reversed phase C18 columns are the most chosen for the separation of Cephalosporins, typically operated at temperatures close to ambient. The flow rate is generally around 1 mL/min, likely because it provides an optimal balance between maintaining reasonable pressure within the column and ensuring efficient separation without overly slow run times.

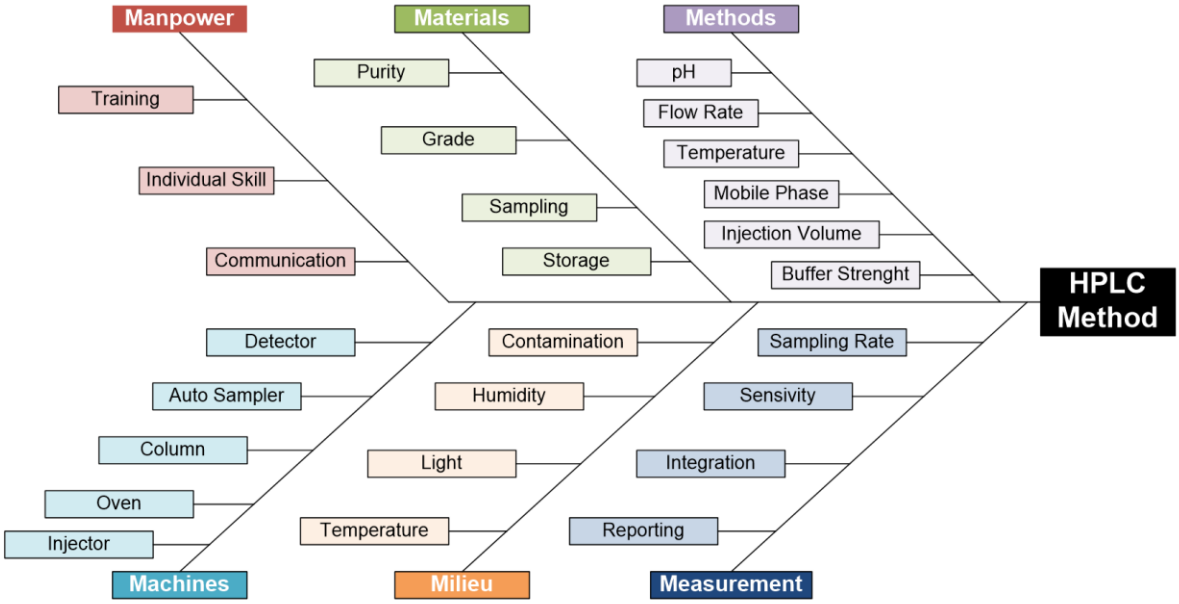


Figure 2.11 - Ishikawa diagram for the 6 M's approach in a HPLC method development [37].

The detection wavelength for Cephalosporins ranges from 220 nm to 285 nm. However, when conducting contamination analysis, it is crucial to carefully consider potential interferences that may arise from selecting a wavelength that is either too low or too high, as this can impact the accuracy of detecting Cephalosporins in the presence of other compounds. The mobile phases predominant in the detection of Cephalosporins, as Tables 2.5 and 2.6 demonstrate, are phosphate-based buffers and ammonium acetate buffers, containing an organic compound such as methanol and acetonitrile.

Using a mobile phase based on a phosphate buffer containing acetonitrile [38], it is possible to successfully detect and separate six of the Cephalosporins produced in Hikma 2. Using gradient altering the percentage of acetonitrile in a 25 mM potassium dihydrogen orthophosphate buffer, CZ, CFT, CP, CZI, CX and TAX were separated [38]. Additionally, a mobile phase composed by 100 mM ammonium acetate with 5% acetonitrile also demonstrates the ability to separate CP, CZI, TAX, CZ and CFT [39]. The separation accomplished with a phosphate buffer solution was performed out on a C18 column, while the separation that used the ammonium acetate buffer was carried out on a C8 column. Notably the pH of the mobile phase differs between these two methods, with values of 4.4 [38] and 5.6 [39].

The ammonium acetate buffer provides good resolution in separating Cephalosporins, with the last Cephalosporin, CZ, eluting at 30 min. Using the same concentration of ammonium acetate buffer but increasing the acetonitrile content to 10% using a C18 column [40], the resolution remains consistent, while the elution time is reduced by nearly half, using as example

TAX, that with 5% acetonitrile in a C8 column has a retention time of 10.94 min [39], and with the C18 column and 10% acetonitrile has a retention time of 5 min [40].

A gradient flow is commonly used because it allows for easier adjustment of the organic phase concentrations at specific times when a compound is expected to elute, optimizing both peak shape and run time. While this approach is beneficial in some cases, it can pose challenges in contamination detection. Contaminants may elute during the changes in the flow, which causes baseline fluctuations in the chromatogram and obstructs detection of the contaminants.

Table 2.5 - HPLC methods for detecting cephalosporins (part 1/2).

Cephalosporins Detected	Flow	Column Temperature	Mobile Phase pH	Detector Wavelength	Injection Volume	Flow Rate	Reference
CFT, CP, CZI	isocratic	25ºC	4.6	254-270 nm	20 µL	1 mL/min	[41]
CZ, CFR, CX, TAX	gradient	35ºC	5	-	10 µL	0.4 mL/min	[42]
CFT, CP, CZI, TAX	gradient	32ºC	3.2	260-285 nm	20 µL	0.85 mL/min	[43]
CZ, CP, CZI, TAX	gradient	40ºC	2	230-260 nm	10 µL	0.5 mL/min	[44]
CZ	isocratic	RT	5.2	254 nm	10-30 µL	2.5 mL/min	[45]
CZ, TAX	gradient	30ºC	8.5	-	10 µL	0.3 mL/min	[46]
CFT, CZI, TAX	isocratic	RT	7.5	270 nm	20 µL	1.5 mL/min	[40]
CP	isocratic	RT	4	280 nm	10 µL	1.5 mL/min	[47]
CP	isocratic	RT	3	270 nm	20 µL	1 mL/min	[48]
CP	isocratic	35ºC	4	280 nm	100 µL	1 mL/min	[49]
CP	isocratic	RT	3	270 nm	5 µL	1 mL/min	[50]
CP, CZI	isocratic	40ºC	4	254 nm	10 µL	1 mL/min	[51]
CZI	isocratic	RT	3	254 nm	10 µL	0.05 mL/min	[52]
CZI	isocratic	RT	7.4	-	20 µL	0.9 mL/min	[53]
CZI	isocratic	RT	5	258 nm	50 µL	1 mL/min	[54]
CZI	isocratic	35ºC	5	258 nm	20 µL	1 mL/min	[55]
CZI, TAX	isocratic	25ºC	7	254 nm	50 µL	1 mL/min	[56]
CZ, CFR, CX, TAX	gradient	20ºC	4.7	-	50 µL	1 mL/min	[57]
CZ, CZI,TAX	isocratic	30ºC	3.6	230 nm	10 µL	0.8 mL/min	[58]
CFT	isocratic	RT	7	267 nm	20 µL	0.3 mL/min	[59]
ZOX	isocratic	RT	7	255 nm	50 µL	1 mL/min	[60]
CZ, CFT, CP, CZI, CX, TAX	gradient	25ºC	4.4	-	20 µL	1mL/min	[38]
CP, CZI	gradient	25ºC	7.4	220-300 nm	40 µL	1 mL/min	[61]
CZ, CFT, CP, CZI, TAX	gradient	25ºC	7	264 nm	-	-	[62]
CZ	gradient	-	-	-	10 µL	0.3 mL/min	[63]
CFT, CZI	gradient	40ºC	-	-	10 µL	0.4 mL/min	[64]
CFT, TAX	isocratic	-	-	-	20 µL	0.8 mL/min	[65]
CFT, CP, CZI, TAX	gradient	-	2	230 nm	10 µL	2.0 mL/min	[66]
CFR, CZI, CX	isocratic	20ºC	5.6	274 nm	50 µL	1 mL/min	[67]
CZI	isocratic	-	5.5	255 nm	100 µL	1 mL/min	[68]
CZI	isocratic	40ºC	-	258.8 nm	40 µL	0.8 mL/min	[69]
CP	isocratic	-	2	263 nm	20 µL	1 mL/min	[70]
CZ, CFT, CFR, CP, CZI, TAX	gradient	20ºC	-	-	5 µL	0.8 mL/min	[71]
CZI, TAX	isocratic	-	2.5	260 nm	100 µL	0.9 mL/min	[72]
CZ, ZOX	isocratic	-	5	262 nm	100 µL	4 mL/min	[73]
CZ, CFT, CFR, CP, TAX	-	20ºC	-	254-366 nm	-	-	[74]
CZ, CX, TAX	gradient		1.5		500 pmol	0.8-1 mL/min	[75]
CP, CZI, TAX, CZ, CFT	isocratic	30ºC	5.6	250 nm	25 µL	0.8 mL/min	[39]

Table 2.5 - HPLC methods for detecting cephalosporins (part 2/2).

Mobile Phase		Column			Ref.
A	B	Type	Dimensions	Particle Size	
50mM potassium phosphate, ACN (80:20, v/v)		µBondapak C18	250 x 4.6 mm	10 µm	[41]
0.1% formic acid in UPW	0.1% formic acid in ACN	Acquity UPLC BEH C18	50 x 2.1 mm	1.7µm	[42]
40mM phosphate buffer	MeOH	Xterra C18	250 x 4.6 mm	5 µm	[43]
10mM phosphoric acid	ACN	Hypersil Gold PFP	100 x 2.1 mm	1.9 µm	[44]
0.01M sodium acetate - ACN - MeOH (91:8:1, v/v)		µBondapak C18	300 x 4 mm	10 µm	[45]
0.1% formic acid in water	MeOH	Acquity BEH Shield RP18	100 x 2.1 mm	1.7 µm	[46]
0.1 M ammonium acetate - ACN (90:10, v/v)		Schimpack GLC-ODS	150 x 6 mm	5 µm	[40]
water - 0.015 M pentane sulfonic acid in ACN (94.5:5.5, v/v)		Perkin Elmer phenyl	100 x 4.6 mm	5 µm	[47]
100mM monosodium phosphoric acid - MeOH (87:13, v/v)		LiChrosorb RP-18	250 x 4.6 mm	5 µm	[48]
20 mM ammonium acetate - ACN (90:10, v/v)		Hypersil Nucleosil C18	100 x 4.6 mm	5 µm	[49]
0.025M sodium phosphate - MeOH (87:13, v/v)		Hypersil BDS C18	250 x 4.6 mm	5 µm	[50]
0.05 M ammonium acetate - ACN (97.2:2.8, v/v)		Hypersil BDS C18	150 x 4.6 mm	5 µm	[51]
MeOH – ACN – 100mM monosodium phosphoric acid (10:10:80, v/v)		Bioanalytical Systems RP-C18 microbore	150 x 1 mm	5 µm	[52]
25 mM KH2PO4/Na2HPO4 buffer - ACN (90:10, v/v)		RP Kromasil C8	250 x 4.6 mm	5 µm	[53]
10mM sodium dihydrogen orthophosphate - ACN (92:2, v/v)		HP ODS	150 x 4 mm	5 µm	[54]
50mM sodium dihydrogenphosphate buffer - ACN (96:4, v/v)		Nucleosil C18	250 x 34.6 mm	5 µm	[55]
25.7 m M KH_2PO_4 in 30 mM Na_2PO_4 - MeOH (85:15, v/v)		Shim-Pack C18	250 x 4.6 mm	5 µm	[56]
0.01 M acetate buffer – MeOH – ACN (87:11:2, v/v/v)	0.01 M acetate buffer - MeOH - ACN (87:2:11, v/v/v)	Nova-Pak C18 Radial-Pak cartridge	100 x 8 mm	4 µm	[57]
MeOH - buffer solution (42:58, v/v)		β-CyD	250 x 4.6 mm	5 µm	[58]
MeOH – ACN – 10 mM phosphate buffer (20:15:65)		Supelcosil LC-18	150 x 34.6 mm	3 µm	[59]
20% MeOH in 40 mM potassium phosphate		Ultrasphere C8	250 x 4.6 mm	5 µm	[60]
ACN	25 mM phosphate buffer	Nucleosil C18	250 x 4.6 mm	5 µm	[38]
ACN	phosphate buffer	Symmetry C8	250 x 4.6 mm	5 µm	[61]
Trifluoroacetic Acid 0.1%	ACN	Kromasil C8	250 x 4.6 mm	5 µm	[62]
0.1% (v/v) formic acid	MeOH	Acquity UPLC BEH C18	100 x 2 mm	1.7 µm	[63]
-	-	Acquity Hclass PLUS	-	-	[64]
CH3CN 1%(v/v) acetic acid (7:13, v/v) (A)	CH3CN and an accelerating solvent (100:1, v/v)	ODS TSK-gel	150 x 2 mm	5 µm	[65]
10 mM phosphoric acid	ACN	Atlantis T3	150 x 4.6 mm	5 µm	[66]
2.5 mM sodium phosphate and MeOH (40:9, v/v)		Lichrospher 100 RP-18	125 x 4.6 mm	-	[67]
(9:91) ACN - phosphate buffer		µBondapak C18	300 x 3.9 mm	10 µm	[68]
25% ACN containing 0.005 M dodecanesulfonate		Ultremex phenyl	150 x 4.6 mm	3 µm	[69]
ACN – 20mM potassium buffer (6:94, v/v)		Supelcosil ABZ1	150 x 4.6 mm	5 µm	[70]
0.1% acetic acid	0.1% acetic acid in ACN	ZORBAX Eclipse XDB-C18	150 x 4.6 mm	5 µm	[71]
10mM potassium phosphate monobasic - ACN (90:10, v/v)		Symmetry C18	250 x 4.6 mm	5 µm	[72]
deionized water - methanol - acetic acid (60:40:0.5, v/v)		ODS C18	150 x 0.3 mm	5 µm	[73]
water and organic modifier (MeOH, ACN and 2-propanol)		RP-18 F254 Merck	100 x 70 mm		[74]
ACN with 1% TFA	water with 1% TFA	capilary AQ-C18	1.5 m x 75 µm	5/10 µm	[75]
0.1M ammonium acetate buffer – ACN (95:5, v/v)		RP Agilent C8	250 x 4.6 mm	5 µm	[39]

2.4 Method Validation

When developing an analytical method for drugs, it is essential to verify the method's reliability, which is achieved through validation. Validation ensures that the method is accurate, precise and linear under the specified conditions, proving confidence in the results obtained. Accordingly, to the ICH (International Council of Harmonization) guidelines Q2(R1) and Q2(R2) [76, 77], the characteristics that should be evaluated consist in:

- Accuracy
- Precision
    - Repeatability
    - Reproducibility
    - Intermediate Precision
- Specificity
- Limit of Detection (LOD)
- Limit of Quantitation (LOQ)
- Linearity
- Range

The characteristics to be addressed during validation depend on the type of analysis the method is intended for. For a method focused solely on identification, only the specificity needs to be assessed. However, if the method is for impurity testing or assay purposes, nearly all validation parameters must be evaluated to ensure the method's reliability across various aspects.

2.4.1 Accuracy

The accuracy of an analytical procedure is assessed by determining how closely the results obtained align with the true or accepted reference value. Accuracy is often expressed as the percentage recovery of known amounts of analyte added to a sample, and it serves as a measure of the exactness of the analytical procedure. Accuracy can be assessed through different approaches, such as determining the analyte’s recovery across the assay range or evaluating the linearity of the relationship between the actual concentrations and the estimated values [76-78].

2.4.2 Precision

The precision of an analytical procedure refers to the consistency of individual results when the procedure is applied repeatedly to multiple samples of a homogeneous sample. The precision of an analytical procedure is expressed as the standard deviation (coefficient of variation), and it can be divided in three different aspects: reproducibility, repeatability and intermediate precision [77, 78].

Precision may be a measure of the degree of either reproducibility or repeatability of the analytical method under normal operating conditions. Reproducibility refers to applying the method across different laboratories, while repeatability refers to using the procedure within the same laboratory over a short period, with the same analyst and equipment. Intermediate precision (or ruggedness) assesses variations within the same laboratory over different days. However, if reproducibility is demonstrated, performing intermediate precision is not mandatory [77, 78].

2.4.3 Specificity

This characteristic determines whether an analytical procedure can accurately and specifically measure the analyte in the presence of other substances, such as placebo or active ingredients. It is often expressed as the degree of bias observed in test results when analysing samples containing these added impurities when compared to test results from samples without added impurities [77, 78].

2.4.4 Limit of Detection

Limit of detection is the lowest concentration of the analyte in a sample that can be detected but not necessarily quantified under the stated experimental conditions. It is expressed as the concentration of analyte (e.g. percentage part per million in the sample). Limit of detection can also be specified based on a slope and a standard deviation of a linear response, as presented in equation 10.

$$LOD = \frac{3.3\sigma}{S} \qquad \text{(eq.10)}$$

σ - standard deviation of the response

S - slope of the calibration curve

The standard deviation can be based on the standard deviation of the blank or on the calibration curve [77, 78].

2.4.5 Limit of Quantitation

Limit of Quantitation is a critical parameter when analysing low levels of compounds, particularly in sample, matrices like impurities in bulk substances and finished dosage forms. It represents the lowest concentration of an analyte that can be both detected and accurately quantified with acceptable precision and accuracy, under specified experimental conditions. The LOQ is expressed as the analyte concentration in the sample, and similar to the LOD, it can be determined using the slope of the calibration curve and the standard deviation of the response in a liner regression model [77, 78], as shown in equation 11.

$$LOD = \frac{10\sigma}{S} \qquad \text{(eq.11)}$$

2.4.6 Linearity

Linearity is a parameter that demonstrates directly or with the application of well-defined mathematical transformations, that the analytical results are proportional to the concentration of analytes within a specified range. When a plot of signal versus analyte concentration visually shows a linear relationship, this observation must be confirmed through appropriate statistical methods, such as linear regression using the method of the least squares. This ensures that the method´s performance is linear across the concentration range being analysed [77, 78].

2.4.7 Range

The range is defined by the interval between the lower and higher concentration of analyte that presents accuracy, precision and linearity [78].

3

MATERIALS AND METHODS

The method development started from the ground up, rather than being a modification of the existing method within the company. For that reason, the initial approach used a simpler method, making it easier to modify if necessary. Literature describes a method able to detect five of the cephalosporins produced in Hikma 2 [39], Cefazolin Sodium, Cefotaxime Sodium, Ceftriaxone Sodium, Cefepime Hydrochloride and Ceftazidime. As such the starting point of the development was based on this method.

3.1 Materials

3.1.1 Reference Substances

Since the work was merely for detection some final products were used as standard:

- Cefepime USP standartd
- Ceftazidime USP standard
- Cefotaxime USP standard
- Cefepime final product
- Ceftriaxone final product
- Ceftizoxime final product
- Cefuroxime final product
- Cefazolin final product
- Cefoxitin final product

3.1.2 Reagents

- Ammonium acetate
- THPAB (tetraheptyammonium bromide)
- Sodium acetate
- potassium dihydrogenphosphate

- Phosphoric acid
- Glacial acetic acid
- ACN (Acetonitrile) HPLC grade
- MeOH (Methanol) HPLC grade
- DIW (Deionized water)

3.1.3 Equipment

- Analytical balance
- Potenciometric pH metre
- Waters alliance HPLC System, with a separation module e2695 and a UV/visible detector 2489
- Ultrassonic bath

3.1.4 Sampling materials

- PVDF disposable filter, pore size 0.45 µm, diameter 25 mm, Whatman®
- Xtra PTFE, pore size 0.45 µm, diameter 25 mm, Chromatil®
- TX 761K swabs, Texwipe®
- Sterile swabs, Cultiplast®

3.1.5 Chromatographic colunms

- Agilent Zorbax RX-C8 5 µm, 4.6 x 250 mm
- Symmetry C18 5 µm, 4.6 x 250 mm
- Xterra MS C18 5 µm, 4.6 x 250 mm
- XBridge BEH C18 5 µm, 4.6 x 250 mm

3.2 Method Development

In addition to detecting all eight cephalosporins, the primary goal of this method development is to achieve complete separation of the cephalosporins. During the development stage, it was necessary to prepare different sample solutions:

- Eight individual solutions, one for each cephalosporin, to identify the peaks, at a concentration of 5 ppm.
- One mixture containing all eight cephalosporins at a concentration of 5 ppm.
- One mixture containing all eight cephalosporins at a concentration of 0.02 ppm.

The 0.02 ppm solution was used because it represents the LOD in the company's current method, and the new method should not have a lower LOD. In the early stages of development, only the 5 ppm concentration solutions were injected, as the primary focus was to first establish a method capable of fully separating the cephalosporins. Once that objective was achieved, the 0.02 ppm solution was injected to assess the method's performance at lower concentration levels.

These solutions were prepared once a week to ensure that the degradation of the cephalosporins wasn't affecting the results.

In total, were prepared 5 different mobile phase buffers based on the best results of the literature review as shown in Table 3.1. To simplify understanding, each mobile phase will be referred to as method A, B, C, D or E.

Table 3.1 - Mobile phase buffer attempts in method development.

Method	Mobile Phase Buffer	pH	Reference
A	100mM ammonium acetate	5.6	[39]
B	100mM ammonium acetate + THPAB	5.6	[39]
C	25mM KH_2PO_4 + 326mM Na_2HPO_4 + tetraheptylammonium bromide	5	[79]
D	10mM sodium acetate	4.7	[57]
E	40mM potassium dihydrogenphosphate	3.2	[43]

Table 3.2 presents the column used for each mobile phase tested, along with the corresponding temperature settings for the column. Every column used had 250 x 4.6 mm and a particle size of 5 µm.

Table 3.2 - Columns used in method development and their temperature settings.

Method	Column	Column Temperature
A, B, C	Agilent Zorbax RX-C8	30ºC
D	Agilent Zorbax RX-C8 XTerra MS C18 Symmetry C18	20ºC
E	XTerra MS C18 XBridge BEH C18	32ºC

An organic component (ACN or MeOH) was included in the mobile phase for every method attempt, except for Method C, where the only variation was the concentration of THPAB) in the mobile phase. However, to simplify the process and avoid the need to prepare a new mobile phase for every trial, separate channels of the HPLC equipment were used for the buffer and the organic phase, similar to a gradient flow but with the same percentage of each channel throughout the entire run. This setup allowed for easier adjustments and blending during the method development. Table 3.3 presents the configurations of concentration for each method.

Table 3.3 - Organic concentrations tested in every method

%MeOH \ %ACN	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	25	30
0	E					A	A	A	A	A B	A E	A E	A E	A D E	A E	A E	A E	A E	A E	A E	A E	C	C
1	E												D E	E	E		E						
2	E												E	D E	E		E						
3	E										D		E	E	E		E						
4	E												E	D E	E		E						
5	E								D				E	E	E		E						
6	E												E	D	E		E						
7	E						D						E		E		E						
8	E												E		E		E						
9	E				D								E		E		E						
10	E												E		E		E						
11	E														E								
12	E	D													E								
13	D E	E	D	E	D E	E	D E	E	E	E	E				E								
14	E					E			E						E								
15	E	E		E	E	E	E	E	E	E	E				E								
16	E					E			E						E								
17	E	E		E	E	E	E	E	E	E	E				E								
18	E				E	E			E						E								
19	E	E		E	E	E	E	E	E	E	E				E								
20	E				E	E			E						E								
21	E	E		E	E	E	E	E	E	E	E												
22	E				E	E			E														
23	E	E		E	E	E	E	E	E	E	E												
24	E				E	E			E														
25	E	E		E	E	E	E	E	E	E	E												
26	E				E				E														
27	E																						
28	E																						

Every injection made during the development stage is presented in Appendix B.

In addition, various solvents used in the preparation of the standards were tested to evaluate their impact on the baseline and determine which solvent would not interfere with the HPLC analysis. Table 3.4 lists all the solvents tested for this purpose.

Table 3.4 - Solvent composition attempts.

DIW (%)	MeOH (%)	ACN (%)
82	13	5
95	0	5
87	13	0
90	5	5
80	10	10
70	15	15
40	30	30
70	20	10
65	20	15
75	15	10
85	10	5

3.2.1 Mobile Phase Preparation

3.2.1.1 Method A

Preparation for 1 L of mobile phase:

- 7.708 g of ammonium acetate, dissolved in 1L of deionized water to obtain the concentration of 100 mM.
- pH adjusted to 5.6 using glacial acetic acid.

3.2.1.2 Method B

Preparation for 1 L of mobile phase:

- 7.708 g of ammonium acetate, dissolved in 1L of deionized water to obtain the concentration of 100 mM. Then added 100 mg of THPAB.
- pH adjusted to 5.6 using galcial acetic acid.

Two more mobile phases were prepared using different amounts of THPAB (one above the concentration already prepared and other below).

Preparation for 1L of the mobile phase with lower concentration of THPAB:

- 7.708 g of ammonium acetate, dissolved in 1L of deionized water to obtain the concentration of 100 mM. Then added 50 mg of THPAB.
- pH adjusted to 5.6 using galcial acetic acid.

Preparation for 1L of the mobile phase with higher concentration of THPAB:

- 7.708 g of ammonium acetate, dissolved in 1L of deionized water to obtain the concentration of 100 mM. Then added 150 mg of THPAB.

- pH adjusted to 5.6 using galcial acetic acid.

3.2.1.3 Method C

Preparation for 1 L of mobile phase:

- 1 g of THPAB, dissolved in 250 mL of acetonitrile. Then added 250 mL of 100 mM of potassium dihydrogenphosphate, 326 mL of 100 mM of sodium hydrogenphosphate and water to make 1000 mL of solution.
- pH adjusted to 5 using acid phosphoric.

3.2.1.4 Method D

Preparation for 1 L of mobile phase:

- 820.3 mg of sodium acetate, dissolved in 1L of deionized water to obtain the concentration of 10 mM.
- pH adjusted to 4.7 using acetic acid.

3.2.1.5 Method E

Preparation for 1 L of mobile phase:

- 5.45 g of potassium dihydrogenphosphate dissolved in 1L of deionized water to obtain the concentration of 40 mM.
- pH adjusted to 3.2 using phosphoric acid.

3.2.2 Standard Solution Preparation

3.2.2.1 5ppm Standard solution

In a 100 mL volumetric flask, 20 mg of each cephalosporin was weighed and dissolved in solvent. To achieve a concentration of 5 ppm, 5 mL of this solution was transferred to a 200 mL volumetric flask and diluted to the mark with solvent.
The solvent used was deionized water.

3.2.2.2 Individual standard for peak identification

The same procedure described in 3.2.1.1 was made for each cephalosporin individually, making a total of eight different solutions.

3.2.3 Sampling

After establishing the conditions necessary to separate all the Cephalosporins, the sampling procedure was tested to determine if the swabs used for surface and hand sampling, as well as the syringe filters use in this procedure, would interfere with the Cephalosporins detection. Two different types of swabs and two different filters were tested for this purpose:

- Swabs: TX 761K (Texwipe®) and sterile swabs (Cultiplast®).
- Filters: PVDF (Whatman®) and PTFE (Chromatil®).

3.2.3.1 Sampling procedure

A solvent solution was prepared, and 10 mL were added to the swab flask. This solution was manually agitated for approximately 10 seconds, followed by an additional 10 seconds in an ultrasonic bath. After agitation, this solution was transferred to an HPLC vial using a syringe with a filter.

3.3 Validation Protocol

The validation protocol was designed to validate HPLC method of analysis for Cephalosporins containment monitoring, surface samples. The validation is to be conducted in accordance with ICH guidelines (ICH Q2) and Hikma Farmacêutica -Portugal validation of analytical procedures SOPSNT16801.

3.3.1 Method of swabing

- Swabbing Area: 100 cm^2
- Swabbing Solvent: Deionized water
- Number and Type of Swab:
  Texwipe Product Number: TX 761K
- Number and Type of Filter:
  25 mm GD/X Disposal Filter Device, PVDF filter media, Whatman®
  Pore Size: 0.45 µm

3.3.2 Mobile Phase Preparation

Dissolve 5.45 g of potassium dihydrogen phosphate to 820 mL of deionized water. Add 130 mL of methanol and 50 mL of acetonitrile, mix well. Adjust pH to 3.2 using phosphoric acid.

3.3.3 Solvent Solution Preparation

Deionized water, methanol and acetonitrile (82:13:5).

3.3.4 Standard Solution Preparation (5 ppm)

Standard Stock Solution: Weight accuratly about the equivelent to 20 mg of each, Ceftizoxime Na, Cefotaxime Na, Cefuroxime Na, Cefazolin Na, Cefoxitin Na, Ceftriaxone Na, Cefepime and Ceftazidime to a 100 mL volumetric flask. Dissolve and dilute with solvent solution.
Standard Solution: Dilute further 5 mL of the standard solution to 200 mL with solvent solution.

3.3.5 Standard Solution Preparation (0.02 ppm)

Dilute 1 mL of 5 ppm working standard to 25 mL and dilute further 1 mL to 10 mL using solvent solution.

3.3.6 Sample solution Preparation

Add exactly 1o mL of a mixture of deionized water, methanol and acetonitrile (82:13:5) to each swab sample under analysis.
Shake the test sample for ten seconds (by hand) then sonicate for anotherr ten seconds and filter through a 0.45 µm filter.

3.3.7 Chromatographic Conditions

Inject 25 µL of each solvent, standard, and sample solutions under the following conditions:

- Column : XBridge C18 5 µm (250 x 4.6 mm)
- Flow rate : 1.2 mL/min
- Wavelength : 260 nm
- Column oven temperature : 32 ºC
- Sample cooler temperature : 8 ºC
- Run time : 30min

3.3.8 System Suitability Requirements

- The %RSD of peak area of each Cephalosporin in six replicate injections of the standard solution should be no more than 10%.
- Resolution between consecutive Cephalosporins should be no more than 1.

3.3.9 Calculations

3.3.9.1 Relative Standard Deviation:

$$\%RSD = \frac{\sigma}{\bar{x}} \times 100 \quad \text{(eq.12)}$$

σ - Standard deviation

$\bar{x}$ - Mean value

$\%RSD$ - Relative standard deviation

3.3.9.2 Standard deviation

$$\sigma = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \quad \text{(eq.13)}$$

x - sample value

n - number of analyses

3.3.9.3 Recovery

$$\%Recovery = \frac{PA_{SMP} \times [STD]}{PA_{STD} \times [SMP]} \quad \text{(eq.14)}$$

$[STD]$ - Concentration of Cephalosporin in the standard, ppm

$[SMP]$ - Concentration of Cephalosporin in the recovery sample, ppm

PA_{SMP} - Peak area of Cephalosporin in the recovery sample, µV.sec

PA_{STD} - Peak area of Cephalosporin in the standard µV.sec

3.3.10 Experimental Determination of System Precision

- Prepare standard solution as per method of analysis section 3.3.4.
- Inject the standard solution 6 multiple times on the HPLC.
- Calculate the average and the %RSD of the areas obtained.

Acceptance criteria

- %RSD of peak area of each Cephalosporin has to be no more than 10.

3.3.11 Experimental Determination of Linearity

- Prepare series of standard solutions of analyte at different levels of the target method concentration using serial dilutions from Standard Stock Solution Section 3.3.4 as demonstrated in Table 3.5.

- Prepare at least 5 different levels of concentration and inject each one three times.

Table 3.5 - Solutions preparation for determination of linearity.

Level (%)	Dilution	Concentration (µg/mL)
150	Transfer 7.5 mL from Standard Stock Solution Section 3.3.4 into 200 mL volumetric flask. Complete volume with solvent and mix well.	7.5
128	Transfer 8 mL from Standard Stock Solution Section 3.3.4 into 250 mL volumetric flask. Complete volume with solvent and mix well.	6.4
100	Transfer 5 mL from Standard Stock Solution Section 3.3.4 into 200 mL volumetric flask. Complete volume with solvent and mix well.	5
10	Transfer 2 mL from Linearity Level 100% into 20 mL volumetric flask. Complete volume with solvent and mix well.	0.5
4	Transfer 4 mL from Linearity Level 100% into 100 mL volumetric flask. Complete volume and mix well.	0.2
0.4*	Dilute 1 mL from Linearity Level 100% into 25 mL with solvent. Further dilute 1 mL to 10 mL with solvent. Mix well.	0.02

* If acceptance criteria fail replace this level with the concentration of the quantitation limit.

- Calculate the %RSD at each level and plot the results (Average Peak Area Vs. Concentration) to obtain the correlation coefficient.

Acceptance Criteria:

- %RSD of peak area of each Cephalosporin has to be no more than 10.
- The correlation coefficient should be greater than 0.98 for each Cephalosporin.

3.3.12 Experimental Determination of Swab Challenge

- Prepare three solutions of different at different levels of concentration: 10%, 4% and 0.4% (if the level with lower concentration fails acceptance criteria may be replaced by the concentration of quantitation limit.
- Dip a swab in glass bottle, add 5 mL of each linearity level prepared, individually, and sonicate for about 10 seconds. Make three preparations of swab for each level.
- Inject each preparation and calculate the percentage of recovery and the percentage of RSD, for each individually recovery level.

Acceptance Criteria:

- The %RSD between the three injections of the same level must be less than 10.
- The percentage of accuracy must be within 100 ± 15 of the actual amount (85% - 115%).

3.3.13 Experimental Determination of the LOQ and LOD

- Prepare different concentration solutions until reach a signal to noise not less than 10 for the LOQ and not less than 3 for the LOD.

Acceptance Criteria:

- The LOQ level must be linear (Section 3.3.11), accurate (3.3.12), and precise (%RSD of three replicates must be no more than 10).
- The LOD level must present a signal to noise about 3.

4

RESULTS AND DISCUSSION

4.1 Development

4.1.1 Method A

First, the mobile phase and conditions for Method A were prepared. The chromatograms showing the results for different organic concentrations in the mobile phase are presented in Appendix C.1. It was determined that the 100 mM ammonium acetate buffer, with 9% of ACN in the composition, could successfully separate all the eight cephalosporins, as demonstrated in Figure 4.1.

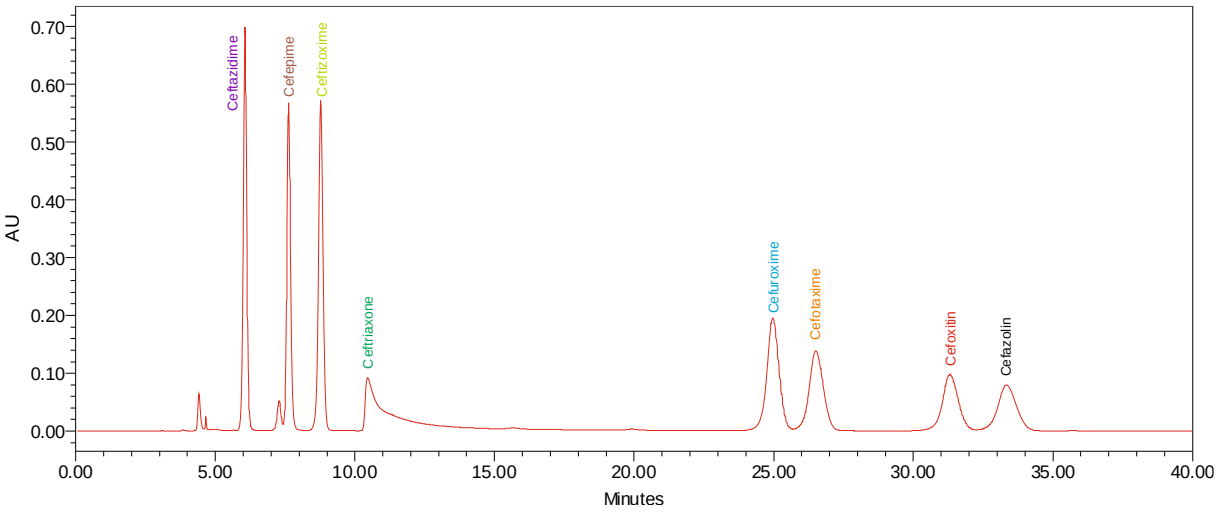


Figure 4.1 - Method A chromatogram of 5 ppm standard, with 9% ACN in the mobile phase.

However, despite achieving the primary objective, two major challenges emerged from these results:

- The Ceftriaxone peak shows major tailing.
- The last cephalosporin is detected at minute 33.

Peak tailing is undesirable because at low concentrations it can make detection challenging, and despite being the most isolated peak, tailing can also negatively impact the resolution of neighbouring peaks. The tailing observed in the Ceftriaxone peak aligns with findings in the literature [40], where Ceftriaxone also shows peak tailing at the same standard concentration. However, the tailing in the literature is less pronounced compared to what is shown in Figure 4.1. A key difference in the pH of the mobile phase, which is 7.5 in the literature [40] compared to 5.6 in this experiment. Additionally, the column used here is a Zorbax-C8, while the literature employs an ODS (octadecylsilane) column (also known as C18 column) [40].

Furthermore, since the last cephalosporin is detected at minute 33, the run time for each injection would need to be at least 50 minutes. This is because the run time is typically set to double the retention time of the last analyte exiting the column to ensure complete detection of all analytes during the run. Having a run time of 50 minutes or more isn't desirable since it would affect heavily the time needed to analyse all samples with this method.

By altering the % of ACN in the mobile phase, it was observed that higher levels of organic content reduced the retention times of the Cephalosporins, as presented in Figure 4.2.

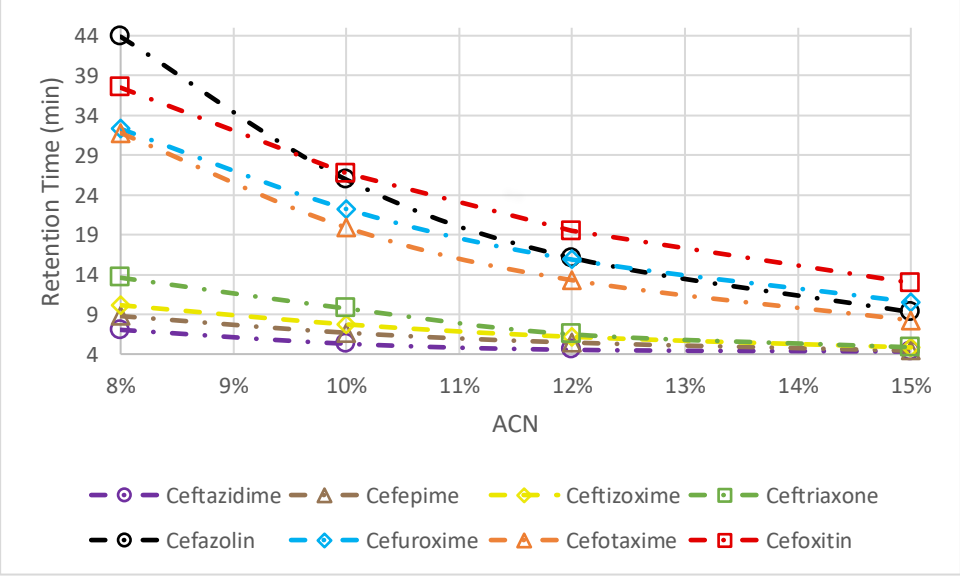


Figure 4.2 - Variation of the retention time with the % of ACN in the mobile phase (100 mM ammonium acetate).

4.1.2 Method B

To overcome the Ceftriaxone peak tailing presented in method A, THPAB was added at the same concentration as used in the current method employed by the company (as in SOP QCC157) [79], and at a lower and higher concentrations as well. This addiction was made to introduce an ionic pair for Ceftriaxone, aiming to increase its stability and therefore reduce its peak tailing. However, the results were inconclusive. As shown in Figure 4.3, the retention times changed drastically with the addition of THPAB making it difficult to establish a clear relationship between the addiction of the ionic pair and the stability of Ceftriaxone.

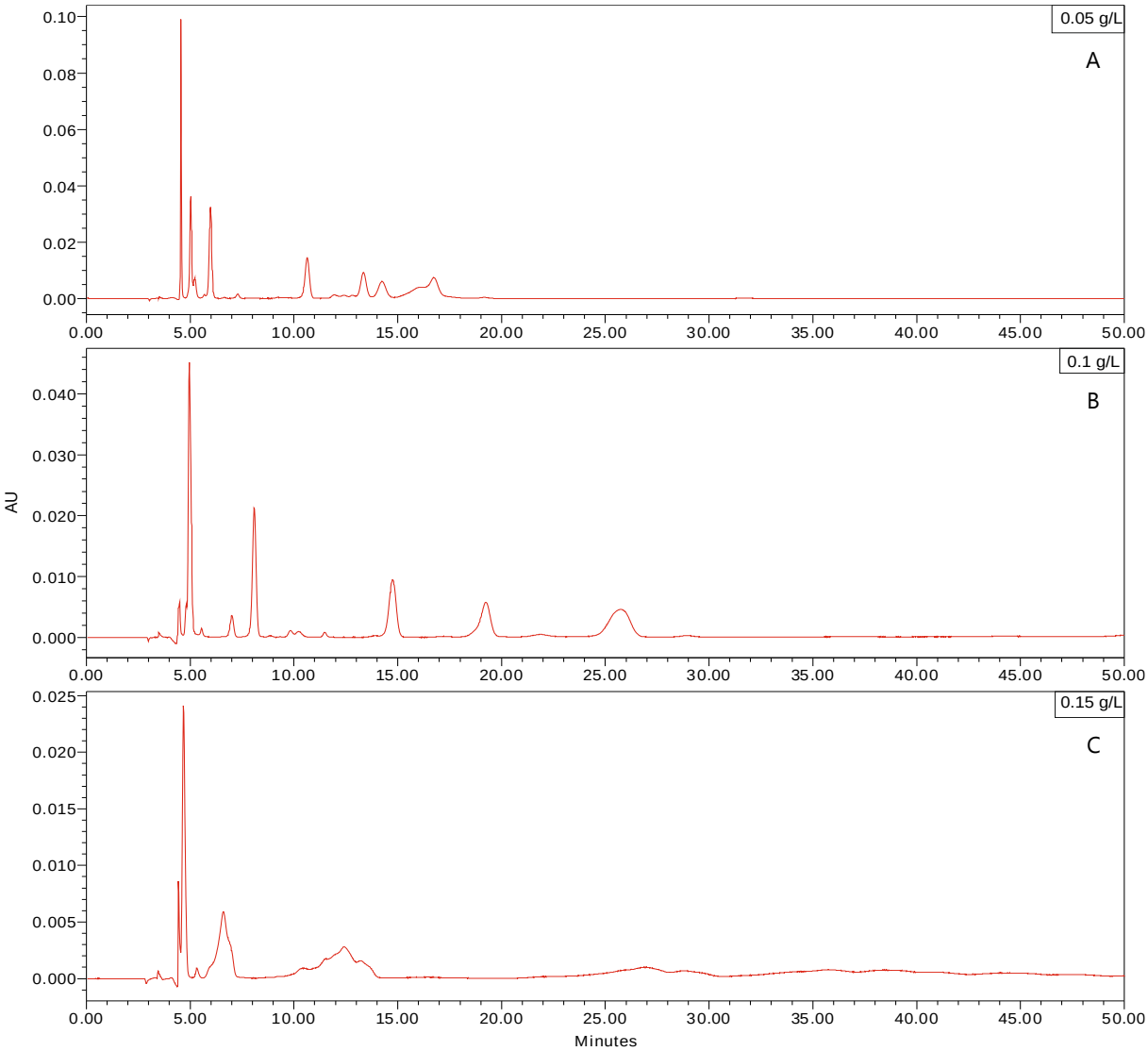


Figure 4.3 - Method B chromatograms of 5 ppm standard: A-mobile phase with 0.05 g/L THPAB; B-mobile phase with 0.1 g/L THPAB; C-mobile phase with 0.15 g/L THPAB.

Since the results were worst compared to Method A, peak identification was not performed, and no further attempts using this mobile phase were pursued.

4.1.3 Method C

This method was just an attempt to use the method already existing in the company altering the column used to an Agilent Zorbax RX-C8.

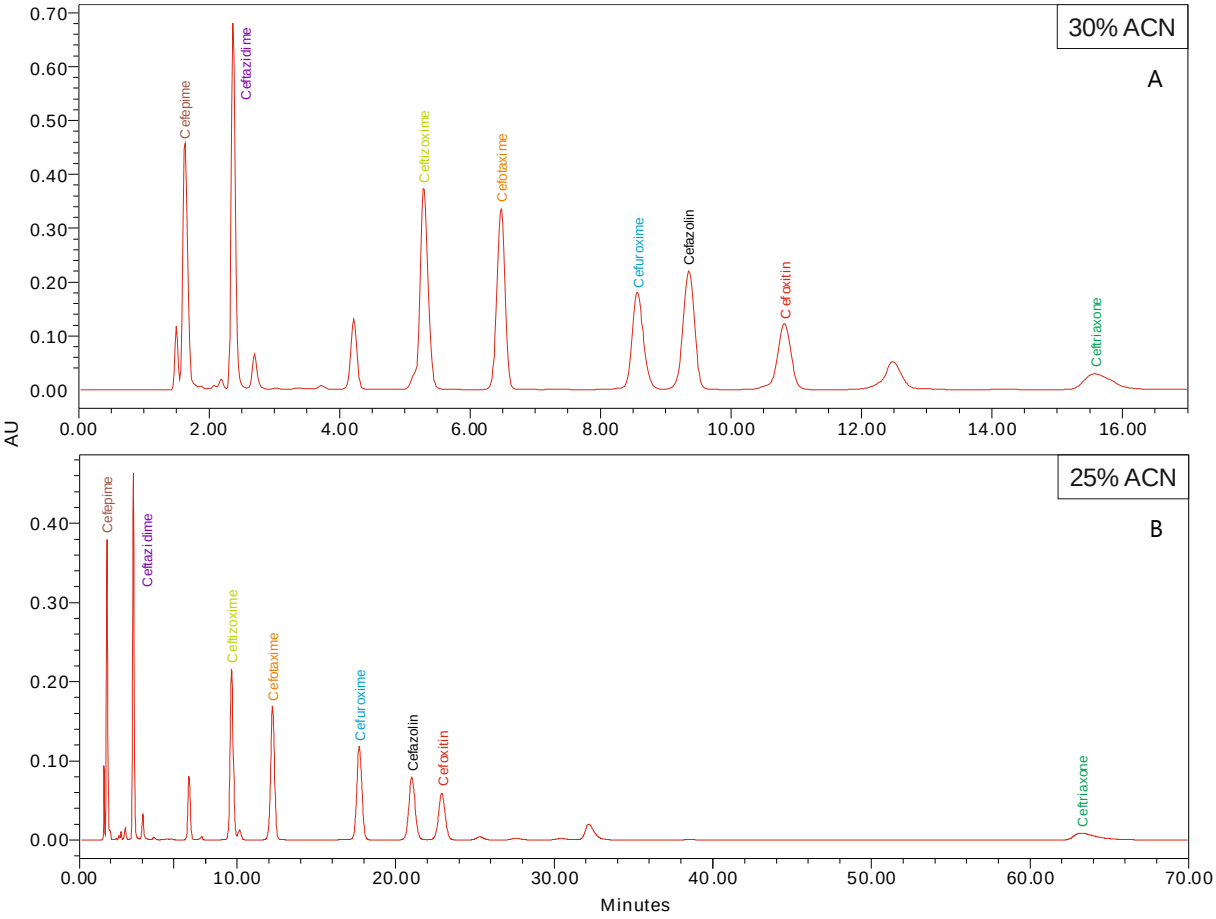


Figure 4.4 - Method C chromatograms of 5 ppm standard, variation of ACN in the mobile phase: A - 30% ACN; B - 25% of ACN.

The results showed in Figure 4.4 demonstrate complete separation of the eight peaks. However, the first two peaks have very low retention times, making it difficult to identify them at low concentrations due to overlap with the injection peak.

It would be possible to adjust the organic content by reducing the percentage of ACN to delay the retention times of the cephalosporins, as seen in Method A (Figure 4.1). However, decreasing the ACN by just 5% causes the last peak to be detected at minute 65, and the retention

times of the first two peaks do not change significantly. Having these results another Method was pursued.

4.1.4 Method D

A completely different method was attempted based on the literature [57].

In this method the approach was mixing two different organics in the mobile phase to have different reactions between the cephalosporins and the mobile phase. In this method, three different columns were tested: Xterra MS C18, Symetry C18 and Agilent Zorbax RX-C8. However, none of these columns produced eight distinct peaks in the results, leading to the method being discarded.

The best result obtained is presented in Figure 4.5 and the rest of the results are presented in appendix C.2.

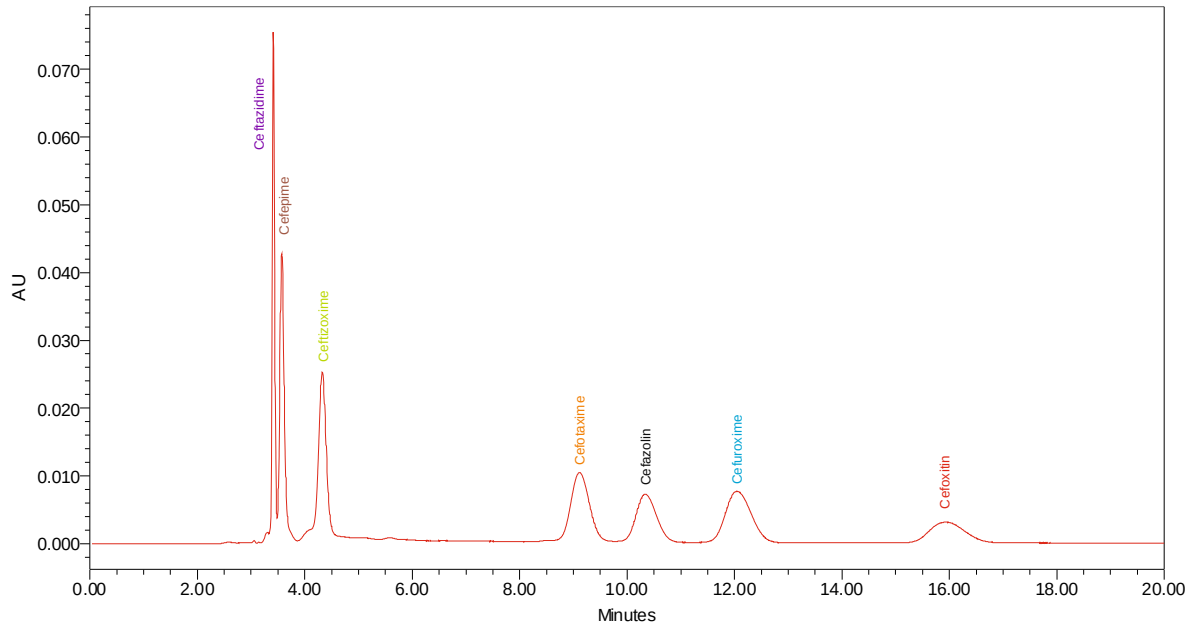


Figure 4.5 - Method D chromatogram of 5 ppm standard, mobile phase with ACN and MeOH (12:1).

The absence of one Cephalosporin peak may be attributed to a reaction between the buffer and the Cephalosporin, potentially extending its retention time beyond the analysis run time, o causing it to co-elute with another Cephalosporin. As peak identification was not performed for this method, further assessments could not be made.

4.1.5 Method E

The final method attempt utilized a 40 mM phosphate buffer solution as the mobile phase and a XTerra MS C18 column. Initially, only ACN was added, and its concentration was varied to observe its effect on the separation. Figure 4.6 illustrates that Ceftazidime and Ceftizoxime are most sensitive to the presence of ACN, with their retention times significantly decreasing as the concentration of the organic component increases. In the other hand Figure 4.7 demonstrates the same sensitivity of Cefazolin and Cefotaxime in the presence of MeOH. All the chromatograms for this variation in both ACN and MeOH are illustrated in Appendix C3.

To optimize separation, the best concentrations of ACN were identified, and MeOH was then added. The concentration of MeOH was varied to achieve better separation while reducing the run time. Similarly, for the best concentrations of MeOH, the concentration of ACN was varied using the same approach.

All the results for these variations are presented in Appendix C3 and the best results obtained in this method are showed in Figure 4.8.

A mobile phase containing 23% MeOH and 1% ACN would significantly reduce the run time of each analysis, but the resolution between Cefotaxime, Cefuroxime and Cefazolin is insufficient. in this context, only the mobile phase with 12% ACN or the one with 13% MeOH and %5 ACN offer acceptable peak resolution.

If resolution were the sole criterion, the mobile phase containing only ACN would be the best option. However, since the method will be used at lower concentrations, it is crucial to evaluate its performance under those conditions. One significant issue at low concentrations is peak tailing, particularly with Ceftriaxone, which exhibits more tailing in ACN-dominant mobile phase. Therefore, the optimal choice is a mobile phase with higher percentage of MeOH to better stabilize the Ceftriaxone peak and minimize tailing.

Finally, the column temperature was varied to assess its impact on the method. Figure 4.9 shows that a 15ºC variation did not significantly affect the results, with only Ceftriaxone and Cefazolin displaying noticeable changes in their retention times.

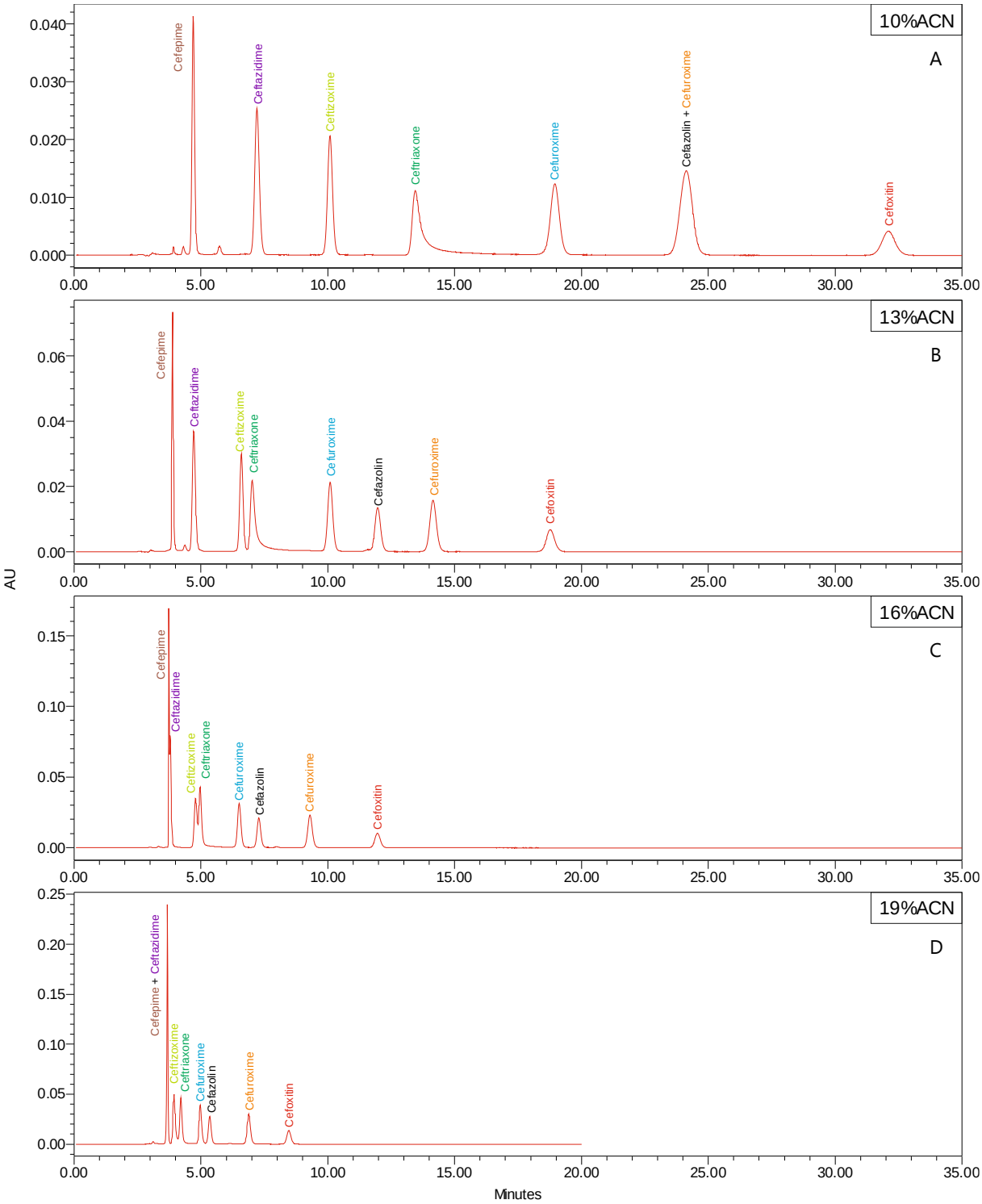


Figure 4.6 - Method E chromatograms of 5 ppm standard, variation of %MeOH in the mobile phase: A - 10% ACN; B - 13% ACN; C - 16% ACN; D - 19% ACN.

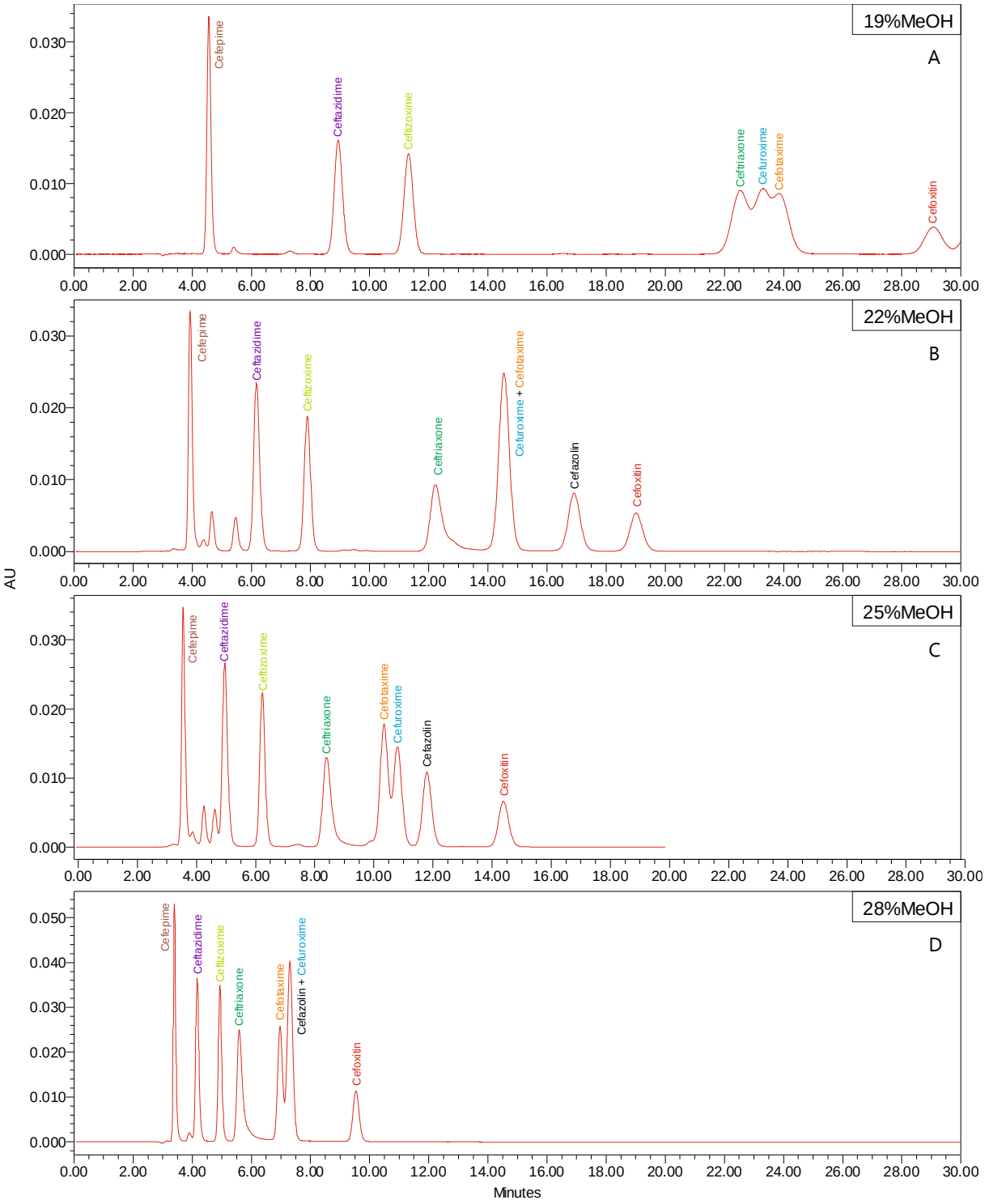


Figure 4.7 - Method E chromatograms of 5 ppm standard, variation of %MeOH in the mobile phase: A - 19% MeOH; B - 22% MeOH; C - 25%MeOH; D - 28% MeOH.

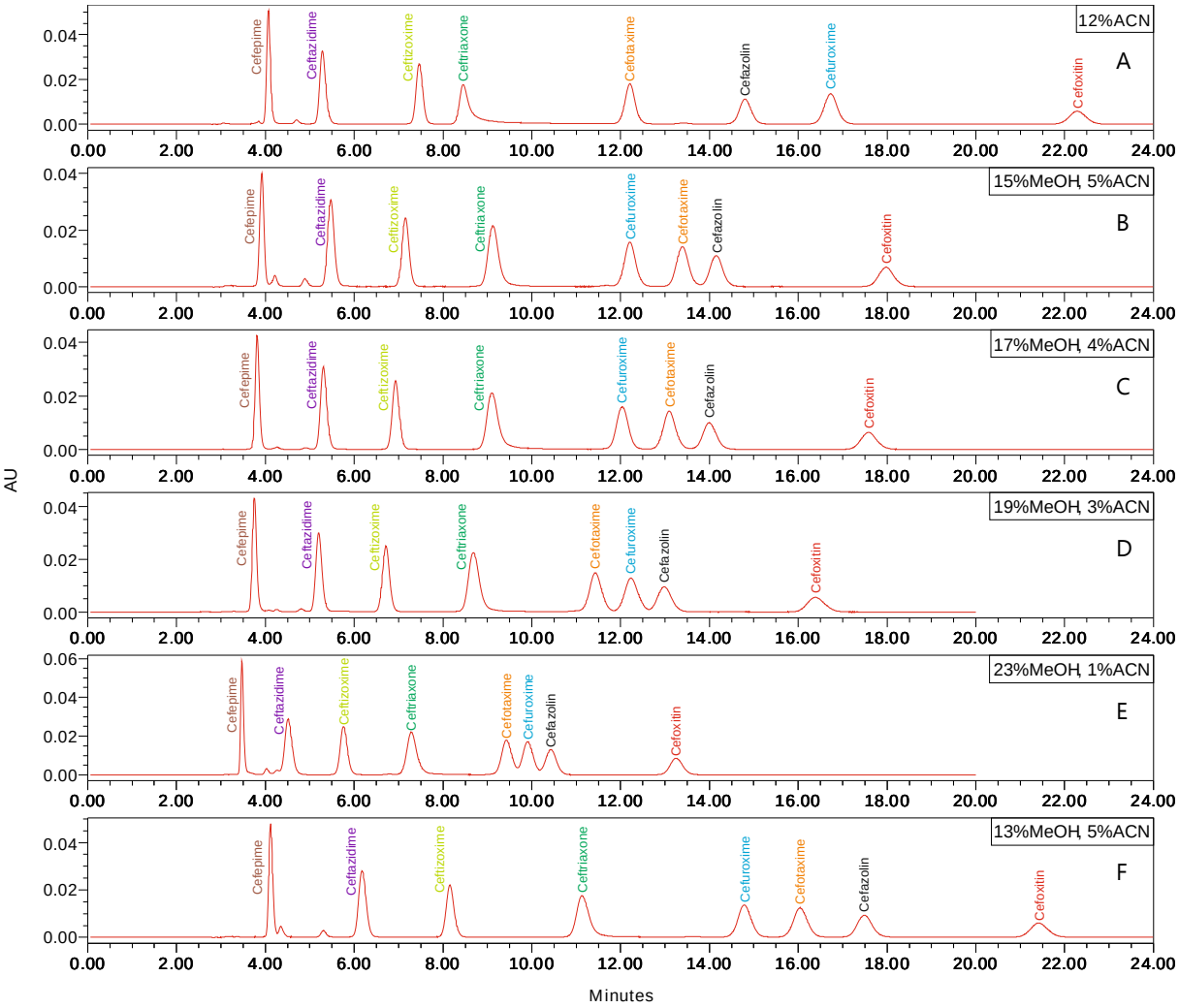


Figure 4.8 - Method E chromatogram of 5 ppm standard, variation of organic in the mobile phase: A - 1 2% ACN; B - 15% MeOH and 5% ACN; C - 17% MeOH and 4% ACN; D - 19% MeOH and 3% ACN; E - 23% MeOH and 1% ACN; F - 13% MeOH and 5% ACN.

With a mobile phase capable of separating all eight Cephalosporins established, the flow rate was increased to shorten the retention time of the las Cephalosporin, allowing it to elute from the column earlier. It is important to emphasize that a 0.02 ppm standard was used when adjusting the flow rate, as all the peaks must elute before the injection peak. If the flow rate is too hight, the Cephalosporins may elute too quickly and overlap with injection peak. Additionally, it is crucial to ensure that the injection peak does not interfere with the first Cephalosporins to elute under this method.

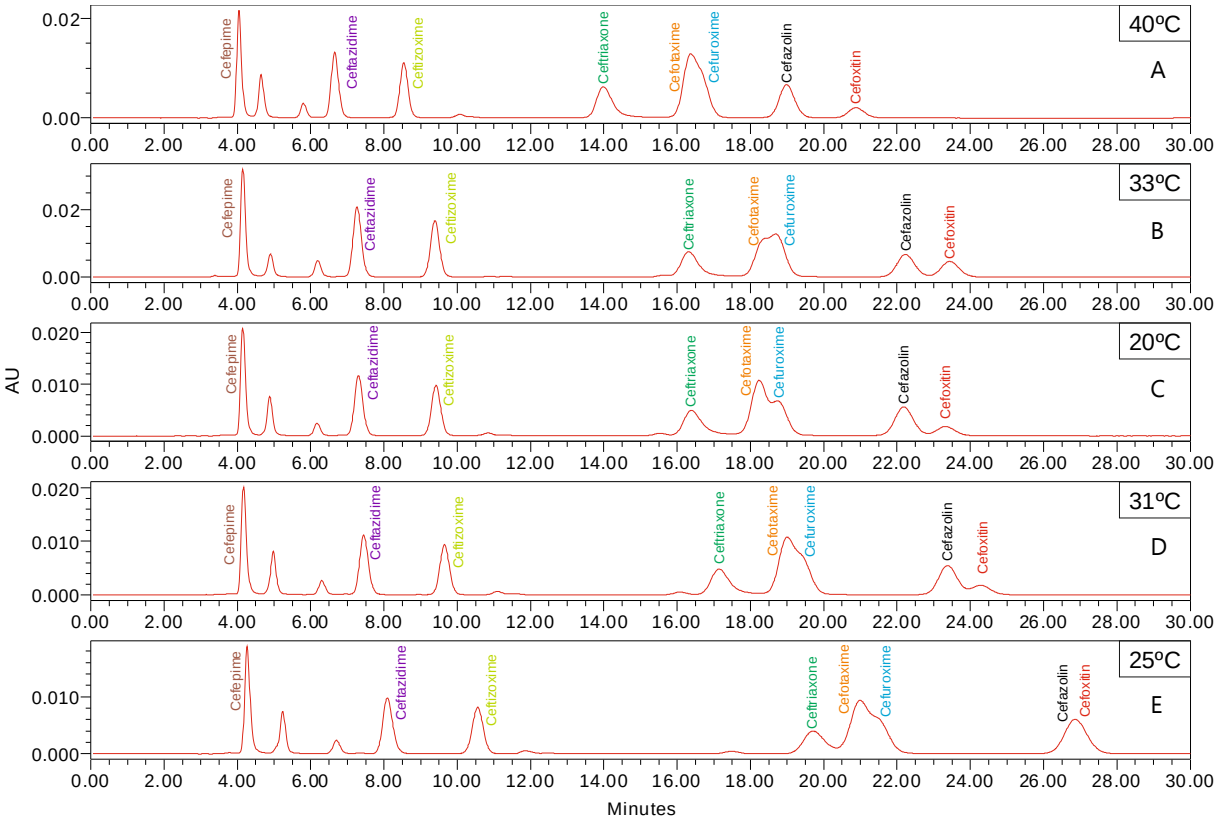


Figure 4.9 - Method E chromatogram of 5 ppm standard, variation of column temperature with 20% of MeOH in the mobile phase: A - 40ºC; B - 33ºC; C - 20ºC; D - 31ºC; E - 25ºC.

Figure 4.10 demonstrates that at a concentration of 0.02 ppm, increasing the flow rate does not significantly affect the distance between the first eluting peak and the injection peak. However, the flow rate was not increased further due to high column pressure, which was already around 2500 psi.

The parameter values for the results shown in Figure 4.10 at a flow rate of 1.2 mL/min are presented in Table 4.1. Analysing the results in table 4.1, it is evident that the plate number for all eight peaks is acceptable, as the minimum requirement according to the US Pharmocopoeia (N > 2000) is met. [80]

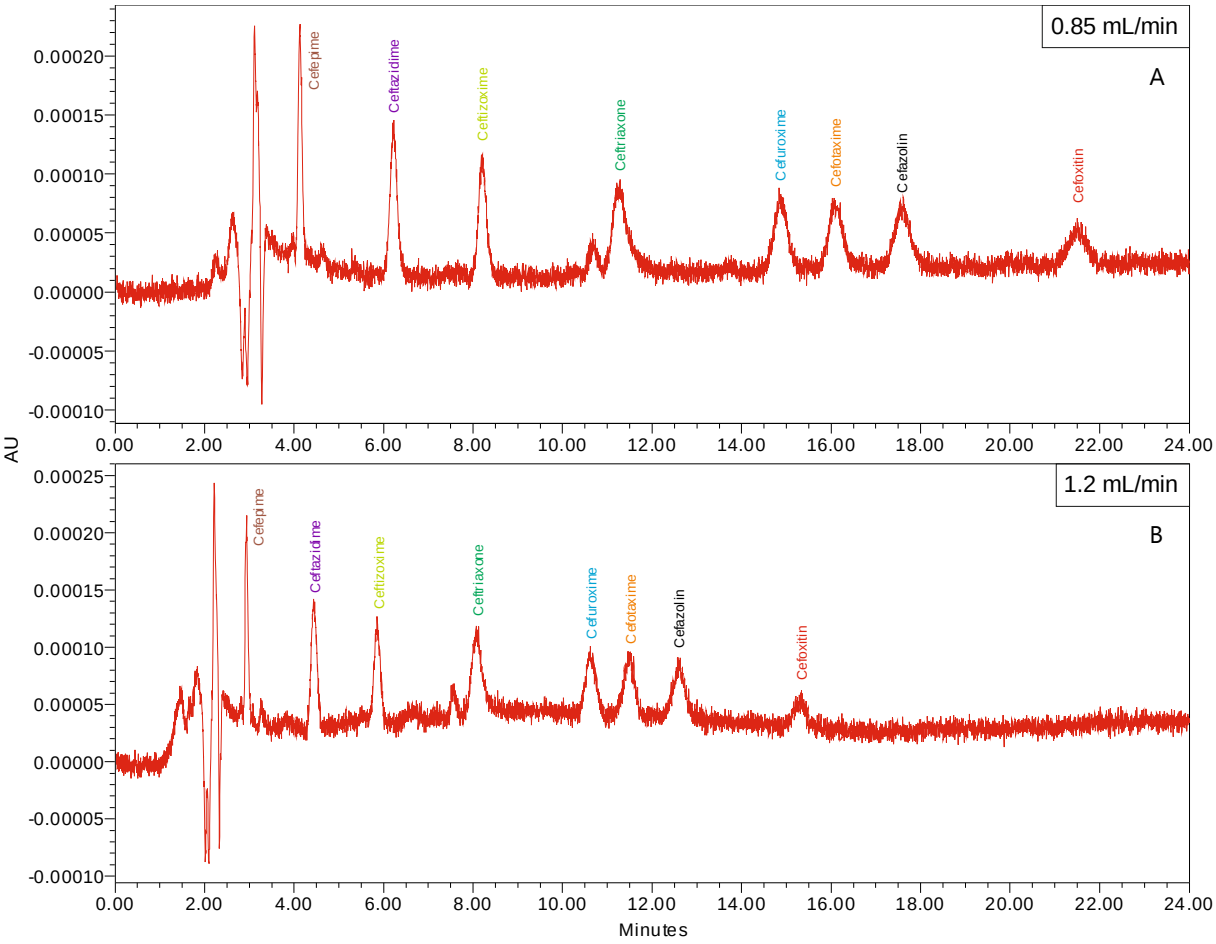


Figure 4.10 - Method E chromatogram of 0.02 ppm standard, variation of flow rate. Mobile phase composition: buffer, MeOH and ACN (82:13:5). A - 0.85 mL/min; B - 1.2 mL/min.

Table 4.1 - Chromatographic parameters for Method E using a mobile phase containing 13% MeOH and 5% ACN with a flow rate of 1.2 mL/min.

Peak Name	Retention Time (min)	Area	Height	Resolution	Plate Count	Tailing	Selectivity
Cefepime	2.8	266	41	-	3977	1.0	-
Ceftazidime	4.6	435	38	7.5	3967	1.3	0.996
Ceftizoxime	7.2	347	24	5.8	6595	0.9	0.996
Ceftriaxone	12.1	491	19	7.9	6846	1.5	0.988
Cefotaxime	14.3	540	16	4.2	80006	0.9	0.994
Cefuroxime	18.5	3674	35	8.4	8937	0.6	0.989
Cefazolin	23.5	300	17	12.6	312667	1.4	0.992
Cefoxitin	25.1	468	17	9.7	407613	3.2	0.996

All peaks exhibit good resolution from one another, with the lowest resolution observed for Cefotaxime, having a value greater than 4. A resolution above 1.5 is sufficient to clearly

distinguish and identify both peaks, although for safeguarding it is best to keep the resolution above 2. [81]

The baseline shown in Figure 4.10 exhibits excessive noise, making smaller peaks like those of Cefoxitin and Cefazolin prone to being mistaken for part of the baseline. To address this issue , the column was switched to an XBridge BEH C18, which is very similar to the XTerra MS C18 but is considered an advanced version of it. Some adjustments were also made in the detection rate of the detector, decreasing the detection to 2 points per second.

The result obtained is demonstrated in Figure 4.11.

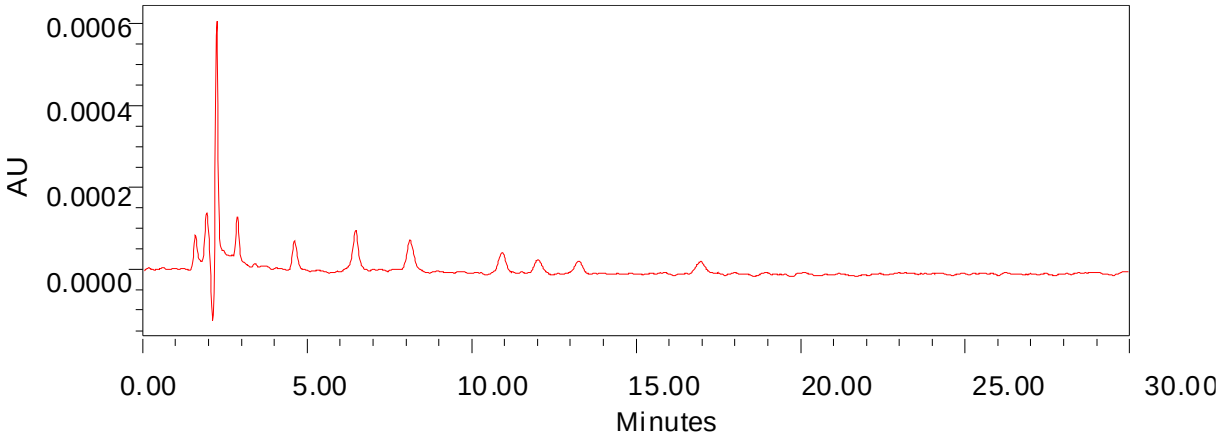


Figure 4.11 - Method E chromatogram of 0.02 ppm standard using a XBridge BEH C18 column, with 13% MeOH and 5% ACN in the mobile phase.

Dissolving Cephalosporins can sometimes be challenging. To address this, a study was conducted to identify the best solvent for preparing the standard solutions. Various concentrations of organic solvents in deionized water were tested (see Appendix C.4), but the ones that showed the least interference with the baseline are presented in Figure 4.12.

The key aspect to focus on here is the injection peak and how it is influenced by different solvents. The solvent with 13% MeOH and 5% ACN (matching the organic concentrations in the mobile phase of the method) shows the least interference with the baseline around the injection peak retention time and for that reason it was the solvent chosen to be used.

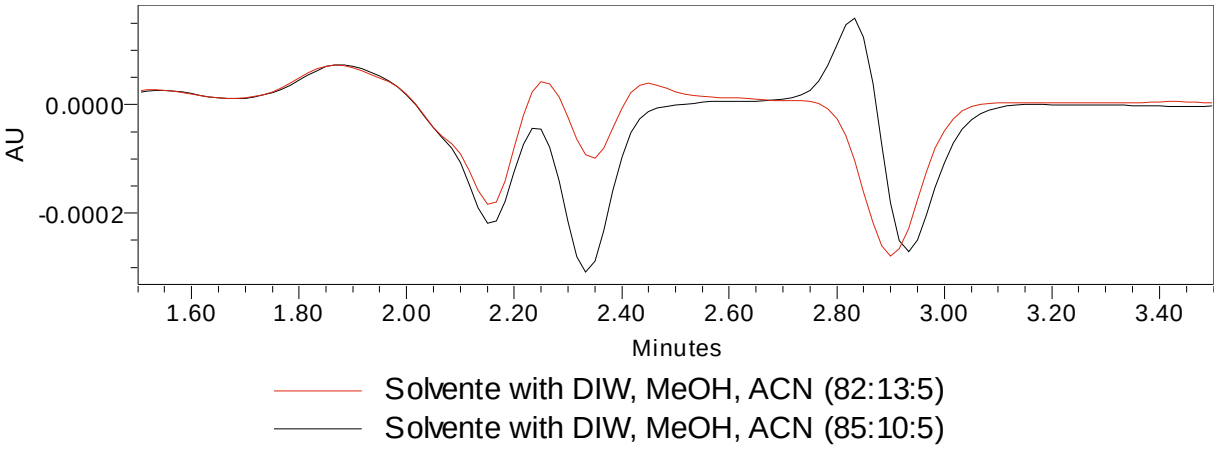


Figure 4.12 - Best solvent chromatogram results.

4.1.6 Sampling

To evaluate the sampling procedure for this method, it was tested two types of swabs, the TX 761K swabs from Texwipe® and the sterile swabs from Cultiplast® and two types of syringe filters, the PVDF from Whatman® and the PTFE from Chromatil®. All the combinations of swab and filter tested are presented in Appendix C.5.

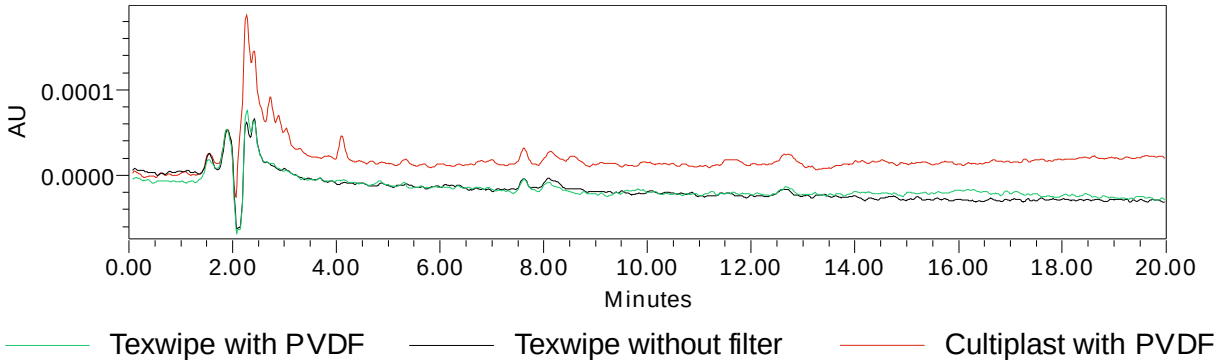


Figure 4.13 - Solvent chromatograms of swab and filter combinations used in sampling procedure.

Figure 4.13 clearly demosntrates that the Cultiplast swabs introduce signifincantly more interference at the start of the solvent baseline, wich is problematic as Cefepime, with a retention time of 2.8 minutes, is affected by this interference. This makes it an unfavorable option for accurate detection.

Using a Texwipe swab, either without a filter or with a PVDF filter, produced similar results. However, it is essencial to use a filter to ensure the sample is properly cleaned and to limit the analysis strictly to detecting Cephalosporins. As such, using the Texwipe swab with the PVDF filter is the best option.

After all this stages of the development, the final method obtained has the following characteristics:

- Column : XBridge BEH C18 5 µm, 4.6 x 250 mm;
- Mobile phase : 40 mM KH_2PO_4, MeOH and ACN (82:13:5);
- Wavelenght detection : 260;
- Flow rate : 1.2 mL/min;
- Column temperature : 32ºC;
- Sample Solvent : DIW, MeOH and ACN (82:13:5);
- Sampling materials : TX 761K swabs and PVDF syringe filters.

4.2 Validation

4.2.1 System precision

The first parameter evaluated in the validation of this method was if the same was reproductable and for that it was evaluated the sistem precison. The results demonstrated in Table 4.2 show the peak area of all the Cephalosporins, Cefepime (CP), Ceftazidime (CZI), Ceftizoxime (ZOX), Ceftriaxone (CFT), Cefotaxime (TAX), Cefuroxime (CFR), Cefazolim (CZ), Cefoxitin (CX).

Table 4.2 - Peak areas for the 6 injections of the 5 ppm solution, made for system precision.

Injection #	Peak Area							
	CP	CZI	ZOX	CFT	TAX	CFR	CZ	CX
1	171926	180423	219921	268971	197679	141046	101745	186009
2	172091	180796	220253	269474	198181	141309	101807	186421
3	171462	181152	219660	268959	197638	141020	101683	185864
4	171257	180573	219517	268465	197566	141143	102079	185873
5	170977	179566	219202	267855	197072	140629	101622	185933
6	171011	180206	219261	268552	197126	140875	101826	185477
Average	171454	180453	219636	268713	197544	141004	101794	185930
%RSD	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2

Since the acceptance criteria require that non of the RSD values exceed 10%, this method demonstrates system precision.

4.2.2 Linearity

To demonstrate the linearity of the method, a linear regression was performed for each cephalosporin, summarizing the data related to peak area and solution concentration. All the results from this linearity test are presented in Appendix C.6.

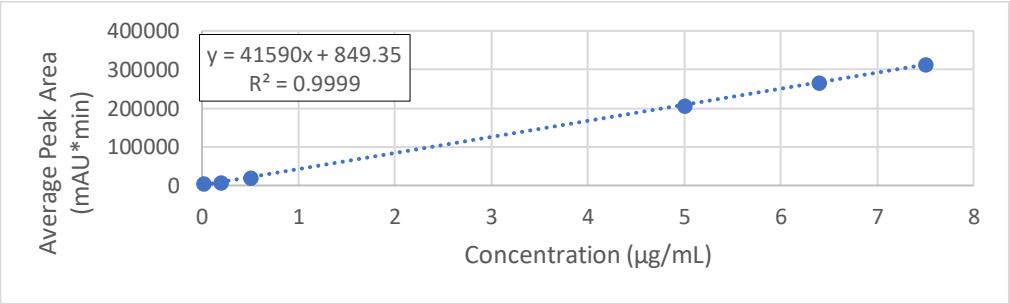


Figure 4.14 - Cefepime linear regression analysis.

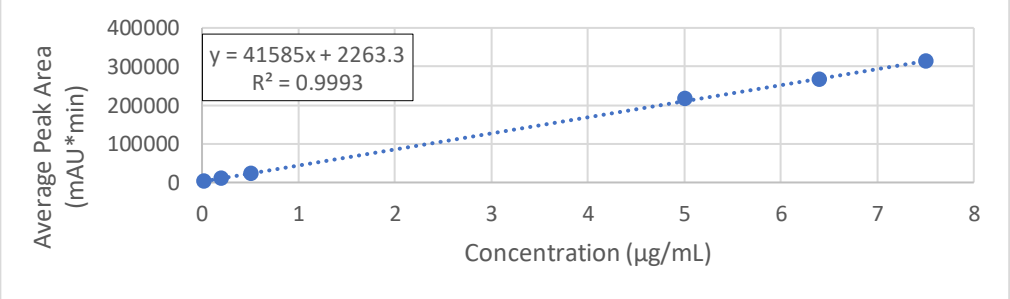


Figure 4.15 - Ceftazidime linear regression analysis.

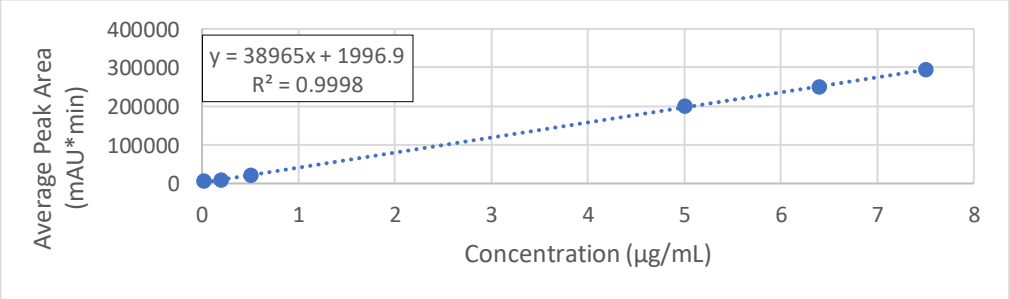


Figure 4.16 - Ceftizoxime linear regression analysis.

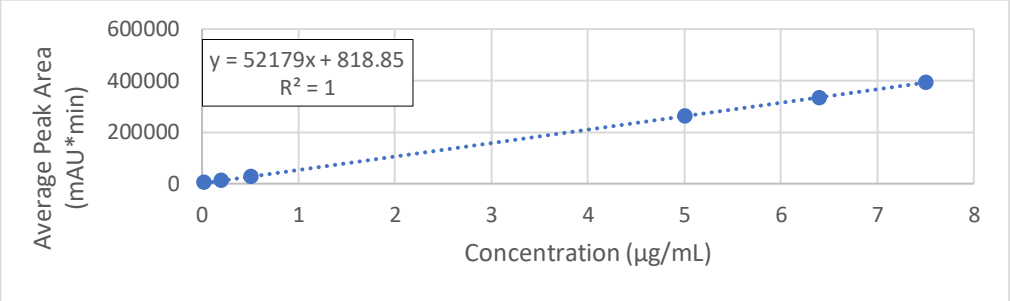


Figure 4.17 - Ceftriaxone linear regression analysis.

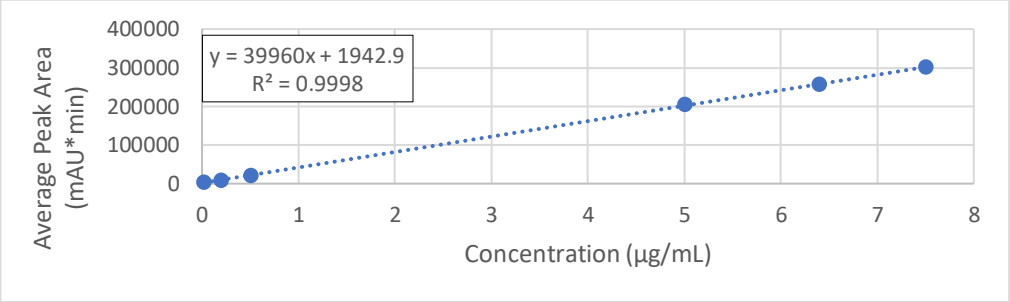


Figure 4.18 - Cefotaxime linear regression analysis.

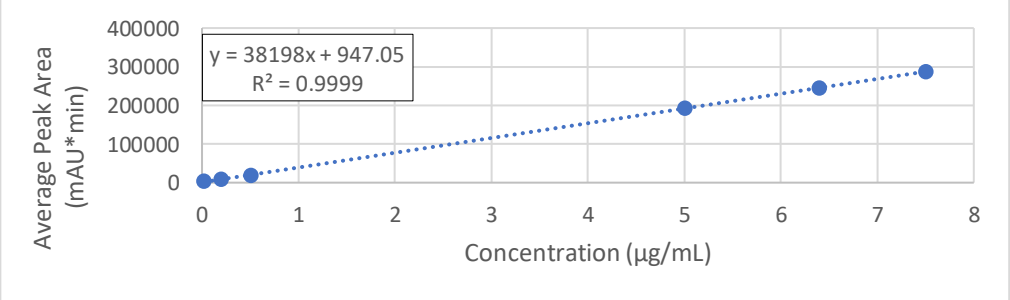


Figure 4.19 - Cefuroxime linear regression analysis.

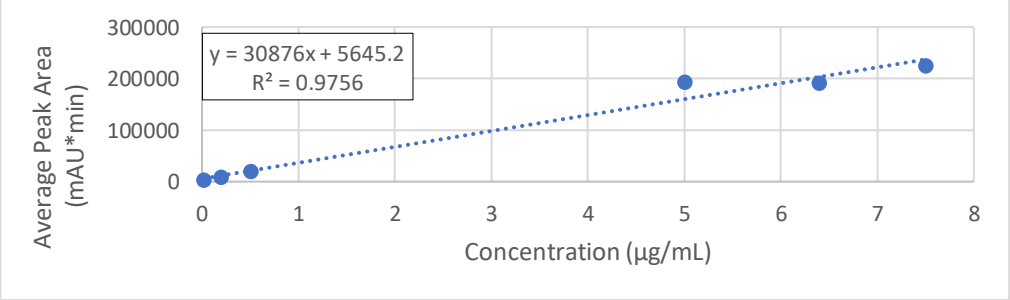


Figure 4.20 - Cefazolin linear regression analysis.

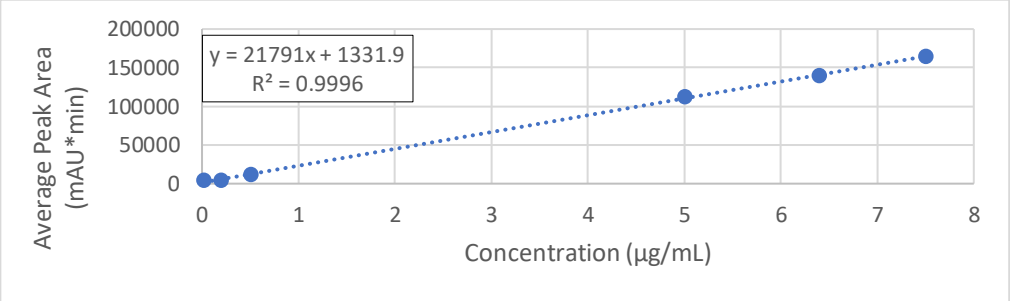


Figure 4.21 - Cefoxitin linear regression analysis.

Table 4.3 gather all the information from the linear regression made for all the Cephalosporins.

Table 4.3 - %RSD between the three injections of all Cephalosporins for the linearity test.

Level (%)	Concentration (µg/mL)	CP	CZI	ZOX	CFT	TAX	CFR	CZ	CX
150	7.5	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1
128	6.4	0.0	0.3	0.0	0.1	0.1	0.1	0.4	0.1
100	5	0.1	0.8	0.2	0.0	0.0	0.1	0.1	0.4
10	0.5	0.2	0.3	0.3	6.0	0.2	0.2	0.5	0.4
4	0.2	5.5	0.8	2.3	4.2	5.2	3.9	2.3	6.9
0.4	0.02	4.7	11.6	17.9	8.9	2.3	11.1	15.6	23.8
Correlation Coefficient		1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00

Although all the Cephalosporins exhibit linearity across the different concentrations, the %RSD for the 0.4% level exceeds 10%, which is one of the acceptance criteria. Therefore, this level was replaced with the LOQ level. Again, a linear regression for each Cephalosporin was made as presented from Figure 4.22 to Figure 4.29.

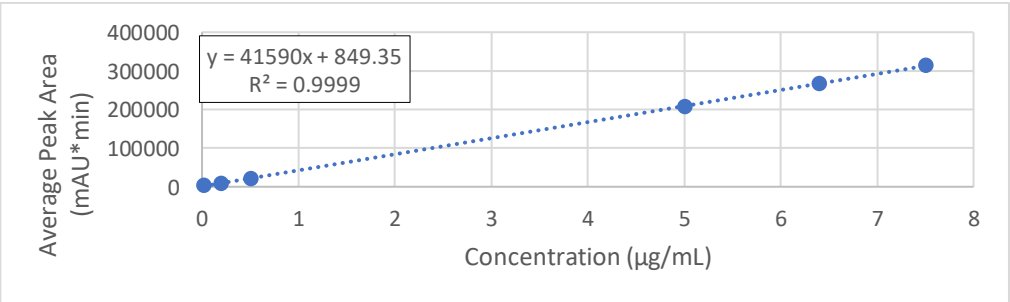


Figure 4.22 - Cefepime linear regression analysis, using the LOQ as the last level.

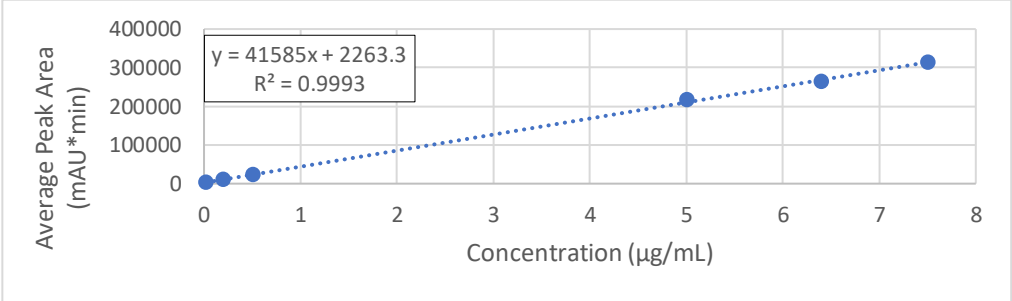


Figure 4.23 - Ceftazidime linear regression analysis, using the LOQ as the last level.

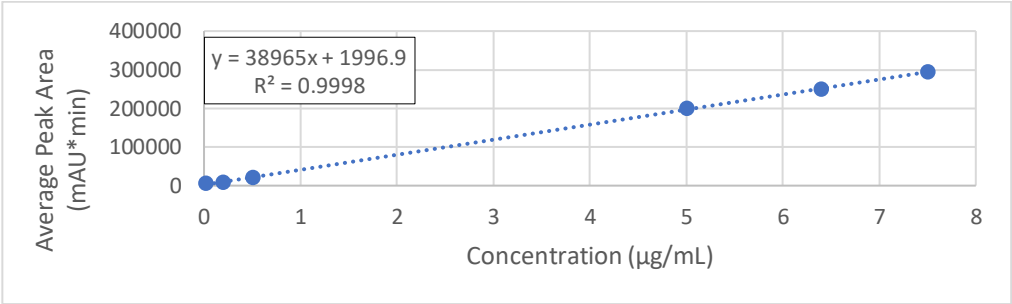


Figure 4.24 - Ceftizoxime linear regression analysis, using the LOQ as the last level.

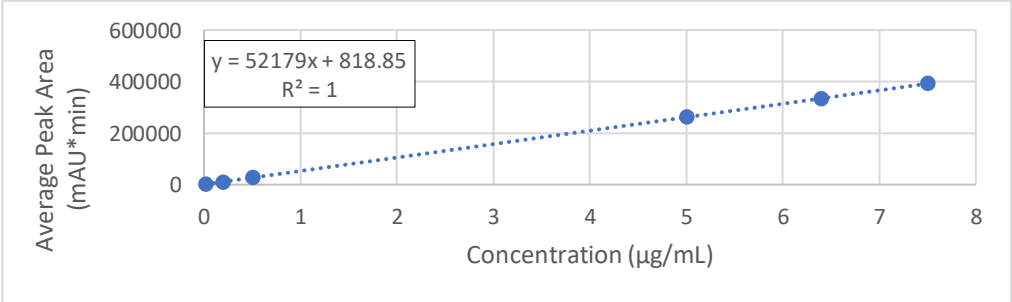


Figure 4.25 - Ceftriaxone linear regression analysis, using the LOQ as the last level.

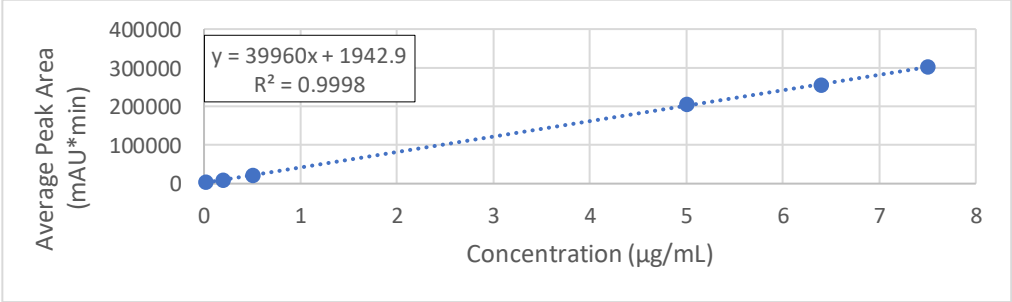


Figure 4.26 - Cefotaxime linear regression analysis, using the LOQ as the last level.

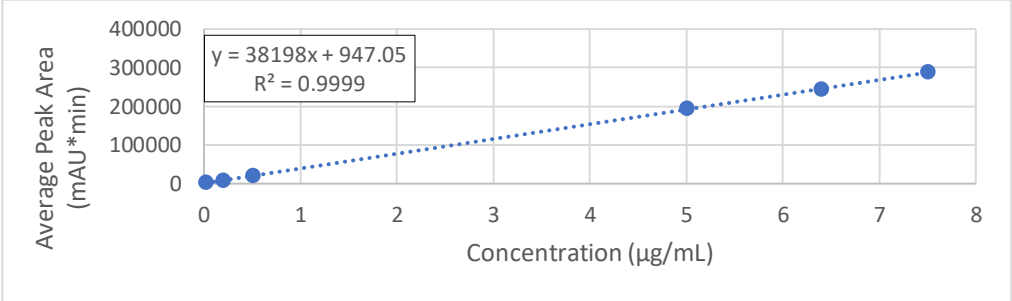


Figure 4.27 - Cefuroxime linear regression analysis, using the LOQ as the last level.

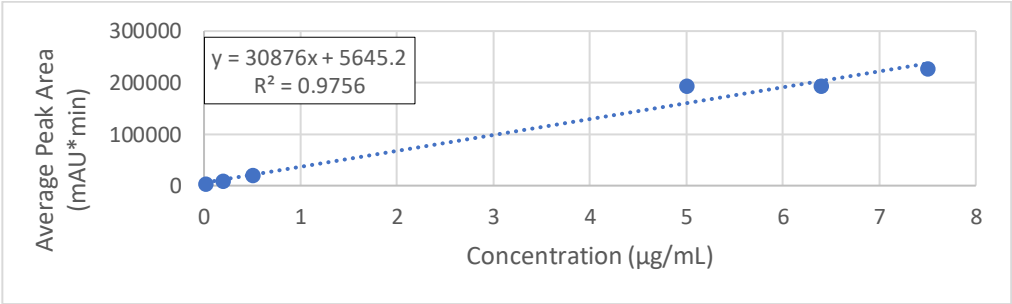


Figure 4.28 - Cefazolin linear regression analysis, using the LOQ as the last level.

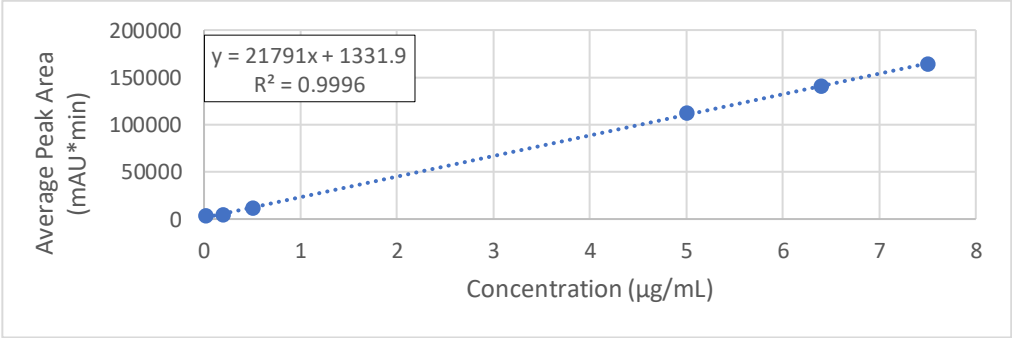


Figure 4.29 - Cefoxitin linear regression analysis, using the LOQ as the last level.

Table 4.4 gather all the information from the linear regression made for all the Cephalosporins. By using the LOQ as the lowest concentration level, all the Cephalosporins demonstrate a RSD below 10% across all the concentration levels, and the correlation coefficient for each Cephalosporin is above 0.98. Since the acceptance criteria are met, the method's linearity is confirmed.

In addition, the range of the method is verified to be between the concentration levels 1.6% (0.08 µg/mL) and 10% (0.5 µg/mL), as these are the concentrations at which linearity and accuracy were confirmed (accuracy confirmed in Section 4.2.5).

Table 4.4 - %RSD between the three injections of all Cephalosporins for the linearity test, using the LOQ as the last level.

Level (%)	Concentration (µg/mL)	CP	CZI	ZOX	CFT	TAX	CFR	CZ	CX
150	7.5	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1
128	6.4	0.0	0.3	0.0	0.1	0.1	0.1	0.4	0.1
100	5	0.1	0.8	0.2	0.0	0.0	0.1	0.1	0.4
10	0.5	0.2	0.3	0.3	6.0	0.2	0.2	0.5	0.4
4	0.2	5.5	0.8	2.3	4.2	5.2	3.9	2.3	6.9
1.6	0.08	1.0	0.2	1.0	0.7	1.7	3.4	1.5	0.7
Correlation Coefficient		1	1.00	1.00	1.00	1.00	1.00	0.99	1.00

4.2.3 Limit of Quantitation

For the limit of quantification, the concentration that would achieve a signal to noise ratio of 10 was estimated using a linear regression. Table 4.5 presents the result of this regression analysis.

Table 4.5 - Signal to noise ratio of all Cephalosporins for all concentration levels used in linearity test.

Level (%)	Concentration (µg/mL)	CP	CZI	ZOX	CFT	TAX	CFR	CZ	CX
150	7.5	5690	3306	3982	3144	2075	1934	1265	1434
128	6.4	4827	2806	3386	2666	1763	1644	1479	1220
100	5	3887	2299	2701	2943	1410	1301	855	967
10	0.5	383	230	271	277	139	129	85	96
4	0.2	1337	92	106	106	56	51	33	38
Correlation Coefficient		0.98	1.00	1.00	0.97	1.00	1.00	0.97	1.00
Slope		33.13	22.06	26.55	21.57	13.84	12.89	9.53	9.56
Intercept		627.48	17.01	7.40	136.41	3.81	1.09	-4.08	1.31

Given the high correlation coefficient, it is possible to estimate the concentration that will produce a peak with a signal to noise ration of 10. The regression analysis of Cefazolin and Cefoxitin were used for this estimation, as they consistently show the lowest signal to noise ration across nearly all concentrations levels.

Accordingly to the linear regression a signal to noise ratio of 10 is achieved at a concentration level of 1.2 for Cefoxitin ((10-1.31) / 9.56 = 1.2) and at a concentration level of 0.6 for Cefazolin ((10+4.08) / 9.53 = 0.6).

As such it was attemped a concentration level of 1% first and then a concentration level of 1.6%.

Table 4.6 - Signal to noise ratio for the concentration levels 1.6% and 1%.

Level (%)	Concentration (µg/mL)	CP	CZI	ZOX	CFT	TAX	CFR	CZ	CX
1.6	0.08	65	80	34	14	63	30	20	25
1	0.05	26	19	15	4	27	16	10	24

As shown in Table 4.6 the best option for the LOQ level is the 1.6% concentration, as the smallest signal to noise ratio obtained is slightly above the LOQ threshold of 10, and the Ceftriaxone peak at the concentration level of 1% shows a signal to noise ratio of 4, very bellow the limit. This LOQ level needs to be reproducible, so three injections were made and the %RSD was calculated as summarized in Table 4.7.

Table 4.7 - Peak Area of the three replicates of level 1.6% of concentration (0.08 µg/mL).

Peak Area								
Injection #	**CP**	**CZI**	**ZOX**	**CFT**	**TAX**	**CFR**	**CZ**	**CX**
1	3879	3398	4579	2953	3820	2628	2060	3555
2	3922	3409	4574	2916	3808	2679	2000	3555
3	3847	3398	4495	2946	3924	2805	2018	3513
Average	3883	3402	4549	2938	3851	2704	2026	3541
%RSD	1.0	0.2	1.0	0.7	1.7	3.4	1.5	0.7

All peaks exhibit a signal to noise ratio above 10, and the %RSD of the three replicates for each Cephalosporin is below 10%, demonstrating that the LOQ is precise. Since this concentration level was used in the linearity test, it also confirms the linearity of this concentration.

4.2.4 Limit of Detection

For the limit of detection, it was unnecessary to prepare different solution concentrations, as the concentration used during the development stage for lower levels already demonstrated a signal to noise ratio of 3, which is the acceptance criteria for the LOD.

Since three injections were already made in the linearity test for the 0.02 ppm concentration solution that presented linearity and precision at this level the only criteria left is the signal to noise ration that is presented in Table 4.8.

Table 4.8 - Signal to noise ratio of the LOD.

Signal to Noise Ratio								
Injection#	**CP**	**CZI**	**ZOX**	**CFT**	**TAX**	**CFR**	**CZ**	**CX**
1	8	8	8	6	6	3	3	6
2	8	7	8	7	6	4	3	8
3	8	8	8	6	6	3	3	5
Average	8	8	8	6	6	4	3	6
%RSD	0.02	0.02	0.01	0.07	0.05	0.09	0.07	0.16

4.2.5 Swab Challenge

The first results obtained didn't show a recovery percentage within the range 100% ± 25%, for some Cephalosporins as it is possible to see from Table 4.9 to Table 4.16. Cefepime and Cefotaxime are the ones that present the worst recovery percentages.

Table 4.9 - Cefepime results for Swab Challenge.

Cefepime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	6311	35	33	6.9
		5503	31		
		5865	33		
4	0.2	1598	23	39	36.4
		3115	44		
		3463	49		
0.4	0.08	328	46	39	14.6
		246	34		
		277	38		

Table 4.10 - Ceftazidime results for Swab Challenge.

Ceftazidime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	14098	81	80	1.6
		13658	79		
		13882	80		
4	0.2	5039	74	82	9.7
		5839	85		
		6083	89		
0.4	0.08	670	84	86	12.3
		601	76		
		767	97		

Table 4.11 - Ceftizoxime results for Swab Challenge.

Ceftizoxime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	20861	96	94	2.5
		20908	96		
		19985	92		
4	0.2	8060	92	96	3.1
		8457	97		
		8556	98		
0.4	0.08	800	77	84	9.3
		961	93		
		860	83		

Table 4.12 - Ceftriaxone results for Swab Challenge.

Ceftriaxone					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	18150	92	97	4.7
		19768	100		
		19653	99		
4	0.2	8024	100	101	0.5
		8067	101		
		8098	101		
0.4	0.08	1010	90	97	10.6
		1028	92		
		1218	109		

Table 4.13 - Cefotaxime results for Swab Challenge.

Cefotaxime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	14522	75	73	3.3
		13625	71		
		13853	72		
4	0.2	4611	59	71	15.0
		5833	75		
		6204	79		
0.4	0.08	535	67	73	6.4
		589	74		
		605	76		

Table 4.14 - Cefuroxime results for Swab Challenge.

Cefuroxime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	13780	100	97	3.5
		13220	96		
		12850	94		
4	0.2	5190	93	95	2.7
		5234	93		
		5459	97		
0.4	0.08	589	100	86	16.2
		425	72		
		503	85		

Table 4.15 - Cefazolin results for Swab Challenge.

Cefazolin					
Level (%)	**Concentra-tion (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	9510	94	94	0.3
		9456	94		
		9477	94		
4	0.2	3852	99	99	2.9
		3733	96		
		3954	101		
0.4	0.08	410	80	100	18.4
		533	103		
		596	116		

Table 4.16 - Cefoxitin results for Swab Challenge.

Cefoxitin					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	17625	100	100	1.0
		17885	101		
		17567	99		
4	0.2	6992	100	101	1.8
		7102	101		
		7254	103		
0.4	0.08	426	78	111	39.3
		518	94		
		877	160		

These results were due to insufficient time in the sonic bath, where the separation of the Cephalosporins from the swab occurs. The time was subsequently increased to 1 minute instead of the initial 10 seconds in the sonic bath. In addition, the detection wavelength was reduced to 240 nm. While this change was not expected to significantly affect the results at higher concentrations, at the LOQ level, it could be sufficient to improve the recovery percentage. Since the recovery percentages of Cefepime were close to 30%, the standard used was switched from a USP standard to the final product. The final product contains arginine, which acts as a buffer to neutralize Cefepime's acidity, making it a better option to stabilize the Cefepime peak.

Due to the high %RSD at the 0.4% concentration level, this level was replaced with the LOQ concentration level.

These alterations permitted a recovery percentage acceptable for all Cephalosporins (see Appendix C.8), but Cefepime continued to demonstrate a recovery below 75% as shown in Table 4.17.

At this point, all the modifications made for this validation step do not impact the previous tests conducted, although it is recommended to repeat the tests under these new conditions. Further alterations to the method would necessitate repeating the entire validation process. Therefore, one modification that would be acceptable without supposedly disrupting the validation, is changing the solvent to deionized water instead of the current mixture of DIW, MeOH and ACN (82:13:5). The results of this alteration for Cefepime are presented in Table 4.18, the results for the rest of the Cephalosporins are shown in Appendix C.8.

Table 4.17 - Cefepime results for swab challenge using Cefepime final product as standard and detecting at a wavelength of 240 nm.

Cefepime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**% Recovery**	**Average**	**%RSD**
10	0.5	14128	67	69	6.2
		14159	67		
		15711	74		
4	0.2	6580	77	83	7.4
		6727	85		
		7168	89		
1.6	0.08	2748	102	104	6.1
		2989	111		
		2659	99		

It can be concluded that this method successfully passed the swab recovery challenge, as all Cephalosporins demonstrated recovery percentages within the acceptable range of 100% ± 25%, and the %RSD among the three preparations did not exceed 10%.

Table 4.18 - Cefepime results for swab challenge using Cefepime final product as standard diluted in DIW, detecting at a wavelength of 240 nm.

Cefepime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	15328	75	69	6.2
		15309	75		
		15711	83		
4	0.2	6180	76	83	7.4
		6727	83		
		7168	89		
1.6	0.08	2659	99	104	6.1
		2989	111		
		2748	102		

5

Conclusions and Future Remarks

This work presents the development and validation of an HPLC method capable of detecting Cephalosporins, used to ensure that no cross-contamination occurs within Hikma Sintra facilities.

During the development stage, all tested mobile phases, except the one composed of sodium acetate buffer, successfully separated the eight Cephalosporins produced at Hikma 2. However, despite the ability of the 100 mM ammonium acetate buffer with 9% acetonitrile to separate all the eight Cephalosporins, the Ceftriaxone peak posed a significant challenge due to severe tailing. This tiling is particularly problematic when detecting low concentrations, as it hinders accurate peak determination. The second method attempted added another buffer to the mobile phase, THPAB, with the aim of reducing the tailing of the Ceftriaxone peak. However, the results obtained were significantly different, and the THPAB appeared to modify the retention times of all the Cephalosporins.

The method currently used by the company, which employs a mobile phase composed of 25 mM potassium dihydrogenphosphate, 365 mM disodium phosphate, and 1 g/L tetraheptylammonium bromide, with 25% acetonitrile, was also able to successfully separate all the Cephalosporins when added more 5% acetonitrile. However, the first two eluting peaks have retention times very close to the injection peak, posing a challenge for detecting these peaks at low concentrations. This proximity can cause them to overlap with the injection peak, making their determination more difficult.

Another attempt was made using a different buffer in the mobile phase, 10 mM sodium acetate. This attempt yielded poor results, as one of the Cephalosporins did not appear in the chromatograms with different variations of concentration of organic in the mobile phase, even

with an extended run time of 120 minutes. Given the impracticality of such a long run time, further pursuit of this method was deemed unnecessary.

The last method attempted uses a 40 mM potassium dihydrogenphosphate buffer in the mobile phase. This method was able to separate all the eight Cephalosporins with different combinations of organic concentrations, using both MeOH and ACN. The combinations of MeOH and ACN, 17:4, 13:5 and 0:12 were the ones that obtained better separation results, being that combination 13:5 was the one that had better resolution between all peaks present a value of 4.2 for the lowest resolution.

In terms of column selection, the first four mobile phase attempts use an Agilent Zorbax RX-C8 column. However, during the fourth attempt, three different columns were tested: Agilent Zorbax RX-C8, XTerra MS C18, and Symmetry C18. The comparison between them revealed that C18 columns are more effective for separating Cephalosporins in a shorter run time while maintaining good efficiency, compared to C8 columns. For the final mobile phase attempt, only two C18 columns were used: XTerra MS C18 and XBridge BEH C18, with the later being considered an advanced version of the XTerra MS C18. The XBridge BEH C18 column demonstrated faster elution of Cephalosporins compared to the XTerra MS C18 column, while also exhibiting less baseline noise at lower concentrations.

Evaluating the flow rate of the mobile phase, a value of 1.2 mL/min was determined to optimize the run time for each analysis while avoiding excessively high-pressure conditions on the column.

The solvent used in the standard sample solution preparations was also tested with varying concentrations of organic components. The goal was to identify the solvent composition that produced a smaller injection peak and minimized baseline noise. The two solvent mixtures that met these criteria were: DIW, MeOH, ACN (82:13:5) and DIW, MeOH, ACN (85:10:5), with the former showing the least interference.

Finally, the sampling procedure was tested to ensure that the swabs and syringe filters used did not impact the results. The best outcomes were achieved using Texwipe swabs without a filter, Texwipe swabs with PVDF filters, and Cultiplast swabs with PVDF filters. Among these, both Texwipe options (with and without filters) exhibited minimal injection peak interference. However, since filtering is necessary to eliminate potencial contaminants that could interfere with Cephalosporins detection, the Texwipe swabs with a PVDF filter proved to be the most suitable choice.

All these considerations led to a method capable of detecting the eight Cephalosporins produced at Hikma 2, under the following conditions: a mobile phase consisting of 40 mM

potassium dihydrogenphosphate buffer, MeOH, and ACN (82:13:5), a flow rate of 1.2 mL/min, using a XBridge BEH C18 column at 32ºC, with detection wavelength of 260 nm. The sample solvent is composed of DIW, MeOH, and ACN (82:13:5), and the sampling procedure involves TX 761K swabs and PVDF syringe filters.

During the method validation, three modifications were made: the sample solvent was changed to DIW, the detection wavelength was adjusted to 240 nm, and the Cefepime standard was replaced with Hikma's final product containing L-Arginine. These changes were implemented to address the low stability shown by Cefepime, and they successfully improved its stability. Although these adjustments were made only during the final validation test, a priori they do not impact the results of the earlier tests conducted throughout the validation process.

The validation confirmed that the method is precise, as six injections of the standard for each Cephalosporin resulted in a %RSD below 0.4%. Linearity was demonstrated across concentrations ranging from 0.02 µg/mL to 7.5 µg/mL, with all Cephalosporins showing a correlation factor of no less than 0.99. The LOQ was determined at 0.08 µg/mL, with a signal to noise ratio of 14, while the LOD, with a signal to noise ratio no less than 3 for every Cephalosporin, was achieved at 0.02 µg/mL. Additionally, the swab recovery test showed recovery rates above 75% for all Cephalosporins at concentrations of 0.5 µg/mL, 0.2 µg/mL, and 0.08 µg/mL. These concentrations also define the method’s range, as they demonstrate linearity, precision, and accuracy.

Some conclusions drawn from this work could benefit from further studies to address and clarify the uncertainties that arose during the process. First and foremost, it is recommended to repeat all validation tests under the new conditions (new Cefepime standard, adjusted wavelength, and new solvent) to ensure that these changes do not affect the method's linearity, precision, LOD, LOQ, accuracy, and range.

The next recommended study could focus on the stability of the sample solution, particularly addressing Cefepime, which presented notable difficulties during the validation process. This research could help identify strategies for extending Cefepime's stability in solution, enabling safer and more accurate analysis over an extended period.

Another area worth exploring is the surfaces that are swabbed and analyzed. For example, the laboratory floors, which are tested for potential cross-contamination, should be studied to determine their capacity to retain Cephalosporins. Additionally, the interaction between the surfaces and the swabs should be investigated to better understand how surface properties may influence the accuracy of the sampling process.

Finally, a test could be conducted to examine the impact of potential contaminants that may not be filtered during the sampling process. This could be done by adding known concentrations of specific contaminants to a standard solution, performing the method, and then comparing the results with those from a pure standard solution and a solution containing only the contaminants. This would help assess the method´s robustness in the presence of unfiltered impurities.

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A

List of pharmacopoeas

Table A.1 - List of pharmacopoeias

National		Regional
Argentina	Korea (Republic of)	Eurasia
Austria	Lativa	European Pharmacopoeia
Belarus	Lithuania	Africa
Belgium	Luxembourg	**International**
Bosnia and Herzegovina	Malta	WHO, Geneva, Switzerland
Brazil	Mexico	
Bulgaria	Montenegro	
Chile	Netherlands	
China	North Macedonia	
Croatia	Norway	
Cyprus	Pakistan	
Czech Republic	Philippines	
Denmark	Poland	
Egypt	Portugal	
Estonia	Romania	
Finland	Russian Federation	
France	Serbia	
Germany	Slovakia	
Greece	Slovenia	
Hungary	Spain	
Iceland	Sweden	
India	Switzerland	
Indonesia	Thailand	
Iran (Islamic Republic of)	Turkey	
Ireland	Ukraine	
Italy	United Kingdom of Great Britain and Northern Ireland	
Japan	United States of America	
Kazakhstan	Vietnam	

B

METHOD DEVELOPMENT

List of all method conditions attempts during the development of the method

Table B.2 - Injection conditions of development stage (1/5).

Buffer	%ACN	%MeOH	Flow Rate (mL/min)	pH	Column			Column Temperature	Injection Volume
					Type	Dimensions	Particle Size		
A	5	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	6	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	7	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	8	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	9	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	10	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	11	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	12	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	15	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	16	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	17	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	18	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	19	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	20	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	5	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
B	9	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
B	9	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
B	9	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	5	0	1.2	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	*	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
A	**	0	0.8	5.6	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ºC	25 uL
E	10	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	11	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	1	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	10	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	2	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	3	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	4	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	5	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL

Table B.2 -Injection conditions of development stage (2/5).

E	12	6	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	7	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	8	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	9	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	0	1	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	12	0	1.125	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	13	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	13	1	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	13	2	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	13	3	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	13	4	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	13	5	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	13	0	1.125	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	1	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	10	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	2	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	3	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	13	1.2	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	13	1	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	13	1.2	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	6	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	7	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	9	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	1	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	10	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	11	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	12	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	13	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	14	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	16	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	18	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	2	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	20	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	3	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	4	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	5	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	6	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	7	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	8	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	14	9	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	14	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	14	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	14	1.125	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	14	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	15	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	1	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	10	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL

Table B.2 -Injection conditions of development stage (3/5).

E	2	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	3	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	6	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	7	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	9	15	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	1	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	10	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	2	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	3	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	4	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	5	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	6	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	7	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	8	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	16	9	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	16	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	16	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	17	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	1	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	10	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	2	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	3	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	17	1	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	17	1.2	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	6	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	7	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	9	17	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	18	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	18	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	18	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	18	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	18	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	19	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	1	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	10	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	2	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	3	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	6	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	7	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	9	19	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	20	0	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	20	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	20	0.85	3.2	Xterra C18	250 x 4.6	5 um	25ªC	25 uL
E	0	20	0.85	3.2	Xterra C18	250 x 4.6	5 um	31ªC	25 uL

Table B.2 - Injection conditions of development stage (4/5).

E	0	20	0.85	3.2	Xterra C18	250 x 4.6	5 um	33ºC	25 uL
E	4	20	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	20	0.85	3.2	Xterra C18	250 x 4.6	5 um	40ºC	25 uL
E	5	20	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	20	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	21	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	1	21	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	2	21	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	3	21	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	21	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	21	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	6	21	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	7	21	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	21	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	9	21	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	22	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	22	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	22	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	22	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	1	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	10	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	2	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	3	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	6	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	7	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	9	23	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	24	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	24	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	24	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	1	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	10	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	2	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	3	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	4	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	5	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	6	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	7	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	9	25	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	26	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	26	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	26	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	26	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	26	0.85	3.2	Xterra C18	250 x 4.6	5 um	20ºC	25 uL
E	0	26	0.85	3.2	Xterra C18	250 x 4.6	5 um	25ºC	25 uL
E	4	26	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	8	26	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	27	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
E	0	28	0.85	3.2	Xterra C18	250 x 4.6	5 um	32ºC	25 uL
D	10	3	1	4.7	Symmetry C18	250 x 4.6	5 um	20ºC	25 uL
D	12	1	1	4.7	Symmetry C18	250 x 4.6	5 um	20ºC	25 uL

Table B.2 - Injection conditions of development stage (5/5).

D	1	12	1	4.7	Xterra C18	250 x 4.6	5 um	20ªC	25 uL
D	13	0	1	4.7	Xterra C18	250 x 4.6	5 um	20ªC	25 uL
D	13	2	1	4.7	Agilent Zobrax RX-C8	250 x 4.6	5 um	20ªC	25 uL
D	13	4	1	4.7	Agilent Zobrax RX-C8	250 x 4.6	5 um	20ªC	25 uL
D	13	6	1	4.7	Agilent Zobrax RX-C8	250 x 4.6	5 um	20ªC	25 uL
D	0	13	1	4.7	Xterra C18	250 x 4.6	5 um	20ªC	25 uL
D	2	13	1	4.7	Agilent Zobrax RX-C8	250 x 4.6	5 um	20ªC	25 uL
D	4	13	1	4.7	Agilent Zobrax RX-C8	250 x 4.6	5 um	20ªC	25 uL
D	6	13	1	4.7	Agilent Zobrax RX-C8	250 x 4.6	5 um	20ªC	25 uL
D	4	9	1	4.7	Agilent Zobrax RX-C8	250 x 4.6	5 um	20ªC	25 uL
D	6	7	1	4.7	Agilent Zobrax RX-C8	250 x 4.6	5 um	20ªC	25 uL
D	8	5	1	4.7	Symmetry C18	250 x 4.6	5 um	20ªC	25 uL
C	25	0	1.5	5	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ªC	25 uL
C	35	0	1.5	5	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ªC	25 uL
C	25	0	2	5	Agilent Zobrax RX-C8	250 x 4.6	5 um	30ªC	25 uL

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C

RESULTS

This Appendix serves as auxiliar to all the results discussed in Chapter 4, covering both the development and validation results. It presents all the chromatogram results for every method attempt in the development stage as well as all the data relative to the validation process. The results from Methods A, D and E are demonstrated in Chapters C.1 to C.3. Chapter C.4 covers all the solvent attempts made in the development phase. All the results obtained in the development of the sapling procedure are demonstrated in Chapter C.5 and Chapters C.6 and C.7 demonstrate all the results obtained in the validation of the method.

C.1 Method A Results

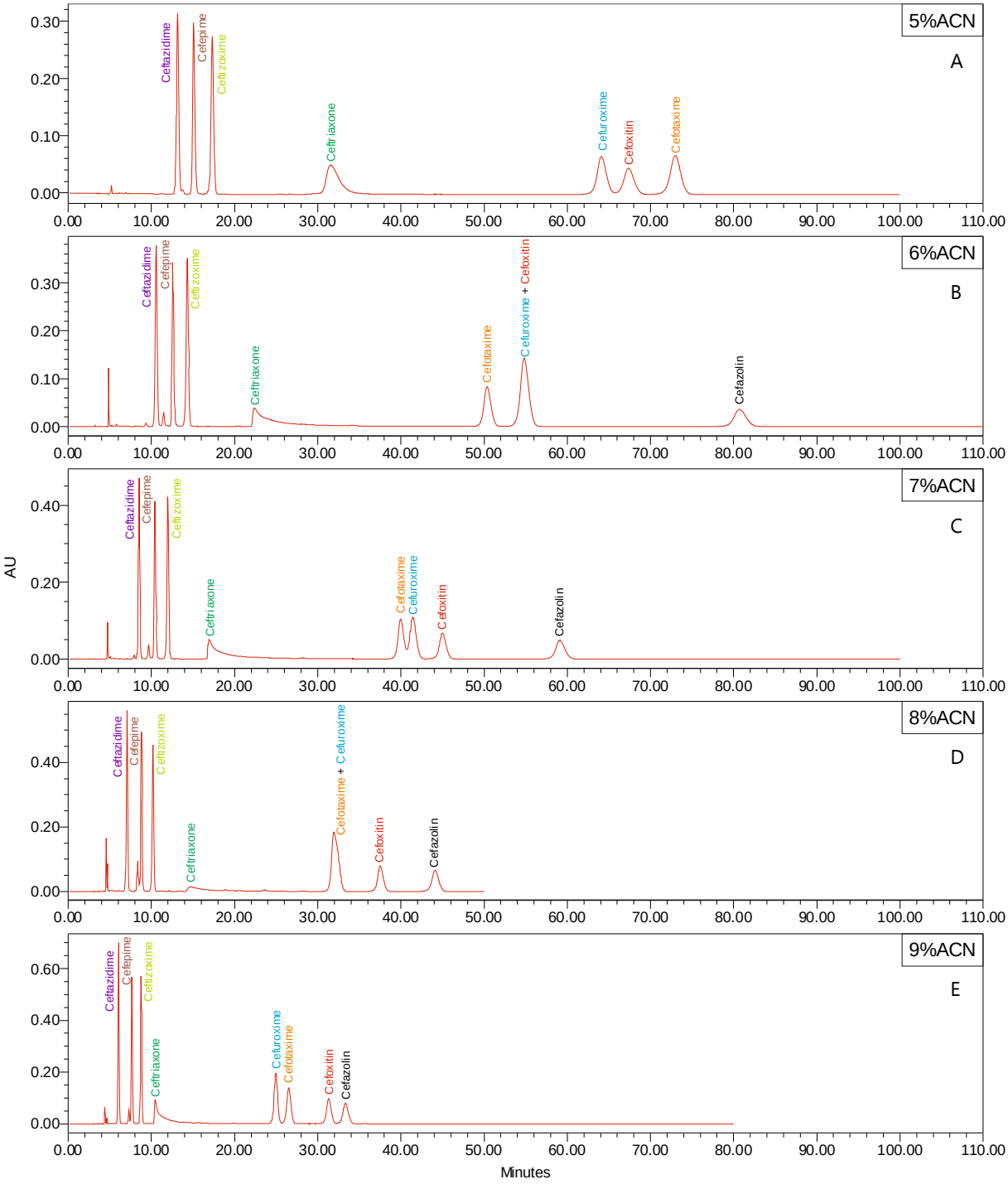

Figure C.1 - Method A chromatograms of 5 ppm standard, variation of ACN in the mobile phase: A - 5% ACN; B - 6% ACN; C - 7% ACN; D 8% ACN; E - 9% ACN.

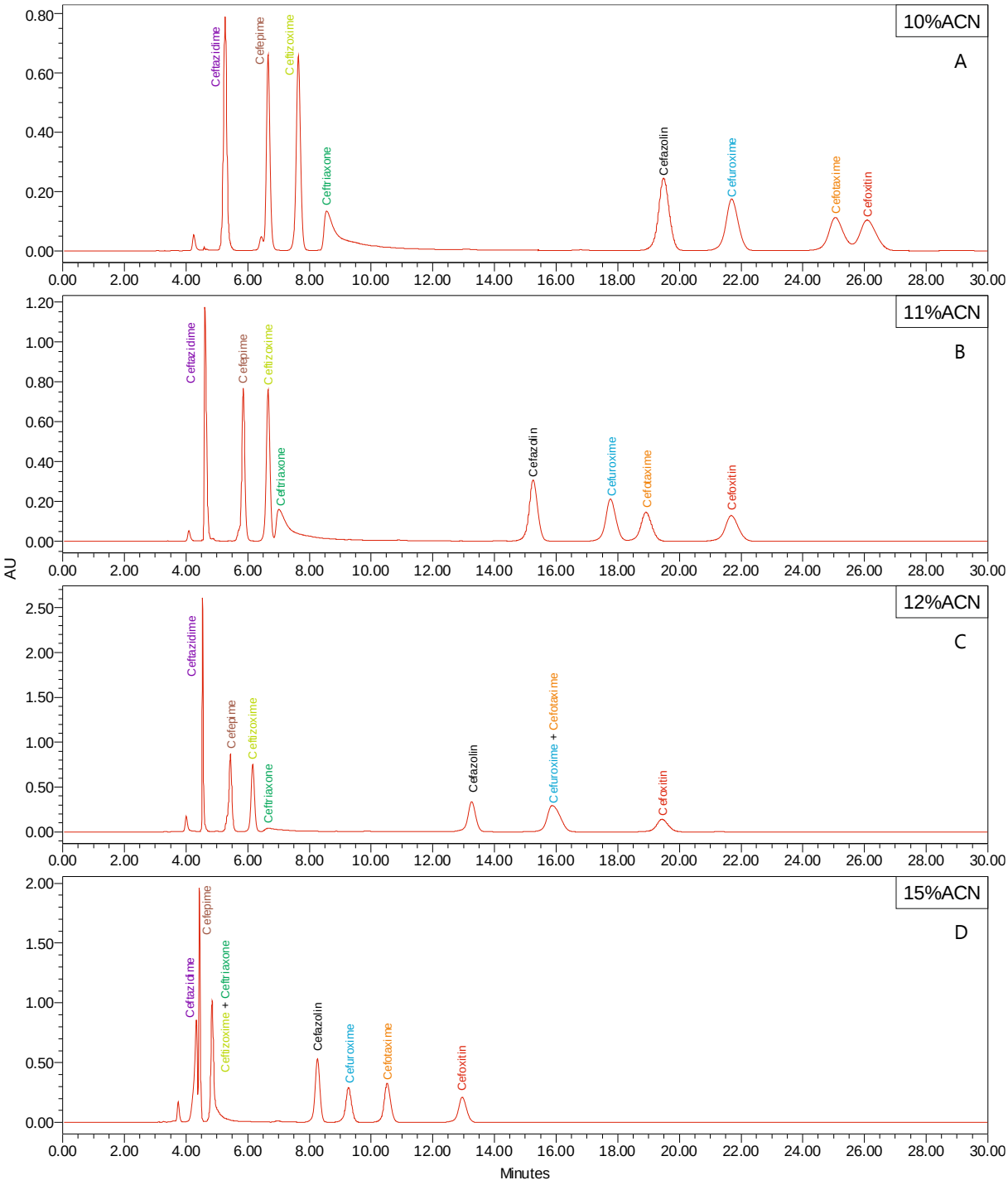

Figure C.2 - Method A chromatograms of 5 ppm standard, variation of ACN in the mobile phase: A - 10% ACN; B - 11% ACN; C - 12% ACN; D - 15% ACN.

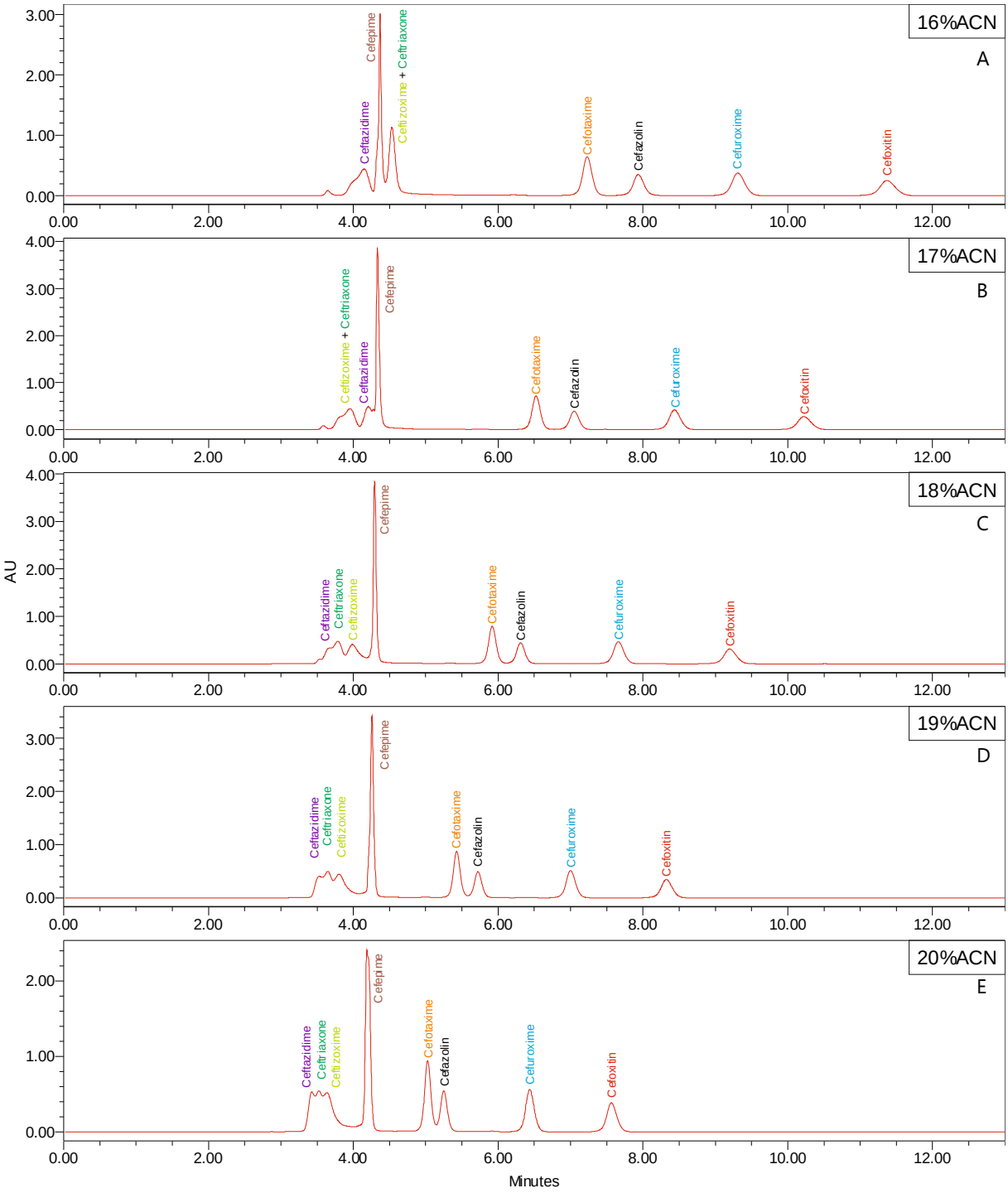

Figure C.3 - Method A chromatograms of 5 ppm standard, variation of ACN in the mobile phase: A - 16% ACN; B - 17% ACN; C - 18% ACN; D 19% ACN; E - 20% ACN.

C.2 Method D Results

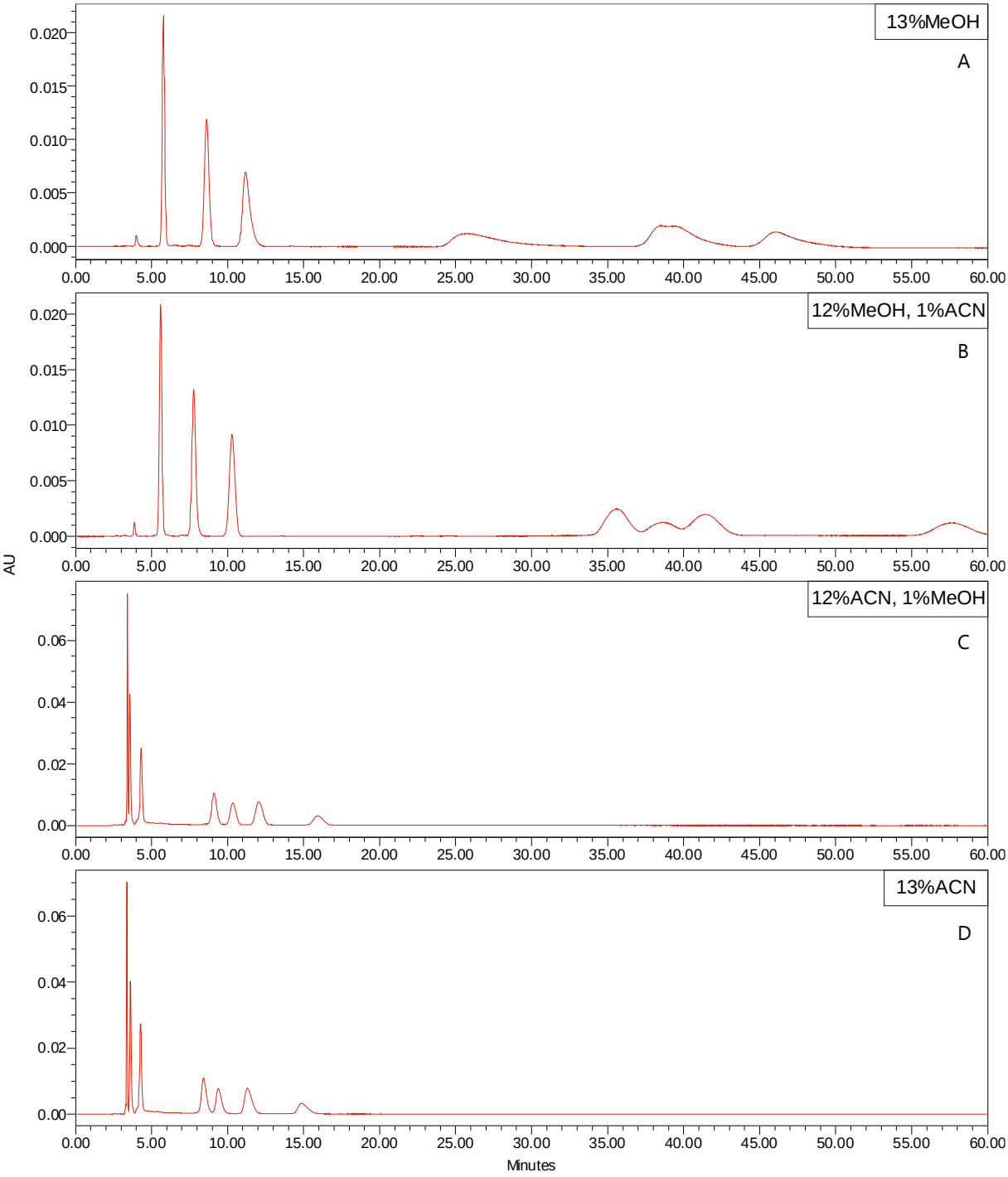

Figure C.4 - Method D chromatograms of 5 ppm standard, using XTerra MS C18 column, variation of organic in the mobile phase: A - 13% MeOH; B - 12% MeOH and 1% ACN; C - 12% ACN and 1% MeOH; D 13% ACN.

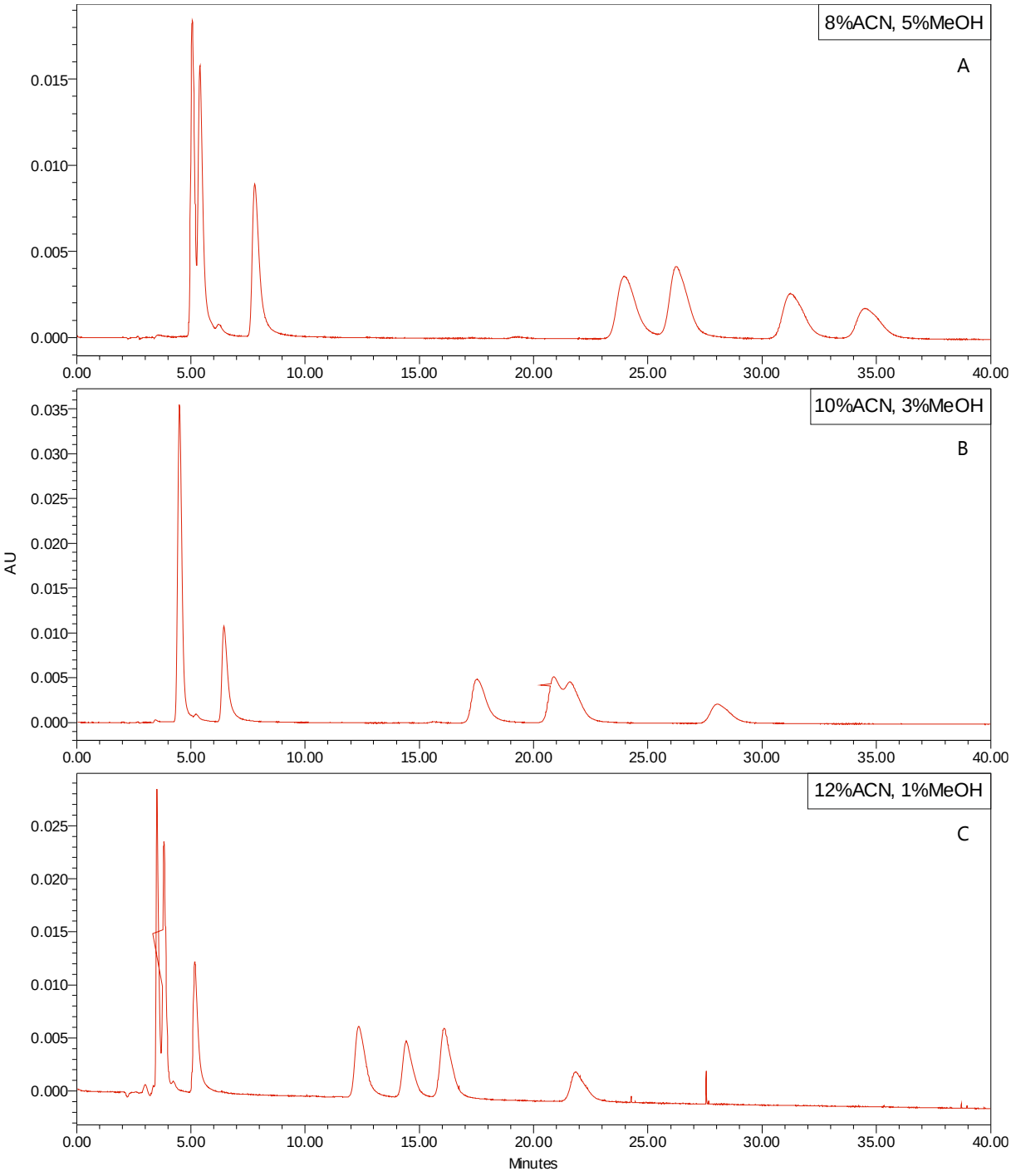

Figure C.5 - Method D chromatograms of 5 ppm standard, using Symmetry C18 column, variation of organic in the mobile phase: A - 8% ACN and 5% MeOH; B - 10% ACN and 3% MeOH; C - 12% ACN and 1% MeOH.

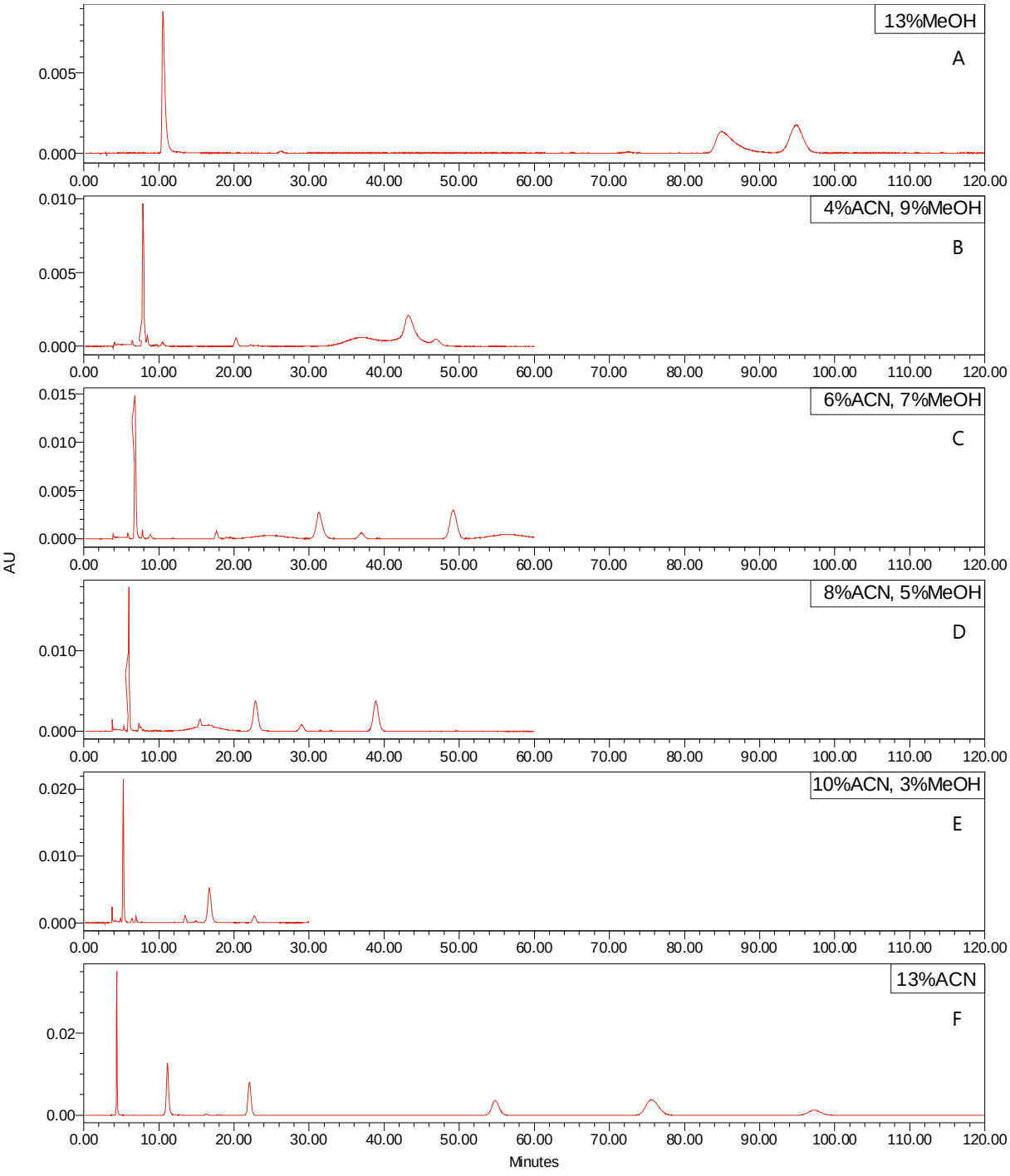

Figure C.6 - Method D chromatograms of 5 ppm standard, using Agilent Zorbax RX-C8 column, variation of organic in the mobile phase: A - 13% MeOH; B - 4% ACN and 9% MeOH and 1% ACN; C - 6% ACN and 7% MeOH; D - 8% ACN and 5% MeOH; E - 10% ACN and 3% MeOH

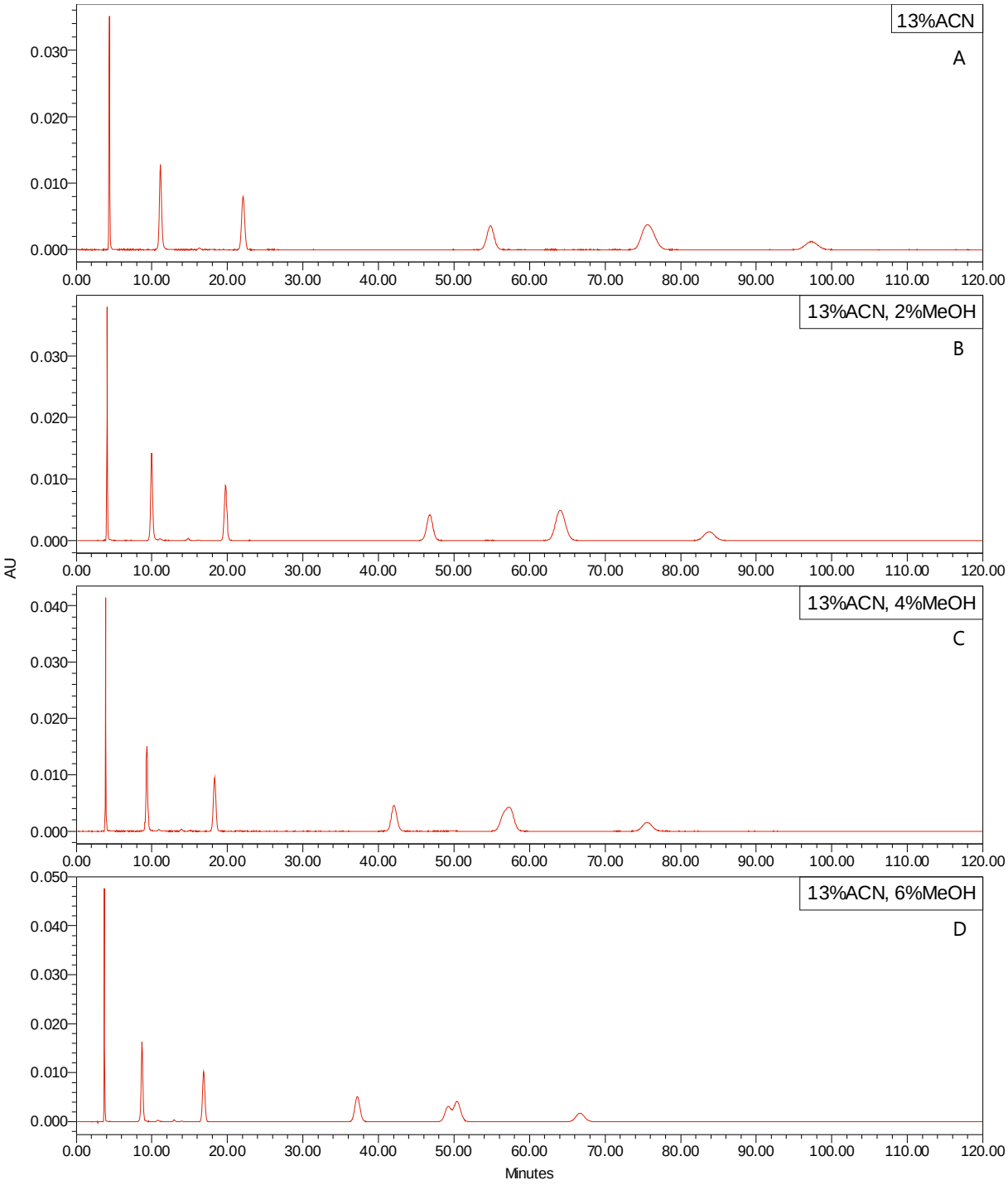

Figure C.7 - Method D chromatograms of 5 ppm standard, using Agilent Zorbax RX-C8 column, variation of MeOH in the mobile phase containing 13% ACN: A - 0% MeOH; B - 2% MeOH; C - 4% MeOH; D - 6% MeOH.

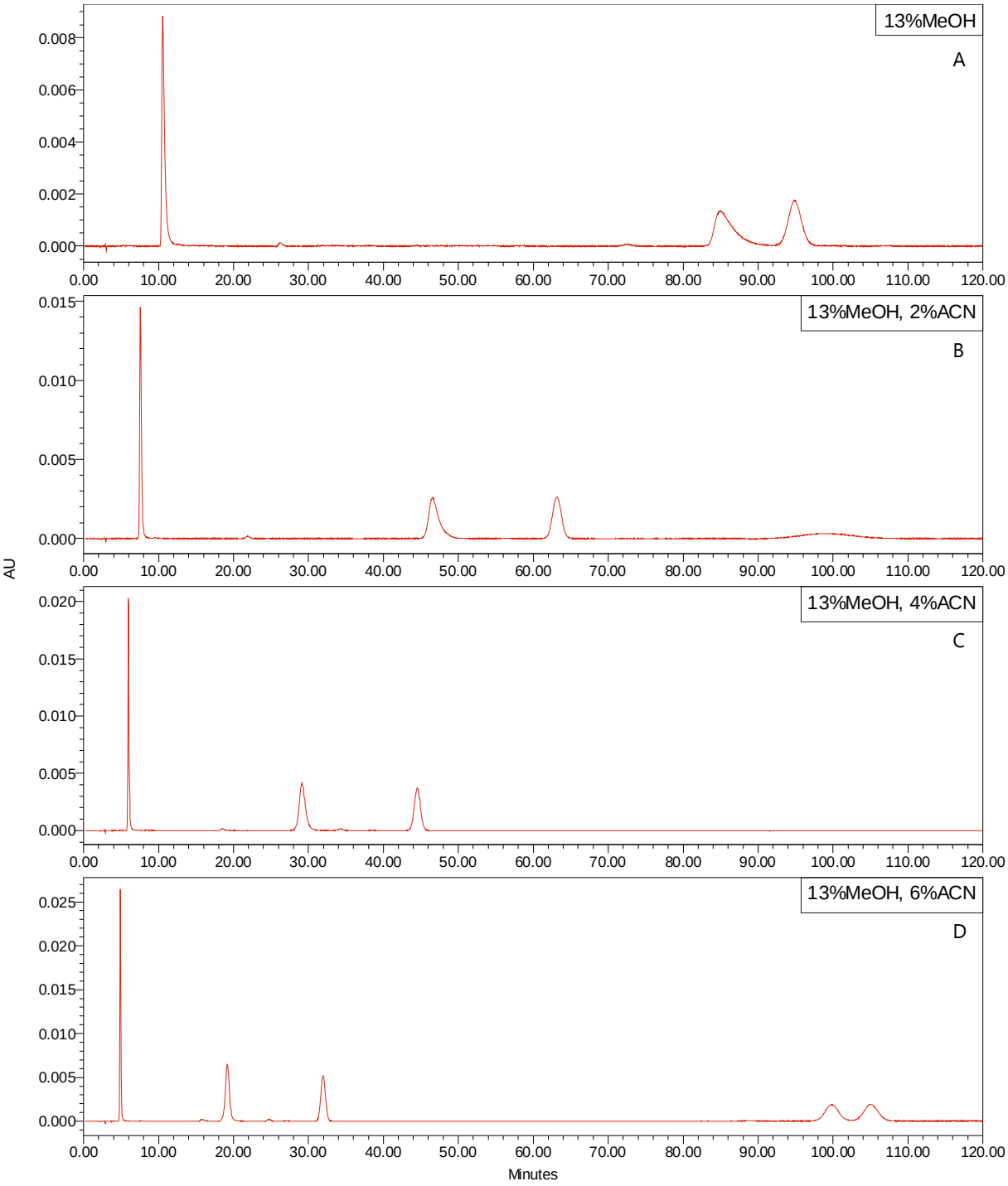

Figure C.8 - Method D chromatograms of 5 ppm standard, using Agilent Zorbax RX-C8 column, variation of ACN in the mobile phase containing 13% MeOH: A - 0% ACN; B - 2% ACN; C - 4% ACN; D - 6% ACN.

C.3 Method E Results

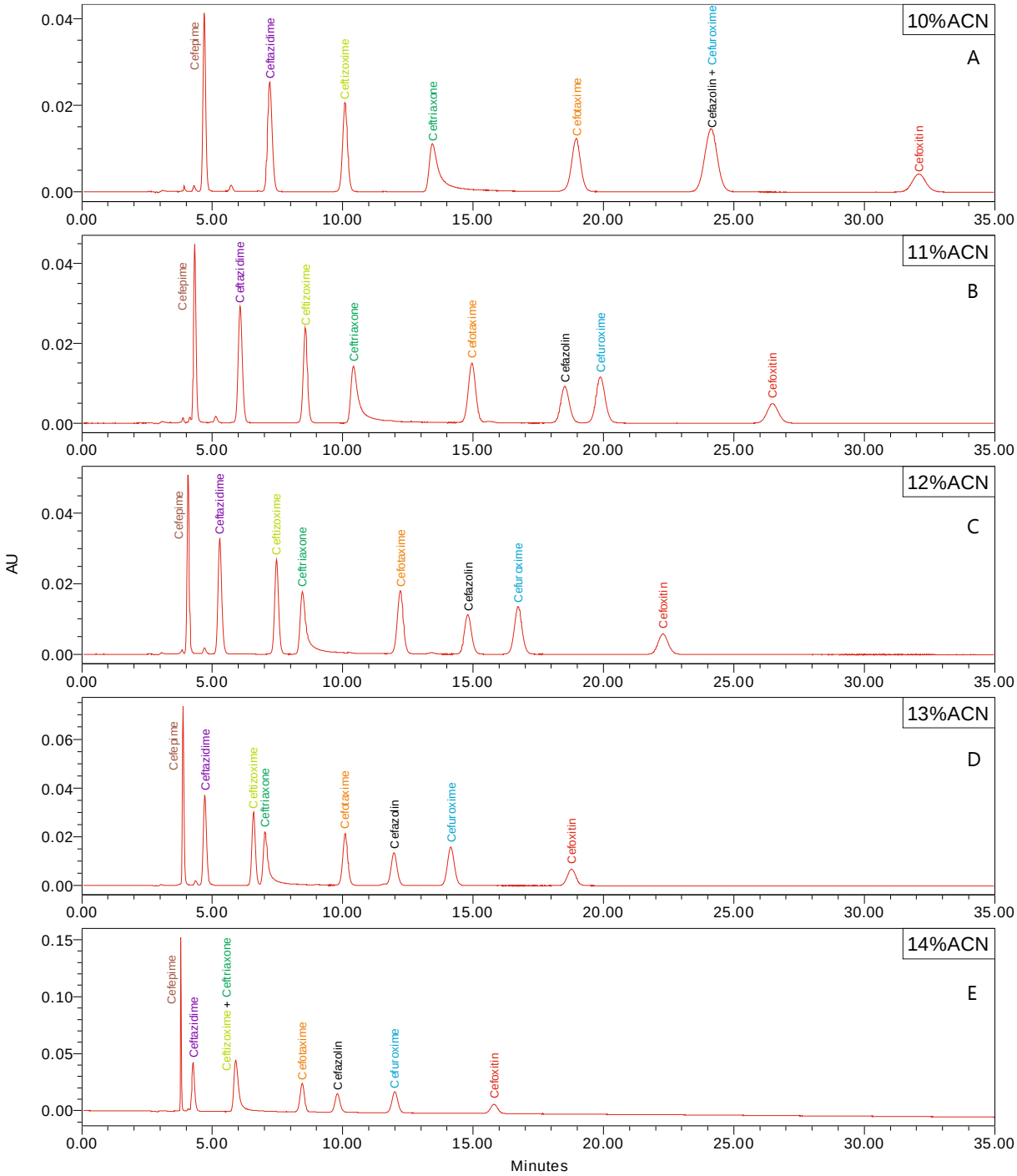

Figure C.9 - Method E chromatograms of 5 ppm standard, variation of ACN in the mobile phase: A - 10% ACN; B - 11% ACN; C - 12% ACN; D - 13% ACN; E - 14% ACN.

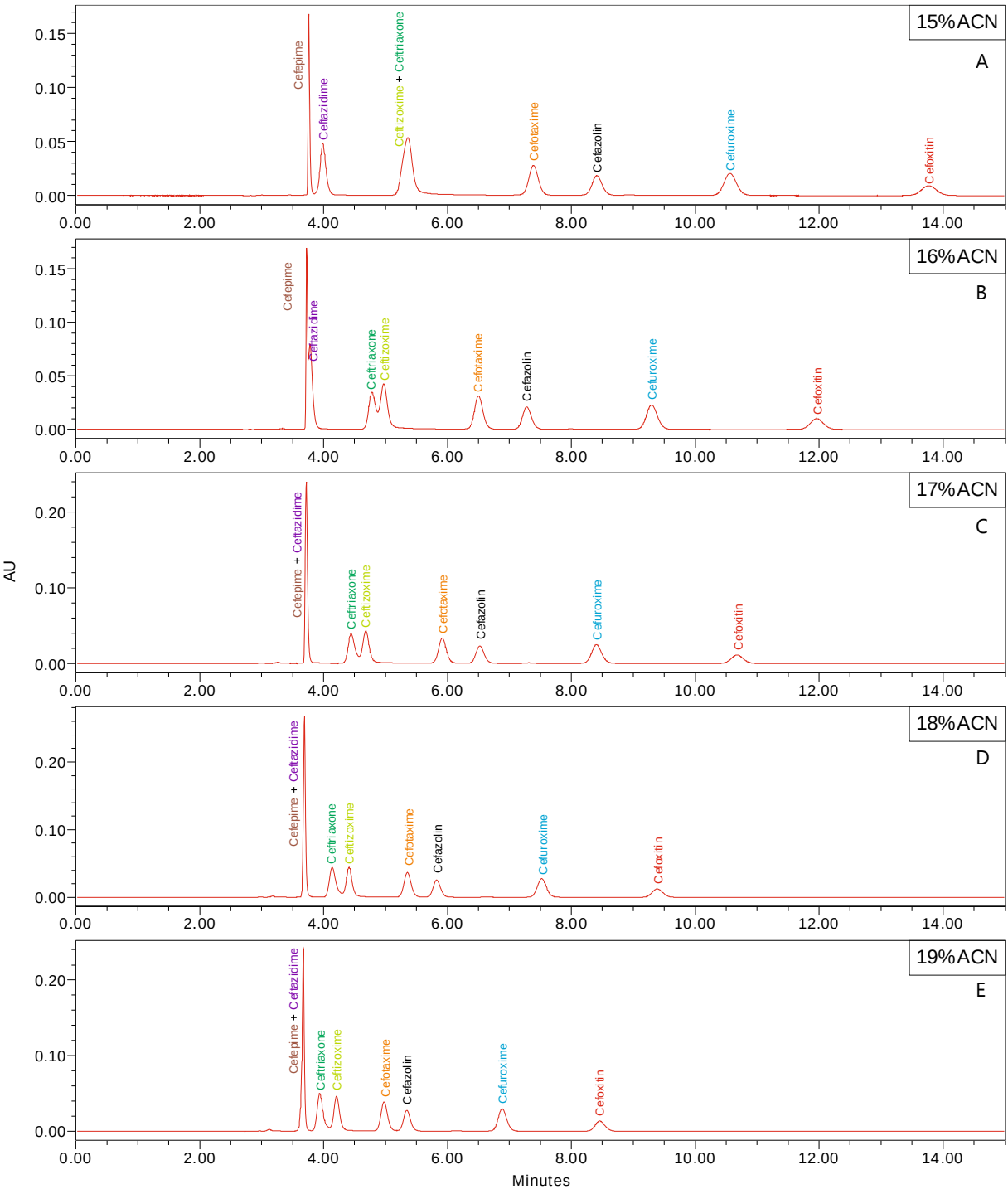

Figure C.10 - Method E chromatograms of 5 ppm standard, variation of ACN in the mobile phase: A - 15% ACN; B - 16% ACN; C - 17% ACN; D - 18% ACN; E - 19% ACN.

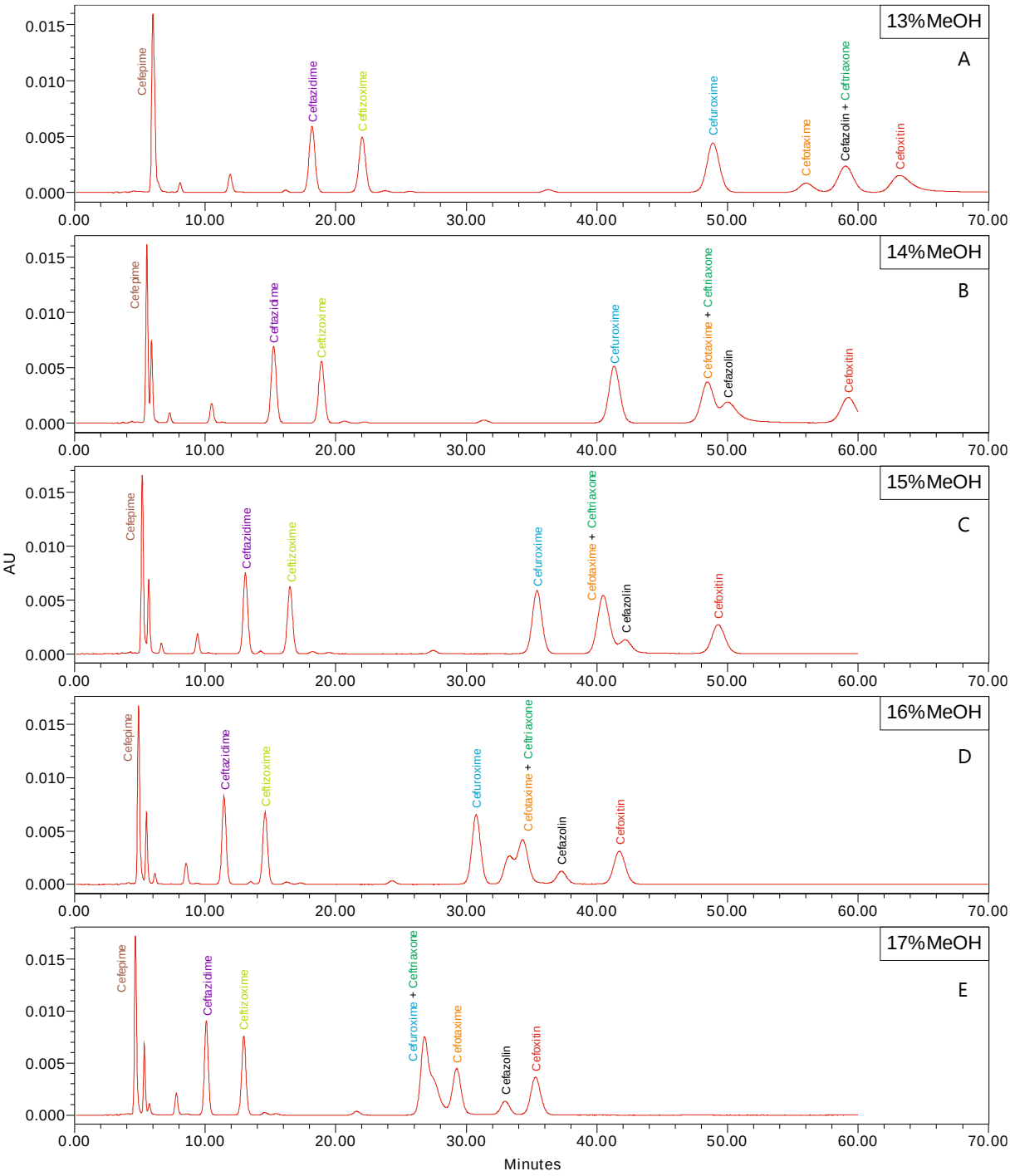

Figure C.11 - Method E chromatograms of 5 ppm standard, variation of MeOH in the mobile phase: A - 13% MeOH; B - 14% MeOH; C - 15% MeOH; D - 16% MeOH; E - 17% MeOH.

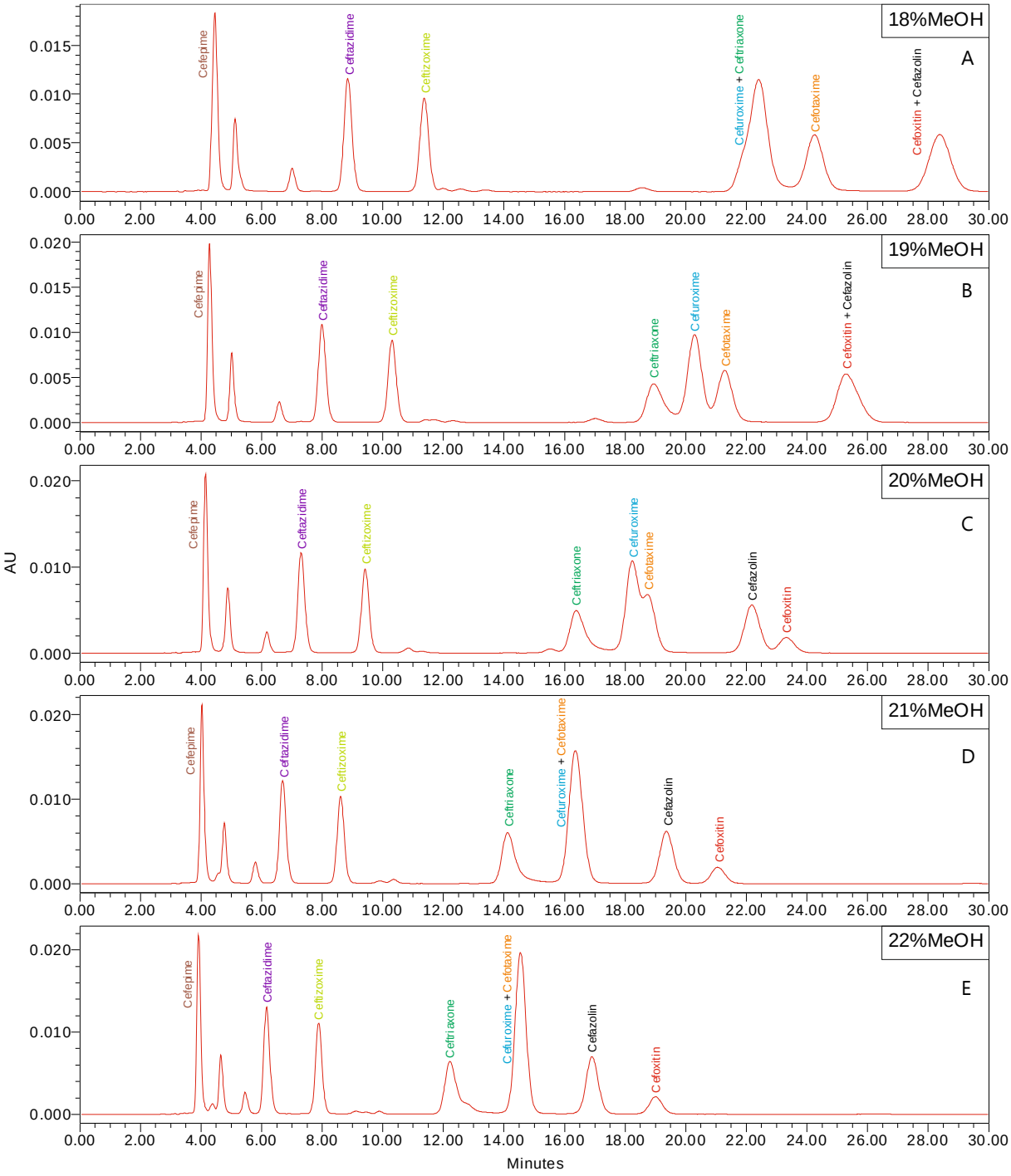

Figure C.12 - Method E chromatograms of 5 ppm standard, variation of MeOH in the mobile phase: A - 18% MeOH; B - 19% MeOH; C - 20% MeOH; D - 21% MeOH; E - 22% MeOH.

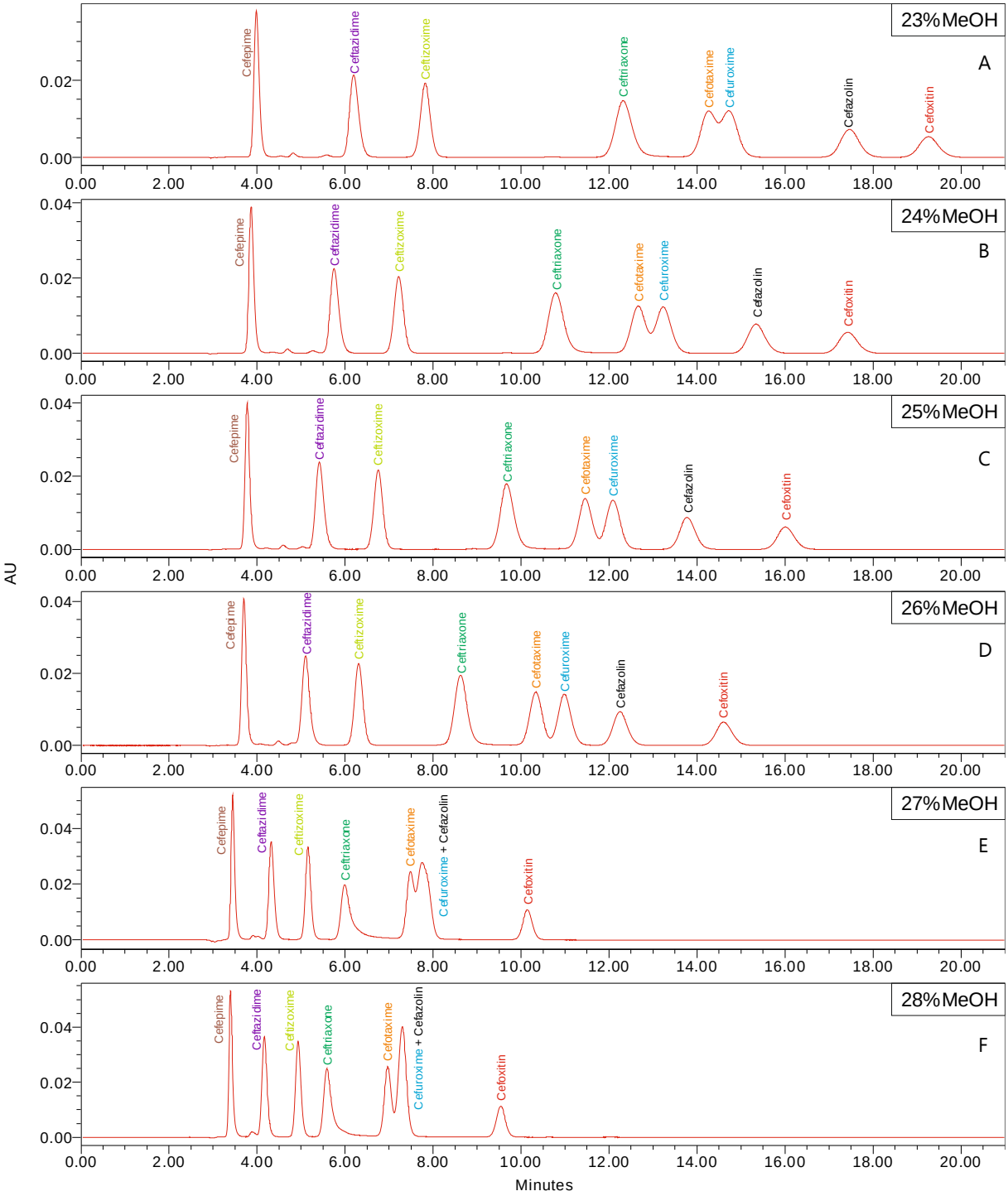

Figure C.13 - Method E chromatograms of 5 ppm standard, variation of MeOH in the mobile phase: A - 23% MeOH; B - 24% MeOH; C - 25% MeOH; D - 26% MeOH; E - 27% MeOH; F - 28% MeOH

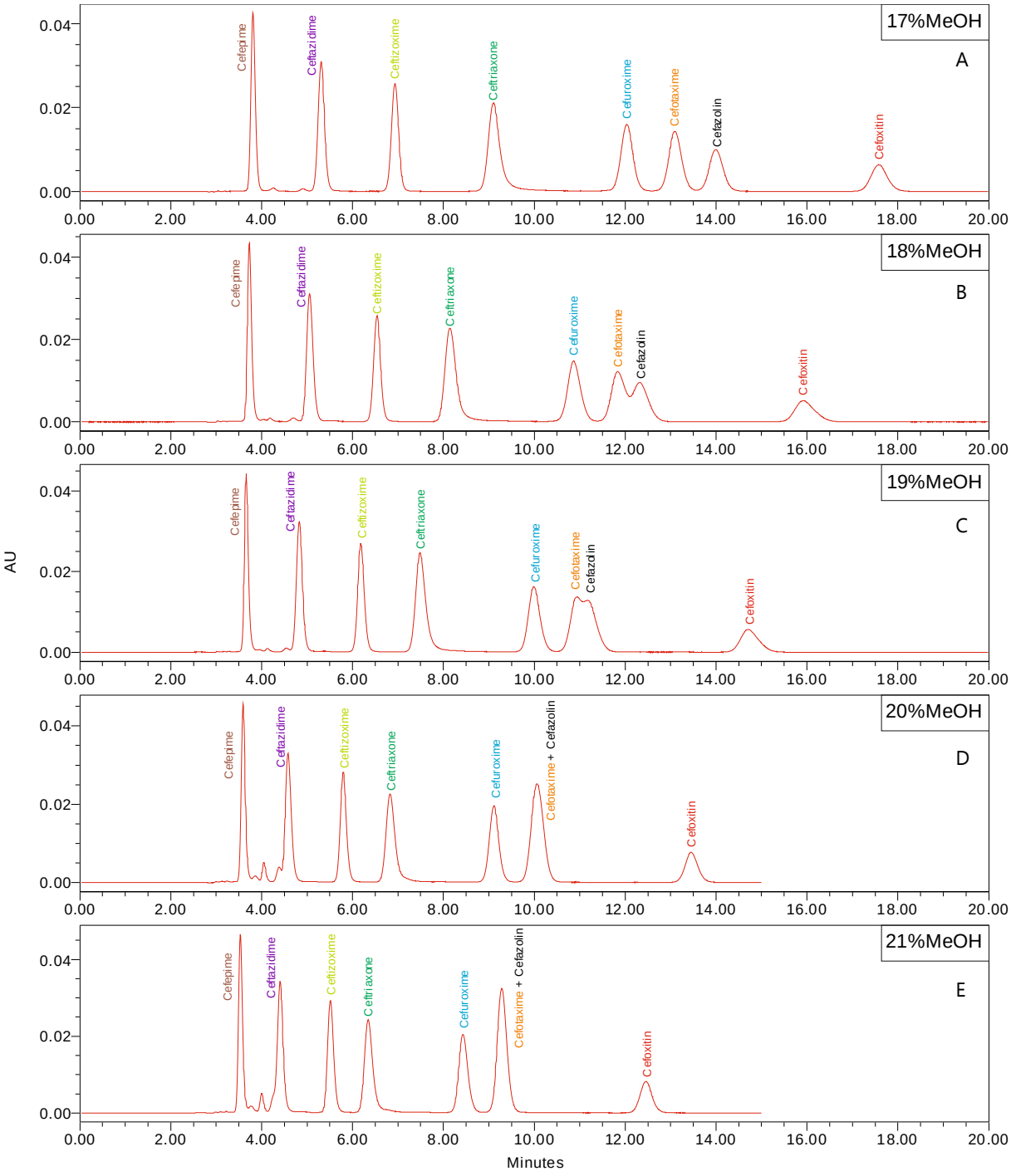

Figure C.14 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 4% ACN: A - 17% MeOH; B - 18% MeOH; C - 19% MeOH; D - 20% MeOH; E - 21% MeOH.

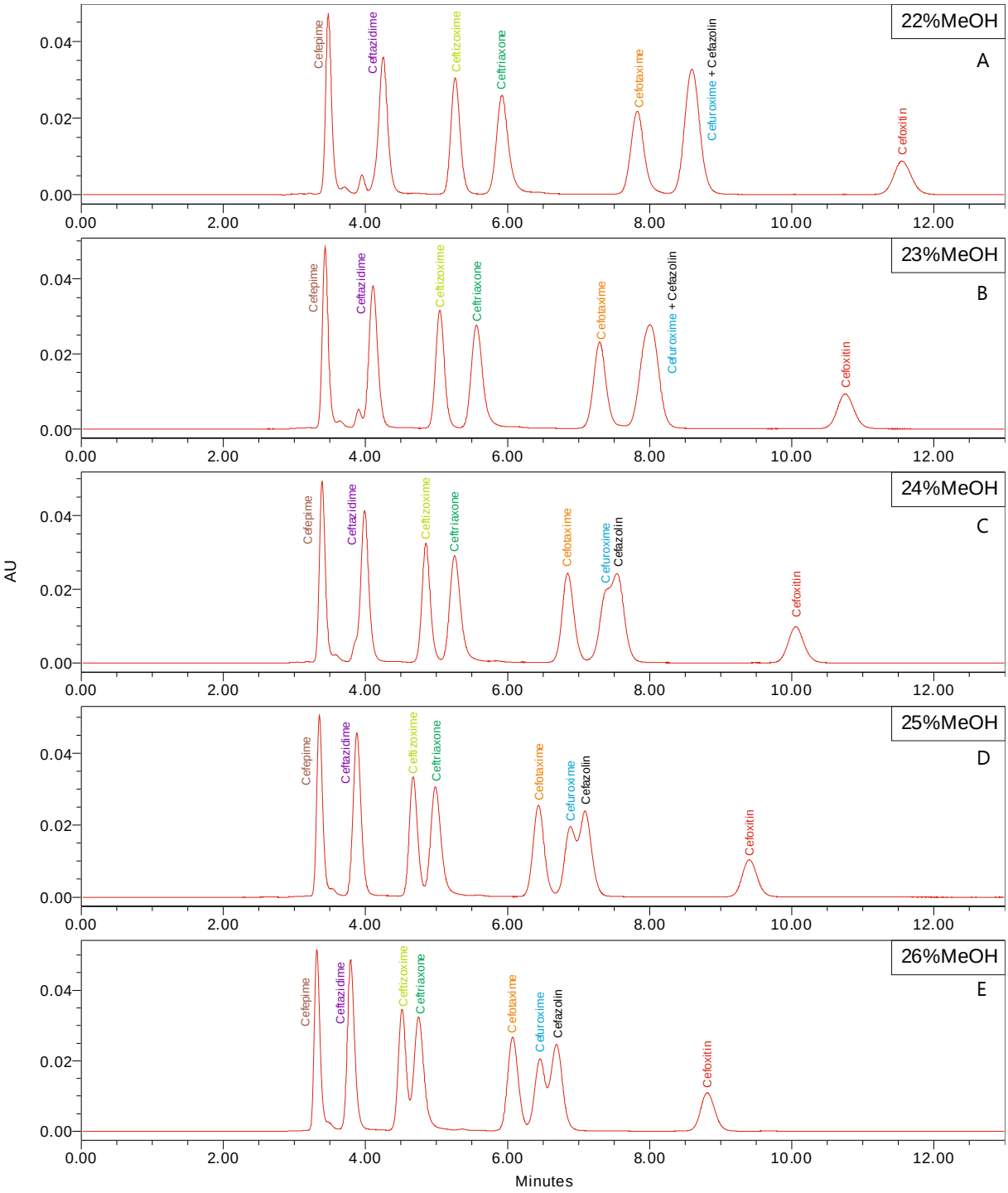

Figure C.15 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 4% ACN: A - 22% MeOH; B - 23% MeOH; C - 24% MeOH; D - 25% MeOH; E - 26% MeOH.

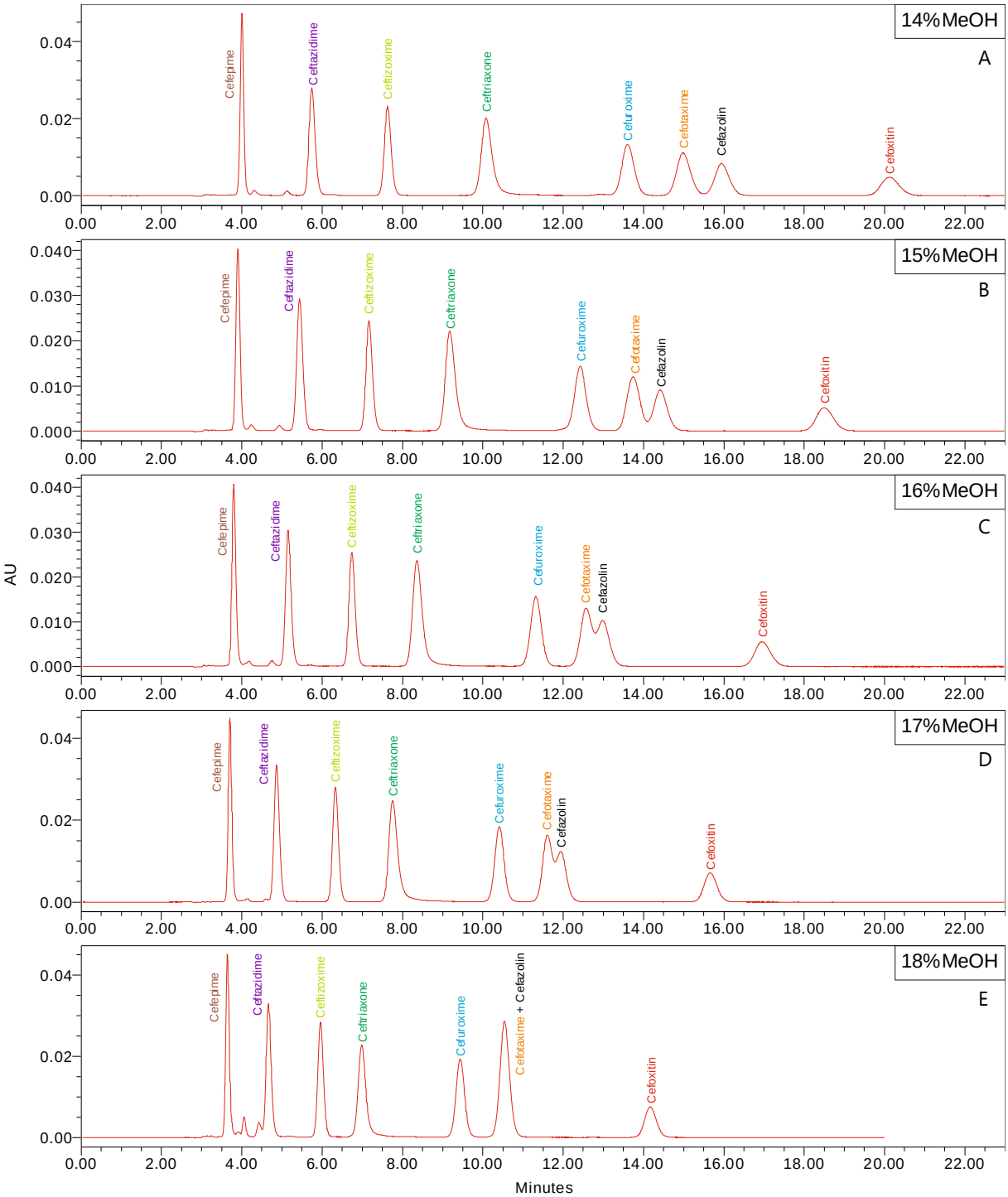

Figure C.16 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 5% ACN: A - 14% MeOH; B - 15% MeOH; C - 16% MeOH; D - 17% MeOH; E - 18% MeOH.

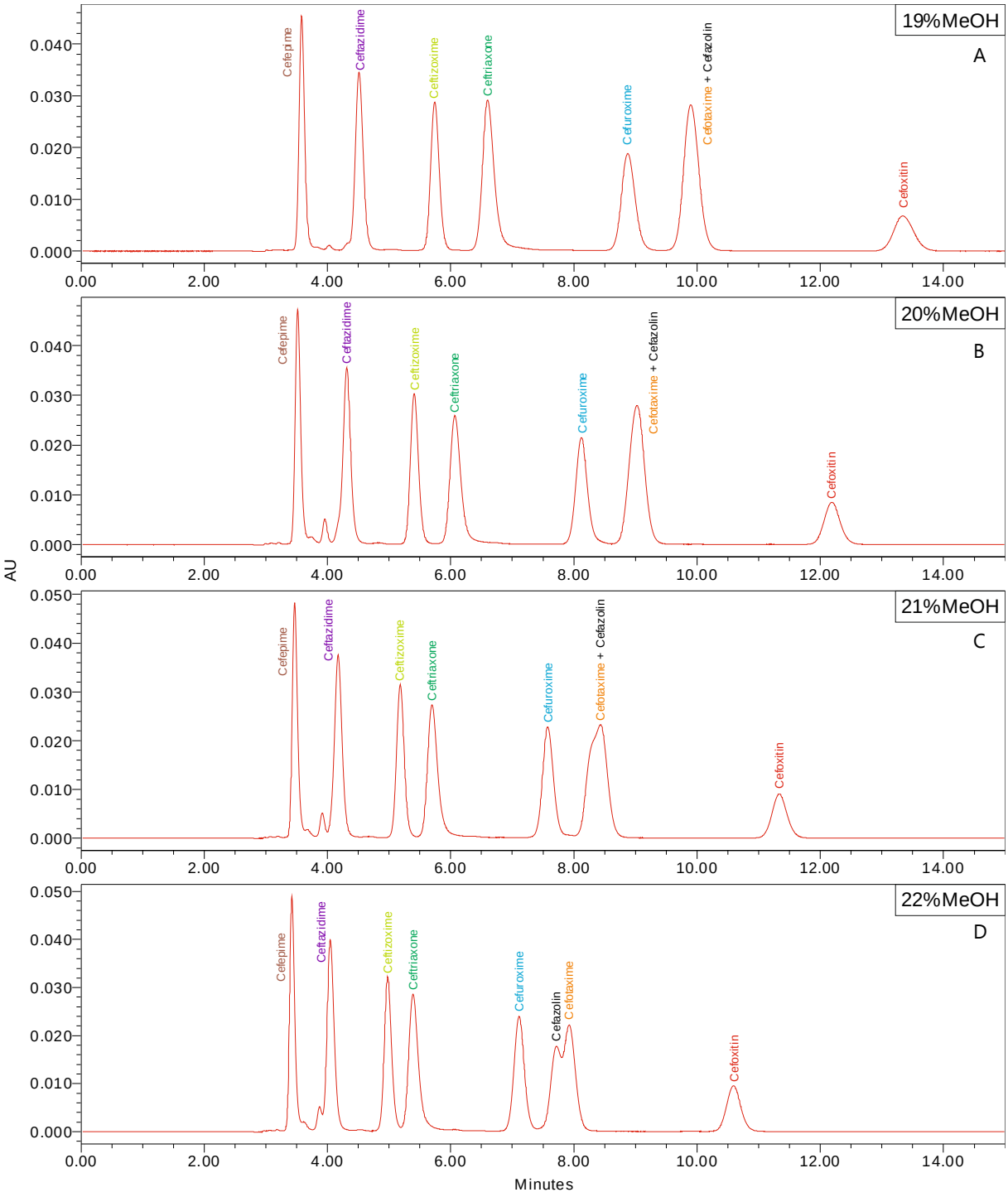

Figure C.17 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 5% ACN: A - 19% MeOH; B - 20% MeOH; C - 21% MeOH; D - 22% MeOH.

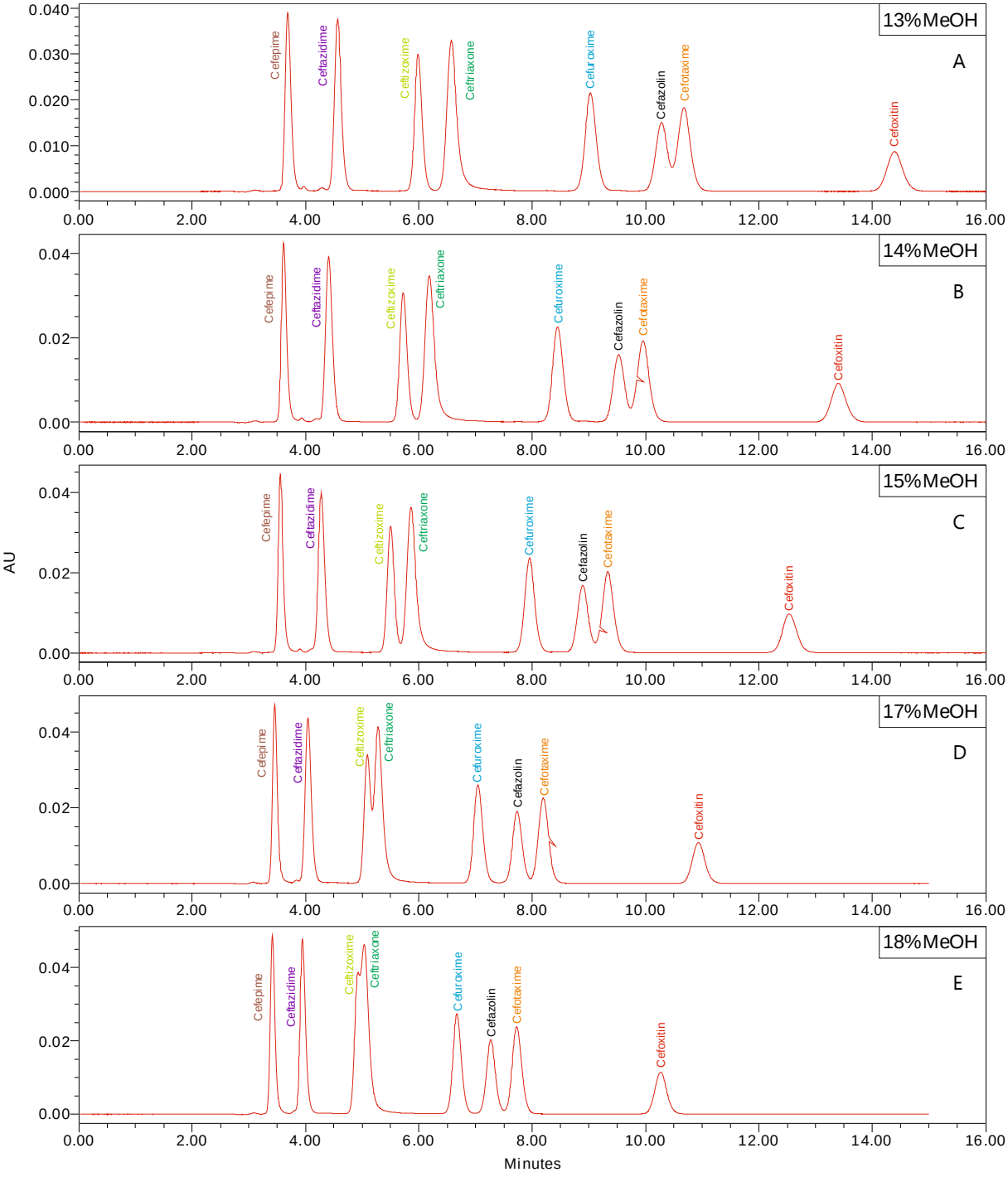

Figure C.18 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 8% ACN: A - 13% MeOH; B - 14% MeOH; C - 15% MeOH; D - 17% MeOH; E - 18% MeOH.

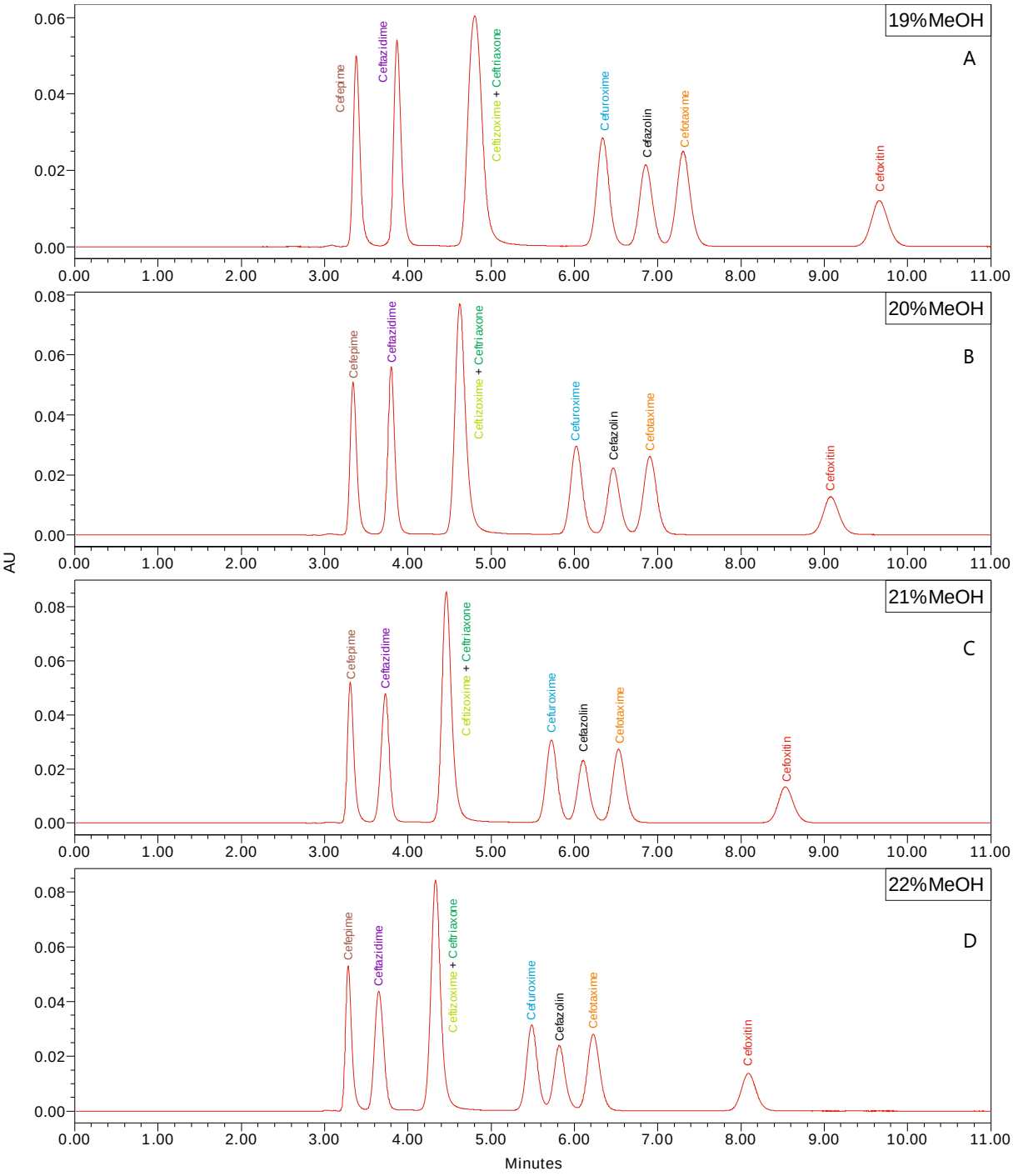

Figure C.19 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 8% ACN: A - 19% MeOH; B - 20% MeOH; C - 21% MeOH; D - 22% MeOH.

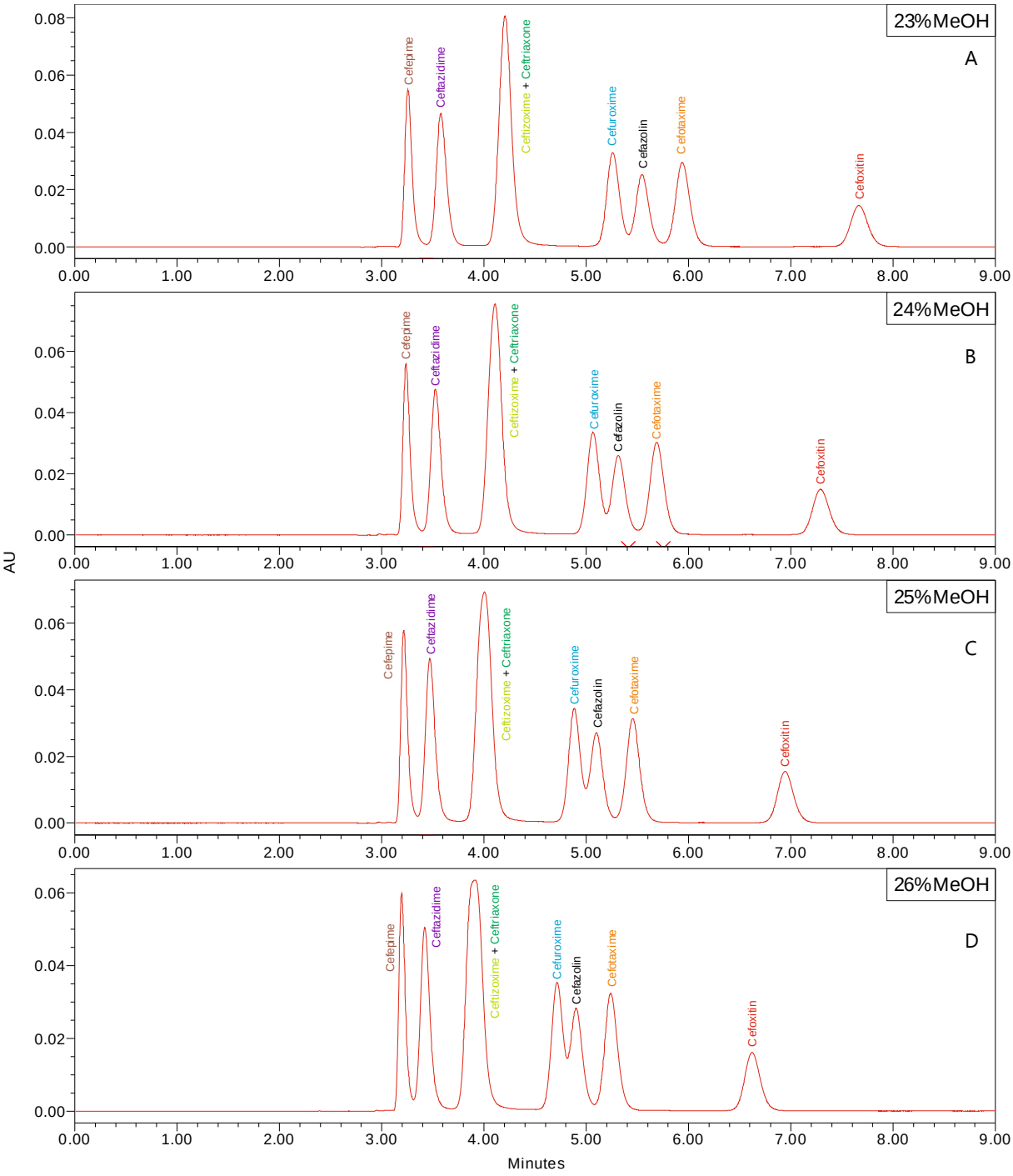

Figure C.20 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 8% ACN: A - 23% MeOH; B - 24% MeOH; C - 25% MeOH; D - 26% MeOH.

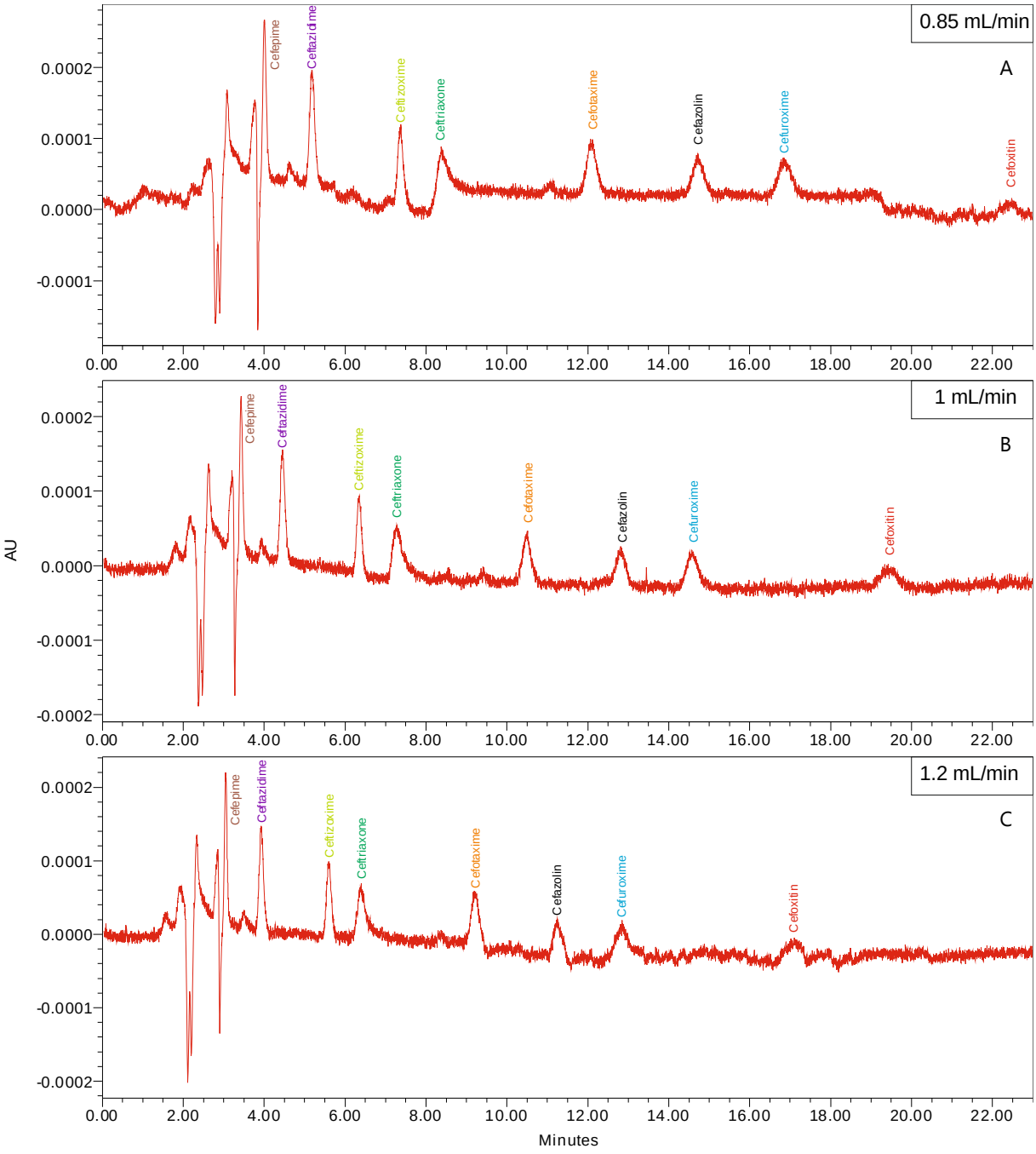

Figure C.21 - Method E chromatograms of 0.02 ppm standard, mobile phase with 12% ACN, variation of flow rate: A - 0.85 mL/min; B - 1 mL/min; C - 1.2 mL/min.

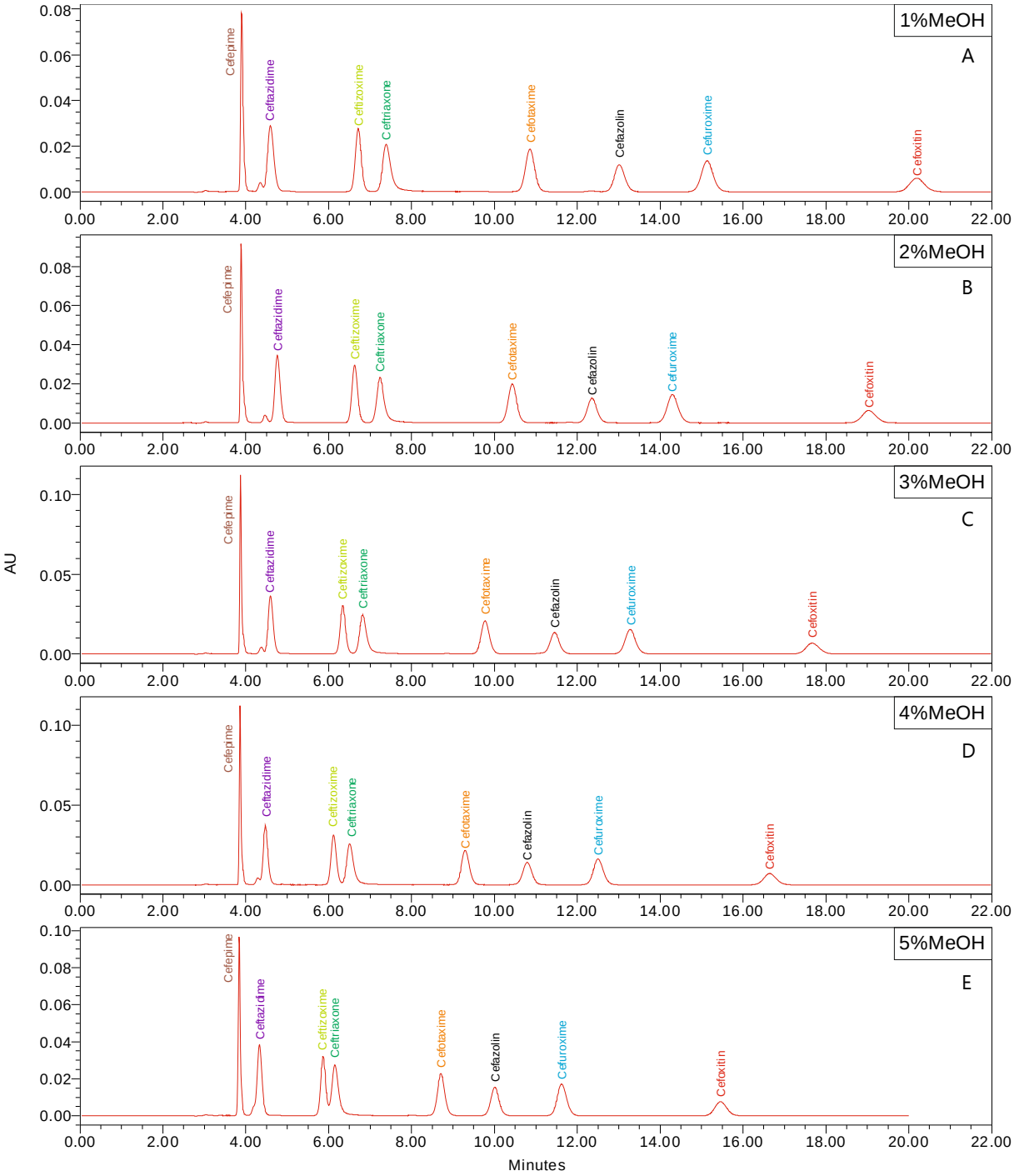

Figure C.22 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 12% ACN: A - 1% MeOH; B - 2% MeOH; C - 3% MeOH; D - 4% MeOH; E - 5% MeOH.

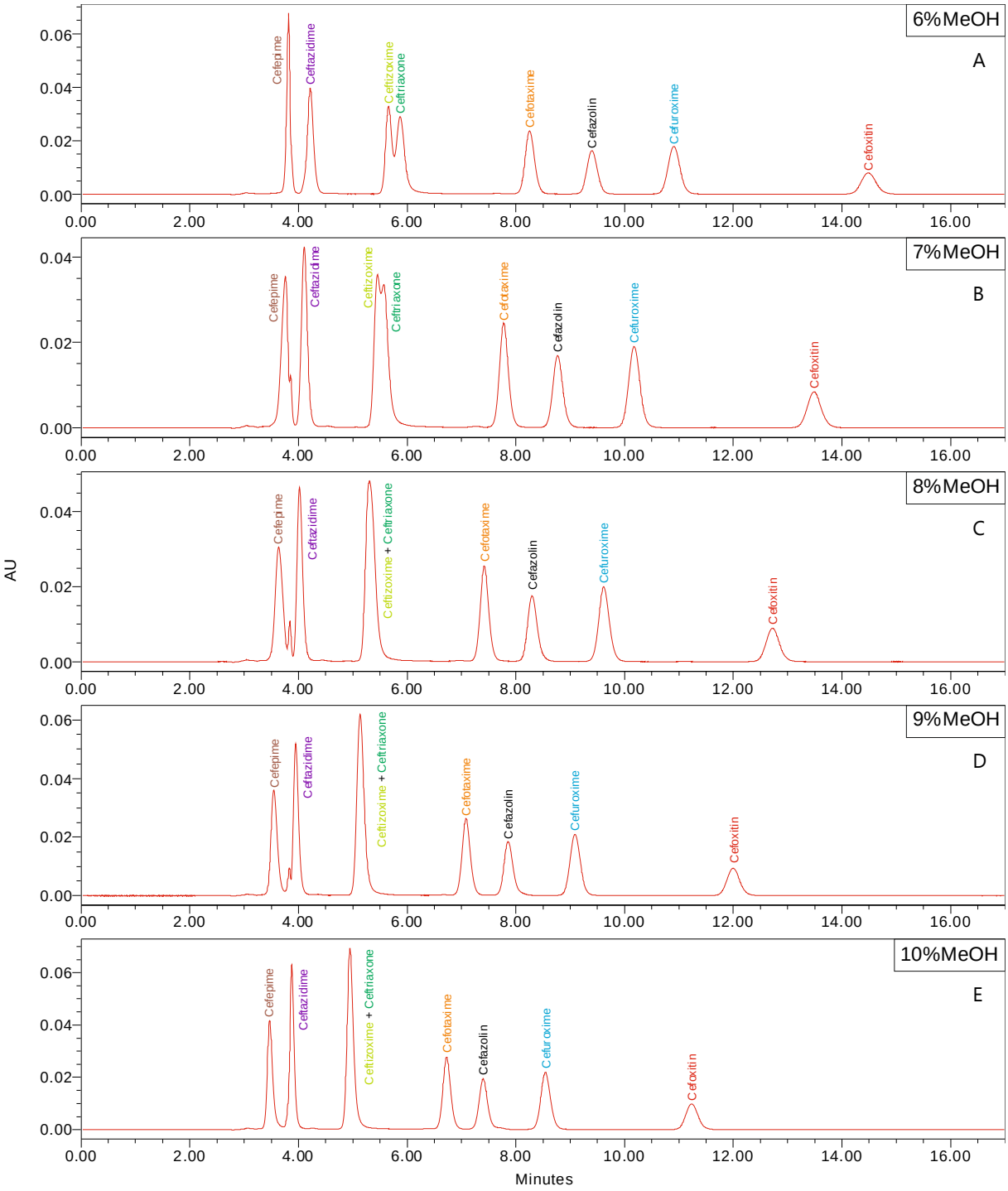

Figure C.23 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 12% ACN: A - 6% MeOH; B - 7% MeOH; C - 8% MeOH; D - 9% MeOH; E - 10% MeOH.

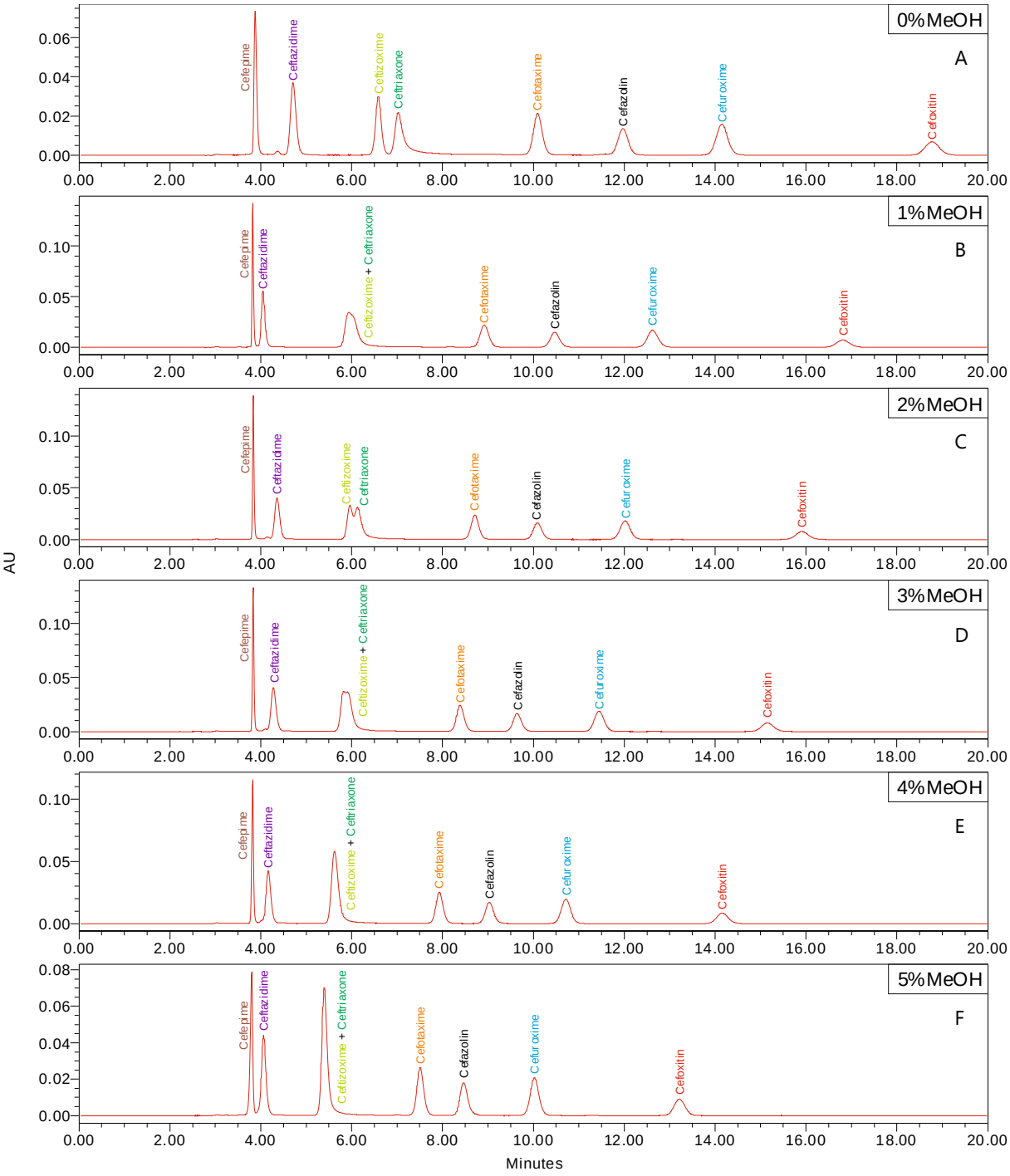

Figure C.24 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 13% ACN: A - 0% MeOH; B - 1% MeOH; C - 2% MeOH; D - 3% MeOH; E - 4% MeOH; F - 5% MeOH

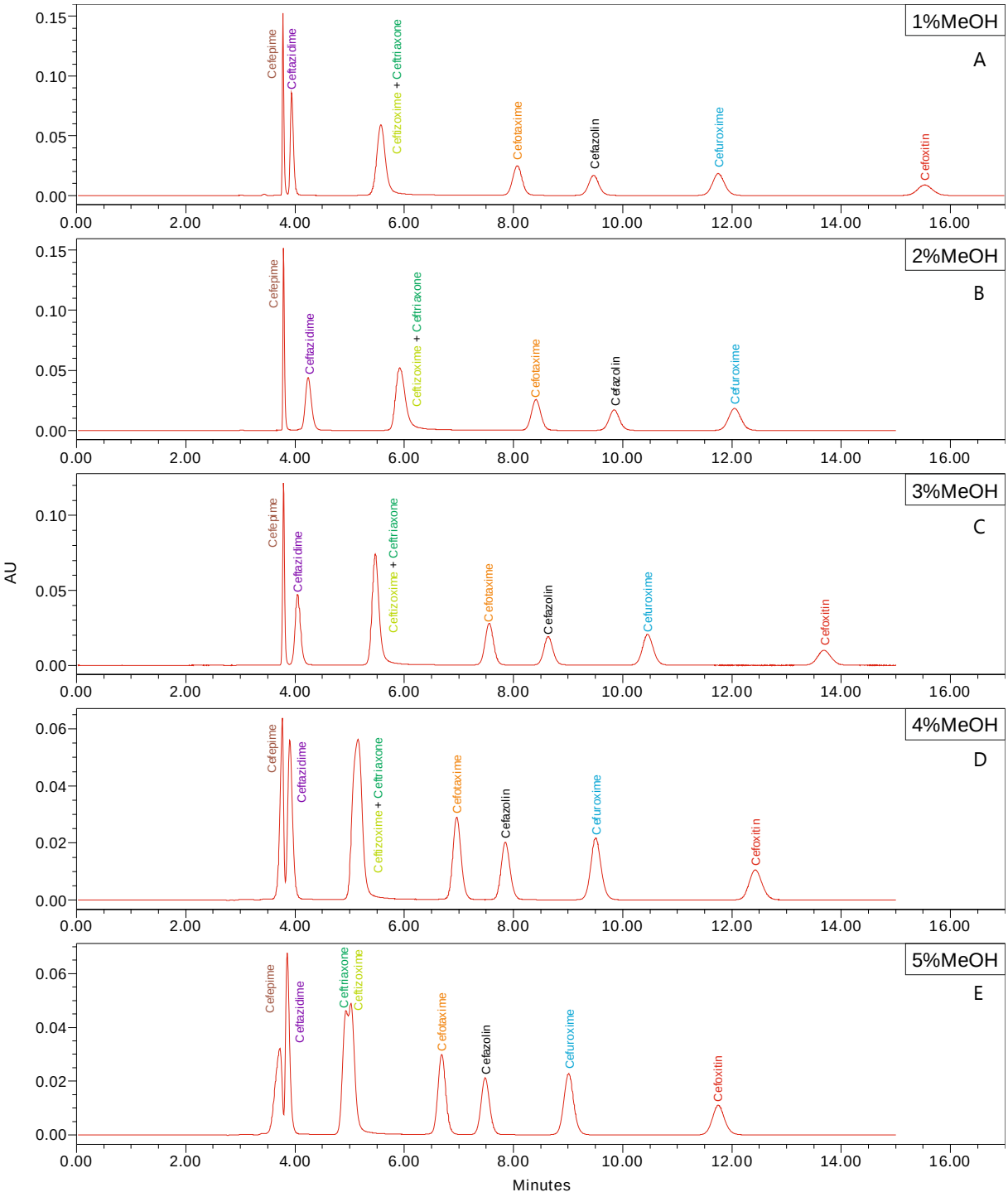

Figure C.25 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 14% ACN: A - 1% MeOH; B - 2% MeOH; C - 3% MeOH; D - 4% MeOH; E - 5% MeOH.

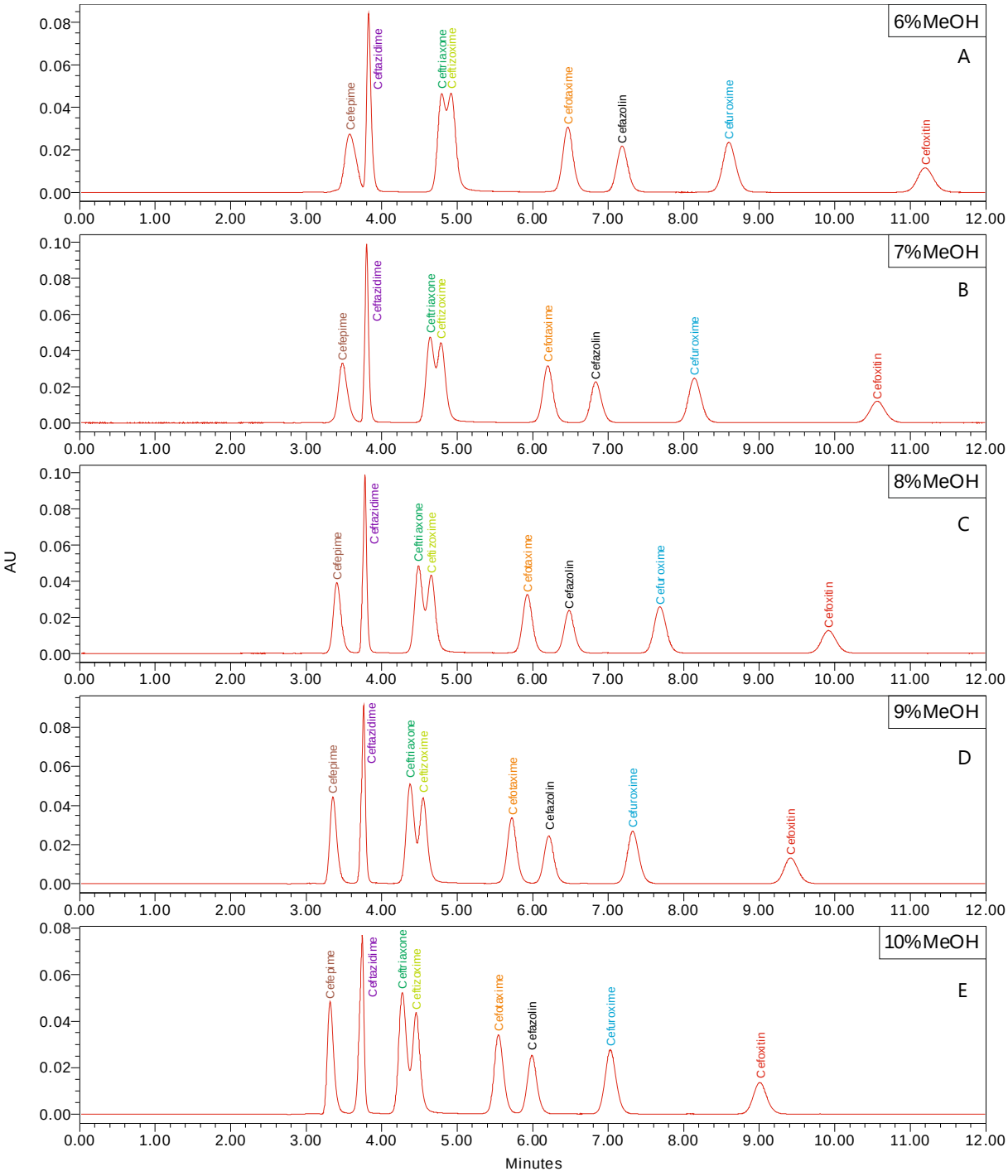

Figure C.26 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 14% ACN: A - 6% MeOH; B - 7% MeOH; C - 8% MeOH; D - 9% MeOH; E - 10% MeOH.

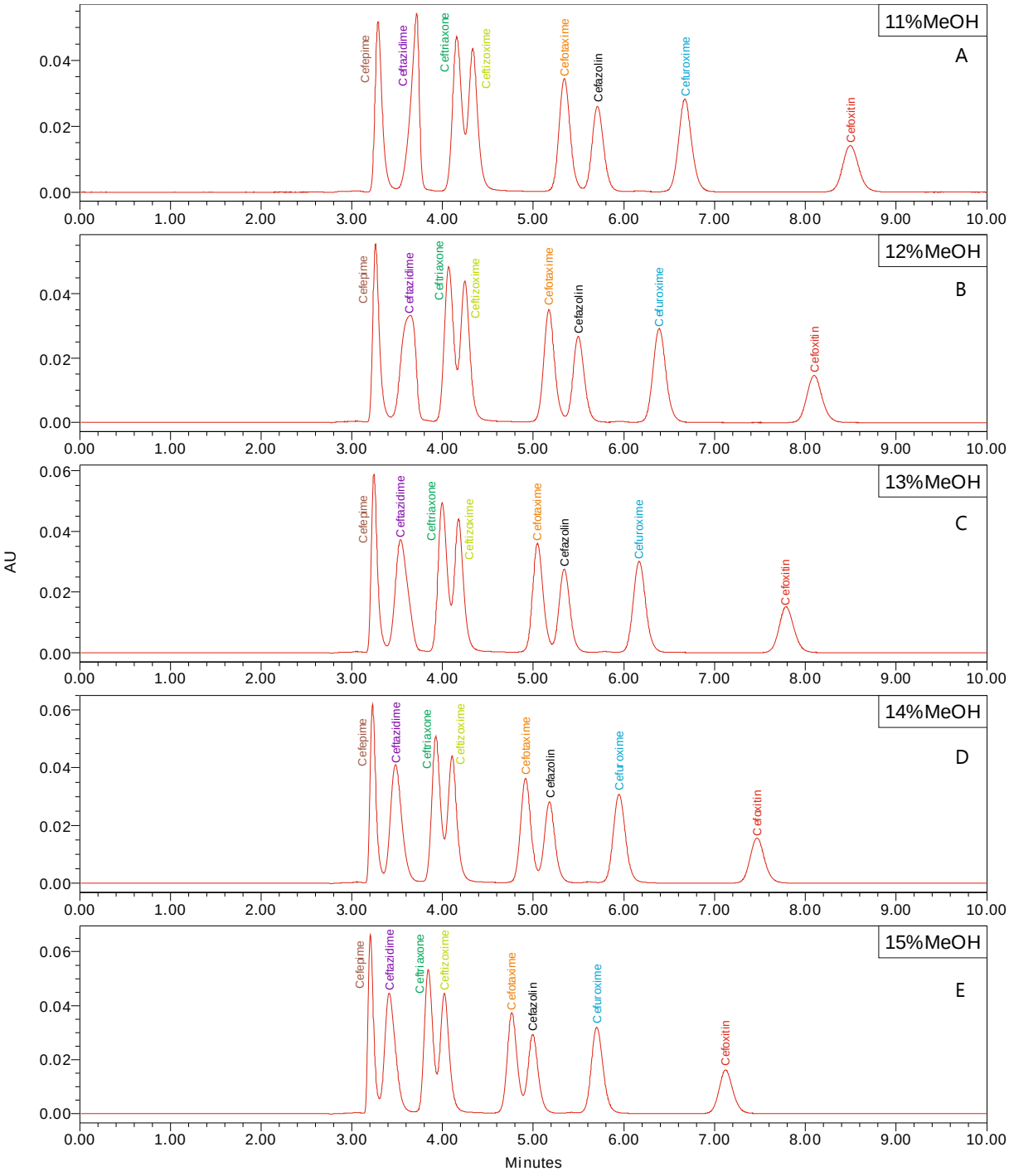

Figure C.27 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 14% ACN: A - 11% MeOH; B - 12% MeOH; C - 13% MeOH; D - 14% MeOH; E - 15% MeOH.

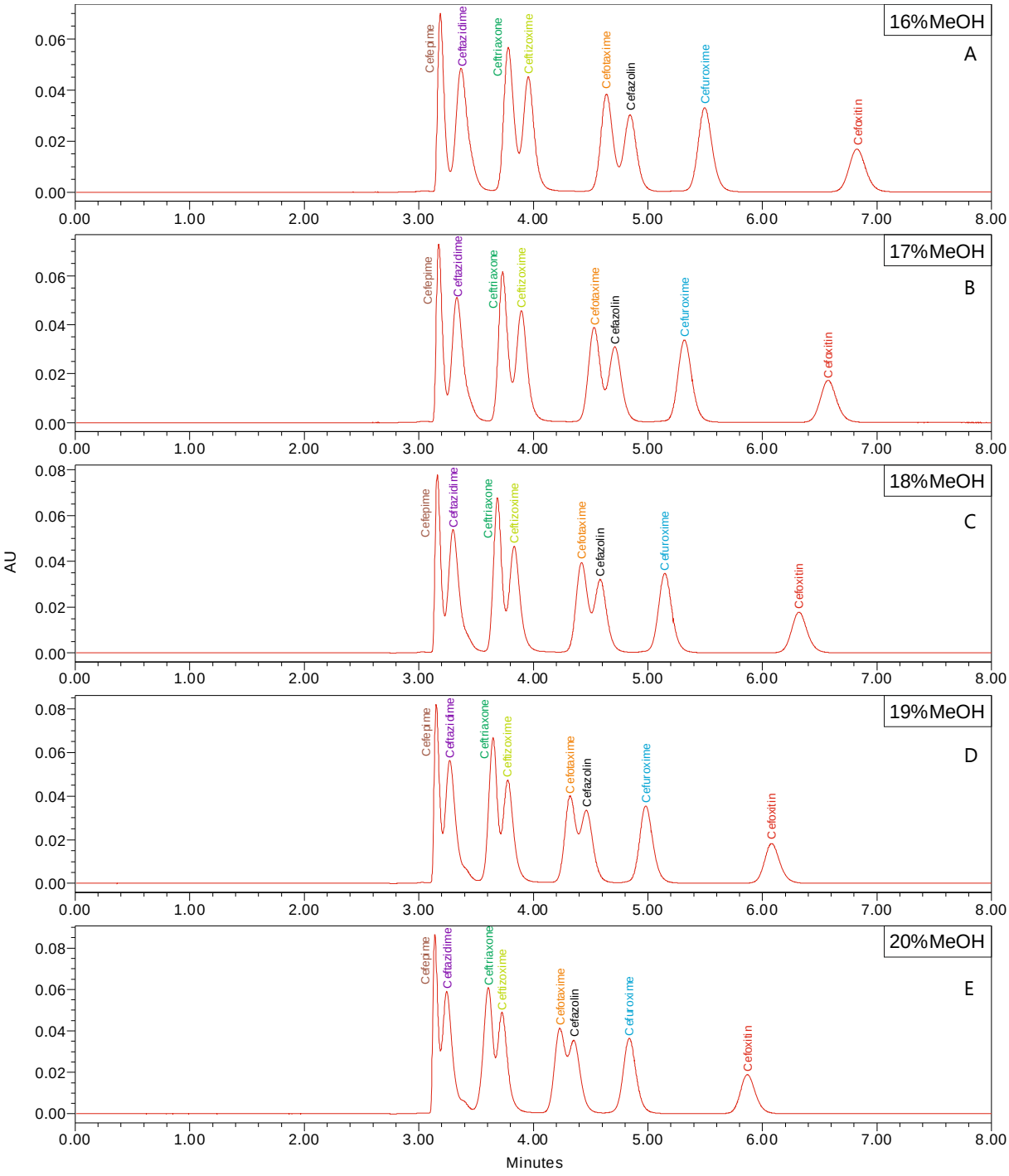

Figure C.28 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 14% ACN: A - 16% MeOH; B - 17% MeOH; C - 18% MeOH; D - 19% MeOH; E - 20% MeOH.

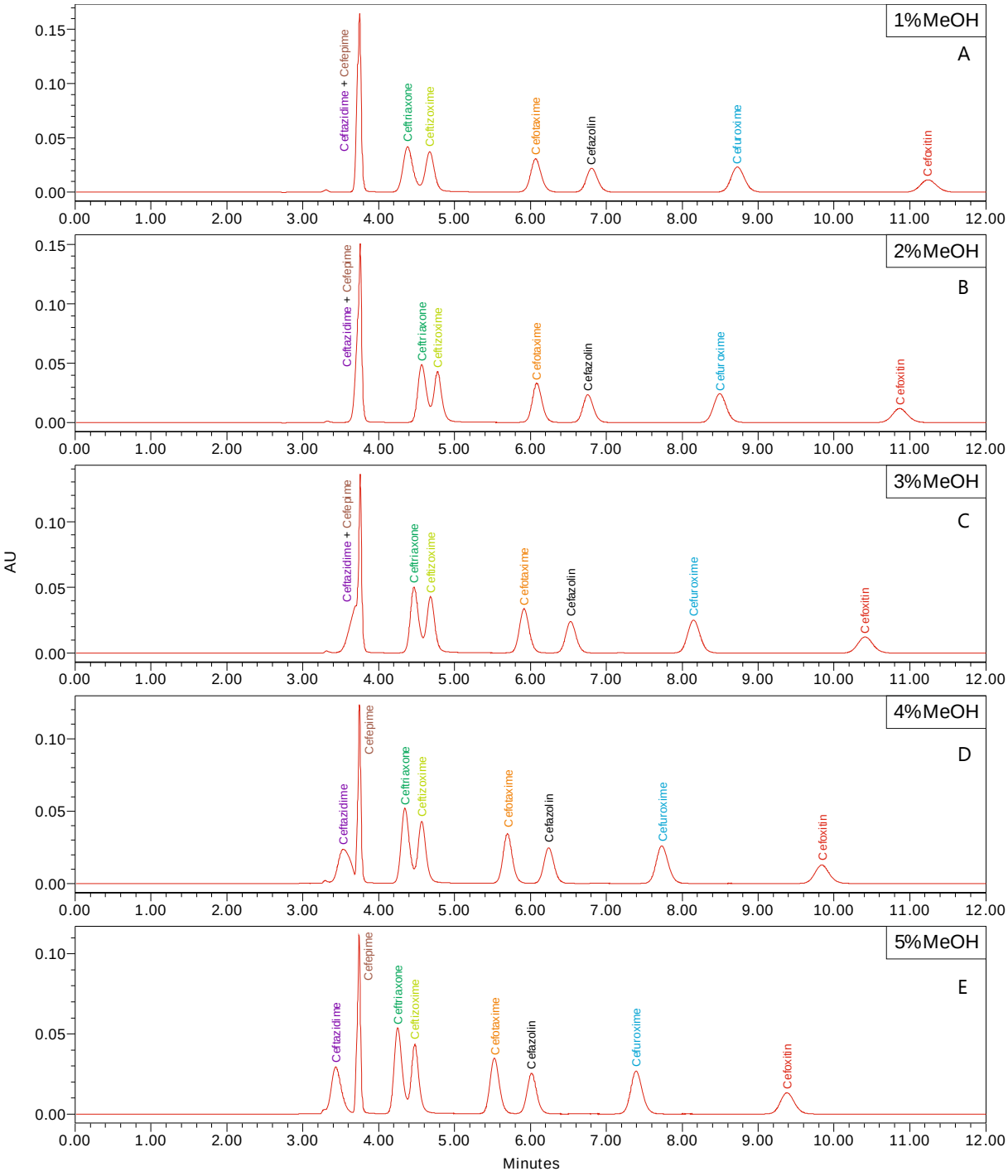

Figure C.29 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 16% ACN: A - 1% MeOH; B - 2% MeOH; C - 3% MeOH; D - 4% MeOH; E - 5% MeOH.

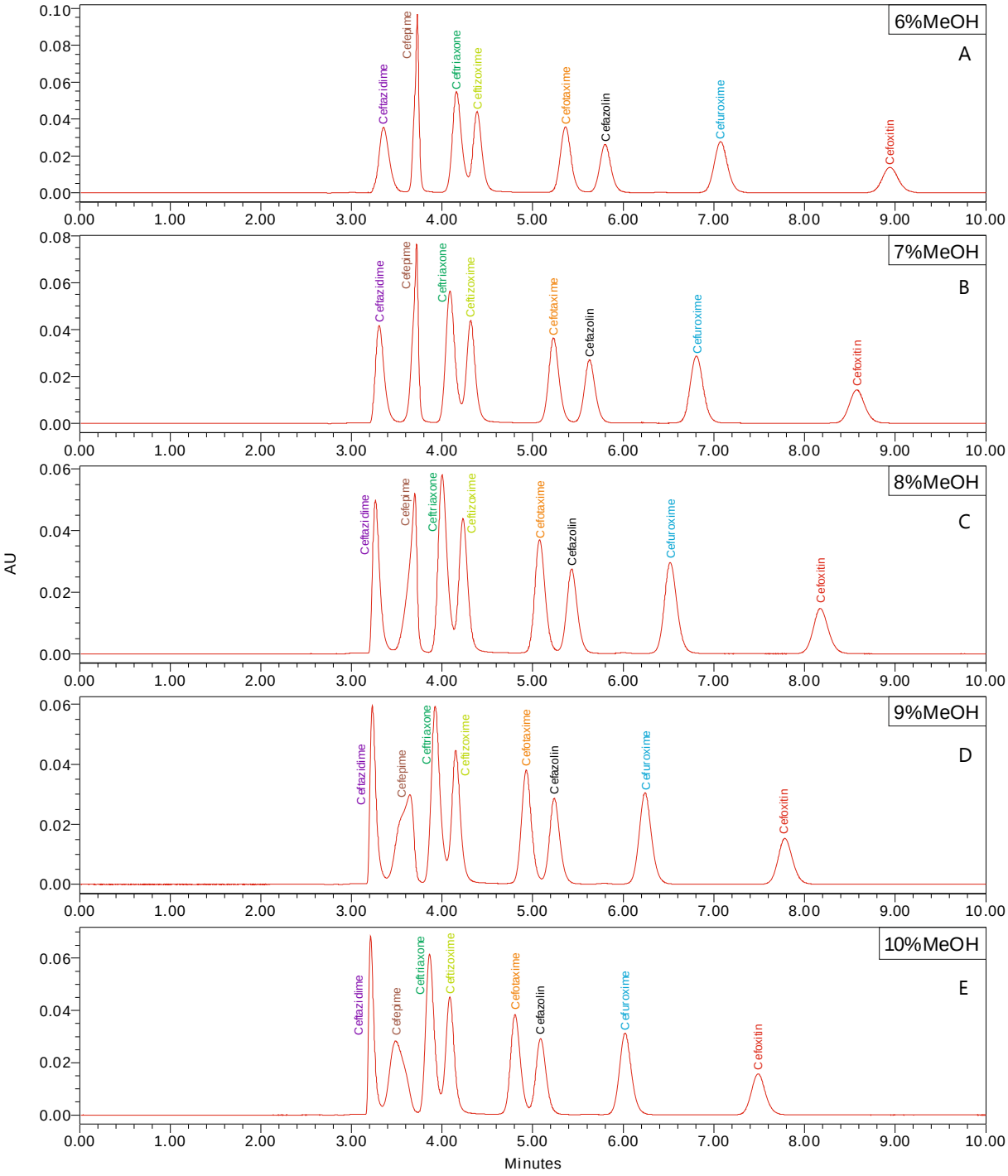

Figure C.30 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 16% ACN: A - 6% MeOH; B - 7% MeOH; C - 8% MeOH; D - 9% MeOH; E - 10% MeOH.

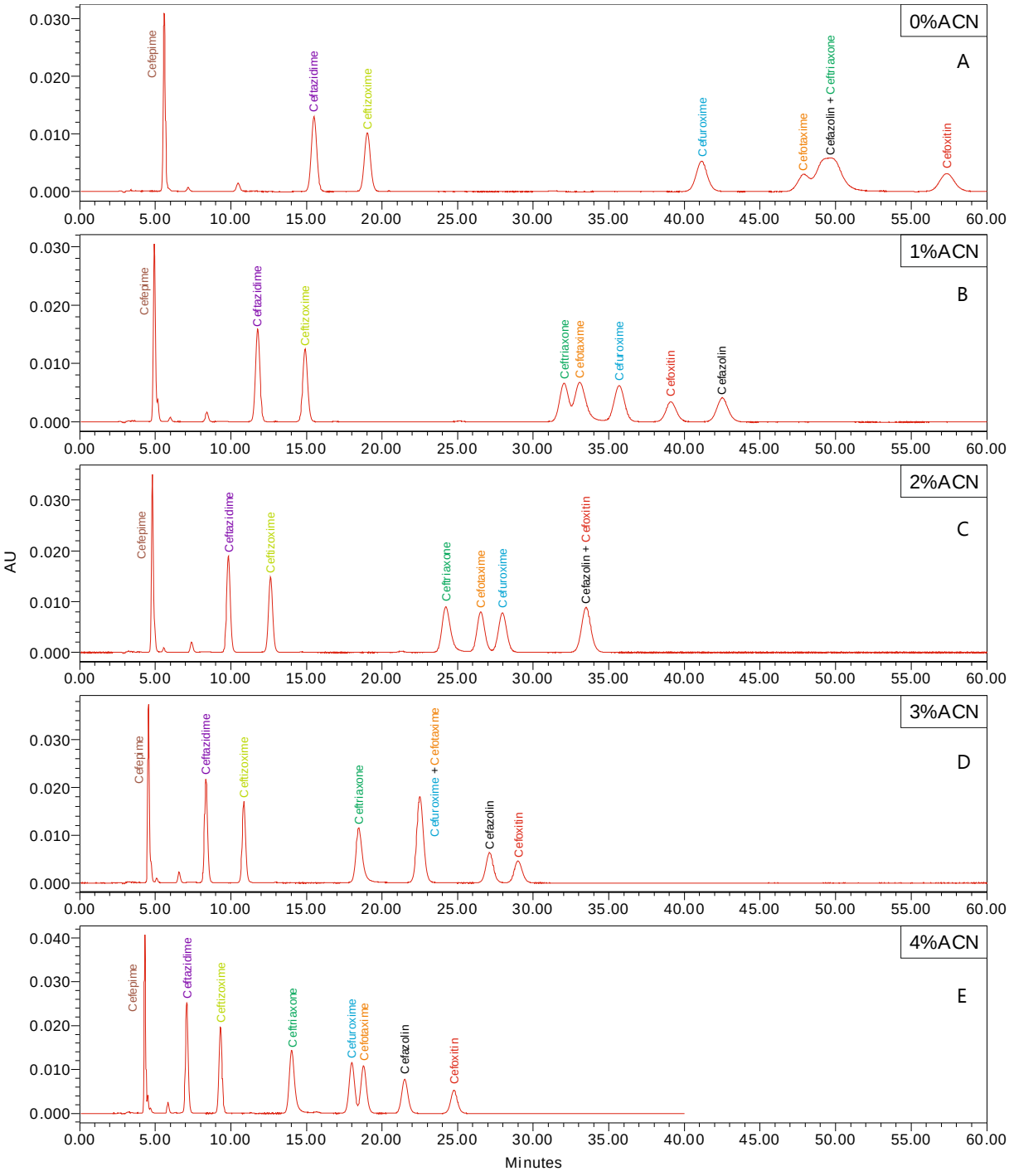

Figure C.31 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 13% MeOH: A - 0% ACN; B - 1% ACN; C - 2% ACN; D - 3% ACN; E - 4% ACN.

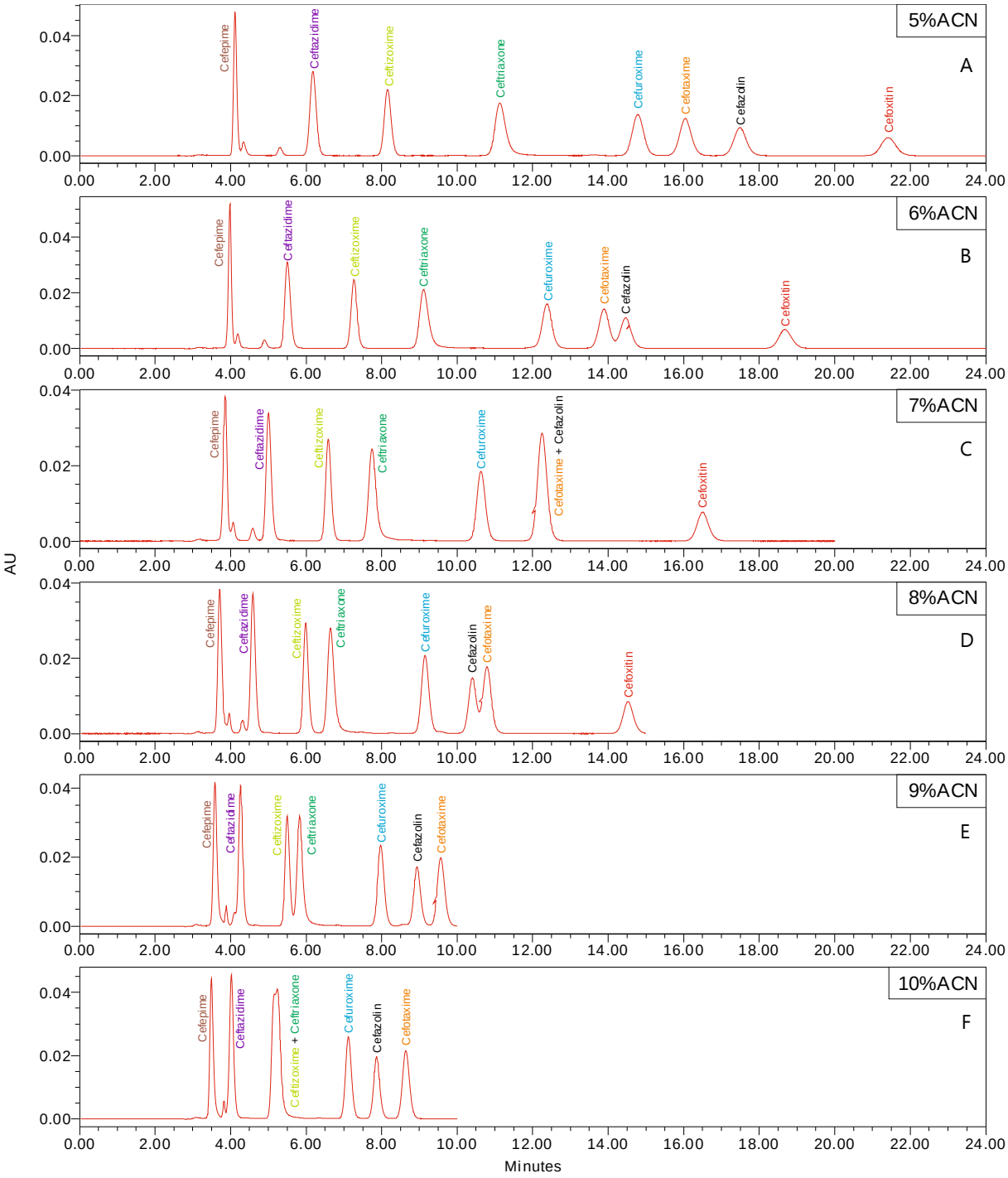

Figure C.32 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 13% MeOH: A - 5% ACN; B - 6% ACN; C - 7% ACN; D - 8% ACN; E - 9% ACN; F - 10% ACN.

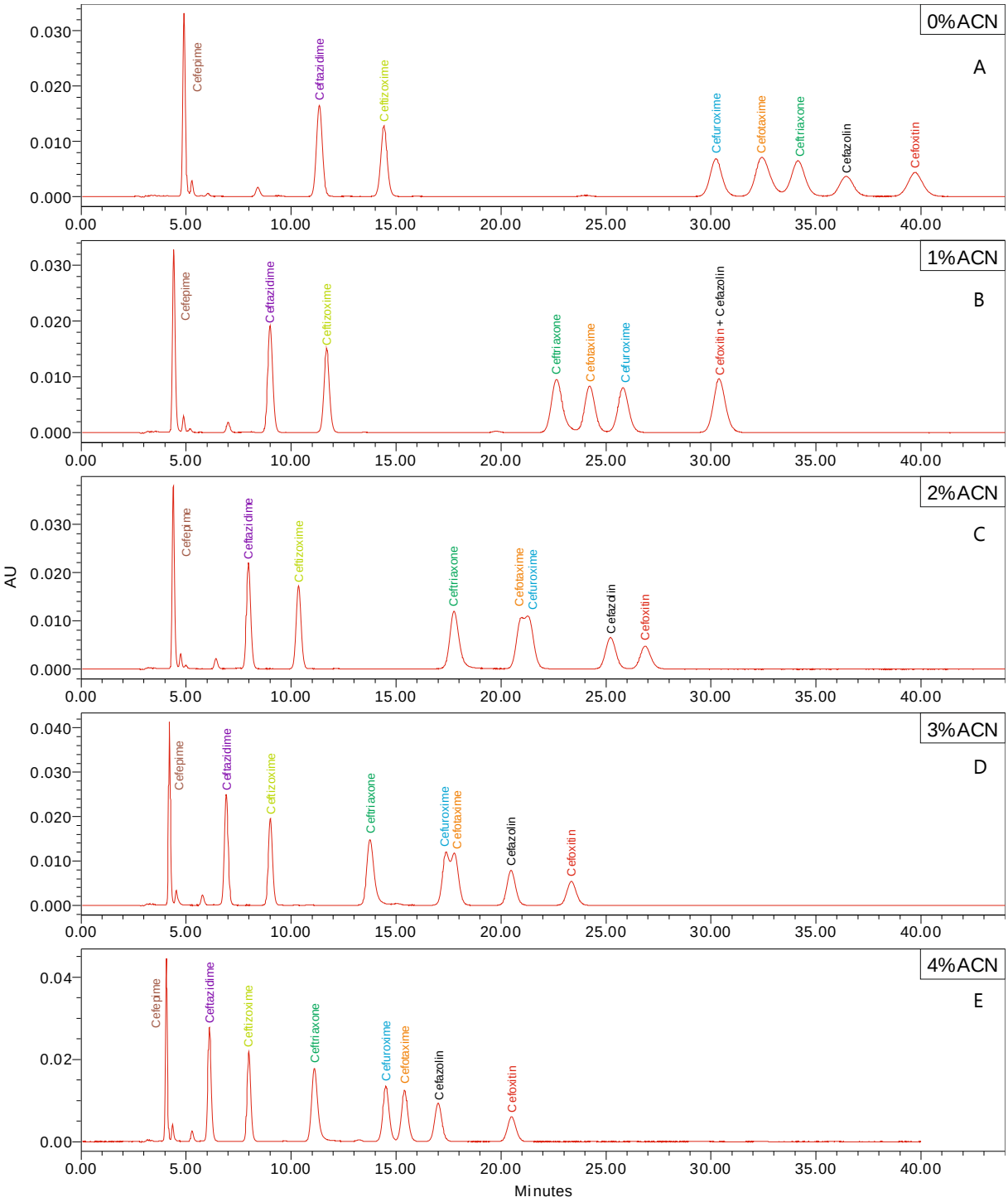

Figure C.33 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 15% MeOH: A - 0% ACN; B - 1% ACN; C - 2% ACN; D - 3% ACN; E - 4% ACN.

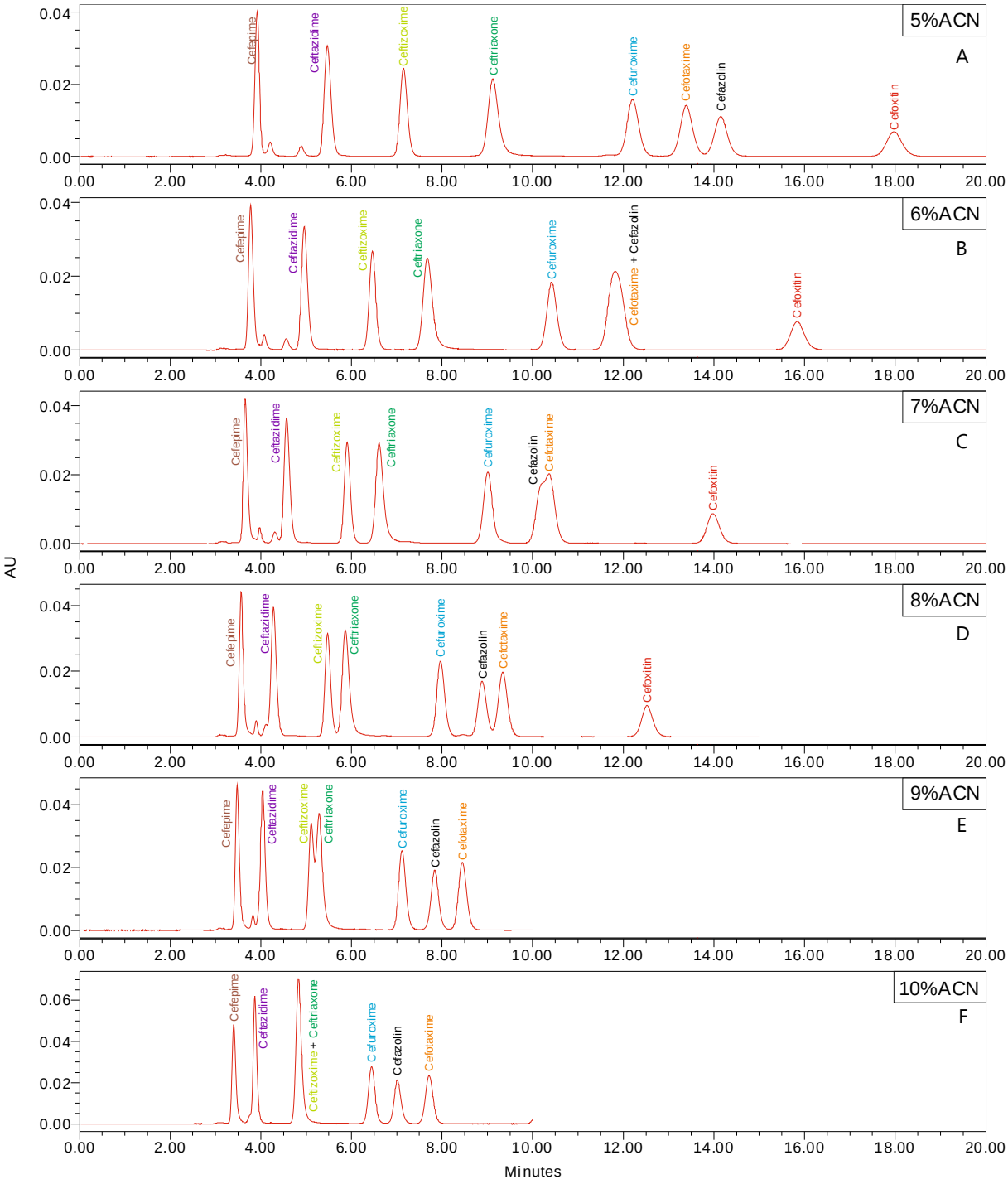

Figure C.34 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 15% MeOH: A - 5% ACN; B - 6% ACN; C - 7% ACN; D - 8% ACN; E - 9% ACN; F - 10% ACN.

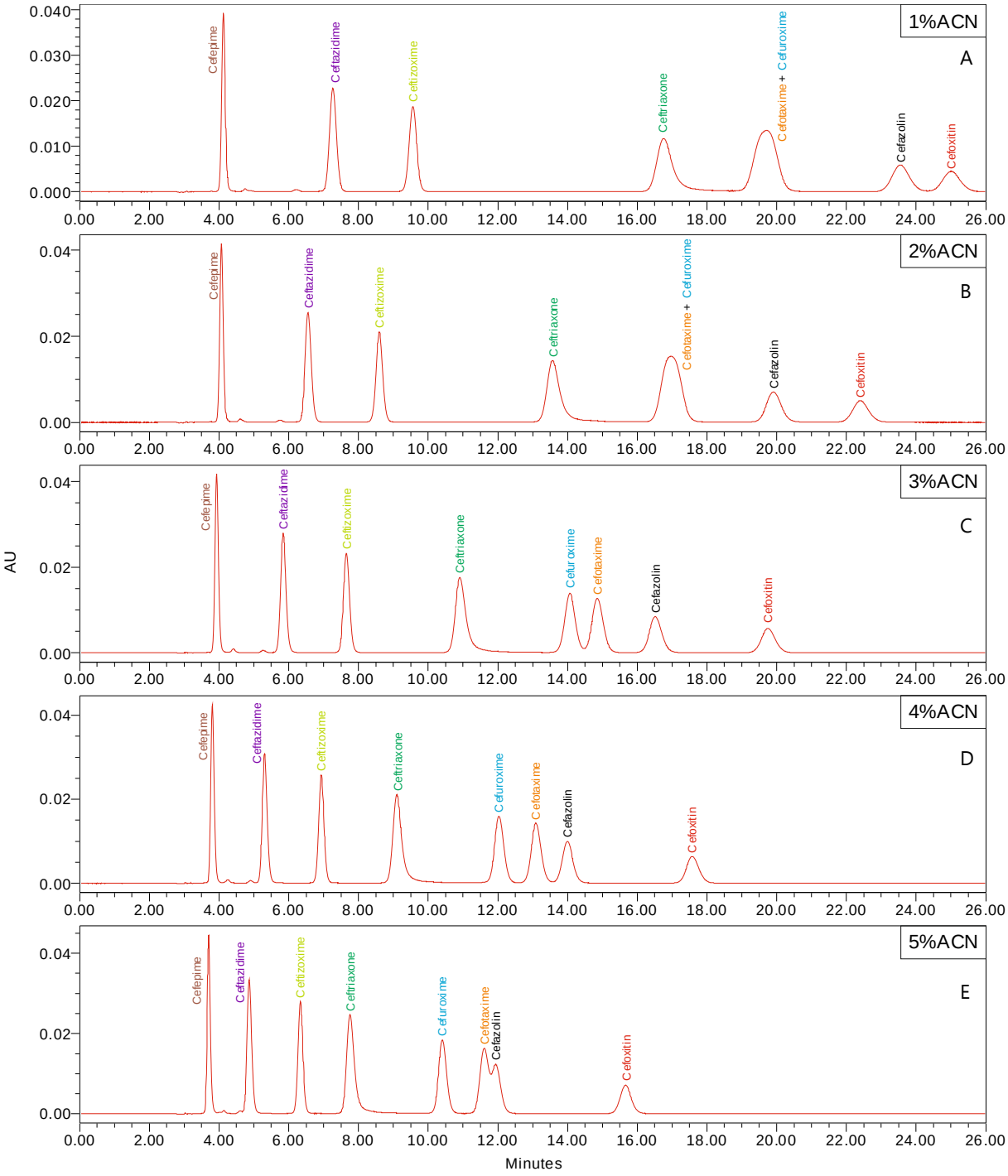

Figure C.35 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 17% MeOH: A - 1% ACN; B - 2% ACN; C - 3% ACN; D - 4% ACN; E - 5% ACN.

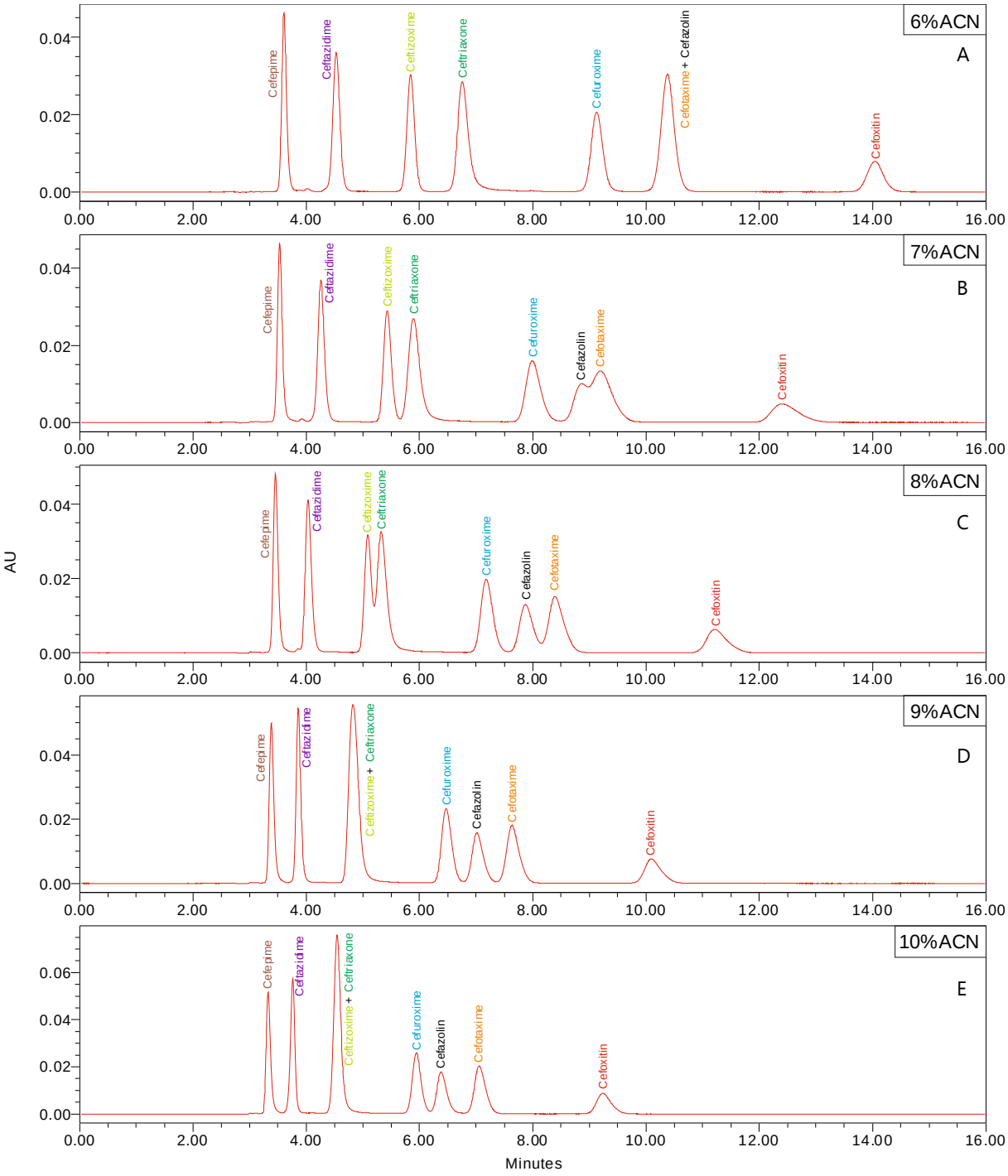

Figure C.36 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 17% MeOH: A - 6% ACN; B - 7% ACN; C - 8% ACN; D - 9% ACN; E - 10% ACN.

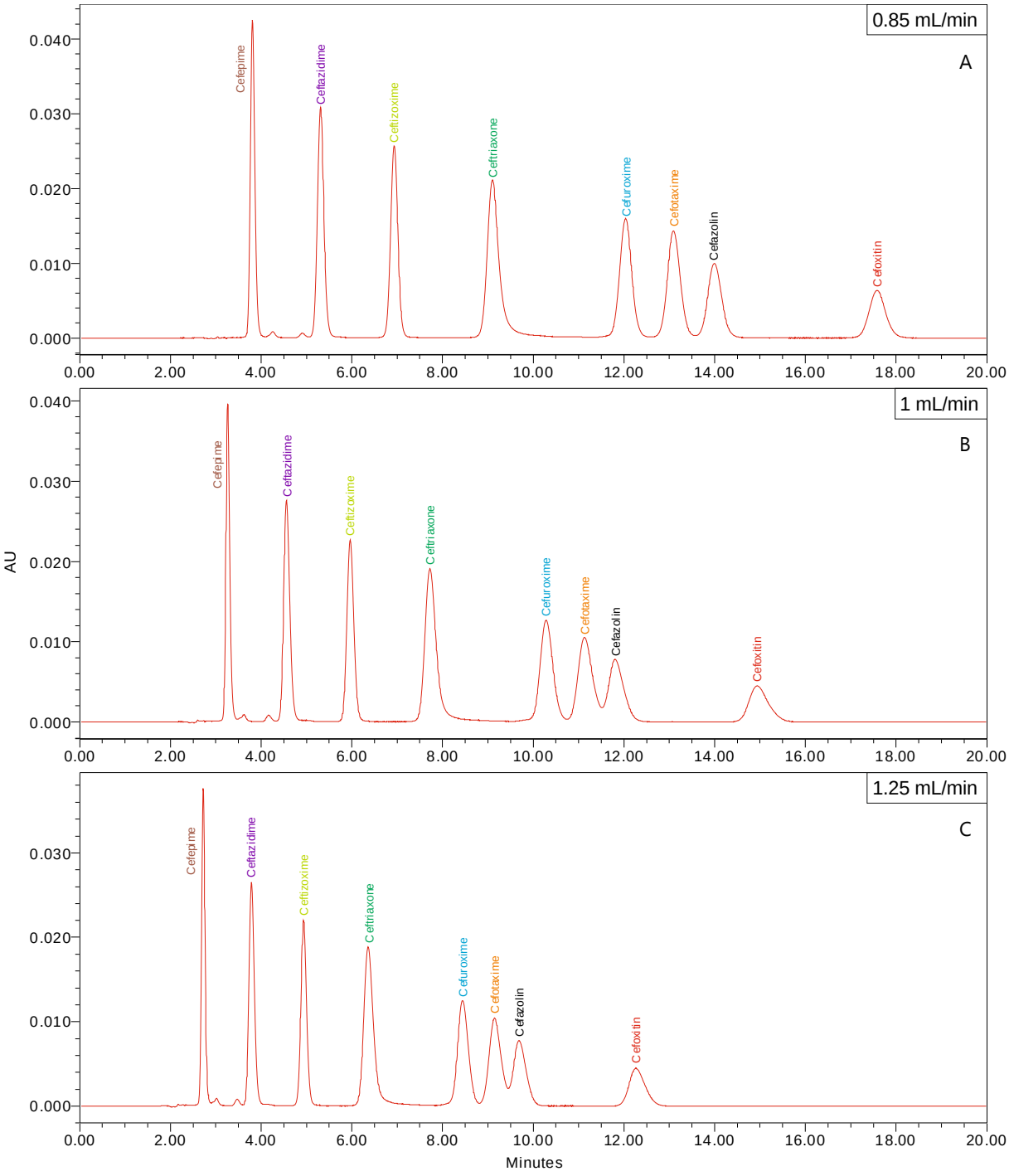

Figure C.37 - Method E chromatograms of 5 ppm standard, mobile phase with 17% MeOH and 4% ACN: flow rate variation: A - 0.83 mL/min; B - 1 mL/min; C - 1.25 mL/min.

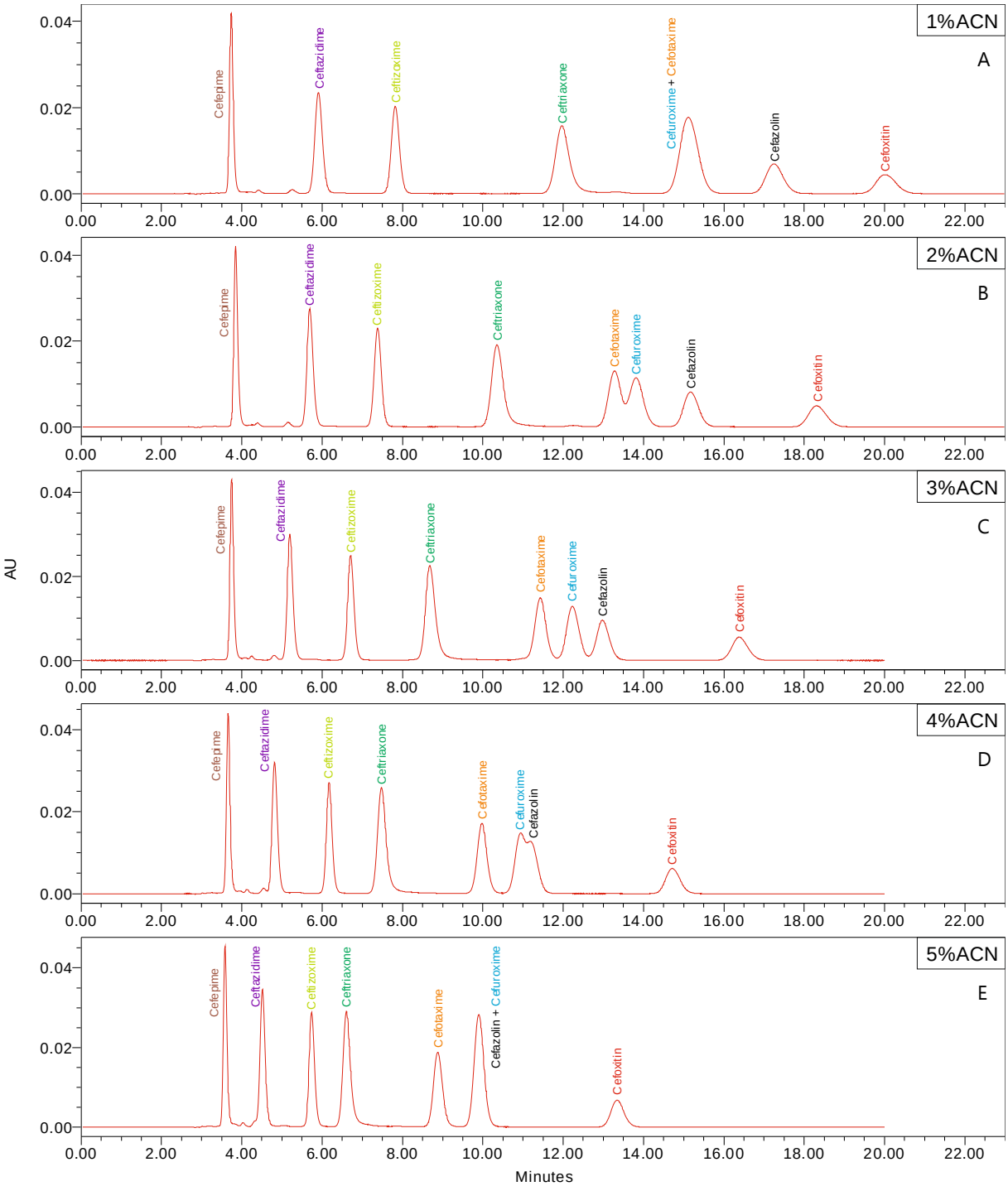

Figure C.38 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 19% MeOH: A - 1% ACN; B - 2% ACN; C - 3% ACN; D - 4% ACN; E - 5% ACN.

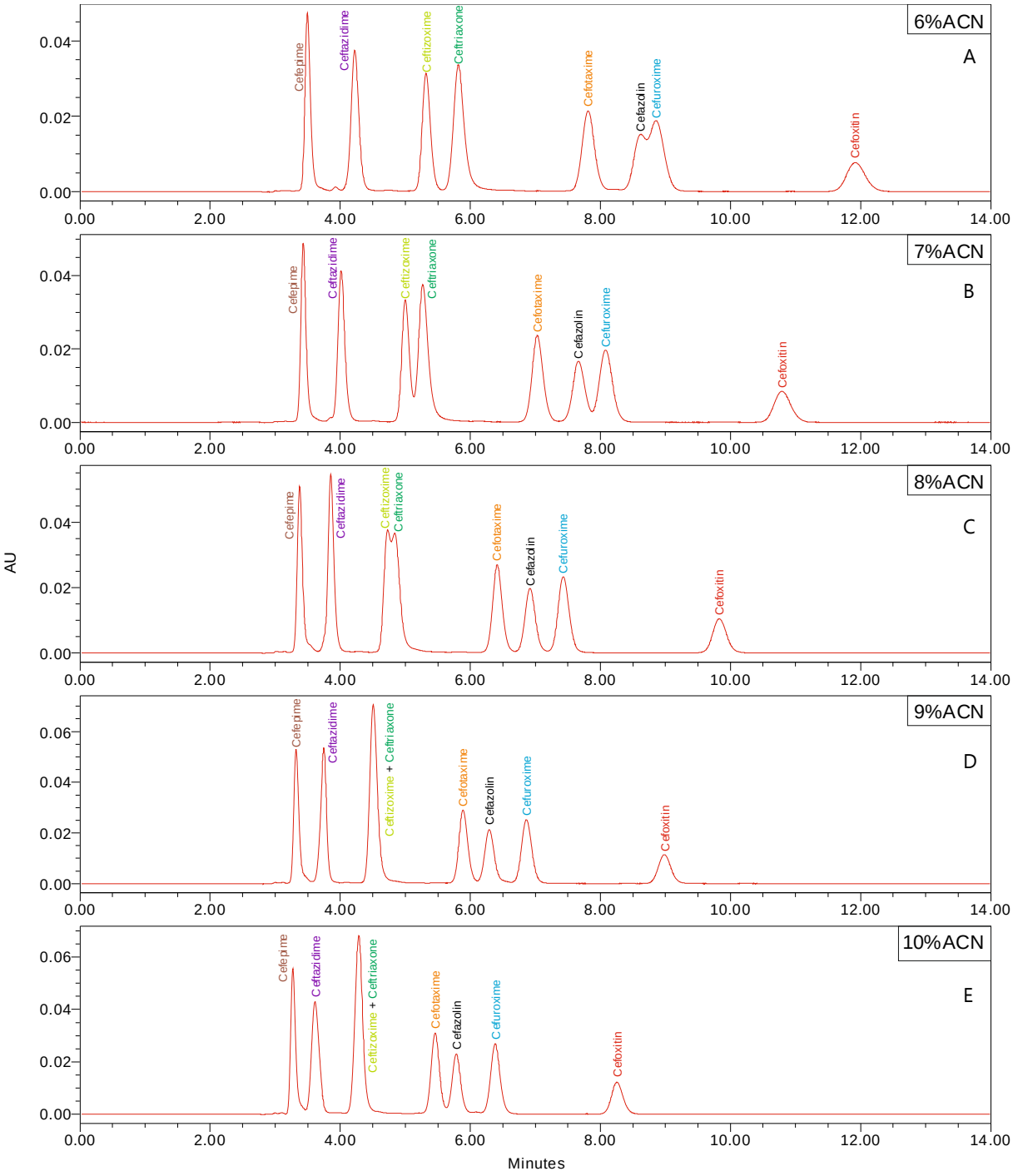

Figure C.39 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 19% MeOH: A - 6% ACN; B - 7% ACN; C - 8% ACN; D - 9% ACN; E - 10% ACN.

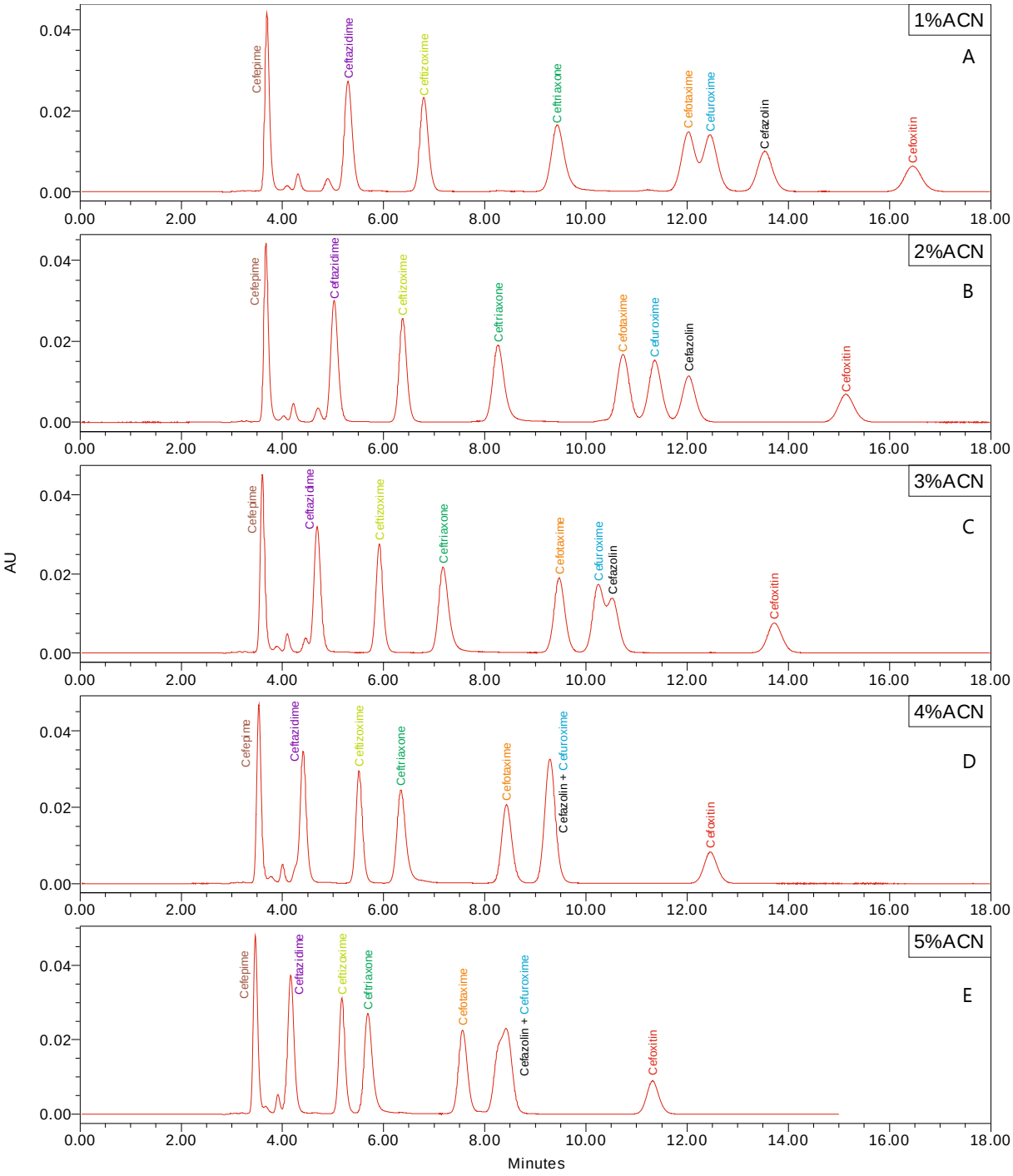

Figure C.40 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 21% MeOH: A - 1% ACN; B - 2% ACN; C - 3% ACN; D - 4% ACN; E - 5% ACN.

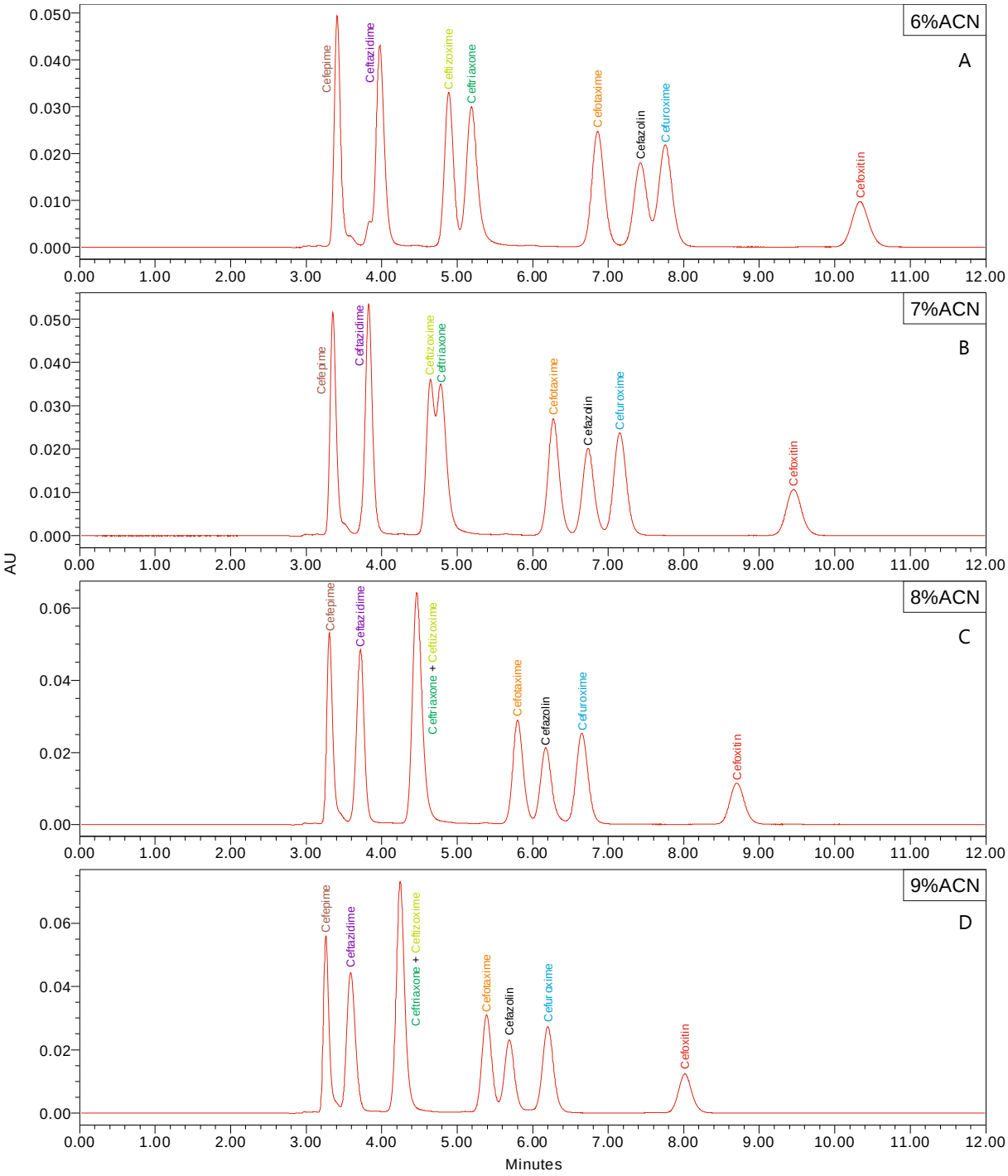

Figure C.41 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 21% MeOH: A - 6% ACN; B - 7% ACN; C - 8% ACN; D - 9% ACN.

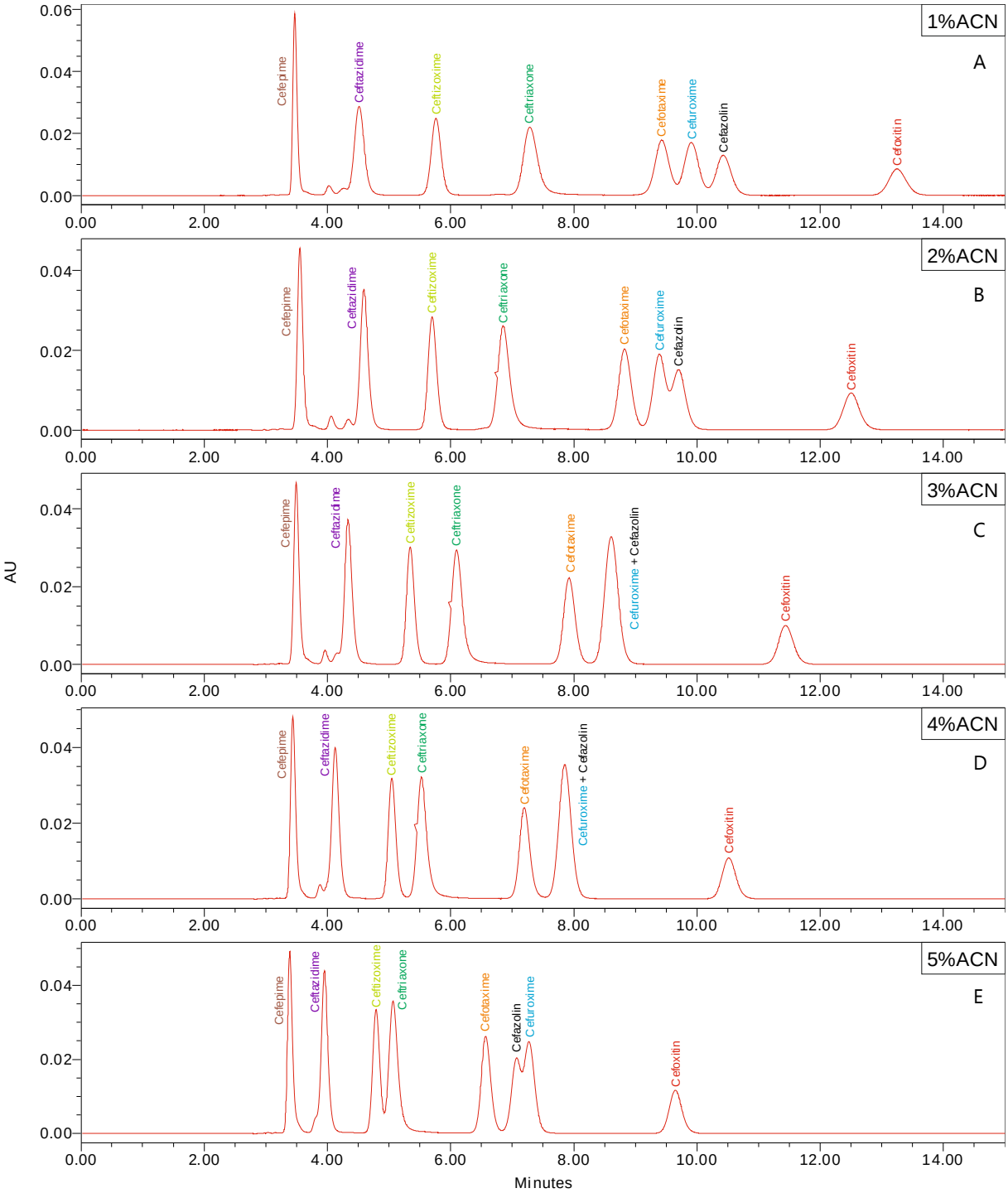

Figure C.42 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 23% MeOH: A - 1% ACN; B - 2% ACN; C - 3% ACN; D - 4% ACN; E - 5% ACN.

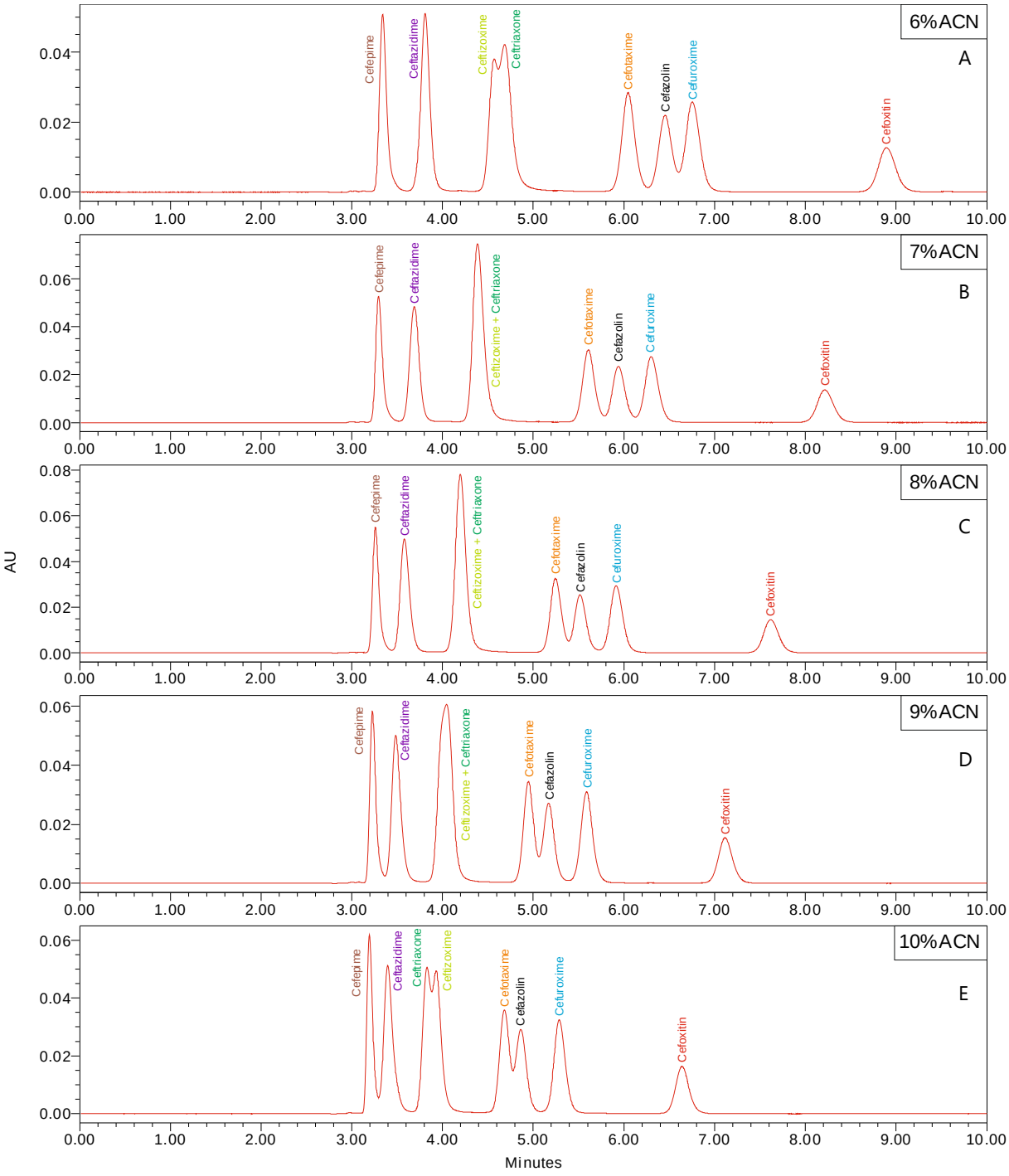

Figure C.43 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 23% MeOH: A - 6% ACN; B - 7% ACN; C - 8% ACN; D - 9% ACN; E - 10% ACN.

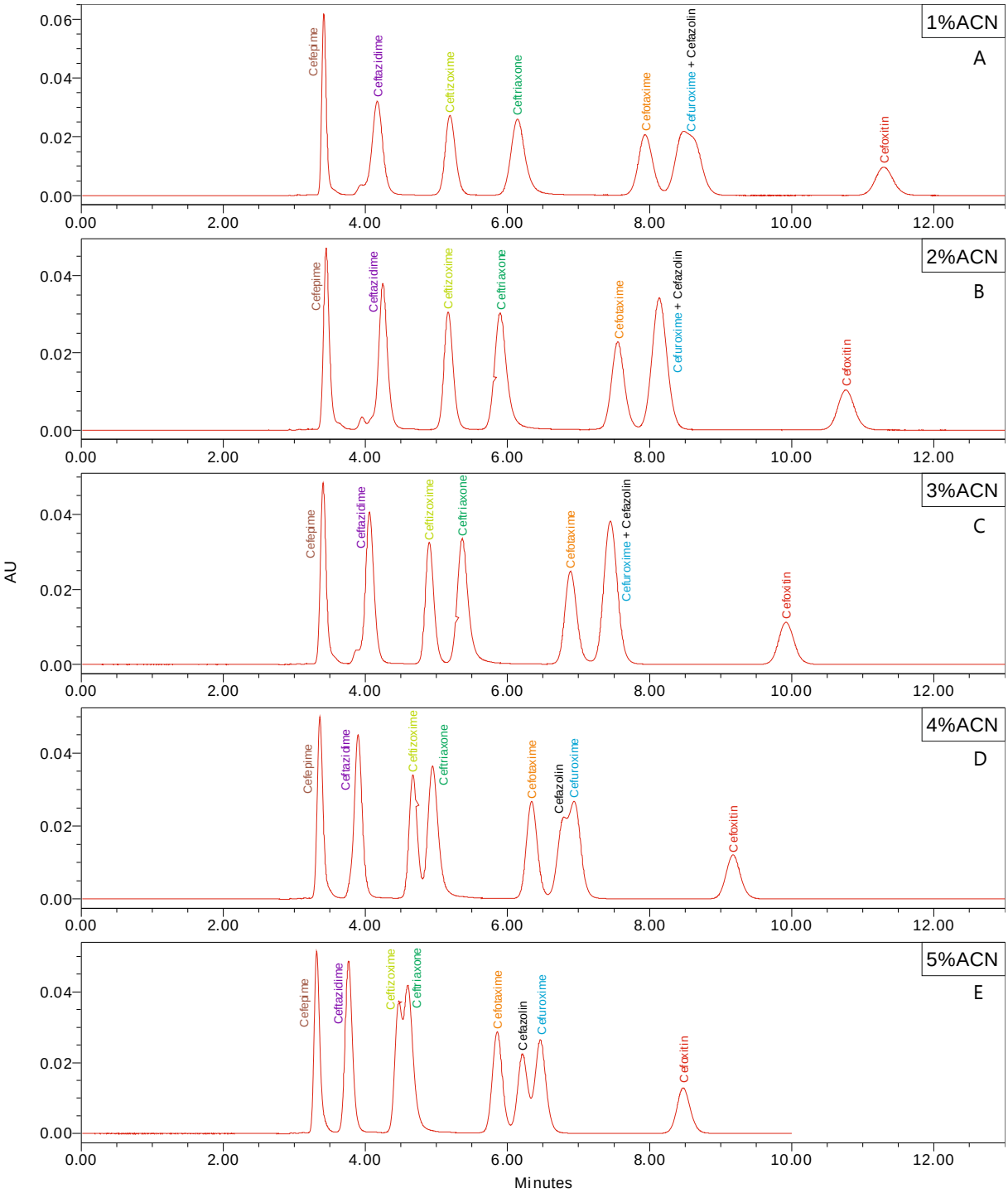

Figure C.44 - Method E chromatograms of 5 ppm standard, variation of ACN in a mobile phase with 25% MeOH: A - 1% ACN; B - 2% ACN; C - 3% ACN; D - 4% ACN; E - 5% ACN.

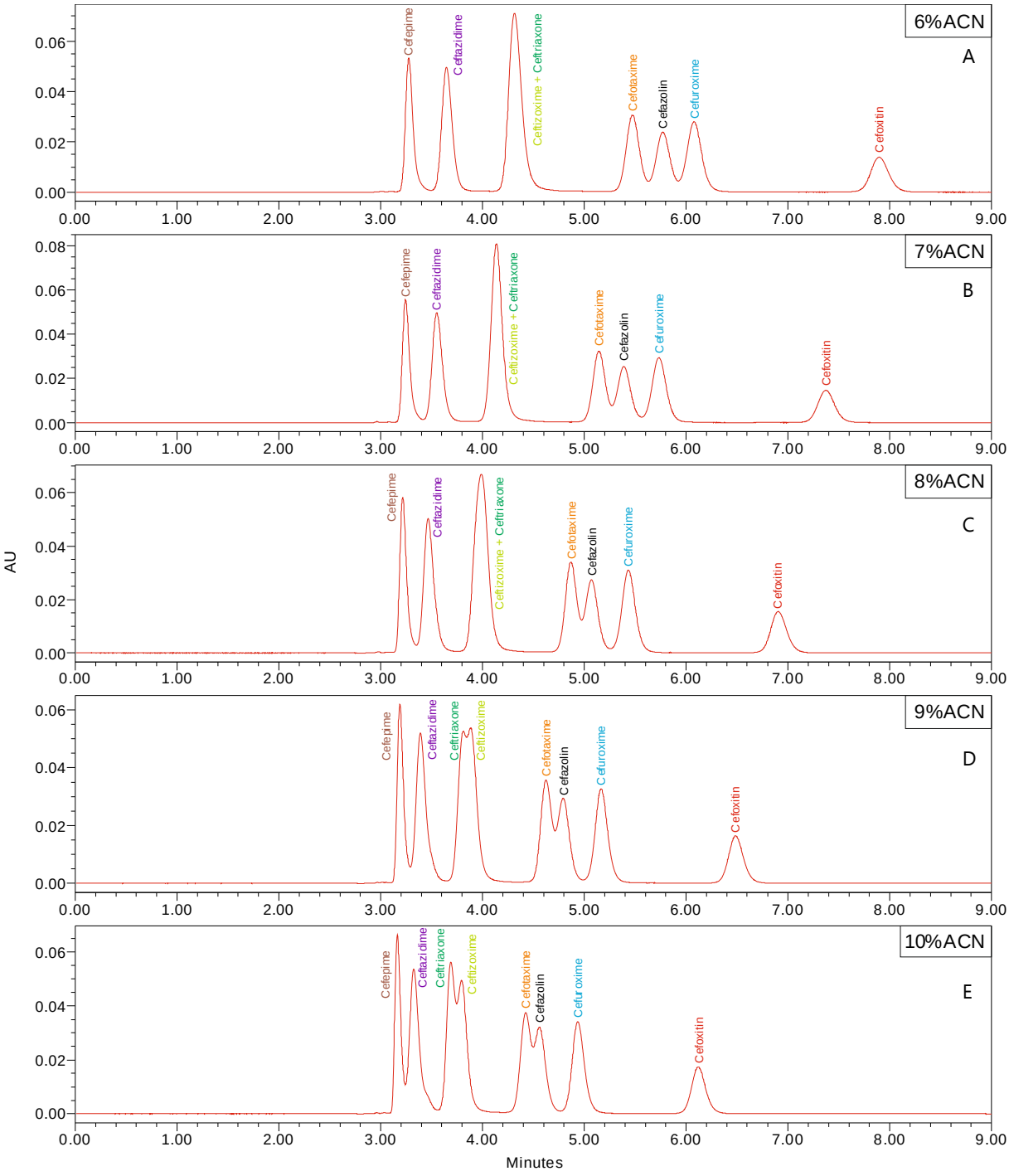

Figure C.45 - Method E chromatograms of 5 ppm standard, variation of MeOH in a mobile phase with 25% MeOH: A - 6% ACN; B - 7% ACN; C - 8% ACN; D - 9% ACN; E - 10% ACN.

C.4 Solvent

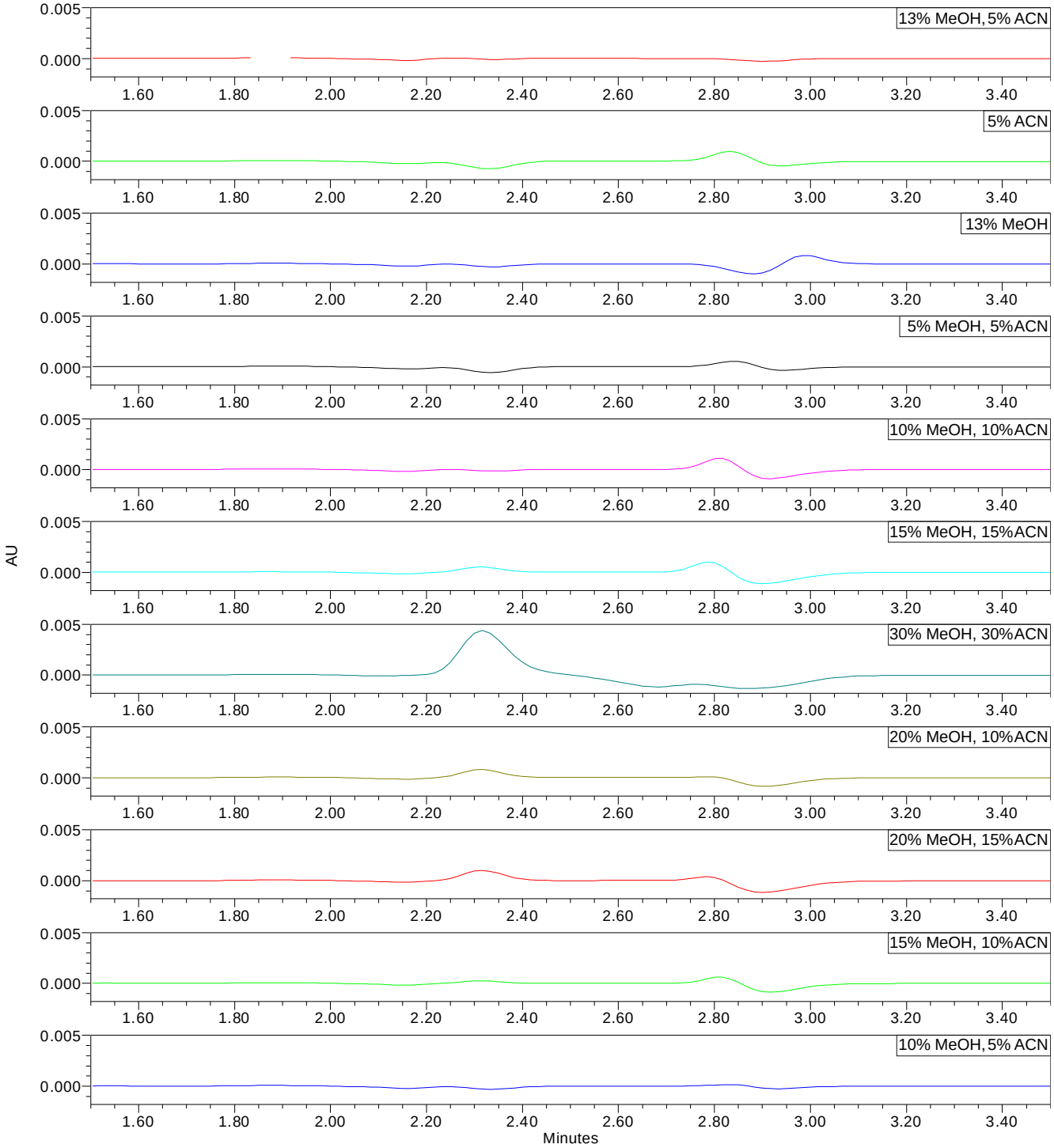

Figure C.46 - Sovent chromatograms variation.

C.5 Sampling procedure

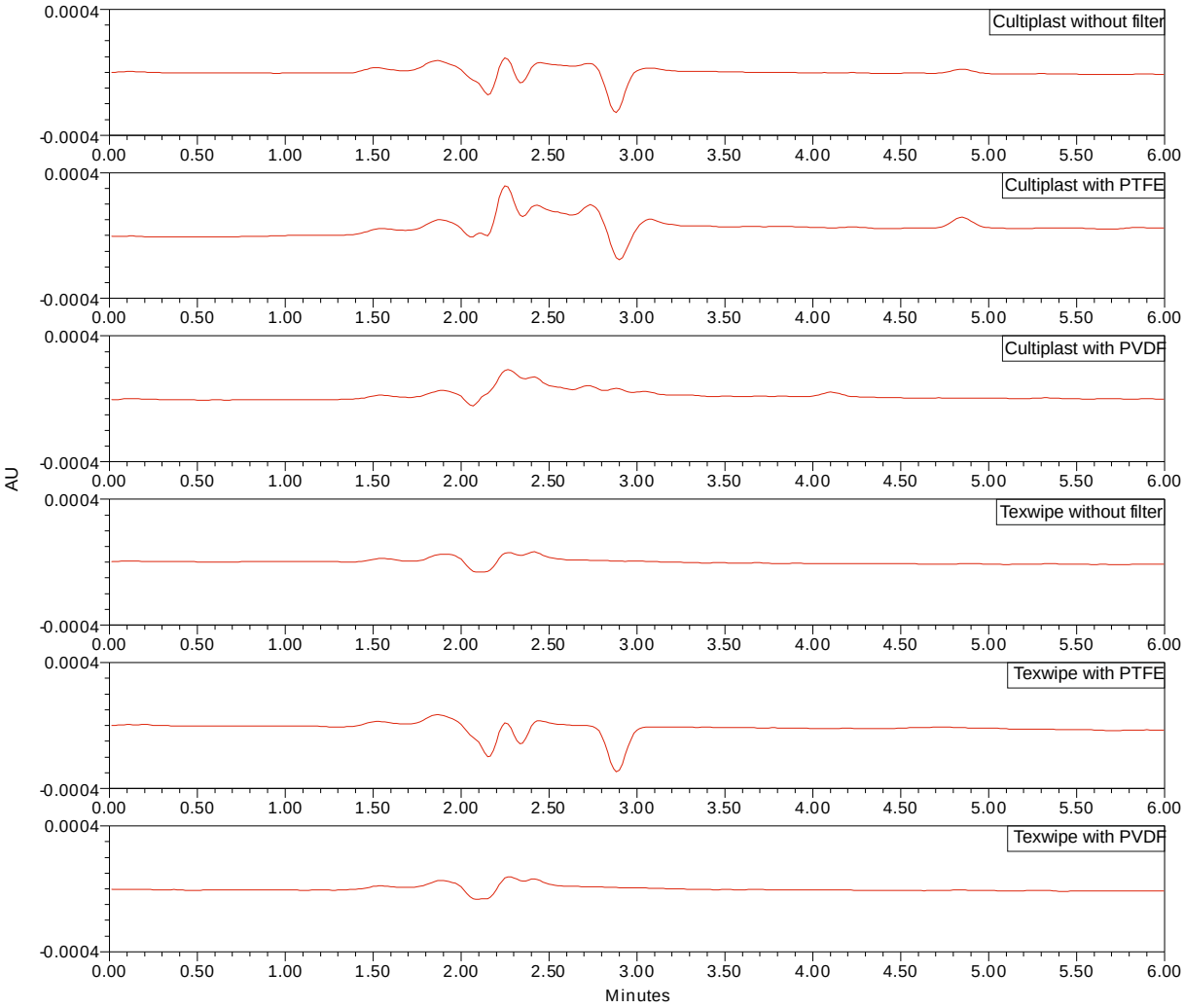

Figure C.47 - Solvent chromatograms of swab and filter combinations used in the sampling procedure.

C.6 Validation - Linearity Test

Table C.3 - Cefepime results for linearity test.

Cefepime					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	314402	313977	0.1
		2	313598		
		3	313931		
128	6.4	1	267027	266930	0.0
		2	266918		
		3	266846		
100	5	1	207578	207240	0.1
		2	207126		
		3	207016		
10	0.5	1	20611	20623	0.2
		2	20587		
		3	20670		
4	0.2	1	8980	8440	5.5
		2	8175		
		3	8164		
0.4%	0.02	1	777	742	4.7
		2	707		
		3	743		
Correlation Coefficient		1			
Slope		41765			
Intercept		-246			

Table C.4 - Ceftazidime results for linearity test.

Ceftazidime					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	312717	312575	0.1
		2	312679		
		3	312330		
128	6.4	1	265919	264923	0.3
		2	264440		
		3	264409		
100	5	1	216147	217165	0.8
		2	219258		
		3	216090		
10	0.5	1	22106	22042	0.3
		2	22005		
		3	22014		
4	0.2	1	9401	9375	0.8
		2	9291		
		3	9433		
0.4	0.02	1	994	946	11.6
		2	820		
		3	1023		
Correlation Coefficient		1.00			
Slope		41722			
Intercept		1407			

Table C.5 - Ceftizoxime results for linearity test.

Ceftizoxime					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	293792	294028	0.1
		2	294045		
		3	294246		
128	6.4	1	249869	249856	0.0
		2	249812		
		3	249887		
100	5	1	199644	199254	0.2
		2	199112		
		3	199006		
10	0.5	1	20094	20118	0.3
		2	20182		
		3	20077		
4	0.2	1	8599	8675	2.3
		2	8903		
		3	8524		
0.4	0.02	1	1212	1086	17.9
		2	1184		
		3	862		
Correlation Coefficient		1.00			
Slope		39158			
Intercept		789			

Table C.6 - Ceftriaxone results for linearity test.

Ceftriaxone					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	392240	392779	0.1
		2	393083		
		3	393014		
128	6.4	1	333282	333608	0.1
		2	333443		
		3	334100		
100	5	1	262210	262317	0.0
		2	262285		
		3	262455		
10	0.5	1	25837	26724	6.0
		2	25751		
		3	28583		
4	0.2	1	9876	10297	4.2
		2	10277		
		3	10737		
0.4	0.02	1	1032	1140	8.9
		2	1153		
		3	1234		
Correlation Coefficient		1.00			
Slope		52279			
Intercept		191			

Table C.7 - Cefotaxime results for linearity test.

Cefotaxime					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	301382	301392	0.0
		2	301381		
		3	301413		
128	6.4	1	255645	255762	0.1
		2	255965		
		3	255676		
100	5	1	204741	204752	0.0
		2	204848		
		3	204668		
10	0.5	1	20543	20487	0.2
		2	20472		
		3	20445		
4	0.2	1	9993	9437	5.2
		2	9075		
		3	9243		
0.4	0.02	1	831	816	2.3
		2	795		
		3	823		
Correlation Coefficient		1.00			
Slope		40129			
Intercept		884			

Table C.8 - Cefuroxime results for linearity test.

Cefuroxime					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	287346	287609	0.1
		2	287749		
		3	287732		
128	6.4	1	243922	244124	0.1
		2	244287		
		3	244163		
100	5	1	193612	193425	0.1
		2	193382		
		3	193282		
10	0.5	1	19358	19382	0.2
		2	19373		
		3	19415		
4	0.2	1	7550	7892	3.9
		2	7977		
		3	8150		
0.4	0.02	1	902	908	11.1
		2	810		
		3	1012		
Correlation Coefficient		1.00			
Slope		38299			
Intercept		321			

Table C.9 - Cefazolin results for linearity test.

Cefazolin					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	225379	225178	0.1
		2	225336		
		3	224820		
128	6.4	1	191938	191896	0.4
		2	191196		
		3	192554		
100	5	1	192958	192951	0.1
		2	193220		
		3	192676		
10	0.5	1	19448	19377	0.5
		2	19421		
		3	19261		
4	0.2	1	8079	8234	2.3
		2	8442		
		3	8180		
0.4	0.02	1	575	696	15.6
		2	728		
		3	784		
Correlation Coefficient		0.99			
Slope		30950			
Intercept		5181			

Table C.10 - Cefoxitin results for linearity test.

Cefoxitin					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	164419	164326	0.1
		2	164319		
		3	164241		
128	6.4	1	140143	140018	0.1
		2	139953		
		3	139958		
100	5	1	111653	112092	0.4
		2	112492		
		3	112131		
10	0.5	1	11192	11139	0.4
		2	11094		
		3	11131		
4	0.2	1	4469	4416	6.9
		2	4090		
		3	4688		
0.4	0.02	1	471	607	23.8
		2	592		
		3	759		
Correlation Coefficient		1.00			
Slope		21954			
Intercept		309			

Table C.11 - Cefepime results for linearity test, with the LOQ as the last level.

Cefepime					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	314402	313977	0.1
		2	313598		
		3	313931		
128	6.4	1	267027	266930	0.0
		2	266918		
		3	266846		
100	5	1	207578	207240	0.1
		2	207126		
		3	207016		
10	0.5	1	20611	20623	0.2
		2	20587		
		3	20670		
4	0.2	1	8980	8440	5.5
		2	8175		
		3	8164		
1.6	0.02	1	3879	3883	1.0
		2	3922		
		3	3847		
Correlation Coefficient		1.00			
Slope		41590			
Intercept		849			

Table C.12 - Ceftazidime results for linearity test, with the LOQ as the last level.

Ceftazidime					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	312717	312575	0.1
		2	312679		
		3	312330		
128	6.4	1	265919	264923	0.3
		2	264440		
		3	264409		
100	5	1	216147	217165	0.8
		2	219258		
		3	216090		
10	0.5	1	22106	22042	0.3
		2	22005		
		3	22014		
4	0.2	1	9401	9375	0.8
		2	9291		
		3	9433		
1.6	0.02	1	3398	3402	0.2
		2	3409		
		3	3398		
Correlation Coefficient		1.00			
Slope		41585			
Intercept		2263			

Table C.13 -Ceftizoxime results for linearity test, with the LOQ as the last level.

Ceftizoxime					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	293792	294028	0.1
		2	294045		
		3	294246		
128	6.4	1	249869	249856	0.0
		2	249812		
		3	249887		
100	5	1	199644	199254	0.2
		2	199112		
		3	199006		
10	0.5	1	20094	20118	0.3
		2	20182		
		3	20077		
4	0.2	1	8599	8675	2.3
		2	8903		
		3	8524		
1.6	0.02	1	4579	4549	1.0
		2	4574		
		3	4495		
Correlation Coefficient		1.00			
Slope		38965			
Intercept		1997			

Table C.14 - Ceftriaxone results for linearity test, with the LOQ as the last level.

Ceftriaxone					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	392240	392779	0.1
		2	393083		
		3	393014		
128	6.4	1	333282	333608	0.1
		2	333443		
		3	334100		
100	5	1	262210	262317	0.0
		2	262285		
		3	262455		
10	0.5	1	25837	26724	6.0
		2	25751		
		3	28583		
4	0.2	1	9876	10297	4.2
		2	10277		
		3	10737		
1.6	0.02	1	2953	2938	0.7
		2	2916		
		3	2946		
Correlation Coefficient		1.00			
Slope		52179			
Intercept		819			

Table C.15 - Cefotaxime results for linearity test, with the LOQ as the last level.

Cefotaxime					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	301382	301392	0.0
		2	301381		
		3	301413		
128	6.4	1	255645	255762	0.1
		2	255965		
		3	255676		
100	5	1	204741	204752	0.0
		2	204848		
		3	204668		
10	0.5	1	20543	20487	0.2
		2	20472		
		3	20445		
4	0.2	1	9993	9437	5.2
		2	9075		
		3	9243		
1.6	0.02	1	3820	3851	1.7
		2	3808		
		3	3924		
Correlation Coefficient		1.00			
Slope		39960			
Intercept		1943			

Table C.16 - Cefuroxime results for linearity test, with the LOQ as the last level.

Cefuroxime					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	287346	287609	0.1
		2	287749		
		3	287732		
128	6.4	1	243922	244124	0.1
		2	244287		
		3	244163		
100	5	1	193612	193425	0.1
		2	193382		
		3	193282		
10	0.5	1	19358	19382	0.2
		2	19373		
		3	19415		
4	0.2	1	7550	7892	3.9
		2	7977		
		3	8150		
1.6	0.02	1	2628	2704	3.4
		2	2679		
		3	2805		
Correlation Coefficient		1.00			
Slope		38198			
Intercept		947			

Table C.17 - Cefazolin results for linearity test, with the LOQ as the last level.

Cefazolin					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	225379	225178	0.1
		2	225336		
		3	224820		
128	6.4	1	191938	191896	0.4
		2	191196		
		3	192554		
100	5	1	192958	192951	0.1
		2	193220		
		3	192676		
10	0.5	1	19448	19377	0.5
		2	19421		
		3	19261		
4	0.2	1	8079	8234	2.3
		2	8442		
		3	8180		
1.6	0.02	1	2060	2026	1.5
		2	2000		
		3	2018		
Correlation Coefficient		0.99			
Slope		30876			
Intercept		5645			

Table C.18 - Cefoxitin results for linearity test, with the LOQ as the last level.

Cefoxitin					
Level (%)	**Concentration (ug/mL)**	**Replicate #**	**Peak Area**	**Average Peak Area**	**%RSD**
150	7.5	1	164419	164326	0.1
		2	164319		
		3	164241		
128	6.4	1	140143	140018	0.1
		2	139953		
		3	139958		
100	5	1	111653	112092	0.4
		2	112492		
		3	112131		
10	0.5	1	11192	11139	0.4
		2	11094		
		3	11131		
4	0.2	1	4469	4416	6.9
		2	4090		
		3	4688		
1.6	0.02	1	3555	3541	0.7
		2	3555		
		3	3513		
Correlation Coefficient		1.00			
Slope		21791			
Intercept		1332			

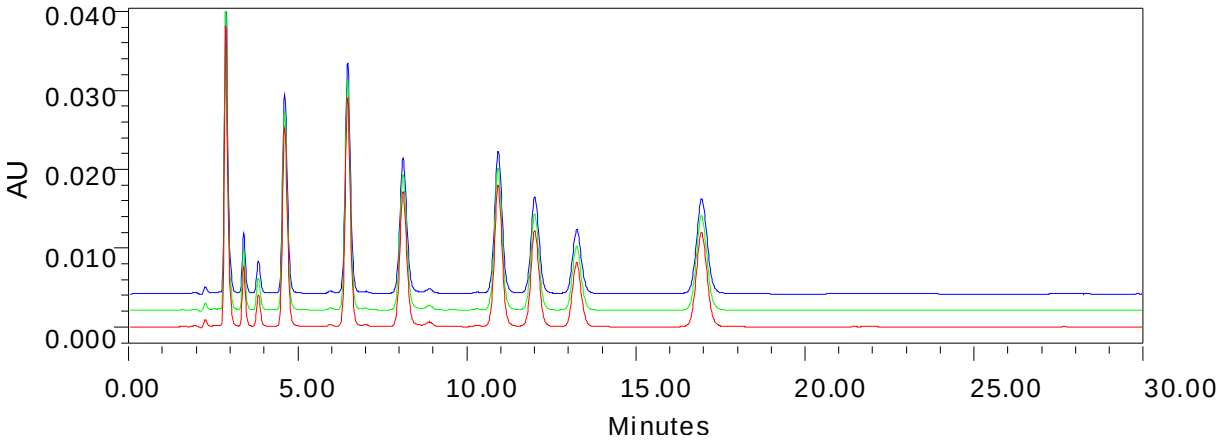

Figure C.48 - Concentration level 150% chromatograms.

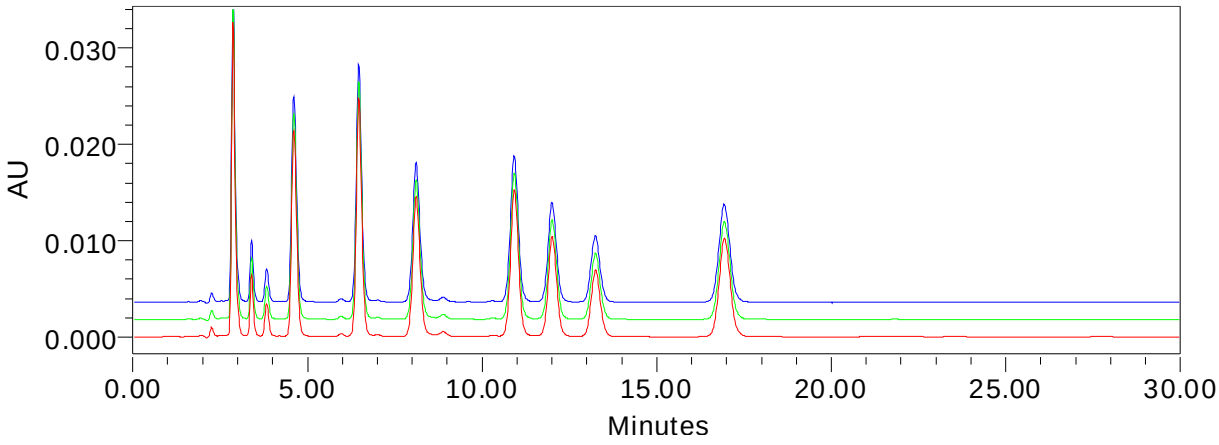

Figure C.49 - Concentration level 128% chromatograms.

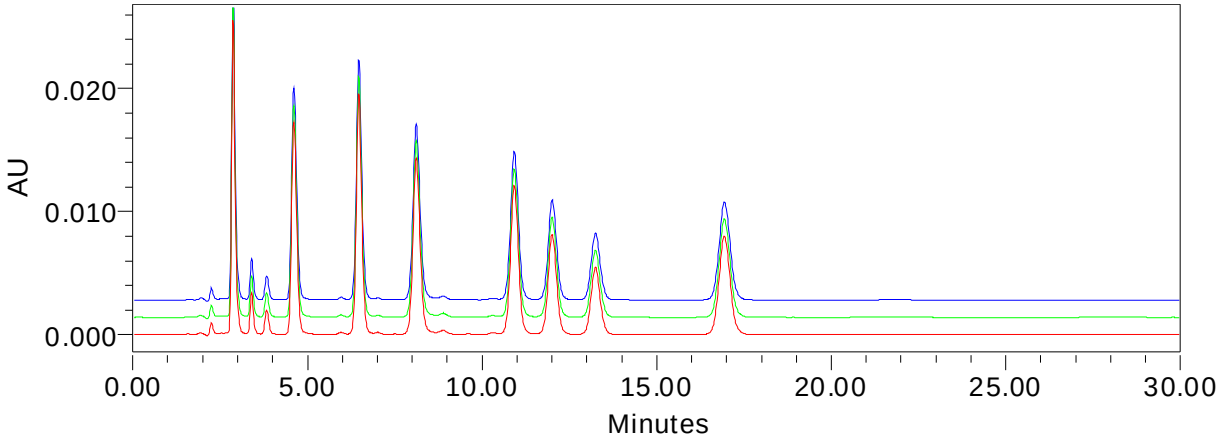

Figure C.50 - Concentration level 100% chromatograms.

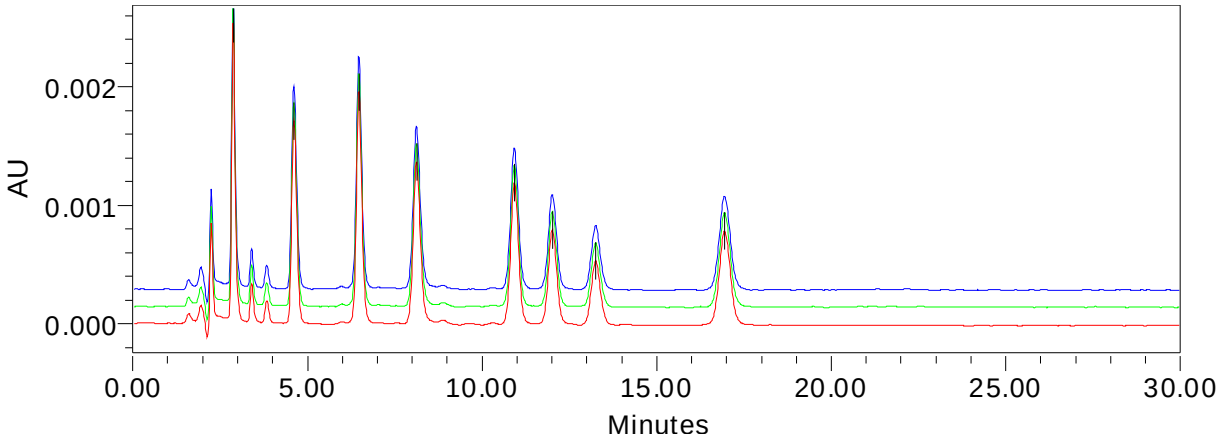

Figure C.51 - Concentration level 10% chromatograms.

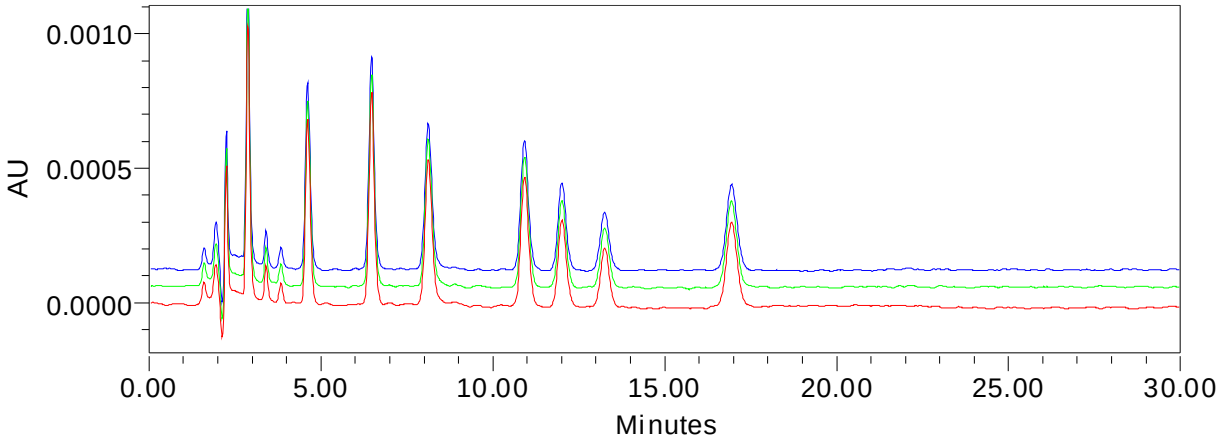

Figure C.52 - Concentration level 4% chromatograms.

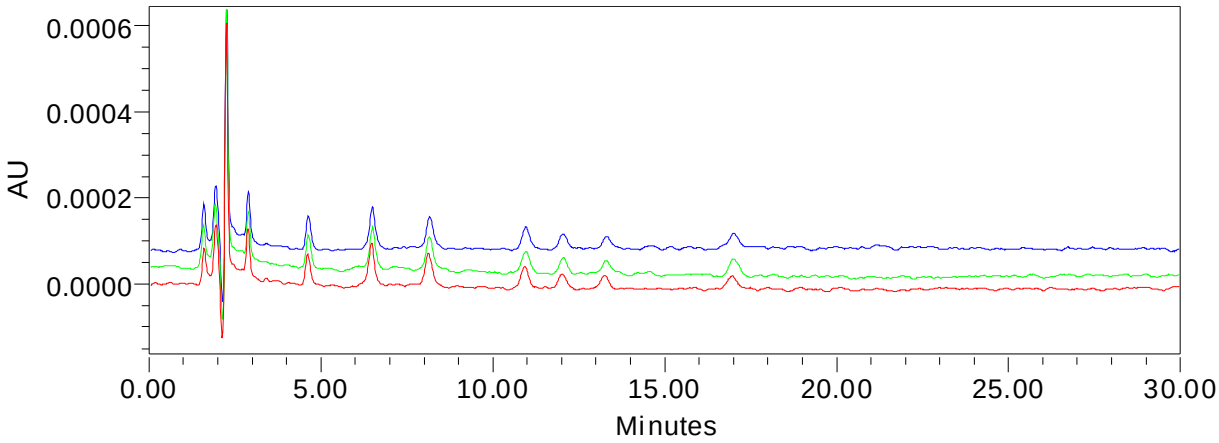

Figure C.53 - Concentration level 0.4% chromatograms.

C.7 Validation - Swab Challenge

Table C.19 - Ceftazidime results for swab challenge using Cefepime final product as standard and detecting at a wavelength of 240 nm.

Ceftazidime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	16468	92	93	1.4
		16582	93		
		16916	94		
4	0.2	6704	95	96	1.1
		6836	97		
		6838	97		
1.6	0.08	2831	92	89	3.7
		2818	91		
		2648	86		

Table C.20 - Ceftizoxime results for swab challenge using Cefepime final product as standard and detecting at a wavelength of 240 nm.

Ceftizoxime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	21416	95	95	0.5
		21577	96		
		21641	96		
4	0.2	9338	101	100	1.1
		9258	100		
		9133	99		
1.6	0.08	3625	107	106	0.3
		3615	106		
		3601	106		

Table C.21 - Ceftriaxone results for swab challenge using Cefepime final product as standard and detecting at a wavelength of 240 nm.

Ceftriaxone					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	23214	98	99	0.7
		23414	99		
		23545	99		
4	0.2	9599	96	99	3.0
		10176	102		
		10013	100		
1.6	0.08	4193	99	101	2.3
		4208	99		
		4370	103		

Table C.22 - Cefotaxime results for swab challenge using Cefepime final product as standard and detecting at a wavelength of 240 nm.

Cefotaxime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	18478	95	96	1.0
		18542	95		
		18812	97		
4	0.2	7271	96	98	2.2
		7511	99		
		7584	100		
1.6	0.08	3255	102	99	3.4
		3042	95		
		3177	99		

Table C.23 - Cefuroxime results for swab challenge using Cefepime final product as standard and detecting at a wavelength of 240 nm.

Cefuroxime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	13432	98	98	1.2
		13312	97		
		13639	100		
4	0.2	5148	93	96	2.7
		5416	98		
		5377	97		
1.6	0.08	2241	98	98	0.7
		2249	98		
		2219	97		

Table C.24 - Cefazolin results for swab challenge using Cefepime final product as standard and detecting at a wavelength of 240 nm.

Cefazolin					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	9919	99	99	0.2
		9889	99		
		9886	99		
4	0.2	3845	94	94	0.8
		3905	95		
		3892	95		
1.6	0.08	1587	101	101	8.4
		1718	109		
		1452	92		

Table C.25 - Cefoxitin results for swab challenge using Cefepime final product as standard and detecting at a wavelength of 240 nm.

Cefoxitin					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	17758	98	99	0.6
		17796	99		
		17963	100		
4	0.2	6850	96	98	2.1
		7123	100		
		7068	99		
1.6	0.08	2985	104	102	2.1
		2948	103		
		2865	100		

Table C.26 - Ceftazidime results for swab challenge using Cefepime final product as standard diluted in DIW, detecting at a wavelength of 240 nm.

Ceftazidime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	16468	92	93	1.4
		16582	93		
		16916	94		
4	0.2	6704	96	97	1.1
		6836	97		
		6838	97		
1.6	0.08	2648	86	89	3.7
		2818	91		
		2831	92		

Table C.27 - Ceftizoxime results for swab challenge using Cefepime final product as standard diluted in DIW, detecting at a wavelength of 240 nm.

Ceftizoxime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	21416	95	95	0.5
		21577	96		
		21641	96		
4	0.2	9338	98	97	1.1
		9258	98		
		9133	96		
1.6	0.08	3601	106	106	0.3
		3615	106		
		3625	107		

Table C.28 - Ceftriaxone results for swab challenge using Cefepime final product as standard diluted in DIW, detecting at a wavelength of 240 nm.

Ceftriaxone					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	23214	98	99	0.7
		23414	99		
		23545	99		
4	0.2	9599	100	104	3.0
		10176	106		
		10013	104		
1.6	0.08	4370	103	101	2.3
		4208	99		
		4193	99		

Table C.29 - Cefotaxime results for swab challenge using Cefepime final product as standard diluted in DIW, detecting at a wavelength of 240 nm.

Cefotaxime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	18478	95	96	1.0
		18542	95		
		18812	97		
4	0.2	7271	93	96	2.2
		7511	96		
		7584	97		
1.6	0.08	3177	99	99	3.4
		3042	95		
		3255	102		

Table C.30 - Cefuroxime results for swab challenge using Cefepime final product as standard diluted in DIW, detecting at a wavelength of 240 nm.

Cefuroxime					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	13432	98	98	1.2
		13312	97		
		13639	100		
4	0.2	5148	94	97	2.7
		5416	99		
		5377	99		
1.6	0.08	2219	97	98	0.7
		2249	98		
		2241	98		

Table C.31 - Cefazolin results for swab challenge using Cefepime final product as standard diluted in DIW, detecting at a wavelength of 240 nm.

Cefazolin					
Level (%)	**Concentra-tion (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	9919	99	99	0.2
		9889	99		
		9886	99		
4	0.2	3845	98	99	0.8
		3905	100		
		3892	100		
1.6	0.08	1452	92	101	8.4
		1718	109		
		1587	101		

Table C.32 - Cefoxitin results for swab challenge using Cefepime final product as standard diluted in DIW, detecting at a wavelength of 240 nm.

Cefoxitin					
Level (%)	**Concentration (µg/mL)**	**Peak Area**	**%Recovery**	**Average**	**%RSD**
10	0.5	17758	98	99	0.6
		17796	99		
		17963	100		
4	0.2	6850	97	99	2.1
		7123	101		
		7068	100		
1.6	0.08	2865	100	102	2.1
		2948	103		
		2985	104		

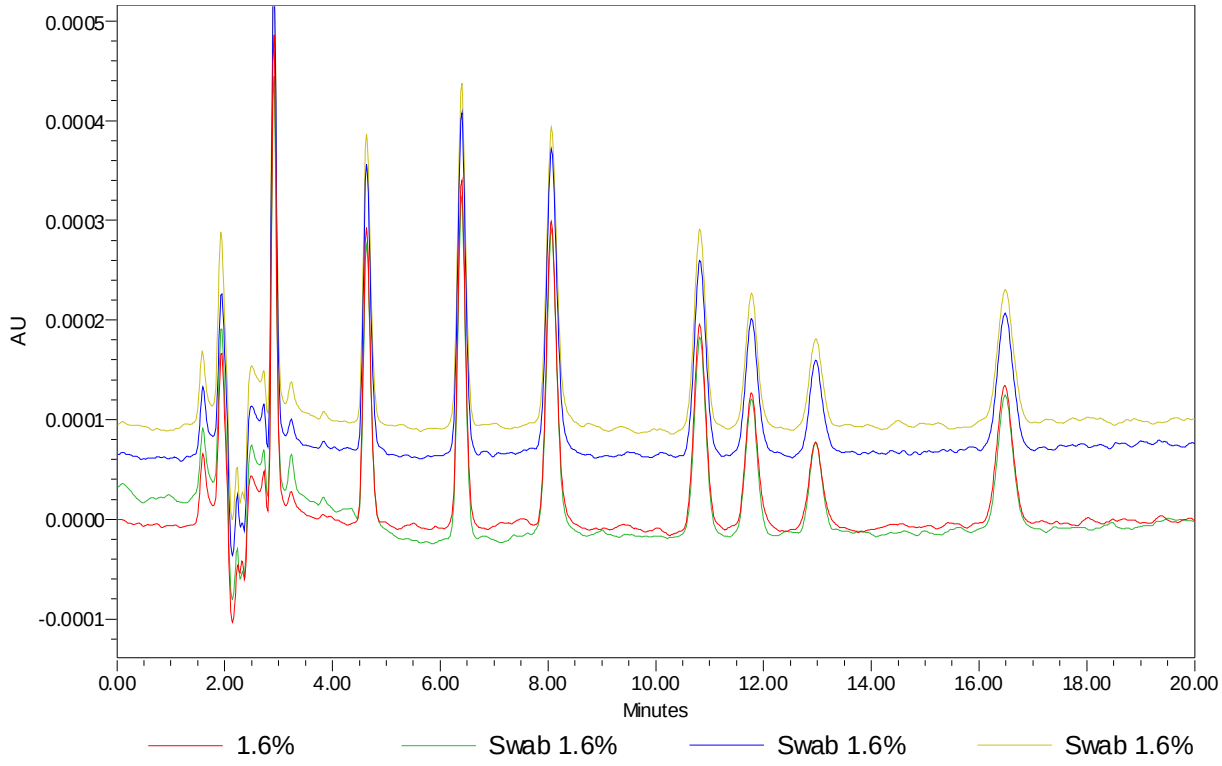

Figure C.54 - Chromatogram results of swab recovery at 1.6% concentration.

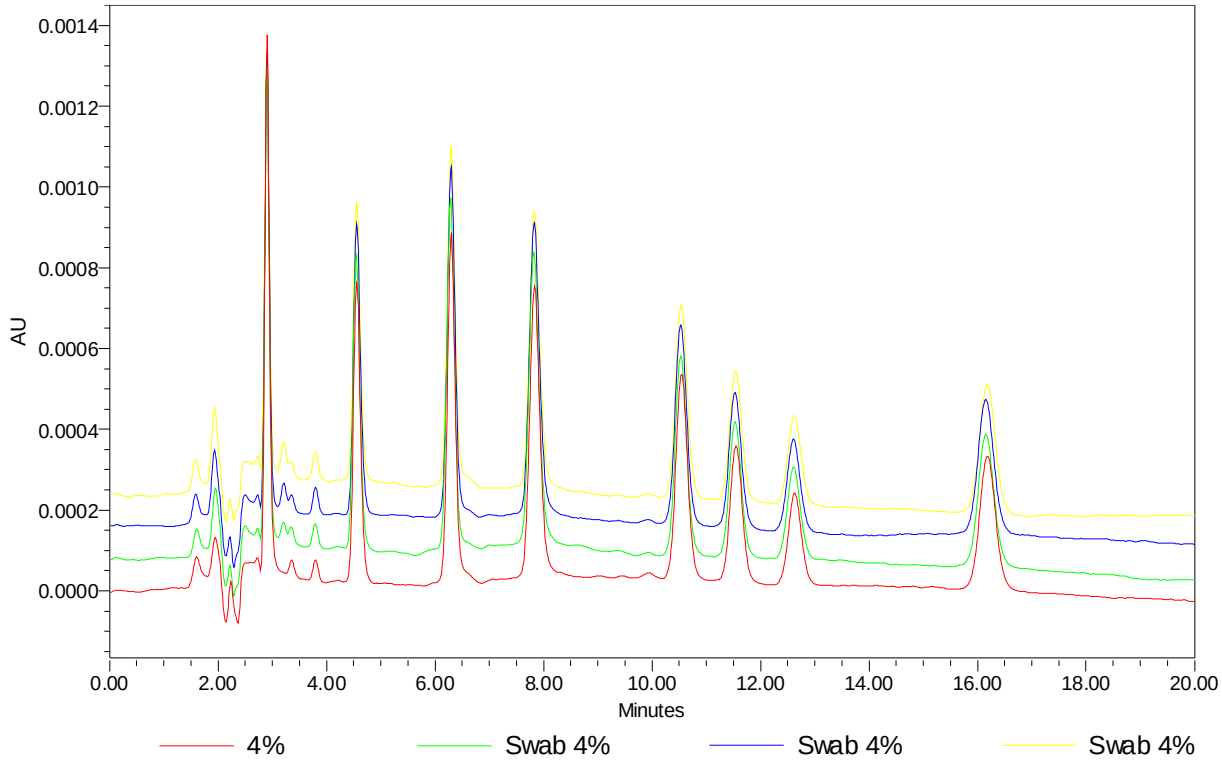

Figure C.55 -Chromatogram results of swab recovery at 4% concentration.

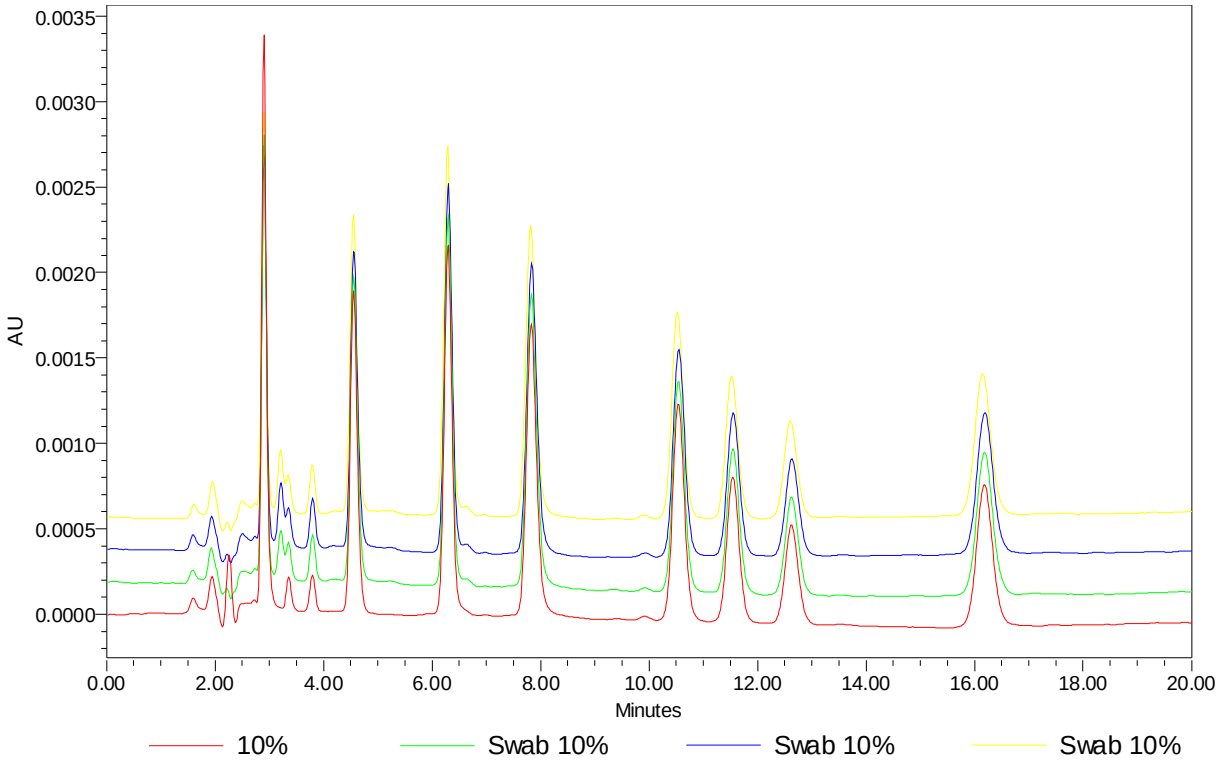

Figure C.56 - Chromatogram results of swab recovery at 10% concentration.