Research & Reviews: Journal of Pharmaceutical Analysis

e-ISSN: 2320-0812
p-ISSN: 2347-2340

Analysis of Sitagliptin On Halogenated Reversed Phase Columns: A Comparative Study

Hebatallah A Wagdy[1] and Hassan Y Aboul-Enein[2]*

[1]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, The British University in Egypt (BUE), Cairo, Egypt

[2]Pharmaceutical and Medicinal Chemistry Department, Pharmaceutical and Drug Industries Research Division, National Research Center (NRC), Dokki, Giza 12622, Egypt

Research Article

Received date: 26/10/2016
Accepted date: 27/10/2016
Published date: 12/11/2016

***For Correspondence**

Aboul-Enein HY, Pharmaceutical and Medicinal Chemistry Department, Pharmaceutical and Drug Industries Research Division, National Research Center (NRC), Dokki, Giza 12622, Egypt

E-mail: haboulenein@yahoo.com

Keywords: Halogenated stationary phases, Pentabromobenzyl phase column, Perfluorophenyl phase column, Sitagliptin, Retention mechanism, Pharmaceutical analysis.

ABSTRACT

This investigation describes the liquid chromatographic behavior in reserved phase mode for the separation of sitagliptin using two halogenated stationary phases namely pentabromobenzyl (PBr) and pentafluorophenyl (PFP) columns as well as the separation mechanism involved.

The optimized separation was achieved using a mobile phase composed of acetonitrile: water: triethylamine: acetic acid (80:20:0.2:0.1; v/v), at a flow rate 1.0 mL/min, at temperature 25°C. The detection wavelength was 268 nm. The quantitative analysis of sitagliptin in bulk and pharmaceutical tablet formulations was performed and validated on PBr column since this column eluted the analyte under study at a shorter retention time than PFP column.

Furthermore, this study led to better understanding of the mechanism of action involved in these newly developed halogenated stationary phases regarding the studied analyte and mobile phase/column parameters. It was found that both columns showed π aromatic- π aromatic, π aromatic- π aliphatic, π aromatic- π cationic interactions, in addition to hydrogen bonding in case of PFP column.

INTRODUCTION

In liquid chromatography (LC), many factors have an impact on the separation and the selectivity. Among these factors, it could be mentioned the following: the stationary phase, the mobile phase, the nature of the analytes and the experimental variables such as pH, flow rates and temperature [1]. It's worth mentioning that the stationary represents one of the most important influences on the separation in LC.

This could be the reason of the numerous numbers of columns commercially available in market. Nevertheless, there is continuous development of stationary phases for separation improvement [2,3]. One of the newly developed stationary phases is the halogenated stationary phases. In this paper, two halogenated stationary phases were investigated namely pentafluorophenyl column marketed under the name "Fluophase PFP" and pentabromobenzyl column marketed under the name "Cosmosil PBr" **(Figure 1)**.

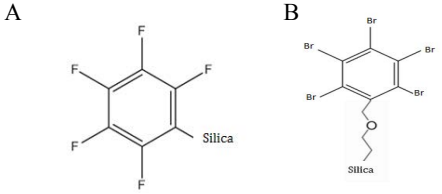

Figure 1. Structures of the two stationary phases under investigation in this study named: Fluophase PFP (A) and Cosmosil PBr (B).

The first study in literature to describe the chromatographic characteristics of flurorinated stationary phase was reported in 1981 [4,5]. Recently, this fluorinated column became widely used as an alternative option for the conventional C18 in reversed

e-ISSN: 2320-0812
p-ISSN: 2347-2340

phase LC. This is due to the high selectivity, especially for halogenated and non- halogenated compounds [6]. The fluorinated phase is characterized by its hydrophobicity and having some polarity [7]. Its retention mechanisms include many interactive forces such as dipole- dipole, π-π interaction, H-bonding and ion exchange [4]. Several reports compared the separation behavior of different fluorinated stationary phase (s) to conventional C18 and other phenyl columns [8-16] or comparing between different fluorinated stationary phases [6,17]. Furthermore, other investigations have attempted to explain the retention mechanism of perfluorophenyl stationary phases by analyzing different compounds [18,19].

Pentabromobenzyl-bonded silica known as Cosmosil PBr had been just recently introduced in the market. It was of interest to study the chromatographic for the separation of sitagliptin on these two halogenated stationary phases. To the best of our knowledge, this is the first publication in literature dealing with this topic.

Sitagliptin [(2R)-1-(2,4,5-trifluorophenyl)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro-[1,2,4]-triazolo-[4,3-a]pyrazin-7(8H)-yl]-butan-2-amine] is an antidiabetic drug. It ameliorates the glycemic control of diabetic patients with type 2 diabetes mellitus by increasing both α and β cell sensitivity to glucose. Sitagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor, which led to prevention of incretins glucagon-like peptide and gastric inhibitory polypeptide. As a consequence, it led to improve the glucose control [20-22]. Few papers have been reported for the analysis of sitagliptin using different techniques such as LC-UV [23], LC-MS [24], NMR [25], spectrophotometric [26]. It's worth mentioning that none of these methods have been used for analyzing sitagliptin on any of the halogenated stationary phases under study in this work **(Figure 2)**.

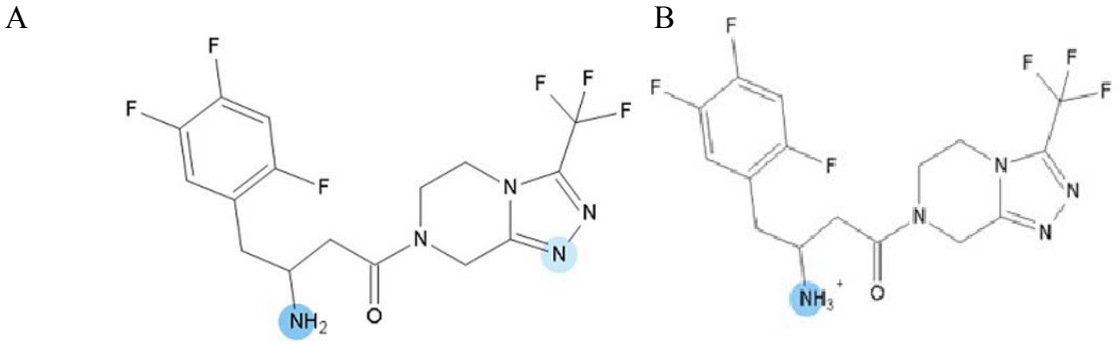

Figure 2. Structure of Sitagliptin (A) and the ionized form under the studied conditions (B) (ACD/Labs®).

The aim of this work is to analyze sitagliptin on both PBr and PFP columns. Subsequently, illustrate a comparison between the mechanisms involved in the separation of sitagliptin on both columns. Based on this comparison, a validated analytical method will be established for the drug under study using the column that provided the best results.

EXPERIMENTAL

Equipment

The HPLC system utilized was Thermo Fisher UHPLC Dionex Ultimate 3000 (Germering, Germany). It consists of pump (ISO-3100SD), autosampler (WPS 3000 SL), column thermostat (TCC-3000 SD) and Diode Array detector (DAD- 3000 RS) (Germering, Germany). The software utilized for data acquisition was Chromeleon 6.8 (Germering, Germany).

The columns used in this study were Cosmosil PBr (250 mm × 4.6 mm, 5 µm particle size), purchased from Nacalai Tesque, (Kyoto, Japan) and Fluophase PFP (250 mm × 4.6 mm, 5 µm particle size), purchased from Thermo Scientific, Germany.

Materials and Reagents

Methanol (MeOH) (HPLC grade), acetonitrile (ACN) (HPLC grade), triethylamine (TEA) and glacial Acetic acid (AC) were purchased from Sigma-Aldrich, Germany.

Sitagliptin was provided by Merck Sharp and Dohme Co. (Cairo, Egypt). Januvia 100 mg (Merck Sharp & Dohme Ltd., Cramlington, UK) was purchased from the local market.

Methods

The standard stock solution (1 mg/mL) was prepared by dissolving 100 mg in 100 mL methanol. For the assay of pharmaceutical tablets (Januvia), ten tablets were weighed and grinded to a fine powder. An amount equivalent to 50 mg; from the grinded powder; was accurately weighed and dissolved into 50 mL methanol. This was followed by sonication of the volumetric flask for 30 min, to ensure complete dissolution. The solution was then filtered using 0.2 µm syringe filter. All the solutions were kept from light by covering it with aluminum foil paper and stored in the fridge.

All the experiments; on both columns; were performed using isocratic elution. The optimum condition was achieved using mobile phase consisting of acetonitrile: water: triethylamine (TEA): acetic acid (AC) (80:20:0.2:0.1; v/v), at a flow rate 1.0 mL/

e-ISSN: 2320-0812
p-ISSN: 2347-2340

min, at temperature 25°C. The UV detector was adjusted at wavelength 268 nm. The mobile phase was filtrated using 0.45 µm membrane filter, and then allowed to remain in ultrasonic bath for 30 min for degassing. The injection volume in all runs; on both columns; was 5 µL.

Validation of the method was performed according to the ICH guidelines [27]. The parameters under investigation included linearity, limit of detection (LOD), limit of quantification (LOQ), precision (inter- and intra-day), accuracy and robustness.

For linearity, a calibration curve was constructed by plotting the peak area against the corresponding concentrations (mg/mL). Nine calibrations standards were prepared by serial dilution of sitagliptin covering a range of 0.4 mg/mL to 1.2 mg/mL. Each concentration was injected 3 times. This range covers 40% to 120% of target concentration, which is 1.0 mg/mL. Results were evaluated by calculating square of the regression coefficient (r^2).

LOD was calculated from the calibration curve using the following equation:

LOD=3.3 (σ/S)

LOQ was calculated from the calibration curve using the following equation:

LOQ =10 (σ/S)

where "σ" is the standard deviation of the response and S is the slope of the calibration curve [27].

Precision is describing the degree of repeatability of an analytical method. In this work, the precision of the methods was evaluated in terms of inter- and intra-day precision. Both were expressed by calculating % relative standard deviation (%RSD) [28]. Intra-day precision was performed by analyzing 3 different concentrations (0.8 mg/mL, 1.0 mg/mL, 1.2 mg/mL) three times on the same day (n=9) while inter-day precision was performed on 3 different days.

Accuracy was performed by spiking sitagliptin pharmaceutical tablet formulation (claim to contain 0.479 mg/mL active ingredient) with known amount of standard 0.30 mg/mL, 0.50 mg/mL and 0.70 mg/mL, so that the final concentrations are 0.779 mg/mL, 0.979 mg/mL and 1.179 mg/mL, respectively. Each sample was injected three times. The results were expressed as percentage recovery (% recovery) and %RSD [29].

Robustness was evaluated by the capacity of the method to remain uninfluenced by small changes in the experimental parameters [27].

RESULTS AND DISCUSSION

Optimization of the Chromatographic Conditions

Pentabromobenzyl (PBr) column

At first, the condition under investigation was MeOH: water: TEA: AC (60:40:0.1:0.1; v/v) at flow rate 0.5 mL/min and temperature 20°C, the peak of sitagliptin eluted at 17.607 min [30]. However, the peak was relatively broad. This was followed by increase in flow rate to 1 mL/min, which led to both decreasing in retention time (tR was 8.747 min) and narrowing of the peak.

When the percentage of the organic phase was increased to 80%, the PBr column showed a typical reversed phase behavior and the tR decreased to be 6.077 min. When the temperature was increased to 25°C caused a slight decrease in tR and enhancement of the peak shape, the tR was 6.007 min as shown in **(Figure 3)** [31].

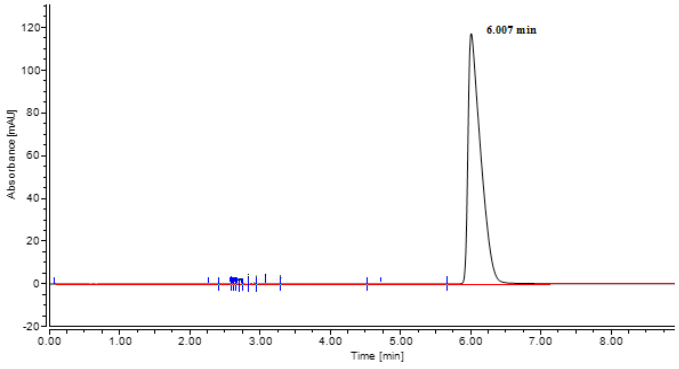

Figure 3. Chromatogram of sitagliptin on PBr column under the condition: Mobile phase MeOH: Water: TEA: AC (80:40:0.1:0.1; v/v); flow rate 1.0 mL/min, T=25°C and UV 268 nm.

To study the effect of ternary eluent on the analysis using PBr column, three different compositions of organic phases were tested using two different organic solvents namely methanol and acetonitrile.

Different % of organic phases were investigated: a) 100% MeOH, b) MeOH: ACN (1:1 v/v) and c) 100% ACN, tR were 6.007

e-ISSN: 2320-0812
p-ISSN: 2347-2340

min, 5.253 min and 4.670 min respectively. **Figure 4** showed the 100% ACN condition. It was observed that increasing the % of ACN resulted into decrease in tR, which is attributed to the stronger eluent strength of ACN relative to that of MeOH.

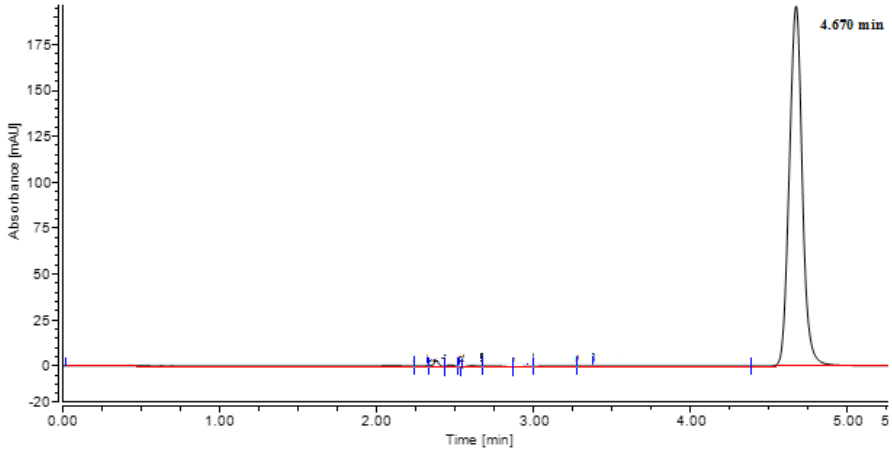

Figure 4. Chromatogram of sitagliptin on PBr column using 100% ACN as organic solvent.

In addition, the effect of TEA percentage and AC percentage on separation was also studied. When the % of AC was increased to be 0.2%, this led to slightly increase in tR to be 4.793 min. While increasing the TEA percentage to be 0.2%, resulted into decrease in tR to be 3.737 min **(Figure 5A)**.

Therefore, the optimum conditions for analysis of sitagliptin on PBr column are: mobile phase consisting of acetonitrile: water: TEA: AC (80:20:0.2:0.1; v/v), at a flow rate 1.0 mL/min, at temperature 25°C. The suggested interactions involved in sitagliptin analysis, on PBr column, were π aromatic- π aromatic and π aromatic- π aliphatic [9] and dispersion force (instantaneous dipole-induced dipole force) which is also known as London dispersion force. This is a weak intermolecular force that results from dipoles temporarily induced from random unsymmetrical electron positions in two adjacent atoms.

The pKa of sitagliptin were measured using ACD/Labs® and were 7.20 ± 0.1 and 8.60 ± 0.4. Under the working chromatographic condition, the amino group in sitagliptin will be ionized, as presented in **Figure 2B** which will result in the π aromatic- π cationic interaction.

Pentafluorophenyl (PFP) column

The optimum condition on PBr column was transferred to PFP column. The mobile phase composition was ACN: water: TEA: AC (80:20:0.2:0.1; v/v), at flow rate 1.0 mL/min and temperature 25°C.

Different % of TEA and AC were investigated. The tR of sitagliptin was 11.363 min when 0.1% TEA: 0.1% AC were added to the mobile phase. When the percent of AC was increased AC (0.2%), the retention time tR was slightly increased be 11.740 min. While, doubling the % of TEA (0.2%), resulted into significant decrease in tR to be 5.347 min **(Figure 5B)**.

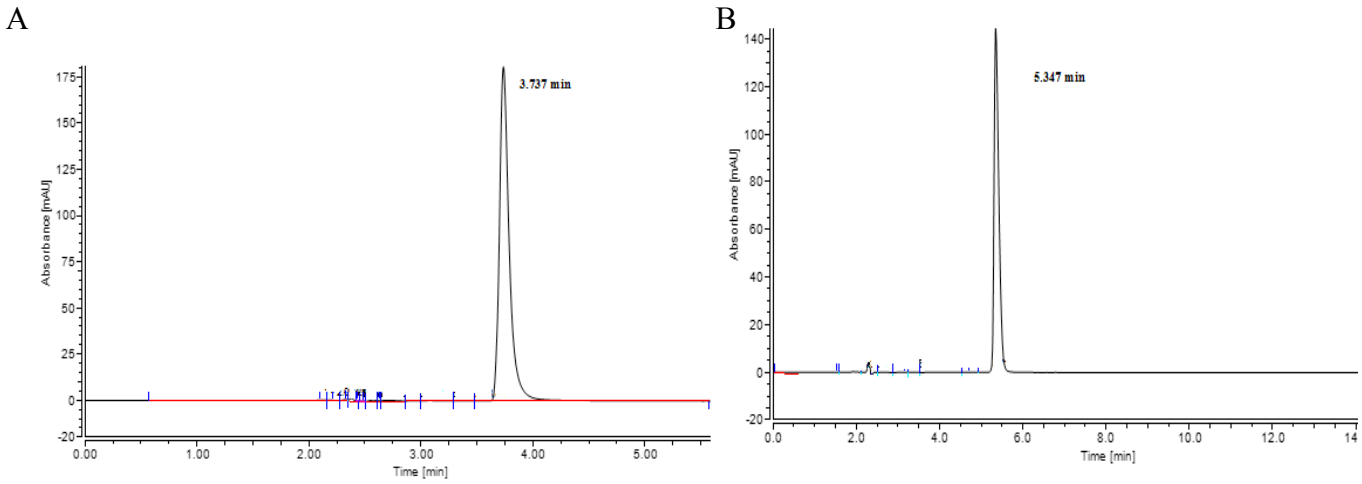

Figure 5. Chromatograms of sitagliptin under the optimum condition named: acetonitrile: water: TEA: AC (80:20:0.2:0.1; v/v), at a flow rate 1.0 mL/min and T 25°C, on PBr column (A) and PFP column (B).

The effect of increasing the percent of TEA or AC was almost similar, on both columns. The increase in % AC led to slight

e-ISSN: 2320-0812
p-ISSN: 2347-2340

increase in tR and the increase in % TEA led to, relatively, significant decrease in tR. It is of interest to mention that the effect of increasing % TEA on decreasing the tR was higher on PFP column than that on PBr column. It's worth mentioning that TEA decreased the tailing effect of the peak.

Under the same chromatographic condition; sitagliptin eluted at less tR on PBr column than PFP column. This could be contributed to two effects.

1. The presence of strong hydrogen bonds in case of PFP which provide more interactions between sitagliptin and the pentafluorophenyl column which led to an increase in retention time while this type of interactions are very weak or even non existing in case of the pentabromobenzyl column.
2. The π-π interactions between the fluorine atoms on PFP and that on sitagliptin are stronger than π-π interactions between the bromine atoms on PBr and the fluorine atoms on sitagliptin since fluorine atom are more electronegative than the bromine.

Since the tR of sitagliptin on PBr column was less than that on PFP column, under the studied conditions, a validated analytical method was developed for the analysis of sitagliptin using the PBr column.

Method Validation

The calibration curve plotted for sitagliptin was linear in the concentration range of 0.4 mg/mL to 1.2 mg/mL. The regression equation for the calibration curve is y =16987x-61.799. The regression coefficient (r^2) was be 0.9991 indicating good linearity of the method in this range. It worth mentioning that the standard deviation of the slope was 2.05 and that of the intercept was 2.12.

The LOQ and LOD were 0.082 mg/mL and 0.027 mg/mL respectively for standard drug solutions. For inter-day precision, was investigated by preparing three concentrations: 0.8 mg/mL, 1.0 mg/mL and 1.2 mg/mL; then each was injected three times and the % RSD was 0.589, 1.069 and 0.263, respectively. The three previously mentioned concentrations were injected over 3 consecutive days to assess the intra-day precision. The % RSD were 0.635, 1.053 and 0.710, respectively **(Table 1)**.

Table 1. Inter-day and Intra-day precision of sitagliptin on PF column.

Concentration (mg/mL)	Inter-Day precision		Intra-Day precision	
	Peak Area*	%RSD	Peak Area*	%RSD
0.8	12593.64	0.589	12414.13	0.635
1	15592.16	1.069	15644.57	1.053
1.2	19458.57	0.263	19128.2	0.71
*Average of 3 preparations each was repeated 3 times.				

For accuracy, the % recovery, ranged from 98.33% to 98.76%, and the %RSD ranged from 0.219 to 0.580. The robustness of the method was assessed by RSD values, by intentionally introducing small change in the following parameters: temperature, wavelengths, %ACN, %TEA and %AC and the results were as follow:

- Temperature 25°C ± 3°C with %RSD 1.96%.
- Wavelength 268 nm ± 3 nm with %RSD 2.57%.
- % Acetonitrile 80% ± 1% with %RSD 3.35%.
- % TEA 2% ± 0.01% with %RSD 0.58%.
- % AC1% ± 0.01% with %RSD 0.87%.

The above results showed the method developed for the analysis of sitagliptin on the PBr column is highly robust **(Table 2)**.

Table 2. Accuracy of sitagliptin on PF column.

Theoretical concentration (mg/mL)	Actual Concentration* (mg/mL)	%RSD	%Recovery*	%RSD
0.779	0.771	0.368	98.33	0.385
0.979	0.972	0.335	98.68	0.219
1.179	1.17	0.476	98.76	0.58
* Average of 3 repetition				

Application on Pharmaceutical Preparation

Ten Januvia® tablets contain 100 mg sitagliptin in each tablet were weighted and finely grinded. An amount equivalent to 50 mg from the grinded powder was accurately weighed and dissolved into 50 mL methanol then sonicated for 30 min followed by filtration using 0.2 µm syringe filter. The filtrate (5 µL) was injected under the optimum chromatographic condition on PBr column described above. The tablet excipients did not interfere with the analysis **(Figure 6)**.

e-ISSN: 2320-0812
p-ISSN: 2347-2340

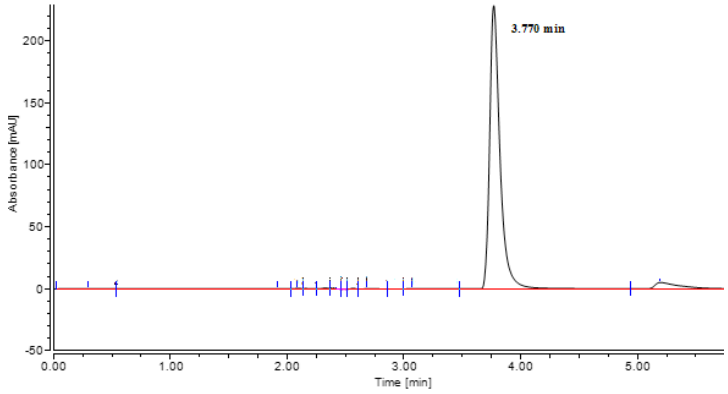

Figure 6. Chromatogram of Januvia® 1 mg/mL on PBr column, under the optimum conditions: Mobile phase ACN: Water: TEA: AC (80:20:0.2:0.1; v/v); flow rate 1.0 mL/min, T=25°C and UV=268 nm.

CONCLUSION

A comparative study between the two halogenated columns namely PFP and PBr was investigated. The interactions contributed in analysis were π aromatic- π aromatic, π aromatic- π aliphatic, π aromatic- π cationic on both columns along with the London dispersion force (instantaneous dipole-induced dipole force).

The results showed under the studied condition that sitagliptin is more retained on PFP column. This was attributed to two effects: 1) The presence of strong hydrogen bonding between the fluorine in PFP stationary phase and sitagliptin and 2) the fact that fluorine atom is more electronegative than bromine, hence, the π- π interactions between PFP and sitagliptin will be stronger than that between PBr and sitagliptin.

A validated method for the analysis of sitagliptin in bulk and pharmaceutical tablet formulate on PBr column was established. The optimum condition was mobile phase consisted of acetonitrile: water: TEA: AC (80:20:0.2:0.1; v/v), at a flow rate 1.0 mL/min, at temperature 25°C and wavelength adjusted at 268 nm. The analysis was conducted in less than 6 minutes.

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