



## Separation of complexed semi-synthetic flavonoids by using fused core column in short time

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### ABSTRACT

Hydroxyethylrutosides (Troxeutin) is a standardized mixture of semi-synthetic flavonoids obtained by substituting hydroxyethyl groups in the naturally occurring flavonol rutin. Because there are four dissociation hydroxyls in rutin, some fifteen kinds of hydroxyethylrutins can be theoretically synthesized. The good quality of the raw material of a drug and the finished product must include the related impurities in an analytical investigation, and this seemed particularly important to the quality control of Troxeutin. According to the literature, it is clear that, the chromatographic analysis of Troxeutin still problematic as its sample can be considered as a complex sample as it contains plenty of different components. In this study, new HPLC technology named fused-core column was used to separate the major components of Troxeutin bulk powder. Fused – Core silica stationary phases represent key technological advancement in the area of fast HPLC separations. These phases are made by fusing 0.5  $\mu\text{m}$  porous silica. The reduced intra – particle flow path of the fused particles provides superior mass transfer kinetics and better performance at high mobile phase velocities, while the fused – core particles provide lower pressure than sub – 2  $\mu\text{m}$  particles.

**Keywords:** Troxeutin, UPLC- like method, Fused core column.

### INTRODUCTION

Flavonoids are formed in plants from the aromatic amino acids phenylalanine and tyrosine, and malonate. the basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6-C3-C6), labeled **A**, **B**, and **C**.

The various classes of flavonoids differ in the degree of oxidation and pattern of substitution of the **C**-ring, while individual compounds within each class differ in the pattern of substitution of the **A** and **B**-rings. The flavonoid classes include flavones, flavanones, isoflavones, flavonols, flavanonols, flavan-3-ols, and anthocyanidins, biflavones, chalcones, aurones, and most of these classes are present in plants as glycosides.

Flavonoids represent a well-known family of compounds and their synthesis has been the object of a great number of studies. In general, procedures for laboratory synthesis of flavonoids are still based on the approaches originally developed by Robinson [1], with other methods including the Baker–Venkataraman rearrangement [2,3], synthesis via chalcones [4], and synthesis via an intramolecular Wittig reaction [5]. Despite the number of steps often involved in these methods, they constitute the most popular methodologies used nowadays for the preparation of flavonoids. The synthesis of flavonoid derivatives is also an area of significant interest and many synthetic routes have been used to add moieties to the natural flavonoids nucleus. The Mannich reaction, for instance, is used for the addition of alkyl amine derivatives [6]. The Aldol condensation reaction is used either for the synthesis of natural

flavonoids or for the addition of alkyl or benzyl moieties [7]. Many publications covering flavonoid biotransformations also focus on oxidation, methylation, glucosidation [8,9,10], and halogenations [11].

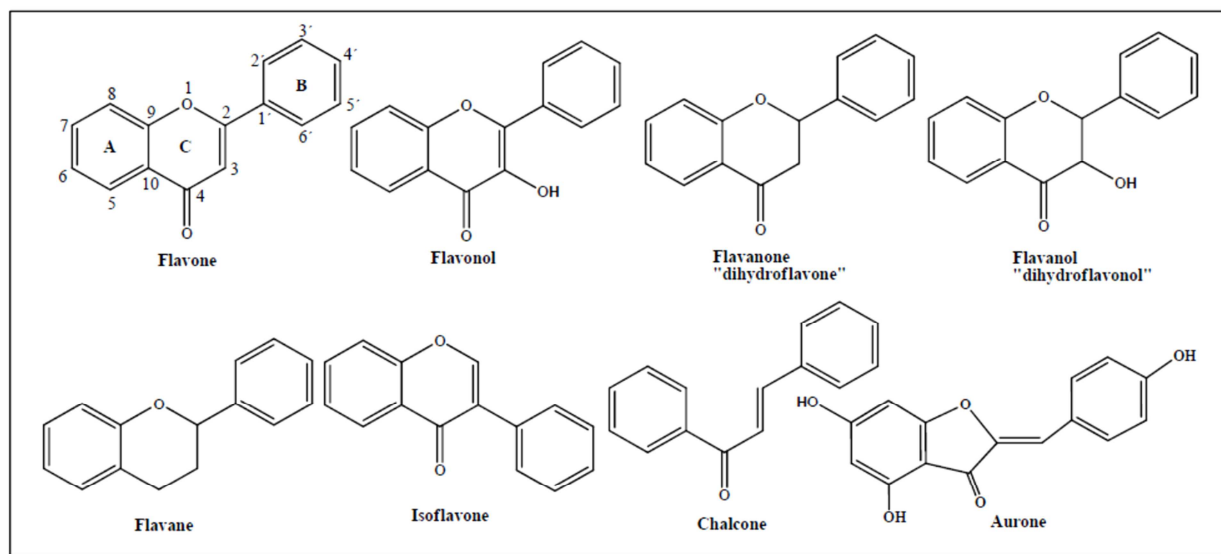


Figure 1. Various classes of flavonoids which differ in the degree of oxidation and substitution

Hydroxyethylrutosides is a standardized mixture of semisynthetic flavonoids obtained by substituting hydroxyethyl groups in the naturally occurring flavonol rutin. It acts primarily on the microvascular endothelium to reduce the hyperpermeability and friability of micrangium, inhibit platelet agglutination and erythrocyte aggregation, prevent the thrombosis and angiosclerosis, and is commonly used for the relief of oedema and related symptoms in patients with chronic venous insufficiency [12]. Because there are four dissociation hydroxyls in rutin, some fifteen kinds of hydroxyethylrutins can be theoretically synthesized (four mono-, six di-, four tri- and one tetra-hydroxyethylrutosides); the proportion of the individual composition in hydroxyethylrutosides is related to the reaction conditions. Other hydroxyethylated constituents, such as tetra-hydroxyethyl-quercetin, in which the sugar moiety is absent, are also present in small amounts. Therefore, 7,3',4'-hydroxyethylrutoside, namely 7,3',4'-tris[O-(2-hydroxyethyl)]rutin (Figure 2) has the highest potency.

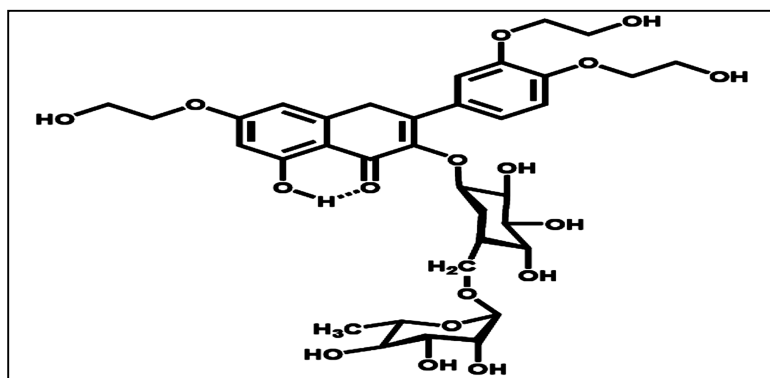


Figure 2: Chemical structure of "Trihydroxyethylrutin; 3',4',7-Tris[O-(2-hydroxyethyl)]rutin"

The good quality of the raw material of a drug and the finished product must include the related impurities in an analytical investigation, and this seemed particularly important to the quality control of Troxerutin. Unfortunately, it is difficult to find the standards of the derivative impurities on the market, which sometimes makes the investigation impossible. HPLC is the most commonly used method to control the quality of Troxerutin and its preparations [13 - 17]. The separation and determination of Troxerutin have been done on a VP-ODS column. The column was thermostatically controlled at 35°C [18]. Some other methods, such as TLC [19] and CE [20] were also reported for Troxerutin analysis. There are some studies with various stationary phases RP-18, RP-8 and RP-2 for analysis of avonoids[21]. Experiments have imposed the conclusion that the longer C-chain, the higher the resolution.

Some of the publications reported pure chromatographic studies on the effects of the stationary and mobile phases [22]. Nedyalka Dimova used gradient elution and RP-column Nucleosil 100 C8 125\_4 mm, 5  $\mu$ m particle size (USA) as stationary phases for analysis [23].

According to the literature, it is clear that, the chromatographic analysis of Troxerutin still problematic as its sample can be considered as a complex sample as it contains plenty of different components. The new stationary phase, monolithic was reported in a combination with capillary electrochromatography for the determination of Troxerutin [24].

Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method was used for the simultaneous quantitative estimation of Calcium dobesilate 500 mg and Troxerutin 500 mg in tablets as per ICH guidelines on Enable C18G (250X4.6 mm; 5 $\mu$ ) column and an isocratic elution [25].

However, there is no new technology as fused core column was used. Therefore, in this study, fused core column with HPLC and TLC methods were developed to separate the major components of Troxerutin bulk powder.

## EXPERIMENTAL SECTION

### 2.1. Material and solutions

All chemicals and reagents used were of analytical grade. Troxerutin (TRX) 90% was obtained from Aldrich chemical, (Milwaukee, USA), and Trihydroxy ethylrutin  $\geq$ 80% HPLC was obtained from Sigma - Aldrich (Belgium). Sodium dihydrogen phosphate was obtained from Germed, (Germany). Acetonitrile HPLC grade was obtained from Baker, (Deventer- Holland). Sodium hydroxide was obtained from Merck, (Darmstadt, Germany). Phosphoric acid was obtained from Riedel – De Haen (Hanover, Germany). Spherisorb ODS2 RP – 18- column (5  $\mu$ m particle size, 250 mm x 4.6 mm, VDS Optilab), while Ascentis Express C18 column (10cm x 4.6 mm, 2.7  $\mu$ m) was obtained from (Sigma Aldrich, USA).

### 2.2. Sample preparation

A 10 mg of each of TRX 90%, Aldrich chemical (Milwaukee, USA) and Trihydroxy ethylrutin  $\geq$ 80% HPLC, Sigma-Aldrich (Belgium) were accurately weighed separately and quantitatively transferred to two different 10 mL volumetric flasks by dissolving each compound separately in small amount of mobile phase and complete to the volume of each flask with the mobile phase to obtain stock (1g. L<sup>-1</sup>) solutions of each compound sample.

Working solutions: Serial dilutions for each compound were done to get the drug concentrations of 50, 100, 500 mg L<sup>-1</sup>.

A 0.1M phosphate buffer for the (Solvent **A**) was prepared by dissolving a 13.8 g of sodium dihydrogen phosphate in 1L distilled water. The pH was adjusted to 4.4 by addition of diluted NaOH solution 2M (8.5 gm/ 100ml water) or phosphoric acid (10%). Where the (Solvent **B**) is acetonitrile HPLC-grade, the mobile phase consists of a mixture of solvent A and B in the ratio 84:16 v/v. The mobile phase was degassed by sonication before use.

All solutions were prepared by using ultrapure MilliQ-water (Millipore, Milford, MA, USA) and were filtered through a 0.2  $\mu$ m membrane filter (Dassel, Germany). The pH value of the buffers was measured and adjusted with the aid of a Digital pH meter pH 522, (WTW, Germany).

### 2.3. Apparatus

The experiments were performed by HPLC system (Merck Hitachi\_Darmstadt, Germany),\_Interface (D-6000 Merck/Hitachi), the mobile phase was delivered by a LC pump (L-6200 Intelligent pump, Merck– Hitachi, Darmstadt, Germany) and samples were injected using an automatic autosampler (Elite LaChrom L-2200, Merck-Hitachi, Darmstadt, Germany). The column was kept in the oven T-6300, Merck/Hitachi, Darmstadt, Germany) to keep the temperature constant at room temperature (20°C). The (L-4500 UV-VIS diode array) was used as a detector. Data acquisition was done by (D7000 HSM, Merck/Hitachi) software.

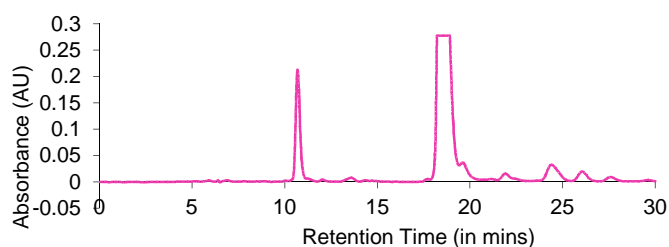
### 2.4. Chromatographic conditions

The separation was performed on fused core, and Spherisorb ODS2 columns with flow rate of 0.5 mL.min<sup>-1</sup>. All separations were performed at ambient temperature and 350 nm was the detection wavelength. Injection volume was 20  $\mu$ L.

**RESULTS AND DISCUSSION****3.1. Transfer of official HPLC method to UPLC-like method:**

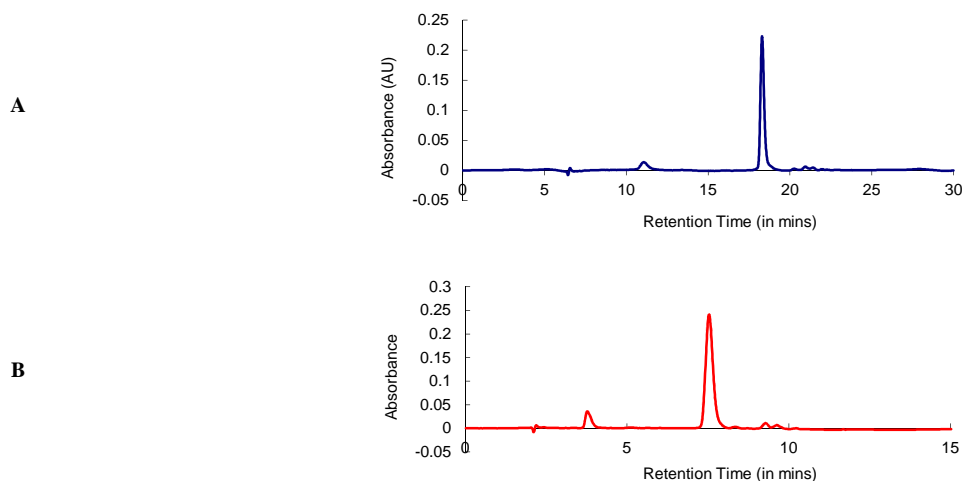
Troxerutin is an official drug as there is a pharmacopoeal analytical method for it however the DAB was the only equivalent reference to the pharmacopoeia which publishes an analytical TLC method for Troxerutin in 2005.

Octadecylsilane (ODS) was used as a HPLC column packing material for the official HPLC analysis of Troxerutin [26] with isocratic elution using a sodium dihydrogen phosphate buffer (pH 4.4) : Acetonitrile (80:20). By the application of the practical official procedure a long chromatographic run was observed with poor resolution and the obtained peaks were with tailing. Therefore experimental trials for optimization of the analytical procedure were tested as changing the ratio of the mobile system components from 80:20 to 84:16 as a result the  $t_R$  of the analyses were decreased with a decrease in the total chromatographic run time from 40 min to 30 min (Figure 3). However the analysis time still long, therefore another trial was tested as a change of the used HPLC column to a fused core column a reduction in the chromatographic run time were observed to just 10 minutes and the obtained peaks were with greater peak heights which indicated a more sensitive detector response.



**Figure 3: Representative chromatogram for optimized HPLC method to analysis of Troxerutin (0.5mg/ml) with conventional column**

It was clearly observed that the chromatographic run time was significantly reduced to about 10 min with the use of fused core column (red chromatogram) compared with the conventional column (blue chromatogram). Moreover the peak height of the obtained components was increased as well by using fused core column which increase the sensitivity of the fused core column HPLC method to the analyses (Figure 4).

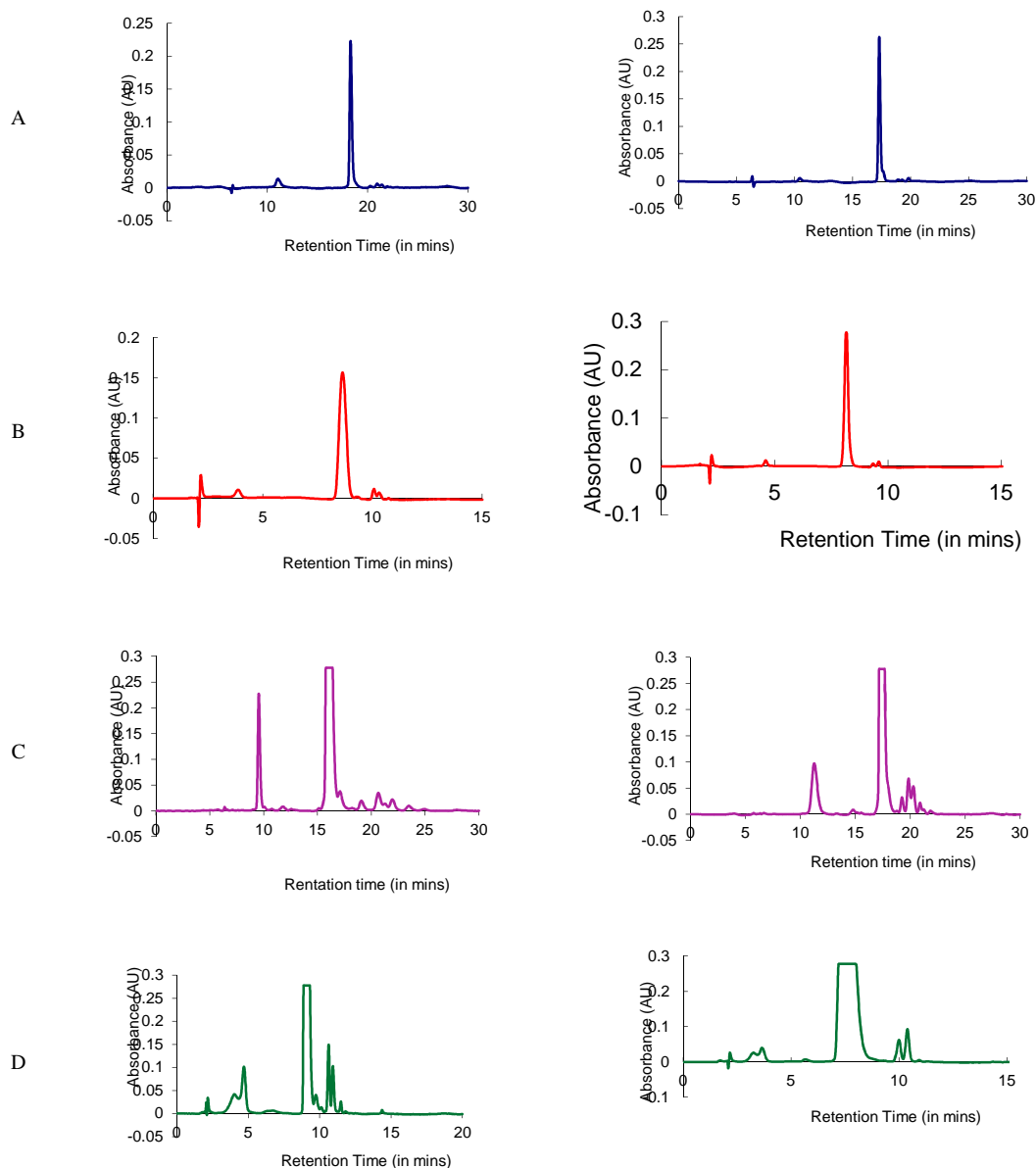


**Figure 4: Representative chromatogram for analysis of Troxerutin by A: conventional column, B: fused core column**

To examine the optimized procedure for the determination of all components in the presence of each other a large amount of Troxerutin (10  $\mu$ g) was injected as a sample and as shown in the obtained chromatogram the separation efficiency of the fused core was much better than the conventional one, as extra peaks were observed with the use of fused core column compared with the conventional one.

It is important to notice the difference in the obtained chromatograms with the different sample sources (manufactures). Although both companies are prestigious companies however both gave different chromatograms even if the used columns, the mobile system and all the analytical procedure were the same as shown in the table. As a conclusion of that difference was the TRX is a difficult mixture of components to be analyzed as a different source

of that analyte gave different components so that gave another challenge in its determination and/or separation of these components (Figure 5).



**Figure 5:** Representative chromatograms for analysis of TRX (0.05 mg/ml) A and B on conventional and fused core columns respectively, (0.5 mg/ml) C and D on conventional and fused core columns respectively. Left hand side; TRX 90%, Aldrich chemical (Milwaukee, USA) and right hand side; Trihydroxy ethylrutin  $\geq 80\%$  HPLC, Sigma-Aldrich (Belgium)

## CONCLUSION

Through the literature of the official and nonofficial HPLC analysis of Troxerutin, conventional HPLC column was the only stationary phase which used for its analysis. There are many disadvantages for the use of that type of columns such as long analysis time. To the best of our knowledge there is no reported UPLC-like method for this drug. UPLC is a new type of fast HPLC technique which characterised by the short analysis time. Therefore it was important to transfer the official HPLC method to UPLC-like method by the use of new analytical column which packed with more advanced stationary phases such as fused core column. Very short analysis time was obtained with the developed method by using fused core column with isocratic elution. Where, the official chromatographic run time for the analysis of Troxerutin was (40 min) and on its transfer to UPLC-like method the analysis time was reduced to (10 min).

Modern column gave more resolution and efficiency than that of the conventional column which can be attributed to the better peak shape and reduced baseline noise. Generally On using fused core column the backpressure was reduced which attributed to the new technology in particles (2.7  $\mu\text{m}$ ) with a high-capacity and very pure porous silica layer which fused to a solid silica core.

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