



SEPARATION, PURIFICATION AND IDENTIFICATION OF FLAVONOID GLYCOSIDES USING REVERSED PHASE HPLC

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Optimal high performance liquid chromatography (HPLC) separation conditions and semi-preparative scale isolation of flavonoid glycosides from three plant species namely *Vitex nagunda*, *Rubus ulmifoliosus* and *Malotus philipensis* is reported. Identification of purified flavonoid glycoside was achieved using spiking technique in HPLC.

Keywords: HPLC, Separation, Purification, Identification, Plant extracts, Flavonoid glycosides

1. Introduction

Flavonoid glycosides have been shown to be an important class of natural products due to their wide range of applications [1-5]. However, these compounds can only be rendered useful when obtained in pure state. The most difficult aspect of flavonoid analysis is the process of their purification. For this purpose a number of chromatographic techniques are available to the plant chemists. However, HPLC has proved to be the method of choice due to its reproducibility, speed, high resolution and sensitivity. The excellent reviews of Kingston [6] and Johnson [7] have presented some selected examples of HPLC separation of flavonoid glycosides.

The successful separation of flavonoid glycosides by HPLC depends largely on the choice of separation conditions which include solvent system, detection, flow rate, column packing material, sample concentration, column temperature, etc. A number of workers have reported the use of different solvent systems [8-11] for the separation of flavonoid glycosides, while others proposed normal phase (NP) [12-13] as well as reverse phase (RP) [14] columns. Similarly variable UV detection wavelength, ranging from 200 – 400 nm has been suggested by other workers [11,15-18]. As far as the column temperature, sample concentration and flow rates

are concerned, no specific literature is available.

The work so far reported for the separation of flavonoid glycosides is specific and can only be applied to one or another type of a mixture of flavonoid glycosides. Moreover, the full potential of HPLC is not very frequently used for the isolation of flavonoid glycosides on semi-preparative scale as well as for their characterization. Therefore, a standard method of separation applicable to various mixtures of flavonoid glycosides. In addition isolation of individual glycoside on semi-preparative scale and their identification using HPLC is also demonstrated.

2. Experimental

2.1 Materials

To determine the optimal HPLC conditions for the separation and purification of flavonoid glycosides, extracts of *Vitex nagunda*, *Rubus ulmifoliosus* and *Malotus philipensis* leaves were prepared. For this purpose leaves of these plant species were collected in May 1999 from Murree Hills, Pakistan. Air-dried leaves of each plant (500g) were repeatedly extracted with 80% methanol (MeOH). Each extract was concentrated under reduced pressure until complete removal of methanol. Non-polar contents in the aqueous extract were removed with chloroform before extraction with ethyl acetate. The ethyl acetate

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extract was evaporated to dryness and the dry residues (3.76, 2.46 and 4.01 g respectively) of flavonoid glycosides were separated by HPLC. The separation parameters used were solvent system, solvent flow rate, detection wavelength and sample concentration. The effect of each of these parameters on the separation of flavonoid glycosides was studied. HPLC grade acetonitrile, methanol, tetrahydrofurane (THF) and propyl alcohol were purchased from Fluka AG, Chem. Fabrik CH 9470 Buchs, Switzerland. Deionized water used was triple distilled.

2.2 Apparatus

The HPLC system consisted of a Shimadzu (Koyoto, Japan) LC-6A, equipped with a dual-piston, solvent-delivery module with a high-sensitivity filter unit, a Shimadzu Model SCL-6A auto-injector with a 100 μ l sampling loop, a Shimadzu Model SIL-6A system controller, a Shimadzu Model SPD-6A variable wavelength UV detector and Shimadzu Chromatopac C-R3A data processor. Shimpack Column ODS C₁₈- 10 μ m (25 cm x 4.6 mm I.D.) was kept in a Shimadzu CTO-6A oven to maintain constant temperature

3. Results and Discussions

3.1 Optimized HPLC separation conditions

There are a number of parameters, on which the separation of constituents of an extract depends in a HPLC analysis. These include solvent system, flow rate, detection wavelength, sample concentration, column and column temperature. In the present analysis the last two parameters were kept constant while the other were varied one by one in search of optimized separation conditions.

3.1.1 Mobile phase

While keeping all other separation conditions constant, six solvent systems: MeOH-acetonitrile-THF-H₂O (70:10:5:15), MeOH-acetonitrile (60:40), MeOH-THF-H₂O (15:65:20), CH₃CO₂H-H₂O (2.5:97.5), Iso-PrOH-H₂O-THF (5:80:15) and MeOH-H₂O-CH₃CO₂H (16:80:4) were used one after the other to monitor the degree of resolution of *Vitex* flavonoid glycosides. Examination of chromatograms recorded in these solvent systems revealed high resolution of the *Vitex* flavonoid glycosides in solvent system. MeOH-H₂O-CH₃CO₂H (16:80:4). This mobile phase had a pH = 6.0.

3.1.2 Solvent flow rate

The solvent flow rate was varied from 1.5 - 2.5 ml min⁻¹ while MeOH-H₂O-CH₃CO₂H (16:80:4) was used as the mobile phase. Chromatograms with flow rates 1.5, 1.9, 2.3 and 2.5 ml min⁻¹ exhibited 9, 10, 10, 9 peaks respectively; while chromatograms with flow rates 1.7 ml min⁻¹ and 2.1 ml min⁻¹ respectively show 13 peaks each, however resolution of peaks with flow rate 1.7 ml min⁻¹ was much better. Thus flow rate 1.7 ml min⁻¹ was retained as the optimized.

3.1.3. Detection

The UV detection wavelength was varied between 254 and 366 nm, using optimized mobile phase and flow rate (Table-1).

Table-1. Detection at variable wavelengths

Detection Wavelength	Number of peaks
254 nm	9
275 nm	15
280 nm	10
290 nm	8
330 nm	3
360 nm	3

The resultant chromatograms indicated that 275 nm is probably the most suitable wavelength and was in complete agreement with that reported by Morve et al [11]. From this experiment it was concluded that 275 nm is the most suitable UV wavelength for the detection of flavonoid glycosides.

3.1.4 Sample concentration

Chromatograms were recorded with variable flavonoid glycoside extract concentrations ranging from 2.8 - 4.0 mg ml⁻¹. As a result of these experiments it was suggested that sample concentration has no marked effect on the resolution of flavonoid glycosides but simply influence the height of the peaks.

For further work the following optimized separation conditions were selected :

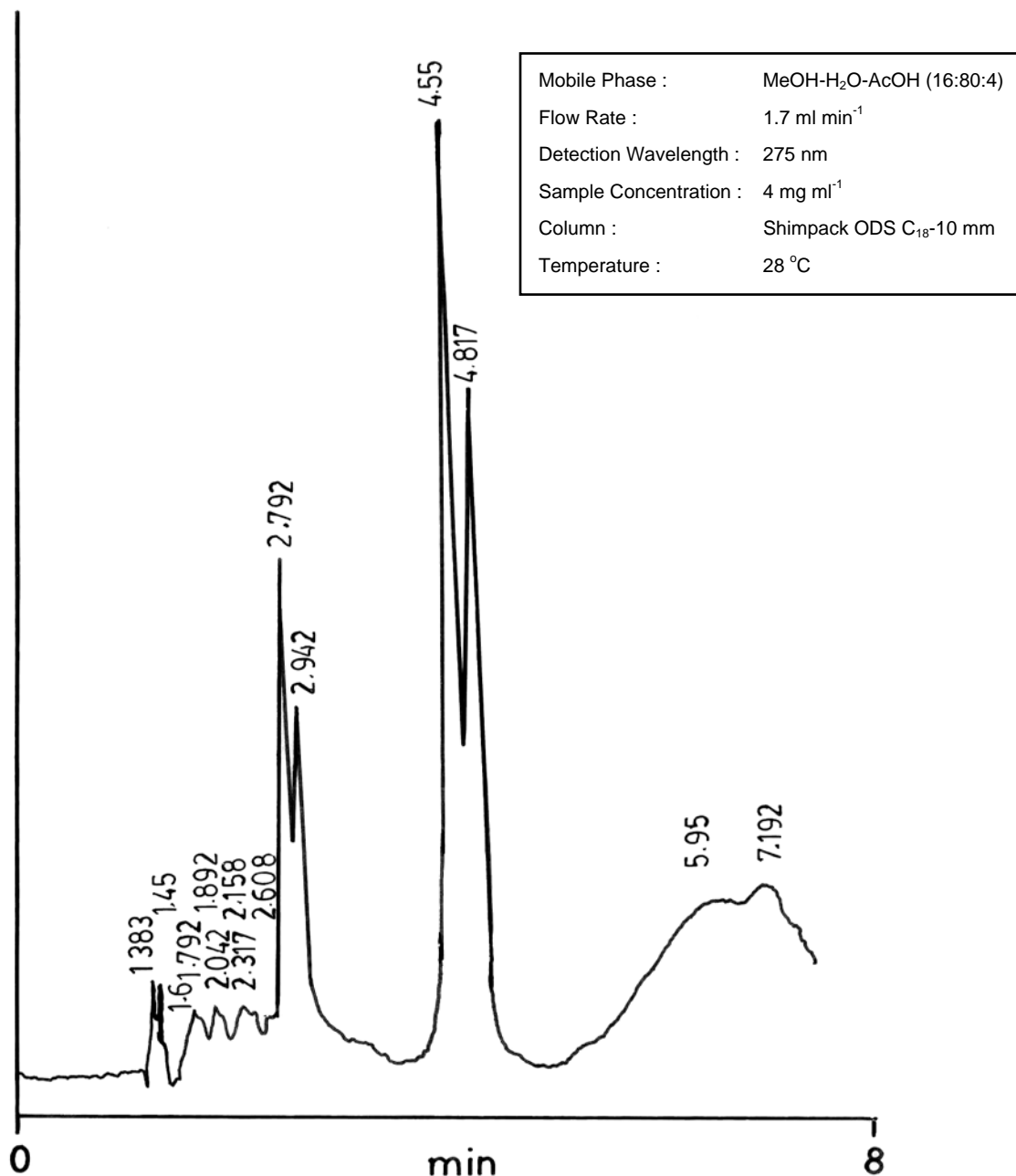


Figure 1. Application of optimized HPLC separation conditions to the flavonoid glycoside extract of *Vitex nagunda*.

Mobile Phase = MeOH-H₂O-CH₃CO₂H (16:80:4)

Flow Rate = 1.7 ml/min

Wavelength = 275 nm

Sample Concentration = 4.0 mg ml⁻¹

Column = Shimpack ODS C₁₈-10 μm (25 cm x 4.6 mm I.D.)

Column Temperature = Ambient (28 °C)

Using optimized separation conditions to the flavonoid glycoside extract of *Vitex nagunda*, produced a chromatogram with 15 partially resolved peaks as shown in Figure 1.

In order to check the effectiveness of the optimized separation conditions, flavonoid

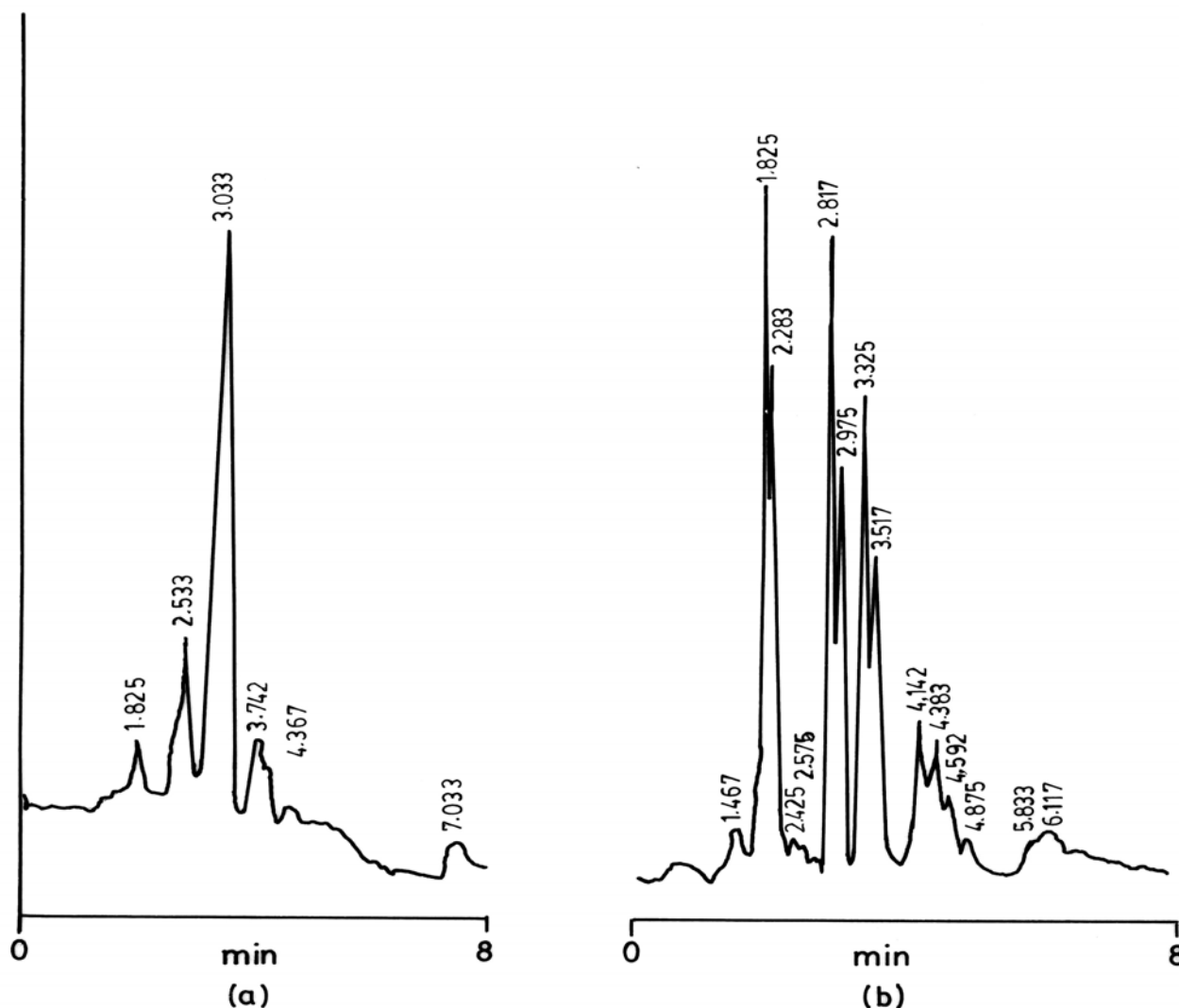


Figure 2. Application of optimized HPLC separation conditions to the flavonoid glycosides extracts of (a) *Rubus ulmifoliosus* (b) *Malotus philipensis*.

glycoside extracts of two other plant species namely *Rubus ulmifoliosus* and *Malotus philipensis* were analyzed under the same separation conditions. In both the cases well-resolved chromatograms were obtained (Figure 2).

3.2. Semi-preparative scale isolation by HPLC

To demonstrate the possibility of isolating an individual component from a mixture of flavonoid glycosides, the peak with retention time (t_R) 3.033 min. (Figure-3a) was selected for isolation by HPLC on semi-preparative scale. For this purpose 50 chromatograms were developed (by injecting 100 μ l of the methanolic solution of flavonoid

glycosides of *Rubus ulmifoliosus*) and each time liquid fraction was collected at its exit. The combined solution was concentrated and then injected onto the column. A single peak at t_R 3.029 min (Figure 3b) demonstrated the semi-preparative scale isolation of one of the components of the flavonoid glycosides mixture. Flavonoid glycoside corresponding to a peak at t_R 3.033 min. was latter identified by well known methods [19] to be 5,7,3',4' tetrahydroxy flavonol 3-O- β -D-rhamnoside (quercetin 3-O- β -D-rhamnoside or quercitrin). Same methodology when applied to other components of a mixture of flavonoid glycosides, helped to obtain purified compounds.

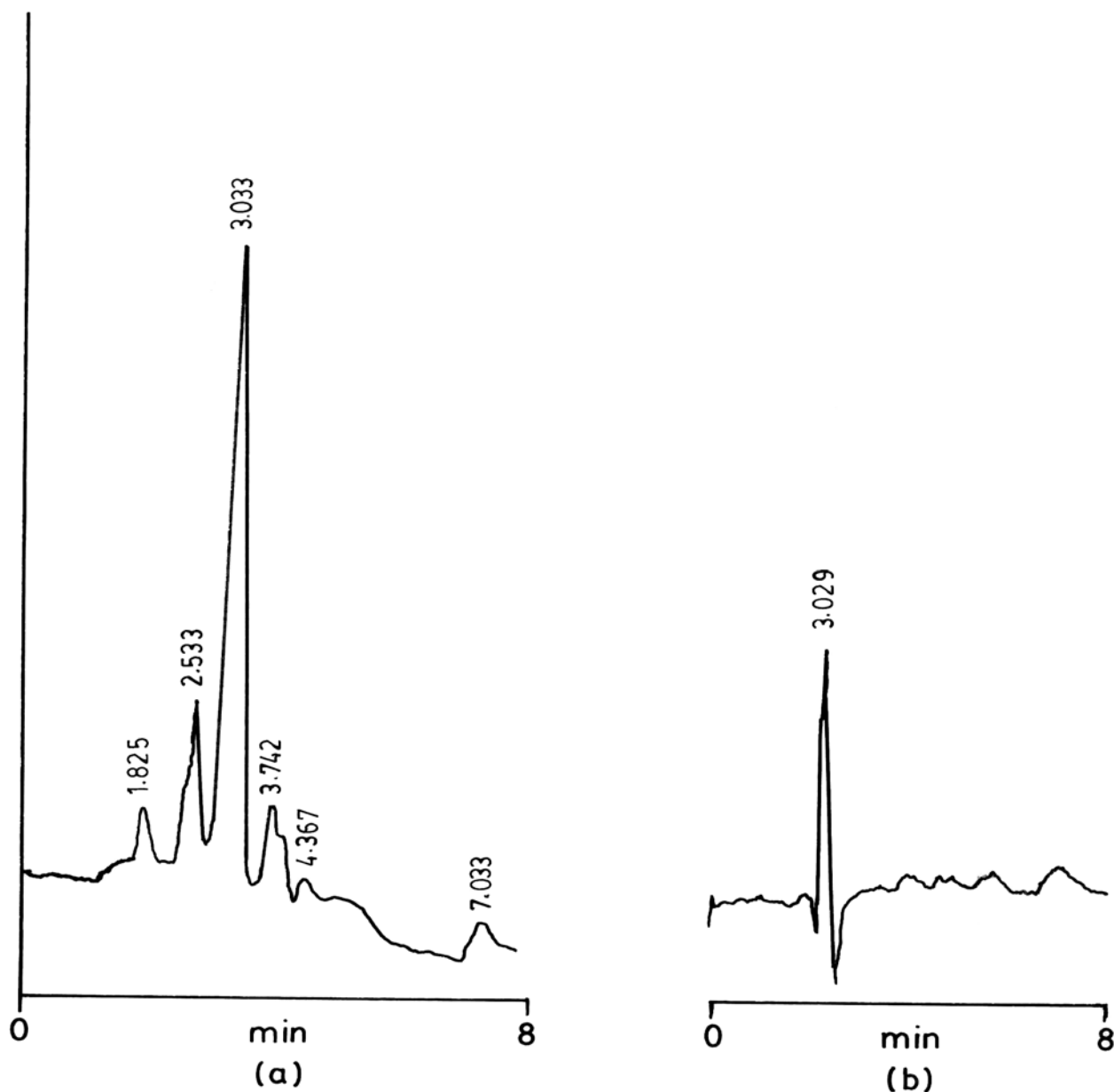
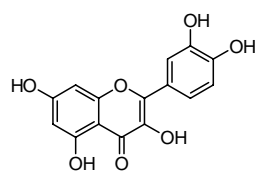


Figure 3. Semi-preparative scale isolation of quercitrin (a) *Rubus ulmifolios* flavonoid glycoside extract (b) purified quercitrin.

3.3 Identification by HPLC

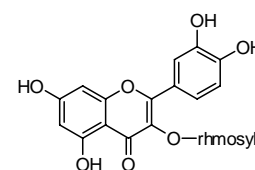
Identification of an individual component of a mixture of flavonoid glycosides can also be achieved by a method called spiking, provided a wide range of flavonoid glycoside markers are available. For the present experiment an authentic marker of quercitrin was mixed with a dilute methanolic solution of flavonoid glycoside extract of *Rubus ulmifolios* and was analyzed. The peak with retention time 3.03 min. was enhanced as shown in Figure 4b, thus identifying and confirming

the presence of quercitrin as one of the flavonoid glycoside in the mixture of *Rubus ulmifolios*.



QUERCETIN

FLAVONOL AGLYCONE



QUERCITRIN

FLAVONOL GLYCOSIDE

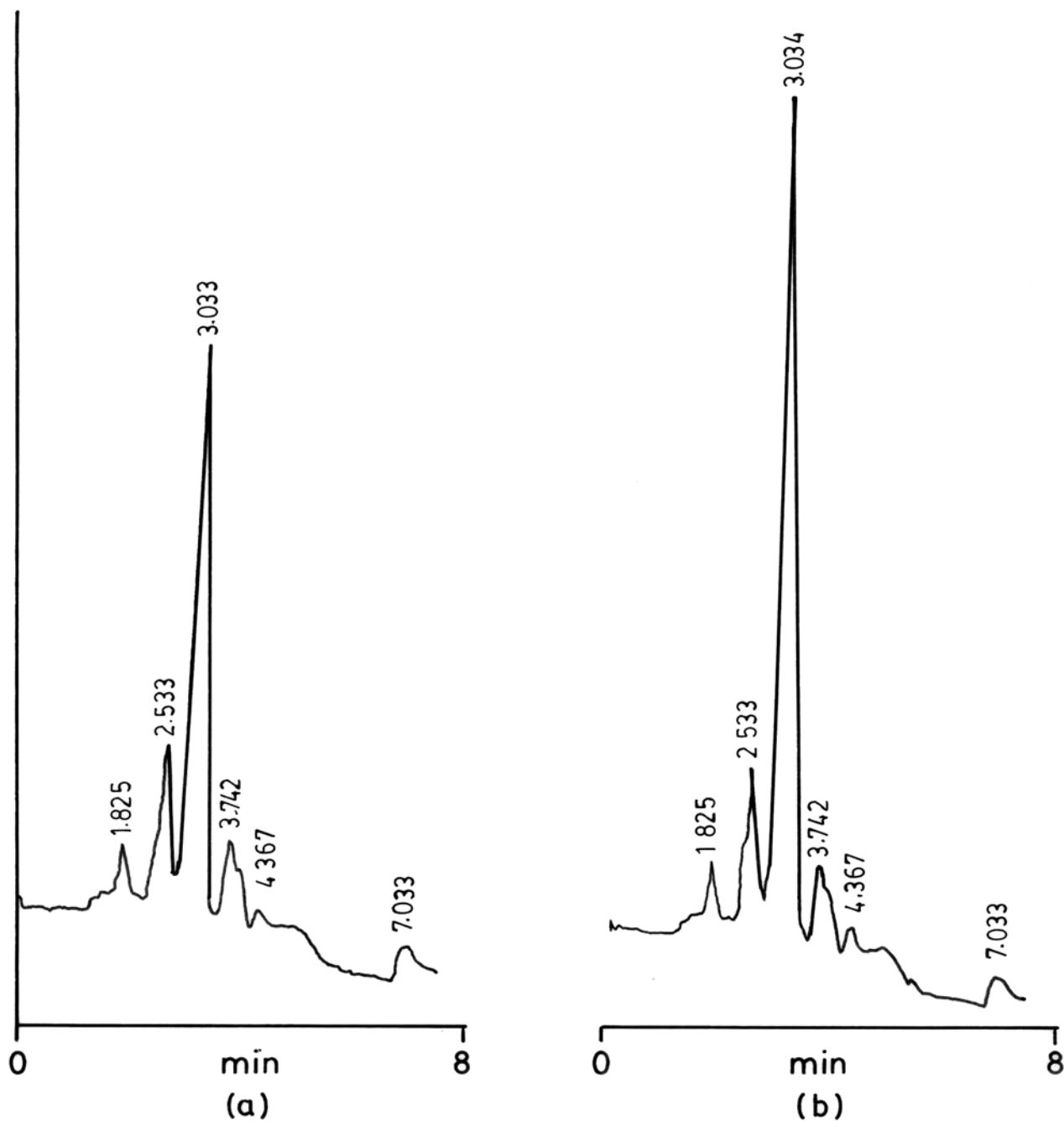


Figure 4. Identification of quercitrin by the method of spiking (a) *Rubus ulmifoliosus* flavonoid glycoside extract (b) Spiked quercitrin.

4. Conclusion

As a result of this investigation it is evident that with the use of only a single technique of HPLC, separation, purification and identification of flavonoid glycosides can be achieved.

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