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Journal of Chromatography A, 1057 (2004) 95–100
JOURNAL OF CHROMATOGRAPHY A
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Purification of the isoflavonoid puerarin by adsorption chromatography on cross-linked 12% agarose

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Received 27 April 2004; received in revised form 8 September 2004; accepted 21 September 2004

Abstract

The isoflavonoid puerarin in extracts of the well-known traditional Chinese drug *Radix puerariae* (root of the plant *Pueraria lobata*) can be separated from other isoflavonoids by adsorption chromatography on the cross-linked 12% agarose gel Superose 12 equilibrated in distilled water. The adsorption is totally quenched by the addition of 50% acetic acid. The separation of the isoflavonoids is tentatively ascribed to interaction with the residues of the cross-linking reagents used in the manufacturing process of Superose 12. Thus, no useful separation can be achieved with non-cross-linked 12% agarose gel media. Symmetric elution profiles at high sample loadings (16 mg on a 24 ml column) suggest linear adsorption isotherms for the isoflavonoids.

Keywords: Isoflavonoid separation; Agarose cross-linker; Puerarin; Acetic acid quenching

1. Introduction

The adsorptive properties of the epichlorohydrin cross-linked dextran gel Sephadex G-25 for certain low molecular weight substances were recognized soon after its introduction in 1959 [1–5]. Aromatic substances in particular showed elution behaviour that differed greatly from what might be expected based on molecular size. Thus, Ruttenberg et al. in 1965 [6] showed that the separation of the antibiotic cyclic decapeptides tyrocidine A, B and C was based on the different tryptophan content of the three peptides (tyrocidine A lacks tryptophan, tyrocidine B contains one and tyrocidine C contains two tryptophan residues) and could be controlled by the acetic acid concentration of the eluent. At 1% acetic acid total adsorption was observed and at 50% acetic acid all three peptides eluted together unseparated. Optimal baseline separation was obtained at an acetic acid concentration of 10%. Eaker and Porath [7] in 1967 published a comprehensive report on adsorption phenomena on the tightly cross-linked dextran gel Sephadex G-10 and Janson [8] published a brief review on adsorption phenomena on Sephadex gels the same year. To the best of our knowledge, no observations of adsorption of low molecular weight aromatic substances have so far been reported for cross-linked agarose gel media.

Extracts of the root of *Pueraria lobata* (Willd.) Ohwi (*Radix puerariae*), a perennial leguminous plant native to eastern Asia, are rich in isoflavones and have been widely used as antipyretic, antidiarrhoetic, diaphoretic, and antiemetic agents in traditional Chinese medicine [9]. Recently, they have also been tried as a possible remedy for alcoholism [10]. Puerarin (daidzein 8-*C*-glucoside), daidzin (daidzein 7-*O*-glucoside), and daidzein are the major isoflavonoids in *P. lobata* extracts [11]. Recently, the separation and purification of puerarin was achieved by adsorption chromatography in the presence of 10% acetic acid on oligomeric β-cyclodextrin covalently attached to Sepharose HP base matrix [12]. The present report shows that separation and purification can also be achieved by isocratic adsorption chromatography on a column packed with the cross-linked

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doi:10.1016/j.chroma.2004.09.068

12% agarose gel, Superose 12, in the presence of distilled water or low concentrations of acetic acid. The adsorption is quenched by the addition of high concentrations of acetic acid.

2. Experimental

2.1. Equipment

HPLC CR-10A (Shimadzu, Japan), Pump (Beijing Xingda Company), UV Detector 8823-A (Beijing New Technology Application Institute), Chromatography Control Unit N2000 (Zhejiang University), Motor Valve MV-7 and chromatography columns (GE Healthcare, Uppsala, Sweden).

2.2. Reagents

Ethanol, acetonitrile, acetic acid, sodium hydroxide, and ammonia solution, were of analytical grade and obtained from Beijing Chemicals Factory, Beijing, China. A crude extract powder of *R. puerariae* called "*Radix puerariae* flavone", and reference puerarin with a purity of >98% were bought from Luye Biology Ltd. Co., Huainan, Anhui Province, China. Superose 12, Superose 12 prep grade, Sephadex G-15, Sephadex G-25, Sephadex LH-20, Phenyl Sepharose HP were obtained from GE Healthcare, Uppsala, Sweden and Superose 12 prep grade base matrix was a gift from GE Healthcare.

2.3. Separation of R. puerariae flavone by adsorption chromatography

The best solvent for the crude *R. puerariae* flavone powder was 20% ethanol. All samples were pre-filtered to remove possible dust before injecting to the chromatographic columns. The injected sample volume varied from 0.5 to 2 ml. The flow velocity used was invariably 1 cm/min and the eluent was monitored using a UV detector at 280 nm. A concentration of 0.5% acetic acid in the elution buffer was chosen to reduce possible growth of microorganisms in the columns. This concentration in the eluent provided elution characteristics for the isoflavonoid components almost identical to those using distilled water as the eluent. The columns were cleaned-in-place using four column volumes of 0.35 M NaOH or four column volumes of 50% acetic acid at a flow velocity of 1 cm/min. The columns were stored in 20% (v/v) ethanol.

2.4. Analysis of puerarin by HPLC

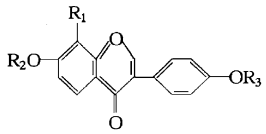
Column: Sephasil C-18, 5 μm reversed phase silica, 250 mm × 4 mm i.d.; mobile phase: (A) 0.1% trifluoroacetic acid (TFA) and (B) 50% acetonitrile in 0.1% TFA; detector: UV 280 nm; flow-rate: 0.5 ml/min; loading volume: 20 μl.

2.5. Synthesis of cross-linked and substituted Superose 12 prep grade

Superose 12 prep grade base matrix was cross-liked and substituted as described in [13,14] using 1,3-bis-glycidoxybutane and epichlorohydrin as reactants, respectively.

3. Results and discussion

The isoflavonoid content of *R. puerariae* flavone is 10–14% (w/v), mainly composed of daidzein, daidzin, puerarin, and daidzein-4′,7-diglucoside, whose structures are shown in Fig. 1. The content of puerarin is approximately 4%. The effect of the acetic acid concentration in the eluent on the degree of adsorption and extent of separation of the isoflavonoid components of the *Radix pueraria* crude extract is shown in Fig. 2. The largest peak is puerarin (daidzein 8-*C*-glucoside). The peak eluting at around 240 min in (A) is daidzin (daidzein 7-*O*-glucoside). The non-glucosylated daidzein is probably much more strongly retarded (elution data not available due to lack of reference standard). The quenching effect of increasing concentrations of acetic acid on the adsorption is obvious. At 50% acetic acid, practically no adsorption occurs. This confirms previous studies on the adsorption of low molecular weight aromatic substances to tightly cross-linked polysaccharide gel media [6–8]. Our studies show that neither Superose 12 prep grade, nor Sepharose HP base matrix possess the ability to separate puerarin from the *R. puerariae* flavone crude extract as efficiently as does Superose 12. Tentatively, this phenomenon is explained by the differences in cross-linking reagents and synthesis procedures, respectively, for these three media. The reagents and procedures for the synthesis of Superose 12 have been published [13,14] but details the synthesis of Superose 12 prep grade and Sepharose HP are still proprietory knowledge. In an attempt to mimic the synthesis procedure for Superose 12 using Superose 12 prep grade base matrix as the



Compound	Group		
	R_1	R_2	R_3
Daidzein	H	H	H
Daidzin	H	Glucose	H
Puerarin	Glucose	H	H
Daidzein-4',7-diglucoside	H	Glucose	Glucose

Fig. 1. The molecular structures of *R. puerariae* flavonoides.

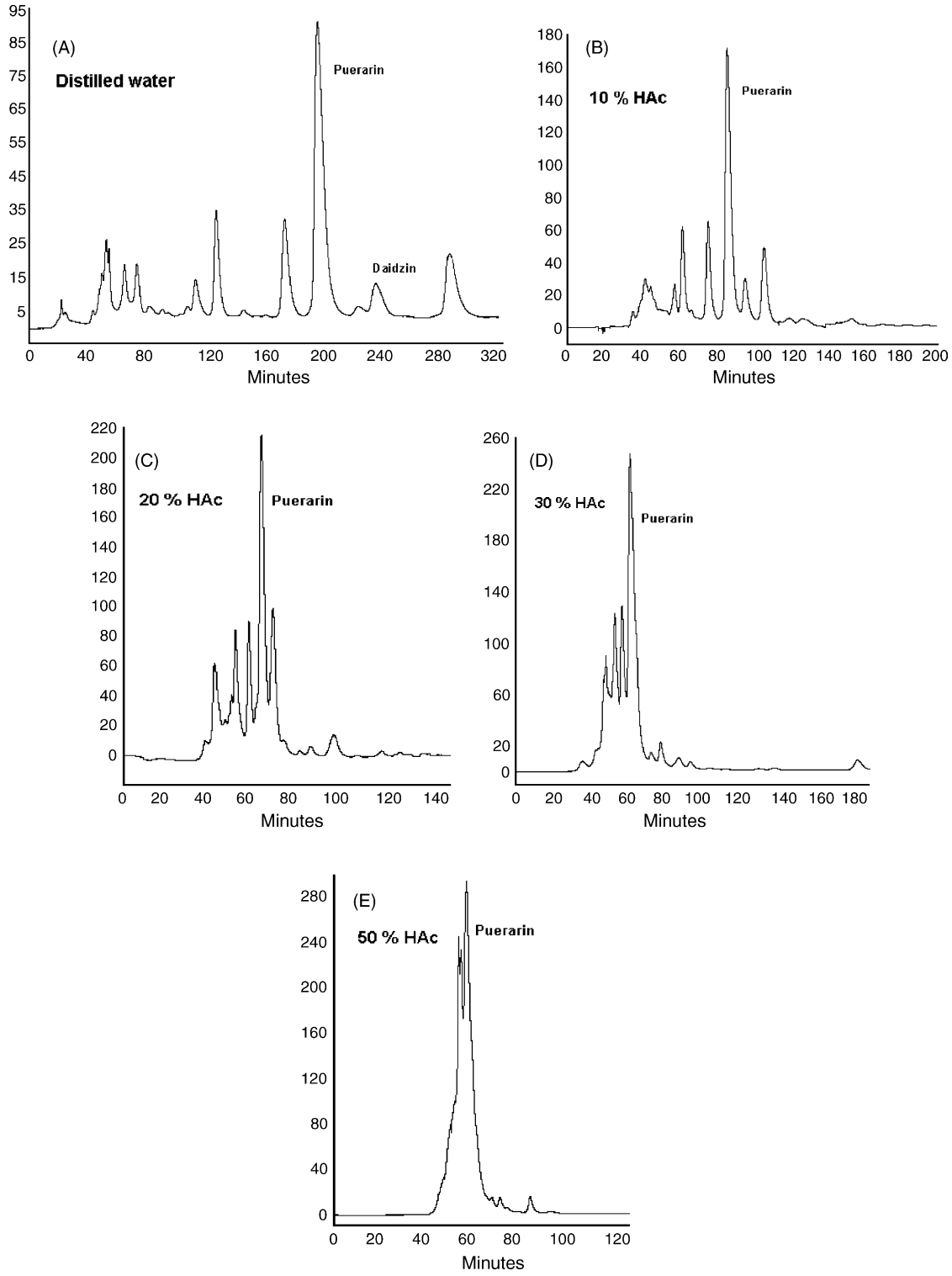


Fig. 2. Result of isocratic adsorption chromatography on Superose 12 HR 10/30 in the presence of different acetic acid concentrations. Sample: crude 20% ethanol extract of *R. puerariae* flavone, 1 mg in 0.5 ml. Flow rate: 1 ml/min. The eluent was monitored using a UV detector at 280 nm.

starting material, i.e. using a combination of long chain bisepoxides and epichlorohydrin as reagents according to the published procedure, a gel was obtained that gave an isoflavonoid separation pattern similar, but not identical to that obtained using Superose 12 (Fig. 3).

When epichlorohydrin reacts with the agarose gel matrix under alkaline conditions, 2,3-epoxypropyl ethers are first formed by reaction with the hydroxyl groups in the galactosyl residues. The immobilized epoxides thus formed, then react with water or with an adjacent hydroxyl group either belong-

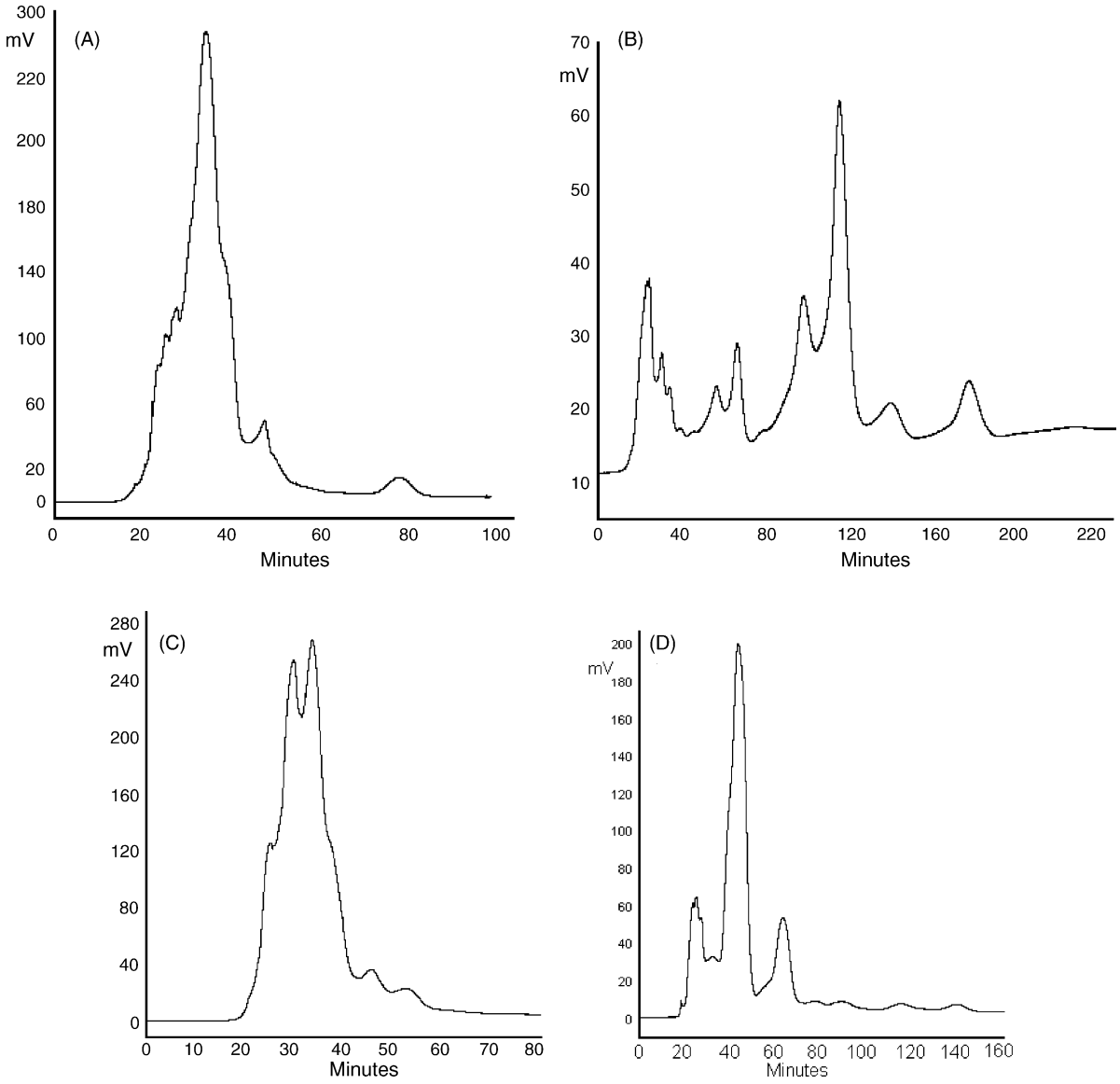


Fig. 3. Result of isocratic adsorption chromatography on different cross-linked and non-cross-linked agarose gel columns (10 mm i.d. and 30 cm bed height) in the presence of 0.5% acetic acid. Sample: *R. puerariae* flavone crude 20% ethanol extract, 1 mg in 0.5 ml. Flow rate: 1 ml/min. The eluent was monitored using a UV detector at 280 nm. (A) Superose 12 prep grade base matrix. (B) Superose 12 prep grade base matrix cross-linked with 1,3-bis-glycidoxybutane and epichlorohydrin and further substituted with epichlorohydrin. (C) Sepharose HP base matrix. (D) Phenyl Sepharose HP.

ing to the same galactosyl residue or to a substituent in the same residue. Alternatively, the hydroxyl group could belong to a galactosyl residue in another galactan polymer chain or to a substituent in that residue. This last reaction would lead to the formation of an interchain cross-link. Subsequently added epichlorohydrin molecules will react with hydroxyl groups formed by water hydrolysis of already coupled epoxides creating polymeric side chains of different lengths. The cross-linking and substitution procedures for agarose gels using epichlorohydrin would thus, lead to a very large number of different structural elements, both cyclic and non-cyclic, and introduce a variety of polar groups, such as ether bonds, as well as hemiacetals and primary and secondary hydroxyl groups. Tentatively, these groups are considered to occur in high concentrations at regular repeating distances on the surface of the gel, allowing cumulative cooperative interaction with the aromatic ring system of the isoflavonoids, and thus, providing a sufficiently high total interaction energy to give rise to chromatographic retardation. This type of short-range aromatic adsorption requires intimate molecular contact between exposed benzene rings of the solute and the cross-link residues of the gel. The different extent and type of glucosidic conjugation of the isoflavonoid ring system (Fig. 1) is thereby believed to affect the degree of adsorption, thus giving rise to differential retardation of the various *R. puerariae* isoflavonoid components on the gel matrix.

As delocalized electrons are also present in acetate ions, a tentative explanation for the quenching effect of high con-

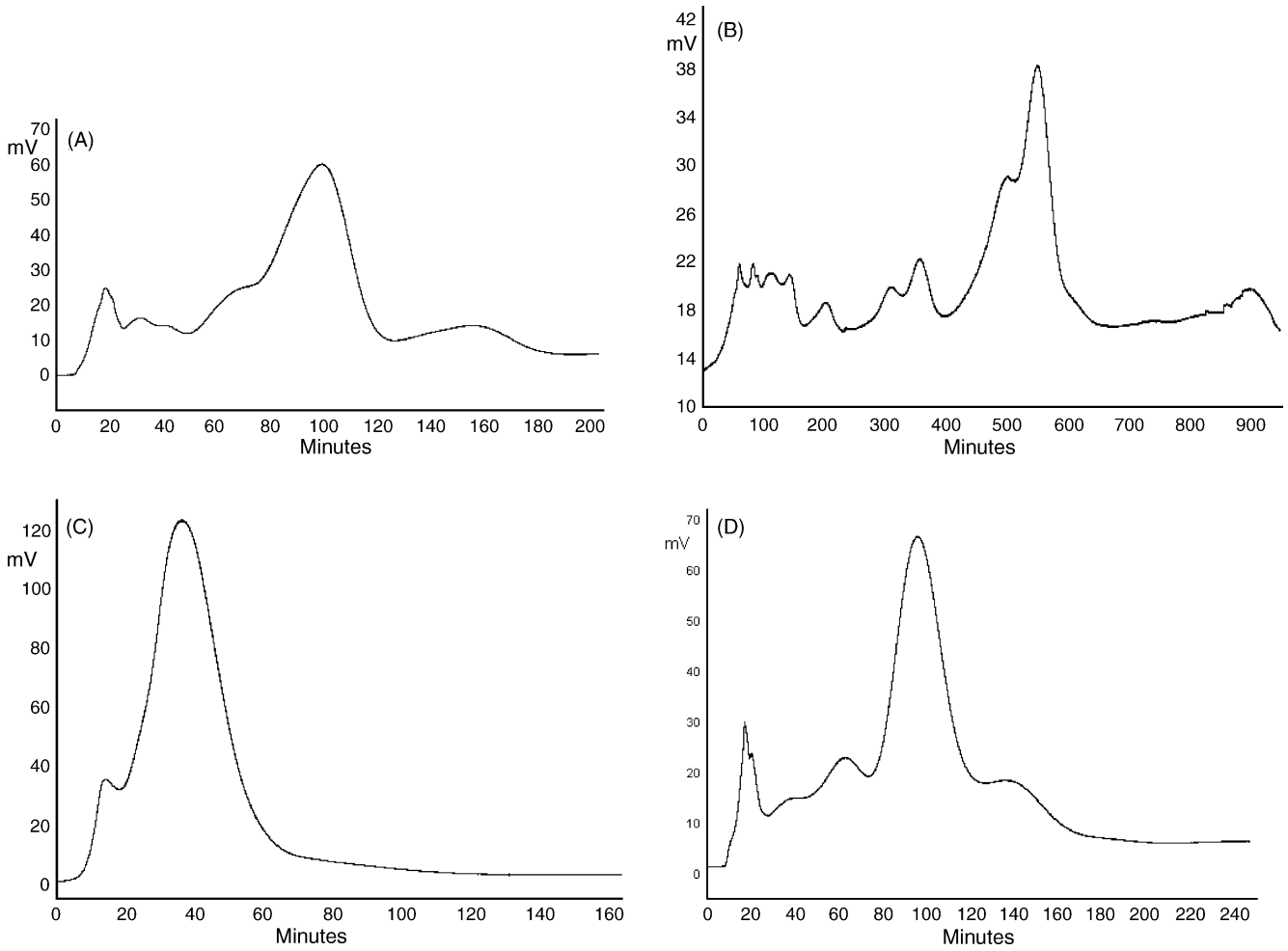


Fig. 4. Result of isocratic adsorption chromatography on different tightly cross-linked dextran gel columns (10 mm i.d. and 30 cm bed height) in the presence of 0.5% acetic acid. Sample: *R. puerariae* flavone crude 20% ethanol extract, 1 mg in 0.5 ml. Flow rate: 1 ml/min. The eluent was monitored using a UV detector at 280 nm. (A) Sephadex LH-20. (B) Sephadex LH-20 dry sieving fraction (particles passing through a 45 μm sieve). (C) Sephadex G-25. (D) Sephadex G-15.

centrations of acetic acid would be that these compete with the aromatic ring system for binding to the cross-linking substituents present on the outer and inner surfaces of the structural network of Superose 12.

In order to demonstrate the unique adsorptive character of Superose 12 for the separation of the *R. puerariae* isoflavonoids a series of comparative separations were carried out with crude 20% ethanol extracts using a variety of cross-linked and non-cross-linked polysaccharide gel media equilibrated and eluted in 0.5% acetic acid. The results of these experiments are shown in Figs. 3 and 4, respectively. Fig. 3A shows the separation obtained using a non-cross-linked agarose gel, Superose 12 prep grade base matrix. No useful separation was obtained. However, as shown in Fig. 3B, after cross-linking with 1,3-bis-glycidoxybutane and epichlorohydrin and further substitution with epichlorohydrin, in order to mimic the synthesis procedure for Superose 12 [13,14], a similar separation to that with Superose 12 (Fig. 2A) was obtained. This indicates that the adsorption characteristics can be ascribed to the unique cross-linking and substitution procedure used in the synthesis of the latter gel. In contrast, Fig. 2C and D, show the separation patterns obtained with Sepharose HP base matrix, cross-linked according to a proprietary procedure and Phenyl Sepharose HP, respectively. These data suggest that neither of these gel media possess the cross-links or substituents that give the desired adsorption characteristics required for efficient separation of the *R. puerariae* isoflavonoid mixture.

Fig. 4 shows the results from experiments with a variety of well-known highly cross-liked and substituted Sephadex media that in previous publications [2–6] have been demonstrated to have separation power for aromatic low molecular weight substances. However, no useful separation was obtained for the *R. puerariae* mixture of isoflavonoids. Although unexpected, one must bear in mind that the average wet particle sizes of these media are considerably larger than that of Superose 12, and their resolving power is thereby considerably lower due to lower efficiency.

The aromatic adsorption property is especially advantageous and useful because the adsorption isotherms are lin-

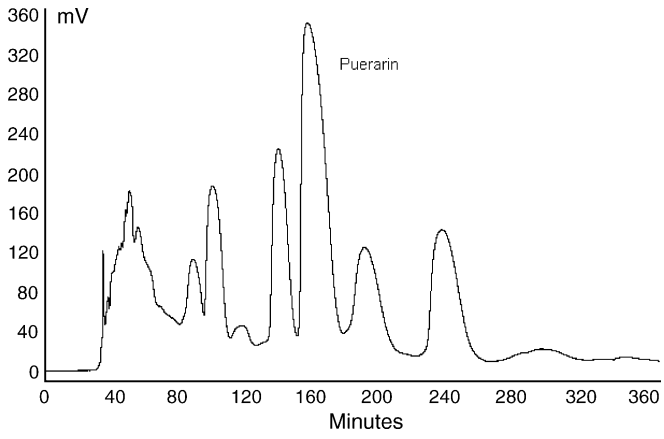


Fig. 5. Result of isocratic adsorption chromatography at high sample loading on Superose 12 HR (10/30) in the presence of 0.5% acetic acid. Sample: *R. puerariae* flavone crude 20% ethanol extract, 16 mg in 2 ml. Flow rate: 1 ml/min. The eluent was monitored using a UV detector at 280 nm.

ear over a wide range of concentrations. Symmetrical peaks are consequently obtained as shown in Fig. 5. This fact was utilized for the one-step preparative purification of puerarin directly from a crude extract. In order to demonstrate the separation power of the 23 ml (10 mm i.d. and 30 cm bed height) prepacked column of Superose 12, a 16 mg sample dissolved in 2 ml 20% ethanol was applied to the column equilibrated and eluted with 0.5% acetic acid. By cutting out the puerarin (main) peak in Fig. 5, a 97% pure puerarin could be recovered with a yield of 94%. HPLC analysis by RPC showed only one sharp peak (data not shown).

4. Conclusion

Isocratic adsorption chromatography on Superose 12, a 10 μm average particle diameter, tightly cross-linked 12% agarose gel, can be used for the one-step preparative separation and purification of puerarin, a Chinese traditional medicine. The conditions for separation were optimized for a crude extract dissolved in 20% (v/v) ethanol. The composition of the mobile phase is an important factor affecting the separation of the isoflavones on this agarose gel. For the prepacked column Superose 12 HR 10/30, distilled water and 0.5% acetic acid gave the best separation of puerarin from neighbouring peaks at a flow rate of 1 ml/min. At a crude sample load of 16 mg in 2 ml (0.7 mg/ml adsorbent) a 97% pure puerarin could be recovered with a yield of 94%.

Acknowledgements

The authors are grateful for the support given by the National Science Foundation of China (Contract Nos. 29976004 and 20136004), the Fok Yingtung Education Foundation (Contract No. 71067), the Teaching and Research Award Program for outstanding Young Teacher in Higher Education Institutes in China and by GE Healthcare, R&D Department, Uppsala, Sweden.

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