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Preparation of polymer based sorbents for solid phase extraction of polyphenolic compounds

Invited Paper

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Abstract: This article discusses the preparation of different polymeric sorbents for solid phase extraction. Various monomers like acrylamide, methacrylic acid and 4-vinylpyridine (VP), cross-linkers such as divinylbenzene (DVB) and ethyleneglycoldimethacrylate (EDMA), porogens like tetrahydrofuran (THF) and dimethylsulphoxide (DMSO) / acetonitrile (ACN) with different ratios were investigated in order to optimize recoveries. Resulting polymers were characterized through scanning electron microscope (SEM) and compared with Oasis HLB (Waters, MA, USA) and Strata-X (Phenomenex, Torrance, USA) on the basis of extraction performance, recovery efficiency and loading capacity. Sample applied was a mixture of flavonoid standards (rutin, myricetin, luteolin, quercetin, apigenin and kaempferol). HPLC hyphenated with PDA was used for the analysis of samples. Results showed that among the prepared SPE materials, 4-VP-co-EDMA produced best results. Comparison of the produced polymers with of Oasis HLB and Strata-X resulted in comparable efficiencies; especially the polymer 4-VP-co-EDMA gave almost similar results for all analytes to those of commercially available SPE materials. A general trend of decrease in retention efficiency with increase in polarity has been observed in both synthesized and already available SPE materials. The newly synthesized polymeric materials can be employed as SPE sorbents for efficient extraction of polyphenolic compounds especially for flavonoid aglycons.

Keywords: *Solid phase extraction • Flavonoid standard mixture • Polymer sorbents • Oasis HLB • Strata-X*

1. Introduction

Solid phase extraction (SPE) is one of the most widely applied purification and enrichment procedures for bioanalytical samples, because of its high pre-concentration level, simplicity, versatility and speed [1,2]. Different sorbents are available, each of which separates different analytes by different chemical mechanism. Most of the stationary phases are silica based, consisting of siloxane chemically modified with molecules having functional groups suitable for the desired application *e.g.* hydrophobic carbon chain for RP-SPE [3]. Sorbents can be classified as silica based sorbents, oxides of metals (*e.g.* aluminium, magnesium, zirconium, titanium, and thorium), graphitized carbon or carbon based and polymer based sorbents [4]. Silica based sorbents and other metal oxides along with advantages like versatility to be derivatized, large surface area *etc.*, also suffer from some disadvantages such as: i) high active sites (ionized silanol groups) found on their surfaces and ii) silica dissolves at high pH and can be used within the pH range of 2 to 8 [4,5]. Another limitation in using the reverse-phase silica in SPE is that it must be conditioned with a wetting solvent and remain wetted before sample application [6]. These disadvantages limit the use of these materials. Polymer based sorbents can be used over the whole pH range. Furthermore, problem of high active sites can be avoided by the use of organic polymer sorbents. Additionally, it has been claimed that these materials are less sensitive to drying out after conditioning. Polymeric sorbents can be made by polymerizing a monomer such as styrene, acrylamide, methacrylic acid or methyl methacrylate; by cross linking with another olefinic compound called the cross-linker

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like divinylbenzene or ethyleneglycoldimethacrylate [4]. Structures of some monomers and cross-linkers are shown in Fig. 1. Their high degree of hydrophobicity gives them a large capacity, which can be moderated by proper choice of reacting educts.

Novel functionalized polymeric sorbents like Oasis (Waters, MA, USA), Strata (Phenomenex, Torrance, USA), Bond Elute PPL (Varian, CA, USA), LiChrolut EN (Merck, Darmstadt, Germany) *etc.* have been introduced. Among these polymeric material co-polymer of polystyrene divinylbenzene has wide application in SPE [2].

Chemically Oasis HLB is a product of polymerization of lipophilic DVB and hydrophilic N-vinylpyrrolidone, making a hydrophilic – lipophilic balance. This material can be used for SPE of polar to apolar organic compounds [6]. Strata-X is a surface modified polymer of styrene and divinylbenzene. During SPE it retains analytes by different mechanism like hydrophilic, hydrophobic and π – π interaction; making it nearly universal SPE material for acidic, basic and neutral analytes [7-9]. These polymeric sorbents are used instead of RP silica, because of their high capacity, requiring less amount of sorbent. Moreover macroporous and wettable materials show a straightforward adsorption mechanism and do not suffer from such problems like metallic impurities, pH stability or active silanol groups, which occur when using RP silica as SPE sorbent [3,6]. Fig. 2 shows chemical structures of Oasis HLB and Strata-X materials.

The purpose of this study was to prepare polymeric SPE materials, characterize these synthesized materials through scanning electron microscope (SEM), evaluate their efficiencies and compare them with commercially available polymeric SPE materials (Oasis HLB and Strata-X) using flavonoid standards.

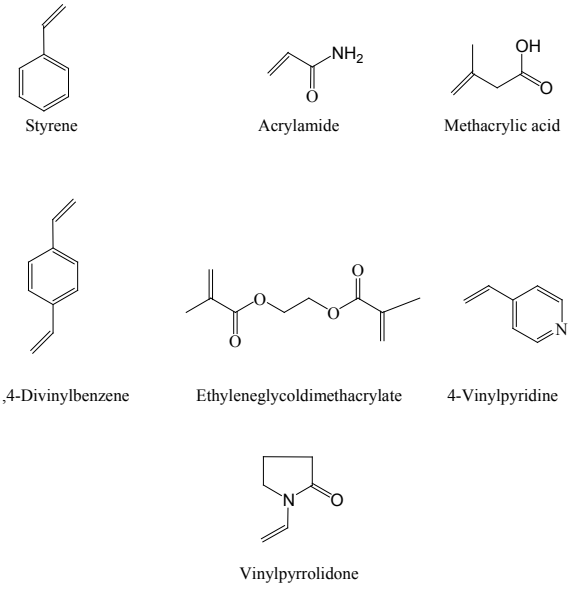

Figure 1. Molecular structures of some monomers and cross-linkers

2. Experimental Procedure

2.1. Chemicals and reagents

Acrylamide (≥98%), methacrylic acid (99%), 4-vinylpyridine (95%), ethyleneglycoldimethacrylate (98%), azobis(isobutyronitrile) (≥98%), dimethysulphoxide, ortho-phosphoric acid (85%) were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol, THF, acetonitrile, acetone and divinylbenzene (~65%) were purchased from Merck KGaA (Darmstadt, Germany). Standards rutin hydrate (min. 95%), quercetin dihydrate (min. 98%), apigenin (≥95%), kaempferol (≥ 96%), myricetin (≥95%) were from Sigma-Aldrich (Steinheim, Germany) and luteolin

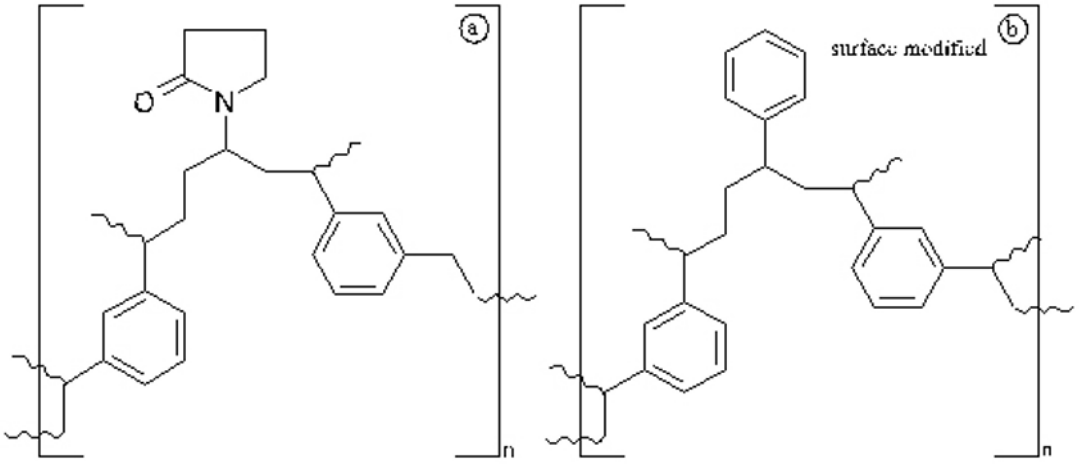

Figure 2. Chemical structures of commercially available SPE materials: (a) Oasis HLB (divinylbenzene-co-N-vinylpyrrolidone); (b) Strata-X (surface modified co-polymer of styrene-divinylbenzene)

(HPLC grade) from Extra-synthese (Genay, France). Oasis HLB (30 mg) was from Waters Corporation (MA, USA) and Strata-X (30 mg) from Phenomenex Inc. (Torrance, USA). Nitrogen gas was purchased from Messer GmbH (Gumpoldskirchen, Austria) and water purified by a Nano Pure-unit (Barnstead, Boston, MA, USA) was used. Methacrylic acid, 4-VP, DVB and EDMA were made inhibitor free by distilling them under high vacuum. AIBN used was re-crystallized in methanol and dried. THF used was dried by distillation over sodium.

2.2. Polymer 1: synthesis of acrylamide-co-divinylbenzene polymer (AA-co-DVB)

This polymer was synthesized according to the method of J. Xie *et al.* with some changes [10,11]. Monomer acrylamide (4 mmol) and the porogen tetrahydrofuran (9 mL) were placed in a glass vial. Cross-linker divinylbenzene (50 mmol) and reaction initiator azobis(isobutyronitrile) (22 mg) were added. After sealing the glass *via*l, the mixture was shaken well to homogenize. Then the mixture was sparged with N_2 for 5 minutes. The polymerisation was carried out in a water bath at 60°C for 24 h. The produced polymer was then ground and sieved through a 0.250 mm sieve. Fine particles were removed by repeated flotation in acetone. Finally the material was dried under vacuum in desiccator.

2.3. Polymer 2A and 2B: synthesis of acrylamide-co-ethyleneglycoldimethacrylate (AA-co-EDMA)

Two types of polymer were prepared using the monomer - cross-linker ratios:

A. 4 + 40

B. 6 + 30

Acrylamide (A. 4 mmol, B. 6 mmol) and tetrahydrofuran (9 mL) were placed in a glass vial. Ethyleneglycoldimethacrylate (A. 40 mmol, B. 30 mmol) and azobis(isobutyronitrile) (22 mg) were added. The rest of the procedure was the same as described for polymer **1** [10].

2.4. Polymer 3: synthesis of methacyrlic acid-co-ethyleneglycoldimethacrylate (MAA-co-EDMA)

Methacrylic acid (8 mmol) and 12.5 mL of mixture of dimethylsulphoxide and acetonitrile (2 + 3) were placed in a glass vial. Ethyleneglycoldimethacrylate (40 mmol) and azobis(isobutyronitrile) (50 mg) were added. The rest of the procedure was the same as described for polymer **1** [12,13].

2.5. Polymer 4: synthesis of 4-vinylpyridine-co-ethyleneglycoldimethacrylate (VP-co-EDMA)

4-vinylpyridine (8 mmol) was used as functional monomer, while the rest of the synthetic procedure was similar to that of polymer **3** [12-14].

2.6. Comparison of prepared polymeric materials with commercially available polymeric based SPE materials (Oasis HLB and Strata-X)

2.6.1 Standards preparation

A mixture of flavonoid standards consisting of rutin, myricetin, luteolin, quercetin, apigenin and kaempferol was prepared in 50% MeOH at a concentration of 0.07 mg mL^{-1}, 0.07 mg mL^{-1}, 0.07 mg mL^{-1}, 0.10 mg mL^{-1}, 0.07 mg mL^{-1}, and 0.14 mg mL^{-1} respectively. Chlorogenic acid solution was prepared having a concentration 1 mg mL^{-1}. Standards mixture was stored at 2 – 8°C.

2.6.2 Solid phase extraction

About 30 mg of the polymeric material was loaded into an empty 1 mL cartridge with a frit at the bottom. Activation of the materials was done with 100% MeOH (1 mL×3) and then equilibrated with water-MeOH (80 + 20) mixture (1 mL×3). One mL of flavonoid standard mixture was loaded on the SPE material drop-wise. After the flow through of the loaded sample, the material was washed with 20% MeOH (1 mL×2) collecting the washing in each step. Retained analytes were eluted with 100% MeOH (1 mL×3) and eluted samples were collected. The same procedure was employed for Oasis HLB (30 mg) and Strata-X (30 mg). Equal volume of internal standard (Chlorogenic acid solution; 1 mg mL^{-1}) of known concentration was added to all the collected samples for the volume correction. The same volume of internal standard was also added to 1 mL of the flavonoid standard mixture. 20 µL of all the samples were injected into the chromatographic column and the analyses were done in triplicate.

2.6.3 Instrumentation

The Shimadzu HPLC system consisted of an online degasser unit (DGU-14A), two solvent delivery pumps (LC-10Advp), an autoinjector (SIL-10ADvp), a column oven (CTO-10Avp) and a system controller (SCL-10Avp). Detection of the analytes was performed using a photo-diode array detector PDA (SPD-M10 Avp). The system control and data analysis was performed using the manufacturer's software packages (LCMS-Solution, version 3 and LCMS-Post run, version 3-H2).

Table 1. Loading capacity ($\mu g\ mg^{-1}$) of synthesized SPE materials

Analyte	Oasis HLB	Strata-X	VEP	MEP	AEP (4+40)	AEP (6+30)	ADP
Rutin	1.17	1.14	0.55	0.30	0.31	0.41	0.31
Myricetin	1.18	1.18	1.15	0.92	1.02	1.14	0.91
Luteolin	1.20	1.20	1.16	0.98	1.04	1.12	1.13
Quercetin	1.68	1.68	1.64	1.45	1.52	1.62	1.55
Apigenin	1.17	1.17	1.14	1.03	1.06	1.12	1.14
Kaempferol	2.35	2.35	2.29	2.16	2.22	2.28	2.30

Table 2. Percentage recoveries of synthesized SPE materials

Analyte	Oasis HLB	Strata-X	VEP	MEP	AEP (4+40)	AEP (6+30)	ADP
Rutin	101.68	95.61	48.92	23.95	30.44	35.94	27.36
Myricetin	99.71	92.53	93.03	73.99	81.42	87.46	67.04
Luteolin	103.01	101.61	96.24	83.08	88.93	93.32	94.61
Quercetin	104.18	102.02	98.42	87.03	91.59	94.73	92.25
Apigenin	103.84	102.99	98.81	89.97	93.10	96.02	98.86
Kaempferol	102.30	102.38	98.34	93.29	95.50	95.89	98.23

The chromatographic separation was performed on a reversed stationary phase column (Hypersil BDS 125×4 mm, 3 µm particle size and 130 Å pore size; Alltech). Gradient elution was carried out using mobile phase A: 900 mL water + 100 mL methanol + 10 mL phosphoric acid and B: 600 mL water + 300 mL tetrahydrofuran + 100 mL methanol + 10 mL phosphoric acid at 50°C and 0.5 mL min^{-1} flow rate [15]. Zero time condition was 10% B. A linear gradient to 60% B was run in 10 minutes. Afterwards elution was made at isocratic condition at 60% B for 35 minutes. After that a linear gradient to 100% B up to 53 minutes was applied. The column was washed at 100% B for 3 minutes. Zero time conditions were gained within 4 minutes. PDA detector was operated in the wave length range of 200 to 600 nm.

2.7 Scanning electron microscopy

Morphological characterization of the five synthesized polymeric SPE materials was performed using JSM-5310LV scanning electron microscope from JEOL (Tokyo, Japan).

3. Results and Discussion

3.1 Preparation of polymer

Polymer based stationary phases for solid phase extraction, were prepared through bulk polymerization using different monomers, cross-linkers, porogens with different ratios. Fig. 3 shows micrographs recorded from SEM of produced polymeric SPE materials. Morphological characterization of polymeric SPE materials through SEM with magnification of 10 µm shows fundamental differences between the materials. Acrylamide-co-DVB (Fig. 3E) shows a very smooth surface; 4-VP-co-EDMA (Fig. 3A) where rough surface is obtained, delivering more possibilities for interaction with analytes.

The flavonoid standard mixture consisting of rutin, myricetin, luteolin, quercetin, apigenin and kaempferol of specific concentrations was used as the sample in order to evaluate the efficiency of prepared SPE materials. For comparative reasons, about 30 mg of stationary phase was employed for SPE. Quantification of analytes was done using HPLC. Fig. 4 shows chromatograms (i – vii) obtained from flavonoid standards mixture, flow through, wash 1 and 2, elution 1, 2 and 3 respectively.

Efficiency of the SPE materials was evaluated based on the retention capabilities of analytes. Tables 1 and 2 show the loading capacities and percentage recoveries of analytes respectively. Results show that the polymeric material of VEP which is a co-polymer of 4-vinylpyridine-EDMA is more efficient than others SPE material synthesized having recovery efficiencies 93.03%, 96.24%, 98.42%, 98.81% and 98.34% for myricetin, luteolin, quercetin, apigenin and kaempferol respectively. Rutin was recovered only up to 48.92%.

Table 3. Comparison of recovery efficiency of VEP and MEP using 30 mg and 110 mg of SPE material

Analyte	VEP (30 mg)	VEP (110 mg)	MEP (30 mg)	MEP (110 mg)
Rutin	48.92	100.47	23.95	102.17
Myricetin	93.03	94.67	73.99	97.65
Luteolin	96.24	101.23	83.08	101.39
Quercetin	98.42	100.25	87.03	98.11
Apigenin	98.81	101.90	89.97	100.46
Kaempferol	98.34	100.19	93.29	97.32

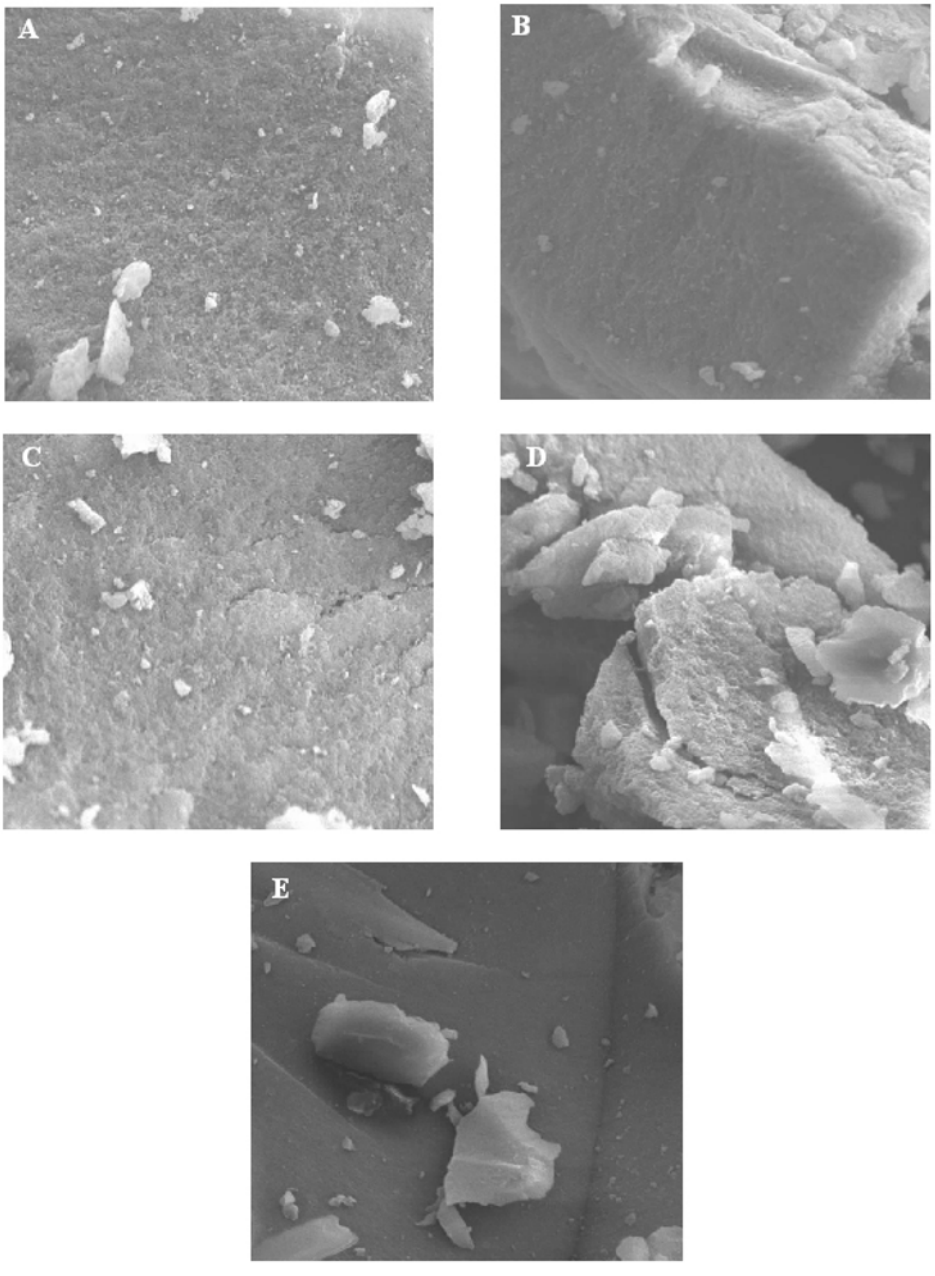

Figure 3. Scanning Electron Microscope Micrographs of polymeric SPE materials: (A) 4-VP-co-EDMA, (B) methacyrlic acid-co- EDMA, (C) acrylamide-co-EDMA (6 + 30), (D) acrylamide-co-EDMA (4 + 40), (E) acrylamide-co-DVB. Magnification of each micrograph is 10 μm.

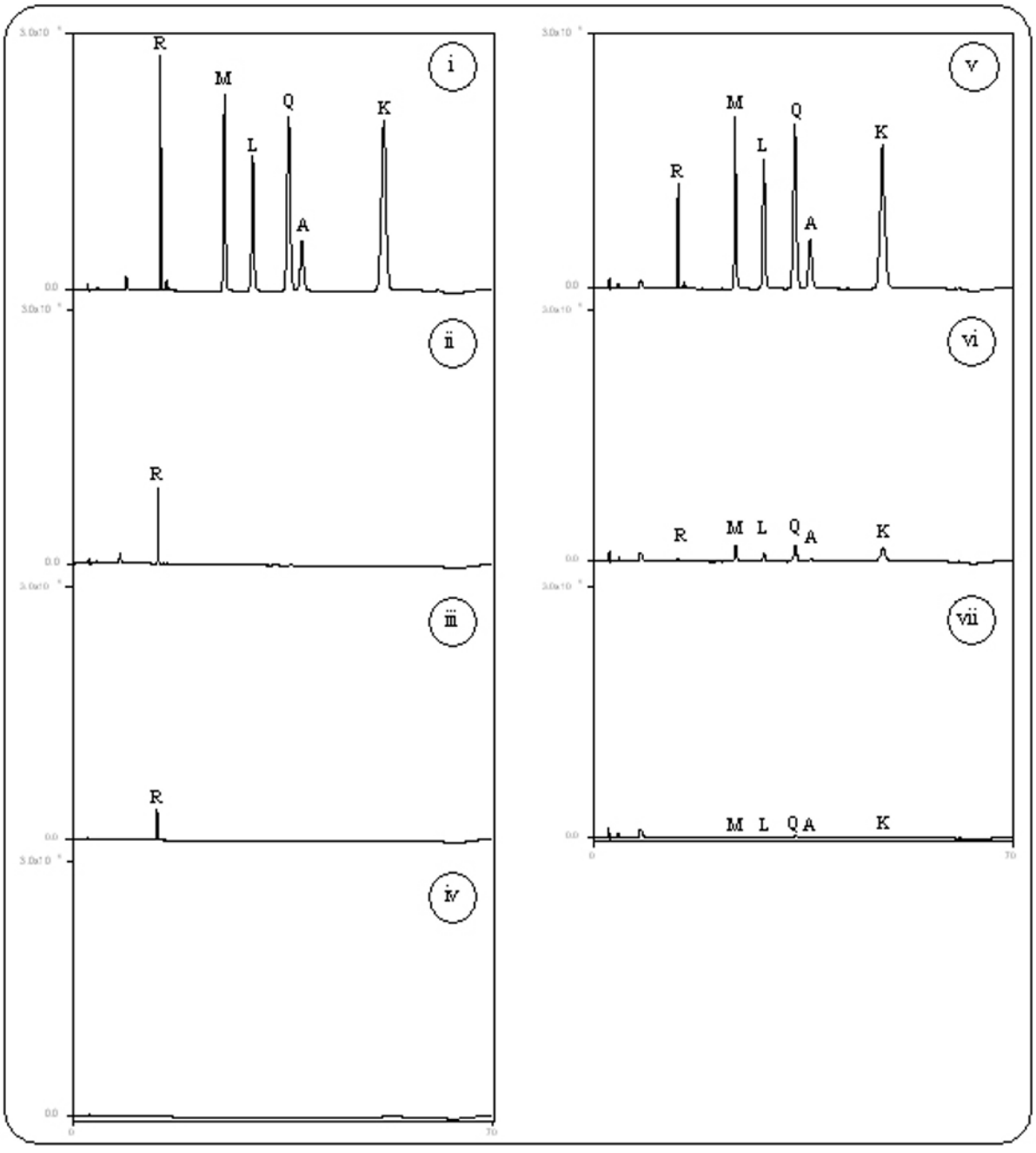

Figure 4. HPLC-PDA chromatograms of samples collected from the SPE of flavonoid standard mixture applied at λ_{max} 370 nm using 4-VP-co-EDMA as SPE sorbent: (i) flavonoid standard mixture, (ii) flow through, (iii) washing 1, (iv) washing 2, (v) elution 1, (vi) elution 2, (vii) elution 3. R = rutin, M = myricetin, L = luteolin, Q = quercetin, A = apigenin, K = kaempferol

3.2.Comparison of synthesized SPE materials with commercially available SPE materials

Commercially available polymeric SPE materials such as Oasis HLB from Waters and Strata-X from Phenomenex were used for comparison. Oasis HLB is a macroporous poly (divinylbenzene-co-N-vinylpyrrolidone) [16] copolymer and Strata-X is a surface modified co-polymer of styrene-divinylbenzene [9].

Among the self synthesized SPE materials, VEP is the most efficient and has comparable recoveries for all the analytes (except rutin) in this study, to those of commercial SPE materials. This could be due to the molecular structure similarities of the 4-VP-co-EDMA polymer with commercial SPE materials. In the case of 4-VP-co-EDMA, functional monomer is 4-VP while it is vinylpyrrolidone in the case of Oasis HLB. It is clear from

the structures of 4-vinylpyridine and vinylpyrrolidone (Fig. 1) that both the monomers are nitrogen containing compounds and both are hydrophilic, in this context rendering hydrophilic character to both the co-polymers.

Loading capacity determines the amount of analyte per specific amount of SPE material. VEP, ADP and in some cases AEP (6 + 30) have comparable loading capacities to those of Oasis HLB and Strata-X except rutin. Similarly, recovery efficiencies of VEP for all analytes under study, except rutin and ADP for apigenin and kaempferol, are very near to those of commercial SPE materials.

The amount of the SPE material used for the retention of analytes, plays an important role. In order to determine this effect, an SPE experiment was performed using 30 mg and 110 mg materials of VEP and MEP. An increase in the amount of sorbent, obviously increases the total surface area which is a source of retention of analytes during the SPE. This effect is very pronounced in the case of rutin which can be recovered only up to 49% and 24% when 30 mg of VEP and MEP SPE materials were used respectively, but the recovery percentage of rutin is increased to 100% using 110 mg of these materials as shown in the Table 3. Other analytes also show higher recoveries when higher amounts of sorbent are used.

4. Conclusions

Polymeric SPE materials are of great potential as they can be used in the whole pH range. A lot of research has been done for the synthesis of polymeric SPE materials and its applications. The present work is a part of this series in which different materials were synthesized, evaluated and compared. From these results 4-VP-co-EDMA was found to have comparable results to those of commercially available SPE materials. The prepared polymeric materials can be used as efficient SPE materials for polyphenolic compounds especially for flavonoid aglycons such as myricetin, luteolin, quercetin, apigenin and kaempferol.

Abbreviations

SPE: solid phase extraction, RP-SPE: reversed phase solid phase extraction, THF: tetrahydrofuran, DVB: divinylbenzene, AA: acrylamide, EDMA: ethyleneglycoldimethacrylate, MAA: methacrylic acid, VP: 4-vinylpyridine, MeOH: methanol, AIBN: azobis(isobutyronitrile), SEM: scanning electron microscope, VEP: 4-vinylpyridine-co-ethyleneglycoldimethacrylate, ADP: acrylamide-co-divinylbenzene, AEP: acrylamide-co-ethyleneglycoldimethacrylate, MEP: methacrylic acid-co-ethyleneglycoldimethacrylate

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