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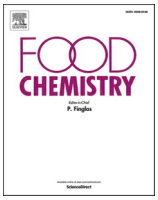

Removal of polyphenols from anthocyanin-rich extracts using 4-vinylpyridine crosslinked copolymers

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ABSTRACT

In this work, new sorbents for the purification of anthocyanin-rich extracts were evaluated. Copolymers of 4-vinylpyridine crosslinked with trimethylolpropane trimethacrylate (poly(4VP-*co*-TRIM)) or 1,4-dimethacryloyloxybenzene (poly(4VP-*co*-14DMB)) were tested for their potential to capture polyphenols. Copolymers were obtained by seed swelling polymerization in the form of microspheres with permanent porous structure – attractive features of sorbents used for sample purification by dispersive solid phase extraction. The microspheres were characterized by AFM, elemental analysis, SEM, and nitrogen adsorption-desorption method. Their capacity to remove polyphenols was evaluated using spectrophotometry, HPLC-DAD, and LC-MS/MS. For proof-of-concept, the aqueous extracts of berries classified into three different groups regarding their anthocyanin composition (strawberries, raspberries, blackcurrants) were selected. It was found that studied microspheres adsorbed flavonoids more effectively compared to primary secondary amine and graphitized carbon black. Copolymers of 4-vinylpyridine also capture anthocyanins and might be used for the purification of extracts of fruits before LC-MS/MS analysis to reduce the matrix effect.

1. Introduction

Anthocyanin-rich fruits play an important role in a well-balanced diet. Fruits and their by-products contain a broad spectrum of phenolic compounds with anti-inflammatory, antibacterial, and anti-cancer properties (Cieślik et al., 2006; Liu et al., 2024; Puupponen-Pimiä et al., 2005; Szajdek & Borowska, 2008). Moreover, polyphenols can bind to biomaterials through various interactions improving their biocompatibility with the human body (Liu et al., 2024). On the other hand, fruits, as well as fruit-derived products (jams, juices, smoothies, fruit beverages, marmalade) can be contaminated with some toxic, mutagenic, and carcinogenic compounds, e.g. mycotoxins, pesticides and their degradation products, dioxins, furans, polychlorinated biphenyls, polycyclic aromatic hydrocarbons. Due to harmful effects on

Abbreviations: 14DMB, 1,4-dimethacryloyloxybenzene; 4VP, 4-vinylpyridine; ACN, Acetonitrile; AFM, Atomic Force Microscope; AIBN, 2,2′-azobis(2-methylpropionitrile); C18, octadecylsilane; CAT, catechin; dMRM, dynamic multiple reaction monitoring; dSPE, dispersive solid-phase extraction; EMR, lipid enhanced matrix removal-lipid; GAE, gallic acid; GCB, graphitized carbon black; HPLC-DAD, high-performance liquid chromatography with a diode array detector; LC-MS/MS, liquid chromatography – tandem mass spectrometry; poly(4VP-*co*-TRIM), copolymer of 4-vinylpyridine crosslinked with trimethylolpropane trimethacrylate; poly(4VP-*co*-14DMB), copolymer of 4-vinylpyridine crosslinked with 1,4-dimethacryloyloxybenzene; PS, polystyrene microspheres; PSA, primary secondary amine; PSD, pore size distributions; QuEChERS, quick, easy, cheap, effective, rugged, and safe; S_{BET}, specific surface area; SEM, scanning electron microscope; TAC, total anthocyanins contents; TFC, total flavonoid content; THF, tetrahydrofuran; TPC, total phenolics content; TRIM, trimethylolpropane trimethacrylate; UHPLC, ultra-high performance liquid chromatograph; V_p, total pore volume; *Z*-Sep, modified silica gel with zirconium oxide.

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living organisms, their content and residue level in a variety of foods should be strictly monitored (Commission Regulation (EC) No 1881/2006 of 19, 2006). The recommended and dedicated approach for the detection and quantification of these toxic compounds is chromatography, especially liquid or gas chromatography coupled with tandem mass spectrometry (LC-MS/MS and GC–MS/MS, respectively). The LC-MS/MS methods have many advantages over other approaches (Azaiez et al., 2014), but might require multi-step sample pre-treatment to reduce the matrix effect (Sadok et al., 2019). The latter leads to inaccurate analytical responses for analytes determined in the presence of sample matrix than in pure solvents (Chambers et al., 2007). The mentioned inconveniences were mainly caused by the complexity of the fruit matrix. Matrix effect may also originate from the sample preparation procedure, quality of chromatographic separation, and ionization type. Furthermore, the matrix effect can be highly fluctuating and difficult to control and predict (Chambers et al., 2007). To minimize the matrix effect, which is crucial for obtaining reliable data and improving measurement sensitivity, proper sample pretreatment should be applied aimed at selective removal of undesirable interfering compounds or/and isolation of analytes. Since fruit matrix components might be co-extracted with the target analyte, purification of the sample extract should be scheduled before analysis.

To reduce the matrix effect, typical QuEChERS (quick, easy, cheap, effective, rugged, and safe) protocols combined with a subsequent sample clean-up employing dispersive solid-phase extraction (dSPE) are commonly used in the analysis of fruits and fruit-based products by LC-MS/MS (Azaiez et al., 2014; Sadok et al., 2023). Preferably, the dSPE sorbent (or a mixture of sorbents) should selectively capture the impurities with negligible loss of analytes. Various commercially available sorbents could be used for this purpose. However, in some applications, the undesirable loss of the analytes during a clean-up step may be observed along with the insufficient reduction of the matrix effect, and new approaches in this field are highly desirable.

Regarding sample type and composition, the purification step can be performed using various bulk sorbents such as primary secondary amine (PSA), octadecylsilane (C18), graphitized carbon black (GCB), enhanced matrix removal-lipid (EMR-Lipid), modified silica gel with zirconium oxide (*Z*-Sep and Z-Sep+) or their mixtures. They can be used to remove potentially interfering compounds from fruit extracts, but with different effectiveness (Mateus et al., 2021). Furthermore, these sorbents can also capture some target compounds, such as mycotoxins, resulting in a decrease in their recovery (Liu et al., 2014; Mateus et al., 2021). The application of polymeric materials based on 4-vinylpyridine (4VP) could provide some advantages in the sample preparation step. 4VP co-polymers are characterized by developed porous structure, spherical shape, uniform size, and other attractive features such as mechanical strength, permanent porosity, high selectivity, and reusability (Grochowicz et al., 2015). Moreover, polymer sorbents stand out among various commercially available materials for sample clean-up (e.g., C18) because of chemical resistance against organic solvents, acids, and bases. Thus, they can be applied in a wide range of pH (Grochowicz et al., 2015, 2022).

Our study aimed to verify the effectiveness of 4VP crosslinked with trimethylolpropane trimethacrylate (poly(4VP-*co*-TRIM)) or 1,4-dimethacryloyloxybenzene (poly(4VP-*co*-14DMB)) microspheres towards clean-up of extracts obtained from anthocyanin-rich fruits. We hypothesized that poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) could remove anthocyanins and other polyphenols from fruit extracts but with different effectiveness. Before application, poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) were characterized in terms of chemical structure (by ATR-FTIR and elemental analysis), morphology (by SEM) and porosity (by the nitrogen adsorption-desorption method). As a model fruit matrices: strawberries, red raspberries, and blackcurrants were selected. According to the classification proposed by Fang (2015), these berries represent the following groups of fruits: (1) containing primarily pelargonidin - strawberry, (2) containing mainly cyanidin/peonidin–red raspberry, and (3) comprising multiple groups of anthocyanidins – blackcurrants. Each fruit matrix was spectrophotometrically characterized by the total content of phenolic, flavonoids, and anthocyanin as well as the individual polyphenols by LC-MS/MS. The inspection of chromatograms acquired for control and pre-treated extracts using high-performance liquid chromatography with a diode array detector (HPLC-DAD) at wavelengths suitable for the detection of a different class of polyphenolic compounds (anthocyanins, flavonols, and phenolic acids) was also performed. The differences between the data obtained for the raw (control) and polymer-treated berry extracts were evaluated. The effectiveness of poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) in polyphenol removal was compared to some commercially available sorbents (PSA, GCB).

2. Materials and methods

2.1. Chemicals, reagents and materials

Standards of (+)-catechin hydrate (CAT) and gallic acid (GAE) were purchased from Chemat (Gdańsk, Poland). *P*-coumaric acid, quercetin-3-glucoside, Folin-Ciocalteu's phenol reagent, LC-MS grade additives for a mobile phase (CH_3COONH_4, CH_3COOH, HCOOH), NaOH, GCB (carbon, mesoporous, nanopowder, particle size <500 nm) and PSA were from Sigma-Aldrich (St Louis, MO, USA), and cyanidin-3-glucoside chloride from MedChemExpress (Sollentuna, Sweden). The reagents for spectrophotometric measurements ($AlCl_3$, $NaNO_2$ and KCl, CH_3COONa, HCl) were supplied by Chempur (Piekary Śląskie, Poland) and Merck (Darmstadt, Germany), respectively. Ultrapure water was produced by a Millipore Direct Q 3UV water purification system (Millipore, Molsheim, France). Acetonitrile (ACN, hypergrade) was from Merck (Darmstadt, Germany).

For polymer synthesis, styrene (99 %), 4VP (95 %), trimethylolpropane trimethacrylate (TRIM), 2,2′-azobis(2-methylpropionitrile) (AIBN) (98 %), and sodium lauryl sulfate (99 %) were obtained from Sigma-Aldrich (St Louis, MO, USA). Toluene, methanol, ethanol, tetrahydrofuran (THF), and acetone were from POCh (Gliwice, Poland). 1,4-dimethacryloyloxybenzene (14DMB) was synthesized following the procedure described earlier (Grochowicz & Gawdzik, 2013). Monomers: 4VP and styrene were purified of inhibitor by vacuum distillation and stored in a refrigerator until use. Other reagents were used as received without further purification.

2.2. The preparation of poly(4VP-co-14DMB) and poly(4VP-co-TRIM) copolymers

The synthesis of poly(4VP-*co*-14DMB) and poly(4VP-*co*-TRIM) was carried out via seed swelling polymerization (Grochowicz et al., 2015, 2022; Unsal et al., 2004). The molar ratio of 4VP to 14DMB and 4VP to TRIM was 2:1; AIBN was used as the initiator of polymerization in the amount of 2 % wt. to the mass of monomers; toluene acted as an activator and pore-forming agent. Polystyrene microspheres (PS) were used as seed polymers. PS particles were synthesized via dispersion polymerization. Obtained PS particles were characterized with $M_w = 19{,}500$ Da and mean bead diameters of about 2 μm. Seed swelling polymerization was carried out in two steps. Firstly, the swelling of PS seed with monomers, initiator, and toluene mixture was made using a mechanical stirrer (IKA, Staufen im Breisgau, Germany) at 30 °C for 24 h at 150 rpm. In the following step, the temperature was increased to 71 °C, and polymerization was carried out for the next 24 h. After the reaction, the obtained microspheres were washed with methanol, hot water, and ethanol, respectively, and finally extracted with boiling THF for 4 h to remove PS seed particles.

2.3. The methods of copolymers characteristics

ATR-FTIR spectra were recorded using the Tensor 27 spectrometer

(Bruker, Mannheim, Germany) equipped with a diamond crystal. The spectrum was made in the spectral range of 600–4000 cm^{-1} with a resolution of 4 cm^{-1} and 16 scans per spectrum. Elemental analysis was performed with an elemental analyzer CHN/CHNS EuroEA3000 (EuroVector, Pavia, Italy). The morphology and internal structure of microspheres were examined using a Quanta 3D FEG scanning electron microscope (FEI, Hillsboro, OR, USA). Polymeric particles were coated with a thin layer of gold. To determine the number of average diameters (D_n) Morphology G3 particle analyzer (Malvern Panalytical, Malvern, UK) was used. The average molecular weight (M_w) of PS particles was determined with the use of a gel permeation chromatography system GPCMax equipped with a triplet detection system TDA 305 (Malvern Panalytical, Malvern, UK). Measurements were performed using THF as a mobile phase at the 1 mL/min flow rate.

Parameters characterizing the porosity of the copolymer were determined by nitrogen adsorption at −196 °C using an ASAP 2420 analyzer (Micromeritics, Norcross, GA, USA). Before measurements, samples were degassed at 80 °C under vacuum. The specific surface area (S_{BET}) was evaluated using the standard Brunauer-Emmett-Teller (BET) method for the nitrogen adsorption data in the range of a relative pressure p/po 0.05 to 0.25, assuming that the area of a single nitrogen molecule is 16.2 $Å^2$. The total pore volume (V_p) was estimated from single-point adsorption at a relative pressure of 0.985. The pore size distributions (PSD) were obtained from the desorption branch of the isotherm using the Barrett-Joyner-Halenda (BJH) procedure (Barrett et al., 1951).

2.4. Collection of fruits and their preparation for experiments

Berries (30 samples) were purchased fresh or frozen (2 frozen and 8 fresh samples of strawberry, 2 frozen and 8 fresh samples of raspberry, and 10 fresh samples of blackcurrant) from various locations in different regions of Poland (supermarkets, street markets, private gardens), and were free of mold. Samples were collected between June 2016 and August 2022. The amount of each fruit sample was about 0.25–1.0 kg. Fruits were homogenized using a food blender (Braun Multiquick Professional, Warsaw, Poland), portioned (from 3 to 10 replicates (each ~90 g), depending on the fruit sample size), and stored at −20 °C in sterile plastic cups until analysis.

For spectrophotometric and LC-MS/MS analysis, extracts of fruit were prepared daily, before analysis. To make the single experimental sample, 10 g of defrosted fruit pulp was treated with 10 mL of distilled water in a centrifugal tube, hand-shaken (2 min), sonicated (5 min) using Polsonic SONIC-6D ultrasonic bath (Warsaw, Poland), vortexed (1 min), and centrifuged (15 min, 8228 ×*g*) using the 5408 Eppendorf centrifuge (Hamburg, Germany). The supernatants were additionally centrifuged for 15 min at 16,000 ×*g* using the 5415R Eppendorf centrifuge (Hamburg, Germany). The extract was then cleaned using the dSPE technique by mixing 1 mL of fruit extract with 5 mg of the proper polymeric sorbent (poly(4VP-*co*-TRIM) or poly(4VP-*co*-14DMB)) in a centrifugal tube. The suspension was shaken for 30 min at room temperature using the IKA MS5 basic (Woburn, Massachusetts, USA) (2000 rpm). Then, after centrifugation (15 min, 16,000 ×g), the collected supernatant was subjected to further experiments. Clean-up of fruit extracts using commercially available sorbents (PSA, GCB) was carried out analogously by applying 5 mg of proper sorbent to 1 mL of sample. Each experiment was repeated twice using the independently prepared sample from a given source.

For HPLC-DAD analysis, control samples (raw fruit extracts) were prepared by mixing 10 g of homogenized fruit pulp with 10 mL of ultrapure water. After 10 min of sonication, samples were mixed by vortex for 2 min, and centrifuged for 5 min at 8228 ×*g*. Polymer-treated extracts were prepared by adding 30 mg of the sorbent into 1 mL of raw fruit extracts, shaken for 10 min (1500 rpm, room temperature) using the IKA shaking machine, and centrifuged for 15 min at 16,000 ×g. The clear extracts were injected (10 μL) into the HPLC-DAD system.

2.5. Determination of total phenolics content and total flavonoid content

The total phenolics content (TPC) and the total flavonoid content (TFC) were measured using the Folin-Ciocalteu method and colorimetric method, respectively using UV–Vis spectrophotometer model U-2900 (Hitachi High-Technologies, Tokyo, Japan). Solutions were prepared and proceeded as previously described (Sadok et al., 2023) adjusting the fruit pulp/water ratios. Samples for spectrophotometric analysis were prepared in duplicate (additionally, three readings were obtained from one sample). The results were expressed as milligram gallic acid equivalent per 100 g of fruit pulp (mg GAE/100 g) for TPC and milligram catechin equivalent per 100 g of fruit pulp (mg CAT/100 g) for TFC.

2.6. Determination of total anthocyanins content

The total anthocyanins content (TAC) in fruit extracts was determined by the pH differential method (Lee et al., 2005). Buffers of pH = 1.0 (0.025 mol/L KCl) and pH = 4.5 (0.4 mol/L CH_3COONa) were prepared in distilled water. pH was adjusted using the SevenMulti™ dual meter pH/conductivity completed with InLab® Expert Pro (Mettler Toledo, Schwerzenbach, Switzerland). One portion of the fruit extract (125 μL) was diluted with 1.125 mL of the buffer of pH = 1.0, and the second portion with 1.125 μL of the buffer of pH = 4.5 (10 times dilution). Spectrophotometric measurements were performed using Hitachi spectrophotometer at two wavelengths: $\lambda_1 = 520$ nm and $\lambda_2 = 700$ nm. Then, a further dilution of the samples was performed, and measurements were repeated until the final results (taking into account the amount of buffer added) were not affected by the dilution factor. Absorbance was measured within 20–50 min of preparation. TAC was calculated using a suitable formula (Lee et al., 2005), and the results (anthocyanin pigment concentration) were expressed as cyanidin-3-glucoside equivalents per 1 g of fruit pulp (mg cyd-3-glu/g).

2.7. HPLC-DAD analysis of fruit extracts

Measurements were performed using an Agilent Technologies 1200 Series high-performance liquid chromatograph equipped with an autosampler, a quaternary pump with a vacuum degasser, and a diode array detector (DAD) (Agilent Technologies, Wilmington, DE, USA). Instrument control and data analysis were carried out using Agilent Mass Hunter Workstation software v.B.06.01 and Mass Hunter Qualitative Analysis software v.B.07.00 (access in September 2024).

Chromatographic separation was achieved using a Zorbax Eclipse Plus-C18 RRHT (4.6 × 100 mm × 3.5 μm) column protected by a Zorbax Eclipse Plus-C18 (2.1 × 12.5 mm × 5 μm) Narrow Bore guard column purchased from Agilent Technologies (USA). The column temperature was set at 30 °C. The mobile phase consisted of H_2O + 5 mmol/L CH_3COONH_4 + 0.1 % (*v/v*) HCOOH +0.2 % (*v/v*) CH_3COOH (solvent A) and ACN + 5 mmol/L CH_3COONH_4 + 0.1 % (*v/v*) HCOOH +0.2 % (*v/v*) CH_3COOH (solvent B) was used. Separation was achieved within 35 min run accompanied by the following gradient program: 0–20 min: solvent B 5–60 %; 20–30 min: solvent B 60–5 % (post run: 5 min) at the flow rate 0.7 mL/min. Anthocyanins were detected at 520 nm, flavonols at 360 nm, phenolic acids and their derivatives at 320 nm, flavan-3-ols at 280 nm (Sokół-Łętowska et al., 2020). The content of anthocyanins, flavonols, phenolic acids and their derivatives, and flavan-3-ols were converted into cyanidin-3-glucoside chloride, quercetin 3-glucoside, (+)-catechin, and *p*-coumaric acid, respectively. Calculations were based on calibration curves obtained for standard compounds.

2.8. Determination of individual polyphenols by LC-MS/MS

Fruit extracts were analyzed using a 1290 infinity ultra-high performance liquid chromatograph (UHPLC) consisting of a degasser, binary pump, autosampler, and column thermostat (Agilent Technologies, Santa-Clara, CA, USA). The UHPLC system was coupled with an Agilent

6460 triple quadrupole mass spectrometer equipped with an electrospray ion source (Agilent Jet Stream). The analytical column was a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm) connected to a Zorbax Eclipse Plus C18 guard column (2.1 × 12.5 mm, 5 μm) both purchased from Agilent Technologies (USA). The column temperature was set at 25 °C and the mobile phase consisted of 1 % (*v/v*) HCOOH in water (solvent A) and 1 % (*v/v*) HCOOH in ACN (solvent B). The separation was achieved within 14 min (post time: 2 min) at flow 0.3 mL/min. The gradient elution started with 5 % B, then changed from 5 to 50 % B up to 12 min, and from 50 to 80 % B for the next 2 min. The injection volume was 10 μL (each sample was injected three times). Ion source parameters were as follows: nebulizer pressure, 241,316.6 Pa; nitrogen gas temperature, 300 °C; nitrogen gas flow, 10 L/min; capillary voltage, 4000 V. Ion acquisition was performed in the dynamic multiple reaction monitoring (dMRM) mode in positive polarity. The monitored ions and other settings of the dMRM method are collected in Table 1. Data were acquired with Agilent MassHunter Acquisition software v.B.08 and analyzed with Agilent MassHunter Quantitative Analysis software v. B.07 (access in February 2023).

2.9. Statistical analysis

The PQStat 1.8.4.162 software was used for the calculations. Data normality was evaluated by the Shapiro-Wilk test. A comparison of the results was done using Kruskal-Wallis one-way ANOVA with post-hoc Dunn Bonferroni or Tukey HSD. The difference between mean values with $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Characterization of poly(4VP-co-14DMB) and poly(4VP-co-TRIM) sorbents

Porous copolymers of 4VP functional monomer in the form of microspheres with the two crosslinkers: aromatic 14DMB and aliphatic TRIM were synthesized. Application of these monomers led to obtaining microspheres with a high degree of crosslinking and a permanent porous structure. Copolymers applied as sorbents must possess functional groups in their chemical structure that can interact with sorbates and improve their retention. In this study, 4VP was chosen as a functional monomer, as it shows a basic character due to the nitrogen atom in the aromatic ring. It can also interact with sorbates by π-π stacking. Moreover, both crosslinkers introduced functional ester groups into the copolymers network, and 14DMB additionally gives an aromatic ring. The chemical structure of both copolymers was confirmed by ATR-FTIR analysis (Fig. 1). Visible on spectra of both copolymers, the absorption bands at 1599 cm^{-1} and 1558 cm^{-1} due to C═C and C═N stretching vibrations of the pyridine ring confirmed that 4VP units are present in the copolymer networks. On the other hand, the absorption bands derived from carbonyl group vibrations (from crosslinkers) are observed at 1745 cm^{-1}. Moreover, the results of the elemental analysis (Table 2) also confirm that 4VP units are present in copolymer networks. Since the intention was to obtain copolymers in the form of microspheres, seed swelling polymerization was chosen as an appropriate method for the production of particles with a spherical shape. This method also allows obtain copolymers with a highly developed permanent porous structure. In Fig. 1 SEM micrographs of poly(4VP-*co*-14DMB) and poly(4VP-*co*-TRIM) are presented. Their average diameter are 9.5 μm and 8.2 μm, respectively. From SEM images it is visible that the surface of the microspheres is porous. Quantitatively, the parameters characterizing the porosity of the obtained microspheres were determined by the low-temperature nitrogen adsorption-desorption method and they are listed in Table 2. The S_{BET} of poly(4VP-*co*-14DMB) is lower than that of poly(4VP-*co*-TRIM). This difference is due to the higher number of methacrylate groups in TRIM monomer than in 14DMB which affects the process of phase separation during polymerization when the porous structure is created (Grochowicz et al., 2015, 2022). The PSD of both copolymers shows two maxima in the region of mesopores, whereas the V_p is about 0.26 cm^3/g.

3.2. Evaluation of changes in TPC, TFC, and TAC in fruit extracts after clean-up with poly(4VP-co-14DMB) and poly(4VP-co-TRIM)

In this section, we attempted to check whether or not poly(4VP-*co*-14DMB) and poly(4VP-*co*-TRIM) show the ability to capture polyphenols from berries extracts. These polymeric sorbents reduced significantly the colour of extracts of red and dark purple-pigmented fruits (Supplementary Fig. 1). It was suspected that poly(4VP-*co*-

Table 1
Settings of the dMRM method applied for polyphenols determination by LC-MS/MS and structures of investigated analytes.

Analyte	Precursor ion [*m/z*]	Production [*m/z*]	Retention time [min]	ΔRetention time [min]	Fragmentor [V]	Collision energy [eV]
gallic acid	169	125 97	1.92	2	60	14 16
4- hydroxybenzoic acid	137	93 65	3.87	2	80	14 14
chlorogenic acid	353	191 85	3.99	2	50	14 50
catechin	289	245 203	4.02	2	60	10 16
caffeic acid	179	135 107	4.43	2	80	14 24
p-coumaric acid	163	119 93	5.61	2	50	14 30
ellagic acid	301	284 229	5.85	2	100	28 28
rutin	609.1	300 271	5.94	2	140	50 60
ferulic acid	193.1	178 134	6.18	2	60	8 10
myricetin	317	179 151	7.36	2	80	16 24
quercetin	301	179 151	9.07	2	100	16 20
naringenin	271	151 119	9.88	2	50	14 24
apigenin	269	151 117	9.94	2	50	20 30

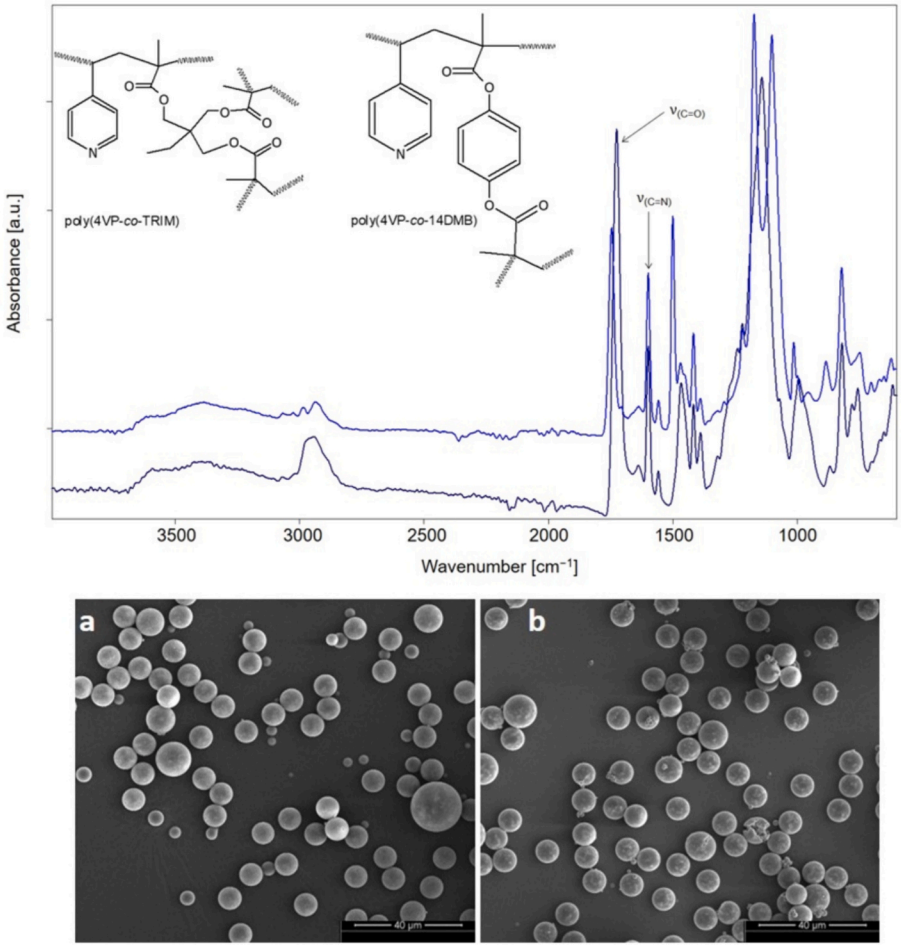

Fig. 1. FTIR spectra of studied microspheres and SEM images of poly(4VP-*co*-TRIM) (a) and poly(4VP-*co*-14DMB) (b) microspheres.

Table 2
Copolymers characterization: porosity, nitrogen percentage (%N), and microspheres diameter (D_n).

Copolymer	%N	D_n [μm]	S_{BET} [m^2/g]	V_p [cm^3/g]	PSD_{max} [nm]
poly(4VP-*co*-14DMB)	5.52	9.5	98	0.26	4/50
poly(4VP-*co*-TRIM)	4.38	8.2	140	0.29	4/34

14DMB) and poly(4VP-*co*-TRIM) capture anthocyanins and flavonoids, which contribute to the red, purple, blue, and yellow colour of fruits (Lu et al., 2021). To verify the hypothesis, changes in TPC, TFC, and TAC were evaluated in fruit extracts before (control) and after dSPE cleaning using tested polymeric microspheres. For each sample, the fold change was calculated by dividing the data obtained for the control by the corresponding polymer-treated sample. All experiments were conducted with the same amount of polymeric material delivered from the same batch with the characteristics provided above. Considering the heterogeneous composition of fruit extracts and to avoid randomness of the results, measurements were repeated for a large number of samples delivered from different sources.

3.2.1. *Changes in TPC*

The lowest TPC in untreated extracts (control) was found in the case of red raspberry samples (mean value 40.99 ± 9.12 mg GAE/100 g f.w.) within the range of 23.41–58.89 mg GAE/100 g f.w. Slightly higher TPC values were determined in the strawberry extracts (mean 42.00 ± 13.56 mg GAE/100 g f.w.) in the range of 21.81–68.38 mg GAE/100 g f.w. As expected, the control blackcurrant extracts were characterized by the highest TPC values in the range of 43.76–91.83 mg GAE/100 g f.w. (mean value: 63.23 ± 13.02 mg GAE/100 g f.w.). Detailed data are available in the OSF repository (https://osf.io/5xrtc/).

Regardless of the fruit matrix evaluated, dSPE purification using poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) resulted in a decrease in TPC values (Fig. 2A and B). For the raspberry matrix, the application of poly(4VP-*co*-TRIM) decreased TPC by 48.00 % ± 12.34 % compared to the control (Fig. 2A). This means that almost half of polyphenols available in the sample were sorbed by the polymer. Similar results were obtained for poly(4VP-*co*-14DMB) (the drop by 46.20 % ± 10.95 %). In the case of the strawberry matrix, TPC values in extracts treated with poly(4VP-*co*-TRIM) amounted to 19.29 ± 7.70 mg GAE/100 g f.w. ($n = 20$), which means that TPC was reduced by 54.77 % ± 4.33 % compared to the control. A comparable drop by ~50 % in TPC values was observed for poly(4VP-*co*-14DMB) (mean TPC: 21.75 ± 9.20 mg GAE/100 g f.w.). Notably, a similar degree of TPC reduction in the red raspberry and strawberry matrices using both 4VP copolymers was observed.

All studied polymers were less effective in the purifying of the blackcurrant extract, since TPC decreased by 21.65 % ± 6.15 % and 21.02 % ± 4.08 % after treatment with poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB), respectively.

For each fruit matrix, dSPE purification with polymeric microspheres resulted in a statistically significant ($p < 0.05$) decrease in TPC values compared to the control (Fig. 2B and Supplementary Fig. 2). Furthermore, the effectiveness of poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) in reducing of TPC values did not differ significantly (Fig. 2B).

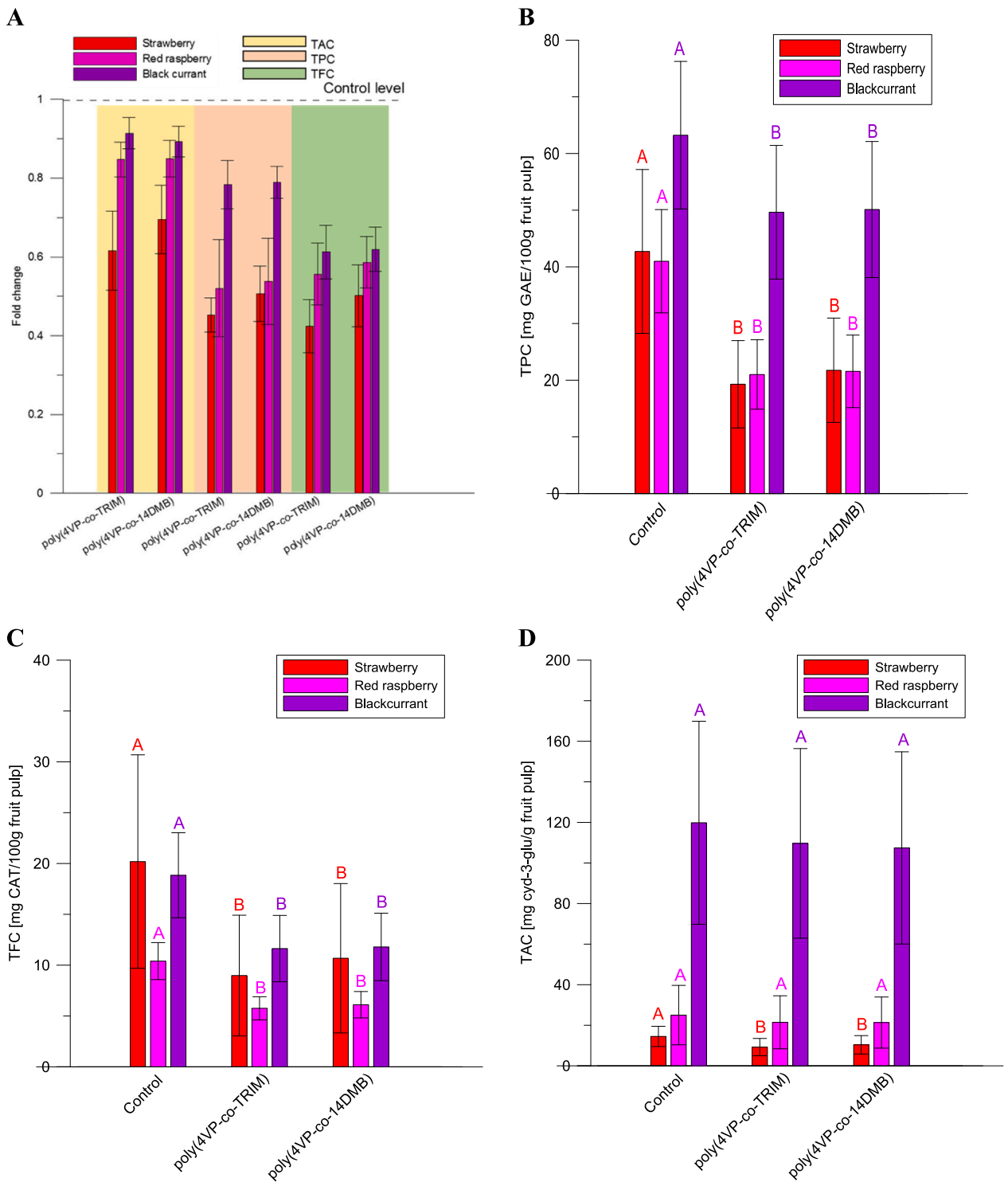

Fig. 2. (A) Fold changes values (dSPE purification of berry extracts using polymeric microspheres vs. the control) of TPC, TFC, and TAC. (B—D) Comparison of TPC, TFC, and TAC values for unpurified (control) fruit extracts and after dSPE clean-up. The different letters in the same colour within a graph indicate a statistically significant difference ($p < 0.05$). Each group consisted of 20 samples.

3.2.2. *Changes in TFC*

Measurements conducted on control extracts have shown that the red raspberry matrix is characterized by significantly lower basal TFC levels compared to strawberry and blackcurrant ones. Furthermore, the TFC obtained for the blackcurrant and strawberry matrices did not differ significantly from each other ($p > 0.05$). The TFCs in the control aqueous extracts were within the range of 7.03–13.88 mg CAT/100 g, 14.40–26.86 mg CAT/100 g, and 9.59–47.25 mg CAT/100 g for red raspberry, blackcurrant, and strawberry, respectively (detailed data are available in the OSF repository (https://osf.io/5xrtc/)). Satisfactory removal of flavonoids by both 4VP copolymers was noted in strawberry and red raspberry matrices since TFC parameter decreased significantly by ~50.0–58.0 % and ~41.0–44.5 % after dSPE purification (Fig. 2A). In blackcurrant matrix, poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) showed the lowest efficiency in flavonoids capture. In this case, TFC values dropped by 38.79 % ± 6.80 % and 38.09 % ± 5.59 %,

respectively. Both polymers were found to significantly decrease TFC values compared to the control extracts ($p < 0.05$, Fig. 2C) in all model fruit matrices (strawberry, red raspberry, and blackcurrant). However, there are no statistically significant differences between the efficiency of the studied polymeric materials ($p > 0.05$).

3.2.3. Changes in TAC

Measurements were performed in both: control (untreated samples) extracts and extracts after treatment with 4VP copolymers. The detailed data were collected in the OSF repository (https://osf.io/5xrtc/). TAC in strawberries' control extracts was in the range of 5.75–24.88 mg cyd-3-glu/g fruit pulp (mean 14.50 ± 5.01 mg cyd-3-glu/g fruit pulp). The major anthocyanin of strawberry fruits is pelargonidin-3-glucoside (~77–90 % of all anthocyanins) (da Silva et al., 2007). In extracts purified with poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB), TAC amounted to 3.31–18.82 mg cyd-3-glu/g fruit pulp (mean 9.23 ± 4.26 mg cyd-3-glu/g fruit pulp) and 3.69–20.21 mg cyd-3-gly/g fruit pulp (mean 10.38 ± 4.53 mg cyd-3-glu/g fruit pulp), respectively. TAC dropped by 38.47 ± 10.02 % in extracts treated with poly(4VP-*co*-TRIM) and by 30.45 ± 8.70 % in extracts purified with poly(4VP-*co*-14DMB), which was presented in Fig. 2A.

The red raspberry samples were characterized by higher TAC than strawberries. TAC of the aqueous fruit extracts amounted from 6.21 ± 0.02 mg cyd-3-glu/g fruit pulp even up to 53.20 ± 0.52 mg cyd-3-glu/g fruit pulp (mean 25.01 ± 14.66 mg cyd-3-glu/g fruit pulp). However, after treatment with poly(4VP-*co*-14DMB) or poly(4VP-*co*-TRIM), TAC decreased by 15.05 % ± 4.62 % and 15.28 % ± 4.36 %, respectively, and amounted to an average of 21.33 ± 12.60 mg cyd-3-glu/g fruit pulp and 21.47 ± 13.06 mg cyd-3-glu/g fruit pulp, respectively (Fig. 2A).

The results from ANOVA showed that TAC in the untreated extract of red raspberry and strawberry matrices did not differ significantly from each other (p was slightly higher than the threshold value 0.085), but TAC in blackcurrant samples stood out from others ($p < <0.05$). In the blackcurrant matrix, the highest TAC was found in the range of 68.13 ± 0.71 mg cyd-3-glu/g fruit pulp to 249.19 ± 7.41 mg cyd-3-glu/g fruit pulp (mean 119.86 ± 50.03 mg cyd-3-glu/g fruit pulp). Simultaneously, the lowest drop of TAC triggered by the purification of the extract with polymers was observed compared to other fruit matrices evaluated. TAC decreased by 10.73 % ± 3.84 % and 8.59 % ± 3.97 % in extracts treated with poly(4VP-*co*-14DMB) and poly(4VP-*co*-TRIM), respectively, compared to the corresponding control sample.

Taking into account the strawberry and red raspberry matrices, a higher drop in TAC was observed after purification with poly(4VP-*co*-TRIM). In the case of blackcurrant, poly(4VP-*co*-14DMB) was more effective in capturing anthocyanins. Regardless of the kind of 4VP copolymer used for dSPE purification, a statistically significant decrease in TACs was observed for the strawberry fruit matrix ($p < 0.05$, Fig. 2D) compared to the control. However, there are no statistically significant differences between the effect of poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) treatment.

3.3. HPLC-DAD analysis of control and polymer-treated fruit extracts

Since the HPLC-DAD method is commonly used to determine phenolics (Bordonaba et al., 2011; da Silva et al., 2007; Hernanz et al., 2007), therefore we attempted to investigate the changes in the chromatograms acquired for dSPE purified extracts in comparison with the control samples. Measurements were carried out for red raspberry extracts at wavelengths recommended for the detection of flavanols (280 nm), phenolic acids and their derivatives (320 nm), flavonols (360 nm), and anthocyanins (520 nm). Most of the signals present in the chromatograms acquired at wavelengths set up for flavanols and phenolic acids and their derivatives were higher in the control extracts than in those purified with poly(4VP-*co*-TRIM) or poly(4VP-*co*-14DMB) (see the chromatograms in Supplementary Fig. 3). Moreover, in the case of flavonols and anthocyanins, the decrease in some signals in red raspberry extract purified with 4VP copolymers was observed. The data suggested the different affinity of 4VP copolymers for the sorption of individual polyphenols.

Relative quantitative analysis was also conducted to investigate changes in the contents of each group of phenolic compounds after dSPE purification (Table 3). The significant drop in the content of anthocyanins, flavonols, phenolic acid, and flavan-3-ols in polymer-treated extracts vs. control proved that studied polymers can be considered efficient sorbents for the adsorption of various polyphenolic compounds. The binding capacity of studied polymers towards a particular group of compounds varied. However, poly(4VP-*co*-14DMB) appeared to be a better sorption material for anthocyanin capture (Table 3).

3.4. Detection of individual polyphenols by LC-MS/MS in fruit extract before and after dSPE clean-up

The study was devoted to investigating the changes in signals of individual polyphenols naturally present in control fruit extracts after purification by dSPE (expressed as a fold change) using the LC-MS/MS method. This method allowed for the detection of 13 different analytes from the flavonoid and phenolic acid groups, which are detailed in Supplementary Table 1. For the study, the effect of dSPE purification with 4VP copolymers on polyphenols signals the fold changes (peak area of the analyte in purified sample/peak area of the analyte in the corresponding untreated sample) were calculated. The fold change amounted to 1 indicating that the analyte did not sorb onto polymers. Values <1 meant analyte sorption, but >1 suggested no sorption onto the 4VP

Table 3
HPLC-DAD data regarding the changes in contents of phenolic compounds in red raspberry fruit matrix after extract clean-up with poly(4VP-*co*-TRIM) and poly (4VP-*co*-14DMB).

	Flavanols [μg/100 g fruit pulp]	Phenolic acids [μg/100 g fruit pulp]	Flavonols [μg/100 g fruit pulp]	Anthocyanins [μg/100 g fruit pulp]
Control (unpurified extract)				
R1 (*n* = 3)	10.29 ± 5.05	5.53 ± 1.25	51.45 ± 13.76	525.73 ± 48.36
R3 (n = 3)	15.38 ± 0.88	12.81 ± 1.34	37.61 ± 1.33	345.99 ± 12.83
R11 (n = 3)	9.25 ± 0.41	8.17 ± 0.35	87.15 ± 0.86	789.82 ± 23.84
poly(4VP-*co*-TRIM)-treated extracts				
R1 (n = 3)	<1.25	<0.5	4.81 ± 0.16	112.56 ± 15.71
R3 (n = 3)	<1.25	1.97 ± 0.20	13.46 ± 1.48	173.92 ± 16.29
R11 (n = 3)	<1.25	1.03 ± 0.11	12.88 ± 0.7	162.23 ± 5.87
poly(4VP-*co*-14DMB) -treated extracts				
R1 (n = 3)	<1.25	<0.27	5.88 ± 2.61	91.62 ± 6.05
R3 (n = 3)	<1.25	2.62 ± 1.53	11.29 ± 6.56	116.92 ± 7.42
R11 (n = 3)	5.87 ± 0.45	1.20 ± 0.42	12.40 ± 1.60	118.85 ± 21.62

copolymer and improved efficiency of analyte ionization by reduction of matrix effect.

3.4.1. Strawberry

In control strawberry samples, several polyphenols were detected, i.e. 4-hydroxybenzoic acid, apigenin, catechin, gallic acid, ellagic acid, naringenin, *p*-coumaric acid, quercetin, and rutin. In the case of both polymeric materials, sorption of all tested hydroxybenzoic acid derivatives from strawberry samples was observed (Table 4). The highest sorption was shown by gallic acid (70.20 % and 63.62 % of the analyte detected in untreated extract sorbed onto poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB), respectively). Then in descending order were ellagic acid (58.52 % and 53.38 %) and 4-hydroxybenzoic acid (34.66 % and 32.84 %). However, among all the detected derivatives of hydroxycinnamic acid, only *p*-coumaric acid was sorbed and the extent of its sorption was very limited (16.92 % and 20.32 %, respectively). From the studied flavonols, the highest signal drop was observed for quercetin (almost 90 % of the analyte was removed). Moreover, other flavonoids were also effectively sorbed onto both polymeric materials. For example, catechin and naringenin signals decreased by 84.57 % and 82.68 %, respectively, in extracts treated with poly(4VP-*co*-TRIM) and by 78.63 % and 81.24 %, respectively, in samples treated with poly(4VP-*co*-14DMB).

Statistical analysis revealed that the basal levels of all detected polyphenols (except *p*-coumaric acid) were significantly reduced ($p < 0.05$) by sorption onto the surface of polymeric microspheres (Supplementary Fig. 4). At the same time, the effectiveness of poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) did not differ significantly ($p > 0.05$). However, in strawberry extracts, poly(4VP-*co*-TRIM) was found to be slightly more effective in removing polyphenols. In the case of *p*-coumaric acid, only the efficiency of poly(4VP-*co*-14DMB) differed significantly from the control and the corresponding extract treated with poly(4VP-*co*-TRIM).

3.4.2. Red raspberry

LC-MS/MS analysis of the control red raspberry extracts has shown the presence of ellagic acid, gallic acid, caffeic acid, and chlorogenic acid, as well as myricetin, quercetin, rutin, catechin, and naringenin. The signal of the hydroxybenzoic acid derivatives (ellagic and gallic acids) decreased significantly by 87.14 % and 77.50 % after sample treatment with poly(4VP-*co*-TRIM), and by 88.64 % and 78.43 % with poly(4VP-*co*-14DMB), respectively (Table 4). Only some hydroxycinnamic acid derivatives were detected and their signals acquired after sample pretreatment with both polymers (poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB)) decreased by 31.47 % and 37.39 % considering caffeic acid as well as 78.91 % and 74.90 % for chlorogenic acid. Moreover, caffeic acid was detected only in 12 red raspberry samples from 20 subjected to analysis (Supplementary Fig. 4). The application of polymeric microspheres caused a significant drop in the flavonols signals, particularly in the case of myricetin (detected in 12 samples), quercetin, and rutin (detected in 19 red raspberry samples). The highest drops considering flavonols were observed for quercetin (86.98 % and 87.55 % for poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB), respectively), and the lowest for myricetin (77.30 % and 77.99 %, respectively). Among other flavonoids, only catechin and naringenin were detected. The decreases in their signals were significant (catechin: the drop was 78.73 % and 74.52 % for poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB), naringenin: 95.73 % and 95.22 %, respectively). The changes between the above-mentioned data were found to be statistically significant ($p < 0.05$).

Unlike the strawberry matrix, poly(4VP-*co*-14DMB) caused a greater decrease in the signals of some analytes than poly(4VP-*co*-TRIM) in red raspberry extracts. However, these differences in the effectiveness of both polymers did not differ significantly ($p > 0.05$).

3.4.3. Blackcurrant

Almost all of the studied analytes (except apigenin) were detected in control blackcurrant samples. Phenolic acids, i.e. 4-hydroxybenzoic acid, ferulic acid, and *p*-coumaric acid sorbed onto the 4VP copolymers less efficiently than naringenin, catechin, and rutin (Table 4). Among all the detected hydroxybenzoic acid derivatives, ellagic and gallic acid were removed in higher amounts from the fruit matrix than 4-hydroxybenzoic acid. 66.80 % and 70.09 % of ellagic acid as well as 50.79 % and 70.71 % of gallic acid were captured by poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB), respectively. Among hydroxycinnamic acid derivatives, the highest sorption was found for chlorogenic acid (62.12 % and 59.21 % on poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB), respectively), followed by caffeic acid (31.15 % and 24.82 %), *p*-coumaric acid (14.26 % and 10.86 %), and ferulic acid (9.79 % and 16.36 %). More than 60 % of all detected flavonols were captured by both polymeric sorbents. Other flavonoids (catechin and naringenin) were also effectively removed because their signal decreased by 78.81 % and 93.20 % after dSPE purification with poly(4VP-*co*-TRIM) and by 72.66 % and 94.88 % after purification with poly(4VP-*co*-14DMB). Notably, the changes between the above-mentioned data were found to be statistically significant ($p < 0.05$) for all detected analytes (Supplementary Fig. 4). Furthermore, the effectiveness of poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) did not differ significantly ($p > 0.05$), but poly(4VP-*co*-14DMB) caused a greater decrease in the signal of more than half of all analytes studied.

Table 4
Fold changes obtained for each detected analyte in the matrices of strawberry, red raspberry, and blackcurrant by LC-MS/MS.

Polyphenol	Strawberry matrix		Red raspberry matrix		Blackcurrant matrix	
	poly(4VP-*co*-TRIM)/ control	poly(4VP-*co*-14DMB)/ control	poly(4VP-*co*-TRIM)/ control	poly(4VP-*co*-14DMB)/ control	poly(4VP-*co*-TRIM)/ control	poly(4VP-*co*-14DMB)/ control
gallic acid	0.31 ± 0.14	0.36 ± 0.19	0.23 ± 0.06	0.22 ± 0.07	0.39 ± 0.22	0.29 ± 0.20
4-hydroxybenzoic acid	0.69 ± 0.17	0.67 ± 0.20	nd	nd	0.81 ± 0.29	0.83 ± 0.36
chlorogenic acid	nd	nd	0.21 ± 0.05	0.25 ± 0.06	0.38 ± 0.21	0.41 ± 0.24
catechin	0.16 ± 0.09	0.21 ± 0.10	0.21 ± 0.13	0.25 ± 0.15	0.21 ± 0.14	0.27 ± 0.20
caffeic acid	nd	nd	0.69 ± 0.24	0.63 ± 0.26	0.69 ± 0.30	0.75 ± 0.30
p-coumaric acid	0.87 ± 0.36	0.80 ± 0.31	nd	nd	0.86 ± 0.52	0.89 ± 0.43
ellagic acid	0.44 ± 0.24	0.47 ± 0.27	0.13 ± 0.22	0.11 ± 0.22	0.31 ± 0.23	0.29 ± 0.25
rutin	0.49 ± 0.19	0.48 ± 0.19	0.18 ± 0.27	0.22 ± 0.24	0.26 ± 0.16	0.26 ± 0.16
ferulic acid	nd	nd	nd	nd	0.90 ± 0.34	0.84 ± 0.38
myricetin	nd	nd	0.23 ± 0.23	0.22 ± 0.16	0.39 ± 0.37	0.27 ± 0.24
quercetin	0.13 ± 0.08	0.14 ± 0.08	0.13 ± 0.06	0.12 ± 0.08	0.32 ± 0.25	0.31 ± 0.29
naringenin	0.18 ± 0.07	0.19 ± 0.08	0.04 ± 0.03	0.05 ± 0.03	0.07 ± 0.12	0.05 ± 0.07
apigenin	0.42 ± 0.26	0.35 ± 0.21	nd	nd	nd	nd

nd-the analyte was not detected

3.5. Comparison of the effectiveness of studied polymeric materials with commercially available sorbents

Finally, we compared the purification effect of studied polymeric materials with commercially available ones. The clean-up step of fruit extracts was performed using 5 mg of PSA or GCB under the conditions applied for 4VP copolymers. Then, TAC, TPC, and TFC were measured in both initial extracts and fruit extracts treated with PSA or GCB, and the percentage of adsorbed groups of compounds was presented in Table 5.

Our results indicated that PSA was less effective in terms of polyphenols removal in comparison to the studied polymeric materials. TAC, TPC, and TFC of extracts treated with PSA decreased by 2.30–10.13 %, 2.84–9.55 %, and 7.07–8.64 %, respectively. GCB showed the highest sorption of anthocyanins and its effectiveness in anthocyanin removal was the greatest among all tested materials. However, in the case of TPC and TFC, the highest efficiency of sample purification was obtained after treatment with poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB). The results indicated that the potential of studied polymeric materials is enormous.

4. Discussion

Many factors (climate, cultivation and harvest practices, storage and processing) can cause the presence of undesired substances in the food. Therefore, fruits as well as fruit-derived products can be inspected for contamination with mycotoxins or pesticides. However, during the sample preparation step towards LC-MS/MS analysis, polyphenols (especially flavonoids) are often co-extracted with target compounds. Sample components can interfere with chromatographic analysis (strong matrix effect, peak overlap, poor sensitivity of the measurement), lower the analyte's recoveries, affect other method performances, and contaminate the instrument. The high content of polyphenolic compounds in fruit extracts and the complexity of the sample can affect the ionization process in the electrospray interface (ESI) which results in the suppression or enhancement of the analyte signal. Thus, it is essential and crucial to simplify the sample matrix composition before LC-MS/MS analysis.

Table 5
The comparison of the sorption effect of poly(4VP-*co*-TRIM), poly(4VP-*co*-14DMB), PSA, and GCB on TAC, TPC, and TFC values.

Studied parameter	Fruit	% of adsorbed groups of compounds on dSPE material			
		poly(4VP-*co*-TRIM)*	poly(4VP-*co*-14DMB)*	PSA**	GCB**
TAC	strawberry	38.47 ± 10.02	30.45 ± 8.70	10.13 ± 0.10	48.02 ± 11.16
	red raspberry	15.28 ± 4.36	15.05 ± 4.62	2.30 ± 0.93	81.44 ± 9.07
	blackcurrant	8.59 ± 3.97	10.73 ± 3.84	7.49 ± 5.01	31.15 ± 8.69
TPC	strawberry	54.77 ± 4.33	49.35 ± 7.09	9.55 ± 0.25	19.63 ± 2.22
	red raspberry	48.00 ± 12.34	46.20 ± 10.95	2.93 ± 0.52	21.78 ± 1.12
	blackcurrant	21.65 ± 6.15	21.02 ± 4.08	2.84 ± 3.87	20.43 ± 3.22
TFC	strawberry	57.61 ± 6.74	49.88 ± 7.82	8.64 ± 1.08	23.65 ± 3.96
	red raspberry	44.40 ± 7.87	41.34 ± 6.48	7.07 ± 1.28	30.20 ± 3.04
	blackcurrant	38.79 ± 6.80	38.09 ± 5.59	7.17 ± 2.89	26.73 ± 5.20

* n (number of samples) = 20
** $n = 2$

At this stage of studies, we attempted to verify the possibility of using poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) microspheres as sorbents for the dSPE purification of red and dark purple-pigmented fruits extracts. These polymers have already been applied as sorbents for ibuprofen and ketoprofen (Grochowicz et al., 2022) and to reduce matrix effect during tryptophan and kynurenine determination in human serum (Sadok, Grochowicz, & Krzyszczak-Turczyn, 2024). The sorption properties of the evaluated polymeric microspheres are influenced by the presence of the basic pyridine group (given by 4VP monomer), the carbonyl groups (from 14DMB or TRIM monomers), and aromatic phenyl ring (from 14DMB monomer) which make them hydrophobic. Furthermore, both polymers contain aromatic pyridine rings in their structure which can form the π-π interactions with the adsorbate (Sadok, Grochowicz, & Krzyszczak-Turczyn, 2024). Here, the utility and flexibility of polymers were evaluated for three model fruit matrices belonging to a different group of fruits. i.e. pelargonidin (strawberry), cyanidin/peonidin (red raspberry), and multiple anthocyanins groups (blackcurrant) due to the most abundant anthocyanin/anthocyanins in the sample (Fang, 2015). From an analytical point of view, strawberries, red raspberries, and blackcurrants belong to the group of food commodities characterized by a high acid and water content (European Commission, 2019).

Differences in the polyphenolic composition of the model fruit matrices are reflected in the determined values of TPC, TFC, and TAC for the control extracts (see dataset in the OSF repository (https://osf.io/5xrtc/). The TPC method is subjected to several interferences due to the presence of chemically similar compounds which not belong to polyphenols, for example, ascorbic acid, tyrosine, formic acid, and acetic acid (Bastola et al., 2017). To avoid making incorrect assumptions, the study enrolled fruit samples gathered from various sources (10 different suppliers for each fruit matrix evaluated). Control (untreated with 4VP copolymers) strawberry and red raspberry extracts showed similar ranges of TPC values with the most significant differences in TFC and TAC values. Blackcurrant control extracts are characterized by significantly higher TPC and TAC values and intermediate TFC values compared to other evaluated fruit matrices. These parameters changed in studied fruit matrices as follows: TPC: red raspberry < strawberry < blackcurrant; TFC: red raspberry < blackcurrant < strawberry; TAC: strawberry < red raspberry < blackcurrant. Notably, in each case, the dSPE purification with poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) caused a statistically significant reduction of TPC and TFC values but at different levels (Fig. 2B and C), which was summarized in Supplementary Fig. 5. A decrease/reduction in TAC values was also observed after dSPE clean-up using both polymeric sorbents, but statisitically significant differences compared to the control (untreated fruit extract) were confirm only for strawberry matrix (Fig. 2D). The studied 4VP copolymers proved to be the most effective at adsorbing flavonoids (~40–50 % decrease in TFC values regardless of the fruit matrix used). For the strawberry and red raspberry matrices, dSPE purification using 4VP copolymers resulted in a comparable decrease in TPC values for both fruit types (~50 %) and better anthocyanins capture in the strawberry matrix. Although the highest TPC and TAC values were noted for control blackcurrant extracts, 4VP copolymers were found to be less effective in reducing these parameters in this fruit matrix. The data suggest that individual polyphenols show different binding abilities to 4VP copolymers, which was confirmed not only by spectrophotometric measurements but also by HPLC-DAD (Table 3) and LC-MS/MS results (Table 4).

Furthermore, we compared the effectiveness of poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) microspheres in the cleaning step with two popular sorbents: PSA and GCB. PSA (3-(N,N-diethylamino)propyltrimethoxysilane) is a silica-based sorbent with ethylene diamine-N-propyl phase that contains primary and secondary amine groups (Oshita & Jardim, 2014). It can be used to remove some sugars, organic and fatty acids, pigments, and other polar components that form hydrogen bonds (Oshita & Jardim, 2014; Qi et al., 2015; Sadok et al.,

2019; Wilkowska & Biziuk, 2011). However, GCB is usually used to eliminate interferences triggered by pigments (Qi et al., 2015; Rahman et al., 2017). These mentioned commercially available sorbents are usually used to purify fruit/food extracts. Thus, they were specifically selected considering the aim of our study. Importantly, both poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) microspheres have shown great effectiveness in the reduction of TPC, TAC, and TFC compared to PSA sorbent in all evaluated berries matrices. Compared to GCB, 4VP co-polymers have shown greater potential regarding the reduction of TPC and TFC values. It seems like poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) can be applied mainly for the removal of flavonoids from fruit extracts.

This hypothesis is strongly supported by HPLC-DAD (Table 3) and LC-MS/MS results (Table 4). Depending on the evaluated fruit matrix, the decrease in signals of individual flavonoids were in the range of about 50 % to 95 %. Regarding phenolic acids, substances from the subgroup of hydroxycinnamic acid derivatives (such as caffeic acid, ferulic acid, and *p*-coumaric acid) showed weaker sorption onto the 4VP copolymers in fruit extracts compared to hydroxybenzoic acid derivatives: ellagic acid, gallic acid (except 4-hydroxybenzoic acid). These differences in the sorption behavior of the investigated polyphenols are probably related to the structure of analytes – the number and the kind of functional groups (Supplementary Table 1), and the pH of the fruit extracts.

The previous studies on ibuprofen and ketoprofen sorption have shown that 4VP-based sorbents are the most effective in an acidic environment of pH 3 (Grochowicz et al., 2022). Under these conditions, the carboxylic groups present in the drug structure were in protonated form and could interact with the sorbent through hydrogen bonding (Grochowicz et al., 2022). The pH of strawberry pulp is ~3.8, while the pH of red raspberry and blackcurrant is ~3 (Sadok et al., 2019). Moreover, in our experiment, the pH of aqueous extracts was about 3.2, 3.0, and 2.8 for strawberry, red raspberry, and blackcurrant, respectively. Thus, considering the pKa of the studied polyphenols (Supplementary Table 1) and pH of the fruit extracts, the carboxylic and hydroxyl groups should be in the protonated form, which allows polyphenols sorption onto polymeric microspheres. Differences in pH of the initial fruit extracts could also explain better sorption of some polyphenols on 4VP copolymers in red raspberry and blackcurrant matrix against strawberry one (Table 4). Furthermore, the worst sorption onto the 4VP copolymers surface was observed for analytes containing in their structure one carboxyl and one hydroxyl group (4-hydroxybenzoic acid, caffeic acid, ferulic acid) compared to analytes having multiple hydroxyl groups (e.g. chlorogenic acid, ellagic acid). A basal level of polyphenols in the control fruit extract could also impact the results. To note, poly(4VP-*co*-TRIM) was found to be slightly more effective in polyphenols removal from strawberry extracts, but poly(4VP-*co*-14DMB) from red raspberry and blackcurrant ones. However, these differences were not statistically significant. It could be explained by the different affinity of 4VP copolymers to capture individual polyphenols present in various amounts in a specific fruit matrix rather than by differences in the surface area of microspheres of both 4VP copolymers (Table 2). Future studies should be devoted to investigating key adsorption parameters (e.g. the adsorbent: solution ratio, the solvent type, pH, equilibrium time), the sorption kinetics, and adsorption isotherms for both 4VP copolymers using standard solutions containing one or the mixture of polyphenols from different classes and sub-groups followed by studies in real fruit matrix. The gained knowledge will be crucial to evaluate the adsorption mechanism of polyphenols on 4VP copolymeric microspheres.

5. Conclusions

Our study aimed to verify the effectiveness of sample pretreatment accompanied by polymeric materials with specially designed architecture towards large groups of polyphenolic compounds present in extracts from anthocyanin-rich fruits. We applied several scientific methods to establish the application potential of poly(4VP-*co*-TRIM) and poly(4VP-*co*-14DMB) microspheres as sorbents for dSPE towards LC-MS/MS determinations of other biologically important compounds. We found that the studied polymeric microspheres show a greater potential for removing flavonoids than anthocyanins. Furthermore, they could provide some benefits at the sample clean-up step compared to commercially available sorbents (PSA and GCB). In future studies, the optimization of the sorption processes towards specified flavonoids will be prepared.

CRediT authorship contribution statement

Agnieszka Krzyszczak-Turczyn: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Marta Grochowicz:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation. **Ilona Jonik:** Writing – review & editing, Investigation. **Ilona Sadok:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ilona Sadok, Agnieszka Krzyszczak-Turczyn, Marta Grochowicz have patent “Method of obtaining purified berry fruit extracts and the use of microspheres based on 4-vinylpyridine for the purification of berry fruit extracts”, P.448492 pending to The Patent Office of the Republic of Poland.

Data availability

The corresponding dataset is available in the OSF repository (https://osf.io/5xrtc/).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141312.

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