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Original Paper

RECOVERY OF SUCCINIC ACID-RICH FRACTION FROM THE JUICE OF *CHAENOMELES JAPONICA* (THUNB.) LINDL. EX SPACH BY ION EXCHANGE FRACTIONATION RESIN

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*Succinic acid (SA) is one of the most demanded and widely used constituents for producing various healthcare and skincare products, biodegradable polymers, and food and feed additives. The present study was undertaken to establish the protocol for selective recovery of SA from a complex matrix of Japanese quince (JQ) (*Chaenomeles japonica*) fruit using ion-exchange fractionation resin DIAION™ UBK550 with negatively charged functional groups cross-linked with a styrene-divinylbenzene matrix. A second SA purification method was used for data comparison using either Supel™-Select HLB or Strata-X columns packed with a hydrophilic modified styrene-based polymer. The analysis of SA accomplished by multiple reaction monitoring (MRM) using triple quadrupole mass selective detection (HPLC-ESI-TQ-MS/MS) revealed the presence of SA in the JQ juice without pretreatment, in the amount of 0.202 mg·100 ml^{-1}. The use of styrene-based polymer Strata-X sorbent for purification of JQ juice led to an increase of 11.0% in the concentration of SA compared with the initial concentration of SA in JQ juice. On the other hand, after purification of JQ juice with Supel™-Select HLB, the concentration of desorbed SA in the eluate fraction was 7.8% lower compared to the initial concentration. The use of ion-exchange fractionation resin DIAION™ UBK550 for purification and concentration of JQ juice ensured up to 152.3 mg of SA in 100 ml^{-1} of syrup-like brown substance, which was much (754.4-fold) higher than that observed in JQ juice itself. All three sorbents ensured the complete removal of mono- and disaccharides from the juice, as no signs of mono- and disaccharides were detected in the eluate fractions.*

Keywords: *bio-based succinic acid, cation exchanger, purification, Japanese quince fruit.*

INTRODUCTION

According to recent reports, the global value of succinic acid (SA) was 131.7 million USD in 2018 and is projected to reach 203.8 million USD by 2026, growing at a compound annual growth rate (CAGR) of 6.8% during the next years (Succinic Acid Market 2017, 2017). A wide range of industrial applications in manufacturing biodegradable polymers, pharmaceuticals, skin and body care formulations, chemicals and food products (Omwene *et al.,* 2021) explains the widespread demand for this multi-functional active pharmaceutical ingredient (API) (Kumar *et al.,* 2020). SA $(CH_2)_2(CO_2H)_2$) (from Latin *succinum*, amber), also known as butanedioic acid, for the first time was isolated as far back as almost five centuries ago in 1546 by Georgius Agricola as a distillate from Baltic amber (Duffin, 2015). Currently, the market offers a wide r ange of SA-based dietary supplements produced through chemical synthesis, and synthetic SA does not seem to have side effects associated with drugs (Ives *et al.,* 2020). However, given the negative impact on human health and the environment and relatively high costs, SA production via chemical routes raises concerns (Merrylin *et al.,* 2020). Furthermore, with technological advancements in analytical chemistry

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and understanding of biochemical reactions, SA production from renewable energy sources is gaining attention for its environmental friendliness (Ives *et al.,* 2020; Ladakis *et al.,* 2020). High production yields of SA and relative lack of side products (contaminants) can be achieved via biorefining of lignocellulose materials by a wide range of microorganisms, such as *Actinobacillus succinogenes* (Salvachúa *et al.,* 2016), *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens*, and various strains of recombinant *Escherichia coli* (Ghayur *et al.,* 2019). Biorefining is an environmentally friendly alternative with a safer profile than conventional production and could represent the future of sustainable SA industrial-scale production. However, as in organic synthesis, bio-based production of SA requires high initial investments in industrial equipment, which explains the relatively high price for this niche product compared to fossil-driven SA (Dickson *et al.,* 2021).

The presence of SA has been documented in a range of fruit and their juices after either liquid-liquid or solid-liquid extraction — these include *Eugenia myrcianthes* (Arena *et al.,* 2023), *Citrus limetta*, *C. aurantifolia*, *C. nobilis*, *C. karna*, *C. medica*, *C. ichangensis*, *C. aurantium* (Sharma *et al.,* 2018), pears (*Pyrus communis* L.), apples (*Pyrus malus*), rhubarb (*Rheum rhabarbarum*) and Hawthorn fruit (*Crataegus curvisepala*) (Cinkmanis *et al.,* 2019), demonstrating diverse fluctuations in concentrations from mg to g per 100 g/ml of sample. Moreover, the fruit of Japanese quince (*Chaenomeles japonica* (Thunb.) Lindl. ex Spach) (JQ) was highlighted as the richest source of organic acids, including malic, quinic, and succinic acids (Granados *et al*., 2003; Antoniewska *et al*., 2019; Hellín *et al*., 2020). Due to the immeasurable effort contributed by breeders Silvija Ruisa and Edīte Kaufmane from the Institute of Horticulture (LatHort) to the field of fruit growing, the planting area of JQ in Baltic countries has been raised four times (Juhnevica-Radenkova *et al.,* 2022). This can be additionally reinforced by data from FAOSTAT 2023, indicating the increase in JQ (including *Cydonia oblonga*, *Chaenomeles japonica*) production quantities in the last five decades by 133.7%, from 298.5 thousand tons in 1961 to 697.5 thousand tons in 2021 (FAOSTAT, 2023). Given the relative availability of bioactives in the fruit of JQ, the present work was undertaken to establish the protocol of selective recovery and analysis of organic acids, SA in particular, through the utilisation of ion exchange resin and the HPLC-ESI-TQ-MS/MS approach, respectively.

MATERIALS AND METHODS

Chemicals and reagents. Commercial succinic acid (SA) standard was purchased from Merck KGaA (Darmstadt, Germany). Methanol (MeOH) and formic acid (HCOOH) (puriss r.a.) of liquid chromatography-mass spectrometry (LC-MS) grade were obtained from the same source. The ion-exchange fractionation resin DIAION™ UBK550 C was provided in kind by the company Mitsubishi Chemical Corporation (Tokyo, Japan) for laboratory purposes. Supel™-Select HLB and Strata-X columns were acquired from Sigma-Aldrich Chemie Ltd., (Steinheim, Germany). Ammonium hydroxide solution (NH_4OH) (25% v/v) was obtained from Chempur (Piekary Śląskie, Silesia, Poland). Hydrochloric acid (HCl) (37% v/v) was purchased from VWR™ International, GmbH (Darmstadt, Germany). Ultrapure water was produced using the reverse osmosis PureLab Flex Elga water purification system (Veolia Water Technologies, Paris, France). Solutions used for washing up and desorption of SA from the cation-exchange resin were as follows:

- Buffer A 10 mM hydrochloric acid solution (pH 2.04);

- Buffer B 2 M ammonium hydroxide solution (11.74).

Preparation of succinic acid for HPLC-ESI-TQ-MS/MS analysis

Purification with cation-exchange resin. Isolation and purification of SA from the matrix of Japanese quince (JQ) juice (*Chaenomeles japonica*) were performed following the ion-exchange principle utilising DIAION™ UBK550 fractionation resin with negatively charged functional groups cross-linked with styrene-divinylbenzene matrix for adsorption of SA below isoelectric point. For this purpose, 60.0 ml of JQ with accuracy ± 0.1 ml was transferred into a 200.0 ml beaker, and 60.0 ml of deionised H_2O was added. Then, the mixture was decolorised by activated carbon during continuous vigorous agitation at 700 rpm and 30.0 ± 1.0 °C for 60 min. The mixture was then centrifuged at 10,280 × g for 10 min at 20.0 ± 1.0 °C in a Hermle Z 36 HK centrifuge (Hermle Labortechnik, GmbH, Wehingen, Germany). The supernatant was decanted and filtered through a 0.20 μm hydrophilised polytetrafluoroethylene (H-PTFE) membrane filter (Macherey-Nagel GmbH & Co. KG, Dueren Germany). Conditioning/equilibration of ion-exchange fractionation resin DIAION™ UBK550 (column volume 12.0 ml) packed with 2.2 mg resin containing negatively charged functional groups (sulfonic acid) was conducted using 24.0 mL buffer A solution at a rate of 1.0 $ml \cdot min^{-1}$ under pressure –1.0 ± 0.1 ″Hg ensured by a 12 port Chromabond solid-phase extraction (SPE) system (Macherey-Nagel GmbH & Co. KG, Dueren Germany) coupled with N 820 LABOPORT vacuum pump (KNF Neuberger AB, Stockholm, Sweden). The loaded sample was washed with 24.0 ml H_2O and the flow-through fraction was collected to estimate the presence of SA. For desorbing SA from the stationary phase of cation-exchange resin, a 24.0 ml buffer B solution was used. The collected eluate fraction was subjected to drying using a "Laborota 4002" rotary evaporator (Heidolph, Swabia, Germany) at 40 ± 1.0 °C and 60.0 ± 2.0 mBar. The obtained dry residues were kept at –18.0 ± 1.0 °C until further analysis and use, for a maximum period of 24 h. A schematic representation of the sample preparation steps is depicted in Figure 1.

Purification with Supel™-Select HLB and Strata-X columns. An alternative SA purification method used for comparative purposes was Supel™-Select HLB or Strata-X columns packed with a modified styrene-based polymer.

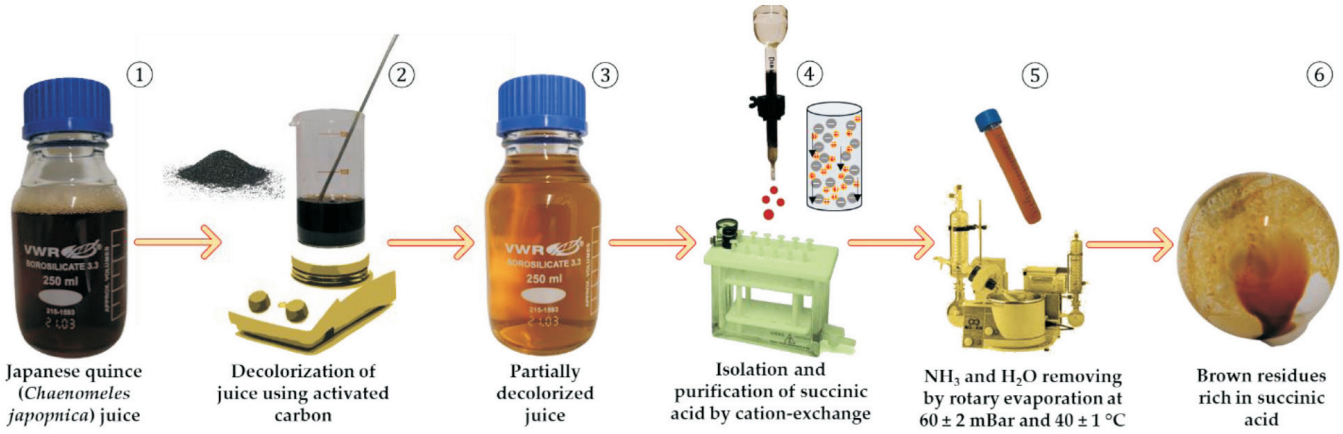


Fig. 1. A schematic representation of the sample preparation steps undertaken for the purification and concentration of succinic acid from the juice of Japanese quince.

Sample preparation for the solid-phase extraction (SPE) was done following the procedure described above for SPE with cation-exchange resin (please refer to Section Purification with Cation-Exchange Resin). The SA isolation was performed according to the protocol developed by Phenomenex and Supelco with some modifications. Briefly, conditioning and equilibration of both sorbents were done using a one-column volume (3.0 ml) of pure MeOH, followed by one-column volume of deionised H_2O. The loaded analytes (3.0 ml) were washed with a two-column volume of deionised H_2O water. The flow-through fraction was collected for qualitative and quantitative chromatographic analysis of SA. To ensure a constant flow of eluate at 1.0 ± 0.2 ml·min^{-1} during SA desorption, a "Chromabond® SPE" (Düren, Germany) SPE vacuum manifold with adjusted pressure —1.0 ± 0.1 ″Hg was applied. The 1.0 ml acidified 100% MeOH solution (MeOH: HCOOH ratio 99:2 v/v) was used as an eluate for desorbing SA from polymers. The resulting fraction was collected and analysed with an HPLC-ESI-TQ-MS/MS HPLC system.

The HPLC-ESI-TQ-MS/MS analytical conditions for analysis of succinic acid. The chromatography analysis of JQ-derived SA (Fig. 2) was conducted using a "Shimadzu Nexera UC" series liquid chromatography (LC) system (Shimadzu Corporation, Tokyo, Japan) coupled to a triple quadrupole mass-selective detector (TQ-MS-8050, Shimadzu Corporation, Tokyo, Japan) with an electrospray ionisation interface (ESI). A sample of 3.0 µl was injected onto a reversed-phase Shim-pack UC-RP column (5.0 µm, 250 × 4.6 mm; Tokyo, Japan) operating at 45.0 °C and a flow rate of 1.0 ml·min^{-1}. The mobile phases used were acidified H_2O (1.0% HCOOH v/v) (A) and acidified MeOH (1.0% HCOOH v/v) (B) in isocratic mode. The MeOH injections were included as a blank run after each sample to avoid the carry-over effect. Data were acquired using the LabSolutions Insight LC-MS version 3.7 SP3 (workstation) (Shimadzu Corporation, Tokyo, Japan), which was also used for instrument control and processing. Ionisation in negative ion polarity mode was applied in this study, while data were collected in profile and centroid modes, with a data storage threshold of 5000 absorbance for MS. The operating conditions were as follows: detector voltage 1.98 kV, conversion dynode voltage 10.0 kV, interface voltage 3.0 kV, interface temperature 300 °C, desolvation line temperature 250 °C, heat block temperature 400 °C,

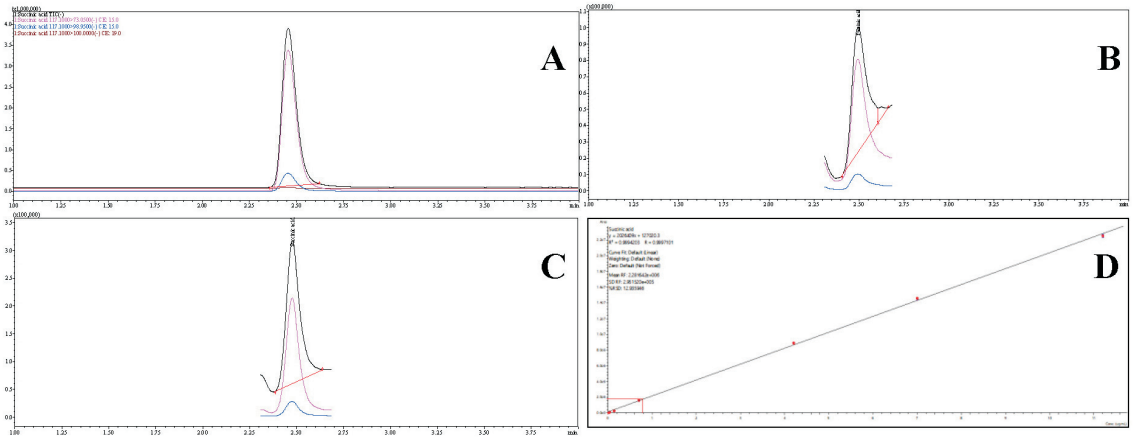


Fig. 2. Selective ion chromatogram of multiple reaction monitoring given for succinic acid standard at the concentration of 0.0122 mg·ml^{-1} (**A**), Japanese quince juice sample before solid-phase extraction (purification) (**B**) and after solid-phase extraction with DIAION™ UBK550 (**C**); The calibration curve plotted from the analytical standard used for determination of succinic acid in the Japanese quince juice samples (**D**). Samples injection volume 3.0 ul (0.037 µg·ml^{-1}).

nebulising gas argon (Ar, purity 99.9%,) at flow 3.0 l min^{-1}, heating gas carbon dioxide (CO_2, purity 99.0%) at flow 10.0 l·min^{-1}, and drying gas nitrogen (N_2, separated from air using a nitrogen generator system from Peak Scientific Instruments Ltd. (Inchinnan, Scotland, UK), purity 99.0%) at flow 10.0 l·min^{-1}. SA was observed in the programmed and optimised multiple reaction monitoring (MRM) mode. The MRM transitions, collision energy, Q1, Q3, and dwell time for SA are depicted in Table 1.

Preparation of standard stock solution. Stock solution containing 1.4 mg SA was prepared in 10.0 ml 100% acidified MeOH solution (MeOH:HCOOH ratio 99:1 v/v). SA was quantified by injecting 3.0 µl at 10 °C of calibration solution with the range from 0.0175 to 11.2 µg·ml^{-1}. The regression coefficient and values for the limit of detection (LOD) and limit of quantification (LOQ) are given in Table 1. The working solution was prepared immediately before being used.

RESULTS

The concentrations of SA in Japanese quince juice obtained using the applied preparation methods are summarised in Table 2. This experiment subjected the JQ to SPE purification using strongly acidic DIAION™ UBK550 ion-exchange fractionation resin. The sorbent selection was based on availability, effectiveness, and simplicity of use since no specific activation apart from conditioning with mild HCl solution was required. No sample acidification prior to SA purification in ion-exchange resin was done since the natural pH of the JQ juice is 2.5–3.0, and most SA is in free form.

The solubility of SA can vary depending on the solvent and the concentration used. Therefore, the removal of sugars and other small molecules present in JQ was accomplished in two steps: activated carbon in the presence of deionised water, followed by washing loaded samples onto sorbents with deionised water. The purity of SA in the collected fraction was studied chromatographically by assessing the presence of side organic acids and mono- and disaccharides.

Preliminary analysis revealed a relatively high concentration of SA in JQ (Table 2). Given the prevalence of other organic acids, such as malic acid and, to a lesser extent, quinic acid, the authors propose the use of JQ in the same way as lemon juice, which could bring additional preservative properties to the food products and maintain its quality during shelf-life. Considerable differences in SA concentration found in JQ may be due to meteorological and agricultural practice and pretreatment techniques applied for the sample preparation and analysis. It is worth noting that during HPLC-ESI-TQ-MS/MS analysis, a relatively good baseline and resolution of SA was achieved (Fig. 2A and 2C) after JQ juice purification with DIAION™ UBK550 fractionation resin. The use of styrene-divinylbenzene-based reversed-phase Strata-X sorbent led to a substantial increase in the concentration of SA, since the amount in the eluate fraction was 11.0% higher than the initial concentration in JQ juice. The concentration of SA in the eluate fraction obtained after SPE with a Supel™-Select HLB delivered a relatively lower amount of SA than Strata-X, since a 7.8% loss of this organic acid was observed compared with the initial concentration. However, it is worth noting that in both cases, no presence of mono- and disaccharides was detected in the eluate fractions.

Table 1. Multiple reaction monitoring succinic acid transitions and corresponding collision energy, Q1, Q3 and dwell time for investigated organic acid

	Optimised MRM parameters								Parameters of calibration			
Organic acid	RT, min	Molecular formula	Ionization mode	MRM transitions	Q1 Pre Bias, V	Collision energy, V	Q3 Pre Bias, V	Dwell time, msec	R^2	RSD, %	LOD, µg·ml^{-1}	LOQ, µg·ml^{-1}
Succinic acid	2.451	$C_4H_6O_4$	$[M–H]^-$	117.1000→ 73.0500	13.0	15.0	13.0	100.0	0.9994	12.93	0.44	1.34
				117.1000→ 98.9500	14.0	15.0	18.0	100.0				

Note: The first MRM succinic acid transitions found were used for quantitative analysis, while the second for qualitative. LOD – limit of detection; LOQ – limit of quantification; RT – retention time; µg – microgram.

Table 2. Concentration of succinic acid in fractions obtained after purification and pre-concentration of Japanese quince juice using three types of sorbents

Organic acid	Mw	Average concentration, mg·100 ml^{-1}			
		1	2	3	4
Succinic acid	118.09	0.202 ± 0.002	0.224 ± 0.024	0.186 ± 0.018	152.3 ± 7.9

Note: Values are means ± SD of duplicate (n = 2). 1 – Japanese quince juice without pretreatment (control), the results expressed in fresh weight; 2 – solid-phase extraction (purification) using styrene-divinylbenzene-based reversed-phase Strata-X (Phenomenex, Torrance, CA, USA) column packed with a sorbent particle size 33 µm, 85 Å, 30 mg 3 ml^{-1}; 3 – solid-phase extraction (purification) using modified styrene-divinylbenzene-based Supel™-Select HLB (Supelco, Bellefonte, PA, USA) column packed with a sorbent particle size of 50–70 µm, 80–200 Å, 60 mg·3 ml^{-1}; 4 – solid-phase extraction (purification) using strongly acidic gel-type poly (styrene-divinylbenzene) cation-exchange DIAION™ UBK550 (Mitsubishi Chemical Corporation, Tokyo, Japan) fractionation resin with a particle size 220 µm, 60 Å, 2.2 mg·12 ml^{-1}.

The yield of the syrup-like brown substance obtained from 60.0 ml of JQ utilising cation-exchange resin made up to 75.7 mg, and this is the only method that delivered a sufficient amount of SA. The concentration of SA in the syrup-like brown substance amounted to 152.3 mg 100 ml^{-1}, which was much (754.4-fold) higher than that observed in JQ. SPE with cation-exchanger, similar to the former two sorbents, ensured 100% removal of mono- and disaccharides (data not shown). To reach the desired purity of SA, a further purification process can be done by directly crystallising SA at 4 °C using a crystalliser, as proposed by Omwene *et al.* (2021) and Xiao *et al.* (2020).

DISCUSSION

In this study, succinic acid (SA) was recovered from the juice of Japanese quince (*Chaenomeles japonica* (Thunb.) Lindl. ex Spach) grown in the orchard of the Institute of Horticulture (LatHort), Latvia. One downstream purification process was developed using activated carbon and strongly acidic DIAION™ UBK550 cation-exchange fractionation resin for decolorisation of JQ juice and recovery of SA from the matrix, respectively. The earlier report of (Benedict *et al.,* 2006) indicated that the solubility of SA in water is insufficient to use as a solvent for preparing a reaction mixture at ambient temperature; therefore, 2 M NH_4OH was used as eluent to desorb retained SA from the cation-exchange fractionation resin. Other researchers (Fechter *et al.,* 1996; Cukier *et al.,* 2018; Xiao *et al.,* 2020) have also made a reasonably similar observation, highlighting the confident effectiveness of 2 M NH_4OH as a solvent in removing carboxylic acids and amino acids from a variety of industrial sources. Up to 152.3 mg·100 ml^{-1} of SA in the syrup-like brown substance was eluted from the cation-exchange fractionation resin. Interestingly, the observed concentration of SA found in the JQ syrup-like brown substance was substantially higher than that detected in raw juices of 19 *Chaenomeles japonica* genotypes, as reported by (Hellín *et al.,* 2020), demonstrating fluctuation over the range from 0.004 to 0.012 mg·100 ml^{-1}. An alternative SA purification method wherein a Supel™-Select HLB or Strata-X columns packed with a modified styrene-based polymer was used for comparative purposes. However, the concentration of SA in eluate fractions obtained was close to those detected in Japanese quince juice without pretreatment. Generally, all three sorbents ensured the complete removal of mono- and disaccharides from the eluate fraction. However, the increased viscosity of syrup-like brown substance obtained by DIAION™ UBK550 cation-exchange resin implies the presence of a high pectin content and other molecules having net positive surface charges at low pH. A further purification process can be done by direct crystallisation of SA to reach the desired purity of SA.

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AR DZINTARSKĀBI BAGĀTĀS FRAKCIJAS ATGŪŠANA NO KRŪMCIDONIJU *CHAENOMELES JAPONICA* (THUNB.) LINDL. EX SPACH SULAS, IZMANTOJOT JONU APMAIŅAS FRAKCIONĒŠANAS SVEĶUS

Dzintarskābe (SA) ir viena no vispieprasītākajām sastāvdaļām, ko plaši izmanto dažādu veselības aprūpes un ādas kopšanas līdzekļu, bioloģiski noārdāmu polimēru, kā arī pārtikas un dzīvnieku barības piedevu ražošanā. Šis pētījums tika veikts, lai izstrādātu protokolu SA selektīvai atgūšanai no krūmcidonijas (JQ) (*Chaenomeles japonica*) augļu kompleksās matricas, izmantojot jonu apmaiņas frakcionēšanas sveķus DIAION™ UBK550 ar negatīvi lādētām funkcionālām grupām, kas šķērssavienotas ar stirola-divinilbenzola matricu. Datu salīdzināšanai tika izmantota otra SA attīrīšanas metode, izmantojot Supel™-Select HLB vai Strata-X kolonnas ar hidrofīlu modificēta stirola polimēra pildījumu. SA analīze, kas veikta, izmantojot jonu pāreju monitoringa režīmu (MRM), izmantojot trīskāršā kvadrupola masselektīvo detektoru (HPLC-ESI-TQ-MS/MS), atklāja SA klātbūtni JQ sulā bez iepriekšējas apstrādes 0,202 mg·100 ml^{-1} apmērā. Uz stirola bāzes izgatavota polimēra Strata-X sorbenta izmantošana JQ sulas attīrīšanai nodrošināja SA koncentrācijas paaugstināšanos par 11,0%, salīdzinot ar sākotnējo SA koncentrāciju JQ sulā. Savukārt pēc JQ sulas attīrīšanas ar Supel™-Select HLB, eluāta frakcijā desorbētas SA koncentrācija bija par 7,8% zemāka, salīdzinot ar sākotnējo SA koncentrāciju. Jonu apmaiņas frakcionēšanas sveķu DIAION™ UBK550 izmantošana JQ sulas attīrīšanai un koncentrēšanai nodrošināja līdz 152,3 mg SA 100 ml^{-1} sīrupam līdzīgas brūnās masas, kas ir 754,4 reizes vairāk nekā atrasts JQ sulā. Visi trīs sorbenti nodrošināja pilnīgu mono- un disaharīdu atdalīšanu no JQ sulas, jo eluāta frakcijā netika konstatētas mono- un disaharīdu pazīmes.