

Extraction of flavonoids from the rind of *Passiflora edulis* f. *Flavicarpa* by pressurized solvent extraction and aqueous two-phase systems

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Abstract

As the rind of passion fruit is rich in flavonoids, it can be used as an alternative to obtain these compounds. This study extracted flavonoids from passion fruit rinds by pressurized solvent extraction. The obtained extract was submitted to aqueous two-phase extraction formed by ethanol and salts for the recovery of flavonoids. Passion fruit rind (PFR) was submitted to pressurized solvent extraction (PSE) using ethanol/water solution at 60:40 (v/v) as a solvent for the extraction of bioactive compounds, showing a polyphenol content of $5.48 \text{ gGAE} \cdot \text{kg}^{-1}$ PFR. Then, aqueous two-phase systems (ATPS) showed that flavonoids and sugars partitioned into opposite phases. The recovery of flavonoids in the top phase was above 98% w/w, and the recovery of sugars in the bottom phase was above 97% w/w. LC–MS was used to evaluate the flavonoid profile in the top phase of ATPS, and the major flavonoids detected were lucenin-2, vicanin, isoorientin, apigenin, schaftoside and vitexin. According to the flavonoid profile by LC–MS and the values of recovery of flavonoids, ATPS proved to be an effective technique for recovery of flavonoids from PSE extraction.

INTRODUCTION

Passion fruit (*Passiflora edulis* f. *flavicarpa*) is a tropical fruit belonging to the family *Passifloraceae*, native to Brazil and widely consumed due to its health benefits and flavor (Nascimento et al. 2012; Oliveira et al. 2012; Wijeratnam 2016). Passion fruit juice is commonly used in the food industry, and its rind represents almost 50% of the fruit. Approximately 40 to 60% of its weight is wasted during passion fruit juice processing, including the rinds (Zeraik et al. 2012; Viganó et al. 2016).

Flavonoids such as C-glycosylflavones are the significant pulp phenolics of *Passiflora edulis*⁵, isoorientin and isovitexin were found in passion fruit pulp (Zeraik and Yariwake 2010), and lucenin-2, vicanin-2, isoorientin, isovitexin, luteolin-6-C-chinovoside, and luteolin-6-C-fucoside were detected in *Passiflora edulis* f. *flavicarpa* (Li et al. 2011). The chemical composition of the rind has the highest nutrient content of all parts of passion fruit and is rich in pectin and soluble and insoluble fibers (Zeraik et al. 2012; Wijeratnam 2016; Souza et al. 2017). In addition to pectin and other polysaccharides, phenolic compounds belonging to the class of C-glycosyl flavonoids are present in passion fruit rind (Viganó and Martinez 2015). Orientin-7-O-glucoside, leucenin II, vicanin II, luteolin-(6-C-pentosyl)-8-C- β -D-glucopyranoside isomer, isoschaftoside, isoorientin, orientin, apigenin-7-rutinoside⁴-methoxyluteolin-8-C- β -D-glucopyranoside, and vitexin were found in extracts of the rind of *Passiflora edulis* obtained by pressurized solvent extraction (PSE) by Souza et al. (2017).

Flavonoids are secondary metabolites synthesized by higher plants whose basic structure is a C-6 (A ring)-C-3 (C ring)-C-6 (B ring) carbon skeleton (He and Giusti 2010), and they have biological activities such as antioxidant (Wang et al. 2015), anxiolytic and sedative (Deng et al. 2010), antifungal (Miles et al. 2013), anticancer (Kumar et al 2013), and antihyperglycemic (Janibekova et al. 2018) activities. *Passiflora* compounds are potentially effective drugs in COVID-19 treatment, and among these compounds, flavonoid ligands bind to the main protease of COVID-19 (Prasansuklab et al. 2021). Flavonoids can reduce inflammation and immune responses in lung infection (Messina et al. 2020). Therefore, they have been studied in recent years as potential drugs against SARS-CoV-2 for the treatment of COVID-19 (Bhati et al. 2021). They present anti-inflammatory and antiviral properties (Jain et al. 2021).

Flavonoids may be extracted by conventional heat-reflux extraction with nonpolar (chloroform) and polar solvents (ethanol, methanol, water), microwave-assisted solid–liquid extraction (Krishnan and Rajan 2016), extraction solvent with EtOH:H₂O (1:1, v/v) (Echeverry et al. 2017), and extraction with ethyl acetate, acetone and methanol using a Soxhlet extractor (Arora and Itankar 2018). Those are time-consuming, and some of them have a high cost. For this reason, techniques such as pressurized solvent extraction using ethanol and water as solvents⁸, aqueous two-phase systems formed by alcohol and salts (Liu et al. 2013; Zhang et al. 2013; Yang et al. 2020), and homogenizer-assisted (HAE), ultrasound-assisted (UAE), and microwave-assisted (MAE) extraction techniques using renewable solvents (Francischini et al. 2020) have been used for the extraction or partitioning of flavonoids.

Pressurized solvent extraction is a technique that uses high temperatures and pressure with liquid solvents to obtain extracts quickly, and fewer solvents are used in the extraction (Arapitsas and Turner 2008; Souza et al. 2017). Due to high pressure, solvents can be used above their boiling point, accelerating the extraction process. High temperature increases solubility and diffusion rates, in addition to decreasing solvent viscosity and weakening solute-matrix interactions (Jentzer et al. 2015). PSE reduces the extraction time and the amount of solvent used (Mustafa and Turner 2011).

The aqueous two-phase system (ATPS) is an effective, versatile, and greener technique to extract or partition bioactive compounds. It occurs when different chemical species, mixed at certain compositions, are immiscible and separate into two phases (Liu et al. 2013). ATPSs formed by alcohol and inorganic salts have been used to extract biological compounds such as flavonoids due to the lower viscosity of phase-forming components facilitating phase separation. This reduces process time, achieving a high recovery in a single step, low cost, easy to scale-up, and mild conditions (Liu et al. 2013; Zhang et al. 2013; Wu et al. 2014).

The present work aimed to extract flavonoids from the rind of *Passiflora edulis* f. *flavicarpa* (an agro-industrial waste) by pressurized solvent extraction, followed by an ethanol/salt aqueous two-phase system (ATPS) to recover flavonoids from the PSE passion fruit rind extract. The flavonoids obtained after ATPS extraction were identified by liquid chromatography–mass spectrometry (UPLC-QTOF).

MATERIALS AND METHODS

Chemicals

Ethanol was obtained from Dinâmica (Brazil, purity 99.5%, PubChem CID: 702), ammonium sulfate ((NH₄)₂SO₄) from Synth (Brazil, purity 99.5%, PubChem CID: 6097028), dibasic potassium phosphate (K₂HPO₄) from Vetec (Brazil, purity 99.5%, PubChem CID: 24450), glucose from Vetec (Brazil, purity 99.5%, PubChem CID: 107526), and gallic acid from Sigma–Aldrich (St. Louis, MO, USA, purity ≥ 98.5%, PubChem CID: 370). All chemicals were of analytical grade. Deionized water was used in the preparation of stock solutions of salts and was used for all experiments.

Sample preparation

Yellow passion fruits (*Passiflora edulis* F. *Flavicarpa*) were obtained from an organic orchard in Fruitessence Frutas Orgânicas, located at Maracanau, Ceará, Brazil. The rinds were chopped into small pieces and dried at 60°C for 48 h in an air circulation oven and mill grounded (Wiley, BT 602 model).

Pressurized solvent extraction

First, the bioactive components were extracted by pressurized solvent extraction (Souza et al. 2017). Briefly, a pressure of 10.342 to 11.721 MPa, temperature 80°C, rinse 5 min, extraction time of 10 min for each cycle, and purge 200 s. The sample (15 g) was weighed and added to 30 g of diatomaceous earth and placed in a stainless-steel extraction cell (66 mL) from an automatic extractor (ASE 350 Dionex, Sunnyvale, CA, USA). Ethanol/water solutions at 60:40 (v/v) were used as solvents. Three extraction cycles per sample were performed, and the extracts were combined and concentrated under reduced pressure.

Aqueous two-phase systems

The aqueous two-phase systems (ATPS) formed by ethanol/(NH₄)₂SO₄ and ethanol/K₂HPO₄ were prepared according to predetermined phase diagrams reported in the literature (Katayama and Miyahara 2006; Wang et al. 2010). The systems were prepared by weighing appropriate forming components in 50 mL graduated Falcon tubes and passion fruit rind extract from pressurized solvent extraction. The mixture was stirred for 1 min in a thermostatic bath at 25°C until phase separation after 24 h. The top phase (ethanol rich phase) and bottom phase (salt rich phase) had their volumes noted on the Falcon tube scale and analyzed for total polyphenol and total sugar concentrations. Partition of flavonoids from PSE extract was evaluated at different ethanol and salt concentrations. The chosen interval was small since the phase diagrams of ethanol/(NH₄)₂SO₄ and ethanol/K₂HPO₄ systems reported in the literature had a small biphasic region.

Performance parameters of ATPS

Total polyphenols were measured by the Folin Ciocalteu method (Larrauri et al. 1997) with some modifications described as follows. An aliquot of sample was added to 1 mL of Folin-Ciocalteu reagent, 2 mL of anhydrous sodium carbonate (20%) and 2 mL of distilled water. The tube was shaken and left to stand in the dark for 30 min. Absorbances were measured in a UV spectrophotometer (Cary 300; Varian, Palo Alto, CA, USA) at 735 nm using gallic acid as a standard.

The phenol–sulfuric acid method was used to analyze the total sugar concentration. Glucose was used as a standard, and the absorbance was measured at 490 nm (Dubois et al. 1956).

Recovery of flavonoids (R_F) and recovery of sugars (R_S) were calculated. The following equation (Cheng et al. 2017) was used:

$$R(\%) = \frac{C_T \times V_T}{C_T \times V_T + C_B \times V_B} \times 100$$

1

where C_T is the concentration of flavonoids or sugars in the top phase, V_T is the volume of the top phase, C_B is the concentration of flavonoids or sugars in the bottom phase, and V_B is the volume of the bottom phase.

The partition coefficient of flavonoids (K_F) and partition coefficient of sugars (K_S) was the ratio of the concentration of flavonoids or sugars in the top and bottom phases, according to the equation below:

$$K = \frac{C_T}{C_B}$$

2

LC–MS analysis

An Acquity® UPLC system coupled to a Quadruple Time-of-Flight (QTOF) system (Waters Corporation, Milford, USA) was used for the identification of flavonoids. The chromatographic conditions were a Waters Acquity® UPLC BEH column (150 × 2.1 mm, 1.7 μm), with a fixed temperature at 40°C,

mobile phases water with 0.1% formic acid (A) and acetonitrile with 1% formic acid (B), gradient ranging from 2–95% B by 15 min, flow rate of 0.4 ml/min and injection volume of 5 µl. The range of ESI in negative mode was 110–1180 Da with a fixed source temperature of 120°C, and the desolvation temperature and desolvation gas flow were 350°C and 500 L/h, respectively. An extraction cone of 0.5 V and 2.6 kV capillary voltages. Leucine enkephalin was used as a lock mass. MSE (high energy mass spectrometry) was the mode of acquisition, and the instrument was controlled by Masslynx® 4.1 software (Waters Corporation, Milford, USA). Flavonoids were tentatively identified compared with data from the literature based on their exact mass.

RESULTS AND DISCUSSION

Recovery of flavonoids from PSE

The solubility of phenolic compounds depends on the polarity of the solvent and affects their recovery (Naczek and Shahidi 2006; Martinez et al. 2012). A previous study extracted flavonoids from yellow passion fruit rind using PSE and concluded that the ethanol/water solution presented higher values of polyphenol content than methanol/water solution. According to the authors, the best proportion of solvent was an ethanol/water solution 60:40 (v/v), which presented a polyphenol content of 4.67 gGAE•kg⁻¹ PFR (Souza et al. 2017). Thus, this proportion was used in this study. The polyphenol content of the PSE extract was 5.48 g GAE•kg⁻¹ PFR, and the recovery of flavonoids from passion fruit rind extract from pressurized solvent extraction with ethanol/water solution at 60:40 (v/v) as solvent was 30.22% ± 0.56.

Effect of phase forming salt

To evaluate the phase-forming salt, experiments were carried out with (NH₄)₂SO₄ and K₂HPO₄ (Table 1). The high partition coefficient values of flavonoids (K_F) indicate that flavonoids preferentially entered the top phase. At the same time, sugars migrate to the bottom phase, resulting in a very low partition coefficient of sugars (K_S). This might be explained by the fact that as sugar molecules are more polar, they have higher affinity to the hydrophilic (salt-rich) phase due to the establishment of a vast number of hydrogen bonds with water (Reis et al. 2013). The recovery of flavonoids (R_F) in the top phase remained the same in both systems, and the recovery of sugars (R_S) in the bottom phase had a slight difference. Thus, further experiments were carried out with both systems.

Table 1
Effect of ammonium sulfate and dibasic potassium phosphate on the partitioning of flavonoids from passion fruit extract

ATPS	Ethanol (%, w/w)	Salt (%, w/w)	K _F	R _F in top phase (%, w/w)	K _S	R _S in bottom phase (%, w/w)
Ethanol/(NH ₄) ₂ SO ₄	30.14	18.50	184.15	99.62 ± 0.22	0.02	97.25 ± 0.31
Ethanol/K ₂ HPO ₄	21.26	22.03	106.86	99.21 ± 0.04	-	98.57 ± 0.33

Influence of phase-forming component concentrations on the partitioning of flavonoids and sugars from passion fruit rind extracts

The effect of ethanol concentration was studied, varying from 26.50 to 32.19% w/w in ATPS ethanol/(NH₄)₂SO₄ and from 19.90 to 25.30% w/w in ATPS ethanol/K₂HPO₄. The results are shown in Table 2. Recovery of flavonoids in the top phase was kept at 100% w/w while varying the ethanol concentration in ATPS ethanol/(NH₄)₂SO₄ and remained practically the same (> 99% w/w) in ATPS ethanol/K₂HPO₄, not influencing the partition of flavonoids.

Table 2
Effect of variation of ethanol concentration on recovery of flavonoids (R_F) in the top phase and recovery of sugars (R_S) in the bottom phase

ATPS	Ethanol (%, w/w)	Salt (%, w/w)	R_F in top phase (%, w/w)	R_S in bottom phase (%, w/w)
Ethanol/ $(\text{NH}_4)_2\text{SO}_4$	26.50	18.50	100	$96,58 \pm 0,18^a$
	28.10	18.50	100	$96,13 \pm 0,24^a$
	30.14	18.50	100	$97,25 \pm 0,31^b$
	32.19	18.50	100	$96,33 \pm 0,37^a$
Ethanol/ K_2HPO_4	19.90	22.03	99.45 ± 0.01	$97,47 \pm 0,40^a$
	21.26	22.03	99.20 ± 0.04	98.57 ± 0.33^b
	23.44	22.03	99.13 ± 0.02	99.12 ± 0.01^b
	25.30	22.03	99.33 ± 0.01	98.51 ± 0.21^b
Means of the same column followed by different letters differ significantly ($p \leq 0.05$)				

Variation in ethanol concentration influenced the recovery of sugars in the bottom phase in ATPS ethanol/ $(\text{NH}_4)_2\text{SO}_4$ when the ethanol concentration was 30.14% w/w, differing significantly from other rescues. The same occurs with ATPS ethanol/ K_2HPO_4 , where recovery of sugars, when ethanol concentration was 19.90% w/w, other rescues from other recoveries. This might be due to the reduction in bottom phase volume with increasing ethanol concentration. The water that dissolves sugars from the bottom phase migrates to the top phase, decreasing the water content in the bottom phase and causing a higher partition of sugars (Liu et al. 2013).

The influence of ammonium sulfate and dibasic potassium phosphate concentration results in the recovery of flavonoids and sugars. Varying the salt concentration is shown in Table 3. The ammonium sulfate concentration rises from 14.86 to 18.50% w/w. Values of salt above 32% cause precipitation in ATPS (Wu et al. 2011). In ATPS ethanol/ K_2HPO_4 , the concentration of salt was between 18.10 and 26.09% w/w. In both systems, varying the salt concentration did not affect the flavonoid content. The salting-out effect promoted by the increase in salt concentration leads to an increase in ethanol in the top phase, and then the flavonoids will be easily diluted in that phase (Liu et al. 2013).

Table 3
Effect of variation in salt concentration on the recovery of flavonoids (R_F) in the top phase and the recovery of sugars (R_S) in the bottom phase

ATPS	Ethanol (%, w/w)	Salt (%, w/w)	R_F in top phase (%, w/w)	R_S in bottom phase (%, w/w)
Ethanol/ $(\text{NH}_4)_2\text{SO}_4$	30.14	14.86	99.67 ± 0.02	92.98 ± 0.39^a
	30.14	15.55	99.60 ± 0.05	95.02 ± 0.39^{ab}
	30.14	16.00	99.31 ± 0.01	95.04 ± 0.63^{ab}
	30.14	17.51	99.90 ± 0.01	96.47 ± 0.85^b
	30.14	18.50	99.62 ± 0.22	97.25 ± 0.31^b
Ethanol/ K_2HPO_4	21.26	18.10	99.35 ± 0.01	96.7 ± 0.40^a
	21.26	20.06	98.99 ± 0.12	98.04 ± 0.10^{ab}
	21.26	22.03	99.21 ± 0.04	98.57 ± 0.33^b
	21.26	24.14	98.71 ± 0.04	98.67 ± 0.16^b
	21.26	26.09	98.72 ± 0.08	98.73 ± 0.55^b
Means of the same row followed by different letters differ significantly ($p \leq 0.05$)				
Means of the same column followed by different letters differ significantly ($p \leq 0.05$)				

Regarding sugar content, as more salt was added to the system, the recovery of sugars increased. Such behavior can be explained by the combined effect of the partition coefficient and the phase volume. Increasing salt concentration would allow water to enter the bottom phase, causing more sugars to be transferred to the bottom phase (salt rich phase) (Cheng et al. 2017).

LC–MS profile

Figure 1a and Fig. 1b show the chromatograms obtained in negative mode (ESI) of flavonoids present in the top phase samples from ATPS ethanol/K₂HPO₄ and ATPS ethanol/(NH₄)₂SO₄, respectively. From the chromatograms, we can see that the flavonoid profile remained similar. UPLC-QToF tentatively identified the flavonoids in the samples. The data for retention time observed mass and calculated mass, empirical formula, error, and their putative names from each peak of the chromatogram are presented in Table 4.

Table 4
Mass spectrometry data for the identification of flavonoids from ethanol/ammonium sulfate and ethanol/dibasic potassium phosphate ATPS of PSE rind passionfruit

Peak	Retention time (min)	[M-H] ⁻ Observed	[M-H] ⁻ Calculated	Product ions (MS/MS)	Empirical Formula	ppm (error)	Putative Name	ATPS Etanol/ K ₂ HPO ₄	ATPS Etanol/(NH ₄) ₂ SO ₄	References
1	3.07	609.1459	609.1456	369.0795; 447.6131; 489.0811; 519.1201; 591.1387	C ₂₇ H ₂₉ O ₁₆	0.5	Lucenin-2	+	+	(Cazarin et al. 2016; Farag et al. 2016)
2	3.35	593.1474	593.1506	353.1226; 383.2011; 473.0666; 503.1519	C ₂₇ H ₂₉ O ₁₅	-5.4	Vicenin	+	+	(Cazarin et al. 2016; Souza et al. 2017)
3	3.70	447.0922	447.0927	327.0522; 357.0639	C ₂₁ H ₁₉ O ₁₁	-1.1	Isoorientin	+	+	(Cazarin et al. 2016; Farag et al. 2016; Souza et al. 2017)
4	4.04	577.1569	577.1557	311.1125; 415,0524	C ₂₇ H ₂₉ O ₁₄	2.1	Apigenin	+	+	(Farag et al. 2016; Souza et al. 2017)
5	4.15	563.1534	563.1401	473.1289; 383.0415; 353.0981	C ₂₆ H ₂₇ O ₁₄	-1.1	Schaftoside	+	+	(Simirgiotis et al. 2013; Farag et al. 2016; Feng et al. 2016)
6	4.58	431.1009	431.0978	341.8860; 311.1777	C ₂₁ H ₂₀ O ₁₀	7.2	Vitexin	+	+	(Cazarin et al. 2016; Farag et al. 2016; Souza et al. 2017)

Peak 1 [*m/z* 609.1459 (C₂₇H₂₉O₁₆)] exhibits MS² spectra with fragmented ions at *m/z* 369.0795 Da [M – H – 240]⁻, *m/z* 447.6131 Da [M – H – 162]⁻, *m/z* 489.0811 Da [M – H – 120]⁻, 519.1201 Da [M – H – 90]⁻ and *m/z* 591.1387 Da [M – H – 18]⁻. This compound was identified as luteolin-6,8-*C*-di-glucoside, or lucenin-2 (Cazarin et al. 2016; Farag et al. 2016).

Peak 2 at *m/z* 593.1474 Da [M – H] was identified as vicenin through the mass values in the profile of MS² fragmentation ions with observed values at *m/z* 473.0666 [M – H – 120]⁻ corresponding to the loss of one molecule of hexose, *m/z* 503.1575 [M – H – 90]⁻, *m/z* 383.2011 Da [M – H – 90–120]⁻ and *m/z* 353.1226 Da [M – H – 120–120]⁻ due to the double loss of *C*-glucoside units, and its molecular formula was obtained with an estimated error of -5.4 ppm (Cazarin et al. 2016; Souza et al. 2017).

Peak 3 at retention time 3.70 showed a deprotonated ion mass of *m/z* 447.0922 and molecular formula C₂₁H₂₀O₁₁. The compound in peak 3 presented MS² fragmentation at *m/z* 357.0639 Da [M – H – 90]⁻ and *m/z* 327.0522 Da [M – H – 120]⁻. This is attributed to the loss of sugars in the structures of *C*-glucoside aglycones. The compound was identified as isoorientin (Cazarin et al. 2016; Farag et al. 2016; Souza et al. 2017).

Apigenin-*C*-deoxyhexoside-*O*-hexoside was identified in peak 4 due to the deprotonated ion at *m/z* 577.1569 Da and at MS², fragmented ions at *m/z* 415.0524 [M – H – 162]⁻ due to the loss of the *O*-hexose moiety Da and *m/z* 311.1125 [M – H – 162–104]⁻ indicating the *C*-dexoyhexoside structure (Farag et al. 2016; Souza et al. 2017).

Peak 5 at a retention time of 4.15 min presented a molecular mass of $C_{26}H_{27}O_{14}$ and deprotonated ions at m/z 563.1534. This compound was identified as schaftoside based on the MS² fragmentation profile at m/z 383.0415 Da $[M - H - 180]^-$, m/z 473.1289 $[M - H - 90]^-$ and m/z 353.0981 Da $[M - H - 210]^-$ (Simirgiotis et al. 2013; Farag et al. 2016; Feng et al. 2016).

Peak 6 (retention time at 4.58 min) was identified as vitexin due to the molecular formula $C_{21}H_{20}O_{10}$ proposed by the Elemental Composition tool. Vitexin presented a deprotonated ion at m/z 431.1009 Da $[M - H]^-$ and by MS/MS experiment, a mass fragment at m/z 341.8860 Da $[M - H - 90]^-$ and m/z 311.1777 Da $[M - H - 120]^-$. The presented fragmentation is related to the loss of *C*-hexosyl units connected to the aglycone unit (Cazarin et al. 2016; Farag et al. 2016; Souza et al. 2017).

CONCLUSIONS

An aqueous two-phase system formed by ethanol/ammonium sulfate and ethanol/dibasic potassium phosphate has successfully separated flavonoids from the pressurized solvent extract of passion fruit rind. The PSE extract had a polyphenol content of 5.79 gGAE•kg⁻¹ PFR and a recovery of flavonoids of 30.22%. After partitioning, in a single-stage extraction, both systems presented recoveries of flavonoids in the top phase higher than 98% w/w and recoveries of sugars in the bottom phase higher than 97% w/w, indicating that flavonoids and sugars went to opposite phases. Through LC-MS, it was possible to putatively identify the flavonoids from ATPS passion fruit rind.

Declarations

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Conflict of interest: The authors declare that they have no competing interests.

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Figures

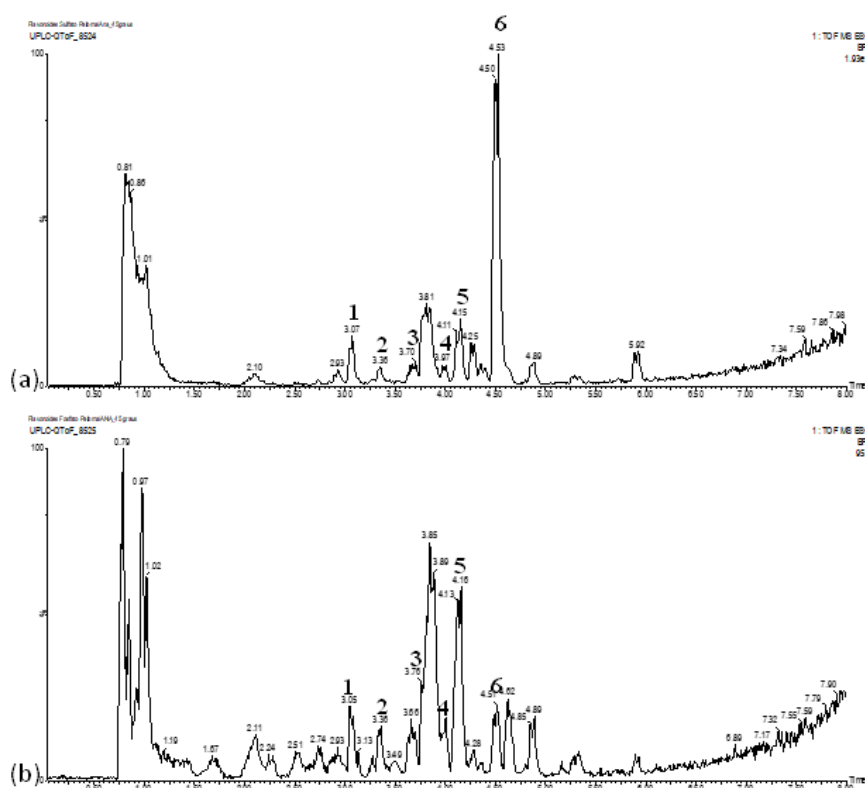


Figure 1

Chromatogram of (a) ethanol/ammonium sulfate ATPS and (b) ethanol/dibasic potassium phosphate ATPS of pressurized solvent extract rind passionfruit