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High-performance liquid chromatography determination of phenolic components in wine using off-line isotachophoretic pretreatment

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Abstract

The off-line combination of isotachophoresis (ITP) and high-performance liquid chromatography (HPLC) to improve sample pretreatment and determination of phenolic compounds in wine was investigated. The ITP system provided an enhanced sample load capacity and served as a sample clean-up technique, HPLC performed a final separation of the analytes presented in samples. The phenolic components were separated by Discovery RP Amid C_{16} chromatography column using water–methanol–acetonitrile–orthophosphoric acid gradient. The identification of phenolic compounds was made by comparison of the retention data obtained for the standard mixture, pretreated sample and the sample spiked standard additions. Satisfactory recoveries for all components analysed were observed between 86.1 and 109.2%.

Keywords: Wine; Food analysis; Isotachophoresis; Sample preparation; Phenolic compounds; Organic acids

1. Introduction

Nowadays high-performance liquid chromatography (HPLC) is a commonly used analytical separation technique that combines high resolution and easy automation with modest sample requirements. In spite of this, the applicability of HPLC may be reduced due to inherently limited sensitivity of the detectors, limited resolution and protection of the equipment and columns from clogging and changes of chromatographic properties. This is frequently a problem for the analysis of analytes in complex matrices (e.g. serum, plasma, urine and extracts of plants), and in these cases, sample clean-up is crucial. For the enrichment and clean-up of the target compounds, either solid-phase extraction (SPE) or liquid–liquid extraction (LLE) is traditionally applied. Another way to solve the above mentioned problem is using isotachophoresis (ITP) as a sample pretreatment technique.

During the isotachophoretic step, focusing of the analytes appears due to local differences in the electric field strength, caused by a discontinuous electrolyte system formed by the leading electrolyte (LE), with high ionic mobility, and the terminating electrolyte (TE), with low ionic mobility. All ionic analytes exhibiting ionic mobility values between that of the leading and terminating ions simultaneously focus into distinct zones, migrating in order of their ionic mobilities in a steady state at equal velocity. In addition to steady-state boundaries exhibiting the self-sharpening effect, the concentration of analytes in their own isotachophoretic zones are adjusted to the composition of the leading zone. For strong uni-univalent electrolytes, the situation is described by the Kohlrausch regulating function. This means that the concentration of the analyte is directly proportionate to the concentration of the leading electrolyte and the function of the mobility of the present ionic species. By this mechanism a diluted sample is concentrated and a concentrated sample is diluted [1–3]. However, the concentration effect is not employed in this paper. Other advantages of this technique are selectivity (only cations or anions migrate), and relatively high sample capacity. Concerning ITP and HPLC, these techniques are based on different physical properties which resulting in a powerful separation. In the literature, only limited numbers of papers have dealt with the combination of isotachophoresis with liquid chromatography [4–9].

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Hydroxybenzoic acids (gallic acid, protocatechuic acid, vanillic acid and syringic acid) hydroxycinnamic acids (caffeic acid, *p*-coumaric acid, ferulic acid) and flavonoids (rutin, quercitrin, myricetin, quercetin and kaempferol) were chosen as target analytes in different types of wines. Most recent studies have reported identification and determination of phenolic compounds in wine using liquid chromatography with spectrophotometry [10–21], fluorimetry [19–22] and mass spectrometry detection [23]. These methods are based mostly on direct injection without any purification [15,16,19,22,23], liquid−liquid extraction [13,17,18,20,21] or solid-phase extraction [13,18] for sample preparation prior proper analyses.

The main aim of this work was to study analytical capabilities of ITP−HPLC combination to separate and determine target components presented in multicomponent ionic matrices as the alternative sample pretreatment technique in connection with the standard analytical method.

2. Experimental

2.1. Chemicals

(−)-Epicatechin, (+)-catechin, gallic acid, protocatechuic acid, vanillic acid, syringic acid, *p*-coumaric acid, caffeic acid, ferulic acid, rutin, quercetin and myricetin were purchased from Sigma–Aldrich (Steinheim, Germany), kaempferol from Roth (Karlsruhe, Germany) and quercitrin from Extrasynthese (Genay, France). Hydrochloric acid, boric acid, Tris(hydroxymethylamino)methane (Tris), barium hydroxide, picric acid were obtained from Lachema (Brno, Czech Republic). Ethanol, methanol and acetonitrile were from Sigma–Aldrich (Steinheim, Germany) and orthophosphoric acid was obtained from Merck (Darmstadt, Germany). All reagents used for the assay were of analytical grade. Pure water was delivered by an Osmium and Elix system (Millipore, MA, USA).

2.2. Equipment

The HPLC system comprised a model LCP 4100 gradient pump (ECOM, Prague, Czech Republic), a Waters 717 plus autosampler and detector Waters 486 (Milford, MA, USA). The detection system was equipped with a DataApex CSW® 1.7. station (Prague, Czech Republic). The column used was a reversed-phase Discovery RP Amid C_{16} (250 mm × 3.0 mm i.d., 5 μm) (Supelco, Bellefonte, PA, USA) with a pre-column Discovery RP Amid C_{16} (2 mm × 2 mm i.d., 5 μm). The ITP experiments were carried out by using the EA 100 Villa Labeco CS Isotachophoretic analyser (Spišská Nová Ves, Slovakia). The preparation column was provided with a 90 mm × 0.80 mm i.d. capillary tube made of fluorinated ethylene–propylene copolymer (FEP) with conductivity detector for monitoring of the separation to achieve proper timing in fractionation procedure. The fraction (53 μl) was eluted from the disconnected column by air zones after passing the detection conductivity sensors. Experimental data were acquired and processed by personal computer software from the Villa company. The samples were injected by microsyringe injection (50 μl). The pH (or pH*) was measured by PHM-220 (Radiometer, France) and pH meter was equipped with pHC 2401-8 combined glass electrode.

Table 1
Gradient program for analysis of phenolic compound in wine

Time (min)	Solvent A	Solvent B	Solvent C
0.00	0	0	100
2.00	0	0	100
4.00	10	5	85
9.00	10	15	75
15.00	5	20	75
17.00	0	30	70
20.00	0	40	60
25.00	0	60	40
30.00	0	80	20
35.00	0	80	20

Solvent A: methanol; Solvent B: acetonitrile; Solvent C: methanol–acetonitrile–aqueous 0.085% orthophosphoric acid (13:7:80).

2.3. Chromatographic condition

Chromatographic separation was carried out using three solvents: (A) methanol, (B) acetonitrile, (C) 0.085% orthophosphoric acid (13:7:80), in a gradient programme shown in Table 1. The flow rate was 0.5 ml/min and the injection volume 10 μl. Peaks were detected at 280 nm.

2.4. Wine samples

A variety of different types of wines were analysed: Sauvignon Blanc (B–Natur s.r.o., Klobouky u Brna, Czech Republic), Blauer Portugieser (František Mádl, Czech Republic) and tawny port wine (Rozès, Vila Nova de Gaia, Portugal).

2.5. Standard solutions

Stock solution of standards containing (15–35 μg/ml) was prepared by dissolving in ethanol. For recovery experiments with a mixture of standards, the stock solution was diluted with water to the final concentration to make 20% (v/v) ethanolic solution of standards. Calibration solutions were prepared by diluting the stock solution with ethanol. Three replicate injections of each standard solution were made on the HPLC and the mean values of peak areas of standards were plotted against corresponding amounts of injected standards.

2.6. Electrolyte solutions

The ITP electrolytes contained 20% (v/v) of ethanol; 2-hydroxyethylcellulose was used as an additive to the

leading electrolyte to suppress electro-osmotic flow. The pH of the electrolytes was adjusted after the addition of ethanol and, therefore, the pH values measured are apparent values. The electrolytes were degassed for 15 min by sonication.

3. Results and discussion

3.1. ITP electrolyte system

Strategy of the sample pretreatment in this study consisted from two main points, to find suitable ITP electrolyte system that ensure of migration both the hydroxybenzoic, hydroxycinnamic acid and flavonoids. The other point was the fractionation of sample without using the fraction valve from the isotachophoretic system. It is very well known that the effective mobility can be influenced through changes in the concentration of the leading electrolyte, the pH of the LE, the complexing agents and the choice of the solvents. From the previous experiences with similar multicomponent ionic matrices [24,25], hydrochloric acid containing Tris as the counter ion as the leading electrolyte was chosen. The migration of flavonoids at relatively low value of pH* ≈ 8.0 was obtained due to the complexing reaction of the 1,2- and 1,3-dihydroxyl structure with borates to form negatively charged stable complexes in alkaline medium [26–29]. Therefore, boric acid of pH* 8.2 (adjusted with barium hydroxide) was used as the terminating electrolyte (Table 2). Barium hydroxide was used to adapt pH and for the purpose of the role of suppressing contributions from the terminating electrolyte to the ubiquitous carbonate contamination arising from dissolved atmospheric carbon dioxide which is encountered when performing ITP at high pH. The disturbances of the zones due to electro-osmosis was prevent by addition of hydroxyethylcellulose (HEC). Fig. 1 show the isotachophoreogram for the sample of the standard mixture and from this run it is shown that between picric acid as frontal coloured marker (M1) and phenol red (M2) hydroxybenzoic and hydroxycinnamic acids are migrated in a mixed zones. The next part of the isotachophoreogram is occupied by the flavonoid zones followed by boric acid, useful as a terminal marker. After the termination of the pre-separation part in which the coloured zones occurred, the target compounds from isotachophoretic system was transferred using very simple, reproducible technique described above in Section 2. The collected samples were analysed by HPLC technique. To improve the pretreatment and fractionation of the phenolic zones from the others, picric acid (1.0×10^{-4} M) and phenol red (2.0×10^{-4} M) were employed as ITP spacers. Finally, phenol red was not used later in order to prevent interference in the HPLC analysis.

Table 2
Operational system for isotachophoresis

Parameter	Electrolyte	
	Leading	Terminating
Solvent	Water–EtOH (4:1)	Water–EtOH (4:1)
Anion	Cl^-	H_3BO_3
Concentration (mM)	10	50
Counter ion	Tris	–
pH*	7.20	8.20 [adjusted by $Ba(OH)_2$]
Additive	HEC	–
Concentration (% (w/v))	0.2	–

HEC: hydroxyethylcellulose; Tris: tris(hydroxymethyl)aminomethane.

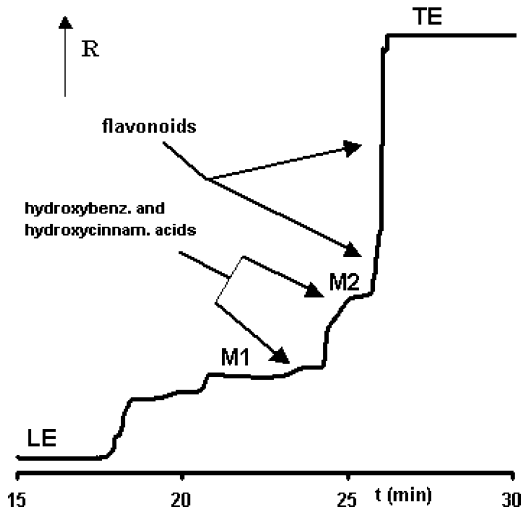

Fig. 1. Isotachophoreogram of the standard mixture from preparative run using discrete spacers (M1, picric acid and M2, phenol red). The driving current was 200 μA. Conditions are given in Table 1. *R*: increasing resistance.

3.2. Chromatography

The chromatographic method was optimised with respect to stationary phase, eluent composition, flow rate and injection volume. For the separation of phenolic components, reversed-phase chromatographic column RP Amid C_{16}, which permitted satisfactory retention was selected. Several experiments were carried out to resolve the phenol mixture using a different acetonitrile–water gradient mobile phase. To increase the retention behaviour of phenolics orthophosphoric acid was used. Consequently, a mixture of acetonitrile–methanol–water was tried to achieve better peak resolution of vannilic acid and syringic acid. Table 1 shows the optimal gradient used in this study. Under the gradient conditions, all compounds were eluted within 35 min. Fig. 2 shows the typical chromatogram of fourteen standards detected at 280 nm. The retention characteristics were : gallic acid (t_r = 4.92 min), protocatechuic acid (t_r = 8.07 min), catechin (t_r = 11.29 min), vanillic acid (t_r = 12.36 min), syringic acid (t_r = 12.63 min), epicatechin (t_r = 13.46 min), caffeic acid (t_r = 14.45 min), *p*-coumaric acid (t_r = 17.75 min), ferulic acid (t_r = 18.57 min), rutin (t_r = 19.56 min), quercitrin (t_r = 23.57 min), myricetin (t_r = 26.96 min), quercetin (t_r = 30.39 min), kaempferol (t_r = 33.05 min). Phenolic compounds with the additional

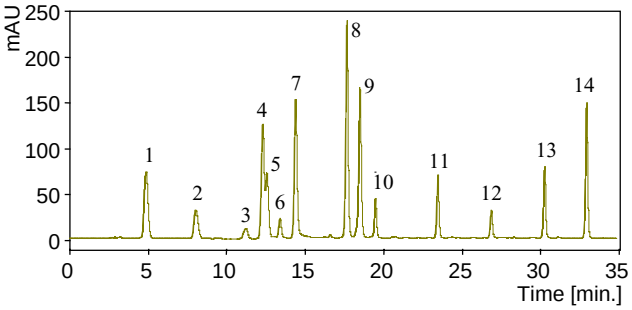

Fig. 2. Chromatogram of standard mixture using gradient elution with detection wavelength set at 280 nm. The peaks correspond to: 1, gallic acid; 2, protocatechuic acid; 3, (+)-catechin; 4, vanillic acid; 5, syringic acid; 6, (−)-epicatechin; 7, caffeic acid; 8, *p*-coumaric acid; 9, ferulic acid; 10, rutin; 11, quercitrin; 12, myricetin; 13, quercetin; 14, kaempferol.

hydroxy groups exhibit increased relative retention on the embedded-polar-group phase. Thus, selectivity differences between traditional alkyl bonded phases and embedded-polar-group bonded phases can be useful in method development. As Fig. 2 illustrates, the most prominent changes is in the retention order the 5–6–7 triplet and the 11–12 duplet in comparison with the C_{18} column [20], in which compounds come out in the order 7–5–6 and compound 12 gets out first with the C_{18} column, but the second with RP Amid C_{16} column. It is apparent that column chemistry changes may yield dramatic selectivity changes. In many cases, when peaks are resolved unsatisfactorily on an alkyl phase, the embedded-polar-group counterpart might be substituted to achieve the desired separation with no mobile-phase adjustment.

3.3. Validation experiments

Calibration graphs were generated using five calibration solutions. All graphs were linear and correlation coefficients of phenolics were better than 0.9991 (Table 3). The precision and accuracy of the method was assessed by repeatedly injecting standard solution on the same day and over a period of 3 days. Table 4 shows that R.S.D. for intra-day precision and accuracy were from 0.6 to 2.7% and recoveries from 92.4 to 104.4%, while R.S.D. for inter-day and accuracy were from 0.8 to 3.4% and recoveries from 91.9 to 105.5%.

3.4. Recovery studies and LOQ and LOD

Recovery studies were performed by extracting the target compounds from standard solution (20% ethanol (v/v)) by using ITP and then HPLC analysis. Mean recovery values were 96.5% for gallic acid (3.7 μg/ml), 105.9% for protocatechuic acid (3.2 μg/ml), 109.2% for (+)-catechin (5.3 μg/ml), 104.7% for vanillic acid (4.8 μg/ml), 101.3% for syringic acid (5.2 μg/ml), 86.1% for (−)-epicatechin (6.1 μg/ml), 100.6% for caffeic acid (5.2 μg/ml), 104.8% for *p*-coumaric acid (3.3 μg/ml), 104.1% for ferulic acid (7.7 μg/ml), 97.9% for rutin (5.5 μg/ml), 94.6% for quercitrin (4.7 μg/ml), 91.7% for myricetin (6.0 μg/ml), 90.2% for quercetin (5.4 μg/ml) and 89.5% for kaempferol (6.7 μg/ml). Limit of detection (LOD) and limit of quantification (LOQ) were determined for each analyte as concentration equivalent to a signal-to-noise ratio of 3 and 10. LOD and LOQ obtained for phenolics are shown in Table 3. As concentration effect was not employed in this work, LOD and LOQ are referred to results obtained from the HPLC technique.

3.5. Analysis of wines

Three brands of wine (white, red and port-wine) were randomly selected from wine shop and analysed using the current method. The peaks were identified by comparing the retention data obtained for the wine, standard mixture and the wine spiked standard additions. The levels of phenolics

Table 3
Analytical characteristics of calibration graphs

No.	Compound	Equation	Linear range (μg/ml)	R^2	LOD[a] (μg/ml)	LOQ[b] (μg/ml)
1	Gallic acid	y=0.7044 × −0.0754	0.4–20	0.9993	0.025	0.083
2	Protocatechuic acid	$y = 0.3772 \times +0.0727$	0.3–15	0.9996	0.065	0.217
3	(+)-Catechin	$y = 0.2267 \times +0.0675$	0.5–25	0.9995	0.112	0.374
4	Vanillic acid	$y = 0.7189 \times -0.0915$	0.5–25	0.9994	0.026	0.087
5	Syringic acid	$y = 0.3828 \times -0.0069$	0.5–25	0.9993	0.054	0.180
6	(−)-Epicatechin	$y = 0.1494 \times -0.0177$	0.6–30	0.9996	0.114	0.380
7	Caffeic acid	$y = 0.7327 \times +0.0123$	0.5–25	0.9996	0.026	0.087
8	*p*-Coumaric acid	$y = 1.6211 \times -0.0490$	0.3–15	0.9993	0.011	0.037
9	Ferulic acid	$y = 0.5094 \times +0.0926$	0.7–35	0.9994	0.037	0.124
10	Rutin	$y = 0.1525 \times -0.0517$	0.5–25	0.9993	0.106	0.354
11	Quercitrin	$y = 0.2737 \times -0.0386$	0.5–25	0.9994	0.062	0.206
12	Myricetin	$y = 0.2652 \times -0.0836$	0.6–30	0.9993	0.158	0.526
13	Quercetin	$y = 0.3016 \times -0.0846$	0.6–30	0.9994	0.072	0.240
14	Kaempferol	$y = 0.4229 \times -0.1062$	0.6–30	0.9991	0.045	0.150

[a] Limit of detection.
[b] Limit of quantitation.

Table 4
Within- and between-day precision and accuracy of assay for determination of phenolics in standard solution

No.	Compound	Injected quantity (μg/ml)	Mean measured quantity (μg/ml)		R.S.D. (%)		Recovery (%)	
			WD	BD	WD	BD	WD	BD
1	Gallic acid	9.34	9.75	9.81	1.6	2.1	104.39	105.03
2	Protocatechuic acid	7.93	7.33	7.29	2.3	2.9	92.40	91.93
3	(+)-Catechin	13.24	13.31	13.26	1.2	1.8	100.53	100.15
4	Vanillic acid	12.05	11.87	11.63	0.9	1.5	98.51	96.52
5	Syringic acid	13.08	13.01	12.85	1.4	1.1	99.46	98.24
6	(−)-Epicatechin	15.25	15.45	15.53	1.7	1.5	101.31	101.83
7	Caffeic acid	12.93	13.21	13.43	2.1	1.8	102.17	103.87
8	*p*-Coumaric acid	8.21	8.51	8.66	1.3	1.5	103.65	105.48
9	Ferulic acid	19.18	19.21	19.15	1.4	0.8	100.15	99.84
10	Rutin	13.72	13.66	13.53	2.7	2.4	99.56	98.62
11	Quercitrin	12.15	12.23	12.02	1.8	2.1	100.66	98.93
12	Myricetin	14.98	14.76	14.13	2.3	3.4	98.53	94.57
13	Quercetin	13.56	13.49	13.40	0.6	0.9	99.48	98.82
14	Kaempferol	16.74	16.94	17.14	1.1	0.8	101.19	102.38

R.S.D.: relative standard deviation; WD: within-day; BD: between-day; mean values obtained from regression straight-line equation for four determinations within-day (WD) and two determinations per day over 3 days (BD).

were as expected according to previous investigations of these compounds in wine above mentioned. Table 5 and Fig. 3a and b show that the predominant phenolic constituents in the commercial wines were gallic acid, vanillic acid and *p*-coumaric acid, followed by protocatechuic acid and caffeic acid. The minor compounds found in wine were ferulic acid and syringic acid. In contrast to previous studies, where quantities of (+)-catechin, (−)-epicatechin, quercitrin, myricetin, quercetin and kaempferol were reported [10,14,16–18], we found all of them below the limit of quantification. The trace amounts of syringic acid, (−)-epicatechin and kaempferol were in white wine with ferulic acid and rutin in red wine and portwine. The lowest content of phenolic compounds was found in white wine, whereas portwine exhibited the highest levels for all analytes studied except caffeic acid, ferulic acid and flavonoids. The contents of *p*-coumaric acid and vanillic acid in portwine were, respectively, 11 and 18 times higher than in white wine. With some technical supplements (fraction valve, microHPLC columns) that are available in the present it will be possible to select more discrete regions of the target zones. Smaller inner diameters of microHPLC columns result in decreased chromatographic dilution and large signal-to-noise ratios compared to conventional columns. Combination with other detection technique could be better for quantitative results, for example diode-array detector that offer advantages to optimise wavelengths for compounds over the course of the run and multi-signal detection can be used for optimum sensitivity over a wide spectral range in connection with checking the peak purity to avoid quantification errors.

Table 5
Phenolics content in wine samples

No.	Compound	*c* (μg/ml)		
		White wine	Red wine	Portwine
1	Gallic acid	NQ	1.367	4.841
2	Protocatechuic acid	0.164	0.251	1.087
3	(+)-Catechin	ND	NQ	ND
4	Vanillic acid	0.186	0.805	3.439
5	Syringic acid	NQ	0.066	0.261
6	(−)-Epicatechin	NQ	ND	ND
7	Caffeic acid	0.458	0.833	NQ
8	*p*-Coumaric acid	0.182	0.813	1.976
9	Ferulic acid	0.285	NQ	NQ
10	Rutin	ND	NQ	NQ
11	Quercitrin	ND	ND	ND
12	Myricetin	ND	ND	ND
13	Quercetin	ND	ND	ND
14	Kaempferol	NQ	ND	ND

ND: not detected; NQ: not quantified.

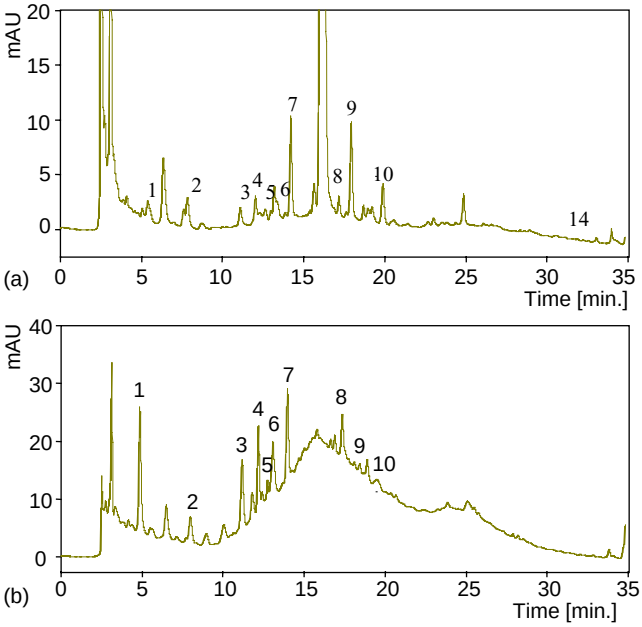

Fig. 3. Typical HPLC chromatograms of: (a) white wine and (b) red wine after ITP pretreatment with detection wavelength set at 280 nm. The peaks correspond to: 1, gallic acid; 2, protocatechuic acid; 3, (+)-catechin; 4, vanillic acid; 5, syringic acid; 6, (−)-epicatechin; 7, caffeic acid; 8, *p*-coumaric acid; 9, ferulic acid; 10, rutin; 14, kaempferol.

4. Conclusion

The results presented provide evidence that the developed ITP–HPLC combination can be an alternative to other frequently applied techniques for sample pretreatment, such as solid-phase or liquid–liquid technique. The method has been applied with acceptable precision and accuracy to the analysis of hydroxybenzoic acids, hydroxycinnamic acids and flavonoids in various types of wine. Overall percentage recoveries after ITP pretreatment were satisfactory (>86%) for the target analytes and detection limits were between 0.011 and 0.158 μg/ml. Future research will aim to optimise the collection of the target analytes by fractionation valve and using HPLC column with less diameter and fluorimetry detection, in order to improve both selectivity and detection limits.

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