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The Application of On-line Microdialysis with High Performance Liquid Chromatography for Determining Catechins in Tea and Tea Drinks

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ABSTRACT

A microdialysis on-line coupled to a reversed-phase liquid chromatography was investigated and applied for analyzing catechins in tea and tea drinks. Catechins in drink samples were diffused into the perfusate through a hollow fiber membrane and then on-line injected into the HPLC system. Catechins were separated with a C8 reversed column by gradient eluting using an acetonitrile/phosphate buffer solution at pH 4.0, and monitored by a UV diode-array detector. Factors affecting the dialysis efficiency such as the conditions in perfusate and in the sample solution were evaluated. The proposed method provided a simple procedure for isolating catechins from tea and tea drink samples. The application was illustrated by the analysis of epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate in tea drinks.

Key words: HPLC, microdialysis, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, tea

INTRODUCTION

Tea is a very popular beverage in the world produced in China, India, Sri Lanka, Kenya, Japan, Taiwan, and many other countries. About 2.5 million tons of tea is produced per year in the world. During the past decade, the beneficial effects of tea on the health of consumers received unprecedented attention[1]. The polyphenols (catechins) are considered to be the important compounds offering these benefits. However, the catechins in tea vary with tea species, horticultural conditions, the seasons, as well as the fermentation level in the production process[2]. In order to understand these beneficial effects, several efforts have attempted to isolate and identify the active components (catechins, etc.) in various tea samples[3-4]. Therefore, establishing a simple and reliable analytical method to determine the level of catechins in tea samples is important for the process in developing high quality tea products.

Many methods have been applied to analyze catechins in tea samples including high performance liquid chromatography-ultraviolet detection (HPLC-UV), gas chromatography (GC), liquid chromatography-mass spectrometry (LC-MS), and capillary electrophoresis[3-10]. Among these, the HPLC method is the most popular and convenient. The organic solvents (chloroform, dichloromethane, acetone, and ethyl acetate) used in sample pretreatment are considered to be significant pollutants, and thus are not recommended now. Although the extracts of tea were filtered to remove rough particles and further filtered through a 0.45 μm membrane filter prior to liquid chromatographic determination, some reactive high-molecular compounds are still in the filtrate and could contaminate the analytical columns. Therefore a convenient clean-up protocol should be applied for the purpose of protecting the columns.

Membrane-based separations have been applied as useful tools in the treatment of complicated samples with the advantages of easy operation, rapid isolation of components from samples, and use little or no organic solvents[11]. Therefore, microdialysis has extensively been applied in biotechnological and biomedical studies[12-14]. It was also applied to determine salicylic acid in emulsified cosmetics, organic acids in fermentation products, and chloroaniline in polymer industrial wastewater[15-17]. In this paper, the microdialysis technique has been proposed to clean up the catechins from tea sample and on-lined to high performance liquid chromatography to develop an analytical protocol for determining epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG), structures as shown in Figure 1, in tea samples.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

The deionized water was produced by EASYpure RF Barnstead ultrapure water system (Barnstead, New York, USA). The 1000 μg/mL of epicatechin, epicatechin gallate , epigallocatechin, and epigallocatechin gallate stock solutions were prepared individually by epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate (Sigma, St. Louis, MO, USA). Fresh working standards (single or mixed) were prepared by appropriate dilutions of the stock solutions. The LC eluent was prepared from LC-

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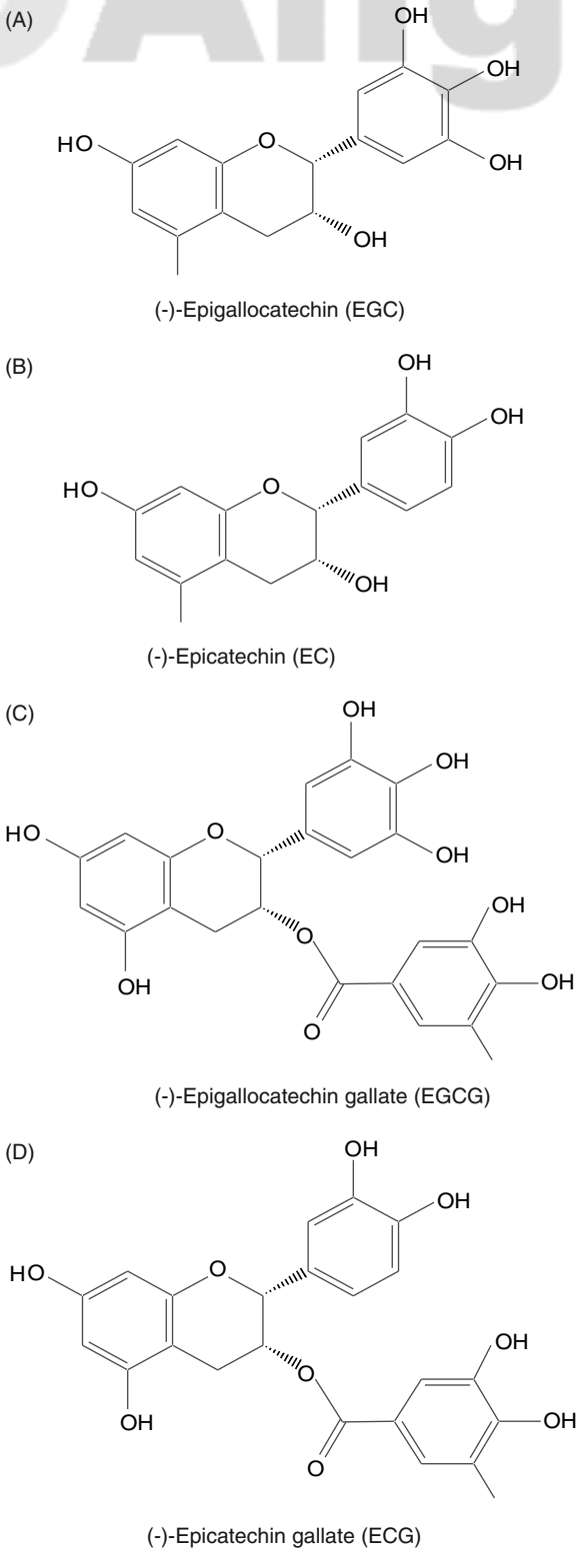

Figure 1. The structures of four catechins. (A) EGC (B) EC (C) EGCG and (D) ECG.

grade acetonitrile (Fisher Scientific, Fair Lawn, N. J., USA) and phosphate buffer (pH 4.0). All eluents were filtered through a 0.45 μm PVDF membrane filter. The tea and tea drinks were obtained from local supermarket in Kaohsiung city.

II. *Apparatus*

The HPLC analyses were performed on an Agilent 1100 liquid chromatograph with a diode-array detector (Waldbronm, Germany) equipped with G1310A pump, G1322A vacuum degasser, and G1315A diode-array detector. A Rheodyne 7725i injector (Cotati, CA, USA) with a 10-μL sample-loop was used to introduce dialysate into the chromatographic system. The ZORBAX Eclipse XDB-C8 reversed-phase column (150 mm × 4.6 mm i.d., 5 μm particle size) (Agilent, USA) was used for separation.

A home-assembled microdialysis sampling system was set up with reference to the literature[18], however, all the parts are commercially available, and can be assembled as shown in Figure 2. The volume of a stainless tube (0.0046" i.d. × 1/16" o.d. × 10 cm) acted as the sampling space. Two three-way unions (PEEK Tee, 0.020" thru-hole, Upchurch, WA, USA) were adapted on two ends of the stainless tube. A linear cellular membrane probe (regenerated cellulose, 13000 Dalton, 200 μm i.d.), taken from Spectra/Por® RC hollow fiber bundles (Spectrum Laboratory, CA, USA) was inserted through the stainless tube and fixed with a Silica SealTight Sleeve (280 μm i.d., 0.011" o.d., Upchurch, WA, USA) onto the three-ways union to form the micro-dialysis sampling system. The quick connect luer adapter was used to connect the syringe (100 μL, Hamilton, Reno, Nevada, USA) of the syringe pump (BSP-99M, Braintree Scientific, MA, USA). The outlet of the system was a needle that was placed in the injector of the LC. The sampling volume of this system is about 0.5 mL.

III. *Procedure*

After introducing a tea sample into the microdialysis sampling system, the perfusate flowed through the membrane to the sample loop of the injector. The injector was in the "load" position to receive the dialysate initially, and then turned to the "inject" position for chromatographic separation after having been equilibrated. At least 3 times of the sample loop volume flowed through the system before injection to the LC system. The HPLC detection was by gradient elution with the eluent (I) acetonitrile and the eluent (II) 1.0×10^{-3} M phosphate buffer (pH 4.0) under the controlled condition.

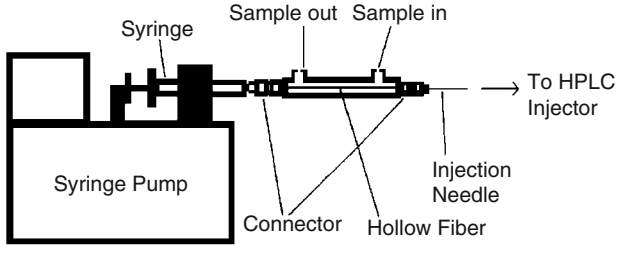

Figure 2. The home-assembled micro-dialysis sampling system.

RESULTS AND DISCUSSION

In order to verify the possibility of the proposed micro-dialysis method to be used in the pretreatment of tea samples, factors that affect the dialysis efficiency such as the flow-rate, pH, and polarity modifier in perfusion, the pH and sodium chloride addition in the sample solution, as well as chromatographic conditions were studied thoroughly to optimize the sampling and analytical conditions.

I. *Optimization of Chromatographic Conditions*

Chromatographic conditions were optimized and built-up prior to the investigation of microdialysis conditions. A ZORBAX Eclipse XDB-C8 column was considered as having the potential to resolute EGC, EC, EGCG, and ECG from other species very well, and was thus used for tests. The detection wavelength was set at 275 nm. From the results of series tests, the optimum eluent condition was 91:9 (v/v) between 0-15 min., then linear gradient to 81:19 (v/v) between 15-25 min, and kept 81:19 (v/v) between 25-30 min. of acetonitrile and 1.0×10^{-3} M phosphate buffer solution (pH 4.0) with the flow rate of 1.0 mL/min. Under these conditions, chromatogram for standard species of EGC, EC, EGCG, and ECG was as in Figure 3. As shown in the chromatogram, peaks 1-4 are related to EGC, EC, EGCG, and ECG, respectively. It is obvious that all species give well separated results within 28 min. The quantitative calibrations of the chromatographic processes were achieved in the concentration range of 10~100 μg/mL. Results are shown in Table 1. The detection limits are based on three times the averaged background noise divided by the detection sensitivity.

II. *Conditions of Perfusate in the Dialysis System*

As described in literatures[15-17], the diffusion efficiency would decrease with the increase of the perfusate flow rate. However, a longer-time collection of perfusate in a limited volume of sample solution decreases diffusion efficiency (dilution effect). The effect is significant as the sample volume is small compared with the perfusate volume. Figure 4 demonstrates the effect of collection time on the detection peak area of perfusate under the flow rate of 1.06 μL/min. It can be seen all the peak areas of 4 catechins increased with collection time and the optimum was reached at about 20 min, then decreased with collection time. However, ECG showed a signicant change and EGC showed very little effect. This can be explained that the diffusion rate of ECG is greater than the flow rate of the perfusate and the diffusion rate of EGC is equivalent or less than the flow rate of the perfusate. The sample was thus collected for 20 min under 1.06 μL/min flow rate.

In a dialysis system polarity modifier (organic solvent) is often added in perfusate to improve the recovery efficiency of analytes[15]. Because the solubilities of EGC, EC, EGCG, and ECG in methanol are higher than in water, methanol was thus selected as the polarity modifier. Figure 5 shows the effect of methanol in perfusate on the detection

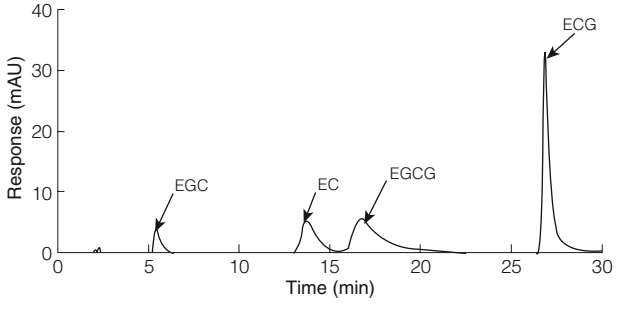

Figure 3. Chromatogram of standard species of EGC, EC, EGCG, and ECG.

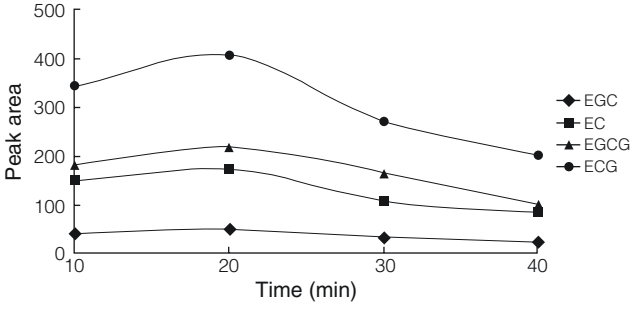

Figure 4. Effect of collection time on the detection peak area of perfusate.

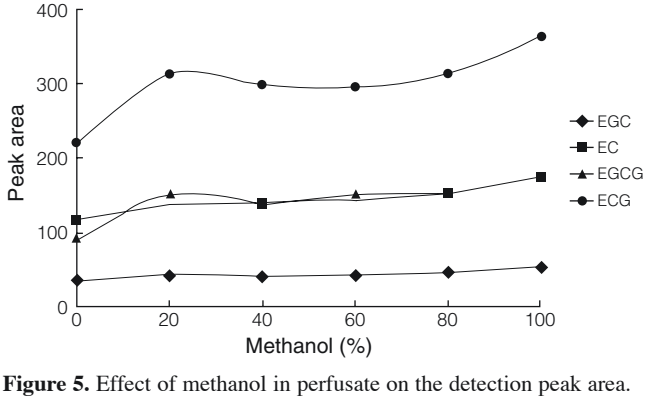

Figure 5. Effect of methanol in perfusate on the detection peak area.

Table 1. Calibration parameters of catechins for direct injection

Catechins	Concentrations (μg/mL)	Linear equation	γ^2	Detection limit (μg/mL)
EGC	10-100	Y = 3.7X - 0.3	0.9999	4.1
EC	10-100	Y = 9.7 X + 1.7	0.9995	4.7
EGCG	10-100	Y = 16.4 X-33.7	0.9992	3.8
ECG	10-100	Y = 26.4X + 4.8	0.9996	4.4

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peak area. It can be seen the peak area increased with methanol content up to 100% methanol. Therefore, methanol was used as the perfusate in the dialysis system.

III. *Conditions in the Sample Solution*

As in other extractions, the pH of the sample solution would affect the dialysis efficiency[18-19]. Figure 6 shows the detection peak areas (related to the dialysis efficiency) under various pH sample solutions. It can be seen, only the dialysis efficiency of ECG slightly increased with the increases of pH, whereas the EGC, EC and EGCG did not show significant increase with the pH. These findings suggested that the pH of the sample solution is not critical in the dialysis process for the range pH 3-6.

Salting out effect is also often applied to increase the recovery of organic species in conventional extraction processes. In the studies, various amounts of NaCl were added into the sample solution to investigate the effect on the diffusion efficiency of catechin species. Results indicated that there was no significant influence of NaCl on dialysis recovery. Thus, the addition of NaCl in the sample solution was not recommended.

IV. *Calibration Plots of Catechins by Direct Injection and Being on-line to Dialysis*

In order to identify the diffusion efficiency at various concentration, calibration plots of EGC, EC, EGCG, and ECG injected directly and on-lined to dialysis were built-up. Table 1 and 2 list the calibration parameters of these four catechins for direct injection and being on-line to dialysis, respectively. It can be seen all of the calibration plots follow very good linear relationship with $\gamma^2 > 0.999$ at 10-100 μg/mL for four catechins, whether being injected directly or on-line to dialysis. In comparison of the slope ratios of the two linear regression equations, the average diffusion efficiency of these four catechins in the concentration ranges under optimum dialysis conditions are 78.6%, 105.4%, 64.0%, and 64.8% for EGC, EC, EGCG, and ECG, respectively. For the 10 μg/mL solutions, the R.S.D. of being on-line to dialysis are 3.6%, 5.0%, 4.6%, and 4.8% for EGC, EC, EGCG, and ECG, respectively.

V. *Analysis of Real Samples*

In order to test the applicability of the proposed method for the determination of EGC, EC, EGCG, and ECG in real samples, three kinds of tea bags were extracted with 100 mL of 95°C hot water for 10 min, then cooled down to room temperature (25°C). The tea extracted from tea bags as well as three tea drinks were on-line dialyzed under the optimum conditions, and then examined by HPLC. The results are listed in Table 3. Obviously, the contents of catechins were different in the various tea samples. The catechins in tea bags are higher than those in tea drinks. In general, green tea has higher amounts of four catechins than Oolong tea and black tea in tea drinks; whereas in tea bags, the green tea has the highest content of EGC and EC than others and Oolong is the highest in EGCG and ECG amounts.

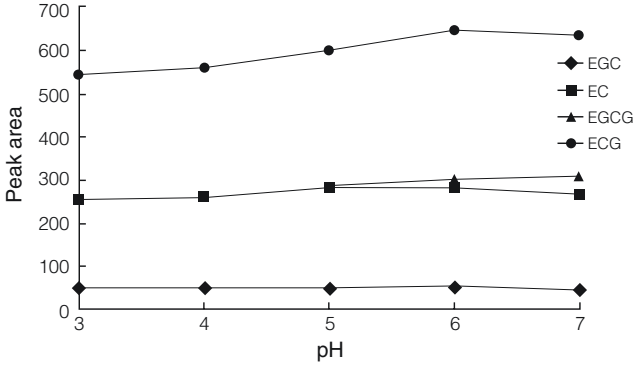

Figure 6. Effect of sample pH on the dialysis efficiency.

Table 2. Calibration parameters of catechins being on-line to dialysis

Catechins	Concentrations (μg/mL)	Linear equation	γ^2	Detection limit (μg/mL)
EGC	10 - 100	Y = 2.9X - 10.0	0.9994	0.7
EC	10 - 100	Y = 10.3 X - 13.8	0.9995	1.2
EGCG	10 - 100	Y = 10.5X -101.3	0.9990	3.2
ECG	10 - 100	Y = 17.1X - 103.4	0.9990	0.6

Table 3. Analytical results of catechins in tea with the proposed method

	EGC		EC		EGCG		ECG	
	Conc. (μg/mL)	R.S.D. (%)	Conc. (μg/mL)	R.S.D. (%)	Conc. (μg/mL)	R.S.D. (%)	Conc. (μg/mL)	R.S.D. (%)
Oolong Tea Bag	191.9*	5.6	38.3	5.1	486.2*	9.1	98.6	6.5
Green Tea Bag	331.6*	4.7	44.0	2.6	341.8*	3.9	73.9	9.3
Black Tea Bag	133.9*	5.2	10.3	1.8	54.5	6.3	70.2	6.5
Oolong Tea Drink	27.2	5.2	6.1	2.0	30.3	9.2	17.9	3.1
Green Tea Drink	85.5	9.1	20.6	7.3	83.7	7.2	36.9	4.5
Black Tea Drink	16.4	4.7	5.7	3.2	31.5	5.1	12.9	1.4

*Sample diluted 10 times before analysis.

CONCLUSION

The study investigated and confirmed the potential of using microdialysis on-line to liquid chromatography to measure catechins (EGC, EC, EGCG, and ECG) in tea samples. Results indicate that the on-line microdialysis technique for determining catechins has the advantages of easy operation requiring fewer staff and use less solvents.

REFERENCES

1. Trevisanato, S. I. and Kim, Y. I. 2000. Tea and health. Nutr. Rev. 58: 1-10.
2. Lin, Y. L., Juan, I. M., Chen, Y. L., Liang, Y. C. and Lin, J. K. 1996. Composition of polyphenols in fresh tea leaves and associations of their oxygen radical absorbing capacity with antiproliferative actions in fibroblast cells. J. Agric. Food Chem. 44: 1387-1394.
3. Horie, H. and Kohata, K. 2000. Analysis of tea components by high-performance liquid chromatography and high-performance capillary electrophoresis. J. Chromatogr. A 881: 425-438.
4. Dalluge, J. J. and Nelson, B. C. 2000. Determination of tea catechins. J. Chromatogr. A 881: 411-424.
5. Goto, T., Yoshida, Y., Kiso, M. and Nagashima, H. J. 1996. Simultaneous analysis of individual catechins and caffeine in green tea. Chromatogr. A 749: 295-299.
6. Khokhar, S., Venema, D., Hollman, P. C. H., Dekker, M. and Jongen, W. 1997. A RP-HPLC method for determination of tea catechins. Cancer Letters 114: 171-172.
7. Zuo, Y., Chen, H. and Deng, Y. 2002. Simultaneous determination of catechins, caffeine and gallic acid in green, oolong, black and pu-erh teas using HPLC with a photodiode array detector. Talanta 57: 307-316.
8. Arts, H. C. W., van de Putte, B. and Hollman, P. C. H. 2000. Catechin contents of foods commonly consumed in the Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. J. Agric. Food Chem. 48: 1746-1751.
9. Zeeb, D. J., Nelson, B. C. and Dalluge, J. J. 2000. Separation and identification of twelve catechins in tea using liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry. Anal Chem. 72: 5020-5026.
10. Lin, J. K., Lin, C. L., Liang, Y. C., Lin-Shiau, S. Y. and Juan, I. M. 1998. Survey of catechins, gallic acid, and methylxanthines in green, oolong, pu-erh, and black teas. J. Agric. Food Chem. 46: 3635-3642.
11. van de Merbel, N. C. 1999. Review membrane-based sample preparation coupled on-line to chromatography or electrophoresis. J. Chromatogr. A 856: 55-82.
12. Torto, N., Gorton, L., Laurell, T. and Marko-Varga, G. 1999. Technical issues of in vitro microdialysis sampling in bioprocess monitoring. Trends Anal. Chem. 18: 252-260.
13. van de Merbel, N. C. 1997. The use of ultrafiltration and column liquid chromatography for on-line fermentation monitoring. Trends Anal. Chem. 16: 162-173.
14. van de Merbel, N. C., Lingeman, H. and Brinkman U. A. Th. 1996. Review sampling and analytical strategies in on-line bioprocess monitoring and control. J. Chromatogr. A 725: 13-27.
15. Jen, J. F., Tsai, Y. Y. and Yang, T. C. 2001. Microdialysis of salicylic acid from viscous emulsion samples prior to high-performance liquid chromatographic determination. J. Chromatogr. A 912: 39-43.
16. Jen, J. F. and Chang, C. T. and Yang, T. C. 2001. On-line microdialysis-high-performance liquid chromatographic determination of aniline and 2-chloroaniline in polymer industrial wastewater. J. Chromatogr. A 930: 119-125.
17. Wei, M. C., Chang, C. T. and Jen, J. F. 2001. Determination of organic acid in fermentation products of milk with HPLC/on-lined micro-dialysis. Chromatographia 54: 601-605.
18. Palmarsdottir, S., Thordarson, E., Edholm, L. E., Jonsson, J. A. and Mathiasson, L. 1997. Miniaturized supported liquid membrane device for selective on-line enrichment of basic drugs in plasma combined with capillary zone electrophoresis. Anal. Chem. 69: 1732-1737.
19. Rosa, M. J. and de Pinho, M. N. 1997. Membrane surface characterization by contact angle measurements using the immersed method. J. Membrane Sci. 131: 167-180.
20. Zupancic, T. and Pihlar, B. 1999. Preconcentration of quinolones by dialysis on-line coupled to high-performance liquid chromatography. J. Chromatogr. A 840: 11-20.