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Short communication

HPLC method validation for *Digitalis* and its analogue by pulsed amperometric detection

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ABSTRACT

We developed a highly sensitive and selective reversed-phase HPLC-pulsed amperometric detection (RP-HPLC-PAD) method for cardiac glycoside detection. Eight cardiac glycosides were completely separated within 45 min on a reversed-phase column using a water–acetonitrile gradient, and were detected using a PAD under NaOH alkaline conditions. The detection ($S/N = 3$) and quantification ($S/N = 10$) limits for the cardiac glycosides were 0.1–0.3 and 0.3–0.8 ng, respectively. The linear regression coefficient was 0.9962–0.9998 for concentrations of 1–25 μg/mL. Cardiac glycosides in the *Digitalis purpurea* leaf displayed intra- and inter-day precisions (RSDs) of <9.30% and average recoveries of 98.63–99.94%. The contents of gitoxin, digitonin, and digitoxin in the *D. purpurea* were 0.197, 0.11, and 0.379 mg/g for leaf dried at 60 °C, 0.058, 0.11, and 0.090 mg/g for leaf dried at ambient temperature, and N.D. (not detected), and 18.379 mg/g, N.D. for seed, respectively. We conclude that our method shows good precision and accuracy.

1. Introduction

Cardiac glycosides in *Digitalis purpurea* and *D. lanata* are widely used to treat congestive heart failure and atrial fibrillation [1]. Digitoxin and gitoxin from the *D. purpurea* leaf are secondary metabolites transformed from the purpurea glycosides A and B [2,3]. Digitonin is contained in the *D. purpurea* seed [4], and digoxin, lanatoside A–C, and deacetyllanatoside C are contained in the *D. lanata* leaf [5,6]. Cardiac glycoside structures containing digitoxose (sugar) and steroid (aglycone) are shown in Fig. 1. The cardiac glycoside composition of *Digitalis* might be variable due to enzyme disintegration, genetic diversity, and/or seasonal effects. The development of a precise analytical method for cardiac glycoside is essential for monitoring the cultivation factors or quality control of *Digitalis*.

The effectiveness of reversed-phase (RP)-HPLC–UV [6–11] or RP-HPLC-fluorescence [12,13] methods for cardiac glycoside detection has been studied in detail. In the RP-HPLC–UV method, cardiac glycosides are detected with low sensitivity because of their weak chromophores. The RP-HPLC-fluorescence method is required for derivatizations. Mass spectrometry [14,15] and immunoassay methods [16] have also been used to analyze cardiac glycosides in blood samples, but have several limitations, including a time-consuming pretreatment step and poor recovery. Therefore, it is difficult to precisely and accurately micro-analyze cardiac glycosides using the above-described methods.

Pulsed amperometric detection (PAD) uses an electrochemical detector to measure the positive potential produced by carbohydrate oxidation on a gold electrode, allowing the direct detection of carbohydrates at low pico-molar levels [17]. High-performance anion-exchange chromatography (HPAEC)-PAD has been used to quantify carbohydrates in plant resources [18–22] because of its strong anion-exchange properties that efficiently separate carbohydrates. However, HPAEC-PAD cannot be used to analyze cardiac glycosides, because of the difficulty in separating cardiac glycosides from other sugars and their digestion products on an anion-exchange column.

The RP-HPLC-PAD method was previously developed to analyze digitoxin and digoxin, but could not be applied to herb samples due to low sensitivity (detection limits of 2.3–14.3 ng) [23]. Here, we developed a highly sensitive and selective RP-HPLC-PAD method for cardiac glycoside detection by optimizing the mobile phase, col-

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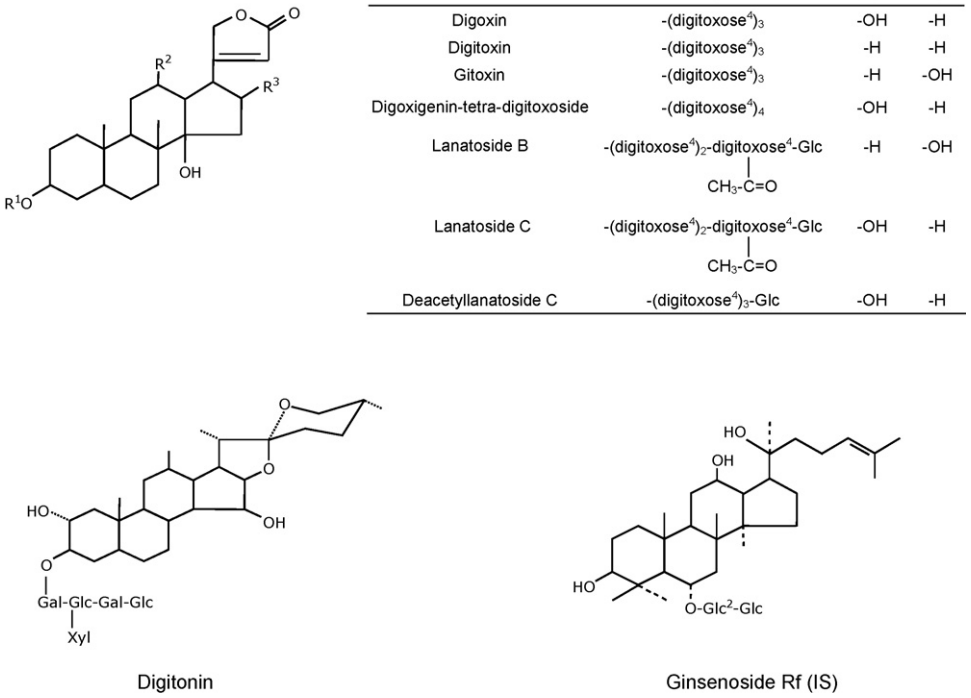

Fig. 1. Chemical structures of cardiac glycosides and ginsenoside Rf (internal standard).

umn, and post-column reagent conditions. Using this method, we successfully determined the cardiac glycosides in the leaf and seed of *D. purpurea*, and determined the temperature-mediated changes of the *D. purpurea* leaf cardiac glycoside content.

2. Experimental

2.1. Materials

Digitoxin and digitonin were purchased from Wako (Tokyo, Japan). Digoxin was purchased from Toronto Research Chemicals (Toronto, Canada). Lanatoside B, lanatoside C, deacetyllanatoside C, gitoxin, digoxigenin-tetra-digitoxoside, and ginsenoside Rf (G-Rf) were purchased from ChromaDex (Santa Ana, CA, USA). HPLC-grade acetonitrile and 50% sodium hydroxide (NaOH) were purchased from Fisher Scientific (Fairlawn, NJ, USA). *D. purpurea* was obtained from an herb farm (Geo-Chang, South Korea) and identified by Prof. Je-Hyun Lee (Dongguk University, Gyeongju, South Korea).

To prepare the standard solutions, sample solutions, and mobile phase, we used 18 MΩ purified water produced by the laboratory water purification system, Automatic Aquarius AW-1001 (Top Trading, Seoul, South Korea). A Millipore membrane filter (type HA, pore size 0.45 μm, Billerica, MA, USA) was used for solvent filtration. All samples were filtered with a disposable syringe filter (PTFE, pore size 0.20 μm, Advantec MFS, Tokyo, Japan) before injection. The weight of each sample was measured on a Mettler Toledo AX 105 DeltaRange (Greifensee, Switzerland).

2.2. Apparatus and high-performance liquid chromatography

HPLC equipment, consisting of a Model Nanospace SI-2/3201 pump, a 3004 column oven with a 10-μL fixed loop, and a 3002 UV detector were purchased from Shiseido (Tokyo, Japan). The Nanospace SI-2/3201 pump has a metal-free head made of polyetheretherketone (PEEK) resin, which resists aggressive chemicals such as alkaline solutions. The PAD from the ICS-3000 series Dionex (Sunnyvale, CA, USA) was equipped with an Au-Flow cell containing a gold working electrode and a solvent-compatible cell containing an Ag/AgCl reference electrode. The potential waveform was as follows: $E1 = -0.2$ V (from 0.00 to 0.04 s); $E2 = 0$ V (from 0.05 to 0.21 s); $E3 = +0.22$ V (from 0.22 to 0.46 s); $E4 = 0$ V (from 0.47 to 0.56 s); $E5 = -2$ V (from 0.57 to 0.58 s); and $E6 = +0.6$ V (0.59 s). Potentials $E1$ and $E2$ were adsorption and delay steps, potentials $E3$ and $E4$ were integration steps, and potentials $E5$ and $E6$ were cleaning activation steps. The data were controlled on a computer running the Chromeleon client program supplied by Dionex.

Chromatographic separation was performed with a Unison UK-C18 column (150 mm × 2.0 mm I.D.; 3 μm, Imtakt, Kyoto, Japan). The mobile phase consisted of 10% acetonitrile (solvent A) and 60% acetonitrile (solvent B). The following program was employed: isocratic elution with A:B (66:34) for 5 min, linear gradient elution from A:B (66:34) to (50:50) from 5 to 30 min, linear gradient elution from A:B (50:50) to (30:70) from 30 to 40 min, linear gradient elution from A:B (30:70) to (10:90) from 40 to 45 min, isocratic elution with A:B (10:90) from 45 to 50 min, A:B (66:34) from 50 to 53 min, and finally equilibration with A:B (66:34) from 53 to 60 min. The mobile phase was made daily by degassing with vacuum filtration, followed by sonication for 20 min before use. The injection volume was 10 μL. The flow rate was 0.2 mL/min, and the separation temperature was 30 °C. A post-column delivery system of 200 mM NaOH at a flow rate of 0.8 mL/min was added to the HPLC system. Throughout the experiment, the post-column delivery system was purged with helium to remove carbonate from the water.

2.3. Preparation of standard solutions

Stock solutions were prepared by dissolving 1 mg of each standard in 1 mL of 50% (v/v) acetonitrile/water in an eppendorf tube. Each stock solution was diluted to create six calibration points (0.25, 0.5, 1, 5, 10, and 25 μg/mL) for calibration curve preparation. The concentration of the internal standard, G-Rf, was 10 μg/mL for all analytes.

Table 1
Linear range, linear equation, correlation coefficient, limits of detection (LOD), and limits of quantification (LOQ) for cardiac glycosides.

Compounds	Linear range (μg/mL)	Linear equation	r^2	PAD		UV at 220 nm	
				LOD (ng)	LOQ (ng)	LOD (ng)	LOQ (ng)
Deacetyllanatoside C	0.4–25	$y = 0.0781x + 0.0075$	0.9998	0.1	0.3	10	30
Lanatoside C	0.4–25	$y = 0.1038x - 0.0179$	0.9972	0.1	0.3	10	30
Digoxin	0.4–25	$y = 0.0918x - 0.0100$	0.9962	0.1	0.3	10	30
Digoxigenin-tetra-digitoxoside	0.5–25	$y = 0.0730x + 0.0158$	0.9970	0.2	0.5	12	40
Lanatoside B	0.8–25	$y = 0.0691x + 0.0213$	0.9998	0.3	0.8	12	40
Gitoxin	0.8–25	$y = 0.1004x - 0.0034$	0.9981	0.3	0.8	12	40
Digitonin	0.8–25	$y = 0.0848x - 0.0098$	0.9978	0.3	0.8	–[a]	–
Digitoxin	0.8–25	$y = 0.0921x - 0.0039$	0.9981	0.3	0.8	10	25

[a] Not detected.

2.4. Preparation of sample solutions

Whole *D. purpurea* plants were divided into two groups. One group was dried at 60 °C for 24 h, while the other was dried at room temperature (RT) for a week. Leaf and seed parts were taken from the whole plants of each group and powdered. Leaf powder (400 mg) from each group was added to 20 mL of 50% acetonitrile, extracted for 10 min under reflux, filtered, and then diluted. The final concentration was 10 mg/mL, including 10 μg/mL of the internal standard (G-Rf). Seed powder (40 mg) from each group was added to 20 mL of 50% acetonitrile, extracted for 10 min under reflux, filtered, and then diluted. The final concentration was 0.4 mg/mL, including 10 μg/mL of the internal standard. The samples were filtered through a disposable syringe filter before HPLC injection.

2.5. Method validation

Linear calibration curves were made at least five times for each reference compound. The regression equation was $y = ax + b$, where y and x were the ratio of the peak area (analytes/internal standard) and sample concentration, respectively. Repeatability was evaluated by performing intra- and inter-day ($n = 4$) assays. Recovery tests for the cardiac glycosides were done by extracting them after adding known amounts of standards (10, 40, and 200 μg) to the leaf powders. Three injections of each sample were performed to measure recovery.

3. Results and discussion

3.1. Optimal conditions of the RP-HPLC-PAD

The RP-HPLC-PAD system focused on detecting sugar moieties in cardiac glycosides. In the acetonitrile-water gradient system, cardiac glycosides were clearly separated on the C18 column. The column effluent was mixed with NaOH before entering the PAD. To determine the optimal analytical conditions, we examined the mobile phase composition, column size, and NaOH concentration in the post-column solution. We first determined whether the PAD sensitivity was affected by the mobile phase. Methanol and tetrahydrofuran could not detect digitoxin because of high currents and severe background noise, while acetonitrile clearly detected digitoxin with low currents and low background noise. Therefore, acetonitrile was chosen as a suitable organic mobile phase.

Some previous papers report that PAD sensitivity is decreased in high acetonitrile concentrations, because the detector response is reduced by acetonitrile adsorbed to the gold working electrode surface [23–26]. Consequently, PAD detection has limited application under conditions of high acetonitrile concentrations (≥30%). To reduce the acetonitrile concentration in the mobile phase, we used a small, 2.0-mm diameter column [26]. As the acetonitrile concentration was increased, the peak sensitivity was nearly constant in the 2.0-mm diameter column.

For post-column delivery system, the range of 150–200 mM NaOH was suitable for detecting most compounds, and 200 mM NaOH had the highest peak sensitivity.

3.2. Relationship between the extraction amount and solvent

To confirm the stability of the standards at high temperature, 1 mg each of digoxin, digitonin, and digitoxin was added to a 50-mL aliquot of 50% methanol or of 50% acetonitrile, refluxed for 1 h, diluted to a final concentration of 5 μg/mL, and then injected into the HPLC system. The cardiac glycosides were stable at high temperatures when cardiac glycosides were extracted using 50% acetonitrile or 50% methanol.

The extraction efficiencies of cardiac glycosides from *D. purpurea* leaf were measured while changing the acetonitrile percentage in the extractant. The extraction efficiency gradually increased with increasing acetonitrile concentration, peaked at 50% acetonitrile, and was relatively constant at a range of 50–100% acetonitrile.

3.3. Analysis of standard cardiac glycosides

The linearity of detection for each analyte was examined using six different standard stock solutions (0.25, 0.5, 1, 5, 10, and 25 μg/mL). A calibration curve was constructed by linear regression of the peak area ratio (analyte/internal standard) *vs.* the analyte concentration. The equations and linear ranges are listed in Table 1. We compared LOD obtained PAD detection and UV detection by using same chromatographic condition. Significantly, the sensitivity of PAD in detecting cardiac glycosides was 33–100 times higher than UV detection at 220 nm. Fig. 2 shows the chromatograms obtained by the UV and PAD methods for eight cardiac glycosides. In the UV method, the cardiac glycoside peaks were too small to be determined because of low sensitivity, and digitonin was not detected. In contrast, using the PAD method, the cardiac glycoside peaks were greatly enhanced by >100 times the *S*/*N* ratio compared to the UV method, and digitonin was detected very well. These results indicate that the cardiac glycosides could be analyzed simultaneously with high sensitivity by our PAD method.

3.4. Analysis of cardiac glycosides in crude drug

To validate our method, we analyzed the reproducibility and recovery of cardiac glycosides contained in the *D. purpurea* leaf dried at 60 °C. The intra- and inter-day precisions (RSDs) were 1.28–9.30% and 6.23–7.80%, respectively. The cardiac glycoside recoveries were evaluated by extracting in 20 mL of 50% acetonitrile after adding the cardiac glycoside mixtures (10, 40, and 200 μg) to 400 mg of the *D. purpurea* leaf sample. The extracted solutions were pretreated with the same method as described in Section 2.4, and subsequently applying 10 μL of the solutions into the HPLC system

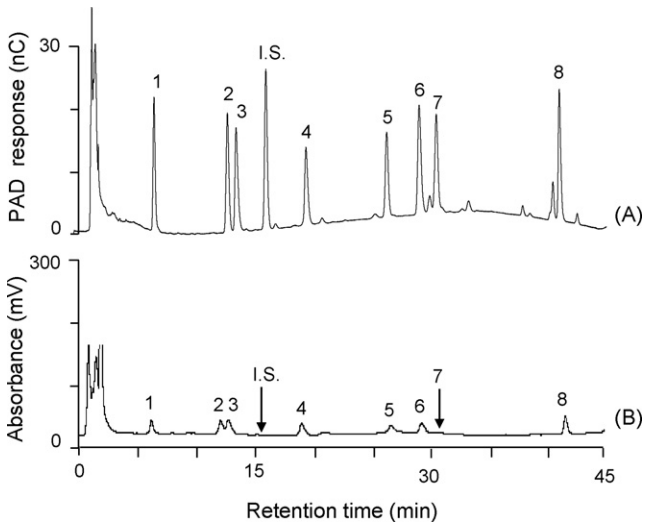

Fig. 2. Detection of standard cardiac glycosides by PAD (A) and UV (B). Peaks: 1, deacetyllanatoside C; 2, lanatoside C; 3, digoxin; 4, digoxigenin-tetra-digitoxoside; 5, lanatoside B; 6, gitoxin; 7, digitonin; 8, digitoxin; I.S., G-Rf. Injected amount: 100 ng.

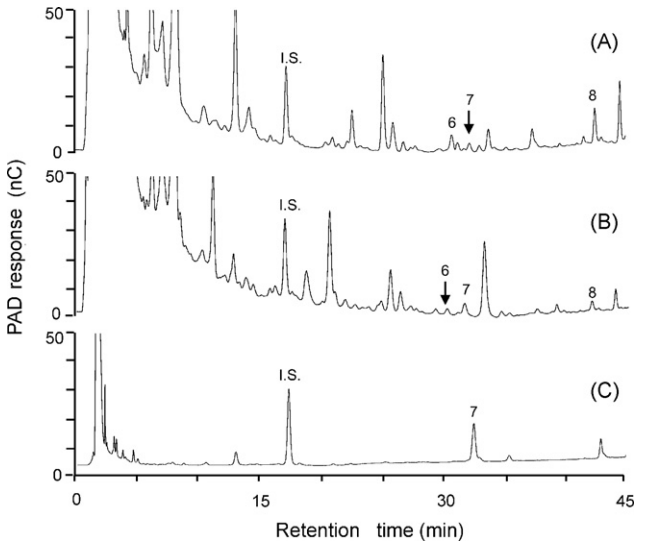

Fig. 3. Chromatograms for *D. purpurea* leaf dried at 60 °C (A) or RT (B), and for *D. purpurea* seed (C). Peaks: 6, gitoxin; 7, digitonin; 8, digitoxin; I.S., G-Rf. Injected amount: 100 μg for leaf (A and B); 4 μg for seed (C).

Table 2
Recovery test for the determination of cardiac glycosides in *D. purpurea* leaf dried at 60 °C ($n = 3$).

Compounds	Added (μg)	Recovery (%)	Mean (%)	RSD (%)
Gitoxin	10	91.439 ± 12.538	95.129	8.029
	40	96.609 ± 8.328		
	200	97.339 ± 0.543		
Digitonin	10	92.356 ± 5.509	94.415	4.618
	40	95.042 ± 6.681		
	200	95.847 ± 0.525		
Digitoxin	10	90.373 ± 11.704	95.684	7.393
	40	99.769 ± 2.749		
	200	96.911 ± 2.114		

(Table 2). The mean recoveries were as follows: gitoxin, 95.13%; digitonin, 94.42%; digitoxin, 95.68%. The RSD ranges for cardiac glycosides were 94.42–95.68% and 4.62–8.03%, respectively.

3.5. Applications

Under optimized conditions, we then analyzed cardiac glycosides contained in *D. purpurea* seed or in leaf dried at 60 °C or ambient temperature (Fig. 3A–C). In *D. purpurea* leaf, gitoxin, digitonin, and digitoxin were detected, but digoxin and the lanatoside series (lanatoside A–C and deacetyllanatoside C) were not. In *D. purpurea* seed, digitonin was detected, but the others were not. Digitonin was present in high amounts (18.379 mg/g) in the seed, and had to be diluted before HPLC injection to be analyzed within the dynamic linear range (0.8–25 μg/mL).

According to Korean Pharmacopoeia (IX), *D. purpurea* leaf is dried at <60 °C. To determine how the cardiac glycoside content of the *D. purpurea* leaf changes with respect to the drying temperature, the cardiac glycoside contents were examined following leaf drying at 60 °C for a day or at ambient temperature for a week (Table 3). The gitoxin and digitoxin contents were 0.197 and 0.379 mg/g for 60 °C, and 0.058 and 0.090 mg/g for ambient temperature, respectively. These results were presumably due to the enzymatic hydrolyzation of gitoxin and digitoxin at ambient temperature, and deactivation of this process under high temperatures. The digitonin contents were the same (0.11 mg/g) for drying at 60 °C or at ambient temperature, indicating that digitonin was not affected by the enzymatic action.

Cardiac glycosides in *D. purpurea* leaf were previously determined by HPLC–UV methods [8,26,27]. Fujii et al. reported that the contents of gitoxin and digitoxin were 0.140 and 0.226 mg/g, respectively [8]. Hagimori et al. reported that the digitoxin contents were 0.21 and 0.11 mg/g for lamina and vein of leaf, respectively [27]. These results agreed with our data. The determination of digitonin has not been reported because of having no UV chromophore. Therefore, digitonin in the leaf or seed of *Digitalis* was determined for the first time through our method.

4. Conclusion

We developed a highly sensitive and selective RP-HPLC-PAD method for cardiac glycoside detection. The 2.0-mm (diameter) column was used to reduce the amount of acetonitrile used in the mobile phase, and 200 mM NaOH was used as a post-column reagent at a flow rate of 0.8 mL/min. Our PAD method displayed sensitive detection comparable to the fluorescence method with derivatization. This method is suitable for the accurate and precise micro-analysis of *D. purpurea* cardiac glycosides, without requiring specific pretreatment processes.

Table 3
Cardiac glycoside contents of three *D. purpurea* types ($n = 4$ samples).

Compounds	Leaf dried at 60 °C		Leaf dried at room temperature		Seed	
	Content (mg/g)	RSD (%)	Content (mg/g)	RSD (%)	Content (mg/g)	RSD (%)
Gitoxin	0.197 ± 0.014	6.923	0.058 ± 0.003	5.764	ND	
Digitonin	0.107 ± 0.008	7.795	0.105 ± 0.007	6.306	18.379 ± 0.758	4.126
Digitoxin	0.397 ± 0.025	6.229	0.090 ± 0.005	6.106	ND	

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References

[1] S. Foster, J.A. Duke, A Field Guide to Medicinal Plants and Herbs of Eastern and Central North America, 2nd ed., Houghton Mifflin Company, New York, 2000.
[2] V. Calcandi, J. Ciropol, Contribution to the study of the cardiac glycosides in the leaves of *Digitalis purpurea* L. stabilized and dried by various methods, Pharmazie 14 (1959) 633–637.
[3] Y. Ikeda, Y. Fujii, I. Nakaya, M. Yamazaki, Quantitative HPLC analysis of cardiac glycosides in *Digitalis purpurea* leaves, J. Nat. Prod. 58 (1995) 897–901.
[4] I. Kawasaki, I. Nishioka, *Digitalis* saponins. I. Seed saponins of *Digitalis purpurea* L. (commercial "digitonin"), Chem. Pharm. Bull. 12 (1964) 1250–1253.
[5] M.V. Balakina, E.N. Zvonkova, K.M. Dyumaev, V.A. Bykov, HPLC control of the quality of *Digitalis lanata* raw material, Pharm. Chem. J. 39 (2005) 587–592.
[6] F. Pellati, R. Bruni, M.G. Bellardi, A. Bertaccini, S. Benvenuti, Optimization and validation of a high-performance liquid chromatography method for the analysis of cardiac glycosides in *Digitalis lanata*, J. Chromatogr. A 1216 (2009) 3260–3269.
[7] F. Orosz, M. Nuridsany, J. Ovadi, Isolation and quantitative determination of some cardioactive glycosides from *Digitalis lanata* by high-performance liquid chromatography, Anal. Biochem. 156 (1986) 171–175.
[8] Y. Fujii, Y. Ikeda, M. Yamazaki, High-performance liquid chromatographic determination of secondary cardiac glycosides in *Digitalis purpurea* leaves, J. Chromatogr. 479 (1989) 319–325.
[9] Y. Fujii, Y. Ikeda, I. Okamoto, M. Yamazaki, Separation and determination of lanatosides in *Digitalis lanata* leaves by high-performance liquid chromatography, J. Chromatogr. 508 (1990) 241–246.
[10] H. Wiegrebe, M. Wichtl, High-performance liquid chromatographic determination of cardenolides in *Digitalis* leaves after solid-phase extraction, J. Chromatogr. 630 (1993) 402–407.
[11] F. Castro Braga, W. Kreis, R. Almeida Recio, A. Braga de Oliveira, Variation of cardenolides with growth in a *Digitalis lanata* Brazilian cultivar, Phytochemistry 45 (1997) 473–476.
[12] K. Belsner, B. Buchele, Fluorescence detection of cardenolides in reversed-phase high-performance liquid chromatography after post-column derivatization, J. Chromatogr. Biomed. Appl. 682 (1996) 95–107.
[13] M.C. Tzou, R.A. Sams, R.H. Reuning, Specific and sensitive determination of digoxin and metabolites in human serum by high performance liquid chromatography with cyclodextrin solid-phase extraction and pre-column fluorescence derivatization, J. Pharm. Biomed. Anal. 13 (1995) 1531–1540.
[14] A. Tracqui, P. Kintz, B. Ludes, P. Mangin, High-performance liquid chromatography–ionspray mass spectrometry for the specific determination of digoxin and some related cardiac glycosides in human plasma, J. Chromatogr. Biomed. Appl. 692 (1997) 101–109.
[15] L. Frommherz, H. Köhler, B. Brinkmann, M. Lehr, J. Beike, LC–MS assay for quantitative determination of cardio glycoside in human blood samples, Int. J. Legal Med. 122 (2008) 109–114.
[16] S.A. Jortani, D. Trepanier, R.W. Yatscoff, R. Valdes Jr., Convergence of three methods to resolve discrepant immunoassay digitoxin results, Clin. Chem. 43 (1997) 1805–1808.
[17] J.S. Jeong, H.R. Yoon, S.P. Hong, Development of a new diagnostic method for galactosemia by high-performance anion-exchange chromatography with pulsed amperometric detection, J. Chromatogr. A 1140 (2007) 157–162.
[18] T.R.I. Cataldi, G. Margiotta, L. Iasi, B.D. Chio, C. Xiloyannis, S.A. Bufo, Determination of sugar compounds in olive plant extracts by anion-exchange chromatography with pulsed amperometric detection, Anal. Chem. 72 (2000) 3902–3907.
[19] C. L'homme, J.L. Peschet, A. Puigserver, A. Biagini, Evaluation of fructans in various fresh and stewed fruits by high-performance anion-exchange chromatography with pulsed amperometric detection, J. Chromatogr. A 920 (2001) 291–297.
[20] T.R.I. Cataldi, D. Nardiello, Determination of free proline and monosaccharides in wine samples by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), J. Agric. Food Chem. 51 (2003) 3737–3742.
[21] Y. Cai, J. Liu, Y. Shi, L. Liang, S. Mou, Determination of several sugars in serum by high-performance anion-exchange chromatography with pulsed amperometric detection, J. Chromatogr. A 1085 (2005) 98–103.
[22] K. Schutz, E. Muks, R. Carle, A. Schieber, Separation and quantification of inulin in selected artichoke (*Cynara scolymus* L.) cultivars and dandelion (*Taraxacum officinale* WEB. ex WIGG.) roots by high-performance anion exchange chromatography with pulsed amperometric detection, Biomed. Chromatogr. 20 (2006) 1295–1303.
[23] K. Kelly, B. Kimball, J. Jonston, Quantitation of digitoxin, digoxin, and their metabolites by high-performance liquid chromatography using pulsed amperometric detection, J. Chromatogr. A 711 (1995) 289–295.
[24] J. Haginaka, Y. Nishimura, J. Wakai, H. Yasuda, K. Koizumi, T. Nomura, Determination of cyclodextrins and branched cyclodextrins by reversed-phase chromatography with pulsed amperometric detection and a membrane reactor, Anal. Biochem. 179 (1989) 336–340.
[25] K. Wasserkrug, Z.E. Rassi, High performance liquid phase separation of glycosides. I. Reversed phase chromatography of cyanogenic glycosides with UV and pulsed amperometric detection, J. Liq. Chromatogr. Relat. Technol. 20 (1997) 335–349.
[26] H.J. Kwon, J.S. Jeong, Y.M. Lee, S.P. Hong, A reversed-phase high-performance liquid chromatography method with pulsed amperometric detection for the determination of glycosides, J. Chromatogr. A 1185 (2008) 251–257.
[27] M. Hagimori, T. Matsumoto, Y. Mikami, Digitoxin biosynthesis in isolated mesophyll cells and cultured cells of *Digitalis*, Plant Cell Physiol. 25 (1984) 947–953.