Separation of digoxin by liquid–liquid extraction from extracts of foxglove secondary glycosides

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Abstract

The present study deals with the extraction of digoxin (Dgx) from chloroform and trichloroethylene extracts of the secondary glycosides of fermented foxglove (*Digitalis lanata* Ehrh.) foliage by liquid–liquid extraction. The extraction degree (*ED*) of Dgx achieved by maceration and percolation using 10 vol.% aqueous ethanol solution was higher than 95%. Using trichlorethylene and chloroform, *ED* of Dgx of about 100 and 96%, respectively, from the liquid ethanolic extracts (macerate or percolate) were achieved by the four-cycle extraction. Fifteen separating funnels were employed for the liquid-liquid extraction. Three different four-component two-phase systems (ethanol:water–chloroform:ethyl acetate, ethanol:water–chloroform:trichloroethylene and ethanol:water–trichloroethylene:ethyl acetate) were tested as an extracting solvent to get the final product having more than 98% of Dgx. The initial amount of the chloroform or trichloroethylene extract in the light phase was varied between 5 and 25 g/L, while the volume ratio of light and heavy phases was in the range of 1:1 to 1:2. The best Dgx yield of 98% was achieved with the system ethanol:water–chloroform:trichloroethylene 35:15:20:30 at the volume ratio of the phases of 1:1.1 and at the initial amount of the extract of 15 g/L. Purity of the separated digoxin was 99.8%.

Keywords: digoxin, foxglove, *Digitalis lanata* Ehrh., liquid–liquid extraction, solid–liquid extraction.

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Bioactive products from plant materials, known as phytochemicals, are often used in manufacturing of phytopharmaceuticals and phytotherapy because of their specific characteristics [1]. Phytopreparations are increasingly being used for various treatments in both human and veterinary medicine, especially for treating the most serious diseases such as: heart disease, cancer, AIDS, herpes, leukemia and viral diseases [1–7]. These substances are rare, expensive and difficult to find on market.

Phytochemicals are obtained from plant materials by specific isolation and purification processes. The most important phases in their isolation from plant raw materials and further purification are solid-liquid extraction [8–14], crystallization [12] and liquid–liquid extraction [10–12]. Highly valuable bioactive substances are usually accompanied with various compounds of similar structure, and are difficult to be isolated in their pure crystalline forms as they crystallize isomorphically [12]. This is also the case with digoxin (Dgx), a highly valuable and efficient secondary cardiotonic glycoside isolated from the fermented foliage of foxglove (*Digitalis lanata* Ehrh.) [15]. This compound, which is irreplaceable drug for a heart disease (so-called elderly heart), has not been synthesized yet because of its complex structure. Besides cardiotoxic effects, Dgx has moderate diuretic effects, as well as anticancer and antiviral activities (herpes) [3–7].

Dgx is, as a rule, found in a mixture of secondary glycosides, such as digitoxin (Dx) and gitoxin (Gx), having very similar structure. One of the methods for isolation of Dgx from this mixture obtained by the extraction from the fermented foliage of foxglove [16–21], with a high degree of purity, is the liquid–liquid extraction [10,11,14]. Pekić [14] studied the batch liquid–liquid extraction of Dgx from the crystallizate of secondary glycosides isolated from the fermented foliage of foxglove.

The present study deals with the isolation of Dgx from chloroform and trichloroethylene extracts of the secondary glycosides of fermented foxglove (*Digitalis lanata* Ehrh.) foliage by liquid-liquid extraction using 15 separating funnel. In a previous procedure, the secondary glycosides were extracted from the fermented foxglove foliage by an aqueous ethanol solution (10 or 50 vol.%) using maceration or percolation. Then, the secondary glycosides were extracted from the aqueous ethanol extract by chloroform or trichloroethylene and were further purified. Finally, three different four-component two-phase systems (ethanol:water–chloroform:ethyl acetate, ethanol:water–chloroform:trichloroethylene and ethanol:water–trichloroethylene:ethyl

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acetate) were tested as an extracting solvent to get the final product having more than 98% of Dgx. The initial amount of the chloroform or trichloroethylene extract in the light phase was varied between 5 and 25 g/L, while the volume ratio of light and heavy phases was in the range of 1:1 to 1:2. The main goal was to define the operating conditions of Dgx isolation (type of extracting system, the initial concentration of the extract, volume ratio of the phases and number of separating stages). Ethyl acetate is used for the first time as a component of an extracting system for Dgx isolation from extracts of secondary glycosides of foxglove by liquid–liquid extraction.

EXPERIMENTAL

Materials

Plant material

Dried foliage of plantation-grown foxglove (*Digitalis lanata* Ehrh.) (Borča, Serbia) was used. Dark green leaves, 10–12 cm in length, about 3 cm wide, hairy on the surface, having characteristic odor, were stored in paper bags. The plant material contained 0.3 to 0.5% of lanatozide C. Identification and quality control of the foliage were done by the official methods [22]. The foliage was chopped in a hammer mill and then sieved. The fraction having the average particle size of 7.0 mm was employed.

Chemicals

Lanatoside C, Dx, Gx, Dgx, digoxigenin, aucubin, acetoside, sodium nitrite, sodium molybdate and sodium hydroxide were purchased from Merck. Chloroform, ethanol, methanol, methylene chloride, acetonitrile, ethyl acetate, anhydrous formic acid, glacial acetic acid, hydrochloric acid, sulfuric acid (98%), lead(II) acetate, ammonium hydroxide, methylene chloride, $CuSO_4$. $5H_2O$, $CoCl_2 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, hexamethylene tetramine, hydrazine sulfate potassium bromide (previously dried for 1 h at 250 °C), silica gel F_{254} and silica gel G were obtained from Fluka. All chemicals were pro analysis quality, unless otherwise indicated. Distilled water was also used.

Reagents

Basic lead(II) acetate solution. Concentrated ammonium hydroxide was added into a solution of lead(II) acetate (30%) until tests with 2–3 drops of this solution in the presence of phenolphthalein turned the color of white wine.

Xanthydrol reagent. Xanthydrol (10 mg) was dissolved in glacial acetic acid (50 mL) in a 100 mL flask, applying slight heating. A hydrochloric acid solution (1 mL, 25%) was added to the flask, which was then filled up with glacial acetic acid to the mark. A fresh solution of xanthydrol was prepared for each determination.

Plant material fermentation

Chopped foliage (5 g) was soaked with distilled water in a mass ratio of 1:2, well homogenized, put in plastic bags, sealed and left to ferment at 37 °C for 48 h [16]. The yields of Dx, Gx and Dgx were about 99–100%. The yields of Dx, Gx and Dgx were defined as the amount of glycoside formed divided by the theoretically amount of glycoside expected to be obtained from lanatozide A, B and C present in the plant material.

Extraction of secondary glycosides from fermented plant material

Secondary glycosides were extracted by maceration and percolation using 10 or 50 vol.% ethanol as an extracting solvent. The Dx, Gx and Dgx extraction degrees (EDs) were defined as the percentages of Dx, Gx and Dgx extracted from the fermented plant material, respectively.

Maceration and percolation

Maceration was carried out according to the modified procedure [16]. The fermented plant material (100 g) and the extracting solvent (10 or 50 vol.% ethanol, 1 L) were added to the extracting vessel equipped with a stirrer. The plant material was macerated at room temperature for 1 h. The liquid extract (macerate) was separated from the plant material by vacuum filtration. The extraction was repeated two more times in the same way. The macerates obtained were mixed in a separating funnel. The filtration cake of the exhausted plant material was washed 3 times with appropriate solvent (100 mL). Washing solutions were added to the total macerate in the separating funnel.

For percolation, a battery of 10 stainless steel percolators (inner diameter: 20 cm; height: 60 cm; empty volume: 18.84 L) having a ball valve at the bottom was employed. The appropriate volume of the extracting solvent was poured in the percolator, and the fermented plant material (particle size: 7.0 mm) was added to the plant layer height of 30 cm. Aqueous solutions of ethanol (10 or 50 vol.%) were used as extracting solvents. The percolation was performed at room temperature at a volumetric flow rate of percolate of 4.0 L/h using a constant volume of the extracting solvent (24 L). The percolate leaving a percolator was used as the extracting solvent in the next percolator. In this way, the percolate leaving the tenth percolator entered the first percolator. When the Dgx extraction degree for the percolate leaving a percolator was higher than 95%, the exhausted fermented foliage was replaced by a fresh plant material, and the process continued further. Samples of the liquid extract (percolate) were taken with the progress of percolation, and the content of Dgx was determined in each sample using the HPLC method.

Extraction of secondary glycosides using chloroform or trichlorethylene

The liquid extracts (macerate or percolate) obtained were treated by liquid-liquid extraction using chloroform or trichlorethylene as the extracting solvent to obtain the liquid fractions of secondary glycosides. The liquid-liquid extraction was performed four times with the extract-to-solvent volume ratio of 1×1:2 and 3×1:4 within 20 min in separating funnels. The extract-to--solvent ratios and the extraction time were previously defined by laboratory tests. The goal of this extraction was to extract more than 95% Dgx from aqueous––alcoholic extracts. The chloroform and trichlorethylene extracts were concentrated by vacuum evaporation to the 1/20 of their initial volume.

Treatment of concentrated extracts with MgO. MgO was added to the concentrated extract (MgO-to-the initial plant material mass ratio 1:10), mixed well and left to stand for 45 min, with occasional stirring. The resulting suspension was filtered under vacuum to separate the concentrate. The MgO cake was washed with the appropriate solvent (chloroform or trichloroethylene, 3×250 mL). The filtrates were added to the concentrate, which was then washed with distilled water (4 times, each with 2 L). The washed concentrate was evaporated under vacuum, and the solid product was further dried at 60 °C under vacuum to the moisture content up to 6%. The contents of Dx, Gx and Dgx in the obtained products were determined by the HPLC method [22].

Liquid–liquid extraction of highly-pure Dgx from purified extracts

Three four-component two-phase systems, namely a) ethanol:water–chloroform:ethyl acetate (EtOH:H_2O––$CHCl_3$:EtOAc), b) ethanol:water–chloroform:trichlorethylene (EtOH:H_2O–$CHCl_3$:TCE) and c) ethanol:water––trichlorethylene:ethyl acetate (EtOH:H_2O–TCE:EtOAc), were used to extract Dgx from the concentrated extracts by liquid–liquid extraction. These systems were at first equilibrated at the volume light (EtOH:H2O)-to-heavy (organic solvent mixture $CHCl_3$:EtOAc, $CHCl_3$:TCE or TCE:EtOAc) phase ratio of 1:1 at room temperature (20–25 °C) in fifteen separating funnels (10 L) and then used to separate Dgx from Dx and Gx. Volume fractions of two-component light and heavy phases were varied from 10:40 to 40:10 vol. to define the optimum composition of both phases. Different amounts (10 to 25 g) the chloroform or trichlorethylene extracts having different contents of Dgx, Dx and Gx were added to the light phase of the extracting system in the first separating funnel.

The light phase (3 L) was poured into 15 separating funnels (10 L). The volume ratio of equilibrated light and heavy phase was varied from 1:1 to 1:2. The heavy phase (3 L) was added to the first separating funnel, mixed (5 min) and left to separate. The heavy phase was transfered to the second sepratating funnel and the procedure (vigorous shaking followed by separation of the phases) was repeated until the last separating funnel. The contents of Dgx, Dx and Gx were determined in both the light and heavy phase after each separation step. For this, samples were taken from both phases of each separating funnel and evaporated to get more than 50 mg of dry residue for determining the secondary glycosides.

The light phases containing Dgx and traces of Dx ili Gx (less than 0.5%) were combined and evaporated to 1/20 of the initial volume under vacuum (100-200 mm Hg). The crystals of Dgx obtained from the concentrated solution of the light phase were separated by vacuum filtration, washed with a small portion of cold ethanol and then dried at 80 °C under vacuum. The ethanol filtrates from washings were collected and evaporated to recuperate the solvent.

The heavy phases containing Dx, Gx and traces of Dgx were also collected and evaporated to dry under vacuum (100–200 mmHg).

Determination of Dx, Gx and Dgx

The basic solution preparation

The fermented plant material (5 g) was transferred into a flask, methanol (50 mL, 50%) was added, and the suspension was shaken for 1 h. Then, while shaken, 5 mL of freshly prepared 30% solution of basic lead(II) acetate was gradually added into the suspension, the content was well shaken, left to rest for 5 min and then the excess of lead(II) acetate was precipitated by adding a sodium sulfate solution (5%). If the extract in the flask gave a positive reaction to Pb^{2+} with a 2% solution of potassium iodide (2–3 drops of clear extract mixed on a watch glass with 2–3 drops of 2% solution of potassium iodide; a yellow precipitate was formed in the presence of Pb^{2+}), the sodium sulfate solution was added until obtaining a negative reaction (no yellow precipitate). After the sedimentation of excess lead(II) acetate, the suspension was filtered through a quantitative filter paper set on a rapid filtration funnel. The filtrate was separated in a separating funnel, and the first turbid filtrate was returned for re-filtration until the filtrate remained totally clear. The precipitate on the filter paper was rinsed 3 times with a methanol solution (50 mL, 50%). The secondary glycosides and the accompanying extraction materials were re-extracted from the combined filtrate with chloroform (once 25 mL and 4 times with 12.5 mL). The collected chloroform re-extracts were passed through anhydrous sodium sulfate (sodium sulfate on the filter paper in the filtration funnel) into a round bottom flask (250 cm^3). Sodium sulfate was rinsed 3 times with 10 mL of chlo-

roform each. The chloroform re-extract, together with chloroform solutions obtained by rinsing, was evaporated to dry under vacuum at 60-70 °C to get a dry chloroform re-extract.

Determination of Dx, Gx and Dgx content

The dry chloroform re-extract (50 mg) was dissolved in 100 mL of methanol (basic solution), and the obtained solution was used to determine the contents of Dx, Gx and Dgx by the HPLC method [22].

Preparation of the standard solution of Dx,Gx and Dgx

Standards of Dx, Gx and Dgx (50 mg), previously dried to constant weight in a vacuum desiccator over phosphorus(V) oxide, was dissolved in methanol (100 mL).

HPLC Analysis of Dx, Gx and Dgx

Apparatus: Agilent 1100 Series. Column: length = 0.15 m, diameter = 3.9 mm, stationary phase: octadecylsilyl–silica gel for chromatography (5 μm). Mobile phase A: acetonitrile:water (10:90, *V*/*V*). Mobile phase B: acetonitrile:water (90:10, *V*/*V*).

Detection: 220 nm. Flow rate: 1.5 ml/min. Volume of injection: Inject in 10 mL basic solution and standard solution. Temperature: room. Retention times and concentrations of mobile phases A and B are given in Table 1.

Calculation (Ph. Eur., 7th ed., 2012, Method 2.2.29) [22]:

$$\%\ \text{Glycoside} = \frac{\text{Ppr} \times Wst \times K}{\text{Pst} \times \text{Wpr}} \cdot \frac{(100 - a_{\text{st}})}{(100 - a_{\text{pr}})}$$

where Ppr is the Dx, Gx or Dgx peak area of the investigated basic solution, Pst is the Dx, Gx or Dgx peak area of the standard solution, Wpr is the weight of the investigated substance (mg), *Wst* is the weight of the standard substance (mg), *K* is the Dx, Gx or Dgx content in the working standard (%), a_{pr} is the drying loss of the investigated substance (%) and a_{st} is drying loss of the standard substance (%).

RESULTS AND DISCUSSION

Extraction of Dgx by maceration and percolation from fermented plant material

The extraction degrees (*ED*) of Dx, Gx, Dgx and total glycoside (*TG*) achieved by maceration and percolation using 10 and 50 vol.% aqueous ethanol solutions were higher than 95% (Table 2). The 10 vol.% aqueous ethanol solution was recommended for extraction at higher scales although it yielded somewhat lower EDs (by 1–2%) than the more concentrated ethanol solution (50 vol.%). However, the use of a smaller amount of ethanol for the Dgx extraction would reduce the extraction costs. The percolation using 10 percolators was as efficient as the three-step maceration, so both techniques could be used for Dgx extraction from the fermented plant material.

Liquid–liquid extraction of secondary glycosides from ethanolic extracts

Chloroform or trichlorethylene was used to extract Dx, Gx and Dgx from the liquid ethanolic extracts (macerate or percolate) by the four-cycle liquid-liquid extraction (the extract-to-solvent volume ratio of 1×1:2 and 3×1:4; 20 min per a cycle). The values of EDs of Dx, Gx, Dgx and total glycosides (TGs) are presented in Table 3. The best *ED* of about 100 and 96% were achieved using trichlorethylene and chloroform, respectively by the four-cycle extraction. The dry chloroform and trichloroethylene extracts had different contents of Dx, Gx, Dgx and TGs (Table 4). The latter extract contained Dx, Gx, Dgx and TGs at higher levels than the former extract. Therefore, trichlorethylene

Table 1. Retention time and the concentration of mobile phases A and B

Time, min	Mobile phase A	Mobile phase B
0 → 5	78	22
5 → 15	78 → 30	22 → 70
15 → 16	30 → 78	70 → 22
16 → 30	78	22

Table 2. ED values (relative to the content in the fermented plant material, %) of Glycoside achieved by maceration and percolation using 10 and 50 vol.% aqueous ethanol solutions (fermented plant material-to-solvent mass ratio: 1:10; extraction time: 3×1 h; average particle size: 7 mm; room temperature)

Glycoside	Maceration		Percolation	
	10 vol.%.	50 vol.%	10 vol.%.	50 vol.%
Digitoxin	96.0	98.0	97.5	98.5
Gitoxin	95.0	96.0	97.0	97.5
Digoxin	97.0	98.0	98.5	99.5
Total glycosides	101.0	102.0	101.5	102.5

Table 3. Values of ED (relative to the content in the macerate or the percolate, %) of secondary glycosides achieved by chloroform and trichlorethylene from macerates and percolates obtained by the 10% vol. aqueous ethanol solution under optimal conditions

Type of extraction	Extraction cycle	Extracting agent							
		Chlorophorm				Trichlorethylene			
		Digitoxin	Gitoxin	Digoxin	Total glycosides	Digitoxin	Gitoxin	Digoxin	Total glycosides
Maceration	1	72	75	80	83	75	82	83	90
	2	86	84	86	88	92	89	87	92
	3	89	88	90	92	90	90	91	95
	4	93	93	94	96	94	95	95	100
Percolation	1	84	79	84	86	83	86	85	91
	2	87	86	88	90	88	90	90	93
	3	90	91	92	96	92	92	93	98
	4	95	96	96	99	100	100	100	102

Table 4. Contents of Dx, Gx, Dgx and TG in dry chloroform and trichlorethylene extracts (Relative to the mass of extract, %)

Glycoside	Extracting agent	
	Trichlorethylene	Chlorophorm
Digitoxin	35.2	12.3
Gitoxin	12.6	5.6
Digoxin	46.2	43.8
Total glycosides	81.8	80.0

was selected as extracting solvent to recover secondary glycosides from the liquid ethanolic extracts.

Separation of Dgx from extracts of secondary glycosides by liquid-liquid extraction

Tables 5–7 presents the results of separating Dx, Gx and Dgx from the trichlorethylene extract by three two-phase systems: EtOH:H_2O–$CHCl_3$: EtOAc, EtOH:H_2O–$CHCl_3$:TCE and EtOH:H_2O–TCE:EtOAc. The optimal systems ensuring the highest Dgx content in the light phase of 78, 76 and 85% were as follows: EtOH:H_2O–$CHCl_3$:EtOAc 25:25:30:20, EtOH:H_2O–$CHCl_3$:TCE 30:20:30:20 and EtOH:H_2O–TCE:EtOAc 35:15:20:30 *V*/*V*, respectively.

Table 5. Separation of Dx, Gx and Dgx by the two-phase system EtOH:H_2O–$CHCl_3$:EtOAc (concentration of trichlorethylene extract in the light phase: 15 g/l; light-to-heavy phase volume ratio: 1:1; volume of phases: 3 L; room temperature)

Volume ratio EtOH:H_2O:$CHCl_3$:EtOAc	Content of glycosides, relative to the content in the initial solution of the trichlorethylene extract from the light phase, %					
	Light phase (EtOH:H_2O)			Heavy phase ($CHCl_3$:EtOAc)		
	Digitoxin	Gitoxin	Digoxin	Digitoxin	Gitoxin	Digoxin
10:40:10:40	45.5	26.7	35.0	64.5	63.3	65.0
10:40:20:30	35.0	28.0	45,5	65.0	72.0	54.5
10:40:30:20	25,7	33.0	42.5	74.3	67.0	57.5
10:40:35:15	22.5	37.0	44.6	77.5	63.0	55.4
10:40:40:10	18.8	41.0	46.0	81.2	59.0	64.0
20:25:10:40	34,6	55.5	48.0	65.4	45.5	32.0
20:25:20:30	28.5	45.7	52.0	71,5	54.3	40.0
20:25:30:20	25.0	34.0	55.5	75,0	56.0	44.5
20:25:35:15	20.4	27.0	60.0	74.6	63.0	55.0
20:25:40:10	17.0	15.5	63.0	83.0	84.5	70.0
25:25:10:40	40.5	45.0	62.0	65.0	55.0	44.5
25:25:20:30	35.0	40.8	64.0	68.0	59.2	55.0
25:25:30:20	22.5	35.0	78.0	72.0	65.0	22,0
25:25:35:15	20.5	30.5	58.0	69.5	69.5	42,0
25:25:40:10	19.0	20.0	60.5	71.0	80.0	39.5

Table 5. Continued

Volume ratio EtOH:H_2O:$CHCl_3$:EtOAc	Content of glycosides, relative to the content in the initial solution of the trichlorethylene extract from the light phase, %					
	Light phase (EtOH:H_2O)			Heavy phase ($CHCl_3$:EtOAc)		
	Digitoxin	Gitoxin	Digoxin	Digitoxin	Gitoxin	Digoxin
30:20:10:40	35.0	30.0	52.5	65.0	70.0	47.5
30:20:20:30	24.0	27.0	48.5	76.0	73.0	51.5
30:20:30:15	15.5	20.0	45.5	84.5	80.0	55.5
30:20:40:10	12.0	17.0	40.5	88.0	83.0	59.5
35:15:10:40	25.0	25.5	55.0	75.0	74.5	45.0
35:15:20:30	21.0	21.0	50.0	79.0	79.0	50.0
35:15:30:20	18.0	16.0	45.5	82.0	84.0	54.5
35:15:35:15	16.5	13.0	42.0	83.5	87.0	48.0
35:15:40:10	15.0	11.5	40.0	85.0	88.5	60.0
40:10:10:40	20.0	20.5	38.0	80.0	79.5	62.0
40:10:20:30	16.0	15.5	35.5	84.0	84.5	64.5
40:10:30:20	14.0	14.0	32.5	86.0	86.0	68.5
40:10:35:15	12.0	12.5	31.0	88.0	87.5	69.0
40:10:40:10	10.0	11.0	30.0	90.0	89.0	70.0

Table 6. Separation of Dx, Gx and Dgx by the two-phase system EtOH:H_2O–TCE:EtOAc (concentration of trichlorethylene extract in the light phase: 15 g/l; light-to-heavy phase volume ratio: 1:1; volume of phases: 3 L; room temperature)

Volume ratio EtOH:H_2O:TCE:EtOAc	Content of glycosides, relative to the content in the initial solution of the trichlorethylene extract from the light phase, %					
	Light phase (EtOH:H_2O)			Heavy phase (TCE:EtOAc)		
	Digitoxin	Gitoxin	Digoxin	Digitoxin	Gitoxin	Digoxin
10:40:10:40	56.5	24.7	30.0	43.5	75.3	70.0
10:40:20:30	45.0	30.0	34.5	55.0	70.0	65.5
10:40:30:20	35.5	35.0	40.5	64.5	65.0	59.5
10:40:35:15	30.5	41.0	46.6	69.5	49.0	53.4
10:40:40:10	28.0	43.0	20.0	72.0	80.0	80.0
20:25:10:40	24.6	20.5	25.0	79.5	79.5	75.0
20:25:20:30	36.5	20.7	38.0	63.5	79.3	62.0
20:25:30:20	35.0	25.0	33.0	65,0	87.0	77.0
20:25:35:15	35.0	30.0	37.0	65.0	70.0	63.3
20:25:40:10	30.0	36.0	40.0	70.0	64.0	60.0
25:25:10:40	50.0	40.0	46.0	50.0	60.0	54.0
25:25:20:30	53.0	42.8	50.0	47.0	57.2	50.0
25:25:30:20	46.0	35.0	45.0	54.0	65.0	55.0
25:25:35:15	38.0	27.5	49.0	72.0	72.5	51.0
25:25:40:10	35.0	20.0	57.0	65.0	80.0	43.0
30:20:10:40	30.0	17.0	65.0	70.0	83.0	35.0
30:20:20:30	25.0	15.0	70.0	75.0	85.0	30.0
30:20:30:20	20.0	12.0	76.0	80.0	88.0	24.0
30:20:30:15	18.5	10.0	68.5	81.5	90.0	31.5
30:20:40:10	15.0	8.0	56.5	85.0	92.0	43.5
35:15:10:40	45.0	37.5	60.0	55.0	62.5	40.0
35:15:20:30	36.0	30.0	50.0	74.0	70.0	50.0
35:15:30:20	30.0	25.0	46.0	70.0	75.0	54.0
35:15:35:15	25.0	20.0	36.0	75.0	80.0	64.0
35:15:40:10	18.0	15.0	30.0	82.0	85.0	70.0

Table 6. Continued

Volume ratio EtOH:H_2O:TCE:EtOAc	Content of glycosides, relative to the content in the initial solution of the trichlorethylene extract from the light phase, %					
	Light phase (EtOH:H_2O)			Heavy phase (TCE:EtOAc)		
	Digitoxin	Gitoxin	Digoxin	Digitoxin	Gitoxin	Digoxin
40:10:10:40	30.0	28.5	48.0	70.0	71.5	62.0
40:10:20:30	22.0	25.0	38.5	78.0	75.0	61.5
40:10:30:20	18.0	20.0	36.5	82.0	80.0	63.5
40:10:35:15	14.0	16.5	33.0	86.0	83.5	67.0
40:10:40:10	12.0	14.0	31.0	88.0	86.0	69.0

Table 7. Separation of Dx, Gx and Dgx by the two-phase system EtOH:H_2O–$CHCl_3$:TCE (concentration of trichlorethylene extract in the light phase: 15 g/l; light-to-heavy phase volume ratio: 1:1; volume of phases: 3 L; room temperature)

Volume ratio EtOH:H_2O–$CHCl_3$:TCE	Content of glycosides, relative to the content in the initial solution of the trichlorethylene extract from the light phase, %					
	Light phase (EtOH:H_2O)			Heavy phase ($CHCl_3$:TCE)		
	Digitoxin	Gitoxin	Digoxin	Digitoxin	Gitoxin	Digoxin
10:40:10:40	36.5	6.7	35.0	65.5	43.3	65.0
10:40:20:30	35.0	60.0	54,5	65,0	40.0	45.5
10:40:30:20	30,5	58.0	48.5	69.5	42.0	41.5
10:40:35:15	26.5	51.0	46.6	73.5	49.0	26.5
10:40:40:10	25.0	42.0	50.0	75.0	58.0	50.0
20:25:10:40	44,6	65.5	60..0	55.4	35.5	40.0
20:25:20:30	38.5	55.7	65.0	61,5	47.3	35.0
20:25:30:20	30.0	43.0	70.0	70,0	67.0	30.0
20:25:35:15	26.5	35.0	67.0	73.5	65.0	43.0
20:25:40:10	21.0	24.5	65.0	79.0	75.5	35.0
25:25:10:40	50.5	57.0	73.0	49.5	43.0	27.0
25:25:20:30	43.0	52.8	78.0	57.0	47.2	22.0
25:25:30:20	30.5	43.0	82.0	69.5	67.0	28,0
25:25:35:15	26.5	37.5	69.0	73.5	62.5	31,0
25:25:40:10	22.0	27.0	66.5	78.0	73.0	33.5
30:20:10:40	40.0	42.0	80.0	60.0	58.0	20.0
30:20:20:30	44.0	37.0	85.5	66.0	23.0	14.5
30:20:30:20	48.0	44.0	89.0	52.0	56.0	11.0
30:20:35:15	26.5	32.0	58.5	73.5	68.0	41.5
30:20:40:10	25.0	29.0	52.5	75.0	71.0	47.5
35:15:10:40	35.0	33.5	60.0	65.0	66.5	40.0
35:15:20:30	30.0	20.0	85.0	70.0	80.0	15.0
35:15:30:20	25.0	22.0	50.5	75.0	78.0	49.5
35:15:35:15	20.0	16.0	48.0	80.0	84.0	88.0
35:15:40:10	18.0	15.0	45.0	82.0	85.0	55.0
40:10:10:40	30.0	28.5	48.0	70.0	71.5	62.0
40:10:20:30	22.0	25.0	38.5	78.0	75.0	61.5
40:10:30:20	18.0	20.0	36.5	82.0	80.0	63.5
40:10:35:15	14.0	16.5	33.0	86.0	83.5	67.0
40:10:40:10	12.0	14.0	31.0	88.0	86.0	69.0

Table 8 represents the EDs of Dx, Gx and Dgx for the optimum light and heavy phases obtained from chloroform and trichlorethylene extracts by liquid-liquid extraction using the three optimum two-phase systems. The highest ED of Dgx in the light phase achieved in the first ten seprataing funnels (Figures 1 and 2) was

in the range between 92.0 and 99.8%, while the contents of Dx and Gx were ignorable (0.0 to 0.5%). The best two-phase system was EtOH:H_2O–$CHCl_3$:TCE 35:15:20:30, which ensured the highest ED of Dgx of 99.5 and 99.8% in the case of the chlorophorm and trichlorethylene extracts, respectively. This system was accepted as the optimum one for separation of Dgx from the chloroform and trichlorethylene extracts.

Table 8. The ED values (extraction degree relative to the content in the initial solution, %) of Dx, Gx and Dgx for the optimum liquid-liquid extraction systems obtained from chloroform and trichlorethylene extracts; C – chlorophorm extract; TCE – trichlorethylene extract

Liquid–liquid extraction system, volume ratio	Phase											
	Light						Heavy					
	Dx		Gx		Dgx		Dx		Gx		Dgx	
	C	TCE	C	TCE	C	TCE	C	TCE	C	TCE	C	TCE
EtOH:H_2O-$CHCl_3$:EtOAc, 25:25:30:20	0.5	0.3	0.0	0.0	92.0	99.5	99.5	97.7	100.0	100.0	8.0	0.5
EtOH:H_2O-$CHCl_3$:EtOAc, 30:20:30:20	0.5	0.5	0.0	0.0	94.0	95.5	99.5	99.5	100.0	100.0	6.0	5.5
EtOH:H_2O-$CHCl_3$:TCE, 35:15:20:30	0.3	0.2	0.0	0.0	99.5	99.8	99.7	99.8	100.0	100.0	0.5	0.2

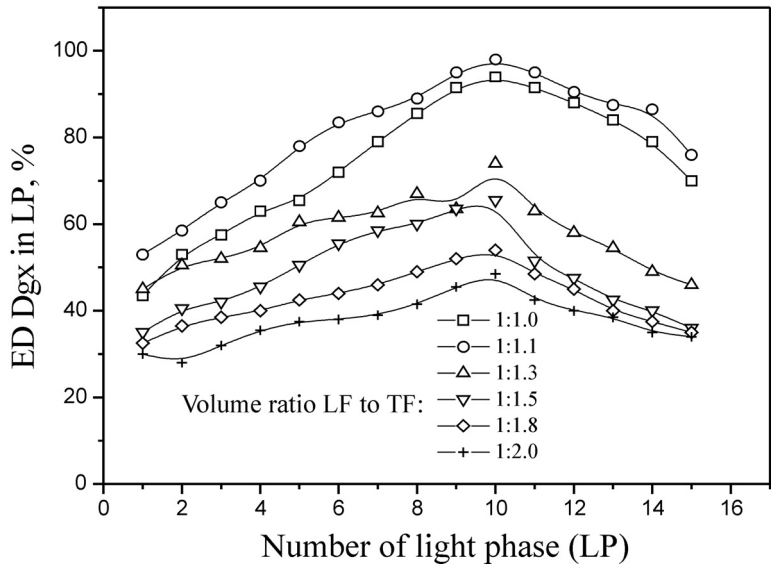

Figure 1. Distribution Dgx in the light phase of the system EtOH:H_2O–$CHCl_3$:TCE, 35:15:20:30 volume ratio, in the separating funnels at different volume ratios of the light and heavy phase (amount of the trichlorethylene extract in the first separating funnel: 15 g/L; volume of the phases: 3 L; room temperature; shaken of the phases in the separating funnels: 5 min).

Figures 1 and 2 shows the distribution of Dgx in the light phase of the optimum system (EtOH:H_2O–$CHCl_3$:TCE, 35:15:20:30 volume ratio) in the 15 separating funnels at different volume ratios of the phases (Figure 1) and at different amounts of trichlorethylene extract in the light phase in the first separating funnel (Figure 2). The ED of Dgx increased with increasing the number of separating funnels up to the tenth funnel and then decreased, independently of the volume ratio of the phases and the initial amount of the glycoside extract (Figures 1 and 2). The highest ED of Dgx was achieved at the volume ratio of the phases of 1:1 (98%, Figure 1) and the amount of the trichlorethylene extract of 15 g/L (99%, Figure 2). Therefore, the volume ratio of the phases of 1:1,1 and the amount of the trichlorethylene extract of 15 g/L were accepted as the optimum ones. The purity of the extracted Dgx product obtained from the concentrate of the combined light phases from the ten separating funnels. The yield of Dgx (with respect to its content in the trihlorethylene extract) was 89%.

CONCLUSION

The optimum operating conditions of the liquid–liquid extraction of Dgx using a four-component system EtOH:H_2O–$CHCl_3$:TCE, 35:15:20:30 volume ratio. Fifteen separating funnels were employed for the liquid–liquid extraction with 3 L of the light and heavy phase each. The volume ratio of the phases was 1.0:1.1. The initial amount of the chloroform or trichlorethylene extract in the light phase of the first separating was 15 g/L. Under these operating conditions, more than 98% Dgx of high purity (over 99%)

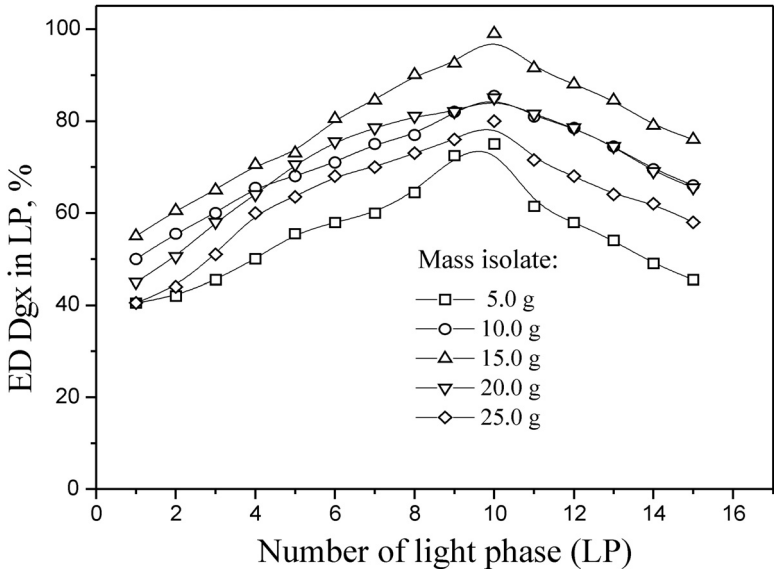

Figure 2. Distribution Dgx in the light phase of the system EtOH:H_2O–$CHCl_3$:TCE, 35:15:20:30 volume ratio, in the separating funnels at different amounts of trichlorethylene extract in the light phase in the first separating funnel (volume ratio of the light and heavy phase: 1:1.1; volume of the phases: 3 L; room temperature; shaken of the phases in the separating funnels: 5 min).

was obtained from chloroform and trichlorethylene extracts of secondary glycoside of foxglove foliage (*Digitalis lanata* Ehrh.).

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REFERENCES

[1] M. Heindich, J. Barnes, S. Gibbons, E.M. Williamson, Fundamentals of Pharmacognosy and Phytotheray, Churchil Livingstone, Elsevier, Amsterdam, 2004, p. 4.

[2] G.S. Joshi, J.C. Burnett, D.J. Abraham, *Cardiac Drugs: Antianginal, Vasodilators, and Antiarrhythmics*, in D.J. Abraham (Ed.), Burger's medicinal chemistry and drug discovery, Sixth ed., Vol. 3: Cardiovascular Agents and Endocrines, John Wiley & Sons, Inc., New York, 2003, pp. 1–54.

[3] W.E. Jr. Cayley, Digoxin in chronic heart failure, Fam. Pract. **21** (2004) 469–472.

[4] Y. Juillière, C. Selton-Suty, Digoxin therapy: A persisting interest despite contrary winds, Arch. Cardiovasc. Dis. **103** (2010) 281–284.

[5] J. Felth, L. Rickardson, J. Rosén, M. Wickström, M. Fryknäs, M. Lindskog, L. Bohlin, J. Gullbo, Cytotoxic Effects of Cardiac Glycosides in Colon Cancer Cells, Alone and in Combination with Standard Chemotherapeutic Drugs, J. Nat. Prod. **72** (2009) 1969–1974.

[6] B. Stenkvist, Cardenolides and cancer, Anti-Cancer Drugs **12** (2001) 635–636.

[7] T. Mijatovic, E. Van Quaquebeke, B. Delest, O. Debeir, F. Darro, R. Kiss, Cardiotonic steroids on the road to anti-cancer therapy, Biochim. Biophys. Acta **1776** (2007) 32––57.

[8] H.-J. Huang, S. Ramaswamy, U.W. Tschirner, B.V. Ramarao, A review of separation technologies in current and future biorefineries, Sep. Purif. Technol. **62** (2008) 1–21.

[9] V.D. Ponomarjev, Ekstragirovanie lekarstvenogo syrya, Medicina, Moscow, 1976.

[10] M. Coulwson, J.F. Richardson, J. R. Backhurst, J.H. Haker, Chemical Engineering, Vol. 2, Fourth ed., Pergamon Press, Oxford, 1991.

[11] Ullmann's Encyclopedia of Industrial Chemistry, E. Müller, R Berger, E, Blass, D. Sluyts Pfennig, Liquid–Liquid Extraction**,** Published Online 2008, http://onlinelibrary.wiley.com/book/10.

[12] N. Qiang, W. Jingkang, Y. Qiuxiang, Separation and purification of two isomorphic steroids by a novel extractive drowning out crystallization, Sep. Purif. Technol. **50** (2006) 342–346.

[13] W. Withering, An Account of the foxglove and some of its medical uses, Practical remarks on dropsy and other diseases, Birmingen, Printed by M. Swinney, London, 1785.

[14] B.S. Pekić, Determination, preparative isolation and chemical transformations of lanatosides from *Digitalis lanata* Ehrh. Foliage, PhD Dissertation, Faculty of Sciences and Mathematics University of Novi Sad, Novi Sad, 1972.

[15] W.L. Xu, Y.B. Huang, J.H. Qian, O. Sha, Y.Q. Wang, Separation and purification of stigmasterol and β-sitosterol from phytosterol mixtures by solvent crystallization method , Sep. Purif. Technol. **41** (2005) 173–178.

[16] M. Stanković, S. Djordjević, S. Stanković, O. Stojanović, Enzymatic transformation of primary glycosides from the *Digitalis lanata* Ehrh., Hem. Ind. **37** (1983) 241–247 (in Serbian).

[17] Ullmann's Encyloppaedie der techischen Chemie, Urban and Schwarzenberg, Muenchen, Bd. **8** (1957) 222.

[18] M. Stanković, D. Stamenković, V. Banković, The extraction of secondary glycosides from the fermentated leaves of *Digitalis lanata* Ehrh. with diluted alcohols and water (In Serbian), Hem. Ind. **35** (1981)128–131 (in Serbian).

[19] B.S. Pekić, M.Z. Stanković, Separation of the secondary glycosides from the leaves of digitalis, Hem. Ind. **31** (1973) 551–553 (in Serbian).

[20] S. Djordjević, M. Stanković, Determination of the conditions of the extraction secondary glycosides from the leaves of *Digitalis lanata* Ehrh. by the mixture of solvents (In Serbian), Hem. Ind. **31** (1977) 633–690 (in Serbian).

[21] V.S. Fonin, A.Y. Khorlin, Preparation of biologically transformed raw material of woolly foxglove (*Digitalis lanata* Ehrh.) and isolation of digoxin therefrom, Biochem. Microbiol. **39** (2003) 588–592.

[22] European Pharmacopoeia, 7th ed., 2012, http://online.edqm.eu.

IZVOD

IZOLACIJA DIGOKSINA EKSTRAKCIJOM TEČNOST–TEČNOST IZ EKSTRAKATA SEKUNDARNIH GLIKOZIDA VUNASTOG DIGITALISA

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(Naučni rad)

Ispitan je uticaj operativnih uslova na efikasnost izdvajanja digoksina ekstrakcijom tečnost-tečnost u levcima za odvajanje iz suvih hloroformskih i trihloretilenskih izolata sekundarnih glikozida vunastog digitilisa. Kao ekstrakcioni rastvarači korišćena su tri četvorokomponentna dvofazna sistema: a) etanol:voda-hloroform:etilacetat, b) etanol:voda-trihloretilen:etilacetat i c) etanol:voda-trihloretilen:hloroform. Ispitivanja su uključila sledeće procesne uslove: sastav ekstrakcionog sistema, koncentracija rastvora suvih hloroformskih i trihloretilenskih ekstrakata sekundarnih glikozida u lakoj fazi prethodno uravnoteženoj teškom fazom u opsegu 5–25 g/L, zapreminski odnos lake i teške faze, broj uravnotežavanja faza i odnos zapremina lake i teške faze 1:1 do 1:2. Definisani su optimalni operativni uslovi za izdvajanje preko 98% digoksina u lakoj fazi, i to: koncentracija sekundarnih glikozida u lakoj fazi 15 g/L; sastav četvorokomponentni sistema etanol:voda–hloroform:trihloetilen, 35:15:20:30; i odnos zapremina lake i teške faze 1,0:1,1. Iz lake i teške faze su, koncentrovanjem i kristalizacijom, izdvajeni digoksin i smeša glikozida digitoksina i gitoksina. Čistoća izdvojenog digoksina je 99,8%.

Ključne reči: Digoksin • Vunasti digitalis • *Digitalis lanata* Ehrh. • Ekstrakcija tečno–tečno • Ekstrakcija čvrsto–tečno