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Optimisation and validation of the high performance liquid chromatography method for determination of biologically active compounds in European centaury herb

INTRODUCTION

Centaurium erythraea Rafn. herb (Picture 1), which is harvested from natural environment, is highly valued bitter raw material applied to the problems with digestive system. Iridoids such as sweroside, swertiamarine, amarogentine and gentiopicroside are the main biologically active compounds of common centaury. There are also flavonoids, phenolic acids, triterpenes, a small amounts of alkaloids, essential oil, mineral salts and resin compounds beside the iridoids [1, 2, 3, 4, 5]. Bitter compounds of common centaury show stimulation action of digestive system. Extracts enhance the production of saliva, bile, digestive juices and facilitate digestion and assimilation of nutrients [1, 2, 6]. Bigger doses of common centaury extracts may irritate the stomach, but can also be used as a anthelmintic and antipyretic drug [7]. In Morocco common centaury is traditionally used in urinary diseases treatment, together with *Rosmarinus officinalis* L. [8]. Recently the research on using *Centaurium erythraea* Rafn. in entomology was provided. In the study on combating *Tribolium castaneum* – an insect, which is dangerous pest of cereal storages, it was found that methanol extract of common centaury was highly toxic to larvae as well as imago [9].

In previous studies on *C. erythraea* Rafn. there was no information about using HPLC method in researches. HPLC is well known method used for identifying and quantifying compounds in biological or chemical samples. HPLC is accurate method, but it requires expensive pure solvents. It is important to keep short time of single analysis reducing the cost. In this study development, optimisation and validation of HPLC method for the raw material were provided.

Pic. 1. Dried common centaury herb (Photo AS)

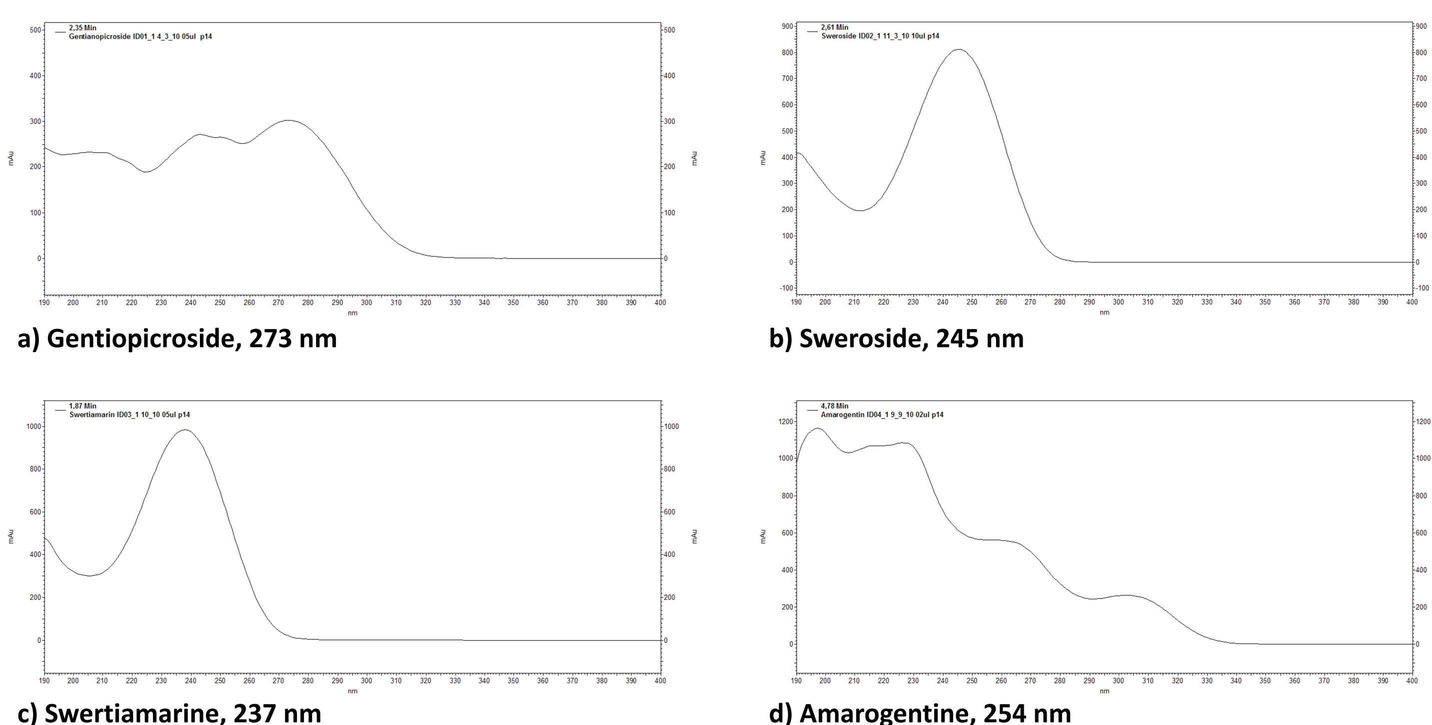
MATERIALS AND METHODS

Plant material was harvested from the WULS-SGGW experimental fields located in Wilanów, Warsaw. The methanol extracts were made using ultrasonic washer. 1 gram of herb was extracted twice with 100 mL of methanol, then it was evaporated to 5 mL, quantitatively transferred to measuring flask for 10 mL and made up to this volume. Standards of iridoids (ChromaDex®) were weighed, dissolved in methanol and also transferred to measuring flask for 10 mL. Six dilution of each standard solution were prepared (Table 1). The calibration curve was made using surface area of different dilutions peaks for every substance. Also the library of absorption spectra (wavelength 190-800 nm) was made (Table 2).

Table 1. Concentration of standards after consideration of purity on 6 levels used to create calibration curves

Compound	Concentration of standard substances [mg×L^{-1}]					
	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Gentiopicroside	11.9	27.7	79.0	158.1	276.6	395.2
Sweroside	50.2	100.3	200.7	571.7	682.4	1003.4
Swertiamarine	47.5	94.9	189.8	474.5	764.3	949.0
Amarogentine	8.8	44.0	88.0	264.0	578.1	939.5

Table 2. Electron absorption spectra of standards dissolved in methanol created using SPD-M10 VP detector controlled with Class CP 7.3 software (name of compound, maximum absorption wavelength nm)



a) Gentiopicroside, 273 nm

b) Sweroside, 245 nm

c) Swertiamarine, 237 nm

d) Amarogentine, 254 nm

The HPLC apparatus, used during the research, consisted of two pumps LC-6A, automatic sampler SIL-20A Prominence, oven CTO-6A, diode detector SPD-M10A VP and system controller SLC-6B, all produced by Shimadzu. The analysis were conducted on the Kinetex® column (Phenomenex®) produced in Core-Shell™ technology. The column is filled with particles with solid silica core covered with homogenous porous shell made of modified silica C18. The diameter of particles is 2.6 µm, the porous diameter – 100 Å, the length of the column is 100 mm and it's diameter – 4.6 mm. The liquid phase consisted of deionized water (produced in our laboratory), acetonitrile (ACN) and methanol (both for HPLC, produced by POCH SA and Sigma-Aldrich®). Aqueous solutions of ACN were acidified with 85% phosphoric acid (produced by Fluka).

The main aim of the HPLC optimisation analysis is to shorten the time of single analysis, while still maintaining good separation of chemical compounds present in the sample. In biological, especially plant, material, there are lots of different substances (flavonoids, phenolic acids, terpens, xanthones, glycosides, etc.), but they may be not relevant as an biologically active compounds. Then it is important to separate peaks of relevant substances (in Centaurium erythraea Rafn. – iridoids) from irrelevant ones (in *C. erythraeae* Rafn. – flavonoids, phenolic acids, triterpenes etc.). Maintaining good separation includes determining the gradient of liquid phases on chosen column, temperature and time of analysis.

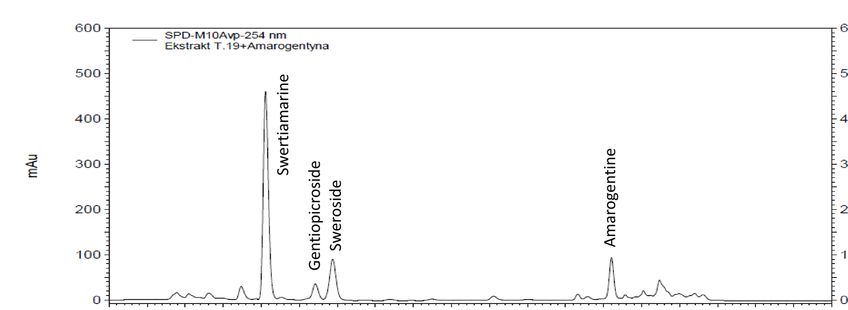
In this study the precision, linearity, measuring range, limits of detection and limits of quantification were checked during the process of validation. Peaks' areas of six repetitions were compared in the process of checking precision. Average – x, standard deviation – S and variation coefficient – Vs were calculated (according to the formula: $Vs = S \times x^{-1}$. The method is precise, when the variation coefficient is lower than 3. Calculation of the limit of detection and the limit of quantification was based on average area of 40 noise peaks around the peak of analyzed substance on the first level. LOD is tripled average area of the disturbance's peaks and LOQ – increased tenfold. The measuring range was simplified to field between the lowest and the highest dilution. Statistical analysis was done in Class VP 7.3 software, Microsoft Excel and the statistical service e-stat, available at http://www.chem.uw.edu.pl/stat/e-stat/ website.

RESULTS

Optimisation

Iridoids are polar substances, easily dissolved in water and alcohols. According to Witkiewicz [10] reversed phase HPLC (RP-HPLC) is proper method to separate these substances and this variant of HPLC was used in the study. Kinetex® column, made in Core-Shell™ technology, used as a solid phase allowed to shorten time of single analysis to 9.5 minutes. As all common centaury's iridoids absorb ultraviolet radiation, it was easy to identify them using DAD detector.

A number of analysis were taken in the study. Starting from gradient C1, which took 9 minutes and characterized with good separation rate of standards, but bad one of substances present in methanol herb extract, throughout gradient C4, which also took 9 minutes, had lower solvent consumption, very good separation rate of standards and quite good one of substances in the methanol extract, to the gradient C6 (Table 3), which took 9.5 minutes, but separation rate of standards and biological active compounds present in common centaury's extract was very good (Picture 2). Also the gradient C6 has the lowest ACN consumption – 1.7 ml per single analysis. Gradient C6 was claimed to be good enough to become a validated method.



Pic.2. Chromatogram of common centaury herb extract, gradient C6

Table 3. Method's C6 gradient

Time	A [%]	B [%]
0.01	88	12
2.50	85	15
4.00	65	35
4.10	65	35
4.20	88	12
9.50	88	12

Validation

Validation was provided for the gradient C6. Precision was checked on every level of the standards (Table 4). Variation coefficient was lower than 3% for every repetition on each level – the method is precise. The Dixon's test was made before checking linearity. No thick errors where found during the test. Linearity was checked twice using Class VP 7.3 software and statistical service e-stat. All calibration curves created in e-stat and Class VP 7.3 had high correlation coefficient. Its value was higher than 0.999, which is needed to provide normal analysis using particular analytical method. Equations of calibration curves created by Class VP 7.3 are presented in Table 5 and calibration curves created by e-stat are in Table 6. Additionally analysis of residual was provided using e-stat. Residual are the differences between experimental and calculated value, using equations of calibration curves. Uniform and random dispersion of residual around zero shows linear relationship between measured analytical signal and content of standard in calibration dilution (data not shown). Results of LOD and LOQ analysis are presented in Table 7. Using different algorithms caused differences in results. LOD and LOQ calculated in e-stat are similar to the lowest concentration of standards and they are much lower, when calculation is based on noise peaks' areas. Results of measuring range are presented in Table 8.

Table 4. Analysis of peaks areas and their diversity for calibration dilutions

Substance	Concentration [mg×L^{-1}]	Average of peaks areas	Standard deviation	Variation coefficient [%]
Gentiopicroside	11.90	13441.33	260.28	1.94
	27.70	28512.83	399.35	1.40
	79.00	71727.67	1126.36	1.57
	158.10	156261.83	288.53	0.18
	276.60	277919.83	1578.72	0.57
	395.20	397873.67	3724.81	0.94
Sweroside	50.20	69766.67	1328.74	1.90
	100.30	136624.50	3677.78	2.69
	200.70	238443.67	2184.56	0.92
	571.70	601784.33	9403.64	1.56
	682.40	733060.33	4300.68	0.59
	1003.44	1017318.33	12627.83	1.24
Swertiamarine	47.45	67708.00	1129.40	1.67
	94.90	104744.33	1352.06	1.29
	189.80	253077.17	5646.44	2.23
	474.50	595939.83	3906.68	0.66
	764.30	980421.17	17148.97	1.75
	949.00	1202494.17	15317.83	1.27
Amarogentine	8.80	16681.50	480.47	2.88
	44.01	56554.33	678.48	1.20
	88.01	101950.50	2351.62	2.31
	264.03	283145.00	6406.57	2.26
	578.10	592802.17	7688.70	1.30
	939.51	951653.33	6733.71	0.71

Table 5. Equations of calibration curves made by Class VP 7.3

Compound	Parameters of calibration curve
Gentiopicroside	Linear Fit ax + b a = 0.00101177 b = 1.28530 Goodness of fit (r^2): 0.999265
Sweroside	Linear Fit ax + b a = 0.00100653 b = -28.0409 Goodness of fit (r^2): 0.999035
Swertiamarine	Linear Fit ax + b a = 0.000768068 b = 6.12057 Goodness of fit (r^2): 0.999391
Amarogentine	Linear Fit ax + b a = 0.00100047 b = -13.4561 Goodness of fit (r^2): 0.999083

Table 6. Sample calibration curve of standards made by e-stat (Gentiopicroside)

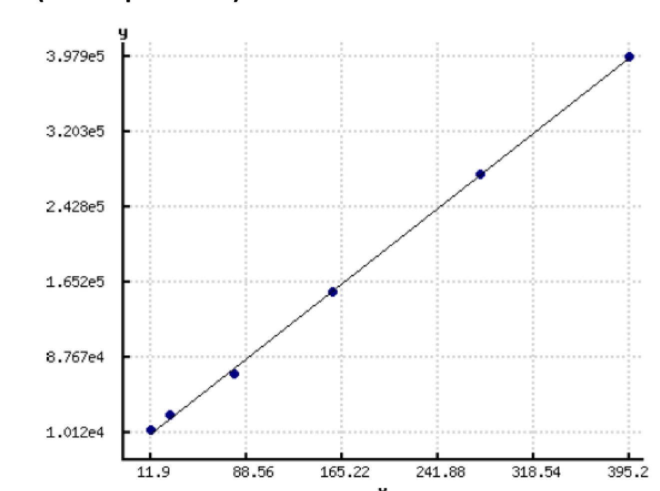


Table 7. LOD and LOQ created by e-stat (a) and calculated using noise peaks' areas (b)

a)

Compound	LOD [mg×L^{-1}]	LOQ [mg×L^{-1}]
Gentiopicroside	13.99	23.31
Sweroside	51.47	85.78
Swertiamarine	42.11	70.19
Amarogentine	13.68	22.81

b)

Compound	Gentiopicroside	Sweroside	Swertiamarine	Amarogentine
Concentration [mg×L^{-1}]	11.90	50.20	47.45	8.80
		Average peak area		
Signal	13441.00	69766.67	67708.00	16917.67
Noise	60.98	70.93	41.45	55.38
LOD	182.94	212.80	124.36	166.14
LOQ	609.79	709.33	414.54	553.79
		Concentration [mg×L^{-1}]		
LOD	0.16	0.15	0.09	0.09
LOQ	0.54	0.51	0.29	0.29

Tabela 8. Measuring range of standards

Compound	Minimum [mg×L^{-1}]	Maximum [mg×L^{-1}]
Gentiopicroside	11.90	395.20
Sweroside	50.20	1003.44
Swertiamarine	47.45	949.00
Amarogentine	8.80	939.51

CONCLUSION

1. Developed method allows precise, fast and cheap determination of sweroside, gentiopicroside, swertiamarine, amarogentine in common centaury herb.
2. Shortening the time of single analysis to less than 10 minutes, reduction of solvent usage, even when working on older apparatus, is possible using modern technology of the column filling.
3. The method is highly precise and sensitive. It is also possible to analyse samples in wide measuring range.

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