Evaluation of the analytical method for a chromatographic profile assessment of the essential oil obtained from *Pimpinella saxifraga* s.l.

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Summary

Pimpinella saxifraga s.l. has been used for medicinal purposes for a long time. The root is a part of medicinal properties composed by essential oils in about 0.4%. The composition of these essential root oils consists of about sixty chemicals.
The aim of the current experiment was assessing the analytical method of chromatographic profile utilizing essential oil of *Pimpinella saxifraga*. This essential oil was isolated with use of Deryng apparatus according to Polish Pharmacopoeia. The method for assessing the chromatographic profile of the essential oil was split-up gas chromatographic capillary column. The quantitative composition of the essential oil was calculated with use of method of normalization. The identity and the compositions of the essential oil from *Pimpinella saxifraga* roots was analyzed on Clarus 500 gas chromatograph equipped with capillary columns with flame ionization detector (FID), automatic dispenser and computer with Total Chrom Navigator software Perkin Elmer. Chromatographic system was characterized by range of retention time, area peaks, tailing factor and compounds' separation. This method of volatile oil substance marking was validated following the ICH standards, by its precision, accuracy and linearity.

Key words: *Pimpinella saxifraga s.l., validation procedure, GC-FID*

INTRODUCTION

Pimpinella saxifraga s.l. belongs to *Apiaceae* family. It is a species of very difficult taxonomic relations. There are two different ideas. According to the first one, there are two subspecies distinguished: *Pimpinella saxifraga* and *Pimpinella saxifraga nigra* (Mill) Gaud [1, 2], the second one considers *Pimpinella saxifraga* L. and *Pimpinella nigra* Willd. as two separate species [3]. *Pimpinella saxifraga* L. occurs all over Poland, while *Pimpinella nigra* grows in a few parts of Poland [4], namely the main regions of the lower Wistula (Mazurian Lake District) and partly in the Małopolska Highland [5]. *Pimpinella saxifraga* s.l. blooms in June to July, and the petals are small, white or pink [2, 3, 6, 7]. Both *Pimpinella saxifraga* and *Pimpinella nigra* have medicinal application [8]. Pharmacologically active substances occur mainly in essential oil from the roots [8-11]. These plants are used to treat respiratory tract diseases [8, 10, 12]. Active substances which occur in the root essential oil have expectorant properties and reduce muscle tension of upper airway. Moreover, pharmacologically active substances may act upon digestive system, stimulating secretion of gastric juices, and they also stimulate the excretion of sweat and urine [13, 14]. Root of *Pimpinella saxifraga* s.l. is used, not only internally, but also externally. Externally application may be by gargling or by addition on the bath [8, 15]. The main aim of the current experiment was making the phytochemical analysis of essential oil of *Pimpinella saxifraga* roots, and assessing the analytical method of marking the chromatographic profile of this essential oil.

EXPERIMENTAL

Reagents and materials

For research 40 samples of *Pimpinella saxifraga* s.l. have been used. They were collected in natural areas near Poznań. To research there were subjected the components of big chemotaxonomic value. The aim of this work is to validate the examination method of listed components of oil obtained from *Pimpinella saxifraga*. The content of individual components in the test sample is presented in the second part of the publication of the variation in themorphological and photochemical analysis of that plant [16]. The reagents were purchased as follows: *n*-hexane pure p.a., xylene pure p.a., sodium sulfate anhydrous pure p.a. from Poch (Poland), α-pinene (puriss p.a. terpene standards for GC), myrcene (≥95%, GC), α-phellandrene (≥95%, GC), limonene (puriss p.a. terpene standard for GC), *p*-cymen (≥99%), sabinene (≥98%, GC) from Fluka (Germany), camphene (95%) and β-pinene (99%) were from Sigma-Aldrich (USA) and γ-terpinene (≥98%, GC) from Chromadex (USA).

Working standards solutions were prepared from abovementioned solutions and afterwards diluted with *n*-hexane prior to analysis. Dissolved 1.00 ml reference solution of α-pinene, β-pinene, camphene, sabinene, myrcene, limonene, α-phellandrene, γ-terpinene and *p*-cymen in *n*-hexane were afterwards diluted to 10.0 ml with the same solvent.

Instrumentation

The *n*-hexane solution of the oil was analyzed with gas chromatography using Perkin Elmer Clarus 500 system. Chromatographic column (Elite-FFAP 30.0 m $\times$ 0.32 mm $\times$ 0.25 μm) was used in a starting temperature of 60°C (2 min), increased by 5ºC/min to 200ºC, then kept constant for 10 min. The flow rate of carrier gas (helium) was set at 0.5 ml/min. The injector had a split ratio of 1:20 mode at 200°C. The volume of each injected sample was 1 μl. The FID detector was operating at 250°C. The components of the tested solution (α-pinene, β-pinene, camphene, myrcene, α-phellandrene, limonene, γ-terpinene, *p*-cymen and sabinene) were located using retention time from the reference chromatogram solutions. The components were quantified according to the normalization procedure.

Sample preparation

In the graduated tube there were used 30.0 g of crushed drugs, transferred to a 500.0 ml flask, with 400.0 ml of water as distillation liquid and 0.30 ml of xylene. This solution was distilled with a Deryng apparatus, at a rate of 3–4 ml/min for 3 h. The essential oil was dissolved in *n*-hexane and diluted to 5.0 ml using the same solvent.

Validation procedure

The detection limit (LOD) and the quantitation limit (LOQ) were expressed as signals based on the mean blank and the standard deviation of the blank responses. LOD and LOQ were converted from signal domain to concentration domain using a calibration function calculated in the concentration range, as demonstrated in table 3. The Barlett and linearity tests were performed at the 95% significance level in order to homoscedasiticity and linearity. Linearity was studied over two orders of magnitude in the concentration range, as shown in Table 3. Precision was calculated respectively. The repeatability was calculated in terms of RSD% (n=6) on two concentration levels for each analyst. The reproducibility were performed on the replicated measurements at the 95% significance level [17].

RESULTS AND DISCUSSION

Method development

In the first step the essential oils were isolated from *Pimpinella saxifraga* s.l. by steam distillation and then analyzed on GC *apparatus* fitted with a *FID* detector. The components of essential oils are compounds separated on Elite–FFAP column. The detector and oven temperatures were set to 250°C and 200°C, respectively. The head pressure, set by the flow rate of the carrier gas (helium), was set at 0.5 ml/min. The injector had a split ratio of 1:20 mode at 200°C. The solvent, column and acquisition parameters were chosen to be a starting point for the method development. The chromatography produced using these starting parameters turned out to be excellent and no further optimization was performed. Chromatographic system was characterized by range of retention time, area peaks, tailing factor and compounds' separation. The precision and linearity studies performed during the method development showed that 1.0μl injection volume was reproducible and the peak response was significant at the analytical concentration.

Furthermore, the stability of standard solutions was evaluated by storing the solution at –10°C for 24 h, and testing both at the beginning and at the end of the storage period. No significant degradation of standards were observed.

Specificity

All standards used for method development were separated. The specificity of the method was demonstrated in figure 1. Obtained profile chromatogram illustrates the complete separation.

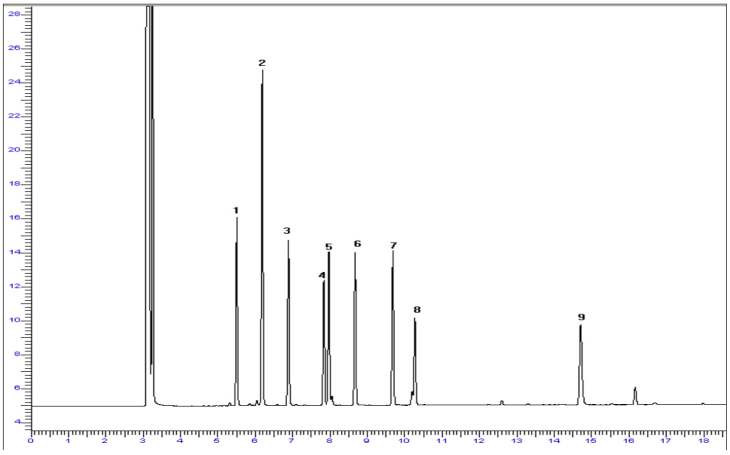

Figure 1. GC chromatographic separation of a mixed standard mixture containing all compounds: (1) α-pinene, (2) camphene, (3) β-pinene, (4) myrcene, (5) α-phellandrene, (6) limonene, (7) γ-terpinen, (8) p-cymen, (9) sabinene

LOD and LOQ determination

In this work the detection limits (LOD) and the quantitation limits (LOQ) of α-pinene, β-pinene, camphene, myrcene, α-phellandrene, limonene, γ-terpinene, p-cymen and sabinene were statistically determined. Table 1 shows the obtained LOD and LOQ values.

Table 1.

Detection limits [LOD] and quantitation limit [LOQ] of the components from *Pimpinella saxifraga*'s oils

analyte	LOD	LOQ
α-pinene [μg/ml]	0.03	0.10
β-pinene [μg/ml]	0.03	0.10
camphene [mg/ml]	0.07	0.19
myrcene [μg/ml]	0.03	0.10
sabinene [mg/ml]	0.03	0.10
limonene [μg/ml]	0.03	0.10
α-phellandrene [μg/ml]	0.03	0.10
γ-terpene [μg/ml]	0.04	0.11
p-cymen [μg/ml]	0.04	0.11

Linearity

The linearity of peak area ratio *vs.* concentration of compounds were studied. Five solutions were prepared corresponding to 20, 60, 100, 140 and 160% of the nominal analytical concentration of utilized stock standards solutions in *n*-hexane. At each level samples were injected and analyzed according to the method previously described and ICH guideline [18, 19]. The regression equation and the determination coefficients (r^2) were calculated for assessing the linearity. The linearity parameters for these compounds are summarized in table 2.

Table 2.

Linearity parameters of essential oil's compounds

analyte	slope	intercept	r^2
α-pinene	3.01×10^{-5}	–0.173199	0.9999
β-pinene	3.09×10^{-5}	–0.027280	1.0000
camphene	3.03×10^{-5}	–0.054760	0.9999
myrcene	4.29×10^{-5}	0.001960	0.9999
sabinene	3.77×10^{-5}	0.109277	0.9995
limonene	3.15×10^{-5}	–0.016679	1.0000
α-phellandrene	3.28×10^{-5}	–0.070856	0.9999
γ-terpene	3.28×10^{-5}	0.032460	0.9999
p-cymen	4.46×10^{-4}	0.178871	0.9993

Precision

The repeatability was determined according to the above described accuracy test by six analysts, following the ICH guideline 18, 19]. The RSD values [%] were also reported in table 3.

Table 3.

GC inter-day repeatability. RSD data

analyte	concentration level	RSD [%]
α-pinene [*μ*g/ml]	1.716	1.50
	13.728	1.62
β-pinene [*μ*g/ml]	1.742	1.16
	13.936	1.91
camphene [mg/ml]	3.323	3.64
	26.587	0.96
myrcene [*μ*g/ml]	1.602	1.79
	12.816	1.72
sabinene [mg/ml]	1.667	1.60
	13.334	2.47
limonene [*μ*g/ml]	1.684	1.36
	13.472	2.05
α-phellandrene [*μ*g/ml]	1.680	1.39
	13.440	1.23
γ-terpene [*μ*g/ml]	1.796	1.87
	14.368	0.96
p-cymen [*μ*g/ml]	1.980	1.77
	15.840	1.66

System Suitability Testing

As system suitability testing is an integral part of method development, these suggested limits were used as a reference to set up the initial system suitability criteria, including injection precision (RSD<3.0%, n=6), retention time (RSD<1.0%), peak area (RSD<2.0%), number of theoretical plate (RSD<2.0%), tailing factor (RSD<2.0%) and peak width (RSD<2.0%). The data are shown in table 4.

Table 4.

System suitability parameters

analyte	RSD [%], n=5				
	retention time [min]	peak area [uV×sec]	number of theoretical plate	tailing factor	peak width [sec]
α-pinene	0.02	1.29	0.43	0.42	0.53
β-pinene	0.02	1.25	0.47	0.38	0.61
camphene	0.03	1.30	0.69	0.50	0.26
myrcene	0.01	1.85	1.63	0.72	1.29
sabinene	0.02	1.07	1.95	2.00	1.84
limonene	0.02	1.41	1.25	0.84	0.56
α-phellandrene	0.54	1.85	1.74	0.11	1.02
γ-terpene	0.02	1.36	1.00	0.72	0.39
p-cymen	0.01	1.56	1.34	0.88	0.44

CONCLUSIONS

Complying the ICH guidelines, the validation criteria such as specificity, range of linearity, precision, detection limit (LOD), quantitation limit (LOQ) and system suitability were considered. Tests proved the method to be specific and linear. The precision testing demonstrated a good degree of reproducibility. Consequently, the results presented in this study indicate that the validated gas chromatographic method can be applied to *α*-pinene, *β*-pinene, camphene, myrcene, *α*-phellandrene, limonene, *γ*-terpinene, *p*-cymen and sabinene assay for the quality control of medicinal products or dietary supplements which may be composed by *Pimpinella saxifraga* s.l.

REFERENCES

1. Adams M, Berset C, Kessler M, Hamburger M. Medicinal herbs for the treatment of rheumatic discorders – A survey of European herbals from the 16th and 17th century. J Ethnopharmacol 2009; 121:343-59.
2. Kubeczka KH, Bohn I, Schultze W. The composition of the essential root oils from Pimpinella saxifraga s.l and chemotaxonomic implication. Zeiteschrift-fuer-Naturforschung- section C – Biosciences 1989; 44c(3-4):177-82.
3. Koczwara M. Pimpinella L. W: Dwuliścienne wolnopłatkowe – dwukwiatowe. Kraków 1960: 65-7.
4. Zając A, Zając M (red). Atlas rozmieszczenia roślin naczyniowych w Polsce. Nakładem Pracowni Chorologii Komputerowej Instytutu Botaniki Uniwersytetu Jagiellońskiego i Fundacji dla Uniwersytetu Jagiellońskiego. Kraków 2001:407.
5. Gawłowska M. *Pimpinella nigra* Wild w Polsce. Dissert Pharm 1964; 16:541-6.
6. Schaffner W. Rośliny lecznicze: chemizm, działanie, zastosowanie. Warszawa 1996:198-9.
7. Lamer-Zarawska E, Kowal-Gierczak B, Niedworok J. Fitoterapia i leki roślinne. Warszawa 2007:244-5.
8. Ożarowski A, Jaroniewski W. Rośliny lecznicze i ich praktyczne zastosowanie. Warszawa 1976:97-9.

9. Stary F, Jivasek V, Severa F, Ostrowski A. Rośliny lecznicze. Warszawa 1976:174.
10. Bagiński S, Mowszowicz J. Krajowe rośliny lecznicze. Łódź 1966:25-6.
11. Kohlmünzer S. Farmakognozja – podręcznik dla studentów farmacji. Warszawa 1998:218.
12. Sarwa AJ. Wielki leksykon roślin leczniczych. Warszawa 2001:292-5.
13. Matławska I (red). Farmakognozja – podręcznik dla studentów farmacji. Poznań 2006: 352-3.
14. Podhajska Z, Rivola M. Wielka Encyklopedia Roślin. Warszawa 1992:216.
15. Borkowski B. Zarys farmakognozji. Warszawa 1974:254-7.
16. Latowski K, Komorowska E, Grys A, Mielcarek S, Buchwald W. Diffrentiation of *Pimpinella saxifraga* L. s.l. in comparative morphological and phytochemical analyziss. Herba Pol 2009; 55(3): 11-9.
17. International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use: Validation of Analytical Procedures: Metrology, ICH-Q2B, Geneva 1996.
18. Namieśnik J, Górecki T. Sample preparation for chromatographic analysis of plant material. *J Planar Chromatography* 2000; **13**:404-13.
19. International Conference on Harmonisation, Guideline on "Validation of Analytical Procedures : Methodology", Federal Register 62, 1997:27463-7.

OCENA METODY ANALITYCZNEJ OZNACZANIA PROFILU CHROMATOGRAFICZNEGO OLEJKU ETERYCZNEGO OTRZYMANEGO Z *PIMPINELLA SAXIFRAGA* S.L.

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S t r e s z c z e n i e

Pimpinella saxifraga s.l. jest rośliną, którą od dawna wykorzystuje się w celach leczniczych. Surowcem *Pimpinella saxifraga* wykorzystanym w lecznictwie jest korzeń, który zawiera od 0,4% olejku eterycznego. Skład olejku eterycznego *Pimpinella saxifraga* jest złożony. W składzie olejku stwierdzono dotychczas około 60 związków chemicznych.
Celem opisywanego doświadczenia była ocena metody analitycznej badania profilu

chromatograficznego olejku eterycznego otrzymanego z *Pimpinella saxifraga s.l.* na aparacie Derynga wg Farmakopei Polskiej VI. Metoda oznaczenia profilu chromatograficznego olejku eterycznego polega na rozdziale metodą chromatografii gazowej na kolumnach kapilarnych i porównaniu z substancją porównawczą metodą wzorca zewnętrznego, oraz oznaczeniu składu ilościowego tego olejku metodą normalizacji. Tożsamość i zawartość składników olejku eterycznego analizowano na chromatografie gazowym Clarus 500 przystosowanym do pracy z kolumnami kapilarnymi, wyposażonym w detektor płomieniowo-jonizacyjny (FID), automatyczny dozownik próbek oraz komputer z oprogramowaniem TotalChrom Navigator firmy Perkin Elmer. System chromatograficzny scharakteryzowano w zakresie oceny powtarzalności czasu retencji, powierzchni piku, współczynnika rozdziału pomiędzy pikami chromatograficznymi oraz symetrii. Opracowaną metodę oznaczania składu olejku eterycznego poddano walidacji zgodnie z wymaganiami ICH. Ocenie poddano precyzję, dokładność i liniowość metody.

Słowa kluczowe: *Pimpinella saxifraga, procedura walidacji, GC-FID (chromatografia gazowa z detektorem płomieniowo-jonizacyjnym)*