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Analysis of Alkaloids in Poppy Straw by High-Performance Liquid Chromatography

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Abstract: - The analytical method was developed for the simultaneous determination of main alkaloids in poppy straw using high-performance liquid chromatography. The optimal chromatographic conditions and the sample preparation technique, including solid phase extraction, were designed and validated. The validation parameter testing included the verification of the repeatability, accuracy, linearity, sensitivity, limit of detection, limit of quantification and robustness of the analytical method. The newly developed method was applied to analyse real poppy straw samples from the harvest of 2013. The results show that this method can be used as a routine, reliable and accurate technique to determine the poppy straw quality in poppy breeding.

Keywords: *- Poppy; Poppy straw; Morphine; Alkaloids; Extraction; HPLC; Validation*

I. INTRODUCTION

The Czech Republic is one of the world´s leading producers of poppy *(Papaver somniferum* L.*)*. Currently, the world´s largest poppy producer is Turkey where white-seed cultivars are grown mainly, the seeds of which are intended for the Indian market in particular. In terms of blue-seed poppy cultivars for the primary use in the food industry, the Czech Republic is the world´s leading producer, accounting for about 60 % of the world´s total production[1]. The poppy growing has had a long-term tradition in the Czech Republic, and the Czech producers master large-scale production technologies. Lately, the poppy growing area has been within the range from 20 000 to 30 000 hectares in the Czech Republic. Only the poppy of the seed (oil) kind is grown in the Czech Republic. In agrobiological terms, it is an annual spring crop. A winter form of poppy is also grown in the Czech Republic in a small scale, the growing areas of which range from about 100 to 400 hectares, depending on the particular year[2-4].

Poppy seeds are characterised by very good dietetic properties. The main component is oil (46 to 50 % of dry matter), in which linoleic acid predominates (60 – 70 %). Therefore, the dietetic properties of poppy oil are similar to those of sunflower oil. The seeds contain, among others, tocopherols, pantothenic acid, niacin, thiamine and a very high content of calcium[3] (1400 mg/100 g).

As mentioned above, the primary purpose of the poppy grown in the Czech Republic is to produce seeds for the use in the food industry. However, due to its content of alkaloids, morphine in particular, poppy is a valuable raw material for the pharmaceutical industry as well. As a source of alkaloids, dry capsules are used, which are harvested together with poppy seeds after they have become ripe, and the meal of them (poppy straw) is separated then. Only the poppy straw from a part of the growing area (about 7 000 hectares currently) is used for these purposes[2, 5-8].

The purchase of poppy straw was carried out without large requirements for quality in the second half of the last century. Gradually, standard quality indicators had been formed for the purchase and assessment of poppy, and the producers responded adequately, so the quality of the poppy straw purchased has risen significantly. The poppy straw being purchased shall be sound, dry, free of fungi and pests, brown-yellow and harvested in the period of biological ripeness. Morphine content, which substantially affects the final purchase price, is an important aspect monitored[9-11].

The morphine content in poppy straw is affected by many factors. Among them, the genetic basis of the variety is the most important one. Therefore, the most efficient tool to intentionally affect this characteristic is a breeding. The assessment of the morphine content in poppy straw is an important part of the breeding process and registration procedure within the process of cultivar approval by the Czech Central Institute for Supervising and Testing in Agriculture. Within the current product range of poppy cultivars, the Opal, the Orbis and the newly registered Opex are the most suitable materials to produce poppy straw. In addition, the final morphine content is affected significantly by the weather during the growth and ripening of poppy and, last but not least, agronomical operations or processes[9-12] (such as nutrition, protection and harvest technology).

Many legal regulations in the Czech Republic relate to morphine content. They are to prevent morphine from being misused for the manufacture of narcotics and addictive substances. It is subject to an obligation to

report, according to which the Customs Office registers poppy growing areas over 100 m^2 and issues a permit to import or export poppy straw. In addition, a new legal regulation coming into effect prohibits producers from growing poppy varieties with the morphine content above 0.8 % in poppy straw. The limit will be observed in the registration procedure by Central Institute for Supervising and Testing in Agriculture for approval of new cultivars[10,2]. Therefore, monitoring of the morphine content in poppy straw during the breeding process becomes more important.

The most important methods for determining alkaloids include thin-layer chromatography, high-performance liquid chromatography, polarography, chiral separation, micellar liquid chromatography, hydrophilic interaction chromatography, ion-exchange chromatography, capillary electrophoresis, gas chromatography and Raman spectroscopy[13-18]. The most frequently used method for determining alkaloids in poppy straw is high-performance liquid chromatography (HPLC), including UV or MS detection. The methods mentioned above differ from one another in the manners of extraction and purification steps[19-23]. A purification of the extract through a solid-phase extraction (SPE)[6,24,25] proved to be an efficient step of preparing the sample for the analysis.

The goal of this study was to develop and establish a method to extract alkaloids from poppy straw and to determine them using high-performance liquid chromatography, including UV/VIS detection. The method developed was validated for determining morphine content as the most important alkaloid that affects the poppy straw quality. Then, the method was used to assess alkaloid contents in poppy breeding at OSEVA PRO limited company.

II. MATERIAL AND METHODS

2.1 Samples

Opium poppy capsules were used for experimental purposes. The plant material was obtained from breeding company OSEVA PRO limited company. Ten cultivars of opium poppy were selected, differing from one another in the content of morphine in poppy straw. They were blue-seed and white-seed cultivars, which differ from one another in their geographical origin, flower colour, plant height, growing season and other agrobiologic properties. The opium poppy cultivars were grown in field small-path experiments conducted by OSEVA PRO s.r.o.; the capsule samples came from the experiments harvested in 2013. At most 7 poppy capsules from the plants of each cultivar were used for the analysis. The capsules were dried to 6 % moisture content. During the plant material sampling, the poppy capsule was broken off of the stalk at the knot, the stigma was cut off and poppy seeds were poured out. The capsule samples, including stigmas (that means poppy straw in the strict sense), were finely ground on a TUBE-MILL Control test mill (IKA, Germany) to a powder sample and pressed through a screen of a mesh size of 0.5 mm.

2.2 Chemicals used

The following chemicals were used for sample preparation and SPE isolation: acetic acid 99.8%, p.a., ammonia 25%, p.a., hydrochloric acid 35 – 36%, p.a., methanol, p.a. (Fisher Scientific, Czech Republic). The following chemicals were used for mobile phase preparation: acetonitrile and methanol for HPLC, glacial acetic acid 100%, chemically pure, triethylamine, GC ≥ 99.5% (all made by Sigma-Aldrich, Germany). Deionised water was prepared using an Aqua Max – Ultra 370 system (Younglin, Korea). The following standards were used for the alkaloid determination, separation optimisation and validation: morphine sulphide pentahydrate, codeine sulphate, hydrochloride papaverine, thebaine and noscapine (HPLC or TLC ≥ 98%; all made by Sigma-Aldrich, Germany). Reserve standard solutions of alkaloids of 1 mg . ml^{-1} (morphine, codeine and thebaine) and 0.125 mg. ml^{-1} (papaverine, noscapine) were prepared by dissolving in methanol and stored at 4 °C. Using the reserve solutions mentioned above, alkaloid calibration solutions were prepared. The concentration ranges (in μg . ml^{-1}) of the alkaloid solutions for calibration were as follows: morphine: 4 – 500; codeine: 1 – 50; thebaine: 0.1 – 8; papaverine: 0.5 – 20; and noscapine: 0.5 – 10.

2.3 Sample preparation and SPE isolation

50 mg of ground plant material were mixed with 5 ml of 5% acetic acid in a centrifuge tube inserted in a TESON 10 ultrasonic bath (Tesla, Czech Republic) for 30 minutes. Then, the sample was shaken for 1 minute in a TE III shaker (Chirana, Czech Republic) at the laboratory temperature and the entire volume of the sample was centrifuged on a K 2015 centrifuge (Centurion Scientific Ltd., Great Britain) for 10 minutes at 3900 rev/min. The extract thus prepared was purified by solid-phase extraction (SPE) on a Mediwax-12 vacuum manifold (Labicom, Czech Republic). 3 types of columns, namely CHROMABOND HR-XC 200 mg/3 ml (Macherey-Nagel, Germany), Strata-X-C 100 mg/3 ml (Phenomenex, USA) and Discovery DSC-MCAX SPE tube 300 mg/3 ml (Supelco, USA), were selected, based on the data from references[6,24], for the SPE isolation. The columns were conditioned by 3 ml of methanol and equilibrated by 3 ml of ultrapure water. Then, 3 ml of the extract were applied on the column and washed with 2 ml of 0.1 M HCl and 2 ml of methanol. The elution

was carried out twice with 2 ml of the mixture (5 % of ammonia in methanol). The eluate was concentrated and the remaining solvent removed on an RV8 rotary evaporator (IKA, Germany). The evaporation residues were dissolved in 3 ml of methanol and sonicated for 3 minutes. The samples thus prepared were used for the chromatographic analysis. Two pieces of each sample were prepared.

2.4 Chromatographic conditions

Chromatographic analyses were carried out on an Agilent 1200 instrument (Agilent, USA). The system is equipped with a quaternary gradient pump, vacuum degasser, autosampler with a feed block, programmable column thermostat and two detectors (UV/VIS and fluorescence). The ChemStation software was used to collect and evaluate the data. The analyses were conducted on an Ascentis Expres F5 column (5 µm, 150 mm x 4.6 mm I. D., Supelco, USA); the mobile phase A contained 5% of acetonitrile and the mobile phase B a mixture of acetonitrile : glacial acetic acid : triethylamine (97.9:2:0.1, v/v); the gradient program is listed in Table 1; flow rate: 1 ml . min^{-1}; column temperature: 30 °C; injection volume: 50 µl; wavelength: 284 nm; run time: 30 minutes.

Table 1 Gradient program for alkaloid separation

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	90	10
5	85	15
10	80	20
20	65	35
30	90	10

2.5 Validation

The following parameters were determined: linearity, accuracy, repeatability, limit of detection (LOD), limit of quantification (LOQ), sensibility and robustness of the method.

III. RESULTS AND DISCUSSION

3.1 Alkaloid extraction and sample purification

The plant matrix processing was based on the procedure described in references[6,24]. Individual steps were modified as needed for the instrumentation used by us. When alkaloids were isolated from poppy straw, different concentrations of acetic acid within the concentration range from 1 to 10 % were tested. In addition, the effect of the duration of the acetic acid extraction in an ultrasonic bath on the total alkaloid yield was tested. The durations of 15, 30 and 60 minutes were selected for verification. To purify the extract, SPE columns with a strongly acidic cation exchanger for basic analyte exchange were selected. The morphine alkaloid yield and the reproducibility of the extraction process were observed for all kinds of column. The method yield was determined by adding external standards. A known quantity of the morphine standard (30; 90; 140 µl) at the concentration of 1 mg . ml^{-1} was added to the extracting solvent sample. The yield was determined three times. All the columns mentioned above showed very similar yield values ranging from 96 to 98 %. The effect of the eluting solvent volume and type on the morphine yield was investigated. The highest yields were obtained for 5% of ammonia in methanol. The combination of a sample dissolved in 5% acetic acid, alkaloid extraction duration of 30 minutes in an ultrasonic bath, Chromabond HR-XC 200 mg . 3 ml^{-1} column and 5% ammonia solution as an eluent was selected as the most suitable one for the next work.

3.2 Chromatographic determination

An important step in developing the chromatographic separation was to determine the most suitable mobile phase composition for the use of the Ascentis Expres F5 column, 5 µm, 150 mm x 4,6 mm. A gradient elution was used, and the effect of the addition of triethylamine and glacial acetic acid as well as the rising concentration of acetonitrile on the alkaloid separation was observed. The best results were obtained when the gradient program listed in Table I was used. In our experimental conditions where stationary phase was used, the elution sequence of alkaloids was as follows: morphine, codeine, thebaine, papaverine and noscapine (show to Fig. 1).

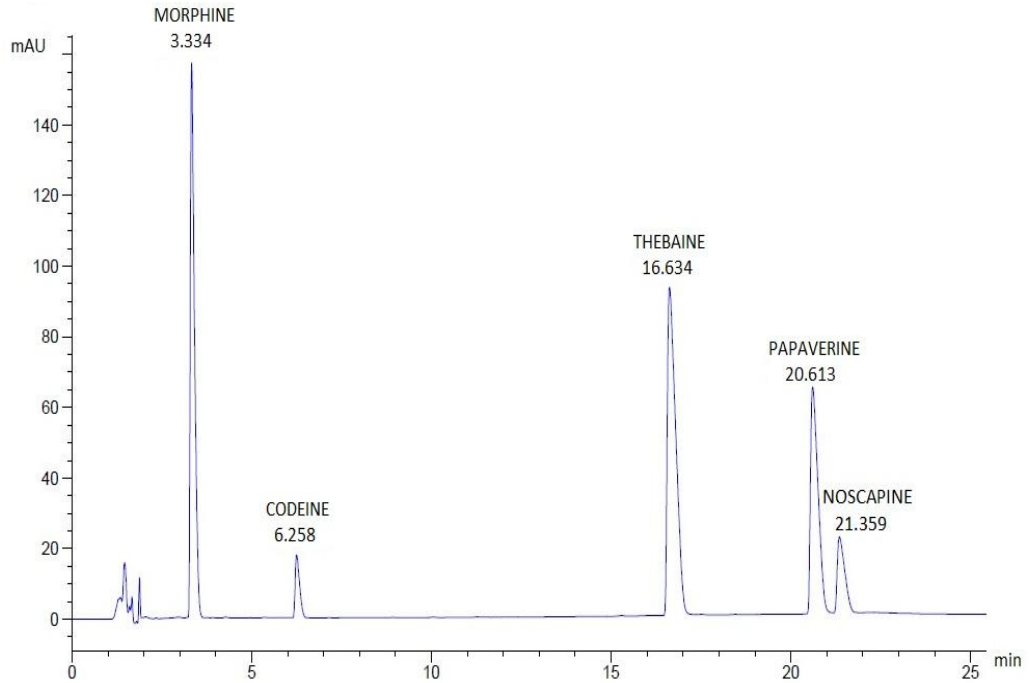

Figure 1 Alkaloid standard chromatogram. Morphine, codeine, thebaine, papaverine and noscapine standards. Ascentis Express F5 column, MF A: 5% acetonitrile, MF B: acetonitrile : glacial acetic acid: triethylamine = 97.9 : 2 : 0.1, 1 ml . min^{-1}, UV/VIS detection, 284 nm.

3.3 Method validation

The method was validated for the determination of morphine content in poppy straw. Validation parameters were determined using the Effi Validation 3.0 software (refer to Table 2). The validation parameter values obtained are summarised in Table 2. Three samples of poppy straw containing different amount of morphine to cover the range required for common morphine concentrations in poppy straw were selected. The samples selected were repeatedly (eight times) extracted using the relevant technique and analysed using HPLC. The repeatability of the analytical method is 4.63 %. Currently, there is no available poppy straw or poppy CRM with a morphine content certified. Therefore, the method accuracy was expressed as a yield, and a morphine standard was used, of which the known quantity was analysed using the method developed. The analysed series of model samples were prepared at three concentration levels to cover the entire range required, and the standards were analysed three times in total. The yield ranged between 95.83 and 101.05 %. The LOD and LOQ were expressed as three times and ten times the baseline noise, respectively. The LOD for determining morphine content in poppy straw is 1.28 μg . ml^{-1} (0.013 %) and the LOQ is 4.22 μg . ml^{-1} (0.043 %). The method linearity was demonstrated using the correlation coefficient and the QC coefficient of the linear regression of the morphine standard peak area dependence on the concentration at levels between the LOQ and 500 μg . ml^{-1}. The method linearity was evaluated based on the values of the correlation coefficient (0.9998) and the QC coefficient (2.54 %). The method sensitivity which is 20 μg of morphine . ml^{-1} as evaluated according to the calibration line slope. The minimum distinguishable difference in concentration is 0.194 μg . ml^{-1}.

Table 2 Validation parameters for determination of morphine in poppy straw using HPLC, including UV/VIS detection

Validation parameter		Value
Linearity	Correlation coefficient	0.9998
	QC coefficient (%)	2.54
Limits	LOD (μg.ml^{-1})	1.275
	LOQ (μg.ml^{-1})	4.215
Repeatability (%)		4.63
Sensitivity (μg.ml^{-1})		1.2
Yield (%)		95.83-101.05

The method robustness was tested against 4 factors. The effects of extraction duration, extracting agent concentration, initial conditions of the gradient and column temperature were observed. The results show only a

minor change in retention times and peak areas, thus proving a high robustness of the method against all the factors tested, which is required for a routine use for the morphine determination in breeding practice.

3.4 Measurement results

10 samples of poppy straw of various cultivars of opium poppy were analysed for alkaloid content. Identification of alkaloids in the samples was carried out by comparing their retention times and spectra to standards. Five alkaloids were identified in the sample chromatogram – morphine, codeine, thebaine, papaverine and noscapine (show to Fig. 2).

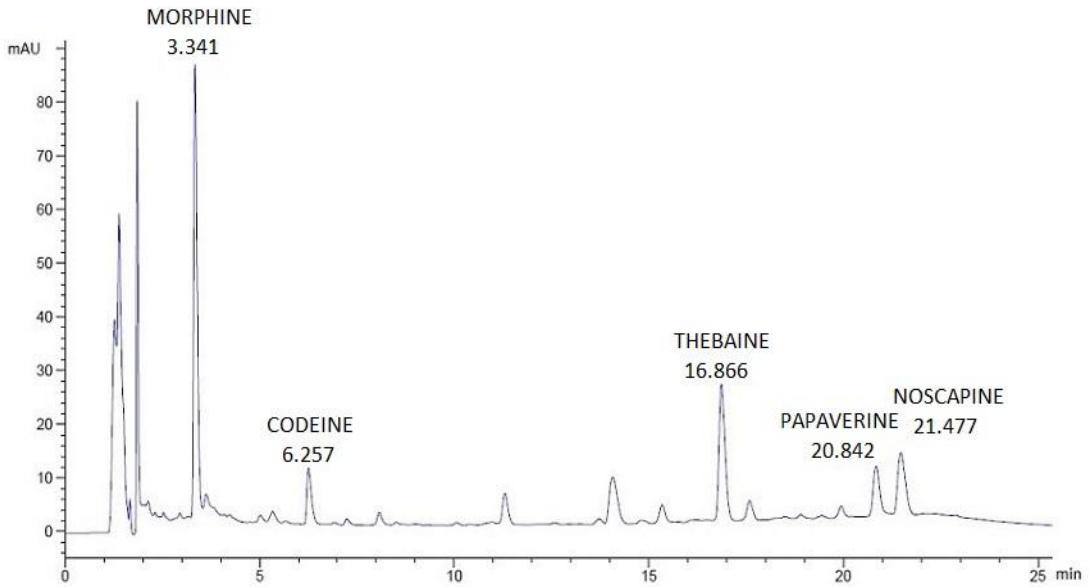

Figure 2 Poppy straw sample chromatogram. Ascentis Express F5 column, MF A: 5% acetonitrile, MF B: acetonitrile : glacial acetic acid: triethylamine = 97.9 : 2 : 0.1, 1 ml . min^{-1}, UV/VIS detection, 284 nm.

Alkaloids were quantified by means of the external standard technique with use of the calibration curves. The calibration series were prepared for each alkaloid to be determined so that alkaloid content in unknown samples could be within the range of the calibration series. The calibration curve for each alkaloid was plotted as a dependence of the peak area on the concentration of the standard. Using the method developed, alkaloids in the poppy straw samples were determined in parallel, and the results expressed in percentage by weight are the average of the two determinations (refer to Table 3). For the newly developed method, the stability of the samples prepared was observed at different time intervals (refer to Table 4). The stability was determined by repeated analyses where the samples were stored in a dark place at 4 °C. The testing of the sample stability shows that samples can be stored for 2 weeks at 4 °C without any occurrence of significant changes in morphine content.

Table 3 Opium alkaloid contents determined in poppy straw samples

Laboratory sample number	Opium alkaloid content (wt. %)				
	Morphine	Codeine	Thebaine	Papaverine	Noscapine
559	0.04	0.01	below LOQ	below LOQ	below LOQ
3977	0.16	0.03	below LOQ	below LOQ	below LOQ
654	0.28	0.10	0.01	0.02	0.03
591	0.39	0.06	below LOQ	0.01	0.04
641	0.44	0.08	below LOQ	0.10	0.03
6087	0.55	0.17	0.01	below LOQ	0.03
6084	0.66	0.19	0.01	below LOQ	0.02
628	0.83	0.17	below LOQ	below LOQ	0.01
6089	0.95	0.14	0.06	0.01	0.02
551	1.47	0.35	0.05	below LOQ	0.02

Table 4 Stability testing of poppy straw samples prepared for HPLC analysis

Laboratory sample number	Morphine content (wt. %)				
	1st day	2nd day	After week	After two weeks	Average
591	0.40	0.41	0.41	0.42	0.41
6089	1.01	1.00	0.99	0.97	0.99

IV. CONCLUSION

The analytical method was developed for the simultaneous determination of main alkaloids in poppy straw using high-performance liquid chromatography. The advantages of the method include particularly the simple isolation of alkaloids from poppy straw, the comparatively short duration of the analysis and the determination of major alkaloids in opium poppy. The optimal chromatographic conditions and the sample preparation technique, including solid phase extraction, were designed and validated. The validation parameter testing included verification of the method repeatability, accuracy, linearity, sensitivity, limit of detection, limit of quantification and robustness for the determination of morphine content. The detection and quantification limits obtained are comparable to those listed in reference[8]. The method was applied to analyse real poppy straw samples from the harvest of 2013. The results show that the developed method can be used as a routine, reliable and accurate technique to determine the poppy straw quality in opium poppy breeding.

V. ACKNOWLEDGEMENT

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