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Comparison of accuracy of screening methods for determination of glucosinolate content in winter rape seed

Porównanie dokładności metod oznaczania zawartości glukozynolanów w nasionach rzepaku ozimego

In 2001 a round robin test of screening for glucosinolate (GSL) contents in 30 samples of winter rape was carried out at several institutes in the Czech Republic and in Germany. In the year 2002 this test was complemented with the determination of GSL content by using the paladium test. The screening method NIRS (Near Infrared Spectroscopy), the glucotest and the paladium test were compared with the reference method HPLC (High Pressure Liquid Chromatography). The highest correlation coefficient was found between HPLC and NIRS (r = 0.7578** and r = 0.7804**), whereas the lowest correlation coefficient was observed between HPLC and the glucotest (r = 0.6346**) and between HPLC and the paladium test (r = 0.6280**). The results suggest a potential of using NIRS for a wide range of GSL contents, whereas the glucotest and the paladium test only help eliminate genotypes with GSL content above the limit.

W roku 2001 zostały przeprowadzone badania porównawcze w kilku laboratoriach na terenie Republiki Czeskiej i w Niemczech nad metodami oznaczania glukozynolanów (GLS) w 30 próbach nasion rzepaku ozimego. Test ten został uzupełniony w 2002 roku badaniami zawartości glukozynolanów metodą palladową. Metody pomiaru zawartości glukozynolanów za pomocą spektrometrii w bliskiej podczerwieni (NIRS) oraz glukotest i test palladowy zostały porównane ze standardową metodą wysokosprawnej chromatografii cieczowej (HPLC). Najwyższy współczynnik korelacji stwierdzono pomiędzy metodą HPLC i NIRS (r = 0,7578** i r = 0,7804**), niższe współczynniki korelacji znaleziono pomiędzy metodą HPLC i glukotestem (r = 0,6346**) oraz pomiędzy HPLC i testem palladowym (r = 0,6280**). Badania wykazały możliwość stosowania metody NIRS w szerokim zakresie zawartości GLS, podczas gdy testy glukozowy i palladowy umożliwiają jedynie eliminację genotypów z zawartością glukozynolanów powyżej pewnej granicy.

In winter rape breeding, the improvement of seed quality has the same priority as the effort to increase seed yield. As for unwanted constituents of rapeseed special emphasis is placed on the determination of glucosinolate contents. These sulphur compounds and their degradation products have an antimicrobial role in plants and are of great importance in plant protection against pests (Giamoustaris, Mithen 1995, Zukalová, Vašák 2002). At present there are several methods for determining total and individual glucosinolate content, which exhibit different demand for equipment and operation, accuracy, rapidity of analysis and costs. The main goal in winter rape breeding is to eliminate materials with high GSL content by application of screening methods and to analyze the resultant smaller set of genotypes by using accurate methods.

The non-destructive screening method NIRS (Near Infrared Spectroscopy) allows determination of GSL content for breeding purposes (Míka et al. 1997, Frauen et al. 1995). Another screening method for pre-selection of plant breeding materials is the glucotest (Kolovrat 1988), or its modification — glucochir. This method is as destructive as the paladium test, which is also simple and of sufficient accuracy (Li-Yan Li et al. 1998, Kolovrat 1988). Among accurate analytical methods is liquid chromatography — HPLC (High Pressure Liquid Chromatography) (Sang et al. 1988, Kolovrat 1988, Hrnčiřík et al. 1998). Unlike the screening methods, which can only be used to determine total GSL content, accurate analytical methods are able to determine the content of individual glucosinolates (aliphatic, aromatic and indole glucosinolates) with high accuracy. Because of the cost and time consumption these analyses are not recommended for routine application on a practical basis in plant breeding. Therefore, it is necessary to start with screening methods for rapid analysis of individual genotypes.

Material and methods

30 samples of winter rape seed harvested from individual plants of homozygous inbred lines in the year 2000 were used for analysis. Internal variability in glucosinolate content among individual seeds in samples was 1.31 µmol GSL $\cdot$ g^{-1} of DM of seed. Moisture content was stabilized at 6%. The samples were analyzed by using 4 methods designed for the determination of glucosinolate content. HPLC and the screening methods such as the glucotest and the paladium test were performed at the VÚOl Opava Institute. NIRS measurements were made at two institutes: VÚRV – VSTE Jevíčko (denoted as NIRS[1]) and at the institute DVS Lippstadt – Thűlle (Germany) where the apparatus was calibrated using a different calibration equation (denoted as NIRS[2]). Samples were analyzed in two replications for all methods of determination of glucosinolate contents. All the measurements are given in µmol GSL $\cdot$ g^{-1} of DM of seed.

1. *Liquid chromatography (High Pressure Liquid Chromatography — HPLC)* was used as a reference method for determining individual glucosinolates following the standard ISO 9167-1. The principle of this method is based on separation of constituents in a two-stage system liquid — liquid. The minimum amount for this method is 200 mg of sample. This method is destructive but it only requires a small amount of sample.

2. *Infrared spectrophotometry (Near Infrared Spectroscopy — NIRS)* is based on the capacity of individual constituents in the seed to absorb infrared radiation. This method employed NIRSystem 6500 with a range of wavelengths of 400–2500 nm and the software ISI 3.10 at the both workplaces. NIRS[1] calibration was covered GSL content 0–43,1 and NIRS[2] was covered GSL content 2–41,2 $\mu mol \cdot g^{-1}$ of DM of seed. Standard error, correlation coefficient and number of samples for calibration are noted in Table 3. For this analysis a portion of 5 grams of seeds was used. This non-destructive method also enables determination of fat content and the content of some fatty acids during a single analysis.

3. *Glucotest* is a method based on the principle of hydrolytic degradation of glucosinolates. Depending on the content in the seed, the enzymatically-released ß-D-glucose will colour the reagent zone of the indication strip (type Glukophan New). After the reagent zone turns green, the strip is visually assessed using a colour scale (a range of 1 to 5). To increase the detection sensitivity activated charcoal was used to remove interfering substances. For conversion to the actual content of GSL in $\mu mol \cdot g^{-1}$ of DM of seed a coefficient k = 8.947 was applied. This method was modified in the Research Institute of Oilseed Crops in Opava for analysis of a minimum number of 10 seeds. Its disadvantage is limited detection sensitivity. With high and low contents of GSL in the seed the bias is even more evident.

4. *Paladium test* — the principle consists in the production of a colour complex between glucosinolates and Na_2PdCl_4 and the measurement of its absorbance at a wavelength of 450 nm. For measurement a spectrophotometer Spekol (VEB Carl Zeiss Jena) was used. For conversion to the actual content of GSL in $\mu mol \cdot g^{-1}$ of DM of seed a coefficient k = 27.47115 was applied.

All 30 samples were put in ascending order according to the results of the reference analysis HPLC, which was used as the standard. The lowest value recorded was 6.17 μmol of GSL $\cdot g^{-1}$ of DM of seed and the highest value was 37.25 μmol GSL $\cdot g^{-1}$ of DM of seed. This ascending series was used for comparison with measurements made by the screening methods.

Correlation coefficients are reported at the level of statistical significance P = 0.01.

Results and discussion

Table 1 gives results from individual analyses. Variation in values according to the reference method was 31.08 μmol GSL $\cdot$ g^{-1} of DM of seed. A high correlation was between HPLC and all the other screening methods at the level of statistical significance P = 0.01 (Table 2).

Table 1

Results of analyses of seed samples from different genotypes of winter rape in ascending order on the basis of GSL content as determined by HPLC — *Wyniki analiz próbek nasion z różnych genotypów rzepaku ozimego w porządku wzrastającym na podstawie zawartości GSL określanej przez HPLC*

Sample no. *Próbka*	Glucosinolate contents [μmol GSL $\cdot$ g^{-1} of DM of seed] Zawartość glukozynolanów				
	HPLC	NIRS[1]	NIRS[2]	glucotest	paladium test
1	6.17	1.74	7.8	15.66	11.54
2	6.38	1.04	10.1	15.66	12.91
3	6.54	6.54	9.6	11.18	10.44
4	6.93	8.81	13.8	13.42	13.19
5	8.99	8.27	14.7	17.89	17.31
6	10.34	11.66	18.7	13.42	17.58
7	10.70	7.81	9.3	13.42	17.03
8	11.77	12.24	15.3	15.66	14.01
9	12.46	10.56	11.9	17.89	17.58
10	12.99	7.02	13.7	17.89	17.86
11	14.18	1.62	9.8	15.66	12.64
12	14.79	6.01	18.8	15.66	16.21
13	15.36	11.89	15.0	13.42	16.21
14	16.26	4.75	14.4	17.89	17.03
15	16.98	13.87	15.0	17.89	16.48
16	18.40	27.90	13.8	20.13	21.98
17	18.41	15.24	14.7	20.13	15.38
18	18.55	12.93	17.8	17.89	15.11
19	18.68	22.24	17.5	17.89	22.25
20	18.81	16.28	17.6	17.89	17.86
21	19.33	10.07	15.2	22.37	20.88
22	20.16	17.33	22.5	20.13	23.08
23	21.42	18.08	21.6	15.66	18.41
24	21.91	14.78	22.4	17.89	20.60
25	22.75	26.23	26.2	20.13	18.41
26	23.94	4.18	22.5	20.13	18.68
27	24.61	19.48	26.3	13.42	18.68
28	31.22	20.23	20.1	22.37	21.43
29	32.65	24.84	19.0	20.13	21.70
30	37.25	35.01	28.3	20.13	16.48
Covar.	48,34	29,93	5,48	5,69	19,03

Table 2

Correlation coefficients between results of analyses by using different methods for determination of GSL contents — *Współczynniki korelacji pomiędzy wynikami analiz zawartości glukozynolanów wykonanych różnymi metodami*

Comparison between *Porównanie pomiędzy*	Correlation coefficient *Współczynnik korelacji*
HPLC : glucotest	0.6346 **
HPLC : NIRS[1]	0.7578 **
HPLC : NIRS[2]	0.7804 **
HPLC : paladium test	0.6280 **

** — significance for $p < 0.01$ — *istotne dla p < 0,01*

Table 3

Standard error, correlation coefficient and number of samples for NIRS calibration
*Błąd standardowy, współczynnik korelacji i ilość próbek dla kalibracji NIRS*

NIRS system	Standard error of calibration *Błąd standardowy dla kalibracji*	Correlation coefficient *Współczynnik korelacji* (r)	N
NIRS[1]	4,61	0,67	227
NIRS[2]	4,12	0,76	304

A correlation coefficient between the reference method HPLC and the glucotest was statistically highly significant ($r = 0.6346$**). The glucotest showed higher accuracy of measurements in median values of GSL content in the seed (Graph 1). This method can be used for the selection of plant materials for GSL content above the limit of 20–25 µmol $GSL \cdot g^{-1}$ of DM of seed with limited sensitivity in lower values of GSL content.

A correlation coefficient between the reference method HPLC and NIRS[1] was statistically highly significant ($r = 0.7578$**), which confirmed the possibility of more accurate determination of a wide range of GSL content in seed (Graph 2). The highest correlation coefficient was found between HPLC and NIRS[2] ($r = 0.7804$**) (Graph 3). According to Leeson et al. (2000) and Míka et al. (1997) the application of this method characterized by high accuracy is only possible when accurate calibration is used, which is also confirmed by the values for correlation coefficients between HPLC and both NIRS measurements. This method is suitable for determining GSL content in the seed after proper calibration based on the results from HPLC.
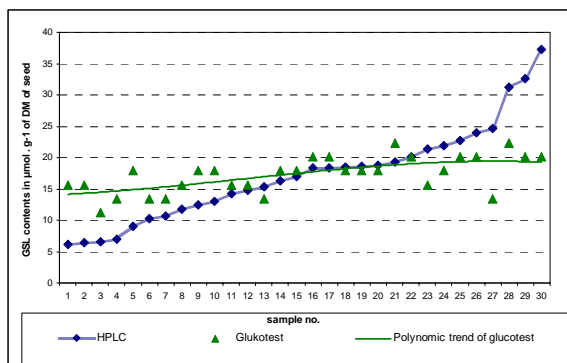
Graph. 1. Comparison of results of measurements by using HPLC and glucotest — *Porównanie wyników pomiarów przy użyciu HPLC i glukotestu*
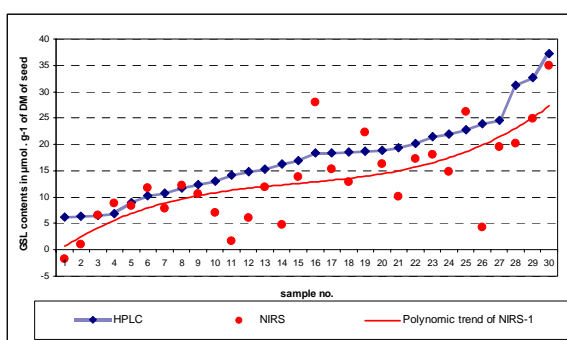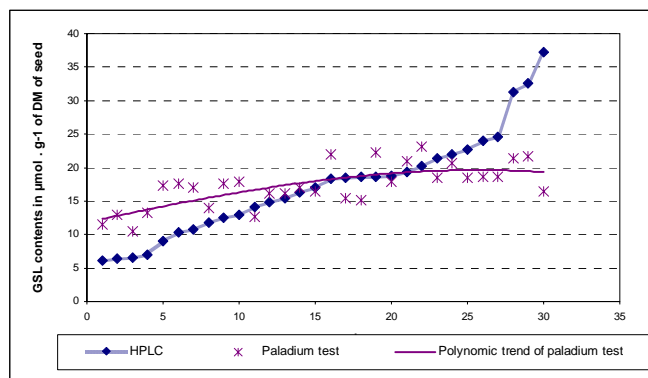

Graph. 2. Comparison of results of measurements by using HPLC and NIRS[1] — *Porównanie wyników pomiarów przy użyciu HPLC i NIRS[1]*
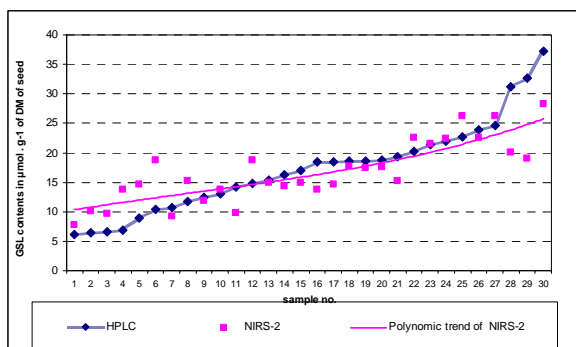
Graph. 3. Comparison of results of measurements by using HPLC and NIRS[2] — *Porównanie wyników pomiarów przy użyciu HPLC i NIRS[2]*

The comparison of the paladium test with the reference method corresponds to the results reported by Mőller et al. (1984). The correlation coefficient $(r = 0.6280^{**})$ confirmed the potential of this method only in a specific area of lower values of GSL content (Graph 4).

Graph. 4. Comparison of results of measurements by using HPLC and palladium test — *Porównanie wyników pomiarów przy użyciu HPLC i testu palladowego*

The glucotest and the paladium test are sufficient for the selection of genotypes above the limit of 20 μmol GSL · g^{-1} of DM of seed. At higher values the sensitivity of the determination of GSL contents by these methods is rather low. These methods are suitable for winter rape breeding on the basis of a selection criterion of GSL content.

Linear regression equations:

1. HPLC (x) and NIRS[1] (y): $y = 0{,}836\ x - 1$
2. HPLC (x) and NIRS[2] (y): $y = 0{,}535\ x + 7$
3. HPLC (x) and glucotest (y): $y = 0{,}236\ x + 13$
4. HPLC (x) and Pd-test (y): $y = 0{,}258\ x + 12$

Conclusion

The obtained results suggest a potential of using NIRS method for rapid and inexpensive detection of a wide range of GSL content, whereas the glucotest and the Pd-test only help to eliminate genotypes with GSL content above the limit of 20–25 μmol GSL · g^{-1} of DM of seed.

Podsumowanie

Otrzymane wyniki wskazują na potencjalne możliwości stosowania metody NIRS dla szybkiego i niedrogiego określania zawartości GSL, podczas gdy glukotest i test palladowy mogą tylko pomóc eliminować genotypy z zawartością GSL powyżej 20–25 µmol GSL · g^{-1} s.m. nasion.

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