RELIABILITY OF ANALYTICAL METHODS FOR QUANTITATIVE DETERMINATION OF INDIVIDUAL GLUCOSINOLATES AND TOTAL GLUCOSINOLATE CONTENT IN DOUBLE LOW OILSEED RAPE

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ABSTRACT

Different analytical methods have been developed and used for determination of glucosinolates in seeds of double low oilseed rape varieties and products thereof. Food and feed produced from varying amounts of these products and therefore containing even lesser amounts of glucosinolates have resulted in increased requirements to reliable and sensitive analytical methods. Determination of individual glucosinolates as well as simple, cheap and quick determination of total glucosinolate contents are a necessity.

The methods investigated for determination of individual glucosinolates comprise: (1) GLC and HPLC of glucosinolate degradation products; (2) GLC of per-trimethylsilylated desulfoglucosinolates; (3) HPLC of desulfoglucosinolates; (4) HPLC of intact glucosinolates. The methods investigated for total glucosinolate determinations include (a) Spectrophotometry based on reaction products formed between thymol-sulphuric acid and intact glucosinolates or desulfoglucosinolates; (b) Glucose determinations following myrosinase catalyzed glucosinolate hydrolysis; (c) Spectrophotometry of Pd-glucosinolate and Pd-desulfoglucosinolate complexes.

The methods have been used to analyze the glucosinolate content in seeds of a great number of different varieties of oilseed rape. The results obtained and the limitations and possibilities of the different methods will be discussed. Methods (1) and (2) are normally sufficient regarding to the assessment of high glucosinolate-containing oilseed rape (single low) and the differentiation between single low and double low varieties. However, advances in production and utilization of oilseed rape demand reliable, fast, simple and cheap methods which can differentiate between double low varieties as well as between products thereof with very low levels of glucosinolates. Establishment of such methods needs discussion.

INTRODUCTION

Glucosinolates co-occur with myrosinase isoenzymes (thioglucoside glucohydrolase, EC 3.2.3.1) in all of the hitherto investigated plants belonging to the order Capparales. Notably the family Brassicaceae possesses vast economic importance owing to e.g. rape and cabbage (Bjerg and Sørensen, 1987a). Hence, much interest is associated with the analytical methodology for isolation, separation, identification and quantification of the individual glucosinolates and degradation products thereof as well as of the total glucosinolate content in different samples. It is, however, absurd to discuss these problems without considering the purpose

of the analytical methodology. It is impossible to solve all problems with only one set of analytical conditions, even if the best known methods are used (Bjerg et al., 1987b). It is also necessary to be familiar with the properties of both myrosinases (Buchwaldt et al., 1986) and glucosinolates since several of them are quite unstable (Figure 1).

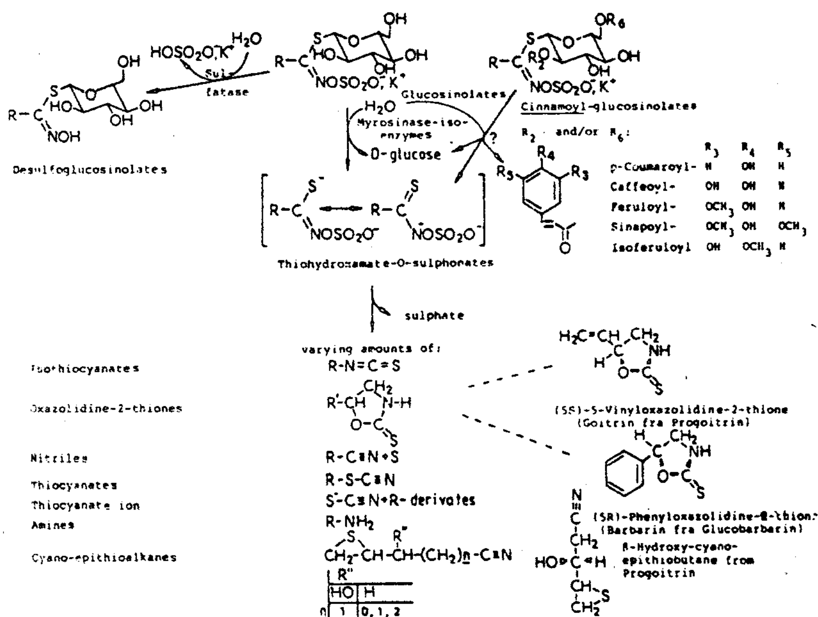


Figure 1. Structures and names of glucosinolates, desulfoglucosinolates and other glucosinolate degradation products.

Glucosinolates are anions with very low pKa value of the sulphate group, implying that it is necessary to work with these compounds as salts (Olsen and Sørensen, 1981). This can be utilized in isolation procedures (Bjerg and Sørensen, 1987a), and it will affect the limitations and possibilities of different methods (Sørensen, 1985).

Today, it is generally agreed that reliable analytical methods for glucosinolates in double low oilseed rape require column chromatographic concentration and purification of the genuine glucosinolates or their immediate derivatives, the desulfoglucosinolates (Figure 1).

ANALYTICAL PROCEDURES

For individual purposes, various techniques are available (Olsen and Sørensen, 1981; Sørensen, 1985). Evaluation of the possibilities of fast

and simple techniques for different purposes requires a consideration of the various experimental steps involved (Figure 2).

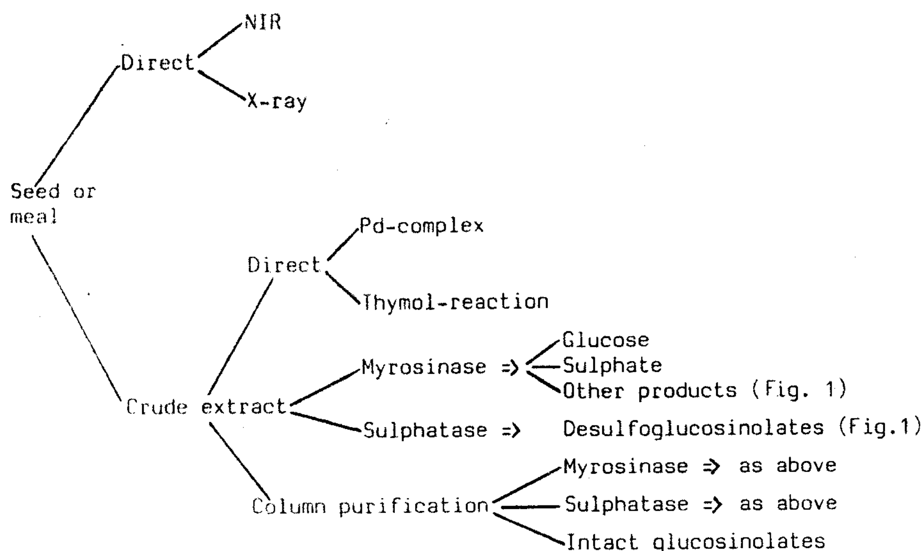


Figure 2. Various experimental steps involved in different analytical procedures.

Determination of total and individual glucosinolates can be performed by various methods. Only some few of these give reliable results when used to determine glucosinolates in double low oilseed rape or products thereof (Sørensen, 1985). All methods require some laboratory experience.

A reliable method for quantification of the glucosinolate level in double low rape can not be based on the various glucosionlate aglucone products (Figure 1) (Nielsen et al. 1979; Olsen and Sørensen, 1981). GLC of per-trimethylsilylated desulfoglucosinolates (vide infra) is also unacceptable since it is impossible to convert all of the important glucosinolates into volatile derivatives(Christensen et al., 1982). Determination of released glucose in myrosinase-catalyzed hydrolyses (Figure 1) is, however, a valuable technique for total glucosinolate determination (vide infra).

Today, HPLC of genuine glucosinolates and/or desulfoglucosinolates are the methods of choice for determination of individual glucosinolates (Bjerg and Sørensen, 1987b) (vide infra). The choice can also fall on

techniques for total glucosinolate determination depending on purpose, requirements to reliability and simplicity, time and price per analysis, available instrumentation, and possibility of carrying out analyses demanding great care.

REFERENCE COMPOUNDS AND PURITY DETERMINATION

Reference compounds are required for several purposes. They are needed for standardizations of analytical methods, and, consequently, the purity should be known. Commercial available glucosinolates are of varying degrees of purity. Methods and techniques for isolation and purity assessment required are available (Bjerg and Sørensen, 1987a).

FAST, SIMPLE AND RELIABLE NON-EXPENSIVE METHODS OF ANALYSIS

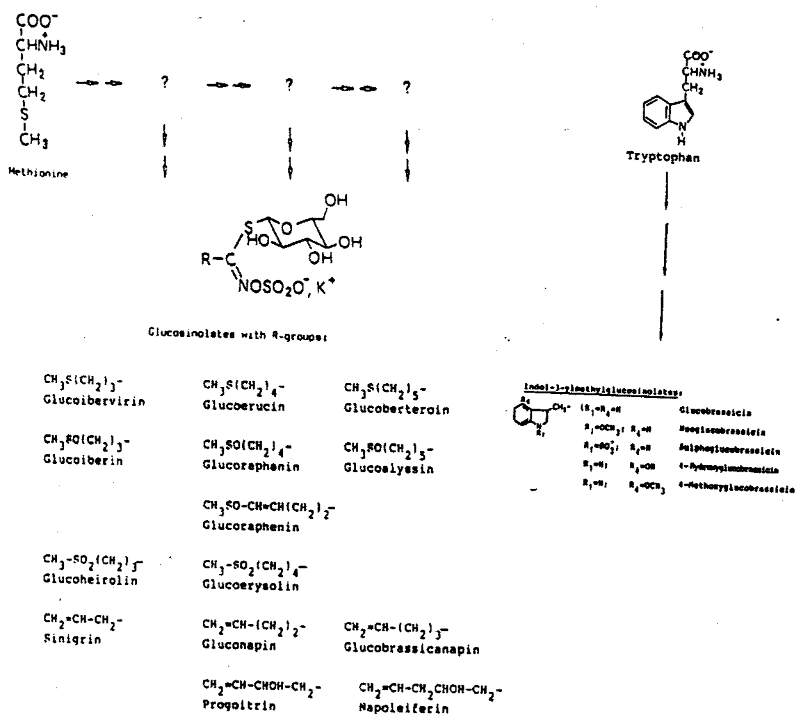
It is difficult to fulfil all of these requirements in a single technique. However, much interest have for obvious reasons been devoted such methods of analysis for determination of glucosinolates. As revealed from Figure 2 the time consuming step (at least 5 minutes) preparing the crude extracts can only be avoided using the direct methods NIR and X-ray. These methods have thus advantages with respect to the time required per analysis. However, for several other reasons, they can not fulfil the requirements for simple, reliable and non-expensive methods usable to differentiate between double low oilseed rape varieties and products thereof with a sufficient low content of glucosinolates (Bjerg et al., 1987c). For these products, serious problems with interfering compounds are to be encountered when methods of analysis based on crude extracts without column purification are used (Figure 2).

Reliable methods of analyses for quantitative determination of glucosinolates in double low oilseed rape and products thereof require concentration and purification on columns. As the next step in alternative and known methods, we can then choose among three possibilities; (1) treatment with myrosinase, (2) treatment with sulphatase, (3) treatment with a buffer solution which removes the charge on the column thereby releasing the intact glucosinolates. For each of these three possibilities, one can choose among several alternatives for the final determination but only some few require attention in relation to the problems attached to double low oilseed rape.

GLUCOSINOLATES IN OILSEED CROPS AND POSSIBILITIES FOR THEIR DETECTION

The analytical methods determine which types of glucosinolates it is possible to detect. Previously, we have used methods based on determination of aglucones produced in myrosinase-catalyzed glucosinolate hydrolysis (Figure 1). It was concluded from these experiments that some types of glucosinolates escaped detection (Nielsen et al., 1979). This is easy to explain when considering the properties of the compounds (Olsen and Sørensen, 1981). In addition, it is also easy to realize that methods based on enzymatic reactions specific for unsubstituted glucose or glucosinolates can not allow detection of some glucosinolates (Figure 1).

The glucosinolates accumulated in seeds of oilseed crops are often quantitatively dominated by glucosinolates derived from methionine and tryptophan (Figure 3).



ure 3. Glucosinolates derived from the amino acids methionine and tryptophan.

The GLC methods (vide infra) have been and are still used in several laboratories even as it is impossible to obtain reliable quantitative results for several glucosinolates and the HPLC technique has several advantages (Sørensen, 1985; Møller et al., 1985a).

TOTAL GLUCOSINOLATE DETERMINATION VIA MYROSINASE-GLUCOSE

The technique based on column purification (Figure 2) followed by hexokinase determination of glucose (Heaney and Fenwick, 1981) gives reliable results for most of the known glucosinolates. When glucose oxidase is used impurities can interfere. Glucosinolates with a substituted thioglucose part (Figure 1) can not be detected. Other drawbacks are that enzymatic reactions impede a fast determination and that the reagents required are relatively expenxive.

TOTAL GLUCOSINOLATE DETERMINATION VIA Pd-COMPLEXES

The technique based an column purification and Pd-complexation (Figure 2) can be used both for intact glucosinolates and desulfoglucosinolates. The latter gives the highest specificity but use of desulphatation impede fast determination. Use of intact glucosinolates, especially in combination with FIA (Møller et al., 1985b), can give a fast and simple method. The slope (α) for standard curves deviates slightly for different glucosinolates which for total determinations of mixtures of glucosinolates are a minor problem. Desulfoglucosinolates gives higher α-values than intact glucosinolates.

Mean values for intact glucosinolates:

$$\frac{1}{\alpha} = 0.23\ \mu\text{mole ml}^{-1}\ (\Delta A_{425})^{-1}$$

Mean values for desulfoglucosinolates:

$$\frac{1}{\alpha} = 0.20\ \mu\text{mole ml}^{-1}\ (\Delta A_{425})^{-1}$$

TOTAL GLUCOSINOLATE DETERMINATION VIA THYMOL REACTION

The technique based on column purification followed by thymol reaction (Figure 2) can be used both for intact glucosinolates and desulfoglucosinolates. The latter gives the highest specificity but use of desulphatation impede a fast determination. The method (Brzezinski and Mendelewski, 1984) is very sensitive to impurities. The colour formation is variable both for different types of glucosinolates and different types of desulfoglucosinolates. Therefore, it can not be recommended as

a general method for determination of response factors to HPLC (Mc Gregor, 1985). The reagents required are unpleasant.

GLC of PER-TRIMETHYLSILYL DESULFOGLUCOSINOLATES

This method (Underhill and Kirkland 1971) have been used successful to several purposes and owing to several problems improvements of this technique have been described (Olsen and Sørensen, 1980; Heaney and Fenwick, 1980 and 1982). It is, however, peculiar that methods based on GLC of per-trimethylsilylated desulfoglucosinolates still are so widespread for quantifying glucosinolates in double low oilseed rape. Several glucosinolates can not be reliably detected, e.g. cinnamoyl-glucosinolates (Figure 1), and some of the glucosinolates derived from methionine and tryptophan (Figure 3) with R-groups; $CH_3S-(CH_2)_n-$, $CH_3SO_2-(CH_2)_n-$ $CH_3SO-(CH_2)_n-$ and indol-3-ylmethylglucosinolates (Sørensen, 1981; Christensen et al., 1982; Heaney and Fenwick, 1982).

Seeds of double low oilseed rape often contain 4-hydroxyglucobrassicin as the quantitatively dominating glucosinolate (vide infra) (Olsen and Sørensen, 1980; Møller et al., 1985a). This compound is known to give problems when the GLC methods are used (vide supra). These problems can be avoided by use of HPLC methods which allow detection of all glucosinolates.

HPLC of DESULFOGLUCOSINOLATES and INTACT GLUCOSINOLATES

Reversed-phase high-performance liquid chromatography has for several years been known as a method for analysis of glucosinolates (Helboe et al., 1980). The preparation of desulfoglucosinolates by enzymatic desulphatation has also been known for several years (Thies, 1978; Rakow et al., 1981). In our first trials we have had problems with impurities in the HPLC desulfotechnique (Olsen and Sørensen, 1983) although this method was succesfully used by other (Minchinton et al.,1982). Both of these two methods have since been much improved (Spinks et al., 1983; Møller et al., 1985a; Bjerg and Sørensen, 1987b). Furthermore, HPLC of desulfoglucosinolates has recently been described as a method representing agreements between a CEC expert group based on a colaboratory study and evaluation of this technique (Wathelet et al., 1987).

Both of these two HPLC methods have advantages and limitations (Bjerg and Sørensen, 1987b). Both methods can be used with gradient technique, resulting in high resolution, but such techniques requires

relatively expensive and complicated instrumentation (Møller et al., 1985a). The desulfotechnique has the highest specificity but some glucosinolates can not be determined (Bjerg and Sørensen, 1987a) and it requires the time consuming desulpatation. The technique based on intact glucosinolates requires a counterion and thereby elevated column temperature (Bjerg and Sørensen, 1987b). It is a simpler technique without requirement of time consuming enzymatic steps, all known glucosinolates can be determined and it can be used as a simple and cheap isocratic technique, which does not require two pumps and system controller units.

In the following tables results obtained by the methods based on glucosinolate aglucone products, GLC of per-trimethylsilylated desulfoglucosinolates and HPLC of intact glucosinolates are presented. The results have been performed on the same seed samples for the three different methods. All results are in µmole/g seed and GLC of per-trimethylsilylated desulfoglucosinolates and HPLC have been performed in two different laboratories. For the latter method some minor peaks have been included in the total. HPLC of desulfoglucosinolates (not shown in the tables) gave nearly results as HPLC of intact glucosinolates.

Variety no.	Official EEC-method The Danish State Seed Testing Station GLC-HPLC analysis of Total glucosinolate degradation products	GLC of per-trimethylsilylated desulphoglucosinolates			
		Gluco-napin	Glucobrassica-napin	Pro-goitrin	Napole-iferin
1	1,7	1,26	0,33	2,15	0,02
2	3,9	2,47	0,49	5,42	0,04
3	1,6	2,06	0,25	3,09	0,02
4	3,7	2,64	0,35	6,40	0,18
5	2,2	1,52	0,40	2,50	0,05
6	2,7	1,27	0,28	2,43	0,03
7	10,6	3,98	0,75	10,50	0,03
8	3,8	2,30	0,32	3,70	0,02
9	5,7	5,55	1,13	7,89	0,06
10	2,8	2,40	0,25	4,10	0,02
11	6,8	4,94	0,69	9,07	0,08
12	6,8	3,05	0,77	6,81	0,02
13	4,4	2,94	0,79	5,78	0,10
14	3,9	2,38	0,46	6,58	0,15
15	2,2	1,85	0,55	4,91	0,18
16	2,3	1,50	0,35	5,79	0,13
Average /1-16/	4,1	2,63	0,51	5,45	0,07
17	65,9	20,10	8,08	70,59	2,71

Variety no.	Total	Gluco-napin	Glucobrassica-napin	Pro-goitrin	Napole-iferin
18	62,0	16,78	9,70	50,26	1,53
19	9,9	3,47	1,08	6,89	0,13
20	23,5	6,72	3,96	17,14	0,99
21	13,6	6,36	2,90	12,13	0,42
22	7,4	3,97	1,40	5,81	0,22
23	7,4	3,92	1,93	10,11	0,37
24	8,7	5,46	2,18	11,73	0,59
25	8,5	4,77	1,94	9,35	0,40
26	9,3	5,98	2,29	14,44	0,53
27	6,3	3,59	1,64	8,36	0,25
28	6,1	3,83	1,89	10,47	0,14
29	6,1	3,32	1,52	10,59	0,28
30	8,2	4,06	1,31	5,82	0,11
31	6,9	3,65	1,59	8,57	0,48
32	8,5	5,60	2,37	11,63	0,41
Average /18-32/	12,8	5,43	2,51	12,89	0,46

HPLC analysis of intact glucosinolates

Variety /No./	Glucoraphanin +Glucoalyssin		Progoi-trin	Napolei-ferin	Gluco-napin	Glucobras-sicanapin	4-Hydroxy-gluco-brassicin	Glucobrassicin+ 4-Methoxygluco-brassicin	Total
1	0,19	0,26	2,43	0,08	0,84	0,24	8,23	0,25	11,68
2	0,17	0,24	6,10	0,19	1,93	0,54	3,46	0,17	14,39
3	0,27	0,35	3,51	0,10	1,89	0,37	3,54	0,24	11,69
4	0,25	0,33	6,33	0,20	1,76	0,28	4,17	0,15	14,84
5	0,21	0,29	3,86	0,17	1,22	0,66	4,26	0,37	12,44
6	0,28	0,36	2,97	0,32	1,73	0,22	1,87	0,16	12,11
7	0,41	0,26	8,86	0,25	2,81	0,61	2,26	0,15	18,77
8	0,37	0,31	4,26	0,37	2,87	0,41	3,20	0,26	15,53
9	0,19	0,43	6,82	0,31	4,63	1,12	3,44	0,46	18,98
10	0,17	0,33	4,20	0,42	1,87	0,18	4,21	0,68	13,49
11	0,42	0,47	8,27	0,10	4,07	0,72	4,17	0,41	20,15
12	0,11	0,15	6,87	0,12	3,10	0,27	6,23	0,14	17,41
13	0,17	0,21	7,94	0,08	2,94	0,60	3,82	0,47	17,26
14	0,10	0,12	6,75	0,15	1,72	0,23	3,67	0,35	14,21
15	0,21	0,28	3,09	0,42	1,02	0,27	4,20	0,67	11,99
16	0,33	0,35	4,94	0,27	0,87	0,31	2,85	0,21	11,97
17	0,89	1,47	60,10	2,10	10,40	3,87	2,10	0,40	82,28

HPLC analysis of intact glucosinolates.

Variety (No.)	Glucoraphanin + Glucoalyssin		Progoitrin	Napoleiferin	Gluconapin	Glucobrassicanapin	4-Hydroxyglucobrassicin	Glucobrassicin + 4-Methoxyglucobrassicin	Total
18	0.46	0.41	47.61	1.00	12.66	1.51	8.76	0.16	81.89
19	0.31	0.32	7.38	0.62	2.77	0.62	3.00	0.20	19.37
20	0.33	0.33	20.32	1.12	4.22	2.11	2.86	0.09	34.75
21	0.41	0.42	13.92	0.61	5.36	1.07	2.58	0.11	30.01
22	0.41	0.43	6.08	0.56	3.50	0.42	4.80	0.57	20.67
23	0.43	0.55	9.61	0.27	2.88	1.40	6.20	0.10	22.87
24	0.42	0.61	18.99	0.62	4.81	0.24	2.75	0.13	30.81
25	0.25	0.46	10.44	0.50	3.66	1.32	2.47	0.11	21.51
26	0.54	0.60	12.63	0.67	4.56	0.58	3.10	0.12	24.93
27	0.11	0.26	8.24	0.48	3.42	0.54	3.47	0.34	19.65
28	0.67	0.56	9.71	0.56	3.20	0.71	2.42	0.43	20.56
29	0.47	0.63	10.81	0.68	3.35	1.02	4.47	0.94	24.45
30	0.31	0.23	9.74	0.53	4.24	0.26	2.71	0.87	20.59
31	0.33	0.36	10.21	0.64	2.86	0.72	3.62	0.12	20.50
32	0.37	0.48	11.43	0.47	4.26	0.81	3.26	0.47	24.12

Comparison of the total glucosinolate content in oilseed rape (µmole/g of seed) determined by different methods of analysis

Variety (No.)	Official EEC-method	GLC method	Pd-glucosinolate method	HPLC of intact glucosinolates
1	1.7	3.8	14.5	11.7
2	3.9	8.4	15.8	14.4
3	1.6	5.4	13.7	11.7
4	3.7	9.6	16.7	14.9
5	2.2	4.5	15.5	12.4
6	2.7	4.0	10.9	12.1
7	10.6	15.3	18.5	18.8
8	3.8	6.3	16.2	15.5
9	5.7	14.7	22.3	19.0
10	2.8	6.8	16.1	13.5
11	6.8	14.0	22.1	20.2
12	6.8	10.7	18.5	17.4
13	4.4	9.6	17.6	17.3
14	3.9	9.6	14.0	14.2
15	2.2	7.5	14.2	12.0
16	2.3	7.8	12.2	12.0
17	65.9	91.5	92.3	82.3
18	62.0	78.9	83.5	81.9
19	9.9	11.6	21.2	19.4
20	23.5	28.8	32.9	34.8
21	13.8	21.8	28.3	30.0
22	7.4	11.4	22.9	20.7
23	7.4	16.3	21.0	22.9
24	8.7	20.0	30.8	30.8
25	8.5	16.5	22.5	21.5
26	9.3	23.2	29.1	24.9
27	6.3	13.8	20.8	19.7
28	6.1	16.3	21.1	20.6
29	6.1	15.7	24.9	24.5
30	8.2	11.3	22.7	20.9
31	6.9	14.3	19.4	20.5
32	8.5	19.8	24.6	24.1
Average (1-16) of "double low" spring rape varieties	4.1	8.7	16.2	14.8
Average (19-32) of "double low" winter rape varieties	9.3	17.2	24.4	24.0

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