Ewald Schnug, Silvia Haneklaus

Emulation and backtracking of HPLC chromatographic profiles for glucosinolate valuation from total sulphur concentrations in oilseed rape seeds*

Emulation und Verifizierung von HPLC Chromatogrammen für die Abschätzung des Glucosinolatgehaltes aus Gesamtschwefelkonzentrationen in Rapssaat

Abstract

The relationship between the total glucosinolate (GSL) concentration calculated from the total sulfur concentration which had been measured by means of X-ray fluorescence spectroscopy and the content of glucobrassicanapine, glucobrassicine, gluconapine, napoleiferine, progoitrine and 4-hydroxiglucobrassicine in the seeds which were measured by chromatographic methods was determined in the line of quality assessment studies of oilseed rape standard reference materials. The constant ratio between individual aliphatic GSLs which is independent of the total GSL content allows to emulating the concentration of individual GSLs from the total GSL content on basis of the total S content. As indol GSLs represent a constant background value of the total GSL content their estimated concentration is added to the calculated sum of aliphatic GSLs in order to obtain an emulated total GSL content. In a simple program written in BASIC the typical background variability of individual GSLs can be randomly added to the results which yields different chromatograms that are statistically not different from true HPLC chromatograms. This may assist in distinguishing true experimental effects in studies targeting effects on individual GSLs from those of background analytical error variability. The program may also be used for an independent verification of HPLC chromatograms of GSLs in oilseed rape as it allows backtracking of a given total GSL content to its expected individual GSL concentrations in chromatographic analysis.

Key words: Glucobrassicanapine, glucobrassicine, gluconapine, glucosinolates, HPLC, napoleiferine, oilseed rape, progoitrine, sulfur, 4-hydroxiglucobrassicine, X-ray fluorescence, X-RF method

Zusammenfassung

Aus Daten zur Zertifizierung der Gehalte an Schwefel, Gesamt- und Einzelglucosinolaten dreier EU Standardreferenzmaterialen aus Rapssaat und Literaturdaten wurden die Beziehungen der Gehalte zueinander und deren methodisch bedingte Variabilität bestimmt. Kernergebnis ist, dass die Gehalte einzelner Glucosinolate (GSL) eine Funktion des Gesamt-GSL-Gehaltes sind. Dabei ist die

*in memoriam Prof. Dr. Richard Marquardt, Giessen (19.05.1938 – 16.12.2010)

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Variabilität der Einzel-GSL-Gehalte signifikant höher als die ihres summarischen Gesamtgehaltes, insbesondere aber signifikant höher als die aus Gesamtschwefelgehalten (mittels wellenlängendispersiver Röntgenfluoreszenzanalyse) berechneten Gesamt-GSL-Gehalte. In diesem Beitrag wird ein BASIC Script vorgestellt, welches aus Gesamtschwefelgehalten den Gesamt-GSL-Gehalt an Hand der für die EU Standardmethode (X-RF Methode) vorgeschriebenen Funktionen berechnet und diesen Gesamtgehalt auf die 6 in der EU HPLC-Standardmethode identifizierten Einzel-GSLe (Glucobrassicanapin, Glucobrassicin, Gluconapin, Glucosinolate, Napoleiferin, Progoitrin, und 4-Hydroxiglucobrassicin) aufteilt. Zusätzlich kann das Programm die Einzel-GSL-Gehalte mit einer aus den analytischen Daten ermittelten, zufällig verteilten, aber methodenspezifischen Variabilität der chromatographischen Analyse ausgeben, oder aus vorgegebenen Gesamt-GSL-Gehalten die zu erwartenden Gehalte an Einzel-GSL berechnen. Das Programm ermöglicht damit die unabhängige Überprüfung von HPLC Analysen. Umgekehrt können mit dem Programm bei Vorgabe eines Gesamt-GSL-Gehaltes die bei chromatographischer Analyse zu erwartenden Konzentrationen an Einzel-GSLen bestimmt werden.

Stichwörter: Glucobrassicanapin, Glucobrassicin, Gluconapin, Glucosinolate, HPLC, Napoleiferin, Progoitrin, Raps, Röntgenfluoreszenz, Schwefel, 4-Hydroxiglucobrassicin, RFA-Methode

Introduction

Double low oilseed rape varieties produce seeds with much lower concentrations in eruic acid and GSLs than found in native genotypes. During the introduction of double low oilseed varieties in agricultural production in the 1980s it became clear that there was a lack of suitable, fast and accurate analytical methods to separate seeds according to their total GSL content (SCHNUG, 1989a, b; WATHELET et al., 1995). The breakthrough was the invention of the so-called X-RF method which relies on the close relationship between total sulfur and total GSL content in oilseeed rape seeds (SCHNUG and HANEKLAUS, 1987). During the rigorous testing of the X-RF method against various competing chromatographic methods it became clear that the variability of the results arising from calculating the total GSL content by means of the total S concentration was significantly smaller than the variability caused by assessing the total GSL content by summing up the concentrations of the six individual GSLs prescribed in the EU HPLC method (glucobrassicanapine, glucobrassicine, gluconapine, napoleiferine, progoitrine and 4-hydroxiglucobrassicine).

This paper describes a BASIC program that calculates not only the total GSL content of oilseed rape seeds by determining the total S concentration, but also permits the differentiation of individual GSLs. In addition, a true HPLC chromatogram can be employed to cross-check for errors in the software-controlled division of individual GSLs. As an extra option the program permits to add typical variability inherent to chromatographic methods which may help to distinguish true experimental effects on individual GSLs in studies from background analytical error variability.

Material and Methods

In general, the relationship between the total S content of oilseed rape seeds and the total GSL content has been checked excessively during the time the EU was seeking for a proper, says fast and accurate method to distinguish between rapeseed batches of different GSL content for granting subsidies (SCHNUG, 1988). The breakthrough in terms of accuracy, repeatability and speed was finally the so-called X-RF (X-ray fluorescence) method. The X-RF method determines the total S concentration in rapeseeds by means of wavelength dispersive X-ray fluorescence analysis in a simple three step procedure (SCHNUG and HANEKLAUS, 1986, 1987a, b). The calculation formulas for computing the total GSL content from the total S concentration provided by SCHNUG and HANEKLAUS (1988) have been verified in a large number of inter-laboratory comparisons (SCHNUG and KALLWEIT, 1987) and these formulas were finally adopted by the EU in combination with wavelength dispersive X-RF as compulsory standard method. The concentration range for total S in rapeseeds is divided in two ranges: above 11 mg/g S the original calibration function of the X-RF method, which has been also verified by stoichiometric assessments (SCHNUG et al, 1992b, ZHAO et al., 1992) is applied (Annex: line 600), but below 11 mg/g S a calibration function considering the non-linear relationship between total S and total GSL content is used (SCHNUG and HANEKLAUS, 1990; annex: line 800). This non-linear function compensates slightly changes of the total protein concentration in seeds with low GSL concentration due to environmental factors like for instance S deficiency in the growth medium.

In line 920 (annex) a correction factor for systematic error deviations of the HPLC method can be brought into consideration if required. In the recent program description the adjustment is made to the latest results of EU standardization.

The analytical data for establishing the regression equations between total GSL analyzed by X-RF and individual GSL concentration in oilseed rape seeds were collected from a number of method inter-comparisons conducted by the Bureau of Standards (BCR) of the European Commission (EU) performed on oilseed rape standard reference materials (SCHNUG et al., 1992; WATHELET et al., 1988, 1991, 1992). The standardized regression equations for calculating individual GSLs from the total GSL content can be found in the program script (see annex) in lines 1000–1600.

During the exhaustive evaluation procedure of the EU standard methods for GSL determination in rapeseeds the urgent need for standard reference materials (SRM)

became an obvious task given to the then operating Community Bureau of Standards (BCR) (Wagstaffe et al., 1992; Wathelet et al., 1988). As a result three SRMs (BCR SRM 190, BCR SRM 366, BCR SRM 367) with certified total contents for GSLs and S were released. Any efforts to certify the concentration of individual GSLs failed utterly. The remarkable phenomenon was that although the sum of 6 individual GSLs analyzed by means of the EU protocol for HPLC was successful, the certification of the individual concentration proved to be not feasible (Wathelet et al., 1987, 1989). The reason is most likely some methodically inherent incapability of the HPLC to differentiate distinctively and sharp enough between individual GSLs.

In the mathematical procedure the variability of the individual GSLs observed in HPLC analysis (Wathelet et al., 1987) was standardized and randomized (see annex lines 2000–4700), and then added to the individual GSL concentrations calculated from the total S content (see annex lines 5300–5800).

In addition to the previously described features the program allows also backtracking of a given total GSL content to its corresponding total S content and based on this data to calculate an expected concentration of individual GSLs (annex line 30000–38735). This procedure is flawed slightly in the lower ranges of GSL concentrations, because the re-calculation is based on an inverted linear calibration function for calculating the total S concentration from GSLs (annex line 34500). This procedure was necessary as the resolution of the cubic function used in line 800 of the annex provided non-conclusive results. This error is, however, well beyond the background error of any HPLC analysis.

Results and Discussion

The program "EMU" (see annex), which performs the above described tasks has been written in BASIC 3.11 in an ancient MSDOS 3.2 environment. However, x86 DOS emulators available from the internet still allow to run this kind of program in recent operation systems (e.g. WINDOWS 10). Installation instructions are provided in the annex.

Table 1 displays in 9 steps example runs with "EMU". The first decision to make is whether a HPLC chromatogram of GSLs in a rapeseed sample shall be emulated from its total S concentration, or if an existing total GSL content in rapeseeds shall be broken down into estimates for individual GSL concentrations (Table 1, step 3).

If the first option (HPLC emulation) is chosen another decision has to be made if the results shall be static or with a random variability conform to the common range of HPLC determination (Table 1, step 4). After entering the total S concentration in mg/g S the results are processed either without or with variability (Table 1, steps 6 and 7). It should be mentioned that emulating GSL concentrations from total S analysis requires S determination which is highly accurate and repeatable, features which are only fulfilled by wavelength dispersive X-RF analysis (Wagstaffe et al., 1992). Energy dispersive X-RF, combustion methods, spectroscopic analyses and gravimetry following wet digestion of the sample do not comply with the quality standards of wavelength dispersive X-RF (Haneklaus et al., 1994), hence the quality of the emulated GSL content in terms of accuracy and repeatability will be significantly diminished when S concentrations obtained by these methods are fed into EMU.

In the "without variability" mode the program will provide for a defined S concentration consistently the same GSL concentration. In case the option "with variability" is chosen (Table 1, step 4) then an emulated amount of variability is added to this concentration, which meets the variability for the analysis of individual GSLs assessed during the fruitless certification attempt by BCR (Wathelet et al., 1987).

Repeated entry of the same S concentration in this mode will generate GSL patterns according to step 6 (Table 1), but with a random amount of variability. For instance: the input of 5 mg/g S at step 5 (Table 1) provides a result of 29.2 μmol/g total GSL when the mode "without variability" had been chosen in step 4 (Table 1); the 10 times repeated input of 5 mg/g S at step 5 gives a series of 31.7, 32.6, 27.0, 26.7, 28.5, 31.7, 28.0, 26.3 and 34.2 μmol/g total GSL. In step 7 the difference between results with and without variability for the particular input at step 4 is shown. With each repetition of the same input of total S the average of the collected results will approximate the value achieved in the mode "without variability", says for an infinite number of repetitions of the same input for mg/g S the deviation of the averaged emulated results from X-RF in the "with variability" mode (Table 1, step 7) approximates zero. One practical application is to check and verify effects on individual GSLs claimed in variety or growth experiments where no sufficient statistics is provided. Many of such effects reported in the literature (e.g. Marquardt and Schlesinger, 1987) fall into the range of uncertainty of the analytical method and thus may become doubtful, at least from a statistical point of view.

Yet another feature of the program is that it permits to backtrack a given total GSL content to its corresponding total S content and to calculate from this data an expected concentration for individual GSLs (Table 1, step 3). An example for the output of the program is given in Table 2.

This procedure is flawed a little in the lower ranges of GSL concentration, because the re-calculation is done by employing the inverted linear calibration function for calculating the total S concentration from GSLs (annex line 34500) and as a matter of fact resolving the cubic function used in line 800 of the annex gives non-conclusive results. But this error shall be well beyond the background error of any HPLC analysis.

The "HPLC-check" modus of "EMU" may be used for an independent verification of HPLC chromatograms of oilseed rape GSLs as it allows sourcing a given total GSL content to its expected individual GSL concentrations in a chromatographic analysis.

Table 1. Operational steps and output of the program EMU for the emulation and backtracking of glucosinolates in oilseed rape

DOSBox 0.74, Cpu speed: 3000 cycles, Framskip 0, Programm BASIC	Step
DOSBox Status Windows DOSBox version 0.74 Copyright 2002–2010 DOSBox Team, published under GNU GPL. --- CONFIG: Loading primary settings from config file C:\User\schnug\AppData\Local\DOSBox\dosbox-0.74.conf MIXER:Can't open audio: DirectSoundCreate: No audio device found, running in no sound mode. MIDI:Opend device:none DOS keyboard layout loaded with mein language code GR for layout gr -- To adjust the emulated CPU speed, use ctrl-F11 and ctrl-F12. To activate the keymapper ctrl-F1. For more information read the README file in the DOSBox directory. Have fun! The DOSBox Team http://www.dosbox.com -- Z:\>SET BLASTER = A220 I7 D1 H5 T6 Z:\>keyb gr Keyboaard layout gr loaded for codepage 437 Z:\>mount c f:\pippa\xrf Drive C is mounted as local directory f:\pippa\xrf\ Z:\>C:\. Direcory of C: EMU BAT 16 08–01–2016 12:36 1 File(s) 16 Bytes. 0 Dir(s) 262,111,744 Bytes free. C:\>_	1: The program is written in BASIC and to run it on any WINDOWS computer first load the emulator software DOSBOX and the BASIC interpreter into the same directory where the script is stored as "EMU.bas". After starting DOSBOX first key in: keyb gr < return > to activate the German keyboard layout and MOUNT the directory where "EMU.bas" is stored to "C". The program starts automatically after the command > basic emu < return > is keyed in. Input is case sensitive and accepts only uppercases for alpha-numeric inputs.
******************************** * HPLC-Emulation for X-RF data * * and HPLC data check by X-RF * * version 4.0 * * (release 31. January 2015) * * copyright by Ewald Schnug * ******************************** Proceed and confirmation press < RETURN>	2: Any alphanumerical input has to be in UPPER CASES! This program script supports no printout. If hardcopies are required you should use the screendump option of your operating system or simply SNIPE and copy it.
modus: HPLC-create (1) HPLC-check (2) **>>: 1_	3: Choose "1" to generate total and individual GSLs from total S input. Choose "2" to estimate individual GSLs from total GSL content.
With variability no (1) or yes (2) **>>: 1	4: Choose "1" to add typical "normal" variability to generate total and individual GSLs from total S input. Choose "2" to estimate individual GSLs from total GSL.
Total sulphur content in seeds (mg/g) **>>: 5_	5: Input is in mg/g total S in air dry seeds (8% H_2O) with 42% fat.

Table 1. Fortsetzung

DOSBox 0.74, Cpu speed: 3000 cycles, Framskip 0, Programm BASIC	Step
Emulation without variability progoitrin:17.10 umol/g napoleiferin:0.59 umol/g gluconapin:7.11 umol/g glucobrassicanapin:1.27 umol/g 4-OH glucobrassicin:3.04 umol/g glucobrassicin:0.13 umol/g total GSL from total S (without var-modus) =: 29.24 umol/g Next S input < RETURN > or back to menu (M) or back to system (S) M_	6: The program calculates the total GSL content from the total S content according to the calibration formulas prescribed for employing the X-RF method according to the EU standard method and the individual GSLs as described in the text.
Emulation without variability progoitrin:16.57 umol/g napoleiferin:0.65 umol/g gluconapin:6.89 umol/g glucobrassicanapin:1.70 umol/g 4-OH glucobrassicin:3.06 umol/g glucobrassicin:0.39 umol/g total GSL from total S (without var-modus)=: 29.25 umol/g total GSL from total S (without war-modus)=: 29.24 umol/g deviation from X-RF : -0.61 umol/g Next S input < RETURN > or back to menu (M) or back to system (S)	7: When “2” for results with variability was chosen in the previous menu, the program adds a random variability to the results which reflects the one observed during ring tests for individual GSLs with HPLC.
modus: HPLC-create (1) HPLC-check (2) **>>: 2 total GLS content in seeds (umol/g) according to EU HPLC reference method **>>: 33_	8: Choosing "2" generates a set of individual GSL concentrations expected at the given input of GSL in µmol/g dry (8% H_2O) seeds with 42% fat.
Check individual GSL progoitrine : 19.09 umol/g napoleiferine : 0.66 umol/g gluconapine : 7.94 umol/g glucobrassicanapine : 1.42 umol/g 4-OH glucobrassicine : 3.06 umol/g gludobrassicine : 0.13 umol/g Checksum : 32.30 umol/g Input EU GSL : 33.00 umol/g Total S calculated (linear calibration approach) (linear calibration approach) : 5.13 mg/g Next GSL input <RETURN> or back to menu (M) or back to system (S) _	9: The result is the expected profile of individual GSLs and the total S content of the seeds.

Table 2. Backtracking individual GSLs in oilseed rape seeds from the total GSL content and comparison of backtracked GSLs with individual GSLs emulated from the total S content by means of the program "EMU"

Glucosinolate	Backtracking* from 33 µmol/g total GSL (µmol/g)	Emulation** from 5 mg/g total S (µmol/g)
Progoitrine	19.1	18.1
Napoleiferine	0.66	0.63
Gluconapine	7.94	7.6
Glucobrassicanapine	1.42	1.4
4-OH-glucobrassicine	3.06	3.1
Glucobrassicine	0.13	0.13
Sum	32.3	30.1

* Program "EMU" Modus "HPLC-check"
** Program "EMU" Modus "HPLC-create, without variability"

Annex

"EMU" program script for BASIC Interpreters (GW-BASIC 3.11, or newer)

The following program emulates HPLC analyses according to the EU standard method from total sulfur analyses in rapeseeds. The program is written in BASIC and to run it on any WINDOWS computer first the emulating software DOSBOX (DOSBOX, 2016) and a BASIC (GW-BASIC, 2016) interpreter have to be loaded in the same subdirectory, where the script is stored as "EMU.bas". The script below must be copied from line 10–40000 and saved in plain ASCII as emu.bas in the same directory as the basic interpreter and a batch file "emu.bat" which contains one line with the command "basica emu" in. After starting DOSBOX first key in: keyb gr < return > to activate the German keyboard layout and then use the BASIC command "MOUNT" to access the directory where your program files are stored.

The program itself starts after the command > basic emu < return > is keyed in. The input is case sensitive and accepts only upper cases for alphanumeric inputs.

If you are unable to retrieve the script in ACII from this file you can download it from this source (DOI: 10.13140/RG.2.1.1128.8089):

https://www.researchgate.net/publication/293146193_Emulation_and_backtracking_of_HPLC_chromatographic_profiles_for_glucosinolate_valuation_from_total_sulphur_concentrations_in_oilseed_rape_Executable_BASIC_program_in_ASCII

The program supports no printer, if hardcopies are required simply SNIPE the result tables and print directly from the SNIPE program provided with the operating system.

"EMU" program script for BASIC Interpreters (GW-BASIC 3.11, or newer)

```
10 REM Script EMU.bas use TRON to assist any debugging
12 PRINT: PRINT: PRINT: PRINT:PRINT: PRINT
20 PRINT" ********************************"
30 PRINT" * HPLC-Emulation for X-RF data *"
40 PRINT" * and HPLC data check by X-RF *"
50 PRINT" * version 4.0 *"
60 PRINT" * (release 31. January 2015)*"
65 PRINT" * copyright by Ewald Schnug *"
70 PRINT" ********************************"
75 INPUT" proceed and confirmation press < RETURN> ",XX
80 PRINT
90 CLS
91 PRINT: PRINT: PRINT: PRINT
95 PRINT" modus: HPLC-create (1) HPLC-check (2) "
```

```
96 INPUT" ****************************************>>: ", MODUS
97 IF MODUS = 2 GOTO 10000
98 IF MODUS = 1 GOTO 190
190 CLS
194 PRINT: Print: PRINT: PRINT
195 PRINT" with variability no (1) or yes (2) "
196 INPUT" ****************************************>>: ",VAR
210 CLS
214 PRINT: PRINT: PRINT: PRINT
240 PRINT" total sulphur content in seeds (mg/g) "
250 INPUT" ****************************************>>: ",S
260 CLS
400 REM GSL calculation according to NCS- or linear system
500 IF S < 10.9999 GOTO 800 ELSE 600
600 RFA = 14.99* S-43.87
700 GOTO 900
800 RFA = −5.6* S + 2.8*(S* S)-.12*(S* S* S)+3.5
900 REM basic calculations for single glucosinolates
910 REM correction to BCR level status may 1990
920 RFA = RFA*.974+.15
1000 REM Routines derived from program > singel.sps < updated for BCR results
1100 PRO= ((.71492* RFA)-4.4234)*.886 + 2.11
1200 GNL=((.0073* RFA)+.46634)* 2.838−1.351300 GNA=((.23856* RFA)-.74791)* 1.111+.023
1400 GBN=((.04147* RFA)+.08421)* 1.18-.29
1500 OH4=((.00069* RFA)+4.2658)* 8−31.25
1600 GBC=((-.00293* RFA)+.35497)*-.179+.177
1700 GSLTOT = (PRO + GNL + GNA + GBN + OH4 + GBC)
1800 REM
2000 REM random functions: time factor bevore rnd = (2* standard deviation)
2100 REM of deviation predicted from measured values)* 10; minus sd* 10 (mean = 0)
3000 REM variability for progoitrine
3100 RANDOMIZE((2211/1000)*(VAL(MID$(TIME$,4,2))* VAL(RIGHT$(TIME$,2))))
3200 VARPRO=((INT(72.72* RND(1)+1))-36.36)/10
3300 REM variability for napoleiferine
3400 RANDOMIZE((1710/1000)*(VAL(MID$(TIME$,4,2))* VAL(RIGHT$(TIME$,2))))
3500 VARGNL=((INT(18.92* RND(1)+1))-9.46)/10
3600 REM variability for gluconapine
3700 RANDOMIZE((79/10)*(VAL(MID$(TIME$,4,2))* VAL(RIGHT$(TIME$,2))))
3800 VARGNA=((INT(18.26* RND(1)+1))-9.16)/10
3900 REM variability for glucobrassicanapine
4000 RANDOMIZE 19834100 VARGBN=((INT(17.48* RND(1)+1))-8.74)/10
4200 REM variability for 4-hydroxy glucobrassicin
4300 RANDOMIZE 1959
4400 VAR4OH=((INT(29.49* RND(1)+1))-14.86)/10
4500 REM variability for glucobrassicine
4600 RANDOMIZE((1954/1000)*(VAL(MID$(TIME$,4,2))* VAL(RIGHT$(TIME$,2))))
4700 VARGBC=((INT(6.76* RND(1)+1))-3.38)/10
4800 REM selection create or check modus
4900 IF MODUS = 1 GOTO 5000
4950 IF MODUS = 2 GOTO 31000
5000 REM selection with or without variability from line 196
5100 IF VAR = 1 GOTO 8191
5200 IF VAR = 2 GOTO 5300
5300 PROV = PRO + VARPRO
5400 GNLV = GNL + VARGNL
5500 GNAV = GNA + VARGNA
5600 GBNV = GBN + VARGBN
```

```
5700 OH4V = OH4 + VAR4OH
5800 GBCV = GBC + VARGBC
5910 IF PROV < 0 THEN PROV=.01
5920 IF GNLV < 0 THEN GNLV=.01
5930 IF GNAV < 0 THEN GNAV=.01
5940 IF GBNV < 0 THEN GBNV=.01
5950 IF OH4V < 0 THEN OH4V=.01
5960 IF GBCV < 0 THEN GBCV=.01
6000 REM output with variability
6100 CLS
6191 PRINT: PRINT: PRINT: PRINT: PRINT
6196 PRINT "E m u l a t i o n w i t h v a r i a b i l i t y "
6197 PRINT
6200 PRINT"progoitrin: ",:PRINT USING"###.##";PROV;:PRINT" umol/g"
6300 PRINT"napoleiferin: ",:PRINT USING"###.##";GNLV;:PRINT" umol/g"
6400 PRINT"gluconapin: ",:PRINT USING"###.##";GNAV;:PRINT" umol/g"
6500 PRINT"glucobrassicanapin: ",:PRINT USING"###.##";GBNV;:PRINT" umol/g"
6600 PRINT"4-OH glucobrassicin: ",:PRINT USING"###.##";OH4V;:PRINT" umol/g"
6700 PRINT"glucobrassicin: ",:PRINT USING"###.##";GBCV;:PRINT" umol/g"
6800 GSLV = PROV + GNLV + GNAV + GBNV + OH4V + GBCV
6900 PRINT
6950 PRINT"total GSL from total S (with var-modus)=: ",:PRINT USING"###.##";GSLV;:PRINT" umol/g"
6970 PRINT"total GSL from total S (without var-modus)=: ",:PRINT USING"###.##";GSLTOT;:PRINT" umol/g"7000
DEVV = GSLV-RFA
7100 PRINT"deviation from X-RF: ",:PRINT USING"###.##";DEVV;:PRINT" umol/g"
7200 PRINT
7300 PRINT
7400 INPUT" next S input < RETURN > or back to menu (M) or back to system (S) ",X$
7500 PRINT
7600 IF X$="M" THEN 90
7700 IF X$="S" THEN 40000
7800 GOTO 210
7900 CLS
8000 REM output without variability
8010 IF PRO < 0 THEN PRO =.01
8020 IF GNL < 0 THEN GNL =.01
8030 IF GNA < 0 THEN GNA =.01
8040 IF GBN < 0 THEN GBN =.01
8050 IF OH4 < 0 THEN OH4 =.01
8060 IF GBC < 0 THEN GBC =.01
8100 CLS
8191 PRINT: PRINT: PRINT: PRINT
8195 PRINT "E m u l a t i o n w i t h o u t v a r i a b i l i t y "
8196 PRINT
8200 PRINT"progoitrine: ",:PRINT USING"###.##";PRO;:PRINT" umol/g"
8300 PRINT"napoleiferine: ",:PRINT USING"###.##";GNL;:PRINT" umol/g"
8400 PRINT"gluconapine: ",:PRINT USING"###.##";GNA;:PRINT" umol/g"
8500 PRINT"glucobrassicanapine: ",:PRINT USING"###.##";GBN;:PRINT" umol/g"
8600 PRINT"4-OH glucobrassicine: ",:PRINT USING"###.##";OH4;:PRINT" umol/g"
8700 PRINT"glucobrassicine: ",:PRINT USING"###.##";GBC;:PRINT" umol/g"
8750 PRINT
8800 PRINT"total GSL from total S (without var-modus)=: ",:PRINT USING"###.##";GSLTOT;:PRINT" umol/g"
8900 PRINT: PRINT
9000 INPUT" next S input < RETURN > or back to menu (M) or back to system (S) ",X$
9612 PRINT
9700 IF X$="M" THEN 90
9800 IF X$="S" THEN 40000
```

```
9850 GOTO 210
9900 CLS
10000 CLS
30000 print: PRINT: PRINT: PRINT: PRINT
32094 PRINT" total GLS content in seeds (ug/g) "
32905 PRINT" according to EU HPLC reference method "
33000 INPUT" **************************************>>: ", EUGSL
34000 REM Total S calculated (linear calibration approach)
34500 Scalc=(EUGSL + 43.87)/14.99
35000 REM Routines derived from program > singel.sps < updated for BCR results
35100 PROT=((.71492* EUGSL)-4.4234)*.886 + 2.11
35200 GNLT=((.0073* EUGSL)+.46634)* 2.838–1.35
35300 GNAT=((.23856* EUGSL)-.74791)* 1.111+.023
35400 GBNT=((.04147* EUGSL)+.08421)* 1.18-.29
35500 OH4T=((.00069* EUGSL)+4.2658)* 8–31.25
35600 GBCT=((-.00293* EUGSL)+.35497)*-.179+.177
38000 REM HPLC Test Output
38010 IF PROT < 0 THEN PRO =.01
38020 IF GNLT < 0 THEN GNL =.01
38030 IF GNAT < 0 THEN GNA =.01
38040 IF GBNT < 0 THEN GBN =.01
38050 IF OH4T < 0 THEN OH4 =.01
38060 IF GBCT < 0 THEN GBC =.01
38070 TOTTEST = PROT + GNLT + GNAT + GBNT + OH4T + GBCT
38100 CLS
38191 PRINT: PRINT: PRINT: PRINT
38195 PRINT " C h e c k i n d i v i d u a l G S L"
38196 PRINT
38200 PRINT"progoitrine: ",:PRINT USING"###.##";PROT;:PRINT" umol/g"
38300 PRINT"napoleiferine: ",:PRINT USING"###.##";GNLT;:PRINT" umol/g"
38400 PRINT"gluconapine: ",:PRINT USING"###.##";GNAT;:PRINT" umol/g"
38500 PRINT"glucobrassicanapine: ",:PRINT USING"###.##";GBNT;:PRINT" umol/g"
38600 PRINT"4-OH glucobrassicine: ",:PRINT USING"###.##";OH4T;:PRINT" umol/g"
38700 PRINT"glucobrassicine: ",:PRINT USING"###.##";GBCT;:PRINT" umol/g"
38710 PRINT
38720 PRINT"Checksum: ",:PRINT USING"###.##";TOTTEST;:PRINT" umol/g"
38725 PRINT
38730 PRINT"Input EU GSL: ",:PRINT USING"###.##";EUGSL;:PRINT" umol/g"
38735 Print
38740 PRINT"Total S calculated (linear calibration approach)"
38750 PRINT"(linear calibration approach): ",:PRINT USING"###.##";SCALC;:PRINT" mg/g"
38800 PRINT
39000 INPUT" next GSL input < RETURN > or back to menu (M) or back to system (S) ",Y$
39100 Print
39700 IF Y$="M" THEN 90
39800 IF Y$="S" THEN 40000
39850 GOTO 10000
40000 SYSTEM
```

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