Rapid separation of seed glucosinolates from *Camelina sativa* by thin layer chromatography

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Abstract

A thin layer chromatography (TLC) method has been developed for the quantitative analysis of glucosinolates extracted from *Camelina sativa*. This procedure resolves the same long-chain glucosinolates as high-performance liquid chromatography but increases simultaneous sample size to 18. The TLC method saves time and solvent, and can be applied to screening of *Camelina*.

Introduction

Camelina sativa, an oilseed crop of the same family as mustard, is currently being grown throughout North America and Europe and crushed to produce biodiesel. In North America, the Great Plains area (Cincinnati, OH, USA) has produced over 10 million road miles of *Camelina* biodiesel to date, and plans to boost production to 100 million gallons by the year 2012.[1] In Europe, the EU Renewable Energy Roadmap (March 2007) set up a directive for the incorporation of a minimum of 10% biofuel by 2020 in total transport fuel use.[2] *Camelina* offers a solution to enable these biodiesel production goals to be met providing a sustainable, low-input biofuel feedstock option that does not interfere with food production. *Camelina sativa* can be harvested and crushed for oil, and the remaining parts can be used to produce high-quality omega 3 rich animal feed, flour, fiberboard and glycerin.[3] The high content of protein in these Camelina oil cakes and flours promotes their potential for use in animal feedstuffs.[3,4] However, the exploitation of *Camelina* cakes is restricted by the glucosinolate (GSL) content. The European Food Safety Authority recommends limiting the total GSL content to 1-1.5 mmol per kg of feed for monogastric animals, and to even lower concentrations in feed for young animals (Directive 2008/76/CE).[5] Instead, the US Food and Drug Administration approved inclusion in up to 10% of the weight of the total ration in the diets of beef cattle and poultry.[6] To increase the use of *Camelina* oilcakes or flour in animal diets, low glucosinolate *Camelina* varieties are required. The total GSL content of samples is usually determined via anion exchange chromatography, the subsequent desulfo-GSL release step with the degrading enzyme sulfatase (EC 3.1.6.1) and, finally, separation by high-performance liquid chromatography (HPLC).[7] This protocol is time-consuming and it is not easy to analyze large sample sizes. In the current work, we present a protocol in which HPLC separation is substituted with thin layer chromatography (TLC) which allows more analysis per time unit.

Materials and Methods

Reagents and plant material

DEAE-Sephadex A-25, sinigrin and sulfatase Type H1 were purchased from Sigma-Aldrich (Milano, Italy). All organic solvents were of analytical grade.

Camelina sativa seeds were sown in the spring at Casazza (Bergamo, Italy). Six different genotypes by origin were sown and harvested: Calena (Germany), Ligena (Germany), CAM40 (unknown), CAM172 (Russia), FF006 (Austria), FF084 (Austria).

Analytical procedures

Camelina seeds were defatted with cold acetone, GSLs were extracted with hot 70% ethanol for 3 h and the samples were then centrifuged for 15 min at 13,000 g. Five hundred µL of ethanol extract were adsorbed onto a small DEAE-Sephadex A-25 column in formate form (100 mg). The column was then washed twice with 1 mL of sodium acetate buffer (20 mM, pH 4.0). Desulfation of GSLs was obtained by 50 µL of sulfatase (500 U) at 37°C overnight. Desulfo-GSLs were eluted from the column with 1 mL of water and dried at 65°C. The samples were resuspended in ethanol before GSL analysis.

Thin layer chromatography procedure

Ethanol samples were loaded with a Linomat IV (Camag, Switzerland) on an HPTLC RP18W plate (Merck, Germany). The plate dimensions were 100×200 mm and, by applying bands and interspaces of 5 mm, it was possible to load up to 18 samples each time. The plate was developed in a horizontal HPTLC Developmental Chamber (Camag, Switzerland) and the mobile phase was acetonitrile/water (4:6, v/v; 15 mL per plate). After the run, the absorbance (229 nm) of each lane was read into a densitometer TLC Scanner II (Camag, Switzerland) using desulfo-sinigrin as standard. GSL data were quantitated by a D-2000 integrator (Hitachi-Merck, Germany). Calibration line with different concentrations of desulfo-sinigrin was calculated by the least squares regression model and t-test for significance.

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Key words: glucosinolate, thin layer chromatography, *Camelina sativa*, oilseed crop, cake.

Contributions: RRu, glucosinolate extraction, TLC, HPLC; RRe, experiment instruction, manuscript preparation.

Conflict of interests: the authors report no conflict of interests.

Acknowledgments: this work was supported by Regione Lombardia, agreement Regione/CNR, project 2 Risorse biologiche e tecnologie innovative per lo sviluppo sostenibile del sistema agro-alimentare.

Received for publication: 22 October 2010.
Revision received: 19 January 2012.
Accepted for publication: 19 January 2012.

International Journal of Plant Biology 2012; 3:e4
doi:10.4081/pb.2012.e4

High-performance liquid chromatography procedure

GSLs were determined according to the method of Kraling *et al*.[7] modified to improve the separation of *Camelina* GSLs. Desulfo-GSLs were separated by gradient HPLC and detection at 229 nm. A 150 mm × 4.6 mm Waters Spherisorb ODS-2 (3 µm) was used for separation. The mobile phase consisted of two eluents: i) water (HPLC-grade); ii) acetonitrile. The flow rate was 0.7 mL min–1. The program started with 95% A and 5% B for 2 min followed by a linear gradient over 25 min to 5% A and 95% B. This was held for 2 min before the program returned to 95% A and 5% B by a linear gradient of 1 min followed by at least 10 min equilibration.

Results

Traditional separation of GSLs by HPLC occurs with a linear acetonitrile gradient on a C18 column in approximately 25-30 min and the system is generally ready for a new run after 35-40 min. In *Camelina*, three main long-chain GSLs were identified by HPLC: 9-methyl-sulfinyl-nonyl GSL (GSL1), 10-methyl-sulfinyl-

decyl GSL (GSL2) and 11-methyl-sulfinyl-undecyl GSL (GSL3).[8,9] In this study, we used HPTLC plates which have an optimized silica 60 layer with 5-6 µm particles modified with the same phase used for HPLC separation (RP-18). Separation of GSLs from *Camelina sativa* Calena was achieved by TLC (Figure 1). The 3 main GSLs migrated on an HPTLC RP18W plate with R_f of 0.58, 0.63 and 0.68. GSL1, GSL2 and GSL3 represented 28.7%, 44.4% and 22.6% of total GSLs, respectively. Traces of other GSLs were only 4%. A similar peak distribution was observed for all the six genotypes (*data not shown*). A comparison was made between the HPLC and TLC procedures for the quantitation of total GSLs in six genotypes of *Camelina sativa* (Table 1). The content of GSLs in the six genotypes ranged from 26 to 29.4 mmol kg^{-1} of defatted flour. There was no statistical difference between those values obtained by TLC and those determined by HPLC analysis.

The linearity and precision of the TLC method were evaluated within the range of interest. The calibration curve of standard desulfo-sinigrin was obtained by plotting peak area against the different concentration (Figure 2). Linear regression was obtained within 0 to 400 nmol of desulfo-sinigrin with a regression coefficient (R^2) of 0.989 and an equation of y=8884 × (P<0.001). The limit of detection was determined empirically by testing dilutions of desulfo-sinigrin standard solutions until no peak could be observed at 229 nm; the limit was 5 nmol.

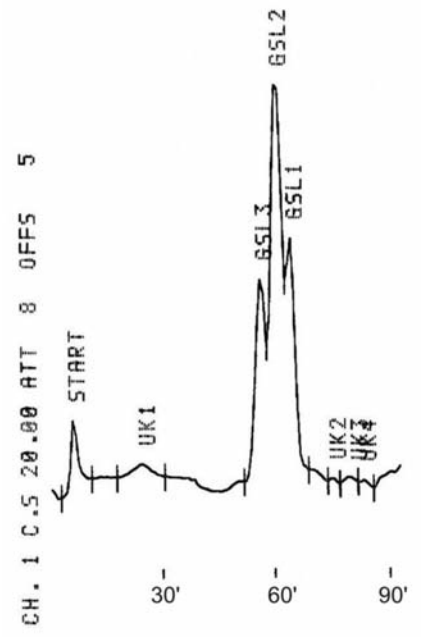

Figure 1. Chromatogram of glucosinolates of Camelina sativa Calena. GSL1, 9-methyl-sulfinyl-nonyl GSL; GSL2, 10-methyl-sulfinyl-decyl GSL; GSL3, 11-methyl-sulfinyl-undecyl GSL; UK, unknown.

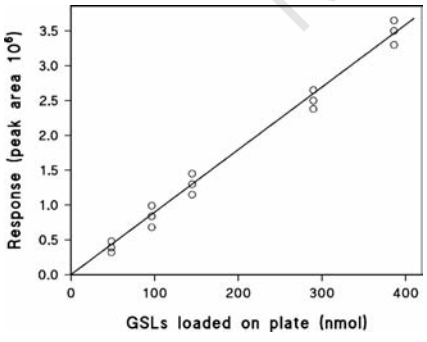

Figure 2. Calibration curve for GSLs: *y*, densitometer response (peak area); *x*, amount of desulfo-sinigrin loaded on plate (nmol). R^2=0.989.

Discussion

Camelina oil and residue of the oil-pressing process are strongly linked such that the success of the oil crop depends on the utilization of both products.[7] Since, according to European and North American guidelines,[5,6] the possibility of using *Camelina* cakes or flours is related to the content of GSLs, it is important to have a fast and reliable method to evaluate these substances. Furthermore, evaluation of genotypic variation for GSL content is considered to be of primary importance for breeding *Camelina* plants low in GSLs. We have developed a rapid and precise procedure for the screening of large plant populations. The TLC protocol allows to up to 18 samples per TLC plate every 90 min to be analyzed, whereas HPLC allows 2 samples every 90 min. This is a great saving in time and, economically significant, also in solvent. Furthermore, the TLC procedure identified the same *Camelina* long-chain GSLs as HPLC (Figure 1) and the results obtained by both methods were similar (Table 1). The TLC method was linear in the range of GSL concentrations normally observed in Camelina extracts (25-30 nmol mg^{-1} of flour). Therefore, TLC represents a fast and economic alternative to HPLC for the determination of *Camelina* GSLs. In the future, efforts will be made to breed *Camelina* genotypes low in GSLs in order to increase the use of *Camelina* cakes and flours in animal feedstuffs. In our institute, a collection of approximately 50 accessions of *Camelina* is now undergoing screening.

Table 1. Glucosinolates content (GSL1+GSL2+GSL3) in flour of six *Camelina sativa* genotypes determined by high-performance liquid chromatography and thin layer chromatography methods.

Genotype	HPLC	TLC
Calena	26.7±1.6	27.0±1.7
Ligena	29.0±1.2	29.4±1.6
CAM40	26.2±0.8	26.4±0.8
CAM172	28.2±1.0	28.5±2.8
FF006	29.2±1.1	28.4±2.2
FF084	27.3±1.0	28.7±2.1

Data are expressed as mmol kg^{-1} defatted flour ± standard deviation and are the mean of 3 independent experiments. HPLC, high-performance liquid chromatography; TLC, thin layer chromatography.

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