J Am Oil Chem Soc
DOI 10.1007/s11746-014-2574-3

ORIGINAL PAPER

A Fluorometric Enzymatic Assay for Quantification of Steryl Glucosides in Biodiesel

Andrés Aguirre · Matías Cabruja · Rodolfo Cabrera · Florencia Eberhardt · Salvador Peirú · Hugo G. Menzella · Rodolfo M. Rasia

Received: 28 June 2014 / Revised: 11 November 2014 / Accepted: 17 November 2014

Abstract Steryl glucosides (SG) are common contaminants in biodiesel that form precipitates, which form and cause problems due to fouling during transport and storage. Therefore, their quantification is necessary to assess the quality of this fuel. The methods currently available for SG analysis require expensive instrumentation, need a previous concentration step by solid-phase extraction (SPE) or are of limited use for the quantitative assessment. We developed an enzymatic method for SG quantification in biodiesel samples based on the hydrolysis of the glucoside catalyzed by a broadly specific beta glucosidase and the subsequent determination of the glucose released by the reaction. The method is non-expensive, sensitive and was adapted to 96-well format fluorescence plate reader, making it useful for the parallel assay of multiple samples. The enzymatic assay presented here represent a valuable tool for both quality control and the development of improved biodiesel production and purification procedures.

A. Aguirre and M. Cabruja have contributed equally to this work.

A. Aguirre · F. Eberhardt · S. Peirú · H. G. Menzella
Facultad de Ciencias Bioquímicas y Farmacéuticas, Genetic Engineering and Fermentation Technology, Universidad Nacional de Rosario, Conicet Suipacha 531, 2000 Rosario, República Argentina

A. Aguirre · F. Eberhardt · S. Peirú · H. G. Menzella
Keclon S.A, Tucumán 7180, 2000 Rosario, República Argentina

M. Cabruja · R. M. Rasia (✉)
Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET-UNR), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Ocampo y Esmeralda, predio CONICET, 2000 Rosario, Argentina
e-mail: rasia@ibr-conicet.gov.ar

R. Cabrera
Unitec Bio S.A, Batalla del Quebracho, s/n Pto. Gral, 2202 San Martín, República Argentina

Keywords Biodiesel · Steryl glucosides · Fluorescence · NADPH

Abbreviations

SG	Steryl glucoside(s)
ASG	Acyl steryl glucoside(s)
TLC	Thin layer chromatography
GC	Gas chromatography
HPLC	High performance liquid chromatography
MS	Mass spectrometry
SPE	Solid-phase extraction
TL	*Thermococcus litoralis* beta-glycosidase
HK	Hexokinase
G6PDH	Glucose-6-phosphate dehydrogenase
MTBE	Methyl *tert*-butyl ether
FID	Flame ionization detector

Introduction

Biodiesels are produced by the transesterification of vegetable or animal derived triacylglycerols with methanol. During the past decade the biodiesel industry has formed and grown as the product represents a promising alternative to petroleum fuels. Biodiesels can be utilized by traditional fuel-burning engines, produce fewer particulates when burnt, have a higher flash point, and are less toxic than petroleum-based fuels. However biodiesels still suffer from some problems derived from their organic origin, mainly due to the formation of precipitates at various points in the production chain and during its transportation and storage [1, 2]. Thus, the content of insoluble contaminants is a closely monitored quality parameter, since an excess of them may cause operational problems in vehicles due to fuel filter plugging by solid materials. In order to meet the

Published online: 04 December 2014

ASTM D6751 [3] and EN14214 [4] specifications, insoluble solids present in biodiesel should not exceed the specification limits of the D7501-12 [5] and EN 12662 [6] methods [1, 2].

It has been reported that the main component of precipitates in biodiesel are steryl glucosides (SG), which consist of a glucose unit glycosidically linked to a sterol [2, 7–9]. SG and acyl SG (ASG) are naturally present in plant tissues and vegetable oils, as derivatives of sterols that are the main unsaponifiable components [10]. In ASG, the 6-position of the sugar is esterified with a long chain fatty acid. Under alkaline conditions, this ester bond between the glucose and the fatty acid is broken, and the ASG are converted to SG. Such a side reaction occurs during transesterification, resulting in an increased SG concentration in biodiesel in comparison to the initial concentration present in the feedstock oil [2]. Due to the loss of the fatty acid chain, SG have an increased polarity making them less soluble than ASG, which explains the formation of precipitates [11]. As new regulations are being set on the tolerable amount of insoluble material, the accurate quantification of SG is important to assess the quality of these fuels.

Several analytical techniques have been proposed to determine both qualitatively and quantitatively SG [7, 8, 11–17]. These include thin layer chromatography (TLC) and high performance TLC (HPTLC), high temperature gas chromatography (GC), high performance liquid chromatography (HPLC) and mass spectrometry (MS). TLC, while fast and non expensive gives only qualitative or semi-quantitative results, whereas HPTLC requires a previous solid-phase extraction (SPE) and elution step and specialized equipment [17, 18]. GC and HPLC require expensive analytic instruments, and also need a previous concentration step by SPE [7, 12, 19], centrifugation [20] or via distillation [2], or alternatively to be equipped with a MS detector [14] to achieve higher sensitivity. In the present work we propose an enzymatic method for quantification of SG in biodiesel. This enzymatic method is non-expensive, its sensitivity falls well within the lowest range of currently observed SG content in the product, and can be run in a high throughput format using a multi-well spectrofluorometric reading system. We show that the results of our method strongly correlate with well-established chromatographic methods when analyzing biodiesel samples produced by local manufacturers.

Experimental Procedures

Materials

Commercial crude soybean biodiesel samples were obtained from Unitec Bio S.A. (Pto. Gral. San Martín, Argentina). Distilled soybean biodiesel was obtained from Intertek Argentina (Rosario, Argentina). A SG standard was purchased from Matreya LLC (Pleasant Gap, PA). The standard cholesteryl-glucoside, the chemicals *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, *p*-nitrophenyl-*β*-*d*-glucopyranoside (pNPG), ATP and NADP+, and the enzymes HK and G6PDH were purchased from Sigma-Aldrich, Co (St. Louis, MO).

Protein Purification

The expression and purification of the broadly specific beta-glycosidase from *Thermococcus litoralis* (TL) was carried out as described in Ref. [20]. Briefly, TL DNA sequence was synthesized by Genscript (Piscataway, USA), cloned into pET28a at NdeI/EcoRI restriction sites to obtain N-terminal fusion to a His_6 tag, and transformed into *E. coli* BL21 (DE3) strain. Cell cultures of this strain were harvested, resuspended in 50 mM phosphate buffer pH 6.8, 150 mM NaCl, and 2.5 % Triton X-100, and disrupted by sonication. After disruption, crude extracts were clarified by centrifugation and purified by affinity chromatography. *β*-Glucosidase activity was assayed as described previously [21]. The reaction mixture (500 µl) contained 465 µl of sodium citrate buffer (25 mM, pH 5.5), 25 µl of 20 mM pNPG, and 10 µl of the appropriate dilution of enzyme-containing sample. After incubation at 80 °C for 5 min, the reaction was stopped by adding 1 ml of cold 200 mM sodium carbonate. The activity of TL was estimated spectrophotometrically by reading the absorbance of the liberated *p*-nitrophenol at 405 nm ($\varepsilon = 18{,}700$). One unit (U) was defined as the amount of enzyme required for the hydrolysis of 1 µmol pNPG/min, under the assay conditions.

Fluorimetric–Enzymatic Determination of Glucose

To set up the method we followed the hexokinase/glucose-6-phosphate dehydrogenase (HK/G6PDH) reaction using different concentrations of glucose dissolved in a biodiesel water extract on a Varian Cary Eclipse spectrofluorometer (Fig. 1, 2, 3 reactions). A reaction mixture containing 0.2 units/ml of HK, 0.1 units/ml of G6PDH, 1 mM ATP, 100 µM NADP+, 5 mM $MgCl_2$ in 2 ml of 100 mM Tris-HCl buffer, pH 7.6, was prepared and the fluorescence intensity at 455 nm (excitation 335 nm) was followed for >100 s in order to obtain a reaction baseline. The glucose sample was then added and the fluorescence intensity was continuously monitored until the reading stabilized. The 96-well format measurements were carried out using a Synergy 2 Multi-Mode microplate reader (BioTek) with reagent dispenser. The calibration curve and the samples to be analyzed (5 µl) were first diluted to 65 µl with H_2O

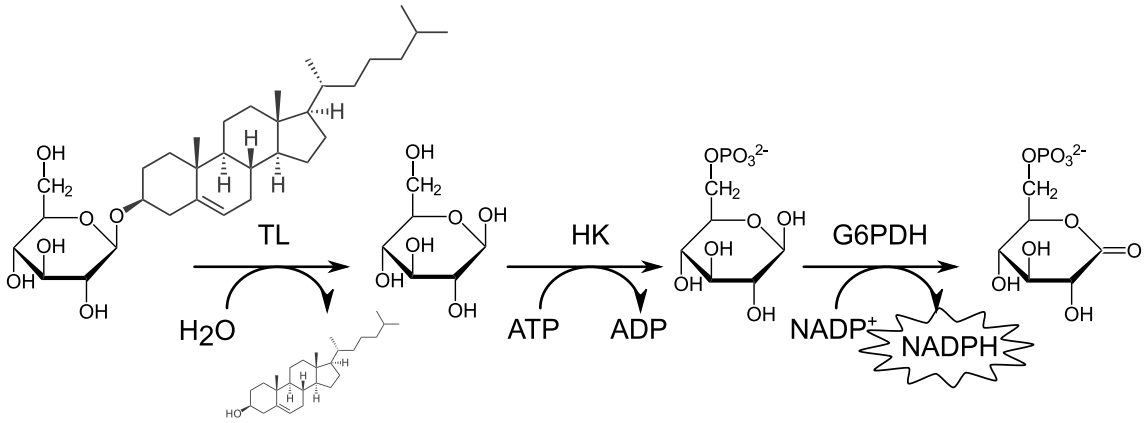

Fig. 1 Schematic representation of the method presented. Enzymes used on each step are indicated on top of the reaction *arrows*. TL, beta-glycosidase from *Thermococcus litoralis*; *HK* hexokinase (Sigma H5000). *G6PDH* glucose-6-phosphate dehydrogenase (Sigma G8529)

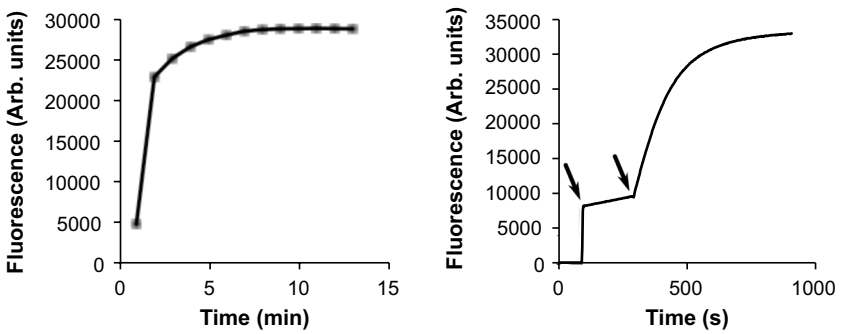

Fig. 2 Typical reaction time courses for glucose quantification by NADPH fluorescence. *Left* reaction monitored on the plate reader. *Right* reaction monitored on the spectrofluorometer. In the *right* panel, the *first arrow* indicates the point where the aqueous extract of reacted treated biodiesel is added, and the *second arrow* marks the addition of the enzymes mix. The extracts produce a background signal that has to be subtracted from the final point to obtain an accurate estimate of the glucose in the mixture

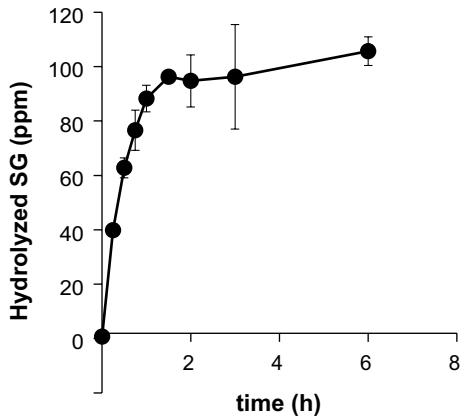

Fig. 3 Time course of the reaction of TL with SG. 100 ppm purified SG was added to distilled biodiesel and the reaction followed for 6 h using the fluorometric assay described here. *Error bars* show the standard deviation of four independent assays

on the wells. A mixture containing NADP+, ATP and the enzyme mix was then added to the wells (final volume 200 µl) using the built-in dispenser of the plate reader and the initial fluorescence was measured immediately on each well.

For quantification of SG in biodiesel an aliquot of the reaction of TL in water:biodiesel medium is centrifuged and the water phase is recovered. Then the glucose released from the SG, which is quantitatively partitioned to the water phase, is quantified using the fluorometric assay described in the previous paragraph.

Enzymatic Hydrolysis of SG in Distilled and Commercial Crude Biodiesel

A fixed amount of purified SG dissolved in 3:1 THF:H_2O was added to 10 ml distilled biodiesel free of SG. TL (10 µg

equivalent to 0, 5 units/ml of biodiesel) was then added in 1.5 ml 100 mM citrate buffer, pH 5.5, to the SG-spiked biodiesel sample and the mixture was thoroughly agitated at 400 rpm in a VP 710 magnetic tumble stirrer (V&P Scientific, San Diego, USA) at 65 °C, in order to maximize the water:biodiesel interface. The reaction was followed for 6 h, taking samples at different intervals. The same protocol was followed for the hydrolysis of SG in commercial biodiesel, except that no additional SG was added.

SPE-GC-Based SG Determination

To determine the initial SG concentration by SPE-GC, aliquots of medium containing 2 g of biodiesel were spiked with 50 ppm of cholesteryl glucoside as internal standard and centrifuged to eliminate water. The biodiesel phase was transferred to a new tube, and residual SG from the interface was extracted three times with 200 µl ethyl acetate, mixed with the biodiesel phase and evaporated to dryness under vacuum. SPE cartridges (Sep-Pak Vac Silica 6 cc, Waters, Milford, MA, USA) were conditioned with 2 ml of hexane, and samples were then quantitatively transferred. Adsorbed SG (including internal standard) was washed with 4 × 3 ml methyl *tert*-butyl ether (MTBE), and eluted with 4 × 3 ml MTBE:acetone 1:1. The eluted fractions were mixed and evaporated to dryness at 50 °C under nitrogen flux. Dried samples were redissolved with 300 µL pyridine and derivatized with 300 µL *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Sigma M7891). The vials were hermetically sealed and incubated at 70 °C for 3 h. A 100-µl aliquot was diluted with 1 mL *n*-heptane, and then a 1 µl of the mixture was automatically injected into an Agilent 7890A gas chromatograph equipped with a temperature programmable capillary injector and a flame ionization detector (FID). Peak separation was achieved with a capillary column, 15 m long, 0.32 mm internal diameter, and film thickness of 0.1 µm, with a 95 % dimethyl-5 % diphenyl-polysiloxane stationary phase (DB5-HT, J &W Scientific Folsom, CA, USA). The oven temperature program was: 50 °C (1 min), 15 °C/min to 180 °C, 7 °C/min to 230 °C, and 10 °C/min to 370 °C. The final temperature was maintained for 15 min. The FID temperature was set to 370 °C and the hydrogen pressure was fixed at 80 kPa. The carrier gas was hydrogen with pressure fixed at 80 kPa (column flow rate 113 ml/min). SG quantification was based on internal standard (cholesteryl glucoside) and a ratio of 1:100 or higher in the peak areas was required.

Results and Discussion

The present method is based on the use of a thermophilic beta-glycosidase [22, 23] followed by glucose quantification. We recently cloned, purified, and characterized TL, a broadly specific beta-glycosidase from TL. The enzyme catalyses the hydrolysis of the beta-glycosidic bond between a carbohydrate and a non-carbohydrate moiety and has a broad specificity for the aglycone part of the substrate [24]. Thus, the enzyme is well suited to carry out the release of glucose from SG for the current purpose. Quantification of SG is performed in two steps. First, the sample is treated with TL in a heterogeneous water:biodiesel medium. After reaction is complete, an aliquot is centrifuged and the lower phase recovered. Next, the glucose released from the SG, which was quantitatively partitioned to the water phase, is quantified through a fluorometric-enzymatic assay (Fig. 1).

We initially sought to set up a sensitive method to measure the glucose released by the reaction of TL with SG. The traditional colorimetric method based on glucose oxidase is not sensitive enough for the range of SG expected in biodiesel samples (4–275 ppm), and we further found interference from unknown components in the water extract that led to irreproducible results [25]. Therefore we resorted to use the coupled reaction of HK with G6PDH that we found to be insensitive to the aforementioned interferences present in the biodiesel water extract [25]. In this reaction, one mole of NADPH is produced per mole of glucose in the sample, and the quantification can be obtained directly by measuring the absorbance of NADPH at 340 nm. Unfortunately, for samples with SG content below 10 ppm (part per million) the expected increase in absorbance would be ~0.09, a value that can be hardly used to obtain an accurate quantification. This problem may be a more serious one when using a microplate reading assay, where the optical path is usually shorter than 1 cm. We therefore decided to measure the fluorescence of NADPH at 460 nm upon excitation at 360 nm, which provides a more sensitive measurement of the extent of reaction.

Fluorescence measurements typically provide higher sensitivity, but are not absolute and require an adequate calibration. To set up the method we followed the reaction of different concentrations of glucose dissolved in a biodiesel water extract on a spectrofluorometer. First a reaction mixture was prepared and the fluorescence intensity at 455 nm (excitation 335 nm) was followed for >100 s in order to obtain a reaction baseline. The glucose sample was then added and the fluorescence intensity was monitored in time until the reading stabilized (Fig. 2). Repeated experiments with different samples showed that the aqueous extract of biodiesel gives variable baseline fluorescence values, indicating that a direct reading of a final point will not be accurate. In order to circumvent this problem, we followed the time course of NADPH production in each reaction. The difference in fluorescence values

between the starting point and the end of the reaction was proportional to the amount of glucose present in the sample. A calibration curve could be obtained by adding known amounts of glucose to an aqueous extract of biodiesel, and following the reaction time course in each case as previously explained.

We next determined the time required to obtain a complete hydrolysis of SG by TL in the heterogeneous medium. The reaction as described in methods section was followed for 6 h, taking samples at different intervals (Fig. 3). Measurement of glucose in the aqueous phase reaches a maximum at 2 h after starting the reaction. Complete hydrolysis of SG extracted from foods was reported recently by Münger and Nyström using inulinase preparations [26]. The enzymatic hydrolysis is used in their work as a previous step to GC-FID in order to accurately determine the sterol profile of SG. Here, in contrast, we use the total amount of glucose released by the enzymatic hydrolysis to quantify the total amount of SG. In addition, to best of our knowledge, this is the first time that the complete enzymatic hydrolysis of SG is accomplished directly from biodiesel samples.

Finally we optimized the assay to measure glucose released from SG in a plate reader. We used a microplate reader with a reagent dispenser module to obtain multiple simultaneous readings and a glucose calibration curve on the same plate in one step. The calibration curve and the samples were aliquoted on the 96-well plates. Next, a mixture containing the reaction mix was added to the wells using the built-in dispenser of the plate reader and the initial fluorescence was then measured immediately on each well. The time course was followed for 15 min, long enough to guarantee the completion of the reaction. The glucose concentration (and the corresponding SG amount) in each sample was then evaluated from the difference in the fluorescence values corresponding to the initial and the final point of the reaction (Fig. 2).

The dose–response curve for the developed method was validated on biodiesel samples supplemented with variable amounts of SG (Fig. 4). Enzymatic measurements were performed on four independent samples and the response was linear over the whole range assayed, up to 100 ppm SG. The lowest limit of quantification for the assay, estimated as five times the standard deviation of the negative control values, is 3 ppm SG.

We finally compared the performance of our enzymatic assay with the well-established SPE-GC-based method [12, 13]. For this purpose, parallel measurements of SG by SPE-GC and by our enzymatic method in four samples (distilled biodiesel, distilled biodiesel spiked with 75 ppm purified SG and two biodiesel samples from local manufacturers) were carried out. The enzymatic assay was performed as described above in the heterogeneous water:biodiesel

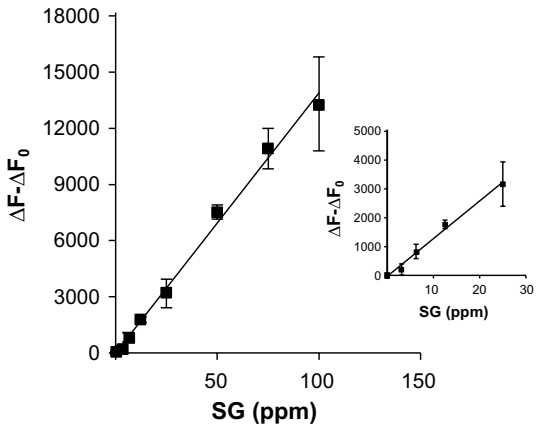

Fig. 4 Dose-response curve of the enzymatic assay. Fixed amounts of purified SG were added to distilled biodiesel, and the SG concentration was measured using the enzymatic assay. Fluorescence is expressed in arbitrary units. The change in fluorescence observed for negative control samples (ΔF0) was subtracted from the change in fluorescence observed on samples containing SG (ΔF). The inset shows an expansion of the first five points. *Error bars* show the standard deviation of four measurements

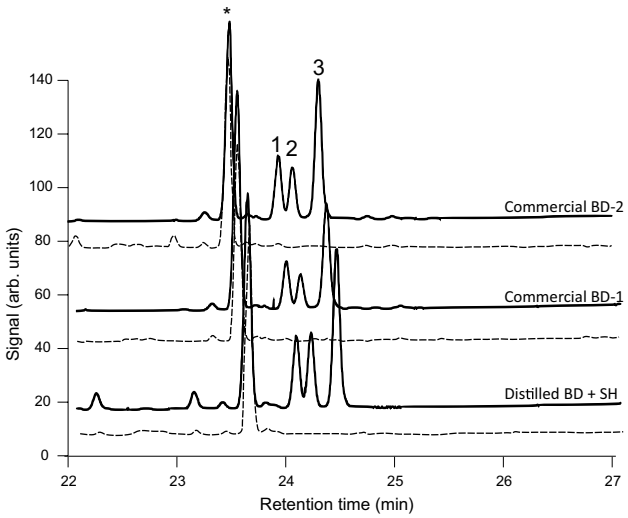

Fig. 5 GC-FID analysis of treated biodiesel samples. From bottom to top, distilled biodiesel spiked with SG, commercial crude biodiesel sample 1 and commercial crude biodiesel sample 2. For each experiment, the *full trace* corresponds to the untreated sample and the *dashed trace* to the sample treated with TL after 6 h hydrolysis. Traces are shifted on both axes for clarity. Peaks are labeled as follows: *Asterisk* cholesteryl glucoside standard; *1* campesteryl glucoside; *2* stigmasteryl glucoside; *3* beta-sitosteryl glucoside. In all samples TL hydrolyses completely all steryl glycosides present

medium. The SPE-GC assay was performed on biodiesel samples before and after treatment with TL, to evaluate if TL was capable of hydrolyzing the different classes of SG present in the samples (Fig. 5). No signal was observed in the chromatograms for any of the three major SG present in

Table 1 Validation against SPE-GC-FID Comparison of SG quantifications (in ppm) obtained using the present method and by SPE-GC-FID. Standard deviations were calculated on four independent samples

Sample	Enzymatic	SPE-GC-FID
Dist. biodiesel	0 ± 0	0 ± 1
Dist. biodiesel + 75 ppm SG	76 ± 2	72 ± 6
Commercial sample 1	52 ± 2	45 ± 3
Commercial sample 2	72 ± 3	69 ± 6

the samples, namely campesteryl-glucoside, stigmasteryl-glucoside and beta-sitosteryl-glucoside, showing that the amount of glucose released corresponds indeed to the total amount of SG present in the samples.

For comparison with the data obtained from the enzymatic assay, the outcome of the GC assay was converted into the equivalent SG concentrations in ppm (Table 1). The enzymatic method consistently gives higher values than the SPE-GC method for all samples. However, the method presented here estimates more accurately the amount of SG present in the spiked sample, containing a known amount of SG, suggesting that SPE-GC slightly underestimates the amount of SG in the samples. Despite these differences, the results of both measurements were in the same range, thus showing that our enzymatic method is reliable and can represent a simpler alternative to the standard assay.

Conclusions

We have described here a novel enzymatic assay to quantify SG in biodiesel samples. The assay is sensitive and reproducible and it can be performed using low-cost instrumentation, in contrast to the GC/HPLC/MS analyses. We show that the assay functions in complex heterogeneous matrixes, as commercial crude biodiesel. The assay can be set up using a standard fluorometer but can also be performed in a plate reader, making it suitable for a high-throughput format. Using the plate reader setup the SG content of 96 samples can be quantified in only 3–5 h, the time required for TL to fully hydrolyze the substrates. The enzymatic assay presented here will add a new tool to those available for quality control of biodiesel samples, and will be helpful in the development of improved biodiesel production and purification procedures as well.

Acknowledgments The authors wish to thank Guillermo Labadie and Sebastián Jäger for their advice on the SPE-GC-FID technique and for providing SG and cholesteryl-glucoside standards. This work was funded by Keclon SA and grants from Agencia Nacional de Promocion Científica y Tecnológica PICT 2010-1157 and PICT 2008-318.

Conflict of interest The authors declare that they have no competing interests.

References

1. Pfalzgraf L, Lee I, Foster J, Poppe G (2007) The effect of minor components on cloud point and filterability. Inf Suppl Biorenewable Resour 4:17–21
2. Hoed V, Zyaykina N, Greyt W et al (2008) Identification and occurrence of steryl glucosides in palm and Soy biodiesel. J Am Oil Chem Soc 85:701–709. doi:10.1007/s11746-008-1263-5
3. American Society for Testing and Materials (ASTM) (2014) D6751-14, Standard specification for biodiesel fuel blend stock (B100) for middle distillate fuels. ASTM International, West Conshohocken, http://www.astm.org
4. European Committee for Standardization(CEN) EN 14214:2012 + A1:2014/AC:2014 Liquid petroleum products: fatty acid methyl esters (FAME) for use in diesel engines and heating applications—requirements and test methods. CEN, Brussels http://www.cen.eu
5. American Society for Testing and Materials (ASTM) (2012) D7501-12a, Standard test method for determination of fuel filter blocking potential of biodiesel (B100) Blend stock by cold soak filtration test (CSFT), ASTM International, West Conshohockenhttp://www.astm.org
6. European Committee for Standardization (CEN) (2008) EN 12662:2014. Liquid petroleum products—determination of total contamination in middle distillates, diesel fuels and fatty acid methyl esters; CEN, Brusselshttp://www.cen.eu
7. Bondioli P, Cortesi N, Mariani C (2008) Identification and quantification of steryl glucosides in biodiesel. Eur J Lipid Sci Technol 110:120–126. doi:10.1002/ejlt.200700158
8. Moreau R, Scott KM, Haas MJ (2008) The identification and quantification of steryl glucosides in precipitates from commercial biodiesel. J Am Oil Chem Soc 85:761–770. doi:10.1007/s11746-008-1264-4
9. Lee I, Pfalzgraf L, Poppe G et al (2007) The role of sterol glucosides on filter plugging. Biodiesel Mag 4:105–112
10. Sugawara T, Miyazawa T (1999) Separation and determination of glycolipids from edible plant sources by high-performance liquid chromatography and evaporative light-scattering detection. Lipids 34:1231–1237
11. Lacoste F, Dejean F, Griffon H, Rouquette C (2009) Quantification of free and esterified steryl glucosides in vegetable oils and biodiesel. Eur J Lipid Sci Technol 111:822–828
12. Gómez-coca RB, Pérez-camino MC, Moreda W (2013) On the glucoside analysis : simultaneous determination of free and esterified steryl glucosides in olive oil. Detailed analysis of standards as compulsory first step. Food Chem 141:1273–1280. doi:10.1016/j.foodchem.2013.03.080
13. Nyström L, Schär A, Lampi A-M (2012) Steryl glycosides and acylated steryl glycosides in plant foods reflect unique sterol patterns. Eur J Lipid Sci Technol 114:656–669. doi:10.1002/ejlt.201200033
14. Pieber B, Schober S, Goebl C, Mittelbach M (2010) Novel sensitive determination of steryl glycosides in biodiesel by gas chromatography-mass spectroscopy. J Chromatogr A 1217:6555–6561
15. Wewer V, Dombrink I, vom Dorp K, Dörmann P (2011) Quantification of sterol lipids in plants by quadrupole time-of-flight mass spectrometry. J Lipid Res 52:1039–1054. doi:10.1194/jlr.D013987
16. Schrick K, Sunitha S, Arpin JC et al (2012) Steryl glucoside and acyl steryl glucoside analysis of Arabidopsis seeds

by electrospray ionization tandem mass spectrometry. Lipids 47:185–193. doi:10.1007/s11745-011-3602-9.Steryl
17. Haupt J, Brankatschk G, Wilharm T (2009) Sterol glucoside content in vegetable oils as a risk for the production of biodiesel—Study of the technological chain impact. Arbeitsgemeinschaft Qual. Biodiesel e. V
18. Lepage M (1964) Isolation and characterization of an esterified form of steryl glucoside. J Lipid Res 5:587–592
19. Philips KM, Ruggio DM, Ashraf-Khorassani M (2005) Analysis of steryl glucosides in foods and dietary supplements by solid-phase extraction and gas chromatography. J Food Lipids 12:124–140
20. Wang H, Tang H, Salley S, Ng KYS (2010) Analysis of sterol glycosides in biodiesel and biodiesel precipitates. J Am Oil Chem Soc 87:215–221. doi:10.1007/s11746-009-1489-x
21. Aguirre A, Peiru S, Eberhardt F et al (2014) Enzymatic hydrolysis of steryl glucosides, major contaminants of vegetable oil-derived biodiesel. Appl Microbiol Biotechnol 98:4033–4040. doi:10.1007/s00253-013-5345-4
22. Moracci M, Ciaramella M, Nucci R et al (1994) Thermostable beta-glucosidase from *Sulfolobus solfataricus*. Biocatalysis 11:89–103
23. Henrissat B, Bairoch A (1996) Updating the sequence-based classification of glycosyl hydrolases. Biochem J 316:695–696
24. Menzella H, Peirú S, Vetcher L (2013) Enzymatic removal of steryl glycosides
25. Okuda J, Miwa I (1973) Newer developments in enzymic determination of D-glucose and its anomers. Methods Biochem. Wiley, pp 155–190
26. Münger LH, Nyström L (2014) Enzymatic hydrolysis of steryl glycosides for their analysis in foods. Food Chem 163:202–211. doi:10.1016/j.foodchem.2014.04.082