§Development of a Solid Phase Array Assay for the Screening of Galactose Oxidase Activity and for Fast Identification of Inhibitors

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Abstract: Galactose oxidase catalyses the highly selective oxidation of terminal galactosides on a wide range of natural glycoconjugates and has found wide applications in biotechnology, particularly in biocatalysis. Here we show that the enzyme is able to oxidise solid gold-SAM surfaces functionalised with galactose. This array platform allows for the fast identification of galactose oxidase substrates and inhibitors from a library of deoxy-fluorinated sugars using MALDI-Tof spectrometry as a label–free read-out method. In addition, the enzymatic reaction enables for the *in situ* activation of sugar-coated surfaces to bioorthogonal aldehydes, which can be used for subsequent chemical modifications.

Keywords: biooxidation, galactose oxidase, biocatalysis, glycan arrays, bioorthogonal chemistry, surface functionalisation, inhibitors, fluorinated sugars

INTRODUCTION

Over the last decade, the interest in biocatalysts and their industrial application has increased dramatically.[1] With the advent of directed evolution as a tool to generate mutants of enzymes with high exploitation potential,[2-4] the need for powerful screening tools has become evident. Particularly attractive are miniaturised screening methods on solid surfaces, which can be used in a microarray format.[5] For enzymes, such technologies require a good understanding of the effects that attachment of substrates has on enzymes activity, making use of model systems.[6]

Galactose oxidase (GOase; EC.1.1.3.9) has been widely exploited in a variety of chemical syntheses,[7-9] analytical applications[10-11] and in the industry.[12] Also, the application in a P450 monooxygenase high throughput screening assay has recently been demonstrated.[13] GOase is copper dependent and uses oxygen to oxidise the C6-primary alcohol of galactose and produces hydrogen peroxide. The enzyme activity can be conveniently assessed by a colorimetric assay.[11] This assay employs horseradish peroxidase (HRP), which oxidises 2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) in the presence of hydrogen peroxide. In a search for GOase inhibitors, molecules similar to galactose are typically screened. However if the molecule bears a primary or secondary alcohol, it could in principle also be a substrate for GOase. In such case, the colorimetric assay is not able to distinguish between substrate and inhibitor, since oxidation of both molecules would result in the formation of hydrogen peroxide (Figure 1A) and hence give a positive signal. Alternative assays are therefore required.

In this paper, we describe the immobilisation of the natural substrate of the enzyme – D-galactose – onto a gold surface functionalised by self-assembled monolayers (SAMs,). A GOase solution (one drop on each spot) is then put in contact with this surface for a certain period of time and then washed away. The action of GOase on the immobilised galactose can then be quantified by a dedicated surface analysis (MALDI-ToF MS, *vide infra*). For inhibitor studies, the enzyme is incubated together with the inhibitor (Fig. 1b). The same analytic method can be used to measure how the enzyme was inhibited in its action toward immobilised galactose. Thus the analysis is not relying on the formation of H_2O_2 and as the measurement only detects conversion of galactose, the inhibition potency of any molecule can be very rapidly determined.

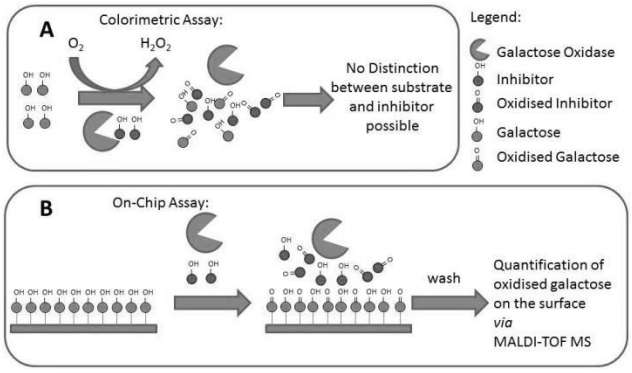


Figure 1. Comparing the colorimetric assay and the on-chip assay for the study of GOase inhibitors/competitors. Competitors having structural similarities with galactose can in principle also be oxidised and yield H_2O_2. The colorimetric assay can thus not distinguish the action of GOase on galactose and on the competitor. In the on-chip assay, the action of GOase on immobilised galactose is specifically probed.

RESULTS AND DISCUSSION

In recent years, our laboratory and others have developed a microarray system, which allows the direct analysis of covalently attached molecules by MALDI-ToF MS and has proven very valuable to study enzyme reactions.[14-15] Self-assembled monolayers (SAMs) were formed on gold surfaces. These SAMs were produced by alkyl thiols which were omega-functionalised with hexaethylene glycol (OEG_6) carboxylic acid.[15] The carboxylic acid was then used to couple aminoethyl galactose[16] to the surface after the activation by *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).[6] The galactose functionalised surface **1** was then treated with one drop (1 µL) of a solution containing the wild type galactose oxidase (0.125 µM at 30 °C, 2 h, 0.1 M phosphate buffer, pH 7). After the reaction, the surface was cleaned and dried. MALDI-ToF MS analysis revealed the oxidation of the galactose on the surface to the aldehyde **2** and the carboxylic acid **3** (Figure 2).

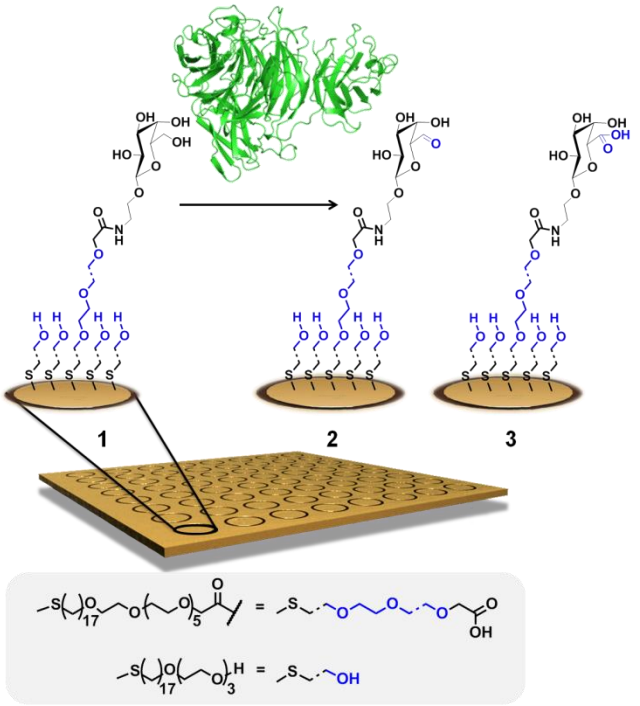


Figure 2. Functionalisation of gold surfaces with galactose and oxidation of galactose by GOase.

To study the activity and hence the feasibility of the system, the GOase reaction was left for different times from 0 to 20 hours and the product formation was quantified by MALDI-ToF MS (Figure 3). The apparent conversion from unmodified galactose **1** to the products **2** + **3** was determined by dividing the peak area of the product by the peak area of reactant and product (**2** + **3**) / (**1** + **2** + **3**).[17] The results showed that our method is suitable for the quantification of enzyme activity on gold surface, and allow us to follow the activity over time (Figure 3). Interestingly, we also used it to analyse the effect of other reaction parameters. We were able to observe the positive effect of adding catalase in the reaction medium and the negative effect of adding H_2O_2.

With a working system in hand, we turned our attention towards the applicability to screen for enzyme inhibitors. Withers *et al.* have reported that fluorinated monosaccharides showed a higher binding to glycogen phosphorylase.[18] Some fluorinated galactose derivatives have previously been shown to be substrates for GOase.[19]2,3-dideoxy-2,2,3,3-tetrafluorogalactose **12** showed an almost nine times higher K_m value than galactose. Therefore we used a set of nine different fluorinated galactose and glucose sugars to screen for potential inhibitors.

First, all fluorinated sugar derivatives were subjected to oxidation by GOase in the standard colorimetric assay (Table 1). The activity for each substrate is expressed relative to the activity of GOase with its natural substrate (Gal) as the reference (set to 100%). Table 1 shows that 2-F-Gal **16** is turned over as fast as Gal. The 3-F-Gal **15** and 4-F-Gal **14** yielded a lower relative activity of 41 and 22%, respectively. A very small activity was measured with 6-F-Gal **10**, attributed to Gal impurities in the commercial sample. As previously reported[19] the heavily fluorinated tetra-F-Gal **12**[20-21] is accepted as a substrate by the enzyme, with a relative activity of 5.5%. The two different anomers α and β *O*-methyl tetra-F-Gal **11** and **13**,[22]

respectively, showed relative activities of 2.2 and 7.5%. As expected from previous studies, GOase showed no activity towards glucose and fluorinated glucose derivatives.

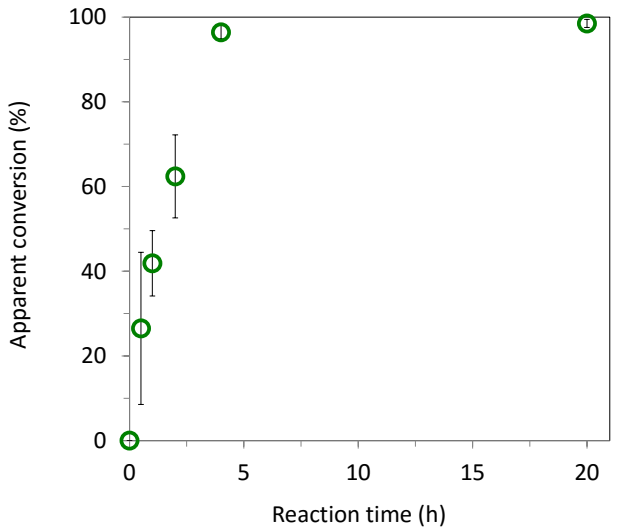


Figure 3. Conversion of immobilised Gal with reaction time (GOase (125 nM) at 30°C).

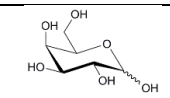
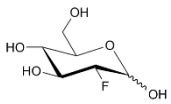
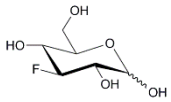
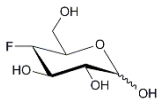
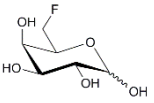
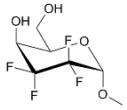
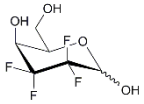
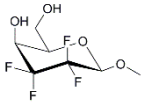
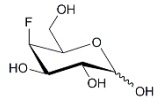
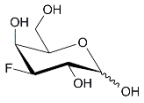
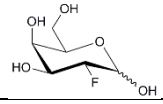
GOase activity for Gal oxidation was then measured by the microarray method in the presence of the various potential inhibitors in the liquid phase (Table 1). With this setup, we are able to measure how the activity of the enzyme towards immobilised Gal is affected by the presence of the fluorinated substrates. The latter could be a true inhibitor (occupying or altering the enzyme without being oxidised) or a substrate (occupying or altering the enzyme but being oxidised by the enzyme). Via the microarray technique, series of potential competitors could be screened rapidly using only very small amounts of enzyme (0.125 pmol per spot) and competitors (0.0125 mmol per spot).

Table 1 shows the relative inhibition provoked by a 62.5 mM concentration of the different competitors on the activity of GOase towards immobilised Gal. Glucose and glucose derivatives did not affect the enzyme at all. This confirms that these sugars obviously do not interact with the enzyme. Galactose and galactose derivatives **16**, **15** and **14** decrease the conversion of attached galactose. Interestingly, the α and the β forms of *O*-methyl tetra F-Gal **11** and **13**, respectively, do not affect the enzyme surface activity to the same extent An inhibition effect is observed with 6-F-Gal **10**. It was verified that this effect cannot be caused by the small (~1%) contamination of 6-F-Gal **10** by Gal because these small Gal amounts (~0.6 mM) only show a 2% relative inhibition effect.

This inhibition effect suggests that the 6-F-Gal **10** does interact with the enzyme. To further study the inhibition effect and to investigate its binding region, 6-F-Gal **10** could be used as a probe for protein crystallisation experiments.

One can also perform the surface reaction in the presence of various concentrations of the competitor. This has been done with 3-F-Gal (**15**).

Table 1 Comparison of the initial activity of GOase towards various substrates and the inhibition by these substrates. Initial activity was measured in the liquid phase assay with a substrate concentration of 62.5 mM and GOase concentration of 5 nM. It is expressed in comparison to the activity towards Gal (set arbitrarily to 100%).

	Substrate	Relative initial activity in solution (%) [a]	Relative inhibition effect on the chip (%)[b]
6		100.0[a]	99 ± 1
7		0.0 ± 0.0	-10 ± 5
8		0.0 ± 0.0	-6 ± 8
9		0.0 ± 0.0	-8 ± 8
10		1.8 ± 0.1	75 ± 10
11		2.7 ± 0.1	20 ± 9
12		5.5 ± 0.9	67 ± 6
13		7.5 ± 0.4	65 ± 12
14		22 ± 1.5	43 ± 11
15		41 ± 2	94 ± 3
16		108± 8	99 ± 1

[a] Gal as the natural substrate was set to 100% and all other reactivities relative to it. Results of triplicate measurements.

[b] The inhibition effect is at a substrate concentration of 62.5 mM was measured on the gold surfaces and is defined as the reduction of activity provoked by the presence of the inhibitor (expressed in % as compared to the activity observed when GOase is incubated alone). Results of quadruplicate measurements.

By measuring the apparent conversion at different competitor concentrations, one can determine an apparent IC_{50} value for the surface reaction (Figure 4). The latter would be defined as the concentration at which the inhibitor/substrate shows a decrease of the enzyme activity of 50%. Our results showed an apparent IC_{50} of 32 mM for **15**. It is here suggested that such quantitative analysis might

be used in further systematic inhibitor screenings to rank the tested compounds based on their apparent IC_{50}.

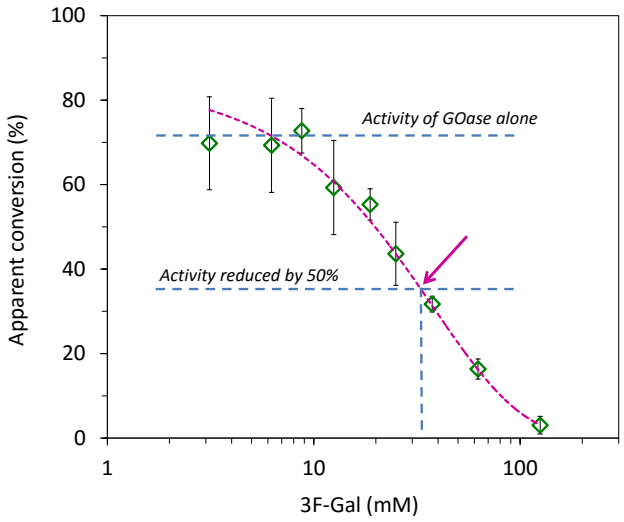


Figure 4 Apparent conversion of the immobilised Gal in 2 hours at 30°C with GOase (125 nM) and in the presence of increasing concentration of 3-F-Gal. The arrow represents the point where the activity of GOase towards immobilised Gal is reduced by 50% as compared to the case where GOase in incubated alone. This can be formalised as the "apparent IC_{50}" for 3-F-Gal.

Finally, MALDI TOF MS data showed that immobilised galactose **1** was oxidised by GOase, yielding not only the aldehyde **2** but also the corresponding carboxylic acid **3**. This finding is of interest because the carboxylic functions could be further used to functionalise the surface. Functionalisation of immobilised carbohydrates or polysaccharides is of high interest for different biotechnological applications.[23] Here, we show that GOase can be used as a biocompatible catalyst to generate functionalised chip surfaces. To demonstrate this concept, the chip was treated with the enzyme and then with the activating agent 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) in MeOH. Analysis by MALDI-ToF MS showed the conversion of the carboxylic acid **3** to the methyl ester **3-OMe**. With these results in hand, the coupling of amines onto the carboxylic acids **3** was investigated. The enzyme-treated surface **3** was thus activated with NHS and EDC and subsequently subjected to amino ethanol. As expected, the formation of amide **4** was observed (Figure 5). Simultaneously, the aldehyde **2** reacted with the amine to form the corresponding imine. Thus, the action of an enzyme towards immobilised surface could be used to functionalise the surface and such surface modifications can be monitored by MALDI TOF MS.

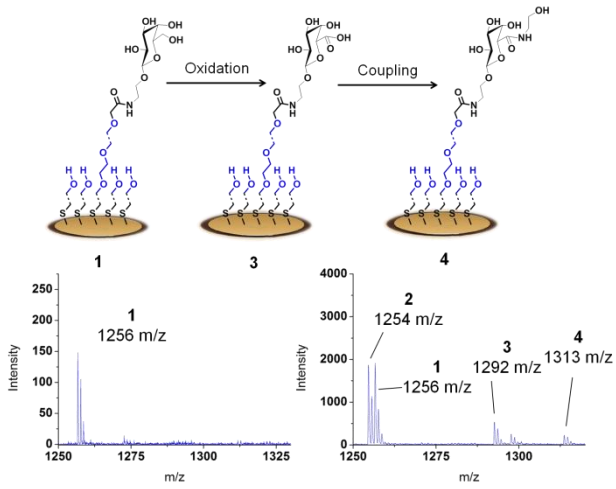


Figure 5 Enzymatic oxidation of attached galactose moieties followed by a chemical functionalisation of the carboxylic acid

CONCLUSION

We demonstrate that we can use galactose oxidase for selective oxidations of galactose-functionalised surfaces. Experiments can be carried out in a miniaturised mode using very little amounts of substrate and enzyme. Moreover, the measurement of competition or inhibition is facilitated by the possibility to measure the activity of the enzyme towards only one substrate in systems where more than one substrate is present. We also showed that GOase can be employed for the functionalisation of array surfaces and provide the possibility for orthogonal coupling of different ligands using both the aldehyde or carboxylic acid functionalities.

REFERENCES

1. Bornscheuer, U. T.; Huisman, G. W.; Kazlauskas, R. J.; Lutz, S.; Moore, J. C.; Robins, K., Engineering the third wave of biocatalysis. *Nature* **2012,** *485* (7397), 185-194.
2. Stemmer, W. P. C., Rapid Evolution of a Protein in-Vitro by DNA Shuffling. *Nature* **1994,** *370* (6488), 389-391.
3. Bougioukou, D. J.; Kille, S.; Taglieber, A.; Reetz, M. T., Directed Evolution of an Enantioselective Enoate-Reductase: Testing the Utility of Iterative Saturation Mutagenesis. *Adv. Synth. Catal.* **2009,** *351* (18), 3287-3305.
4. Reetz, M. T.; Wang, L. W.; Bocola, M., Directed evolution of enantioselective enzymes: Iterative cycles of CASTing for probing protein-sequence space. *Angew. Chem. Int. Ed.* **2006,** *45* (8), 1236-1241.
5. Gray, C. J.; Weissenborn, M. J.; Eyers, C. E.; Flitsch, S. L., Enzymatic reactions on immobilised substrates. *Chem. Soc. Rev.* **2013,** *42* (15), 6378-6405.
6. Sardzik, R.; Green, A. P.; Laurent, N.; Both, P.; Fontana, C.; Voglmeir, J.; Weissenborn, M. J.; Haddoub, R.; Grassi, P.; Haslam, S. M.; Widmalm, G.; Flitsch, S. L., Chemoenzymatic Synthesis of O-Mannosylpeptides in Solution and on Solid Phase. *J. Am. Chem. Soc.* **2012,** *134* (10), 4521-4524.
7. Siebum, A.; van Wijk, A.; Schoevaart, R.; Kieboom, T., Galactose oxidase and alcohol oxidase: Scope and limitations for the enzymatic synthesis of aldehydes. *J. Mol. Catal. B: Enzym.* **2006,** *41* (3-4), 141-145.
8. Butler, T.; Schumacher, T.; Namdjou, D. J.; Gallego, R. G.; Clausen, H.; Elling, L., Chemoenzymatic synthesis of biotinylated nucleotide sugars as substrates for glycosyltransferases. *Chembiochem* **2001,** *2* (12), 884-894.

9. Staniland, S.; Yuan, B.; Gimenez-Agullo, N.; Marcelli, T.; Willies, S. C.; Grainger, D. M.; Turner, N. J.; Clayden, J., Enzymatic Desymmetrising Redox Reactions for the Asymmetric Synthesis of Biaryl Atropisomers. *Chemistry-a European Journal* **2014,** *20* (41), 13084-13088.
10. Rannes, J. B.; Ioannou, A.; Willies, S. C.; Grogan, G.; Behrens, C.; Flitsch, S. L.; Turner, N. J., Glycoprotein Labeling Using Engineered Variants of Galactose Oxidase Obtained by Directed Evolution. *J. Am. Chem. Soc.* **2011,** *133* (22), 8436-8439.
11. Escalettes, F.; Turner, N. J., Directed evolution of galactose oxidase: Generation of enantioselective secondary alcohol oxidases. *Chembiochem* **2008,** *9* (6), 857-860.
12. Delagrave, S.; Murphy, D. J.; Pruss, J. L. R.; Maffia, A. M.; Marrs, B. L.; Bylina, E. J.; Coleman, W. J.; Grek, C. L.; Dilworth, M. R.; Yang, M. M.; Youvan, D. C., Application of a very high-throughput digital imaging screen to evolve the enzyme galactose oxidase. *Protein Eng.* **2001,** *14* (4), 261-267.
13. Weissenborn, M. J.; Notonier, S.; Lang, S. L.; Otte, K. B.; Herter, S.; Turner, N. J.; Flitsch, S. L.; Hauer, B., Whole-cell microtiter plate screening assay for terminal hydroxylation of fatty acids by P450s. *Chem. Commun.* **2016,** *52* (36), 6158-6161.
14. Weissenborn, M. J.; Wehner, J. W.; Gray, C. J.; Sardzik, R.; Eyers, C. E.; Lindhorst, T. K.; Flitsch, S. L., Formation of carbohydrate-functionalised polystyrene and glass slides and their analysis by MALDI-TOF MS. *Beilstein Journal of Organic Chemistry* **2012,** *8*, 753-762.
15. Weissenborn, M. J.; Castangia, R.; Wehner, J. W.; Sardzik, R.; Lindhorst, T. K.; Flitsch, S. L., Oxo-ester mediated native chemical ligation on microarrays: an efficient and chemoselective coupling methodology. *Chem. Commun.* **2012,** *48* (37), 4444-4446.
16. Sardzik, R.; Noble, G. T.; Weissenborn, M. J.; Martin, A.; Webb, S. J.; Flitsch, S. L., Preparation of aminoethyl glycosides for glycoconjugation. *Beilstein Journal of Organic Chemistry* **2010,** *6*, 699-703.
17. Min, D. H.; Su, J.; Mrksich, M., Profiling kinase activities by using a peptide chip and mass spectrometry. *Angewandte Chemie-International Edition* **2004,** *43* (44), 5973-5977.
18. Street, I. P.; Armstrong, C. R.; Withers, S. G., Hydrogen-Bonding and Specificity - Fluorodeoxy Sugars as Probes of Hydrogen-Bonding in the Glycogen-Phosphorylase Glucose Complex. *Biochemistry* **1986,** *25* (20), 6021-6027.
19. Ioannou, A.; Cini, E.; Timofte, R. S.; Flitsch, S. L.; Turner, N. J.; Linclau, B., Heavily fluorinated carbohydrates as enzyme substrates: Oxidation of tetrafluorinated galactose by galactose oxidase. *Chem. Commun.* **2011,** *47* (40), 11228-11230.
20. Golten, S.; Fontenelle, C. Q.; Timofte, R. S.; Bailac, L.; Light, M.; Sebban, M.; Oulyadi, H.; Linclau, B., Enantioselective Synthesis of Dideoxy-tetrafluorinated Hexoses. *J. Org. Chem.* **2016,** *81* (11), 4434-4453.
21. Timofte, R. S.; Linclau, B., Enantioselective synthesis of tetrafluorinated glucose and galactose. *Org. Lett.* **2008,** *10* (17), 3673-3676.
22. Linclau, B.; Golten, S.; Light, M.; Sebban, M.; Oulyadi, H., The conformation of tetrafluorinated methyl galactoside anomers: crystallographic and NMR studies. *Carbohydr. Res.* **2011,** *346* (9), 1129-1139.
23. Parikka, K.; Leppanen, A. S.; Pitkanen, L.; Reunanen, M.; Willfor, S.; Tenkanen, M., Oxidation of Polysaccharides by Galactose Oxidase. *J. Agric. Food. Chem.* **2010,** *58* (1), 262-271.