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A new HPTLC platformed luminescent biosensor system for facile screening of captan residue in fruits

Yisheng Chen[a,b], Caihong Huang[b], Bernhard Hellmann[c], Zhengyu Jin[a,b], Xueming Xu[a,b,*], Guosheng Xiao[d]

[a] The State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[b] School of Food Science and Technology, National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China
[c] Institute of Nutritional Science, Justus Liebig University Giessen, Wilhelmstraße 20, 35392 Giessen, Germany
[d] College of Biology and Food Engineering, Chongqing Three Gorges University, Wanzhou 404100, China

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ABSTRACT

This study presented a HPTLC platformed luminescent biosensor system for screening captan residue. First, the potential bio-effects of layers materials on the detectability of a luminescent bacteria *Photobacterium phosphoreum* (ATCC 11040) as the sensor cell were assessed. From comparison, it was noteworthy that the combination of sensor cells with normal silica gel layer exclusively gave outstanding detectability (< 10 ng/zone). On this basis, HPTLC mediated separation and biosensing was further optimized. Then, the obtained graphic results were digitally quantified via software processing, offering satisfactory selectivity, linearity (R^2 = 0.9901 within 10–80 ng/zone) and sensitivity (0.5 mg/kg against MRLs ≥ 6 mg/kg). Additionally, the performance of the established method was validated with different fruits (recover rates 75–96%, RSD < 11.8%). Meanwhile, it was demonstrated that detectability of this hybrid system would be tuneable by altering the combination of bacteria strains and layer materials, which was meaningful to strengthen the usability of microbial biosensors.

1. Introduction

Captan (*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide) was one of the most widely used broad-spectrum fungicides. Especially for a large variety of fruits, captan was commonly used as an effective preserver, displaying remarkable efficacy to maintain their bright and health-looking outward appearance during storage. Whereas, the abusing of captan was an emerging problem and consequently posed serious challenge to human health. Captan had been the most frequently detected fungicide in some market basket studies and its residue in apple without post-harvest preparation could be up to 5.1 mg/kg (Rawn et al., 2008). Apart from acute toxic symptoms like dermatitis, vomiting and conjunctivitis, chronic exposure to captan even at trace level may also lead to cytotoxicity toward mammalian cells and human reproductive system (Inoue, Kinoshita, Oyama, Kamemura, & Oyama, 2018; Nesakumar, Sethuraman, Krishnan, & Rayappan, 2015). Therefore, the rapid, simple and reliable screening of captan in fruits was urgently needed.

Nevertheless, the analysis of captan was a challenge to conventional methods, because of its thermal instability (Oulkar et al., 2019). This implied that considerable part of captan may be lost during its injection into GC-liner. To circumvent this problem, uncommon instrument configurations like cold on-column injection, must be used for its analysis by GC. Apart from that, special attention was also required to circumvent the degradation of captan that was pH sensitive, during sample preparation and cleanup (EURL-SRM, 2017). More specifically, the cleanup of sample extracts with primary-secondary amine sorbents may cause pH rise to neutral or weak alkaline conditions (pH = 7–9), which eventually results in extensive degradation of captan.

Biosensors in combination with high performance thin-layer chromatography (HPTLC) as converging technology was excellent tools for identification of bioactive compounds in sophisticated sample matrices, which was fostering a new frontier of analytical chemistry (Wang, Xu, Chen, Ren, & Liu, 201; Wang et al., 2019; Xie et al., 2017; Zhang et al., 2017). Apart from intrinsic advantages like high simplicity, throughput, matrix-tolerance and image-giving (Kokotkiewicz, Migas, Stefanowicz, Luczkiewicz, & Krauze-Baranowska, 2017; Li, Yu, Yang, & Liu, 2018; Mikropoulou et al., 2019; Pedan et al., 2018), HPTLC additionally offered an tailored platform for microbial biosensors, achieving ideal cooperation of chromatography and biosensing at ease (Chen & Morlock, 2016; Chen & Schwack, 2015; Krüger, Bergin, & Morlock, 2018; Ristivojević & Morlock, 2018). More specifically, such hybrid

* Corresponding author.
E-mail addresses: chen.yisheng@jiangnan.edu.cn (Y. Chen), xmxu@jiangnan.edu.cn (X. Xu).

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system enabled facile separation of the analyte and interfering sample matrices prior to biosensing, thus not only significantly saving workload for sample cleanup, but also substantially enhancing the selectivity of detection.

Among the broad diversity of microbial biosensors that can be coupled to HPTLC, bioluminescent bacteria was paid specially attention, because its sensing mechanism was based on the toxicity signature of the analyte. Therefore, any chemicals causing disorder of cell physiological state may be detected, reflected in the change of bioluminescence intensity (Gaudin, 2017; Schoenborn et al., 2017; Szczepańska, Kudłak, & Namieśnik, 2018). Obviously, this was particularly suitable for untargeted screening. In addition, using bioluminescent bacteria in combination with HPTLC also benefited higher sensitivity, compared to conventional cuvette assays (Schulz, Weiss, Weber, & Winzenbacher, 2017). Noteworthy as well, exposure of whole-cell biosensors to HPTLC layer materials may result in variation of cell physiological activity (van Loosdrecht, Lyklema, Norde, Schraa, & Zehnder, 1987; van Loosdrecht, Lyklema, Norde, & Zehnder, 1990) and therefore their overall detection performance. This point, neglected by previous publications dealing with HPTLC-microbial biosensors, was firstly discussed in our previous work (Chen & Morlock, 2016; Chen & Schwack, 2014).

The objective of this work was to establish a facile and reliable method for fast screening of captan in different fruits. With this regard, the combination of different layers with a new bioluminescent bacteria *Photobacterium phosphoreum* was for the first time evaluated to achieve sensitive and selective visualization and quantitation of the analyte on HPTLC plates, as schematically illustrated in Fig. 1. After chromatography development and biosensing optimization, the established method was successfully validated with real samples.

2. Material and methods

2.1. Chemical and apparatus

The reference standard of Captan (> 99% purity) was purchased from Aladdin (Shanghai, China). Other chemical reagents and solvents of analytical purity were purchased from Sigma Aldrich (Shanghai, China). Glass backed silica gel G60 F_{254} plates (layer thickness 0.2 mm, 10 × 20 cm, batch No. 1.05626.0001) were from Merck (Darmstadt, Germany), NH_2 bonded silica gel plates F_{254} (layer thickness 0.2 mm, 10 × 20 cm, batch No. A011401/02730) from Macherey-Nagel (Düren, Germany), acidified and neutral aluminium dioxide plates (layer thickness 0.1 mm, 10 × 10 cm) from Huanghai (Qingdao, China). Prior to using, all plates were pre-washed by developing with methanol to the top, then dried at 120 °C by TLC heater III (CAMAG, Muttenz, Switzerland) for 20 min, and stored in a airproof ziplock. A Millipore Synergy system (Schwalbach, Germany) was used to prepare Ultra-pure water (18 MΩ cm). The bioluminescent bacteria strain *Photobacterium phosphoreum* (ATCC 11040) was from Food Inspection and Quarantine Center, Shenzhen Customs. Fruit samples including Fresh apple, pear, cherry, peach and plum samples were purchased from local supermarket.

2.2. Preparation of bacteria suspension

The method for preparing bacteria suspension was principally based on that we previously established (Chen & Schwack, 2014). Frist, 1 L water was added with 30 g NaCl, 6.1 g $NaH_2PO_4{\cdot}H_2O$, 2.75 g KH_2PO_4, 0.204 g $MgSO_4{\cdot}7\ H_2O$, 0.5 g $(NH_4)\ H_2PO_4$, 3 mL glycerol, 5 g peptone, and 0.5 g yeast extract. After mixing, the liquid medium was adjusted to pH = 7.0 with sodium hydroxide solution (25% in water), and then autoclaved at 121 °C for 20 min. After cooling down to room temperature, 100 mL of this liquid medium was added in a triangle flask, to which a single bacterial colony from an agar plate was seeded. Then, the flask was covered with aluminium foil that will not only prevent microorganism contamination but also let oxygen in. The medium was cultivated at 20 ± 3 °C on a rotary shaker set to 100 rpm. To monitor the growth of bacteria, the medium was sampled every 2 h for absorbance measurement with a UV–Vis spectrometer at 600 nm, shown in Fig. S1. After 12 h cultivation, the bacterial suspension with strong bioluminescence was harvested and further diluted with another 100 mL fresh medium.

2.3. Preparation of standard solution

Stock solution of captan was prepared by dissolving 10 mg reference standard in 100 mL methanol containing 0.4% acetic acid (v/v) and stored at 4 °C in darkness. Working solution of captan was obtained by further diluting the stock solution to 0.01 and 0.001 mg/mL with methanol containing 0.4% acetic acid (v/v).

2.4. Sample preparation and cleanup

As for the sample preparation, a simplified QuEChERS procedure in accordance with the EN 15,662 protocol was used, however with some modification. Briefly, 10 g fruit sample was homogenized with 10 mL acetonitrile containing 1% formic acid instead of pure acetonitrile for

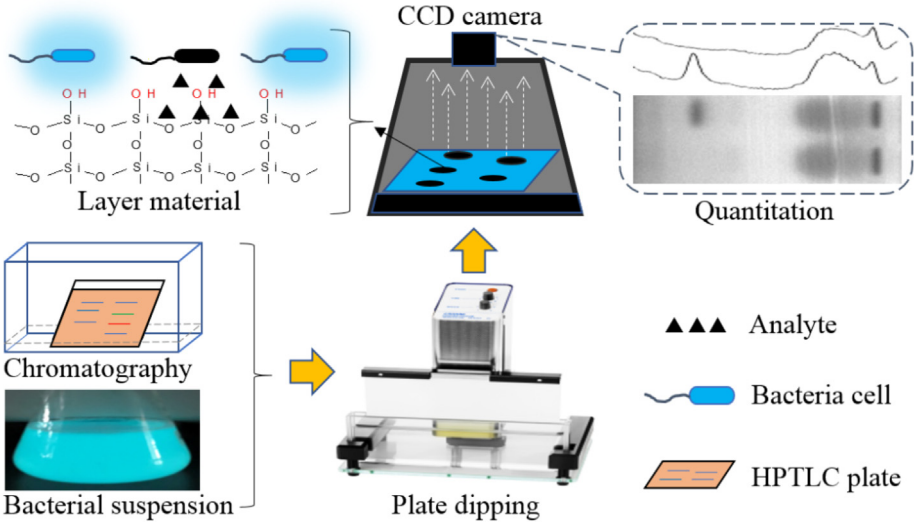


Fig. 1. The schematic illustration of the work-flow of the developed biosensing system.

extraction. If necessary, 0.2- or 0.5-mL stock solution of captan (0.1 mg/mL) was spiked into blank homogenates, respectively, resulting in 2 or 5 mg/kg artificial contamination. Then 4 g $MgSO_4$ and 1 g NaCl was added for partitioning. After shacking for 3 min, the mixture was centrifugated at 5000 rmp for 3 min, from which 2 mL of supernatant were filtered through disposable polyester syringe filters of 0.45 μm pore size. The obtained extract was directly analysed without further cleanup.

2.5. HPTLc

Carried by a 0.5 MPa nitrogen gas stream, 0.5–10 μL working solution of captan and 5–10 μL sample extracts were spotted by Limonat 5 (CAMAG) onto the plate surface as 6 mm bands with automatically calculated intervals, initially 20 mm from the left side, and 8 mm distance from the bottom. Separation of samples to 60 mm was performed by ADC-2 (CAMAG) filled with 10 mL the mixture of ethyl acetate/toluene (8/2, v/v) as the mobile phase, following 2 min moisture adjusting (34% relative humidity) and 10 min pre-condition. To eliminate residues of organic solutions, the developed plate was dried in vacuum for 10 min.

2.6. Bioluminescence documentation and quantitation

The dried plate was dipped into the bacteria suspension, via a TLC Immersion Device III (CAMAG), at a vertical speed of 2 cm/s and 2 s immersion time. After that, the plate was immediately transferred to Biovisualizer (CAMAG) equipped with a cooled CCD camera and images were captured with exposure time of 30 s, sequence display delay 250 ms, and automatic gain and offset, at interval of 2 min. Quantitation of the graphical results was realized by using Videoscan software (CAMAG) that was tailored to measure and process the gray value of digital pixels.

3. Results and discussion

3.1. Evaluation bio-effects of layer materials

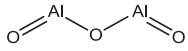
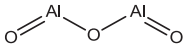
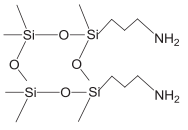
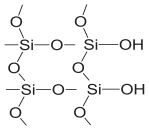
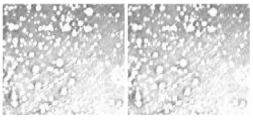
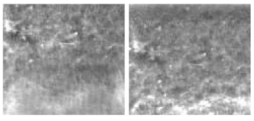
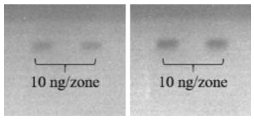
First of all, the potential effects of layer materials on the performance of the sensor cells was evaluated. For comparison, three major types of HPTLC, including aluminium oxide, NH_2 bonded silica gel and silica gel, were exemplarily tested. In order to quantify the strengthen of such effects, plates spotted with bands (6 × 1 mm size) of captan at 10 ng/zone was directly dipped into bacteria suspension and measured. As comparatively summarized in Table 1, it was apparent that the bioluminescence responses of bacteria cells displayed strong layer-dependence. Drastic suppressing effects of aluminium oxide layers, either acidified or neutralized, on bacteria cells was observed, resulted in noisy backgrounds. It implied that these layer materials were not compatible to this whole-cell biosensor. In contrast, silica gel-based layers did not cause interfering backgrounds for bioluminescence signals, but the obtained detectability differed dramatically. On normal silica gel, outstanding sensitivity was achieved in the initial minutes after dipping, while no visible inhibition induced by captan can be observed on the NH_2 bonded silica gel layer, even extending the exposure time to 30 min. These results diametrically opposed previous observation with *Aliivibrio fischeri* (Chen & Morlock, 2016; Chen & Schwack, 2014), suggesting such bio-effects may be not only layer material-dependent, but also cell type-dependent. More importantly, the combination of bacteria cells and silica gel plate was of superior practicability as well, in the view of cost-efficiency, because silica gel plate much less expensive than NH_2 bonded silica gel plates. In light of this finding, silica gel plates were fixed for further chromatographic and biosensing development.

3.2. Biosensing conditions

Compared to other devices designed for reading the bioluminescence responses, the most featuring superiority of HPTLC was that it offered graphic results impressive and straightforward to eye-inspection (Chen & Morlock, 2016; Chen & Schwack, 2014; Froehner, Backhaus, & Grimme, 2000; Galarce-Bustos, Pavón-Pérez, Henríquez-Aedo, & Aranda, 2019; Hernando, De Vettori, Martínez Bueno, & Fernández-Alba, 2007; Ristivojević & Morlock, 2018; Roda, Cevenini, Michelini, & Branchini, 2011). In order to document the weak bioluminescence of bacteria cells, the CAMAG Bioluminzer integrated a deeply cooled high resolution CCD camera was used for documentation after dipping. Like many other biosensors, the response of cell bioluminescence to chemicals on HPTLC plates was a dynamic process, implying that cell incubation was essential to satisfactory sensitivity.

However, the major problem associated with cell incubation on HPTLC was the diffusion of zone shape. Driven by capillary force, the sharp bands spontaneously expanded to spreaded spots being wetted, leading to the merging of targeted bands with noises. To avoid this problem, images of bioluminescence should be taken as soon as possible after dipping. Here, zones of captan at 3 different concentrations (20, 40 and 60 ng/zone) were used to profile the intensity changes of cell bioluminescence in the initial 20 min of exposure. As quantitatively shown in Fig. 2(a), the combination of bacteria cells and silica gel layer benefited remarkably instant visualization of the targeted compounds. Immediately after dipping into bacteria suspension, the bioluminescence inhibition strength within captan zones drastically raised. The fourth minute marked a turning-point, because after that signal intensities became steady, however accompanied with increasingly insignificant zone shape diffusion. In light of this observation, the exposure time for image documentation was therefore fixed at 4 min after dipping.

Table 1
Characterization of the bio-effects of layer materials on the detectability of sensor cells.

Layer materials	Acidified aluminium oxide	Neutral aluminium oxide	NH_2-bonded silica gel	Silica gel
Structure				
Exposure	0 min 4 min	0 min 4 min	Exposure 0 min	4 min 0 min
Inhibition pattern				
Detectability	N/A	N/A	No	Yes

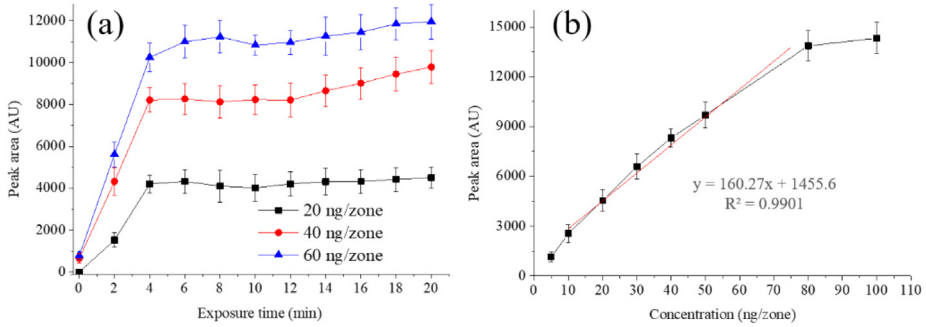


Fig. 2. (a) Time-dependent variation of bioluminescence inhibition intensity within captan zone at three different concentrations (20, 40 and 60 ng/zone); (b) does-response curve of the bioluminescent results by digital evaluation.

3.3. Quantitation via pixel scanning

In order to get quantitative data from the graphic results, digital scanning of the pixels was performed with the software Videoscan tailored for HPTLC imaging evaluation. Compared to other software (MATLAB or ImageJ for instance) with similar function, CAMAG Videoscan featuring in superior user-friendliness and automation, which was of crucial importance to the calculation precision. When the captured image was saved, the grayscale intensity of each pixel was digitalized in the range from 0 (Black) to 256 (White). Through digital scanning, the grayscale intensity values of pixels were extracted from the defined track-lines, based on which densitogram was built. Then, signals of interests can be located and calculated automatically.

A major challenge in the quantification of bioluminescent inhibition was that the reading values from cuvette assays normally obeyed a polynomial regression, inconvenienced calculation based on calibration curves (Froehner et al., 2000; Hernando et al., 2007). Aiming to further assess the quantitation capacity of this detection, here the does-response curve was established depending on 8 calibration points within a broad range from 5 to100 ng/zone. As presented in Fig. 2**(b)**, it was clear that a good linearity (R^2 = 0.9901) of inhibition signals can be obtained within the range of 10–80 ng/zone, evidencing the applicability of pixel scanning for quantifying captan.

3.4. Chromatographic separation

Apart from detection sensitivity, it was important to keep in mind that bioactive compounds in fruit samples would be co-extracted with the analyte, necessitating the separation of crude extracts in advance to analysis. Here, the principle of HPTLC development prior to bioautography was not to achieve full separation of all compounds, but to create specific windows for the targeted compounds, free from interfering co-extracts. From a screening test, the mixture of ethyl acetate/toluene (8/2, v/v) that delivered the best separation was chosen as the mobile phase. Illustrated in Fig. 3**(a)**, a sharp window for captan at 0.7 Rf was resulted from chromatographic development, while interfering matrices were left behind within the range of 0–0.5 Rf. Additionally, the differences between blank and spiked samples can be further discriminated by comparing corresponding chromatogram, as displayed in Fig. 3**(b)**. Through simply reading these straightforward results, a "Yes/No" judgement can be instantly reached, which was a convincing proof for the outstanding selectivity and convenience of this method for fast screening of captan in fruits.

3.5. Method validation

To estimate the feasibility of the established biosensing system for practical analysis, performance was validated with six different fruits, including apple, peer, plum, apricot, cherry and peach. For the limit of detection, inhibition patterns of three matrix-matched zones with

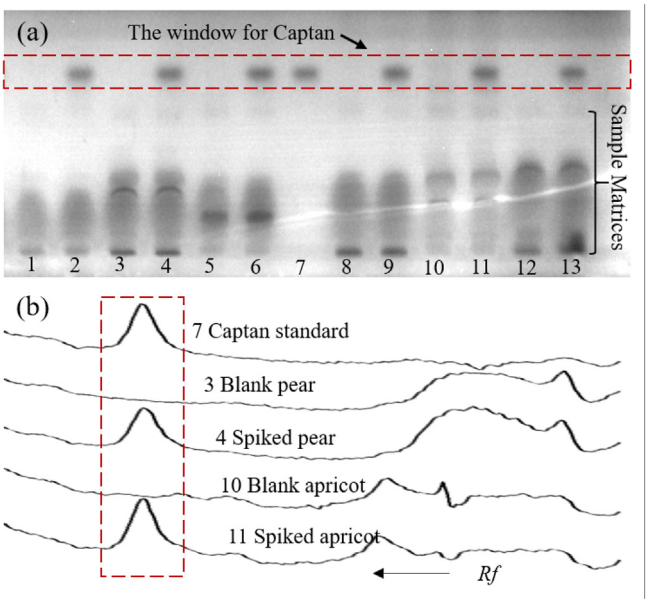


Fig. 3. (a) Image of the bioluminescence inhibition of separation results, with track assignment: 1 apple, 2 apple spiked with captan, 3 pear, 4 pear spiked with captan, 5 plum, 6 plum spiked with captan, 7 peach, 8 peach spiked with captan, 9 captan standard, 10 apricot, 11 apricot spiked with captan, 12 cherry, 13 cherry spiked with captan. *Note*: spiking level at 20 ng/zone, equal to 2 mg/kg. (b) Chromatogram converted from digital images via videoscan, examplarily shown with track 7, 3, 4, 10 and 11.

descending concentrations (5, 3 and 1 ng/zone) were evaluated. It was detected that the inhibition effect disappeared when captan concentration was reduced from 5 to 3 ng/zone. This implied that the fruit samples containing at least 0.5 mg/kg captan were detectable by this method, if 10 µL extracts were applied. Though still not comparable to the detectability (LOQ = 0.01 mg/kg) of LC-MS/MS (Oulkar et al., 2019), this method was still useful, since the MRLs of captan stipulated by EU Commission Regulation 2016/452 (European-Union, 2016) in many fruits were higher than 6 mg/kg. Meanwhile, the method accuracy was assessed by determining the recovery rates of captan spiked into fruit samples at two critical levels (2 and 5 mg/kg). As presented in Table 2, the recovery rates with insignificant sample-dependence located within the range of 75–96% with maximally 11.8 RSD%, which should be suitable for fast screening.

4. Conclusions

By jointly using HPTLC and bioluminescent cells, a hybrid biosensor system tailored for screening captan residue in different fruits was developed and validated in this work. Under optimized chromatographic

Table 2
Recovery rates of analyte spiked into different sample matrices.

Sample matrices	MRL (mg/kg)	LOD (mg/kg)	Spiked (mg/kg)	Detected (mg/kg)	Recovery rates (%)
Apple	10	< 0.5	2.0	1.7 ± 0.2	85 ± 11.8
			5.0	4.5 ± 0.3	90 ± 6.7
Pear	10	< 0.5	2.0	1.5 ± 0.3	75 ± 6.7
			5.0	4.8 ± 0.1	96 ± 2.1
Plum	10	< 0.5	2.0	1.9 ± 0.2	95 ± 10.5
			5.0	4.7 ± 0.2	94 ± 4.3
Apricot	6	< 0.5	2.0	1.8 ± 0.1	90 ± 5.6
			5.0	4.6 ± 0.3	92 ± 6.5
Cherry	6	< 0.5	2.0	1.8 ± 0.2	90 ± 11.1
			5.0	4.8 ± 0.4	96 ± 8.3
Peach	6	< 0.5	2.0	1.6 ± 0.2	80 ± 6.3
			5.0	4.7 ± 0.1	94 ± 2.1

and biosensing conditions, selective visualization of the analyte can be accomplished within 4 min, offering remarkable quantitative and throughput capacity. In addition, the layer-dependence of cell responses observed in this study sparked reconsideration on the function of HPTLC layers in bioassay. This analytical strategy presented here could be applied in any controlling laboratory with little microbiological effort. The type of HPTLC layer material can be customized according to the bioactivity of analyte, enabling efficient screening with both high selectivity and detectability.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125691.

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