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Short communication

A molecular imprinted SPR biosensor for sensitive determination of citrinin in red yeast rice

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ABSTRACT

A novel and sensitive molecular imprinted surface plasmon resonance (SPR) biosensor was developed for selective determination of citrinin (CIT) in red yeast rice. Firstly, the gold surface of SPR chip was modified with allyl mercaptane. Then, CIT-imprinted poly(2-hydroxyethyl methacrylate–methacryloylamidoglutamic acid) (p(HEMA–MAGA)) film was generated on the gold surface modified with allyl mercaptane. The unmodified and imprinted surfaces were characterized by Fourier transform infrared (FTIR) spectroscopy, atomic force microscopy (AFM) and contact angle measurements. The linearity range and the detection limit were obtained as 0.005–1.0 ng/mL and 0.0017 ng/mL, respectively. The SPR biosensor was applied to determination of CIT in red yeast rice sample.

1. Introduction

Mycotoxins appear in the food chain as a result of fungal infection of crops. Due to their resistance to decomposition, they remain in meat and dairy products. The results show that the temperature treatments such as cooking and freezing cannot destroy some mycotoxins (Robbins, Swenson, Nealley, Kelman, & Gots, 2000). CIT, one of the major groups of mycotoxins, is highly toxic, mutagenic and carcinogenic metabolite. CIT (4,6-dihydro-8-hydroxyl-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid) (Fig. S1) was first isolated from *Penicillium citrinum* and implicated as a causative agent in human hepatic and extrahepatic carcinogenesis (Li, Zhou, Yang, & Ou-Yang, 2012; Zhou, Fu, & Li, 2015). The contaminations of CIT in various food such as fermented maize, cheese, corn, wheat, barley, red yeast rice, apples, brewed beer and cereal products have been reported (Arévalo, Granero, Fernández, Raba, & Zón, 2011; Guo et al., 2010; Markov et al., 2013; Singh et al., 2014). In addition, the ingestion of CIT is harmful effects to humans and animals. It causes serious health problems such as liver and kidney diseases, nervous system damage and carcinogenicity (Arévalo et al., 2011; Bennett & Klich, 2003; Wang, Kong, Yang, & Xin, 2005). Because of these reasons, the determination of CIT is very significant due to their negative effects on human health.

Recently, SPR sensors have attracted attention (Yola, Atar, & Eren, 2014; Yola, Eren, & Atar, 2014a). SPR, an optical phenomenon, occurs when a p-polarized light goes through a prism. Then, it hits a metal layer covering the prism surface at a particular angle (Englebienne, Hoonacker, & Verhas, 2003). SPR sensors have important applications such as determination of affinity-binding constants (Zhang, Liu, & Wang, 2008) and genotype analyzing (Hayashi, Hagihara, & Nakatani, 2008). In addition, molecular imprinting technique is effective method in terms of specific and selective recognition. The method relies on the molecular recognition. It is a kind of polymerization which is formed around the target molecule. Hence this technique forms specific cavities in the cross-linked polymeric matrices (Gupta, Yola, & Atar, 2014; Yola, Uzun, Özaltin, & Denizli, 2014). Hence, the sensors based on molecular imprinting polymers have many additional advantages including high sensitivity, selectivity and favorable portability in comparison with other analytical systems (Yola, Eren, & Atar, 2014b, 2015).

Several analytical methods such as high performance liquid chromatography (HPLC) (Franco et al., 1996; Vazquez et al., 1996), liquid chromatography–mass spectrometry (LC–MS) (Blaszkewicz, Muñoz, & Degen, 2013), thin-layer chromatography (TLC) (Gimeno & Martins, 1983; Xu et al., 2006) enzyme immunoassays (EIA) (Abramson, Usleber, & Martlbauer, 1995; Duan et al., 2009), micellar electrokinetic capillary chromatography (MEKC) (Nigović, Sertić, & Mornar, 2013) and microsphere-based flow

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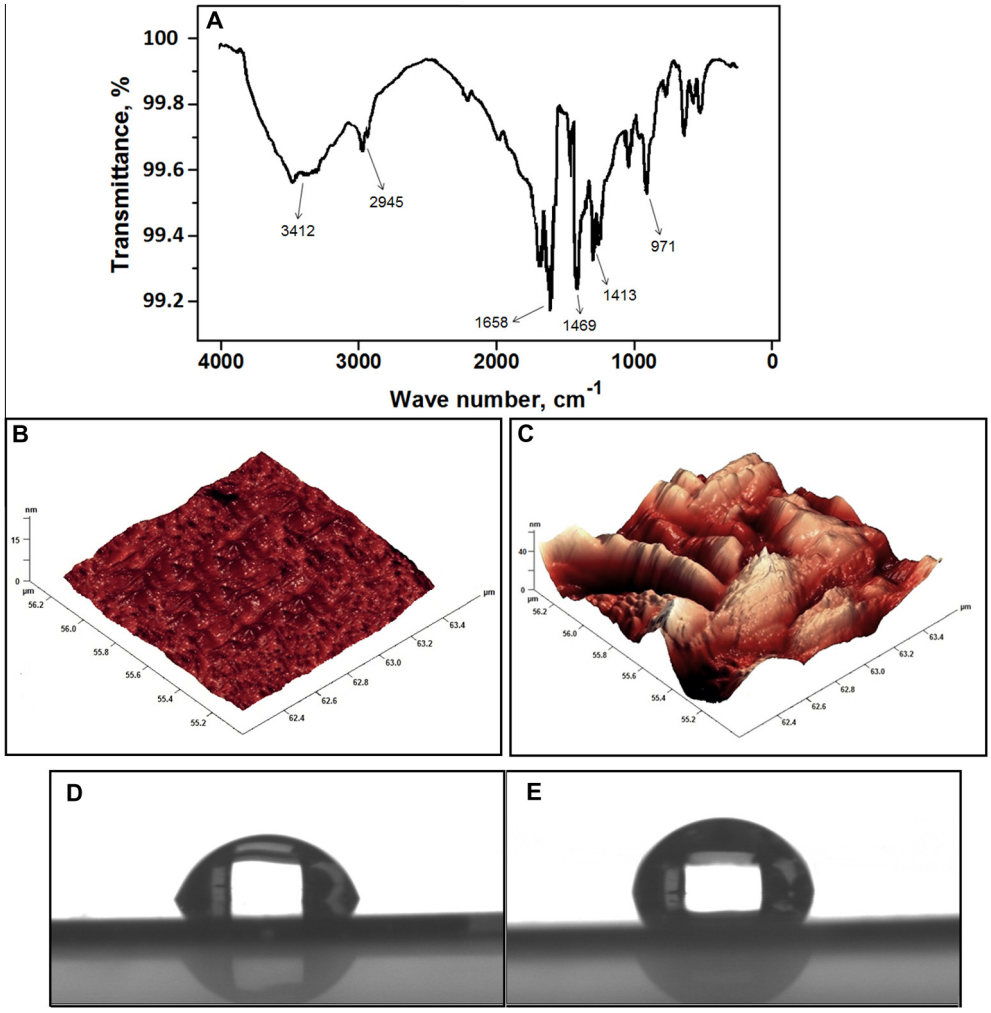

Fig. 1. (A) FTIR spectra of the CIT-imprinted p(HEMA–MAGA) film; AFM images of (B) non-modified SPR chip; (C) CIT-imprinted p(HEMA–MAGA) film; contact angle measurements of (D) non-modified; (E) CIT-imprinted p(HEMA–MAGA) film on SPR chip.

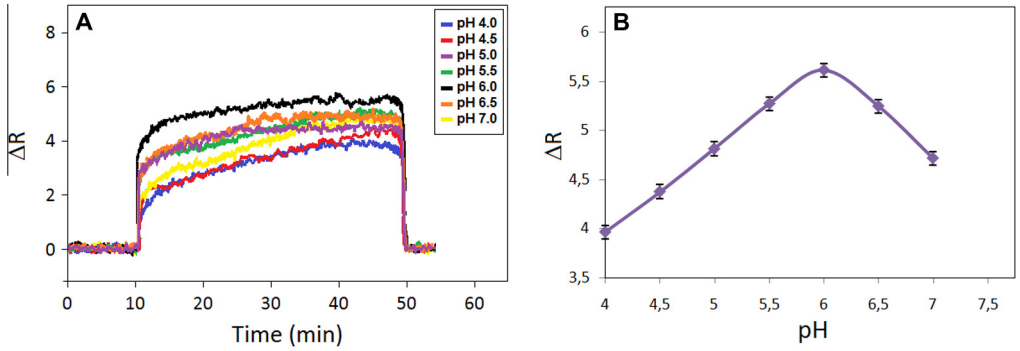

Fig. 2. (A) The sensorgrams for the interaction between 0.250 ng/mL CIT in different pHs and (B) effect of pH on CIT-imprinted SPR biosensor.

cytometric immunoassay (Li, Wu, Guo, Zheng, & Guo, 2012) have been reported for determination of CIT. However these methods need expensive equipment and include time consuming extraction steps to eliminate contaminants. These problems can easily be solved by molecular imprinted sensors.

In this paper, the CIT-imprinted SPR biosensor was developed for the first time. The prepared molecular imprinted polymers were characterized by using FTIR, AFM and contact angle measurements. The CIT-imprinted SPR biosensor was successfully applied to red yeast rice sample for the determination of CIT with high sensitivity and selectivity.

2. Experimental

2.1. Materials

CIT, Zearalenone (ZEA), Lovastatin (LOV) and Ochratoxin A (OCT) were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA) and used as received. The stock solution of CIT (1.0 mM) was prepared with 50 mL of ultra-pure quality water. The working solutions were prepared by diluting the stock solution with 0.10 M phosphate buffer (pH 6.0). Allyl mercaptane (CH_2CHCH_2SH), 2-hydroxyethyl methacrylate (HEMA), ethylene

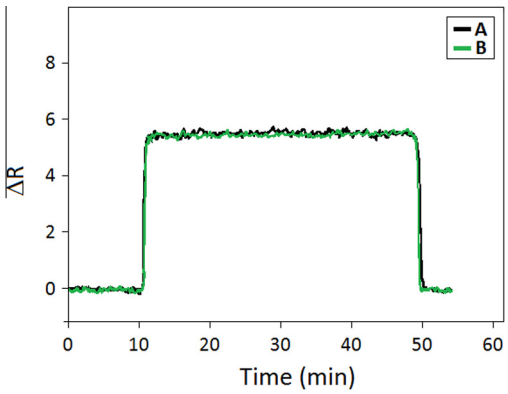

Fig. 3. The sensorgrams for (A) long-term and (B) short-term stabilities of CIT.

glycol dimethacrylate (EGDMA), N,N′-azobisisobutyronitrile (AIBN), trichloroacetic acid (TCA), sodium chloride (NaCl) were obtained from Sigma–Aldrich. MAGA was obtained Nanoreg Ltd. Şti., Ankara, Turkey.

2.2. Surface modification of the SPR chip

SPR chip modified with CH_2CHCH_2SH was prepared according to our previous works (Gupta et al., 2014). CIT-imprinted p(HEMA–MAGA) film on SPR chip modified with CH_2CHCH_2SH was prepared according to this protocol: firstly, CIT and MAGA monomer were mixed with 500 μL of phosphate buffer (pH 6.0) at room temperature for 3 h. The molar ratio of MAGA–CIT was 2:1. After that, 5.0 mg of AIBN as initiator was dissolved in 1250 μL of HEMA and 500 μL of EGDMA. 200 μL of MAGA–CIT complex was added into this solution to prepare stock monomer solution. The solutions were passed with nitrogen gas for 15 min. Then, 20 μL of aliquot was taken from the stock monomer solution and dropped onto the SPR chip modified with CH_2CHCH_2SH by using *spin coating method*. The method is used to deposit uniform thin films. After 10 s, The SPR chip was removed from spin coater and polymerization was started by UV light (100 W, 365 nm). After 60 min, SPR chip with polymer was washed with ethanol three times, then and dried in vacuum oven (Yola et al., 2014a).

2.3. CIT removal from SPR chip surface

There are the electrostatic interactions and hydrogen bonding between the carboxylic acid groups of MAGA monomer and polar groups of CIT molecules. In order to break the interactions, we used 1.0 M NaCl solution in water as a desorption agent. Firstly, the removal study of CIT was performed via batch system. CIT-imprinted p(HEMA–MAGA) surface was dipped into 25 mL of desorption agent. The SPR chip was swinged in bath (200 rpm) at room temperature. After CIT removal, the SPR chip was washed with ultra pure quality water and dried with nitrogen gas under vacuum (200 mmHg, 25 °C).

2.4. Sample preparation

The red yeast rice samples were bought from local supermarket. The extraction and dilution procedures of the samples were as follows: 0.1 g of ground red yeast rice sample (<0.2 mm) was mixed with 1.0 mL of TCA (10% m/v) by a vortex mixer for 25 s and centrifuged at 4500 rpm for 15 min. The supernatant was transferred to another centrifuge tube and the precipitate was treated twice as mentioned above. The collected supernatant was centrifuged again at 4500 rpm for 5 min and filtrated by a 0.45-μm syringe filter. The filtrate was directly used as the sample solution. The filtrate was diluted with 0.1 M phosphate buffer (pH 6.0) for analysis.

The real time determination of CIT was performed using SPR system (GenOptics, SPRi-Lab, Orsay, France) according to our previous report (Yola et al., 2014a).

3. Results and discussion

The CIT-imprinted p(HEMA–MAGA) film was characterized by FTIR spectrophotometer (Thermo Fisher Scientific, Nicolet iS10, Waltham, MA, USA). After CIT removal from chip surface, the specific bands of the polymeric structure such as O–H stretching of HEMA and MAGA at 3412 cm^{-1}, saturated C–H stretching of MAGA at 2945 cm^{-1}, carboxyl–carbonyl stretching of MAGA at 1658 cm^{-1} have been observed in Fig. 1A. In addition, the absorptions at 1469 and 1413 cm^{-1} are corresponded to –COO stretching peaks of MAGA monomer.

AFM images of SPR surfaces were obtained in non-contact mode (Nano Magnetics Instruments, Oxford, UK). The values of surface deepness for non-modified (Fig. 1B) and CIT-imprinted p(HEMA–MAGA) film on CH_2CHCH_2SH modified SPR chips (Fig. 1C) are 3.20 ± 1.10 and 25.33 ± 1.60 nm, respectively. These results indicate that the surface deepness of the CIT-imprinted p(HEMA–MAGA) film on CH_2CHCH_2SH modified SPR surface increased. These results confirmed that polymerization could be accomplished on the SPR surface.

As seen in Fig. 1D and E, contact angle values of non-modified and CIT-imprinted p(HEMA–MAGA) SPR surfaces were obtained as 81.06 ± 2.04 and 68.56 ± 3.24, respectively. The decrease in surface contact angles indicated that hydrophilicity or polarity of

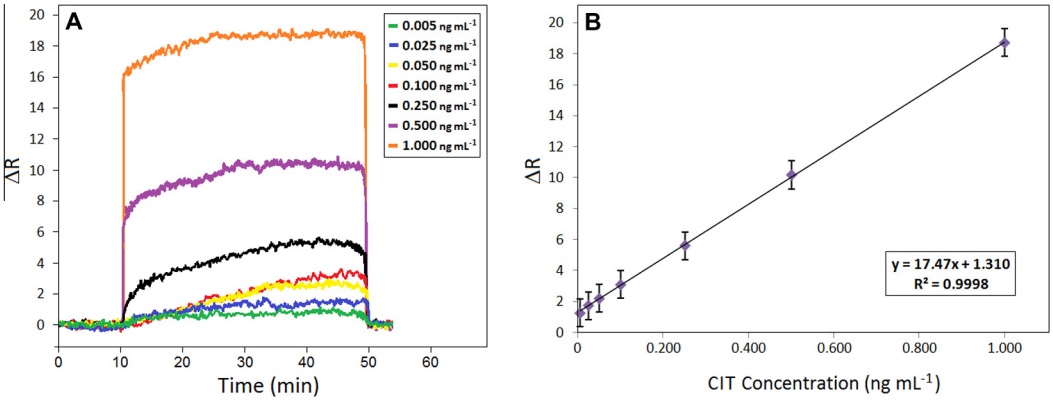

Fig. 4. (A) Effect of concentration on SPR response of CIT-imprinted biosensor and (B) the calibration curve of CIT.

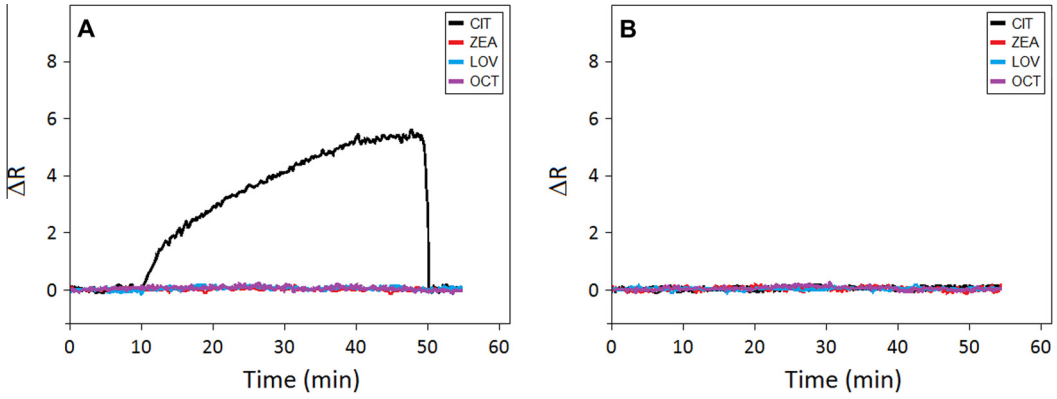

Fig. 5. Comparison of selectivity of SPR biosensors: the SPR responses of (A) CIT, ZEA, LOV and OCT (each of 0.250 ng/mL) on CIT-imprinted biosensor; (B) CIT, ZEA, LOV and OCT (each of 0.250 ng/mL) on nonimprinted SPR biosensor.

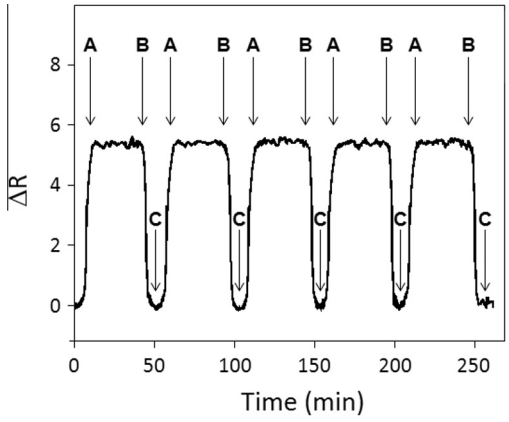

Fig. 6. Repeatability of CIT-imprinted SPR biosensor: (A) adsorption; (B) desorption; (C) regeneration.

surface increased. The increase of hydrophilic character of surface is expected due to the hydrophilic structure of MAGA monomer containing two carboxylic acid groups.

A series of 0.10 M phosphate buffer with different pH was tested in the presence of 0.250 ng mL^{-1} CIT in Fig. 2A. The binding to SPR surface increases gradually up to pH 6.0. This situation is related to the structure of MAGA monomer. MAGA monomer is based on glutamic acid and has two pKa values (pKa_1: 2.10, pKa_2: 4.07). The increase in pH value caused the carboxylic acid groups of MAGA monomer loading negatively. These negative groups interacted efficiently with –OH groups of CIT. Hence, the affinity of sensor-analyte increased.

When pH exceeds 6.0, the concentration of the anion form of CIT increases. Hence the binding to SPR surface could be difficult. Hence, pH 6.0 (ΔR = 5.613) was selected as optimum pH (Fig. 2B).

The stock solutions of CIT were hidden at two different temperatures such as 4 °C for 45 days (long-term stability) in Fig. 3A and 25 °C for 24 h (short-term stability) in Fig. 3B. The important differences in SPR responses were not found for 0.250 ng mL^{-1} CIT. Thus, these results indicate that CIT is highly stable.

The sensorgrams with increasing CIT concentration (Fig. 4A) show that the signal of SPR biosensor increased linearly with concentration. The SPR responses are linear with CIT concentrations from 0.005 to 1.000 ng mL^{-1}. This situation is directly related to basic adsorption phenomena which describes concentration difference between liquid and solid phases. These phases drive CIT molecules through the surface to interact with specific ligands/cavities. The regression equation of CIT (Fig. 4B) is $y = 17.47x + 1.310$. Each point of the calibration graph is corresponded to the mean value obtained from 6 independent measurements.

LOD and LOQ were experimentally calculated from the analysis of samples spiked with a standard mixture of the analytes at serially diluted concentrations. The minimum concentration which gave a signal-to-noise ratio higher than 3 is LOD. LOQ was then calculated on the basis of a minimal accepted value of the Signal/Noise (S/N) of 10 (Yola & Özaltin, 2011a, 2011b). LOQ and LOD for CIT were found to be 0.0050 and 0.0017 ng/mL, respectively (Table S1). As seen in Table S2, the CIT-imprinted SPR biosensor shows lower LOD with high sensitivity.

In addition, the recovery experiments were performed with different CIT concentrations. The results are presented in Table S3. Closeness of the results to 100.00% showed that recovery of the developed CIT-imprinted SPR biosensor was very good. A rapid, simple and sensitive LC–MS/MS method was employed as a comparison to evaluate the validity of the developed SPR biosensor. Table S4 gives the results obtained by the two methods for the determination of CIT in red yeast rice sample. These results were compared by Wilcoxon test and there was no significant difference between the obtained results of SPR and LC–MS/MS ($T_{calculated} > T_{tabulated}$, $p > 0.05$).

To confirm the selectivity of the CIT-imprinted SPR biosensor in the presence of ZEA, LOV and OCT as competitors in Fig. 5A, the samples were applied to the CIT-imprinted SPR biosensor. The selectivity coefficients (k) and relative selectivity coefficients (k') values are given in Table S5. CIT-imprinted SPR biosensor was 11.7, 12.2 and 11.0 times more selective for CIT than ZEA, LOV and OCT, respectively. The results show that because of selective cavities in the polymer structure, CIT-imprinted SPR biosensor has higher adsorption capacity (ΔR values) for CIT in comparison to ZEA, LOV and OCT. CIT-imprinted SPR biosensor shows low non-specific responses for ZEA (ΔR = 0.480), LOV (ΔR = 0.460) and OCT (ΔR = 0.510). These responses resulted from the structural and physico-chemical similarities between CIT and the other mycotoxins (ZEA, LOV and OCT).

To display the specificity of CIT-imprinted SPR biosensor, nonimprinted SPR biosensor (NIP) was also prepared and the signals of nonimprinted SPR biosensor against CIT, ZEA, LOV and OCT were obtained as 0.150, 0.120, 0.140 and 0.130, respectively in Fig. 5B. The selectivity coefficients for nonimprinted SPR biosensor in respect to ZEA, LOV and OCT were calculated as 1.3, 1.1 and 1.2, respectively. The results showed that CIT-imprinted SPR biosensor was 7.9, 43 and 12.7 times more selective in comparison to ZEA, LOV and OCT, respectively.

In order to show the repeatability of CIT-imprinted SPR biosensor, five equilibration–adsorption–regeneration cycles were

repeated in presence of 0.250 ng mL^{-1} CIT. As seen in Fig. 6, CIT-imprinted SPR biosensor has demonstrated repeated reflectivity response during the cycles.

4. Conclusion

We developed molecular imprinted SPR biosensor for the sensitive determination of CIT in red yeast rice. The non-imprinted and CIT imprinted p(HEMA–MAGA) surfaces were characterized by using con-tact angle measurements, FTIR and AFM. According to the results, polymerization was accomplished on the SPR surface. The developed imprinted biosensor showed high sensitivity towards CIT with a detection limit of 0.0017 ng/mL. In addition, CIT-imprinted SPR biosensor was 11.7, 12.2 and 11.0 times more selective for CIT than ZEA, LOV and OCT, respectively. In particular, the CIT-imprinted SPR biosensor offers the advantages of simplicity and efficiency in target detection from food samples. A high percentage of recovery shows that the CIT-imprinted SPR biosensor can be used to quantify CIT without interference. In conclusion, the CIT-imprinted SPR biosensor is sensitive, rapid, cheap and easy to use and might be preferred to the other methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.03.065.

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