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Hydrogen sulphide (H_2S) releasing capacity of essential oils isolated from organosulphur rich fruits and vegetables

Dong Liang [a], *Chenhui Wang* [a], *Restituto Tocmo* [a], *Haixia Wu* [a], *Lih-Wen Deng* [b,*], *Dejian Huang* [a,c,**]

[a] *Food Science and Technology Program, Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore*

[b] *Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, 8 Medical Drive, 117597, Singapore*

[c] *National University of Singapore (Suzhou) Research Institute, 377 Linquan Street, Suzhou Industrial Park, Jiangsu 215123, China*

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ABSTRACT

Hydrogen sulphide (H_2S) is a gaseous signalling molecule with multiple biological functions in the human body. Previous research demonstrates that diallyl trisulphide (DATS) and diallyl disulphide (the two major compounds of garlic oil) can be metabolized to generate H_2S in biological conditions and modulate cell signalling. In this paper, a fluorescent method for quantification of H_2S releasing capacity in cell lines was developed. MCF-7 cells in 96 well plate were incubated with a H_2S probe BCu for 3 h, then treated with H_2S donors for 2 h, which would be metabolized by the cells and produce H_2S. The resulting H_2S was captured by the probe and turn on its fluorescence, which was measured with a microplate reader. Linear dose response could be established between organosulphur concentration and fluorescence intensity, and the H_2S releasing capacity of a sample was determined by comparing the slope of the regression curve with that of a DATS standard obtained in parallel. With this method, the H_2S releasing capacity of ten organosulphur rich fruits and vegetables (garlic, red onion, yellow onion, scallion, shallot, leek, spring onion, Chinese chives, durian, and stinky beans) were evaluated and ranked. Stinky beans (a vegetable from Southeast Asia) topped the ranking with very high H_2S releasing capacity, followed by garlic and yellow onion. Our work provides new thinking on the health benefits of organosulphur-rich foods.

* *Corresponding author*. Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, 8 Medical Drive, 117597, Singapore. Tel.: +65 6516 1239; fax: +65 6779 1453.
E-mail address: lih_wen_deng@nuhs.edu.sg (L.-W. Deng).
** *Corresponding author*. Food Science and Technology Program, Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore. Tel.: +65 6516 8821; fax: +65 6775 7895.
E-mail address: chmhdj@nus.edu.sg (D. Huang).

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1. Introduction

Hydrogen sulphide (H_2S), a toxic gas with the characteristic foul odour of rotten egg, has been recognized as a gaseous signalling molecule (Polhemus & Lefer, 2014; Wang, 2012). Along with nitric oxide (NO) and carbon monoxide (CO), H_2S is the third and newest member of gasotransmitters (Łowicka & Bełtowski, 2006; Wang, 2002, 2010). Pharmacologists in the past decade have shown that H_2S might be beneficial to human health via multiple mechanisms, including blood vessel relaxation (Hosoki, Matsuki, & Kimura, 1997; Yang et al., 2008; Zhao, Zhang, Lu, & Wang, 2001), cardioprotection (Calvert et al., 2009; Geng et al., 2004), neuroprotection (Kimura, Dargusch, Schubert, & Kimura, 2006; Kimura & Kimura, 2004), antiinflammation (Sivarajah et al., 2009; Whiteman et al., 2010), antioxidant (Kimura et al., 2006; Kimura, Goto, & Kimura, 2010) and anticancer (Lee et al., 2014). In mammals, H_2S is produced endogenously from L-cysteine catalysed by four enzymes, including cystathionine γ-lyase (CSE, EC 4.4.1.1), cystathionine β-synthase (CBS, EC 4.21.22), 3-mercaptopyruvate sulphur transferase (3-MST, EC 2.8.1.2) and cysteine aminotransferase (CAT, EC 2.6.1.3) (Abe & Kimura, 1996; Shibuya et al., 2009; Stipanuk & Beck, 1982). In recent years, many H_2S donors have been developed for therapeutic and research purposes (Song et al., 2014; Zhao, Biggs, & Xian, 2014); however, most of them are synthetic compounds that are not suitable as active ingredients of functional foods. On the other hand, there are wide ranges of dietary organosulphur compounds found in vegetables and fruits that may have potentials as H_2S donor but little research is carried out.

Garlic has been used as an herbal remedy for thousands of years (Chen, Kao, Tseng, Chang, & Hsu, 2014; Kim, Kang, & Gweon, 2013; Ota et al., 2012); while its health benefits especially the cardioprotective effects have been documented in some clinical studies (Qidwai & Ashfaq, 2013; Rohner, Ried, Sobenin, Bucher, & Nordmann, 2014; Stabler, Tejani, Huynh, & Fowkes, 2012), the mechanisms remain elusive. Benavides and co-workers (Benavides et al., 2007) provided a completely new mechanism by showing that garlic extracts, as well as its major compounds diallyl trisulphide (DATS) and diallyl disulphide (DADS), could be converted into H_2S by human red blood cells or by rat aorta, and the resulting H_2S was able to relax rat aorta ring. Another group reported that S-allyl cysteine, the major water soluble compound of aged garlic extract, was protective against myocardial infarction through an H_2S dependent mechanism (Chuah, Moore, & Zhu, 2007). These studies suggest that H_2S might be the mysterious key molecule responsible for the health benefits of garlic.

Allium genus are known to contain organosulphides (Wang & Huang, 2014); these include garlic, onion (*Allium cepa*), scallion (*Allium fistulosum L.*), shallot (*Allium ascalonicum auct.*), leek (*Allium porrum L.*), and Chinese chives (*Allium tuberosum L.*). Other notable foods of tropical origin, i.e. durian (*Durio zibethinus Murr.*) and stinky beans (*Parkia speciosa*) are known for their extraordinary high content of organosulphur compounds responsible for their offensive smells when uncooked (Block, Naganathan, Putman, & Zhao, 1992; Cai et al., 1994; Gmelin, Susilo, & Fenwick, 1981; Tocmo, Lin, & Huang, 2014; Weenen, Koolhaas, & Apriyantono, 1996). We hypothesize that organosulphides in these foods, when consumed in our diet, could also be metabolized and generate H_2S in physiological conditions and exert certain health benefits.

H_2S has a short half-life (ranging from seconds to few minutes) under physiological conditions (Polhemus & Lefer, 2014); as a result, the accurate detection and measurement of H_2S in biological samples is challenging. We recently succeeded in developing an H_2S selective and sensitive turn-on fluorescent probe, BCu, which takes advantage of a Cu(II)–cyclen complex as a reaction centre for H_2S and as a quencher of the fluorescence of (boron-dipyrromethene) based fluorophores (BODIPY) (Fig. 1) (Wu et al., 2014). BCu has been shown to be highly selective and sensitive to H_2S and was suitable for detection of H_2S in cells. Taking advantage of the ideal property of BCu probe, we developed a fluorescent method for quantification of H_2S releasing capacity of dietary organosulphides in a cell line model. The H_2S releasing capacity of essential oils obtained from ten commonly consumed organosulphur-rich fruits and vegetables was evaluated. Our work may provide new insights into the health benefits of these flavourful foods.

2. Materials and methods

2.1. Chemicals and materials

H_2S probe BCu was synthesized as previously reported (Wu et al., 2014). Diallyl trisulphide (DATS) was purchased from Abcam (Hong Kong, China) Ltd. Sodium sulphide nonahydrate ($Na_2S{\cdot}9H_2O$), anhydrous sodium sulphate (Na_2SO_4), dipropyl trisulphide, glutathione (GSH) and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich, Singapore. Diethyl ether

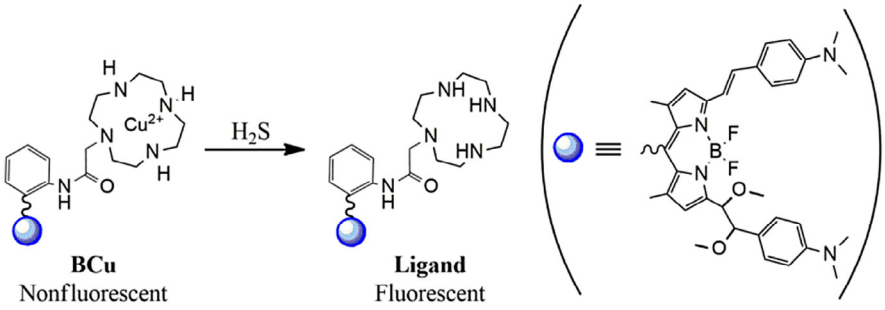

Fig. 1 – The structure and mechanism of H_2S probe BCu.

and dichloromethane were obtained from Merck Pte. Ltd. (Singapore). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Polar Lipids, Inc., Alabaster, AL, USA. Dulbecco's Modified Eagle Medium (DMEM) was purchased from HyClone Laboratories, Inc., Logan, UT, USA. Foetal bovine serum (FBS) was purchased from HyClone Ltd. (Cralington, UK). Penicillin–streptomycin was purchased from PAN Biotech (Adenbach, Germany). Phosphate buffered saline (PBS) was purchased from Vivantis Technologies Sdn. Bhd. (Selangor Darul Ehsan, Malaysia). Garlic, red onion, yellow onion, scallion, shallot, leek, spring onion, Chinese chive, durian, and stinky beans were purchased from local stores in Singapore.

2.2. *Cell culture*

Human breast cancer MCF-7 cells were purchased from American Type Culture Collection (ATCC), and were grown in DMEM medium, supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and were maintained at 37 °C and 5% CO_2.

2.3. *Extraction of essential oil*

The Likens–Nickerson simultaneous distillation and extraction apparatus (Yu, Wu, & Liou, 1989) was employed to obtain the volatile compounds from the materials. One kilogram of the foods were cleaned, cut, and blended into puree. The puree was mixed with deionized water (800~1000 mL), and the mixture was incubated under 25 °C for 30 min so that volatile organosulphur compounds could be generated through the enzyme-modulated reactions. The incubated homogenate was then subjected to Likens–Nickerson simultaneous distillation and extraction for 3 h, starting from the boiling of the homogenate. Diethyl ether (40 mL) was used as the extraction solvent and heated to 40 °C on water bath. The resulting diethyl ether extract was dried with anhydrous sodium sulphate before the solvents were evaporated at 45 °C. The obtained oil was weighed and stored under –20 °C for further use.

2.3.1. *Probe selectivity* in vitro

BCu in DMSO (1 mM, 100 μL) was vortexed, then an aliquot of 20 μL was added to 3.98 mL PBS buffer (10 mM, pH = 7.4) containing 0.5% Tween 20 in a glass cuvette and vortexed to give working solution of the probe (5 μM). Then the samples dissolved in 100 μL DMSO (DATS, dipropyl trisulphide, garlic oil, yellow onion oil, and stinky beans oil) or in 100 μL PBS buffer (Na_2S and GSH) were added. Control cuvette was added with equal amount of PBS buffer. The fluorescence intensity (λ_{ex} at 620 nm, λ_{em} at 680 nm) at designated intervals was measured with a HORIBA Jobin Yvon Fluoromax-4 fluorometer (Edison, NJ, USA).

2.4. *H_2S releasing capacity measurement*

The DOTAP liposome was used to deliver BCu into the cells. The BCu–DOTAP complex was prepared according to the previous method with slight modification (Yan et al., 2013). Briefly, BCu solid in dichloromethane (0.1 mM, 100 μL) was mixed with DOTAP (14 μL, 25 mg/mL in chloroform), and the solution was mixed thoroughly by pipetting. The volatiles were evaporated with a gentle argon stream. The film obtained was further dried under vacuum for 5 min to remove trace solvent, then hydrated with de-ionized water (0.5 mL) and sonicated for 10 min to give a clear bluish BCu–DOTAP solution, which was subsequently diluted with 4.5 mL of cell culture medium to generate a medium containing 20 μM working probe.

MCF-7 cells were seeded at a density of 2×10^4/well on a 96-well plate in 100 μL culture medium and incubated for 22 h. The surrounding 36 wells of the plate were filled with 100 μL culture medium to avoid edge effects. After incubation, the medium was changed with 100 μL freshly prepared medium containing 20 μM BCu probe in DOTAP liposome. After 3 hours of incubation, the probe-containing medium was drained, and cells were washed with 200 μL of PBS. Afterwards, 100 μL freshly prepared culture medium containing samples to be tested at designated concentration were added to each well and incubated for 2 hours. The fluorescence intensity (λ_{ex} at 620 nm, λ_{em} at 680 nm, sensitivity was set at 100, with lag time of 10 min) of each well was measured with a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) under 37 °C.

DATS and essential oil stock solutions were dissolved in DMSO at concentrations 100 times higher than their highest concentrations tested and were diluted with growth medium to desired concentrations immediately upon use. One per cent DMSO was used as control. Four replicates were set for each concentration, and at least three independent assays were carried out for each sample.

2.5. *Statistical analysis*

The results are given as means ± standard error of the mean (SEM). Data analysis was done in Origin 8.5 software (Northampton, MA, USA).

3. Results and discussion

3.1. *Selectivity of H_2S probe BCu*

The selectivity of BCu is crucial for this method. Previously we have shown that BCu demonstrated excellent selectivity to H_2S against a wide variety of biologically relevant reactive oxygen species (ROS) and reducing agents, including NO, nitric dioxide (NO_2), hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), peroxynitrite (ONOO-), superoxide (O_2-), hydroxyl radical (HO•), peroxyl radical (ROO), ascorbic acid, Trolox, L-cysteine, and GSH (Wu et al., 2014). Here, the selectivity of BCu towards sulphides has been studied. As shown in Fig. 2, treatment with 40 μM Na_2S (an inorganic H_2S donor) led to an instant 20 fold turn on of fluorescence, while 1.0 mM DATS and dipropyl trisulphide failed to lead to a fluorescence turn on. The reactivity of BCu towards three representative essential oil samples was also tested. The essential oil from garlic, yellow onion, and stinky beans, which represent a source of alkenyl, alkyl (Block, 1992) and cyclic (Gmelin et al., 1981) polysulphides, respectively, also had negligible effects on the fluorescence intensity of BCu, even at fairly high concentrations (100 μg/mL). These results confirmed that BCu is selective to H_2S, thus could be used in the study.

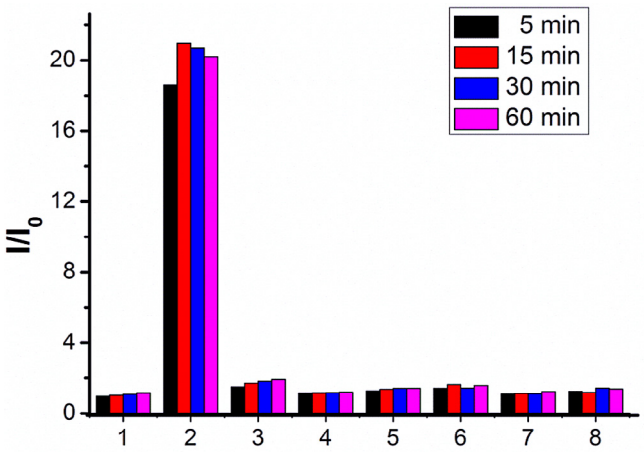

Fig. 2 – Selectivity of BCu towards organosulphides and essential oil in PBS buffer (10 mM, pH = 7.4). Bars represent the relative response of BCu at 680 nm after the addition of various analytes (λ_{ex} 620 nm). (1) control, (2) 40 μM Na_2S, (3) 5 mM GSH, (4) 0.5 mM dipropyl trisulphide, (5) 0.5 mM DATS, (6) 100 μg/mL garlic oil, (7) 100 μg/mL yellow onion oil, (8) 100 μg/mL stinky beans oil.

3.2. *Dose response in MCF-7 cells*

After confirming its selectivity, the probe was delivered into MCF-7 cells with the help of DOTAP liposome; our previous data showed that treatment with BCu–DOTAP complex for 3 h led to negligible cytotoxicity to the cells (Wu et al., 2014). As shown in Fig. 1A, both inorganic H_2S donor Na_2S and organic H_2S donor DATS increased the fluorescence intensity in cells linearly. Meanwhile, essential oil samples such as leek oil or stinky beans oil also elevated the fluorescence intensity in cells in a dose-dependent manner; however, the amount of fluorescence intensity gain were dramatically different, as shown in Fig. 1B and C, and 30 μg/mL of leek oil increased the fluorescence intensity for less than 3000, while 2 μg/mL stinky beans oil increased the fluorescence intensity to nearly 6000. Throughout the experiment, all the samples were loaded at appropriate concentrations determined from pre-experiments to allow the fluorescence intensity fall in the range of 6000~15,000, which is within the linearity range of the fluorescence. These results confirmed that these essential oils are H_2S donors.

3.3. *Determination of H_2S releasing capacity in cells*

To avoid run to run variation and to enable data comparison among different groups, we introduced the concept of DATS equivalent (DATS-E) to this assay. In practice, each plate was divided into two zones each with 48 wells; one zone was loaded with different concentrations of samples, the other with different concentrations of DATS. Regression curve for sample oil and DATS were obtained in parallel from the same plate (Fig. 3), and DATS-E was defined as:

$$DATS-E = \frac{S_{sample}}{S_{DATS}}$$

where S_{sample} and S_{DATS} are the slopes of the linear fitting line of the dose response curves of the sample and DATS respectively.

To normalize the H_2S releasing from each sample, the DATS-E of fresh fruits and vegetables was calculated by taking into account the essential oil yields; the results were shown in Table 1. Stinky beans, a culinary ingredient unique in Southeast Asia, topped the list with very high DATS-E value of 158.0, which means the H_2S released from oil distilled from one kilogram of fresh stinky beans equals to that from 158.0 mmol (or 28 g) of DATS. The high value of DATS-E for stinky beans was largely attributed to its high DATS-E value (94.3) of the essential oil. It has been reported that stinky beans contain mainly cyclic sulphur compounds (Gmelin et al., 1981), such as lenthionine, 1,2,4-trithiolane, 1,2,4,5-tetrathiane. These compounds have very high (>80%) content of sulphur. However, the high DATS-E value for stinky beans oil could not be explained solely by its high sulphur content since DATS also contains 54% (96/178) of sulphur. The other and likely more important reason should be the differences in their metabolism by cells; perhaps cyclic sulphur compounds could be converted into H_2S in a more efficient way than linear compounds. Garlic ranked second in the list with a DATS-E value of 18.53, which is much lower than that of stinky beans, albeit much higher than the rest samples. To our surprise, durian, which is notorious for its strong sulphury note, has the lowest DATS-E

Table 1 – H_2S releasing capacity of essential oil from organosulphur rich foods.

Rank	Name	Essential oil yield (g of oil/kg raw material)	DATS-E of oil (mmol DATS/g of oil)[a]	DATS-E of raw material (mmol DATS/kg of raw material)[a]
1	Stinky beans (*Parkia Speciosa*)	1.67	94.3 ± 4.39	158 ± 7.35
2	Garlic (*Allium sativum*)	5.29	3.50 ± 0.23	18.5 ± 1.21
3	Yellow onion (*Allium cepa*)	0.64	7.22 ± 0.20	4.59 ± 0.13
4	Scallion (*Allium fistulosum L.*)	0.97	2.21 ± 0.49	2.13 ± 0.47
5	Red onion (*Allium cepa*)	1.04	1.84 ± 0.26	1.92 ± 0.27
6	Leek (*Allium porrum L.*)	1.05	1.52 ± 0.29	1.59 ± 0.30
7	Spring onion (*Allium fistulosum L.*)	1.17	1.05 ± 0.14	1.23 ± 0.17
8	Shallot (*Allium cepa* var. *aggregatum*)	0.77	0.97 ± 0.09	0.75 ± 0.07
9	Chinese chive (*Allium tuberosum L.*)	1.6	0.46 ± 0.04	0.73 ± 0.06
10	Durian (*Durio zibethinus Murr.*)	1.2	0.33 ± 0.01	0.40 ± 0.01

[a] Data are expressed as mean ± SEM (n = 3~4).

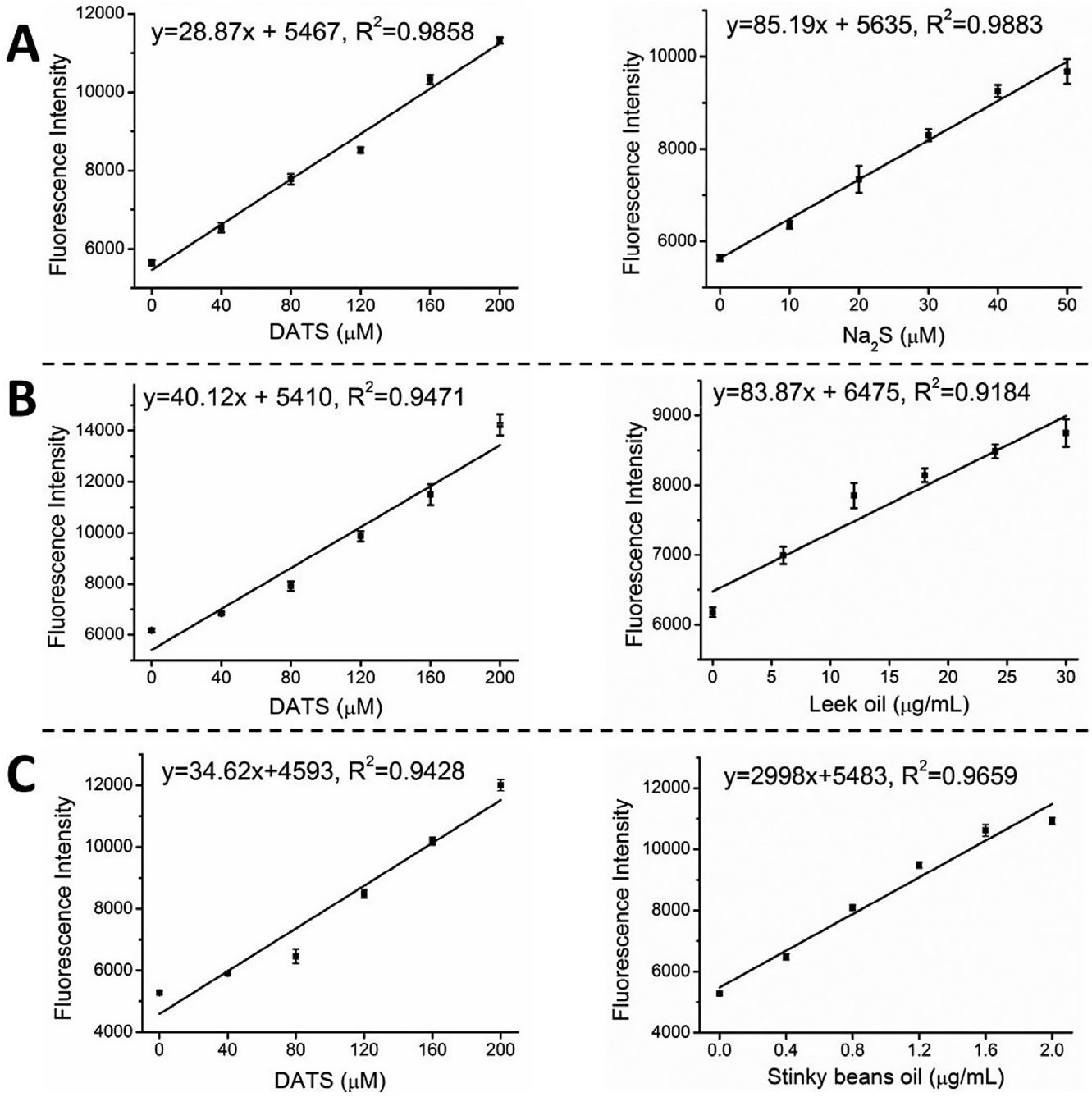

Fig. 3 – Representative curves of H_2S releasing assay for (A) DATS and Na_2S, (B) leek oil, (C) stinky beans oil in MCF-7 cells. Dash lines are used to separate data from different plates. Data are expressed as mean ± SEM of 4 replicates.

value, probably because of the existence of large amount of non-sulphur compounds in durian volatile (Weenen et al., 1996). Understandably, the flavour activity of the organosulphur compounds is not correlated with the H_2S releasing activity. The 3rd to 9th spots were occupied by other *Allium* species with DATS-E values ranging from 0.4 to 4.59. It should be pointed out that the high H_2S releasing activity may not be necessarily desired because slow but long-lasting H_2S donors could be desired under conditions where maintaining low but steady concentrations of H_2S is needed to keep the healthy level of H_2S as high burst of H_2S may have toxic effects.

Organosulphides are known for their intertransformation upon processing either in industrial scale or in household kitchen (Tocmo, Liang, Lin, & Huang, 2015). Heating pure DADS at 150 °C for less than 10 min gave significant amount of diallyl sulphide (DAS), DATS, diallyl tetrasulphide (5:5:1 ratio) and trace amount of other sulphides (Block et al., 1988). Previous study reported that diallyl sulphide did not release H_2S, while DATS released about three times H_2S more than DADS when reacted with glutathione (Benavides et al., 2007). Work from our group demonstrated that processing method (crushing, autoclaving, boiling, and freeze drying) and media pH had significant

influences on the quantitative and qualitative profiles of organosulphides obtained from shallot (Tocmo et al., 2014). Therefore, the results reported here only apply to the specified conditions employed in this study. How processing methods affect the H_2S releasing capacity of each foods warrant further study in order to optimize the potential health benefits of these vegetables as a naturally-occurring H_2S donor.

In summary, we reported a novel method for H_2S releasing capacity measurement in cells, and this method was applied to determine the H_2S releasing from ten commonly consumed organosulphur rich foods. We found that essential oil from stinky beans is a very strong H_2S donor, with a DATS equivalent of 94.3 mmol DATS/g or 1.7 times more active than DATS on equal weight basis. Fresh stinky beans are about 9 times higher than garlic in terms of H_2S releasing. This coupled with the unique cyclic sulphides found in stinky beans warrants further study to clarify their organosulphur profile and H_2S releasing activity and mechanism before their potential health benefits can be established.

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