A Novel Proof-of-Concept Sandwich Immunoassay for Screening Microcystin in Cyanobacteria Based on Michael Addition Reaction

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We present an innovative concept of a screening tool for detecting free microcystin in cyanobacteria using a sandwich immunodetection format, based on Michael addition reaction between α,β-unsaturated carbonyl moiety of microcystin and thiol of coating substance. This proof-of-concept immunoassay was developed using bovine serum albumin as a microcystin-binding model, and was tested with toxic Microcystis samples. The preliminary results indicate that the proposed Michael addition-based immunodetection is promising and can be used as a platform for further development to become a useful tool for free microcystin analysis in various samples in the future.

Keywords Microcystin, free form, sandwich immunoassay, Michael addition reaction, ELISA

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Introduction

Microcystin is a potent liver toxin mainly produced by freshwater bloom-forming cyanobacteria and poses a serious threat to public and ecosystem health.1 Structurally, microcystin is a cyclic heptapeptide typically consisting of Ala, Z, MeAsp, X, Adda, Glu, and Mdha, in which X and Z are two variable amino acids, MeAsp is erythro-β-methylasparte, Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, and Mdha is N-methyldehydroalanine (Fig. 1). The presence of α,β-unsaturated carbonyl moiety in Mdha enables microcystin to react with the thiol of amino acid, peptide, or protein via Michael addition reaction (Fig. 1),2,3 which has various physiological significances. In mammals, conjugating of microcystin with the cysteine residue of regulatory enzymes, protein phosphatases 1 and 2A eventually causes severe liver damage.4,5 In mammals and aquatic organisms, conjugating of microcystin with glutathione and cysteine involves microcystin detoxification.5,6 More recently, intracellular microcystin in the toxic cyanobacteria was found to covalently conjugate with protein. Such transformation of intracellular free microcystin form to the conjugated form was suggested to be physiologically important in protecting the microcystin producer from oxidative damage.7,8 Therefore, both free and conjugated microcystins coexist in the toxic cyanobacteria (e.g. Microcystis). However, a conjugated form is often less toxic than a free form.9,10 To accurately evaluate the toxic effect and biofunction of microcystin associated with toxic cyanobacteria blooms, differentiating and quantifying free and conjugated microcystin in toxic blooms are of great importance.

Traditional immunodetection, such as enzyme-linked immunosorbent assay (ELISA), detects total microcystin and cannot differentiate between the free and conjugated forms of microcystin.11–14 Chromatographic techniques, such as high performance liquid chromatography (HPLC) coupled with UV detector, or liquid chromatography coupled with mass spectrometry (LC-MS and LC-MS/MS), can be used to detect free microcystin variants and small, extractable microcystin conjugates that were formed from peptide and amino acid (when the corresponding standard is available),5,8,15,16 but are not applicable for detecting unextractable protein-conjugated microcystins that can account for a large proportion of total microcystin in Microcystis.5 In a pioneer work, Kaya et al. developed a set of approaches (colorimetry, HPLC/UV, and LC/MS) to determine free common microcystins with Mdha or Dha residue, via quantifying the related product of the conjugated complex of microcystin with glutathione (GSH) and trinitrobenzene sulfonate (TNBS).17 Their approaches were accurate, but required multiple rounds of chemical reactions and matrix cleanup steps. Recently, traditional ELISA and chromatographic techniques have been applied to detect total and free microcystin in toxic cyanobacteria, respectively, and the conjugated microcystin was indirectly determined by comparing the difference from the two methods.8 Although chromatographic techniques have excellent accuracy in free microcystin analysis, they require skilled personnel, expensive instruments, complex steps of matrix cleanup, and high-cost materials18,19 as well as necessary microcystin standards that are not always available. Microplate-based colorimetric immunoassay has been widely used as a conventional screening tool because of its outstanding advantage in easy and quick operation, and high-throughput detection as well as

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reasonable cost. Therefore, developing a facile and inexpensive microplate-based immunoassay for screening free microcystin in toxic cyanobacteria blooms is needed urgently.

In this study, we proposed a concept of a screening tool for free microcystin using a sandwich immunoassay based on the Michael addition reaction between the \( \alpha,\beta \)-unsaturated carbonyl moiety of free microcystin and the free thiol group of the coating proteins (most proteins harbor thiols). To demonstrate the proof-of-principle, a common protein, bovine serum albumin (BSA) that harbors thiol groups was chosen as a microcystin-binding model. The principle of the proposed novel sandwich immunoassay was depicted in Fig. 2. Briefly, BSA is coated on the microplate and further reduced with dithiothreitol to enable more free thiols to only bind with free microcystin, but not with the conjugated microcystin that has been bound with thiol-containing compounds. The free microcystin bound with coating protein on the microplate is subsequently recognized by primary antibody, followed by immunoreaction with peroxidase-labeled secondary antibody and subsequent color development. The output signal would be directly proportional to the concentration of free microcystin in a sample. The newly proposed method, together with a traditional ELISA, was tested with toxic *Microcystis* samples. Advantages, disadvantages, and suggestions for further improvement of the immunoassay were discussed.

**Experimental**

**Reagents and chemicals**

Microcystin standard, MC-LR (\( \geq 95\% \) purity, HPLC-grade) was purchased from Beagle Bioproducts Inc. (OH, USA). Mouse monoclonal antibody against the Adda of microcystin (AD4G2) was purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). The Nunc MaxiSorb ELISA microplate, bovine serum albumin (BSA), stabilized peroxidase conjugated goat-anti-mouse (H+L), 1-StepTM Ultra 3,3’,5,5’ tetramethylbenzidine (TMB) solution, and phosphate-buffered saline (PBS, 100 mM, pH 7.4) were purchased from Thermo Scientific (Waltham, MA, USA).

**Microcystis culture and field samples**

For this proof-of-concept study, three microcystin-producing *Microcystis* laboratory cultures (LE2, LE3, and LE4) were originally isolated from western Lake Erie where annual toxic *Microcystis* blooms have been observed since the mid-1990s. These isolated strains were cultivated in CT medium at 25°C under continuous light irradiance and their microcystin-producing genotype was validated with PCR using mcyA-specific primer set (mcyA-Cd1F/mcyA-Cd1R). In addition, two bloom samples were collected from the western basin of

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**Fig. 1** Michael addition reaction between \( \alpha,\beta \)-unsaturated carbonyl group (marked in red) in *N*-methyldehydroalanine (Mdha) of common microcystin and the thiol group of protein.

**Fig. 2** Schematic illustration of the novel sandwich immunoassay for microcystin detection based on the Michael addition reaction between free microcystin and thiol-containing protein (e.g. BSA).
Lake Erie during bloom season and were chosen for evaluating the natural microcystin transformation using the newly developed method and traditional ELISA. Without microcystin extraction and matrix cleanup procedures, Microcystis samples were directly subjected to five cycles of freezing-thawing. After centrifugation at 5000g, supernatant was collected, and the pH was adjusted to 9.6 and stored at −20°C until further microcystin analysis.

**Apparatus**

The absorbance of the final reaction solution in the immunoassay was read at 450 nm on the Dynex MRX microplate reader (Dynex Technologies Inc, Chantilly, VA, USA).

**New proof-of-concept sandwich immunoassay for free microcystin analysis**

As illustrated in Fig. 2, the microplate was coated with BSA (150 μL, 1%, in PBS buffer, pH 7.4) at 37°C for 1 h. After washing three times with PBS, dithiothreitol (DTT) (100 μL, 10 mM in 10 mM Tris-HCl buffer, pH 8.0) was added and incubated at 37°C for 30 min; after PBS washing, 100 μL of MC-LR standards (10^4, 10^3, 10^2, 10 μg/L) in 50 mM carbonate buffer, pH 9.6 or the supernatant samples of Microcystis were added and incubated for 45 min. The carbonate buffer (50 mM, pH 9.6) was used as a blank control. After washing, 100 μL of anti-Adda monoclonal antibody (1:2500 dilution with 10 mM PBS, pH 7.4) was added and incubated at 37°C for 30 min. After washing three times with PBST [10 mM PBS, pH 7.4, Tween-20 (0.05%, v/v), horseradish peroxidase (HRP)-labeled antibody AD4G2, which exhibits good cross-reactivity with common microcystin variants,12,13 was utilized to recognize the free microcystin bound to the coated BSA on the microplate.

**Traditional ELISA for total microcystin analysis**

The concentration of total microcystin in the Microcystis sample was evaluated using a commercial Microcysts/ Nodularin (ADDA) ELISA kit (Product No. 522011, Abraxis, Warminster, PA, USA) according to the manufacturer’s instruction. This kit utilizes an indirect competitive immunoreaction format with MC-LR as the working standard (0.15–5 μg/L). A sample with the microcystin concentration above the upper detection limit (5 μg/L) was further diluted to fall within their measurable range.

**Statistical analysis**

The relative difference of microcystin concentration from Microcystis samples detected by the proposed method and the commercial ELISA kit was evaluated using the following equation: the relative difference (%) = 100 × (CeLISA − Cnew) / CeLISA, in which CeLISA and Cnew denoted the microcystin concentration detected by the ELISA kit and the proposed proof-of-concept method, respectively. The difference in microcystin concentration detected by the two methods was statistically evaluated using SPSS software 22.0 (IBM Corporation, USA) with paired Student’s t-test at a significant level (p <0.05).

**Results and Discussion**

To demonstrate the proof-of-concept of the screening tool for free microcystin using the Michael addition reaction-based sandwich immunoassay, BSA, which is one of most common protein (easily available) and contains one free cysteine and 17 intramolecular disulfides,23 was selected as a microcystin-binding model protein. The BSA was further reduced with dithiothreitol to generate more microcystin-reactive thiols. MC-LR was chosen as a free microcystin standard and monoclonal antibody AD4G2, which exhibits good cross-reactivity with common microcystin variants,12,13 was utilized to recognize the free microcystin bound to the coated BSA on the microplate.

**Standard curve**

The standard curve of the proof-of-concept immunooassay was obtained from a serial dilution of MC-LR standard solution (10^4, 10^3, 10^2, and 10 μg/L). The response (%) was calculated according to the following equation: Response = OD_{sample}/OD_{max}, in which OD_{sample} and OD_{max} was the optical density (OD) of the reaction solution at 450 nm for the test sample and 10^5 μg/L of MC-LR (generating the maximum OD signal), respectively. Figure 3 shows the standard curve of the newly developed immunooassay for MC-LR (10^4, 10^3, 10^2, and 10 μg/L), which was based on the experiments in triplicate. The response was positively proportional to the MC-LR concentration. The newly developed method had a wide linear quantitative range (10^2 - 10^5 μg/L, R^2 = 0.9478) with the detection limit at 100 μg/L. Using one of the most common protein BSA, this study has succeeded to develop a proof-of-concept immunooassay for screening free microcystin based on the Michael addition reaction between microcystin and protein. Moreover, this immunooassay was sufficiently sensitive to screen free microcystin in most of toxic Microcystis laboratory cultures and field bloom samples since they had microcystin concentrations typically higher than 100 μg/L. Indeed, high microcystin concentrations (>100 μg/L) were often documented in various Microcystis laboratory cultures,8,9,23 and was also frequently detected in algal bloom samples elsewhere. For example, over 300 μg/L of microcystin was previously detected in the Microcystis bloom sample of western Lake Erie (OH, USA),25 and up to 8428.6 μg/L was also documented in the toxic cyanobacteria bloom samples in a western Australian wetland.26 The developed proof-of-concept immunooassay would be particularly suitable for screening free microcystin in laboratory and algal bloom samples of cyanobacteria typically containing high concentrations of microcystins without the requirement of tedious blind dilution.
detects total microcystin (free and conjugated microcystin). This study detects free microcystin whereas the commercial ELISA kit detects total microcystin (free and conjugated microcystin). In principle, the proof-of-concept immunoassay proposed in this study detects free microcystin whereas the commercial ELISA kit detects total microcystin (free and conjugated microcystin).

Currently, it is acknowledged that both free and conjugated microcystins coexist in toxic cyanobacteria. Indeed, several studies recently utilized the immunoblotting technique to demonstrate the presence of a significant fraction of microcystin covalently bound with protein in the laboratory cultures and field samples of Microcystis from Lake Erie. Indeed, several studies recently utilized the immunoblotting technique to demonstrate the presence of a significant fraction of microcystin covalently bound with protein in the laboratory cultures and field samples of Microcystis.

Because of the importance and urgency of understanding the biofunction of microcystin and evaluating the risk of microcystin to public health, laboratory cultures and field bloom samples of toxic cyanobacteria are being frequently studied by traditional ELISA and chromatographic techniques (HPLC, LC-MS, and LC-MS/MS). However, because of the unextractable characteristic, the protein-conjugated microcystins in toxic Microcystis can be neglected in the HPLC and LC-MS analysis whereas the free microcystin form in toxic Microcystis can be overestimated in the traditional ELISA. Therefore, this proof-of-concept immunoassay would be a simple and useful alternative tool for direct screening of free microcystin in bloom samples without requiring microcystin extraction and matrix cleanup as well as blind dilutions, thus, it can significantly save analysis time. Combining the newly developed sandwich immunoassay with traditional ELISA would be an alternative for indirect screening of the conjugated form in the samples.

### Application of the proof-of-concept immunoassay to real toxic cyanobacteria samples

The newly developed proof-of-concept immunoassay together with traditional competitive ELISA was further tested with Microcystis samples (3 laboratory cultures and 2 algal bloom samples). As shown in Table 1, the detection results of the proposed method (192–1018 μg/L) were consistently and significantly lower than that detected by the traditional ELISA (239–1779 μg/L) (p <0.05, paired Student’s t-test). The relative difference of detection results between traditional ELISA and the proposed method ranged from 16 to 43% (mean = 29%). Considering that (i) conjugated microcystin form cannot react with the thiol-containing protein as previously demonstrated, and (ii) microcystin in the Microcystis samples of western Lake Erie consisted of common variants MC-LR and MC-RR, seldom included the rare microcystins that do not harbor the α,β-unsaturated carbonyl, or the rare microcystins that have Mdhb or Dhb residue (non-thiol-reactive), the difference observed between the traditional ELISA and the newly proposed assay was most likely associated with the presence of a considerable proportion of conjugated microcystins in both laboratory and field bloom samples of Microcystis from Lake Erie. Indeed, several studies recently utilized the immunoblotting technique to demonstrate the presence of a significant fraction of microcystin covalently bound with protein in the laboratory cultures and field samples of Microcystis.

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### Table 1: Microcystin concentrations in Microcystis samples evaluated by a commercial ELISA kit and the proposed method

| Sample code | Sample type | Commercial ELISA kit Mean ± SD/μg L⁻¹ | Proposed method Mean ± SD/μg L⁻¹ | Relative difference, % |
|-------------|-------------|-------------------------------------|----------------------------------|-----------------------|
| 1           | Microcystis LE2 | 239 ± 15                           | 192 ± 157                       | 20                    |
| 2           | Microcystis LE3 | 559 ± 15                           | 469 ± 47                        | 16                    |
| 3           | Microcystis LE4 | 1779 ± 174                         | 1018 ± 121                      | 43                    |
| 4           | Field sample 1  | 302 ± 1                            | 227 ± 70                        | 25                    |
| 5           | Field sample 2  | 995 ± 124                          | 573 ± 44                        | 42                    |

- In principle, the proof-of-concept immunoassay proposed in this study detects free microcystin whereas the commercial ELISA kit detects total microcystin (free and conjugated microcystin).
- SD, standard deviation.

Advantages, disadvantages, and perspective of the proof-of-concept immunoassay

While various types of immunodetection approaches have been developed for detecting total microcystins in the past decades, until this study, no previous studies were designed in an attempt to screen free microcystin. This study proposes here a brand new concept of screening for the free microcystin form using a sandwich immunodetection format based on Michael addition reaction between free microcystin and thiol of protein. To demonstrate the concept, a common protein, BSA, was selected as a microcystin-binding model protein. The results are promising and demonstrate the feasibility of developing a Michael addition reaction-based immunoassay for screening free microcystin. The proof-of-concept immunoassay based on the BSA was sufficiently sensitive (~100 μg/L) for screening free microcystin in the toxic Microcystis cultures and bloom samples, which have typically high microcystin concentrations and are frequently studied. In addition, the proposed Michael addition reaction-based immunodetection has the following promising features: 1) inexpensive—all the required reagents and instruments are easily available and inexpensive, for example, thiol-containing compound (e.g. BSA) is very common and the TMB/H₂O₂-based colorimetric detection system is the most widely used in immunoassays; 2) simple and fast—the proposed immunoassay is simple and easy to operate, and does not require microcystin extraction and matrix clean-up steps, thus saving time for analysis time; and 3) versatile—a wide variety of thiol-containing compounds can potentially bind with free microcystin via Michael addition reaction, thus one can expect a wide choice of microcystin-binding compounds, which can be used as a platform for further developing various types of Michael addition-based immunoassays in screening of free microcystin.

This proof-of-concept immunoassay was not as sensitive compared to conventional competitive ELISA that typically detects ~0.1 μg/L of total microcystin in various sample types. This can be due to the limited microcystin-binding capacity of the coating protein BSA used in the present proof-of-concept experiment. Indeed, only one native cysteine residue and 17 intramolecular disulfides are present in the native BSA, which was subsequently reduced by dithiothreitol to enhance the number of microcystin-reactive thiols.

Theoretically, according to the principle of Michael addition reaction, the thiol only binds to free common microcystins (e.g. [Mhda¹ or Dha¹] microcystin) that harbors α,β-unsaturated carbonyl moiety, rather than the unusual microcystins that have a saturated moiety at unit 7 or the conjugated form of common microcystin with thiol-containing substance. Indeed, this was recently confirmed in multiple papers. Kaya et al. showed that the thiols of cysteine and glutathione did not attack the unusual [Dhb¹] microcystin and [L-Ser² or L-Ala³] microcystin, and that only the common microcystins were labeled. Zilléges et al. demonstrated that only free common microcystin, rather than the conjugated form, can react with thiol-containing protein. Therefore, the proposed Michael addition reaction-based immunoassay in this study should exclusively screen the free normal microcystins that harbor Mdhb or Dhb residue, rather than the conjugated form of common microcystin and the rare microcystins that harbor Mdhb or Dhb residue, or saturated moiety at unit 7 (non-thiol-reactive).

The aim of this study was to demonstrate the feasibility of a Michael addition reaction-based immunodetection for free microcystin. While the present proof-of-concept immunoassay is promising, further improvement, especially the sensitivity of the method, is certainly warranted in the future. As the detection...
limit of such type of Michael addition reaction-based immunoassay largely depends on the capacity of the used substance to bind with microcystin, one can anticipate that a more sensitive sandwich immunoassay can be developed if a new type of microcystin-binding substance (e.g., with more microcystin-reactive thiol(s)) is optimized in the future. Therefore, prior to application of the proposed immunodetection format to various types of samples, future systematic investigation should focus on i) improving the sensitivity via optimizing the microcystin-binding substances and the reaction condition, and ii) validating the proposed Michael addition-based immunoassay with chromatographic techniques (e.g., LC-MS/MS) and the free microcystin-selective method developed by Kaya et al. as well as using a large sample size.

Conclusion
Most microcystin variants harbor α,β-unsaturated carbonyl moiety in the Mdha or Dha residue, which can covalently bind with free thiols of amino acid, peptide, and protein via Michael addition reaction. Both free and conjugated forms of microcystin coexist in toxic cyanobacteria and the free microcystin form is typically more toxic than the conjugated form. Thus, developing an immunoassay for screening free microcystin is in urgent need. To our knowledge, this study makes the first effort to develop a microplate-based immunoassay for screening the free form of microcystin. This study proposed a new concept of screening for the free microcystin using a sandwich immunodetection format based on Michael addition reaction between the α,β-unsaturated carbonyl moiety of free microcystin and thiol of coating protein on the microplate. The newly developed assay was sufficiently sensitive to screen microcystins in laboratory cultures and field bloom samples. While the present proof-of-concept immunoassay is promising, future studies should focus on improving the detection sensitivity and validating the newly developed method with chromatographic techniques and other free microcystin-selective methods.

Based on the promising detection features (versatile, simple, facile, and inexpensive) of the Michael addition reaction-based immunodetection, one can anticipate that the new Michael addition reaction-based immunodetection proposed in this study can be a useful tool for free microcystin screening.

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References
1. J. Lee, S. Lee, and X. Jiang, Annu. Rev. Food Sci. Technol., 2017, 8, 281.
2. F. Kondo, Y. Ikai, H. Oka, M. Okumura, N. Ishikawa, K. Harada, K. Matsuura, H. Murata, and M. Suzuki, Chem. Res. Toxicol., 1992, 5, 591.
3. R. W. Mackintosh, K. N. Dalby, D. G. Campbell, P. T. Cohen, P. Cohen, and C. Mackintosh, FEBS Lett., 1995, 371, 236.
4. M. Runnegar, N. Berndt, S. M. Kong, E. Y. Lee, and L. Zhang, Biochem. Biophys. Res. Commun., 1995, 216, 162.
5. M. Dai, P. Xie, G. Liang, J. Chen, and H. Lei, J. Chromatogr. B, 2008, 862, 43.
6. F. Kondo, H. Matsumoto, S. Yamada, N. Ishikawa, E. Ito, S. Nagata, Y. Ueno, M. Suzuki, and K. Harada, Chem. Res. Toxicol., 1996, 9, 1355.
7. Y. Zilliges, J. C. Kehr, S. Meissner, K. Ishida, S. Mikkat, M. Hagemann, A. Kaplan, T. Börner, and E. Dittmann, PLoS One, 2011, 6, e17615.
8. S. Meissner, J. Fastner, and E. Dittmann, Environ. Microbiol., 2013, 15, 1810.
9. N. Wei, L. Hu, L. Song, and N. Gan, Toxins, 2016, 8, 293.
10. J. S. Metcalf, K. A. Beattie, S. Pflugmacher, and G. A. Codd, FEMS Microbiol. Lett., 2000, 189, 155.
11. C. Hu, N. Gan, Z. He, and L. Song, Int. J. Environ. Anal. Chem., 2008, 88, 267.
12. A. Zeck, M. G. Weller, D. Bursill, and R. Niessner, Analyst, 2002, 126, 2002.
13. M. G. Weller, A. Zeck, A. Eikenberg, S. Nagata, Y. Ueno, and R. Niessner, Anal. Sci., 2001, 17, 1445.
14. M. G. Weller, Sensors (Basel), 2013, 13, 15085.
15. L. A. Lawton, C. Edwards, and G. A. Codd, Analyst, 1994, 19, 1525.
16. K. M. Karlsson, L. E. Spoof, and J. A. Meriluoto, Environ. Toxicol., 2005, 20, 381.
17. K. Kaya, T. Sano, H. Inoue, and H. Takagi, Anal. Chim. Acta, 2001, 450, 73.
18. H. Li, X. Wei, C. Gu, K. Su, H. Wan, N. Hu, and P. Wang, Anal. Sci., 2018, 34, 893.
19. Y. Lv, X. Zhao, L. Yang, X. Zhang, and Y. Bai, Anal. Sci., 2018, 34, 421.
20. X. Yin, S. Wang, X. Liu, C. He, Y. Tang, Q. Li, J. Liu, H. Su, T. Tan, and Y. Dong, Anal. Sci., 2017, 33, 659.
21. M. M. Watanabe and T. Ichimura, Bull. Jpn. Soc. Phycol., 1977, 25(Suppl), 371.
22. M. Hisbergues, G. Christiansen, L. Rouhiainen, K. Sivonen, and T. Börner, Arch. Microbiol., 2003, 180, 402.
23. S. T. Kelly and A. L. Zdyney, Biotechnol. Bioeng., 1994, 44, 972.
24. T. Bui, T. Dao, T. Vo, and M. Luerling, Toxins, 2018, 10, 123.
25. C. Hu, C. Rea, Z. Yu, and J. Lee, J. Appl. Microbiol., 2016, 120, 138.
26. A. Kemp and J. John, Environ. Toxicol., 2006, 21, 125.
27. J. Dyble, G. L. Fahnenstiel, R. W. Litaker, D. F. Millie, and P. A. Tester, Environ. Toxicol., 2008, 23, 507.