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Safety evaluation of the food enzyme xylose isomerase from the genetically modified *Streptomyces rubiginosus* strain DP-Pzn37

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Abstract

The food enzyme is a D-xylose aldose-ketose-isomerase (EC 5.3.1.5) produced with the genetically modified *Streptomyces rubiginosus* strain DP-Pzn37 by Danisco US Inc. Although the production strain contains antibiotic resistance genes, the food enzyme was shown to be free from viable cells of the production organism and its DNA. The food enzyme is intended to be used in an immobilised form for the isomerisation of glucose for the production of high fructose syrups. Residual amounts of total organic solids (TOS) are eliminated by the use of an immobilised food enzyme and further removed by the purification steps applied during the production of high fructose syrups using the immobilised enzyme; consequently, dietary exposure was not calculated. Genotoxicity tests did not raise safety concerns. The systemic toxicity was assessed by a repeated dose 90-day oral toxicity study in rats. The Panel identified a no observed adverse effect level of 85.2 mg TOS/kg body weight (bw) per day, the highest dose tested. Similarity of the amino acid sequence to those of known allergens was searched and no match was found. The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions by dietary exposure cannot be excluded, but the likelihood is considered to be low. Based on the data provided, the immobilisation process and the removal of total organic solids during the production of high fructose syrups, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

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1. Introduction

Article 3 of the Regulation (EC) No 1332/2008[1] provides definition for 'food enzyme' and 'food enzyme preparation'.

> 'Food enzyme' means a product obtained from plants, animals or micro-organisms or products thereof including a product obtained by a fermentation process using micro-organisms: (i) containing one or more enzymes capable of catalysing a specific biochemical reaction; and (ii) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport or storage of foods.
>
> 'Food enzyme preparation' means a formulation consisting of one or more food enzymes in which substances such as food additives and/or other food ingredients are incorporated to facilitate their storage, sale, standardisation, dilution or dissolution.

Before January 2009, food enzymes other than those used as food additives were not regulated or were regulated as processing aids under the legislation of the Member States. On 20 January 2009, Regulation (EC) No 1332/2008 on food enzymes came into force. This Regulation applies to enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food, including enzymes used as processing aids. Regulation (EC) No 1331/2008[2] established the European Union (EU) procedures for the safety assessment and the authorisation procedure of food additives, food enzymes and food flavourings. The use of a food enzyme shall be authorised only if it is demonstrated that:

i) it does not pose a safety concern to the health of the consumer at the level of use proposed;
ii) there is a reasonable technological need;
iii) its use does not mislead the consumer.

All food enzymes currently on the European Union market and intended to remain on that market, as well as all new food enzymes, shall be subjected to a safety evaluation by the European Food Safety Authority (EFSA) and approval via an EU Community list.

The 'Guidance on submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) lays down the administrative, technical and toxicological data required.

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background as provided by the European Commission

Only food enzymes included in the European Union (EU) Community list may be placed on the market as such and used in foods, in accordance with the specifications and conditions of use provided for in Article 7 (2) of Regulation (EC) No 1332/2008 on food enzymes.

Five applications have been introduced by the companies "BASF Enzymes LLC1" for the authorisation of the food enzyme Alpha-amylase from a genetically modified strain of *Pseudomonas fluorescens* (BD15754), "DSM Food Specialties B.V." for the authorisation of the food enzyme Phospholipase C from a genetically modified strain of *Pichia pastoris* (PRF), and "Danisco US Inc." for the authorisation of the food enzymes Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (DP-Dzb25), Xylose isomerase from a genetically modified strain of *Streptomyces rubiginosus* (DP-Pzn37), and Alpha-amylase from a genetically modified strain of *Bacillus amyloliquefaciens* (DP-Czb53).

Following the requirements of Article 12.1 of Commission Regulation (EU) No 234/2011[3] implementing Regulation (EC) No 1331/2008[2], the Commission has verified that the five applications fall within the scope of the food enzyme Regulation and contain all the elements required under Chapter II of that Regulation.

[1] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on Food Enzymes and Amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97. OJ L 354, 31.12.2008, pp. 7–15.

[2] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, pp. 1–6.

[3] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 64, 11.3.2011, p. 15–24.

1.1.2. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out the safety assessments on the food enzymes Alpha-amylase from a genetically modified strain of *Pseudomonas fluorescens* (strain BD15754), Phospholipase C from a genetically modified strain of *Pichia pastoris* (strain PRF), Alpha-amylase from a genetically modified strain of *Bacillus licheniformis* (strain DP-Dzb25), Xylose isomerase from a genetically modified strain of *Streptomyces rubiginosus* (strain DP-Pzn37), and Alpha-amylase from a genetically modified strain of *Bacillus amyloliquefaciens* (strain DP-Czb53) in accordance with Article 17.3 of Regulation (EC) No 1332/2008 on food enzymes.

1.2. Interpretation of the Terms of Reference

The present scientific opinion addresses the European Commission's request to carry out the safety assessment of food enzyme xylose isomerase from a genetically modified *S. rubiginosus* (strain DP-Pzn37).

2. Data and methodologies

2.1. Data

The applicant has submitted a dossier in support of the application for authorisation of the food enzyme xylose isomerase produced with a genetically modified *S. rubiginosus* strain DP-Pzn37. The dossier was updated on 28 July 2016.

Additional information was requested from the applicant during the assessment process on 29 May 2017, 28 November 2018 and 25 June 2019, and was consequently provided (see 'Documentation provided to EFSA').

Following the reception of additional data by EFSA on 26 April 2018, EFSA requested a clarification teleconference, which was held on 16 May 2018; after which the applicant provided additional data on 1 June 2018.

Following the requests for additional data sent by EFSA on 29 May 2017, 28 November 2018 and 25 June 2019, the applicant requested clarification teleconferences, which were held on 20 June 2017, 20 May 2019 and 5 July 2019.

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA 'Guidance on transparency in the scientific aspects of risk assessment' (EFSA, 2009b) as well as in the EFSA 'Statement on the characterisation of microorganisms used for the production of food enzymes' (EFSA CEP Panel, 2019) and following the relevant existing guidance's of EFSA Scientific Committees.

The current 'Guidance on the submission of a dossier on food enzymes for safety evaluation' (EFSA, 2009a) has been followed for the evaluation of the application with the exception of the exposure assessment, which was carried out in accordance to the methodology described in the CEF Panel statement on the exposure assessment of food enzymes (EFSA CEF Panel, 2016).

3. Assessment

IUBMB nomenclature:	Xylose isomerase
Systematic name:	D-xylose aldose-ketose-isomerase
Synonyms:	Glucose isomerase; D-xylose isomerase; D-xylose ketol-isomerase
IUBMB No:	EC 5.3.1.5
CAS No:	9023-82-9
EINECS No:	232-944-6.

The xylose isomerase catalyses the conversion of D-xylose to D-xylulose and of D-glucose to D-fructose. It is intended to be used for the isomerisation of glucose for the production of high fructose syrups. The food enzyme is intended to be used only in an immobilised form.[4] Based on its technical application, the term glucose isomerase is used throughout this dossier.

[4] Technical dossier/Additional data April 2018.

3.1. Source of the food enzyme

The glucose isomerase is produced with a genetically modified bacterium *S. rubiginosus* strain DP-Pzn37, which is deposited at the Westerdijk Fungal Biodiversity Institute (The Netherlands) with the deposit number [redacted][4]. The taxonomic identification of the production strain was performed on the basis of [redacted].

The whole genome sequence of the production strain was analysed for the presence of antimicrobial resistance genes. [redacted][5]

3.1.1. Characteristics of the parental and recipient microorganisms

The parental microorganism is the Actinobacterium *S. rubiginosus* strain [redacted].

The recipient strain *S. rubiginosus* [redacted] was derived [redacted][6]

3.1.2. Characteristics of the introduced sequences

The donor for the glucose isomerase encoding gene is [redacted][7]

[redacted][8]

3.1.3. Description of the genetic modification process

The production strain *S. rubiginosus* DP-Pzn37 was developed from the recipient strain [redacted][8]

[redacted]

3.1.4. Safety aspects of the genetic modification

The technical dossier contains all necessary information on the recipient microorganism, the donor organism and the genetic modification process.

The production strain DP-Pzn37 differs from the recipient strain [redacted][7]

Genetic stability was demonstrated [redacted][9]

[redacted][5]

3.2. Production of the food enzyme

The food enzyme is manufactured according to the Food Hygiene Regulation (EC) No 852/2004[10], with food safety procedures based on hazard analysis and critical control points, and in accordance with current Good Manufacturing Practice.[11]

[5] Technical dossier/Additional data May 2019/Annex AH.
[6] Technical dossier/1st submission/Annex S and T.
[7] Technical dossier/1st submission/Annex S and Technical dossier/2nd submission/Annex U.
[8] Technical dossier/1st submission/Annex S.
[9] Technical dossier/2nd submission/Annex Z.
[10] Regulation (EC) No. 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of food additives. OJ L 226, 25.6.2004, pp. 3–21.
[11] Technical dossier/2nd submission/p. 40.

The production strain is grown as a pure culture using a typical industrial medium in a submerged, batch or fed-batch fermentation system with conventional process controls in place. After completion of the fermentation, the cells are lysed and the enzyme is immobilised [redacted] The immobilised food enzyme preparation is then separated from the liquid fraction by filtration. Afterwards, the preparation is extruded, dried and sieved.[12] The applicant provided information on the identity of the substances used to control the fermentation and in the subsequent downstream processing of the food enzyme.[4]

The Panel considered that sufficient information has been provided on the manufacturing process and the quality assurance system implemented by the applicant to exclude issues of concern.

3.3. Characteristics of the food enzyme

3.3.1. Properties of the food enzyme

The glucose isomerase is a single polypeptide of [redacted] amino acids.[13] The molecular mass, derived from the amino acid sequence, was calculated to be [redacted] kDa.[14] The food enzyme was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A consistent protein pattern was observed across all batches. The gels showed a single major protein band corresponding to an apparent molecular mass of about [redacted] kDa, consistent with the expected mass of the enzyme.[15] No other enzymatic side activities were reported.

The in-house determination of free glucose isomerase activity is based on the conversion of glucose to fructose (reaction conditions: pH 6.85, 30°C, 30 min). The enzymatic activity is determined by measuring the fructose formed by high-performance liquid chromatography (HPLC). The glucose isomerase activity is quantified relative to an internal enzyme standard and expressed in glucose isomerase units (GIU)/g.[16]

The free food enzyme has a temperature optimum around 80–85°C (pH 7.5) and a pH optimum around pH 8.0–8.5 (60°C). Thermostability was tested after a pre-incubation of the food enzyme for 32 min at different temperatures (pH 7.5). The glucose isomerase activity decreased rapidly above 85°C, showing no residual activity above 95°C.[17]

3.3.2. Chemical parameters

Data on the chemical parameters of the food enzyme were provided for five food enzyme batches, three batches used for commercialisation and two batches produced for the toxicological tests (Table 1).[18] The average total organic solids (TOS) of the three food enzyme batches for commercialisation was 14.4%. The average enzyme activity/TOS ratio of the three food enzyme batches for commercialisation is 21.2 GIU/mg TOS.

[12] Technical dossier//1st submission/Annex K and S and Additional data May 2019/Annex AA.
[13] Technical dossier/1st submission/Annex G.
[14] Technical dossier/Additional data May 2019.
[15] Technical dossier/2nd submission/p. 30.
[16] Technical dossier/1st submission/Annex C.
[17] Technical dossier/2nd submission/p. 34–36.
[18] Technical dossier/Additional data June 2018/Annexes F, G, H, L and M.

Table 1: Compositional data of the food enzyme prior to immobilisation and batches used for toxicological studies

Parameter	Unit	Batches				
		1	2	3	4[(a)]	5[(b)]
Glucose isomerase activity	GIU/g batch[(c)]	2,831	3,238	2,980	434.32	3,530
Protein	%	12.30	14.47	11.37	4.79	20.19
Ash	%	0.17	0.73	0.37	2.30	9.61
Water	%	85.77	82.26	87.48	89.18	44.00
Total organic solids (TOS)[(d)]	%	14.06	17.01	12.15	8.52	46.39
Glucose isomerase activity/mg TOS	GIU/mg TOS	20.14	19.04	24.53	5.10	7.61

(a): Batch used for the Bacterial reverse mutation test and Repeated dose 90-day oral toxicity study in rodents.
(b): Batch used for the *in vitro* chromosomal aberrations test.
(c): GIU/g: glucose isomerase units/g (see Section 3.3.1).
(d): TOS calculated as 100% – % water – % ash.

3.3.3. Purity

The lead content in the three commercial batches and in the two batches used for toxicological studies was below 0.05 mg/kg which complies with the specification for lead (< 5 mg/kg) as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006).[18,19]

The food enzyme complies with the microbiological criteria as laid down in the general specifications and considerations for enzymes used in food processing (FAO/WHO, 2006), which stipulate that *Escherichia coli* and *Salmonella* species are absent in 25 g of sample and total coliforms should not exceed 30 colony forming units (CFU) per gram.[18] No antimicrobial activity was detected in any of these batches (FAO/WHO, 2006).[20]

The Panel considered that the information provided on the purity of the food enzyme is sufficient.

3.3.4. Viable Cells and DNA of the production strain

The absence of the production strain has been demonstrated [redacted] The production strain was not detected [redacted][21]

No recombinant DNA was detected [redacted][22]

3.4. Toxicological assessment

A battery of toxicological tests including a bacterial gene mutation assay (Ames test), an *in vitro* mammalian chromosomal aberration test, and a repeated dose 90-day oral toxicity study in rats has been provided. The batches 4 and 5 (Table 1) used in these studies have lower specific activity compared to the batches used for commercialisation, and thus are considered suitable for toxicological testing.

3.4.1. Genotoxicity

3.4.1.1. Bacterial reverse mutation test

A bacterial reverse mutation assay (Ames test) was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (OECD, 1997a) and following Good Laboratory Practice (GLP).[23] Five strains of *Salmonella* Typhimurium (TA 98, TA 100, TA 102, TA

[19] LoD: Pb = 0.05 mg/kg.
[20] Technical dossier/Additional data June 2018/Annex I.
[21] Technical dossier/2nd submission/Annex E and Technical dossier/Additional data April 2018.
[22] Technical dossier/Additional data September 2019.
[23] Technical dossier/1st submission/Annex M.

1535, TA 1537) were tested in the presence or absence of metabolic activation applying the 'treat and plate' assay. Two experiments were carried out using five different concentrations of the food enzyme for strains TA 98, TA 100, TA 102, TA 1535 (50, 166, 500, 1,660 and 5,000 μg total protein/plate, corresponding to 88.9, 295, 889, 2,953 and 8,894 μg TOS/plate), and three experiments using ten different concentrations for strain TA 1537 (0.166, 0.5, 1.66, 5, 16.6, 50, 166, 500, 1,660 and 5,000 μg total protein/plate, corresponding to 0.30, 0.89, 2.95, 8.89, 29.5, 88.9, 295, 889, 2,953 and 8,894 μg TOS/plate). Growth reductions of the background lawn were observed, in some cases severe, due to toxicity. Upon treatment with the food enzyme, there was no significant increase in revertant colony numbers above the control values in any strain with or without S9-mix.

The Panel concluded that the food enzyme glucose isomerase did not induce gene mutations under the test conditions employed in this study.

3.4.1.2. *In vitro* mammalian chromosomal aberrations test

The *in vitro* chromosomal aberrations test was carried out according to the OECD Test Guideline 473 (OECD, 1997b) and following GLP.[24] The food enzyme was tested for its ability to induce chromosomal aberrations in human peripheral blood lymphocytes with and without metabolic activation (S9-mix). Based on the results obtained in a dose-range finding test, the cells were treated with 1,250, 2,500 and 5,000 total protein μg/mL (corresponding to 2,872, 5,744 and 11,488 μg TOS/mL) applying a 4 + 20 h short-term treatment and recovery in the presence and absence of S9-mix, and a continuous treatment (20 + 0 h) in the absence of S9-mix. In all the tested conditions, the frequency of cells with structural and numerical chromosomal aberrations in treated cultures was comparable to the values detected in negative controls and within the range of the laboratory historical negative control data.

The Panel concluded that the food enzyme glucose isomerase did not induce chromosomal aberrations under the test conditions employed for this study.

3.4.2. Repeated dose 90-day oral toxicity study in rodents

The repeated dose 90-day oral toxicity study in rodents was performed in accordance with OECD Test Guideline 408 (OECD, 1998), and following GLP.[25] Groups of 10 male and 10 female Ntac:SD Sprague–Dawley rats received the food enzyme by gavage in doses corresponding to 21.3, 42.6 and 85.2 mg TOS/kg body weight (bw) per day. Controls received the vehicle (saline solution 2% NaCl).

There were three unscheduled deaths. Two deaths (one control female and one high-dose male) were related to misdosing. The third animal (low-dose male) was found dead and the cause of death could not be established at necropsy due to autolysis.

A statistically significant increase in monocytes in mid-dose females in comparison to the control group was observed. However, in the absence of a dose response and similar finding in males, this finding was considered to be incidental.

Among clinical chemistry parameters statistically significant differences to controls included increased serum sodium concentration in high-dose males and increased cholesterol and albumin concentrations in high-dose females. As these findings lacked consistency between sexes, and the values were within the historical control range (sodium and cholesterol) for the laboratory or slightly higher (albumin: 49.5 ± 2.1 g/L compared to 95% interval of historical control data of 37.89–48.7 g/L) they were considered of no toxicological significance.

Ophthalmological examination was not performed. However, no changes in the eyes were recorded during weekly clinical examinations, at necropsy and by microscopy of the eyes of control and treated groups.

No other significant effects were observed.

Overall, the Panel identified a no observed adverse effect level (NOAEL) of 85.2 mg TOS/kg bw per day, the highest dose tested.

3.4.3. Allergenicity

The allergenicity assessment considers only the food enzyme and not any carrier or other excipient, which may be used in the final formulation.

[24] Technical dossier/1st submission/Annex N.
[25] Technical dossier/1st submission/Annex O.

The potential allergenicity of the glucose isomerase produced with the genetically modified *S. rubiginosus* strain DP-Pzn37 was assessed by comparing its amino acid sequence with those of known allergens according to the scientific opinion on the assessment of allergenicity of genetically modified plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms (EFSA GMO Panel, 2010). Using higher than 35% identity in a sliding window of 80 amino acids as the criterion, no match was found.[26]

No information is available on oral sensitisation or elicitation reactions of this glucose isomerase. In addition, no allergic reactions upon dietary exposure to any glucose isomerase have been reported in the literature. Therefore, it can be concluded that allergic reactions upon oral ingestion of this glucose isomerase, produced with the genetically modified *S. rubiginosus* strain DP-Pzn37 cannot be excluded, but the likelihood of such a reaction to occur is considered to be low.

Quantifying the risk for allergenicity is not possible in view of the individual susceptibility to food allergens. Allergenicity can be ruled out only if the proteins are fully removed. Considering that the food enzyme is only used in immobilised form and in the glucose isomerisation for the production of high fructose syrups, experimental data showed a significant removal (below LoDs) of protein. However, traces of protein could be present in high fructose syrups.

The Panel considered that, under the intended conditions of use, the risk of allergic sensitisation and elicitation reactions upon dietary exposure to this food enzyme cannot be excluded but the likelihood of such reactions occurring is considered to be low.

3.5. Dietary exposure

3.5.1. Intended use of the food enzyme

The food enzyme is intended to be used to isomerise glucose for the production of high fructose syrup at the maximum use level proposed by the applicant of 1 mg TOS/kg glucose syrup derived from cereals,[27] for the immobilised food enzyme only.

As the food enzyme is intended to be used only in its immobilised form, the transfer of TOS into the final product, i.e. high fructose syrups, is expected to be negligible. Additionally, experimental data have been provided showing low ash contents (less than 0.01 g/100 g dry matter syrup), and protein, fat and fibres contents in the final high fructose syrup, after purification steps are applied (i.e. ion exchange chromatography, treatment with active carbon), are not detectable (Annex B in EFSA CEF Panel, 2016a). Amounts of [redacted] in the final food samples have been experimentally shown to be not detectable.[28,29] The Panel considers that the residual amount of TOS (including substances other than proteins, such as DNA fragments) in the final high fructose syrups will be removed.

3.5.2. Dietary exposure estimation

Considering that the food enzyme is intended to be used only in its immobilised form (see Section 3.5.1), and as residual amounts of TOS are removed by the purification steps applied during the production of high fructose syrups, a dietary exposure was not calculated.

4. Conclusion

Based on the data provided, immobilisation of the food enzyme and the removal of TOS during purification steps applied during the production of high fructose syrups, the Panel concluded that the food enzyme glucose isomerase produced with the genetically modified *S. rubiginosus* strain DP-Dzn37 does not give rise to safety concerns under the intended conditions of use.

The production strain of the food enzyme contains a known antimicrobial resistance gene in a multicopy plasmid together with sequences showing homology with genes known to confer resistance to macrolides. However, based on the absence of viable cells and DNA from the production organism in the food enzyme, this is not considered to be a risk.

[26] Technical dossier/1st submission/Annex R.
[27] Technical dossier/2nd submission/p. 74 and Additional data May 2019.
[28] Technical dossier/1st submission/Annex W and Additional data May 2019/Annexes AB, AC and AD.
[29] LoDs: [redacted].

Documentation provided to EFSA

1) Technical dossier 'Application for authorisation of xylose isomerase from a genetically modified strain of *Streptomyces rubiginosus* (DP-Pzn37)'. January 2016. Submitted by Danisco US Inc.
2) Additional information. April 2018. Submitted by Danisco US Inc.
3) Additional information. June 2018. Submitted by Danisco US Inc.
4) Additional information. May 2019. Submitted by Danisco US Inc.
5) Additional information. September 2019. Submitted by Danisco US Inc.

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OECD (Organisation for Economic Co-Operation and Development), 1998. OECD Guideline for the testing of chemicals, Section 4 Health effects, Test No. 408: Repeated dose 90-day oral toxicity study in rodents. 21 September 1998. 10 pp. Available online: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en

[redacted]

Abbreviations

bw	body weight
CAS	Chemical Abstracts Service
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
CFU	colony forming units
EINECS	European Inventory of Existing Commercial Chemical Substances
FAO	Food and Agricultural Organisation
GIU	glucose isomerase units
GLP	Good Laboratory Practice
GMO	EFSA Panel on Genetically Modified Organisms
HPLC	high-performance liquid chromatography
IUBMB	International Union of Biochemistry and Molecular Biology

LoD	limit of detection
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Cooperation and Development
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TOS	total organic solids
WHO	World Health Organisation