Application Note

Food safety, Agriculture

Multielement Analysis and Selenium Speciation in Cattle and Fish Feed using LC-ICP-QQQ

Authors

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Verified for Agilent 8900 ICP-QQQ

Introduction

In the USA, animal feed is subject to regulation under the Federal Food, Drug, and Cosmetic Act (FFDCA), which defines food as "articles used for food or drink for man or other animals". The US Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) is responsible for regulations relating to the safety of feed intended for animals (including but not limited to horses, cattle, swine, poultry, and fish), under the Animal Feed Safety System (AFSS). The regulations control many aspects of the production, storage, and labeling of animal feed, and the permitted levels of additives and contaminants, such as potentially toxic heavy metals.

Selenium (Se) has been approved by the FDA as a supplement for animal feed since the 1970s. The FDA's current method for Se quantification uses ICP-MS with helium collision/reaction cell (CRC) for control of interferences [1], but the sensitivity of this method is affected when high levels of interferences are present.

Selenium is an essential trace nutrient for vertebrates, and is involved in several vital metabolic processes. The recommended human dietary intake is approximately 55 µg Se per day, which most people acquire through the consumption of plant-based foods, such as cereals. However, Se in the soil is not evenly distributed geographically, so dietary supplementation is commonplace in some parts of the world. This approach requires caution though, as Se is toxic in excess, with a tolerable upper intake level of about 200 µg/day depending on gender and age [2].

Animal feeds including cattle feed and fish feed are often supplemented with selenium. Supplementation may be in the form of sodium selenite/selenate, which is approved by the European Food Safety Authority (EFSA) as a food additive for all animal species [3]. Selenized yeast is also commonly used as an additive; if properly produced, the fortified yeast should contain primarily selenomethionine (SeMet). It is noted that the US regulations for supplemented selenium applicable for cattle feed and other livestock do not apply to fish feed.

Total and speciation analysis of selenium

Determination of total Se concentration is commonly carried out as part of a multielement analysis using ICP-MS. More recently, Se analysis has benefited from the lower detection limits and greater freedom from spectral interferences provided by triple quadrupole ICP-MS (ICP-QQQ) [4, 5]. However, the toxicity of Se depends on the chemical form or species in which the Se occurs, so total elemental concentrations do not provide a complete picture of the element's potential toxicity. As a result, separation and detection of the individual species (speciation) is required. The major Se species that occur naturally in the types of crop plant used to produce animal feed include two inorganic species, selenite (Se(IV)) and selenate (Se(VI)), and some selenoamino acids: selenocystine ($SeCys_2$), selenomethionine (SeMet), and Se-methyl selenocysteine (MeSeCys). Selenoamino acids are considered to be less toxic than the inorganic forms, with Se(IV) being the most toxic species [6]. In addition to the naturally occurring selenium species, animal feeds may contain various selenium compounds added during production to raise the level of total selenium in the animals' diet.

Se speciation analysis typically uses the well-established analytical method of HPLC (to separate the various Se-containing species) coupled to ICP-MS (to identify and quantify the individual Se species). HPLC-ICP-MS has been widely employed for analyzing various sample types [7] but there are few studies on selenium speciation in animal feed [8].

In this study, we developed an extraction and analytical method for the measurement of total Se using ICP-QQQ, and for Se speciation using LC-ICP-QQQ. The method provides low background and high sensitivity enabling low detection limits for total Se and Se compounds to be achieved. We then applied the speciation method to evaluate the selenium content and species distribution in cattle feed and fish feed.

Experimental

Samples and sample preparation

Two commercial cattle feeds and four commercial aquaculture feeds were bought from local stores.

Total Se (and multi-element) analysis of feeds

Cattle and fish feed samples were weighed to approximately 100 mg dry mass and microwave digested in a 1:1 mix of trace metal grade HNO_3 and distilled de-ionized (DDI) H_2O. A solution containing various internal standards was added before digestion, giving an internal standard concentration of 5 ng/g in the final diluted solutions as analyzed. The microwave program consisted of a first step at 300 W with a 10 min ramp to 95 °C and a second step at 300 W including a 10 min ramp to 200 °C, followed by a 20 min hold time. After cooling, 1 mL of 30% H_2O_2 was added and a second digestion was performed using the same heating program. The sample digests were diluted with DDI water to give a final acid concentration of approximately 2% HNO_3. Two certified reference materials (CRMs) NIST 1547 Peach Leaves (NIST, Gaithersburg, MD USA) and SELM-1 Selenium Enriched Yeast (National Research Council of Canada) were prepared as quality control samples.

Enzymatic extraction procedure for Se speciation in feeds

An extraction solution containing 50 mM ammonium phosphate monobasic was prepared and the pH was adjusted to 7.5. Feed samples were weighed to approximately 200 mg with 3 mL of the extraction solution [9]. Samples were sonicated using QSonica sonication probe. The sonication program consisted of a 2 second pulse, followed by a 3 second rest at 60% amplitude for 2 minutes. Following sonication, approximately 20 mg of protease type XIV (from Streptomyces griseus, Sigma-Aldrich) and 10 mg of lipase (from Candida rugose, Sigma-Aldrich) dissolved in buffer solution were added to each sample and placed on a hot block for 12 hours at 37 °C. After 12 hours, the samples were sonicated again at 30% amplitude for 30 seconds and placed on the hot block for a further 12 hours.

The "enzyme extract" was filtered with a 0.45 m Spin-X Centrifuge Tube Filter (Costar, USA). The resulting sample was then diluted 1:1 with mobile phase 1 ready for analysis by reversed phase ion pairing LC-ICP-QQQ.

Instrumentation

An Agilent 8800 Triple Quadrupole ICP-MS (ICP-QQQ) equipped with an Agilent SPS4 autosampler was used for analysis of total selenium and other elements in cattle and fish feed samples. Instrument operating conditions are given in Table 1.

Table 1. ICP-QQQ operating conditions.

Parameter	No gas	He	O_2
Spray chamber temp (°C)	2		
RF power (W)	1550		1600
Sampling depth (mm)	8.5		8
Carrier gas (mL/min)	1.00		
Make up gas (mL/min)	0.10		0.15
Cell gas (mL/min)	0.0	3.4	0.5 (30%*)

** Indicates % of full scale flow rate, as displayed in the ICP-MS MassHunter Tune screen*

Agilent ICP-MS MassHunter software was used for the setup and operation of the ICP-QQQ for total Se and multielement data analysis. ICP-MS MassHunter with the optional Chromatographic Analysis module was used for combined instrument control and sequencing of the LC-ICP-QQQ Se-speciation study.

For the speciation studies in cattle and fish feed, an Agilent 1100 Series HPLC was coupled to the ICP-QQQ. Chromatographic separations were performed using an Agilent ZORBAX Extend column (80 Å C18, 4.6 x 250 mm, 5 µm). Details of the HPLC method used for Se speciation analysis of cattle and fish feed are given in Table 2.

The six Se species of interest, Se(IV), Se(VI), selenocystine ($SeCys_2$), selenomethionine (SeMet), methyl selenocysteine (MeSeCys), and selenomethionine-Se-oxide (SeOMet), were calibrated using mixed standard solutions containing each of the Se species at levels from 1 to 50 ng/g.

In common with any ion paring method, column equilibration is crucial to ensuring long-term reproducibility when using this LC-ICP-QQQ method. Equilibration is important after cleaning or when the column has been stored for a long time. To prevent deterioration of the column, 2 mM TBAH was added to the 65% acetonitrile storage solution. Following storage or cleaning, the column was equilibrated for 20 mins with 3x75 µL injections of 25 mM TBAH dissolved in the mobile phase.

Table 2.HPLC method used for the analysis of cattle and fish feed sample extracts.

Method	Salt gradient; reverse-phase ion-pairing		
Injection volume	25 µL		
Mobile phase 1	5 mM ammonium acetate, 2 mM ammonium phosphate, 2 mM TBAH, 2% MeOH, pH 6.5		
Mobile phase 2	15 mM ammonium acetate, 5 mM ammonium phosphate, 2 mM TBAH, 2% MeOH, pH 6.5		
Method	Minute	% Mobile phase 2	Flow rate (mL/min)
	5	0	1
	10	100	1
	16	100	1
	20	0	1
	21	0	1.5
	40	0	1.5
	45	0	1

Interference removal

Routine determination of total selenium concentrations or analysis of Se species using LC-ICP-MS does not necessarily require the use of ICP-QQQ. Conventional quadrupole ICP-MS (ICP-QMS) fitted with a CRC is able to resolve the $^{40}Ar^{38}Ar^{+}$ dimer interference on ^{78}Se sufficiently well to give acceptable results for Se speciation analysis in many sample types [7]. However, doubly-charged ion interferences such as $^{156/160}Gd^{++}$ and $^{156/160}Dy^{++}$ on $^{78/80}Se$ can lead to positive bias in samples containing relatively high levels of the rare earth elements (REEs) [5]. In these sample types, ICP-QQQ is able to completely resolve the doubly charged REE interferences along with other spectral interferences, giving lower detection limits and better accuracy than ICP-QMS for Se (and As).

Results and Discussion

Multielement analysis

The multielement analysis results including total Se content of the feed and CRM samples are summarized in Table 3. The measured value for total Se in Se-yeast SELM-1 CRM was in good agreement with the certified value at 94% recovery. The results validate the sample preparation method and the accuracy of the ICP-QQQ results. Accurate recovery of Se in NIST 1547 Peach Leaves was also obtained (102%, relative to the original 1991 certified value). This indicates the effective control of interferences including doubly charged rare earth elements, as NIST Peach Leaves contains up to 10 mg/kg (ppm) of the light rare earth elements. Table 3 also includes instrument detection limits (IDLs) demonstrating low ng/L (ppt) detection limits for most analytes.

Table 3. Total selenium dry weight concentration (mg/kg) determined in cattle feed, fish feed, and CRMs analyzed by ICP-QQQ.

Element	Tune	Q1→Q2 Set Mass	Cattle Feed 1	Cattle Feed 2	Fish Feed 1	Fish Feed 2
Mg	He	24–>24	2,278 ± 49	2,762 ± 26	1,873 ± 37	2,419 ± 17
K	He	39–>39	8,771 ± 77	9,556 ± 60	7,064 ± 129	10,080 ± 196
V	He	51–>51	1.37 ± 0.05	0.28 ± 0.01	0.30 ± 0.03	0.36 ± 0.02
Cr	He	52–>52	1.79 ± 0.07	0.90 ± 0.03	1.16 ± 0.06	0.74 ± 0.06
Fe	He	56–>56	392 ± 21	166 ± 25	432 ± 10	255 ± 3
Co	He	59–>59	0.66 ± 0.02	1.3 ± 0.1	0.127 ± 0.001	0.67 ± 0.04
Cu	He	63–>63	31.1 ± 0.8	26 ± 2	8.68 ± 0.08	66 ± 2
As	O_2	75–>91	0.21 ± 0.02	0.11 ± 0.01	1.14 ± 0.09	0.30 ± 0.05
Se	O_2	78–>94	0.86 ± 0.04	0.69 ± 0.03	1.07 ± 0.08	0.98 ± 0.05
Sr	He	88–>88	11 ± 2	11.4 ± 0.5	49 ± 1	16.4 ± 0.6
Mo	He	95–>95	1.35 ± 0.03	2.17 ± 0.01	0.77 ± 0.01	1.52 ± 0.02
Cd	He	111–>111	0.094 ± 0.004	0.072 ± 0.003	0.40 ± 0.02	0.049 ± 0.002
Pb	No gas	208–>208	0.24 ± 0.03	0.12 ± 0.01	0.38 ± 0.06	0.23 ± 0.09

Table 3 continued...

Element	Tune	Fish Feed 3	Fish Feed 4	NIST 1547[a]	SELM-1[a]	IDL, ppb
Mg	He	2,152 ± 29	1,586 ± 56	4,406 ± 72 (98)		0.2116
K	He	11,298 ± 110	7,520 ± 66	22,167 ± 364 (91)		7.16
V	He	0.42 ± 0.01	1.14 ± 0.08	0.341 ± 0.006 (93)		0.0123
Cr	He	2.37 ± 0.01	1.10 ± 0.05	1.067 ± 0.009 (107[b])		0.0044
Fe	He	204 ± 15	642 ± 50	225 ± 3 (102)		0.1027
Co	He	0.146 ± 0.002	0.20 ± 0.01	0.068 ± 0.002 (97[b])		0.0005
Cu	He	16.2 ± 0.3	10.42 ± 0.08	3.8 ± 0.2 (101)		0.027
As	O_2	0.171 ± 0.009	0.81 ± 0.03	0.08 ± 0.02 (133[c])		0.0035
Se	O_2	0.55 ± 0.02	1.05 ± 0.04	0.122 ± 0.003 (102[c])	1911 ± 97 (94)	0.0031
Sr	He	40 ± 2	32 ± 2	62 ± 1 (117)		0.018
Mo	He	1.67 ± 0.03	1.11 ± 0.03	0.063 ± 0.006 (104)		0.002
Cd	He	0.074 ± 0.004	0.056± 0.006	0.028 ± 0.001 (107)		0.0039
Pb	No gas	0.25 ± 0.04	0.41 ± 0.03	0.82 ± 0.03 (94)		0.1946

a. Values enclosed in parenthesis are recoveries of the certified value of reference material.

b. Recovery determined relative to a non-certified, information value.

c. Recoveries for As and Se are calculated relative to the original certified values (1991 revision). These certified values have subsequently been removed from the certificate (2017 revision) so may not be reliable.

In the United States, the Association of American Feed Control Officials (AAFCO) 2011 Guidelines [10] and the US FDA's 21 CFR Part 573, Section 573.920 (Selenium) [11] state animal feeds intended for chickens, turkeys, swine, beef cattle, dairy cattle (and in the AAFCO Guidelines, bison, sheep, goats, llamas, alpacas, and horses) may contain selenium yeast at a level not to exceed 0.3 ppm (mg/kg) of selenium based on the complete feed [10]. Furthermore, the level of inorganic species should not exceed 2% of the total Se content in the final yeast product. Our results show that the two cattle feeds contained Se significantly above the 0.3 mg/kg limit, at 0.86 and 0.69 mg/kg Se. Similar results were found in the four fish feeds tested, which contained concentrations between 0.55 and 1.07 mg/kg Se.

All feeds contained at least twice the maximum Se concentration of 0.3 ppm (mg/kg) in selenium supplemented feeds. The feeds were likely supplemented by the addition of "antioxidants" including Se-yeast to increase the selenium content. To further investigate the Se content of the feeds, speciation analysis was performed to separate and quantify the individual Se species present in the feed samples.

Selenium speciation analysis

Selenium speciation analysis was performed using LC coupled to ICP-QQQ. Se was measured using the oxygen cell gas tune mode as for Se in the multielement analysis. The selenium species concentrations were calibrated using mixed standard solutions containing each of the Se species at levels from 1 to 50 ng/g. The integrated peak areas for each species were plotted versus the standard concentrations to generate calibration curves covering the required calibration range.

The chromatogram shown in Figure 1 was obtained from the analysis of a mixed standard containing each Se species at 25 ng/g. The chromatogram demonstrates good sensitivity and peak separation for all species. Peak identities were confirmed by retention time (RT) matching and/or the use of standard spikes added to the extracts.

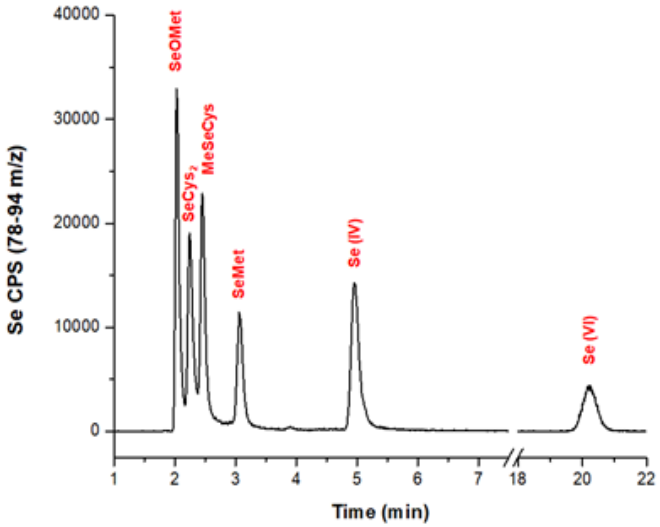


Figure 1. LC-ICP-QQQ chromatogram of standard containing six selenium species at 25 ng/g.

Se species in cattle feed samples

The two cattle feed samples were analyzed using LC-ICP-QQQ. The overlaid chromatograms in Figure 2 show that both samples contained primarily SeMet, while cattle feed 2 also contained significant levels of Se(VI). Other species were present at trace levels. The source of SeMet was likely to be selenized yeast, which is often added intentionally to enrich the feeds. However, natural sources, such as grains and soybeans, are common additives that have been found to accumulate SeMet when supplied with inorganic Se sources [12–14]. Plants naturally uptake Se from soils, and inorganic Se species tend to be more mobile, which leads to increased plant uptake compared with organic forms. Depending on soil conditions, either Se (IV) or Se (VI) may be the major Se source for plants [15]. When Se (IV) is the main source of selenium, it gets metabolized to organic Se compounds; while Se (VI) uptake generally results in higher accumulation without transformation [12, 14]. It can be concluded that the Se (VI) found in the cattle feeds for this study likely originated from the addition of high-selenium plant additives often used in feed production.

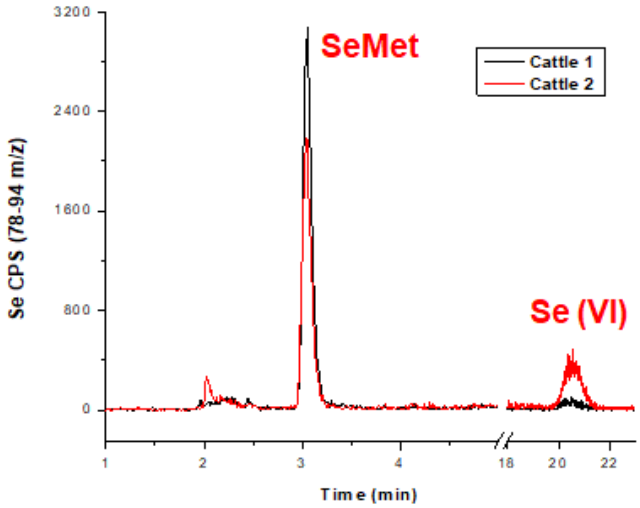


Figure 2. LC-ICP-QQQ speciation analysis of two cattle feed samples.

Se species in fish feed samples

Figure 3 shows chromatograms for the four fish feed samples. As with the cattle feeds, a variety of Se species were observed in these samples. SeMet was the primary species in all feeds, but inorganic forms such as Se(IV) and Se(VI) were also present. The quantitative results in Table 4 show that Se(IV) was considerably higher in fish feed 1 compared to the other samples, while Se(VI) was higher in the cattle feeds than the fish feeds.

Selenium supplementation has been shown to improve growth and antioxidant status for fish reared in the crowded conditions that are typical of mass production methods [16]. Previous aquaculture studies have shown supplementation with inorganic forms of Se, mainly Se(IV), leads to inferior bioavailability compared to SeMet or selenoyeast [17, 18]. Due to greater accumulation of Se in fillets and whole body, many studies currently use organic Se for aquaculture and supplementation research [19, 20].

Table 4. Enzymatic extraction and quantification of Se species for cattle and fish feeds using LC-ICP-QQQ.

Sample	Extraction total (µg/kg, ppb)	Extraction efficiency (%)*	Quantification of known species (µg/kg, ppb)					
			SeOMet	SeCys	MeSeCys	SeMet	Se (IV)	Se (VI)
Cattle feed 1	386 ± 3	45 ± 1	2 ± 1	44 ± 41	5 ± 1	112 ± 8	0 ± 0	72 ± 3
Cattle feed 2	346 ± 14	50 ± 2	6 ± 3	4 ± 2	3 ± 1	97 ± 3	24 ± 27	128 ± 15
Fish feed 1	648 ± 32	61 ± 3	5 ± 2	19 ± 3	12 ± 1	79 ± 11	61 ± 8	23 ± 3
Fish feed 2	484 ± 90	49 ± 9	2 ± 1	14 ± 6	9 ± 1	117 ± 11	10 ± 3	22 ± 4
Fish feed 3	363 ± 103	66 ± 19	4 ± 2	17 ± 3	8 ± 2	222 ± 8	3 ± 5	30 ± 7
Fish feed 4	710 ± 43	68 ± 4	2 ± 1	27 ± 1	14 ± 1	293 ± 39	14 ± 3	31 ± 1

** Compared to total Se concentration (shown in Table 3)*

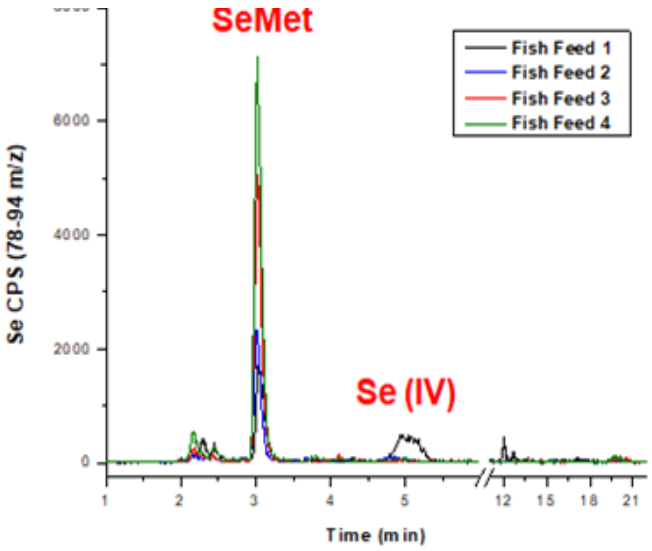


Figure 3. LC-ICP-QQQ speciation analysis of four fish feed samples.

Conclusions

Total concentrations of several elements, including selenium, were determined in cattle and fish feed sample-extracts, using the Agilent 8800 ICP-QQQ. In all samples, the concentration of Se was above the maximum of 0.3 ppm (µg/kg) Se recommended in the AAFCO and FDA guidelines for selenium supplemented feeds.

Reversed phase ion pairing LC-ICP-QQQ was then used successfully to separate and detect the selenium species at low mg/kg levels in the feed samples (low µg/L in the solutions analyzed). The method provided valuable information on the Se species present in the feeds. SeMet was found to be the predominant species, although the toxicologically relevant inorganic forms of Se (Se(IV) and Se(VI)) were also found to be present in most of the samples.

More Information

For a full account of part of this application, see A. F. Oliveira, J. Landero, K. Kubachka, A. R. A. Nogueira, M. A. Zanetti and J. Caruso, Development and application of a selenium speciation method in cattle feed and beef samples using HPLC-ICP-MS: evaluating the selenium metabolic process in cattle. *J. Anal. At. Spectrom.*, **2016**, 31, 1034. DOI: 10.1039/c5ja00330j

References

1. Overview of FDA's Animal Feed Safety System, accessed August 2017, http://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AnimalFeedSafetySystemAFSS/UCM277673.pdf
2. Selenium in Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium and Carotenoids, Institute of Medicine, Washington, DC, 2000
3. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Safety and efficacy of selenium compounds (E8) as feed additives for all animal species: sodium selenite, EFSA Journal 2016; 14 (2): 4398, www.efsa.europa.eu/efsajournal
4. B.P. Jackson, A. Liba, and J. Nelson, Advantages of reaction cell ICP-MS on doubly charged interferences for arsenic and selenium analysis in foods. *J. Anal. At. Spectrom*. **2015**, 30, 1179–1183
5. N. Sugiyama, The accurate measurement of selenium in twelve diverse reference materials using on-line isotope dilution with the 8800 Triple Quadrupole ICP-MS in MS/MS mode, Agilent publication, 2012, 5991-0259EN
6. C. B'Hymer and J. A. Caruso, *J. Chromatogr. A*, **2006**, 1114, 1–20.
7. Handbook of ICP-MS Hyphenated Techniques, 2nd Edition, Agilent publication, 2015, 5990-9473EN
8. M. Stadlover, M. Sager and K. Irgolic, *Die Bodenkultur*, **2001**, 52, 233–241

9. L. H. Reyes, J. L. Guzmán Mar, G.M. Mizanur Rahman, B. Seybert, T. Fahrenholz, H.M. Skip Kingston, *Talanta* 78, 3, **2009**, 983–990

10. Association of American Feed Control Officials: Official Publication, ed. M. K. Walsh, 2011, ch. 5, pp.216–353.

11. Code of Federal Regulations Title 21, Volume 6 [Revised as of April 1, 2017] 21CFR573.920

12. H.F. Li, S. P. McGrath, and F. J. Zhao. Selenium uptake, translocation and speciation in wheat supplied with selenate or selenite, *New Phytologist* 178.1, **2008**, 92-102.

13. Q. Chan, S. E. Afton, and J. A. Caruso. Selenium speciation profiles in selenite-enriched soybean (Glycine Max) by HPLC-ICPMS and ESI-ITMS. *Metallomics* 2.2, **2010**, 147-153.

14. P. D Whanger, Selenocompounds in plants and animals and their biological significance. *Journal of the American College of Nutrition*, **2002**, 21(3), pp.223-232.

15. S. Eich-Greatorex et al. Plant availability of inorganic and organic selenium fertiliser as influenced by soil organic matter content and pH. *Nutrient Cycling in Agroecosystems*, 79.3, **2007**, 221-231.

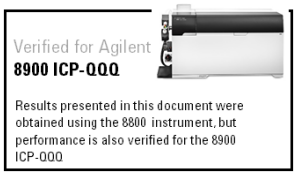
16. F. Z. Küçükbay et al. The effects of dietary organic or inorganic selenium in rainbow trout (Oncorhynchus mykiss) under crowding conditions. *Aquaculture Nutrition*, 15.6, **2009**, 569-576.

17. C. Wang and R. T. Lovell. Organic selenium sources, selenomethionine and selenoyeast, have higher bioavailability than an inorganic selenium source, sodium selenite, in diets for channel catfish (Ictalurus punctatus). *Aquaculture* 152.1-4, **1997**, 223-234.

18. M. Lorentzen, A. Maage, and K, Julshamn. Effects of dietary selenite or selenomethionine on tissue selenium levels of Atlantic salmon (Salmo salar). *Aquaculture* 121.4, **1994**, 359-367.

19. M. Abdel-Tawwab, M.A.A Mousa and F.E. Abbass. Growth performance and physiological response of African catfish, Clarias gariepinus (B.) fed organic selenium prior to the exposure to environmental copper toxicity. *Aquaculture* 272.1, **2007**, 335-345.

20. S. Lee et al. Dietary selenium requirement and toxicity levels in juvenile Nile tilapia, Oreochromis niloticus. *Aquaculture* 464, **2016**, 153-158.



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