National Reference Laboratory Feed Additives – Control and Authorisation

End of Year Report
2018 - 2019

Report reference: CP-2019-13

Prepared by:

Kirstin Gray

Project Manager – Food and Referee Analysis

Approved by:

Michael Walker

Head of the Office of the Government Chemist

Date: 21 May 2019

Contents

1. Introduction

Regulation (EC) No. 882/2004 *on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules* established a network of European and National Reference laboratories. Regulation (EC) No 882/2004 was repealed with effect from 14 December 2019 and replaced by Regulation (EU) 2017/625 *on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products*. Regulation 2017/625 supplements Regulation (EC) No 178/2002 *laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety*, aims for a high level of:

- protection of human, animal and plant health and of the environment via veterinary and phytosanitary measures;
- consumer protection in the internal market; and
- animal welfare along the agri-food chain.

In each area of food and feed control an EU Reference Laboratory (EURL) is identified to coordinate activities in this area. They are supported by a network of National Reference Laboratories (NRLs) which co-ordinate activities within their own member state and contribute to the European wide activities. NRLs are nominated by the Competent Authorities in the respective Member state. In the UK, the Competent Authority for feed additives is the Food Standards Agency (FSA.

The duties of the EURLs and NRLs are set out in legislation however their principal role is to provide analytical and scientific support to ensure that food and feed control is carried out effectively and in a harmonised manner, across the EU member states.

Article 94 of Regulation (EU) 2017/625 describes the responsibilities and tasks of EURLs as follows:

> 1. EURLs shall contribute to the improvement and harmonisation of methods of analysis, test or diagnosis to be used by official laboratories designated in accordance with Article 37(1) and of the analytical, testing and diagnostic data generated by them.
>
> 2. EURLs designated in accordance with Article 93(1) shall be responsible for the following tasks insofar as they are included in the reference laboratories' annual or multiannual work programmes that have been established in conformity with the objectives and priorities of the relevant work programmes adopted by the Commission in accordance with Article 36 of Regulation (EU) No 652/2014:
>
> (a) providing NRLs with details and guidance on the methods of laboratory analysis, testing or diagnosis, including reference methods;
>
> (b) providing reference materials to NRLs;
>
> (c) coordinating the application by the NRLs and, if necessary, by other official laboratories of the methods referred to in point (a), in particular, by organising regular inter- laboratory comparative testing or proficiency tests and by ensuring appropriate follow-up of such comparative testing or proficiency tests in accordance, where available, with internationally accepted protocols, and informing the Commission and

the Member States of the results and follow-up to the inter-laboratory comparative testing or proficiency tests;

(d) coordinating practical arrangements necessary to apply new methods of laboratory analysis, testing or diagnosis, and informing NRLs of advances in this field;

(e) conducting training courses for staff from NRLs and, if needed, from other official laboratories, as well as of experts from third countries;

(f) providing scientific and technical assistance to the Commission within the scope of their mission;

(g) providing information on relevant national, Union and international research activities to NRLs;

(h) collaborating within the scope of their mission with laboratories in third countries and with the European Food Safety Authority (EFSA), the European Medicines Agency (EMA) and the European Centre for Disease Prevention and Control (ECDC);

(i) assisting actively in the diagnosis of outbreaks in Member States of foodborne, zoonotic or animal diseases, or of pests of plants, by carrying out confirmatory diagnosis, characterisation and taxonomic or epizootic studies on pathogen isolates or pest specimens;

(j) coordinating or performing tests for the verification of the quality of reagents and lots of reagents used for the diagnosis of foodborne, zoonotic or animal diseases and pests of plants;

(k) where relevant for their area of competence, establishing and maintaining:

(i) reference collections of pests of plants and/or reference strains of pathogenic agents;

(ii) reference collections of materials intended to come into contact with food used to calibrate analytical equipment and provide samples thereof to NRLs

(iii) up-to-date lists of available reference substances and reagents and of manufacturers and suppliers of such substances and reagents; and

(l) where relevant for their area of competence, cooperate among themselves and with the Commission, as appropriate, to develop methods of analysis, testing or diagnosis of high standards.

3. EURLs shall publish the list of the NRLs designated by the Member States in accordance with Article 100(1).

Article 101 of Regulation (EU) 2017/625 describes the responsibilities and tasks of NRLs as follows:

(a) collaborate with the EURLs, and participate in training courses and in inter-laboratory comparative tests organised by these laboratories;

(b) coordinate the activities of official laboratories designated in accordance with Article 37(1) with a view of harmonising and improving the methods of laboratory analysis, test or diagnosis and their use;

(c) where appropriate, organise inter-laboratory comparative testing or proficiency tests between official laboratories, ensure an appropriate follow-up of such tests and inform the competent authorities of the results of such tests and follow-up;

(d) ensure the dissemination to the competent authorities and official laboratories of information that the EURL supplies;

(e) provide within the scope of their mission scientific and technical assistance to the competent authorities for the implementation of MANCPs referred to in Article 109 and of coordinated control programmes adopted in accordance with Article 112;

(f) where relevant, validate the reagents and lots of reagents, establish and maintain up-to-date lists of available reference substances and reagents and of manufacturers and suppliers of such substances and reagents;

(g) where necessary, conduct training courses for the staff of official laboratories designated under Article 37(1); and

(h) assist actively the Member State having designated them in the diagnosis of outbreaks of foodborne, zoonotic or animal diseases or of pests of plants and in case of non-compliance of consignments, by carrying out confirmatory diagnoses, characterisation and epizootic or taxonomic studies on pathogen isolates or pest specimens.

LGC currently holds the NRL roles for feed additives – control and authorisation. Regulation (EC) No. 1831/2003 on additives for use in animal nutrition describes 'feed additives' as substances, micro-organisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform, in particular, one or more of the following functions:

(a) favourably affect the characteristics of feed,

(b) favourably affect the characteristics of animal products,

(c) favourably affect the colour of ornamental fish and birds,

(d) satisfy the nutritional needs of animals,

(e) favourably affect the environmental consequences of animal production,

(f) favourably affect animal production, performance or welfare, particularly by affecting the gastro-intestinal flora or digestibility of feedingstuffs, or

(g) have a coccidiostatic or histomonostatic effect.

Feed additives should not:

(a) have an adverse effect on animal health, human health or the environment,

(b) be presented in a manner which may mislead the user,

(c) harm the consumer by impairing the distinctive features of animal products or mislead the consumer with regard to the distinctive features of animal products.

Antibiotics, other than coccidiostats or histomonostats, are not authorised as feed additives.

Feed additives play an important role in animal nutrition, addressing various aspects such as feed safety, reduction of environmental emissions and sustainability in livestock farming. Before placing feed additives on the market, authorisation must be obtained as specified in Regulation (EC) No 1831/2003. A summary of the authorisation process is given in Appendix 1.

Pursuant to Regulation (EC) No 1831/2003, a list of the currently permitted feed additives can be found in the European Union Register of Feed Additives. The latest edition can be found at:

https://ec.europa.eu/food/safety/animal-feed/feed-additives/eu-register_en (accessed 21 May 2019)

This report provides an update for the National Reference Laboratory role for Feed Additives – Control and Authorisation for the year April 2018 to March 2019.

2. EURL Training 2018

Following on from the proficiency test (PT) the EURL ran in 2015, in June 2018 the EURL provided hands-on training to NRLs for the determination of carotenoids in feed. Training was deemed to be useful by the EURL as the results from the 2015 PT were not satisfactory. Of the labs that submitted results, 67 % (4 out of 6) obtained satisfactory results for astaxanthin and only 17 % (1 out of 6) obtained satisfactory results for canthaxanthin.

Malvinder Singh, LGC attended the training held at the Joint Research Centre (JRC), Geel on 5th – 6th June 2018. The training involved the simultaneous determination of carotenoids in samples of fish feed and poultry feed. The carotenoids determined during the training were astaxanthin, canthaxanthin and adonirubin.

The principle of the method is enzymatic proteolysis at 50°C followed by extraction with acetone, centrifugation and analysis by reverse phase HPLC with UV detection at 410 nm. The full method provided by the EURL is given in Appendix 2.

3. EURL Proficiency Test 2018

In 2018 the EURL for Feed Additives – Control ran a PT for NRLs for total selenium in compound feed.

An invitation to participate in the PT was received on 26 March 2018 and one sample of compound feed was received on 26 May 2018. The deadline for submission of results was 30 June 2018.

The EURL was approached and, after discussions with the European Commission, agreed that UK official control laboratories (OCLs) could participate in the PT. Four labs registered to participate.

The results submitted by all of the participants, together with the associated uncertainties and z-scores are presented in Table 1 and Figure 1. LGC is lab C-05 and obtained a satisfactory z-score of 0.4.

The four UK OCLs which participated in this PT are lab numbers O-06, O-11, O-23 and O-24. The z-scores obtained by these labs were 24.7, 0.0, 1.8 and 0.0 respectively. The high z-score was followed up informally with the Head of the laboratory concerned who noted the issue was investigated.

Assigned range: x_{pt} = 0.469 ± 0.092 $U(x_{pt}, k = 2)$; and σ_{pt} = 0.070
(all values in mg kg^{-1}, relative to a feed with a moisture content of 12 %).

Lab Code	x_i	±	k	Technique	$u(x_i)$	z' score	ζ score	unc.	Comply
A-01	0.504	0.061	2	ICP-MS	0.031	0.4	0.6	b	Yes
A-03	0.58	0.16	2	ICP-MS	0.080	1.3	1.2	a	Yes
A-07	0.42	0.19	2	ICP-MS	0.095	-0.6	-0.5	c	Yes
A-08	0.431	0.104	2	ICP-MS	0.052	-0.5	-0.5	a	Yes
A-17	0.390	0.059	2	ICP-OES	0.030	-0.9	-1.4	b	No
A-19	0.472	0.079	2	ICP-MS	0.040	0.0	0.1	a	Yes
A-21	0.432	10	2	HG-AAS	5.000	-0.4	0.0	c	Yes
A-25	0.42	0.06	2	ICP-MS	0.030	-0.6	-0.9	b	
C-02	0.79	0.15	2	HG-AAS	0.075	3.8	3.7	a	Yes
C-04	0.51	0.2	2	ICP-MS	0.100	0.5	0.4	c	Yes
C-05	0.50	0.13	2	ICP-MS	0.065	0.4	0.4	a	Yes
C-09	0.59	0.12	2	ET-AAS	0.060	1.4	1.6	a	Yes
C-10	0.46	0.184	2	ICP-MS	0.092	-0.1	-0.1	c	
C-12	0.512	0.102	2	HG-AAS	0.051	0.5	0.6	a	Yes
C-13	0.54	0.12	2	ICP-MS	0.060	0.8	0.9	a	Yes
C-14	0.4	0.1	2	ICP-MS	0.050	-0.8	-1.0	a	Yes
C-15	0.447	0.067	2	ICP-MS	0.034	-0.3	-0.4	b	Yes
C-16	0.442	0.033	2	ICP-MS	0.017	-0.3	-0.6	b	Yes
C-18	0.60	0.28	2	ICP-MS	0.140	1.6	0.9	c	Yes
C-20	0.433	0.108	2	ICP-MS	0.054	-0.4	-0.5	a	Yes
C-22	0.458	0.184	2	ET-AAS	0.092	-0.1	-0.1	c	Yes
O-06	2.542	0.112	2	ICP-OES	0.056	24.7	28.7	a	Yes
O-11	0.47	0.13	2	HG-AAS	0.065	0.0	0.0	a	
O-23	0.62	0.04	2	ICP-MS	0.020	1.8	3.0	b	No
O-24	0.465	0.05	2	ICP-OES	0.025	0.0	-0.1	b	Yes

Performance scores: satisfactory (green); questionable (yellow); unsatisfactory (orange)

Measurement uncertainty: a: $u(x_{pt}) \leq u_i \leq \sigma_{pt}$; b: $u_i < u(x_{pt})$; and c: $u_i > \sigma_{pt}$

Table 1: Summary of results from selenium proficiency test run by the EURL

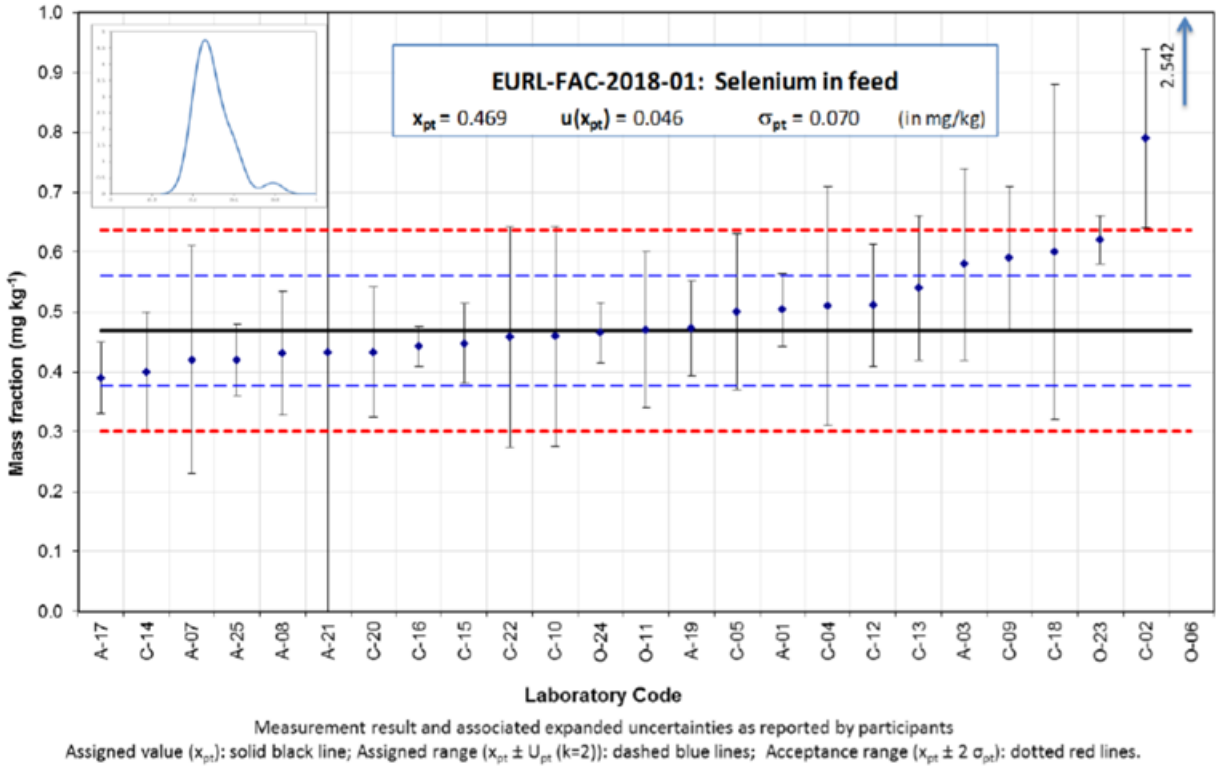

Figure 1: Results of selenium PT with associated uncertainties

The full report for the proficiency test can be found on the EURL-FA website at: https://ec.europa.eu/jrc/en/publication/eurl-fa-control-proficiency-test-report (accessed 21 May 2019)

4. EURL Proficiency Tests 2019

Carotenoids

Further to the training provided in 2018, in 2019 the EURL has organised a PT for the determination of the carotenoids astaxanthin, canthaxanthin and adonirubin in samples of compound feed. The invitation to register for the PT was received on 13 February 2019, with the samples being received on 26 March 2019. The deadline for submission of results is 26 April 2019.

The letter of invitation to participate stated that a limited number of places were available to OCLs if they wished to take part (the total number of participants able to take part was stated as being 25). The invitation was forwarded to the UK OCLs, together with a copy of the EURL's method (Annex 2), no responses were received.

Coccidiostats

On 30 April 2019 an invitation to participate in the EURL's second PT of 2019 was received. This PT focusses on the determination of coccidiostats in compound feed, with the

coccidiostats of interest being diclazuril, nicarbazin and narasin. The invitation implied that the coccidiostats could be at authorised or carry-over concentrations. This invitation also stated that the PT was open to OCLs, with a maximum total number of participants of 25. The invitation was forwarded to UK OCLs, one laboratory indicated an interest in participating. The samples are expected to be dispatched in May 2019 with a deadline for submission of results of 15 June 2019.

5. OCL Training

In the first week of April 2019 a week long residential course was held at Reading University on the Examination and Analysis of Foods. One of the sessions in the course was on feeds and feed additives with presentations given by Kevin Wardle of Public Analysts Scientific Services and Mark Bond, Food Standards Agency, respectively. Both lectures were well received. The course is organised by the Government Chemist programme and the APA Educational Trust and accredited by the RSC. The course consists of a mix of lectures, laboratory practical sessions and interactive exercises and the participants are mainly from UK Public Analyst laboratories.

The UK OCLs are regularly reminded that one of the roles of the NRL is to organise training exercises for OCLs and they have been asked if they have any current training needs. It has been explained that training provided by the NRL does not necessarily have to take the form of a formal PT but could be more specific or individual training if it would be of benefit. The OCLs have also been asked if, as an alternative to analysis, there were any areas where an advice note or guidance would be useful, for example with the calculation and application of measurement uncertainty, interpretation of any specific legislation or application of tolerances. To date, no requests for training have been received.

In another area, we have been working on a validated method for the determination of theobromine in feed for CEN 327 working group 5. The method together with a paper on the validation procedure are expected to be published shortly and include the results of a collaborative trial in which the UK OCLs were well represented.

6. Meetings and advice

An update of NRL activities was presented to the Food Standards Agency on 1 August 2018.

Michael Walker, Association of Public Analysts (APA) Training Officer, attended APA Training Committee meetings and gave updates on NRL activities when required.

Throughout the year, advice was provided following enquiries by the Food Standards Agency in these areas:

- Determination of selenium
- Tolerances for amino acids
- Nitrites in feedstuffs

On 23 August 2018 Kirstin Gray and Mark Bond, FSA, attended a meeting in Brussels to review Regulation 152/2009 on laying down the methods of sampling and analysis for the

official control of feed. Discussions centred on the Annexes of the Regulation and whether the described methods were still fit for purpose or needed to be amended / removed.

Information has also been provided and discussions held as to the possible effects on the UK on exit from the EU with or without a deal. On 26 November 2018 Kirstin Gray attended a meeting held by Defra and the FSA to define the problems and identify the solutions for the potential loss of access to EURLs. A detailed summary of the authorisation process for feed additives was also provided to help highlight gaps in the process, for example access to EFSA, that might arise following EU exit.

Following on from enquiries made last year with regards to possible issues with low recoveries and high variation between replicate analyses for the determination of vitamin A, a further enquiry was made by one of the OCLs. The EURL has been contacted again for an update but, as yet, no response has been received.

7. EURL Workshops

The 18th EURL-Feed Additives Authorisation workshop was held in Brussels on 17th – 18th October 2018. The 7th EURL-Feed Additives Control workshop was held in Brussels on 18th – 19th October 2018. Michael Walker attended both workshops.

Summary

Please refer to the full proceedings for detailed coverage of the meetings. These were useful meetings with information exchange on potential problems and the availability of new methods, ideas and sources of assistance were key features, as well as networking with colleagues from across Europe engaged in similar activities.

Some areas of analysis remain problematic:

- Accurate determination of urea in pet food and other feedstuffs, see the full report of the Controls workshop, with information on enzymatic and LC-MS/MS methods.
- Identification and enumeration of probiotics.
- Single analyte v's multi-analyte methods.

The meeting included:

- Insights into Feed Additive production and routine QC.
- Approaches adopted by related areas e.g. the European Pharmacopoeia.
- Exploration of UK attendance at EURL feed additive authorisation and control workshops post UK exit from EU. The EURL members and other NRLs were sympathetic, citing the attendance and participation of Norway, albeit no voting rights would be accorded.
- The recent denial of authorisation of a riboflavin FA owing to presence of recombinant DNA and antibiotic resistant *B.subtilis*.
- Assessment of botanical extract feed additives and their analysis.
- Toxicological assessment of mixtures of compounds, e.g. flavourings and botanicals.
- Increased efficiency of working by EFSA in face of more legislation and internal protocols and fewer resources.

The outcomes of the Optiphos collaborative trial were discussed. Six RMs were produced, consensus phytase activity was achieved, robust RSD_r and robust RSD_R, and a robust Opiphos conversion factor with its MU were elaborated.

EURL-FA Future Perspectives

Christoph von Holst (JRC-Geel) discussed several topics, (a) new colleagues,(b) the future format of this workshop and (c) rapporteur tasks.

The current workshop structure is a mix of general and specific topics; there is limited involvement of NRLs despite appeals. Suggested for future workshops were: (a) invited presentations from NRLs about their activities, (b) joint discussion of selected dossiers, (c) training. A questionnaire will be sent out to gauge appetite.

The tasks of Rapporteurs are detailed in Art. 5 of Regulation 378/2005. When reviewing the dossier the scientific comments from the NRL's expertise are appreciated. But also valuable would be editorial proof reading – addressing such questions as 'is the text understandable?', even if the subject matter is not in the NRL's immediate field of expertise. Even a response that the NRL is unable to evaluate as NRL does not have relevant expertise would be of assistance.

Communications

The new CIRCA website was discussed; emails should be considered as the exception. NRLs invited to explore the new system and report any difficulties. NRLs will be asked to update their expertise, a survey will be launched via CIRCA soon after the workshop.

The executive summary of the proceedings from the Feed Additives Authorisation and Control workshops are provided in Appendix 1 and Appendix 2 respectively.

Glossary

FA: Feed Additive

LC-MS/MS: liquid chromatography with tandem mass spectrometry

MU: Measurement Uncertainty

Optiphos: a feed additive containing a specific phytase

Opiphos conversion factor: a conversion factor between the "Optiphos" unit and the "international phytase" unit that would allow the application of an ISO method for determining phytase activity in matrices containing the Optiphos® products.

Phytase: an enzyme that releases phosphate bound to the cereal compound phytic acid and hence less bioavailable

RM: Reference material

RSD_r: Relative within laboratory standard deviation

RSD_R: Relative between laboratory standard deviation

8. EURL Work Programme for 2019

The work programme for 2019 for the EURL Feed Additives – Control was described at the annual workshop in October. The main items are:

- Maintenance and update of the web-based methods overview.
- Help NRLs in obtaining pure standards of active substances, when not commercially available and when needed, and legally required, for enforcement of official control.
- To organise proficiency tests for:
    - Carotenoids at authorised levels
    - Coccidiostats at authorised and / or cross contamination levels.
- Follow-up with underperforming laboratories from the 2018 PT for selenium.
- Analytical method development for the determination of p-phenetidine in feedingstuffs and the organisation of a corresponding interlaboratory study.
- Establish a MS/MS semi-targeted profiling database of feed additives for market control.
- Annual workshop (provisional dates 21 – 25 October 2019).

9. NRL Forward Workplan

Secretariat services
The NRL will continue to disseminate information, as appropriate, between the EURL, FSA and OCLs.

Advice and representation within the UK and internationally
Advice will be provided, as requested. The NRL will be represented at relevant meetings, for example, the EURL workshops, for as long as the UK is invited to attend.

Production of standard operating procedures, codes of practice and guidance documents
As requested by the FSA, contribution will be made to the development of standardised operating procedures, relevant codes of practice and guidance documents for use by OCLs and other relevant laboratories.

Compliance assessment via audits and ring trials
The NRL will participate in relevant PTs and method validation studies and co-ordinate the participation of UK OCLs, as appropriate. Training exercises for the UK OCLs will be planned and organised as and when they are deemed necessary.

Co-ordination within the UK of international initiatives
Where appropriate, the recommendations of international organisations related to the standardisation of testing methods will be co-ordinated.

Communication of results and data use
The NRL will provide updates to the FSA, including an annual report.

10. NRL website

LGC's website has been updated and information about our NRL roles can now be found at:

https://www.lgcgroup.com/what-we-do/national-laboratory-and-government-roles/national-laboratory-roles/national-reference-laboratories/ (accessed 21 May 2019)

11. UK OCLs accreditation status

One of the NRLs roles is to maintain a list of the accreditation status of relevant OCLs. Table 2 presents the accreditation status with regards to feed and feedingstuffs as of October 2018, according to the schedules published on the UKAS website.

Laboratory	UKAS accreditation status - Feed additives
Hampshire Scientific Services	No reference to feed / feed additives apart from melamine in feed.
Kent Scientific Services	The following are accredited in animal feedingstuffs: Aflatoxins B1, B2, G1 and G2, Ash Content, Citrinin, Crude Fibre, Copper, Inorganic Arsenic, Lead and Cadmium, Moisture, Nitrogen, Oil, Vitamin A and Vitamin E, Total Mercury, Fumonisins, B1 and B2, Ergot Alkaloids (Ergocornine, Ergocristine, Ergocryptine, Ergometrine, Ergosine, Ergotamine), Deoxynivalenol, Arsenic, Histamine, Mercury, Additives, colourings, preservatives and related contaminants & composition
Lancashire County Scientific Services	The following are accredited in animal feedingstuffs: Ash, Crude oil and fat, Fibre, Moisture, Protein (calculated value), Vitamin A, Vitamin E, Cadmium, Cobalt, Copper, Iron, Lead, Magnesium, Manganese, Zinc
Worcestershire Scientific Services	The following are accredited in animal feedingstuffs: Crude oil and fat, Moisture, Nitrogen and crude protein, Ash, Crude Fibre, Aflatoxins G1, G2, B1, B2, Ash, Dietary fibre, Energy content (by calculation), Fat, Moisture, Nitrogen and crude protein, Ochratoxin A, pH, Salt (expressed as Sodium Chloride), Sodium, Sorbic and Benzoic acid, Sulphur dioxide, Total volatile bases/Total volatile nitrogen
Aberdeen Scientific Services	The following are accredited in animal feedingstuffs: Aflatoxins - B1, B2, G1 and G2 and total aflatoxins, Arsenic and Selenium, Ash, Calcium and Magnesium, Iron, Copper, Manganese, Zinc, Lead, Cadmium, Nitrogen, Oil, Protein, Crude fibre, Vitamin A and E
Dundee City Council Scientific Service (Tayside Scientific Services)	The following are accredited in animal feedingstuffs: Additives, colourings, preservatives and related contaminants, Composition, Aflatoxin B1, B2, G1, G2 and Ochratoxin A, Deoxynivalenol, Zearalenone, Ash, Crude Fibre, Moisture, Oil (total), Nitrogen and crude protein, Nitrogen and Protein, Vitamins A and E, Cobalt, Copper, Iron, Manganese, Zinc, Cadmium, Lead, Arsenic, Selenium
Edinburgh Scientific Services	The following are accredited in animal feedingstuffs: Aflatoxins - B1, B2, G1, G2, Ochratoxin A, Ash, Acid insoluble ash, Crude Fibre, Oil/Fat, Moisture, Nitrogen, Protein, Arsenic, Cadmium, Cobalt, Copper, Lead, Mercury, Selenium and Zinc, Vitamins A, and E
Glasgow Scientific Services	The following are accredited in feedingstuffs: Ash, Crude fibre, Fat/Oil, Crude Oils and Fats, Moisture, Nitrogen, Protein content of feeding stuffs, Crude protein, Cadmium, Copper, Lead, Selenium, Zinc, Calcium, Iron, Magnesium, Manganese, Phosphorus, Vitamin A, Vitamin E
Public Analyst Scientific Services, Wolverhampton	No specific reference to feed / feed additives.
Minton, Treharne and Davies Limited, Carmarthenshire	The following are accredited in animal feedingstuffs: Ash, Copper, Crude Fibre, Lead and Cadmium, Moisture, Nitrogen and Protein, Oil, Generic protocol for the development of methods of analysis

Table 2: Accreditation status of UK OCLs as of October 2018

Appendix 1: Summary of current authorisation process for feed additives

The procedure for authorisation of feed additives is described in Commission Regulation (EC) 1831/2003 on additives for use in animal nutrition https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1558444348834&uri=CELEX:02003R1831-20151230 (accessed 21 May 2019)

In summary, the authorisation procedure involves the following steps:

- The applicant sends a declaration form to the EURL who check the information. The appropriate fee is calculated by the EURL and paid by the applicant.
- The completed application form is sent by the applicant to the European Commission (DG Santé).
- Reference samples are sent to the EURL by the applicant.
- The Commission (EFSA) makes all the information provided by the applicant available to the EURL who evaluate the technical dossier (see below for information provided). At the same time EFSA carry out a scientific assessment.
- The Rapporteur (EURL or a NRL) prepares an initial report which is made available to NRLs for peer review. Comments received are incorporated into a revised version of the evaluation report which is sent, via the EURL if they were not the Rapporteur, to EFSA, DG Santé and the applicant.
- If the evaluation report indicated that further testing and / or validation is required, the EURL provide a detailed work-plan.
- The Commission prepare a draft Regulation to grant or deny authorisation.

Authorisations are valid for 10 years and applications can be made to the European Commission to renew authorisation.

EFSA are required to provide an opinion within 6 months of receipt of a valid application and the Commission is required to prepare a draft Regulation to grant or deny authorisation within 3 months or receiving EFSA's report.

Guidelines to the authorisation process have been published on the EURL Feed Additives webpage: https://ec.europa.eu/jrc/en/eurl/feed-additives/guidance-for-applicants (accessed 21 May 2019)

The applicant is required to send the following information to EFSA who then forward it to the EURL for review:

(a) name and address;

(b) the identification of the feed additive, a proposal for its classification by category and functional group under Article 6 of Commission Regulation (EC) 1831/2003, and its specifications, including, where applicable, purity criteria;

(c) a description of the method of production, manufacturing and intended uses of the feed additive, of the method of analysis of the additive in feed according to its intended use and, where appropriate, of the method of analysis for the determination of the level of residues of the feed additive, or its metabolites, in food;

(d) a copy of the studies which have been carried out and any other material which is available to demonstrate that the feed additive satisfies the criteria laid down in Article 5(2) and (3), i.e. that it is safe and has the necessary favourable characteristics;

(e) proposed conditions for placing the feed additive on the market, including labelling requirements and, where appropriate, specific conditions for use and handling (including known incompatibilities), use levels in complementary feedingstuffs and animal species and categories for which the feed additive is intended;

(f) a written statement that three samples of the feed additive have been sent by the applicant directly to the Community reference laboratory;

(g) for additives which, according to the proposal under point (b), do not belong to either category (a) or category (b) referred to in Article 6(1) (technological additives and sensory additives respectively), and for additives falling within the scope of Community legislation relating to the marketing of products consisting of, containing or produced from GMOs, a proposal for post-market monitoring;

(h) a summary containing the information provided under points (a) to (g);

(i) for additives falling within the scope of Community legislation relating to the marketing of products consisting of, containing or produced from GMOs, details of any authorisation granted in accordance with the applicable legislation.

The fees for the authorisation of a new feed additive are detailed in Commission Regulation (EC) No 378/2005 (https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1558444527803&uri=CELEX:02005R0378-20151022) (accessed 21 May 2019) and are EUR 6000 for one feed additive.

According to the EURL for feed additives authorisation's activity report for 2017, 31 applications were evaluated during the year.

Appendix 2: EURL method for the determination of carotenoids

Determination of Carotenoids in animal compound feed and premixtures by Reverse Phase High Performance Liquid Chromatography – UV detection (RP-HPLC-UV)

Ursula Vincent, Federica Serano

Version: February 2018

Determination of Carotenoids in animal compound feed and premixtures by Reverse Phase High Performance Liquid Chromatography – UV detection (RP-HPLC-UV)

1. Scope

This analytical procedure specifies a reverse phase high performance liquid chromatographic – UV detection (RP-HPLC-UV) method for the simultaneous determination of four authorised carotenoids in fish compound feed, namely astaxanthin (AXN), canthaxanthin (CXN), adonirubin (ADR) and astaxanthin dimethyldisuccinate (AXN DMDS), and of six authorised carotenoids in poultry feed, namely canthaxanthin (CXN); capsanthin (CSN), ethyl ester of beta-apo-8'-carotenoic acid (BACARE), citranaxanthin (CIXN), lutein (LUT) and zeaxanthin (ZEA) at levels ranging from 3 to 3000 mg kg^{-1}(depending on the carotenoid). beta-carotene (BCAR), which is authorised as colorant in canaries and as vitamin and provitamin in all animal species, was also added to the scope. The analytical procedure is fit for the purpose of quantitation of declared carotenoids and labelling confirmation. The procedure applies for natural and synthetic feed additives.

This procedure does not involve a saponification. Therefore, xanthophyll esters like those of lutein, zeaxanthin and capsanthin possibly present in the basic feed are not covered part of the scope of this method.

2. Normative References

The following referenced documents are indispensable for the application of this protocol. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6498:2012, Animal feeding stuffs – Guidelines for sample preparation
ISO 6497:2002, Animal feeding stuffs – Sampling

3. Principle

The carotenoids are first disclosed through an enzymatic reaction at 50 °C. The samples are extracted with acetone by means of a pressurized liquid extraction instrument or by liquid solid extraction. The extracts are centrifuged and analysed by reverse phase HPLC with Diode Array Detection. The isosbestic wavelength of 410 nm is selected for the determination. The quantitation is performed through external calibration.

4. Reagents and materials

WARNING –
1 – Carotenoids are subject to light degradation. Protect analytical work adequately from day light, and keep standard solutions protected from light by using amber glassware, amber vials or aluminium foil.

2 – Avoid inhalation of and exposure to the toxic standard materials and solutions thereof. Work under fumehood when handling the solvents and solutions. Wear safety glasses and protective clothing.

3 - Always wear a safety mask when handling Hydromatrix®.

Use only reagents of recognized analytical grade, unless otherwise specified.

4.1 Alcalase/protease/ Multifect PR 6L.

4.2 Purified water, e.g. Milli-Q or equivalent.

4.3 Butylated hydroxytoluene BHT.

4.4 Hydromatrix®, bulk support material.

4.5 Acetone, HPLC grade.

4.6 Acetonitrile, HPLC grade.

4.7 Methyl tert-butyl ether tBME, HPLC grade.

4.8 Tetrahydrofurane stabilised with 250-350 ppm BHT, HPLC grade.

4.9 n-Hexane, spectroscopic grade[1].

4.10 light petroleum 40-60%, spectroscopic grade [1]

4.11 Ethanol, spectroscopic grade[1]

4.12 cyclohexane, spectroscopic grade[1]

4.13 Mobile phases for HPLC

4.13.1 Phase A: acetonitrile:methyl tert-butyl ether:water mixture approximately 70:20:10; v:v:v, stabilised with 1000 ppm BHT

Transfer 695 ml of acetonitrile (4.6) into a 1000 ml bottle. Add 200 ml of methyl tert-butyl ether (4.7) and 105 ml water (4.2). Add 1.0 g of BHT (4.3). Perform

[1] Depending on the carotenoid for which the UV standardisation of the standard solution is performed (4.17.2)

mixing and degassing for 10 min in an ultrasonic bath (5.11). This mobile phase is stable for 28 days.

NOTE 1: The retention time of the carotenoids is strongly influenced by slight differences in the composition of mobile phase A. The use of an HPLC quality control sample (8.1) is crucial for the correct signal allocation. Furthermore, the presence of non-targeted carotenoids may interfere with the signals of the targeted analytes when the composition of mobile phase A deviates from 4.11.1.

4.13.2 Phase B: acetonitrile:methyl tert-butyl ether mixture (70:30; v:v)

Transfer 700 ml of acetonitrile (4.6) into a 1000 ml -bottle. Add 300 ml methyl tert-butyl ether (4.7). Add 1.0 g of BHT (4.3). Perform mixing and degassing for 10 min in an ultrasonic bath (5.11). This mobile phase is stable for 28 days.

4.14 Reference standards

Guaranteed purity is required for each lot of reference standard.

4.14.1 Astaxanthin (AXN), minimum 97 % purity

4.14.2 Canthaxanthin (CXN), minimum 97 % purity

4.14.3 Adonirubin (ADR), minimum 97 % purity

4.14.4 Astaxanthin dimethyldisuccinate (AXN DMDS), minimum 95 % purity

4.14.5 Capsanthin (CSN), minimum 95 % purity

4.14.6 Ethyl ester of β-apo-8'-carotenoic acid (BACARE), minimum 95 % purity

4.14.7 Lutein (LUT), minimum 95 % purity

4.14.8 Citranaxanthin (CIXN), minimum 95 % purity

4.14.9 Zeaxanthin (ZEA), minimum 95 % purity

4.14.10 beta-carotene (BCAR), minimum 95 % purity

4.15 Standard solutions

Protect all standard solutions from daily light.

4.15.1 Carotenoid[2] stock standard solution, ca. 200 µg ml^{-1}.

4.15.1.1 When the storage container contains more than 1.0 mg of the selected carotenoid [3], accurately weigh 1.0 mg of this carotenoid (note down the weight of standard) into a 5 ml volumetric flask. Dissolve and make up to the mark with THF/BHT (4.8). Mix well using a vortex mixer (5.27) and an ultrasonic bath (5.11). The accurate concentration needs to be standardized using a spectrophotometer (5.2) as described in detail in 4.15.2.

4.15.1.2 When the storage container specifies a nominal content equal to or less than 1.0 mg of the selected carotenoid [3], transfer quantitatively the whole content in a 5.0 ml volumetric flask. Dissolve and make up to the mark with THF/BHT (4.8). Mix well using a vortex mixer (5.27) and an ultrasonic bath (5.11). The accurate concentration needs to be standardized using the spectrophotometer (5.2) as described in detail in 4.15.2.

Prepare fresh and measure immediately.

4.15.2 UV standardisation of the standard solutions.

Pipette 100 µl of the selected carotenoid stock standard solution (4.15.1) into a 10 ml volumetric flask and make up to the mark with the appropriate solvent. The nominal value of the obtained solution is ca. 2 µg ml^{-1}. Measure the absorption of this solution against the pure solvent at the maximum of absorption (scan the maximum), using the spectrophotometer (5.2). The maximum is solvent specific and is given as approximate value in Annex I.
The exact content of the selected carotenoid is given by equation 1 (Eq. 1).

Carotenoid µg ml^{-1} = $E_{\lambda max}$ × 10000/ $E^{1\%}_{1cm}$ Eq. 1

e.g. for all-E AXN, the solvent suggested in the table in Annex I is n-hexane. For this solvent, the wavelength of measurement is approximately 470 nm and the $E^{1\%}_{1cm}$ (or $A^{1\%}_{1cm}$) is 2100.

Pipette with a suited pipette 100 µl of the astaxanthin stock standard solution (4.15.1) into a 10 ml volumetric flask and make up to the mark with n-hexane. The nominal value of the obtained standard measuring solution is ca. 2 µg ml^{-1}. Measure the UV spectrum of this solution against pure n-hexane, using the spectrophotometer (5.2) set at the wavelength of maximum absorption (approx. 470 nm).[4]

AXN µg ml^{-1} = E_{max} × 10000/2100

A solution of the same concentration, 100 µl of the astaxanthin stock standard solution (4.15.1) pipetted into a 10 ml volumetric flask and made up to the mark with acetone (4.5), shall be injected simultaneously in the HPLC (see 4.15.3.1)

---

[2] astaxanthin (AXN), canthaxanthin (CXN), adonirubin (ADR), astaxanthin dimethyldisuccinate (AXN DMDS), capsanthin (CSN), ethyl ester of beta-apo-8'-carotenoic acid (BACARE), citranaxanthin (CIXN), lutein (LUT), zeaxanthin (ZEA) and beta-carotene (BCAR)
[3] 4.14.1 or 4.14.2 or 4.14.3 or 4.14.4 or 4.14.5 or 4.14.6 or 4.14.7 or 4.14.8or 4.14.9 or 4.14.10 depending on the carotenoid selected.
[4] The solvent of the measured solution is not pure as there's a small presence of THF/BHT (4.8). However we consider the effect negligible and we apply the same tabulated extinction coefficient given that the contribution of THF/BHT (4.8) is very small (1%).

4.15.3 HPLC standard calibration curve

4.15.3.1 Carotenoid standard calibration curve

Pipette with a suited pipette 100 µl of carotenoid stock standard solution (4.15.1) into a 10 ml volumetric flask and make up to the mark with acetone.

5. Apparatus

Usual laboratory apparatus and, in particular, the following:

5.1 HPLC system consisting of the following:

5.1.1 Pump, pulse free, flow capacity 0.1 ml min^{-1} to 2.0 ml min^{-1},

5.1.2 Injection system, manual or autosampler,

5.1.3 UV/VIS detector, variable wavelength, suitable for reliable measurements at 410 nm, or UV/VIS photodiode array detector (DAD),

5.1.4 Computer data system,

5.1.5 Analytical column, Supelco® Suplex pKb-100 5 µm, 250 x 4.6 mm, or equivalent,

5.1.6 Guard column, 5 µm, 2 cm x 4 mm SUPELCOSIL™ Suplex™ pKb-100 Supelguard™ Cartridge or equivalent.

5.2 Spectrophotometer, with 1 mm apertures.

5.3 Grinding instrument and

5.4 Sieve, with 1 mm apertures.

5.5 Balances, one analytical, of 10 g capacity or greater with 0.1 mg readability, and one, of 100 g capacity or greater with 0.01 g readability.

5.6 Polypropylene containers, 100 ml with lids.

5.7 Variable-volume positive displacement piston pipettes, suitable for pipetting volumes ranging from 50 to 100 µl.

5.8 Glass volumetric flasks of 1.0, 100, 500 and 1000 ml.

5.9 Round bottom flasks of 500 ml.

5.10 Allhin condenser.

5.11 Ultrasonic bath, temperature controlled.

5.12 Flat spatulas in polypropylene.

5.13 PLE, pressurized solvent extraction system, ASE 300 Dionex®, Büchi SpeedExtractor E-914 or equivalent.

5.14 PLE cells, suitable for the extraction unit used, 66 ml or larger.

5.15 Cellulose filters for PLE cells.

5.16 PLE vials, for the extraction unit used, at least 240 ml.

5.17 **Graduated cylinder,** 250 ml.

5.18 **Microcentrifuge safe-lock tubes,** 1.8 ml, Eppendorf® tubes or equivalent.

5.19 **Microcentrifuge.**

5.20 **1.5 ml HPLC amber glass vials.**

5.21 **HPLC glass vials crimper**

5.22 **Centrifuge**

5.23 **Centrifuge tubes,** 100 ml

5.24 **Ultraturrax mixer**

5.25 **PLE Funnels for PLE cells.**

5.26 **Powder funnels**

5.27 **Vortex mixer**

5.28 **Amber glass vials,** 5 or 10 ml, with hermetic stopper

5.29 **Heating mantle or heated bath**

5.30 **Magnetic stirrer**

5.31 **150 ml volumetric flasks**

6. Sampling

It is important that the laboratory receives a sample that is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this protocol. A recommended sampling method is given in EN ISO 6497.

7. Preparation of test sample

7.1 General

Prepare the test sample in accordance with EN ISO 6498.

7.2 Laboratory sample

Grind the laboratory sample (usually 50 g) so that it passes completely through a sieve with 1 mm apertures. Mix thoroughly.

7.3 Test sample

The test sample consists of a representative and homogenised aliquot of the ground laboratory sample of at least 10 g.

7.4 Test portion

Accurately weigh 5.0 g to the nearest 0.1 g of the thoroughly mixed test sample into a 100 ml polypropylene container (5.6) for extraction procedure 8.3.1. or into a 100 ml centrifuge tube (5.23) for extraction procedure 8.3.2. Note down the mass expressed in g. Submit it to the analysis procedure (8).

8. Procedure

The complete procedure (8.2 to 8.4) should be applied to two test portions (7.4) of the same test sample in order to perform two independent and parallel determinations.

8.1 HPLC quality control standard solution (QCS)

The QCS shall be injected in duplicate before and after the complete sequence samples each day of analysis, in order to assess the suitability of the HPLC/DAD system.

The QCS contains two carotenoids: CXN[5], a stable and more soluble carotenoid and AXN, a less stable and less soluble carotenoid. The mixture is isomerised until a constant ratio of the isomers is reached. This solution, when protected from oxygen, is stable for several months.

Weigh approximately 0.4 mg CXN (4.14.2), 0.8 mg AXN (4.14.1) and 0.5 g BHT (4.3) in a round bottom flask (5.9). Add 200 ml of an Acetone (4.5): Water (4.2) 75:25 v:v solution. Fit a condenser (5.10) to the flask and immerse the flask in a heating mantle or heated bath (5.29) with a magnetic stirrer (5.30). Heat to boiling and allow to refluxing for about 1.5 to 2 hours[6]. This reaction will cause the formation of a stable isomers ratio. Transfer to a 500 ml volumetric flask (5.8) and make up to volume with acetone (4.5). Mix well and transfer

[5] Depending on the analytes to be determined a relevant carotenoid should be added to this mix.
[6] Alternatively, the mixture can be heated at 80°C in a pressure-resistant tube.

immediately in HPLC vials. The filling should be fast and each vial should be immediately closed to avoid evaporation. Store the vials at room temperature and away from light.
Inject one vial. Record the areas of the two peaks and their retention time. These values will be considered as the REFERENCE ($area_{reference\ i}$ and $Rt_{reference\ i}$ with i being 1 or 2 (2 being the total number of carotenoids in the QCS solution).

8.2 Enzymatic disclosure

Add 0.2 g of BHT (4.3), 100 µl of enzyme (4.1) and 15 ml of purified water (4.2) to the test portion (7.4). Close tightly and shake vigorously to ensure that all the feed is permeated with water. Place in an ultrasonic bath (5.11) with the temperature set at 50 °C for 15 to 20 minutes. Shake every 5 minutes.

8.3 Extraction

8.3.1 Extraction using PLE

Remove the lid of the test portion after 8.2, taking care of not losing sample drops. Add approximately 13 g Hydromatrix® (4.4), close tightly and hand-shake vigorously until the wet feed sample looks all adsorbed on the Hydromatrix beadlets and detaches well from the polypropylene container's wall[7]. Open the containers carefully, remove the sample particles from the lid into the container using a flat spatula (5.12). Mix well with the spatula (5.12), making sure that there are no sample clusters left. Place 2 cellulose filters (5.15) on the bottom of the PLE cell (5.14). Transfer all the material in the PLE cell (5.14) using a funnel (5.25). Top up with Hydromatrix (4.4) if needed. Close the cell and extract according to the following method

- Extraction solvent: acetone (4.5)
- Pressure: 103.4 bars (1500 psi)
- Temperature: 58 °C
- Preheat time: 0 min
- Heat time: 0 min
- Static time = 7 min
- Flush volume 120%
- Purge time = 60 sec
- Static cycles = 3

Each feed extraction lasts approximately 30 minutes. The instrument can run up to 12 samples in an automated way, e.g. overnight.

Take the tube containing the raw extract. Mix well and record the total volume (V_{ext}) of extract using a graduated cylinder (5.17).

[7] stomping the container on the table can help

8.3.2 Conventional Liquid Solid Extraction

Add 50 ml of acetone (4.5) and shake for 1 minute. Centrifuge (5.22) at 1850 g for 10 minutes. Collect the supernatant (first extract) and transfer into a 150 ml volumetric flask (5.31).

Add 50 ml of acetone (4.5) to the solid residue of the first extraction. Use the ultra-turrax mixer (5.24) if needed to achieve dissolution and shake for 1 minute. Centrifuge (5.22) at 1850 g for 10 minutes. Collect the supernatant (second extract) and pool with the first extract in the 150-ml volumetric flask.

Add 40 ml of acetone (4.5) to the residue of the second extraction and shake for 1 minute. Centrifuge (5.22) at 1850 g for 10 minutes. Collect the supernatant (third extract) and pool with the two first extracts in the 150 ml volumetric flask. Make up to volume with acetone and shake vigorously.

Transfer a 1.8 ml aliquot of the raw extract into a microcentrifuge safe-lock tube (5.18).

Centrifuge (5.19) for 1 minute at high speed (e.g. 13000 g)[8]. Transfer the supernatant to a HPLC amber vial (5.20) and proceed to the HPLC analysis (8.5.4).

8.4 HPLC Analysis

8.4.1 Analytical conditions

The following conditions are provided for guidance. Other conditions may be used provided they yield to equivalent results.

8.4.1.1 HPLC column: as in 5.1.5.

8.4.1.2 guard column: as in 5.1.6.

8.4.1.3 mobile phase: as in 4.13, flow rate: 0.5 ml min^{-1}.

8.4.1.4 injection volume: 5 µl.

8.4.1.5 column temperature: 20°C.

8.4.1.6 detection wavelength: isosbestic wavelength 410 nm[9].

Perform the elution of the carotenoids in gradient mode. The gradient to be applied is displayed in Table 1.

[8] Alternatively, filter the extract through a 0.45 µm membrane filter (suitable for acetone).
[9] K. Mitrowska et al., *Journal of Chromatography A*, 1233 (2012) 44– 53

Table 1: elution gradient

Time (min)	% of mobile phase A	% of mobile phase B
0	100	0
10	100	0
14	40	60
28	40	60
28.1	100	0
45	100	0

Using these conditions the retention times for AXN DMDS, AXN, ADR, CXN, CSN, CIXN, BACARE, LUT, ZEA and BCAR should approximately be 8.0, 13.5, 14.5, 15.8, 18.6, 20.9, 21.4, 24.5, 25.8, and 32 min respectively.

8.4.2 External calibration curves

Inject each calibration solution (4.15.3) immediately after preparation in the HPLC system (5.1) and in parallel with the spectrophotometric standardization (4.15.2). Note the area of the peak, A. The concentration of this solution, c, correlated to the area of the peak is the exact content determined using the spectrophotometer (in µg ml^{-1}).
The calibration regression line is plot with the following two points:

- Calibration point 1: concentration 0 µg ml^{-1}; area 0 (Arbitrary Unit AU)
- Calibration point 2: concentration c = exact content determined using the spectrophotometer in µg ml^{-1}; determined area A (AU).

Determine the equation of the calibration regression line (Eq. 2).

$$A = a \times c \qquad \text{Eq. 2}$$

with a being the slope coefficient of the calibration curve obtained from the standard solutions.

8.4.3 Quality Control Sample

Inject the quality control sample QCS (8.1) and verify the suitability of the HPLC system as a whole. Record and plot the QCS areas and retention times obtained and compare to the reference values (area and retention times) (8.1). Inject a new QCS vial at the end of the sample sequence to verify that the suitability of the HPLC is still acceptable.

8.4.4 Sample extracts

Inject each sample extract obtained in (8.3.1) or (8.3.2) in duplicate (the determined area should be stable). Determine the mean peak area for the carotenoids signals.

8.4.5 Criteria for acceptance of the analytical result

8.4.5.1 Run acceptance

A QCS must always be injected before and after an analysis is performed. Samples' results can only be calculated if the following acceptance criteria are satisfied:

a) The areas of the QCS AXN and CXN peaks shall be equal (± 5 %) to the respective areas of the reference sample (8.1).

b) The retention time of the QCS AXN and CXN peaks shall be equal (± 5 %) to the respective retention times of the reference sample (8.1).

If the criteria are not met, a new calibration is needed[10].

c) The retention time of the AXN and CXN peaks in the sample extract (8.4.4) shall be equal (± 5 %) to the respective retention times in the QCS (8.4.3).

d) The retention time of the AXN and CXN peaks in the first sample extract (8.4.4) in the sequence shall be equal (± 2 %) to the respective retention times in the last sample extract (8.4.4)[11].

The samples should pass the identification criteria (8.4.5.2).

8.4.5.2 Identification of the analytes

The identity of the analytes is confirmed if the following three criteria are satisfied:

a) The retention time of the sample peaks shall be equal (± 5 %) to the retention time of the standard peaks. In case of doubt, standard addition (standard material added to the sample) shall be performed.

b) Assess the purity of the sample peak on the basis of the conformity of the difference spectra, recorded at the apex and at up-slope and down-slope inflection points. For those parts of the spectra with a relative absorption of at least 10 % (between 10% and 100% in a normalized spectrum), at each wavelength the relative absorption shall be equal (within 25%) for all spectra.

c) The difference spectra of the sample and standard peaks recorded at the peak apex shall not be visually different for those parts of the spectra with a relative absorption of at least 10 % (between 10% and 100% in a normalized spectrum). This criterion is met when:

- the same maxima are present within a margin determined by the resolution of the detection system (4 nm).

---

[10] When the system is stable, it can be run smoothly without performing a new calibration for at least 3 months.
[11] For very long sequences, a QCS sample should be inserted as a new reference for retention times' comparison. The next sample extract injected therefore would also become the new reference for retention times' comparison. The criterion is valid when no change is made, e.g. the same mobile phase batch is used along the whole sequence.

- at those particular maxima wavelengths, the relative deviation, in comparison with the absorbance of the standard analyte, between the two spectra (sample - standard) does not exceed 25 %.

9. Calculation of results

Calculate the target carotenoid content in the unknown samples by comparing the peak area of the injected sample extract with the peak area of the standards used for the external calibration curve (8.4.2).

Use the following equation [5] for the calculation of target carotenoid content (C_{TC}) of the sample in milligrams per kilogram (mg kg^{-1}).

$$C_{TC} = \frac{A_{TC}}{a} \times \frac{V}{m} \qquad \text{Eq. 3}$$

where

C_{TC} is the initial concentration of the target carotenoid in the feed sample (mg kg^{-1})

A_{TC} is the area of the HPLC peak for the target carotenoid (AU)

a is the slope coefficient of the calibration curve obtained from the standard solutions (Eq. 4)

V is the final volume of extract (ml)

m is the test portion mass (g)

10. Test report

The test report shall specify:

- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- all information necessary for the complete identification of the calibrants;
- the test method used, with reference to this protocol;
- all operating details not specified in this protocol, or regarded as optional, together with details of any incidents which may have influenced the test result(s);
- the test result obtained and the units in which the test result(s) have been expressed;
- if the repeatability has been checked, the final quoted result obtained,
- if the trueness has been checked, the final quoted result obtained.

Annex I

I – Absorption coefficient values

- Carotenoids in solution obey the Beer-Lambert law, namely their absorbance is proportional to the concentration.
- Values of absorption coefficients $E^{1\%}_{1cm}$,(or $A^{1\%}_{1cm}$) specified for a particular solvent, are published in literature (e.g. Britton G, Liaaen-Jensen S, Pfander H. 2004. Carotenoids Handbook or USP monographs or JECFA monographs).
- For convenience, the absorption coefficient values used during the in-house validation, the respective solvent, the λ_{max} and the typical coefficient of the calibration curve obtained are reported in the table below. Other values, extracted from published articles or review or experimentally determined, may be equally suitable to determine the exact concentration.

Analyte	Solvent used	$A1^{\%}_{1cm}$ or $E1^{\%}_{1cm}$	approx. λ_{max} (nm)
AXN	n-Hexane [1]	2100	470
AXN DMDS	n-Hexane[2]	1519	470
CXN	Cyclohexane[3]	2200	466
ADR	Cyclohexane[4]	2150	476
CIXN	Cyclohexane[5]	2680	473
CSN	Acetone[6]	2157	470
LUT	Ethanol[7]	2550	445
ZEA	Ethanol[8]	2480	450
BACARE	Cyclohexane[9]	2550	449
BCAR	Cyclohexane[10]	2500	455

[1] Britton G, Liaaen-Jensen S, Pfander H. 2004. Carotenoids Handbook (No 404 - 406).
[2] Derived from $A1^{\%}_{/1cm}$ of astaxanthin considering the different molecular weights.
[3] JECFA monograph - 51st JECFA (1998), published in the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1 (2005). Corrected at the 69th JECFA.
[4] Derived from the A1%/1cm-values of astaxanthin and canthaxanthin.
[5] JECFA Monograph prepared at the 31st JECFA (1987), published in FNP 38 (1988) and in FNP 52 (1992).
[6] alternative to the use of benzene ($E1^{\%}_{1cm}$ 2072, approx. λ_{max} (nm) 483).
[7] US-Pharmacopoeia, 2009 USPC Official 8/1/09 - 11/30/09 Dietary Supplements: Lutein.
[8] Britton G, Liaaen-Jensen S, Pfander H. 2004. Carotenoids Handbook (No 119).
[9] 74th JECFA (2011) and published in FAO Monographs 11 (2001), superseding specifications prepared at the 28th JECFA (1984), published in the combined compendium of feed additive specifications, FAO JECFA Monographs 1 (2005).
[10] JECFA Monograph prepared at the 31st JECFA (1987), published in FNP 38 (1988) and in FNP 52 (1992).

Appendix 3: Executive summary of the proceedings of the Feed Additives Authorisation workshop

Proceedings of the 18th workshop of the EURL & NRLs for Feed Additives authorisation

EURL
European Union Reference Laboratory
for Feed Additives

Report prepared by:	M. J. Gonzalez de la Huebra
Report checked by:	Z. Ezerskis
Report approved by:	C. von Holst

Executive Summary

The 18th workshop (WS) of the EURL Feed Additives (EURL-FA) authorisation was organised and held in Brussels on October 17 - 18, 2018. A total of thirty five participants, representing 21 National Reference Laboratories (NRLs), DG SANTE, EFSA, and the EURL-FA, took part in the workshop. In addition, the representative from feed industry association FEFANA and the external expert from the European Directorate for the Quality of Medicines (EDQM) of Council of Europe attended the public part of the event.

C. von Holst, operating manager of the EURL-FA, started the event by introducing the agenda of the workshop to the participants.

Z. Ezerskis (EURL-FA) gave the presentation on EURL-FA authorisation activities and deliverables of 2018 such as management of declaration forms and feed additive samples, evaluation of dossiers and drafting evaluation reports, the progress of the project related to the publishing on the EURL website the recommended analytical methods for official control of feed additives, annual report on EURL activities in 2017, evaluation of the methods for determination of urea in pet feed on special request of DG SANTE and few other topics the EURL contributed to.

C. von Holst (EURL-FA) continued by presenting the EURL activities of the standardisation work in the frame of CEN mandate concerning analytical methods for certain feed additives such as carotenoids, vitamins A, E and D, coccidiostats, organic acids explaining prospects and concerns of single versus multi-analyte methods and concluding that the work is in good progress. In addition he gave a summary of the revision of the CEN methods for the determination of probiotics.

L. Arnaud (FEFANA) gave an overview of industry experience on analytical aspects of feed additives describing the requirements for the methods proposed for official control, then focusing on the methods used for internal quality control and applicability issues of the standard methods for enumeration of yeasts (both for non-coated and coated formulations) used as feed additives. The presentation also included the examples of the methods for other feed additives such as biotin, silage enzymes and selenised-yeast.

U. Rose (EDQM) gave an overview of the EDQM organisation, presented the work program and procedures of European Pharmacopeia which represents one the departments of EDQM, afterwards focusing on requirements and validation aspects of the analytical methods of the European Pharmacopeia. The presentation included multiple examples of the specific methods demonstrating how the individual method performance characteristics are applied for the identification tests and for the assay of main active substances and quantification of impurities in pharmaceutical substances and medicinal products.

A. Rodriguez (DG SANTE) presented the current status concerning the applications for re-authorisation of several products of Vitamin B_2 and B_{12} produced using genetically modified (GM) strains, pointing out that the products produced with/by GM organisms are not supposed to contain traces of recombinant DNA and an additional authorisation under GMO legislation is not necessary. Then, the case study was presented concerning the vitamin B_2 product which was not supposed to contain the recombinant GMO but after laboratory test showed the presence of GMO and finally after negative EFSA opinion the existing authorisation was denied. Finally, it was concluded that the vitamin B_2 and B_{12} products with favourable EFSA opinions will be eventually authorised while the existing

products on the market not in compliance with the additives authorised will be withdrawn.

S. Bellorini (EURL-FA) presented an overview of botanical flavourings dossiers, outlining analytical aspects of these feed additives, particularly focusing on the requirements of the methods for the determination of phytochemical markers in the feed additives as described in the EURL technical guide for the applicants, which was presented in more details.

M. Innocenti (EFSA) updated on the workload of EFSA in terms of dossiers, mandates and opinions. Then, the work on chemically and botanically defined flavourings was outlined by focusing on prospects and concerns of the two - whole mixture and component based - safety assessment approaches of the flavourings for target species. Afterwards, the new structure of FEEDAP panel was presented by pointing out that it helps to streamline the work flow in more efficient way. Finally, the new ways of collaboration with EURL were mentioned in evaluating the analytical methods for characterisation of feed additives and the methods for residues in tissues.

M.J. Gonzalez de la Huebra (EURL-FA) presented the final outcome of the Optiphos project. The presentation focussed on the results of the intercomparison assay organised by the EURL. The materials (feed additive and feedingstuffs) analysed in the frame of this exercise by using the VDLUFA/ISO method and the Optiphos method led to precision values in agreement with the reproducibility values obtained in the frame of previous phytase interlaboratory studies (ILC) and selected as fitness-for-purpose criteria for this project. The project has been successfully finished by the establishment of a robust conversion factor that would allow the NRLs to screen the Optiphos products by using the VDLUFA/ISO method in the frame of labelling controls, however a sound 'implementation strategy' should be agreed to ensure an harmonised approach.

Finally, C. von Holst (EURL-FA) concluded the workshop and outlined the prospectives for the next year by pointing out that for the next workshop it would be useful that NRLs present newly about their updated organisational structure and activities. Also joint discussion on selected dossiers could be envisaged for the next time. In addition, the important aspects for the rapporteurs and the duties of NRLs to comment the EURL evaluation reports as well as the request to NRLs to update their competence profile were pointed out.

The overall feedback from participants was very positive, the feedback on the relevance of the program of the workshop and on the balance between formal presentations and discussions was very well perceived.

Appendix 4: Executive summary of the proceedings of the Feed Additives Control workshop

European Commission

JRC CONFERENCE AND WORKSHOP REPORTS

Proceedings of the 7th EURL-FA Control Annual Workshop

Vincent, U., Serano F., Chedin, M., von Holst, C.

Executive Summary

The 7th workshop of the EURL for Feed Additives Control (EURL-FA Control) and the consortium of National Reference Laboratories (NRLs) was held at the Albert Borschette meeting Centre in Brussels on October 18-19, 2018. A total of thirty-five participants, representing 24 National Reference Laboratories (NRLs), 1 National Official Control Laboratory (OCL), DG SANTE, and the EURL-FA attended the event. In addition, two representatives from FEFANA were invited to give a presentation to the consortium.
This 7th workshop was on one side the concluding event for the organisation of the first PT exercise conducted by the EURL-FA Control for the determination of total selenium in animal feed. The results from the PT exercise were presented to all participants and discussion including compliance assessment was carried out.
In addition the annual workshop also gave the opportunity to exchange with the NRLs on activities carried out in the field of analysis of feed additives, to get update and clarification from DG SANTE on specific issues such as the on-going revision on Commission Regulation (EC) 152/2009 or the control of labelling. This specific discussion slot was also devoted on clarifying the new cascade approach and on identifying the next steps to tackle the determination of urea both as additive in ruminant feed or as in other feeds. An important outcome of the latter discussion was that there is a need for a revision of the official method for the determination of urea as described in Commission Regulation (EC) 152/2009. Upon request of DG SANTE, the EURL-FA Control will perform a study aiming at (i) determining the suitable methods for the determination of urea both as additive in ruminant feed and as undesirable substance in non-ruminant feed and (ii) optimising and validating such method(s). Following this work, decision regarding the revision of the Commission Regulation will be taken by DG SANTE.
An update on the activities performed by the EURL-FA Control and by another NRL in the frame of the collaboration with the CEN on the development of EN standards for the determination of carotenoids in feed and for the determination of vitamins A, E and D in feed respectively was also given to all participating NRLs.
Finally, the other activities performed by the EURL-FA Control in 2018 were reviewed (including the outcome of the first hands-on training organised for the NRLs for feed additives Control at JRC-Geel for the determination of carotenoids in feed) and the work program for 2019 was presented. The NRLs were informed that in 2019, two PTs will be organised (1) on the determination of carotenoids in feed and (2) on the determination of coccidiostats in feed at cross-contamination and / or additive levels.
The mandatory follow-up for underperforming laboratories during the 2018 PT exercise on cobalt determination will also be carried out.

Finally the participants were encouraged to give feedback to the EURL-FA Control regarding their needs and priority list, for PTs for the period 2020 and beyond and training for the period 2019-2020. The feedback could be given at the workshop but also by electronic mail or using the dedicated web platform for discussion and exchange CIRCA-BC. The participants were also encouraged to use the CIRCA-BC platform in particular for discussion fora or to notify the EURL-FA Control if this tool was considered as not relevant.

A satisfaction survey on the organisation of the PT, the hands-on training and of the annual workshop was conducted. The response rate for the complete survey was 100%. The overall satisfaction rate was of 98% and 89% of the answers were above 85% of satisfaction. The overall satisfaction rate for the 2018 hands-on training was 100% with 100% of these positive answers being above 85% of satisfaction. For the PT, these rates were respectively 100 % and 88 %. Finally, as for the organisation and structure of the workshop, the results were also very good with a 100% satisfaction rate and 92% above 85% of satisfaction.

Highlights from the workshop

The different topics addressed at the workshops are displayed in the enclosed workshop programme and in the slides of all presentations included in these proceedings. In complement to these slides, the highlights presented below could be extracted.

Collaborative trials – reproducibility and fitness for purpose criteria

The results of the collaborative trial carried out for the validation of an analytical method for the determination of vitamins A, E and D, in the frame of the CEN mandate were presented. The method principle and development were recalled; cold or hot saponification can be used and advantages of the cold saponification were emphasized. The speaker signalled analytical problems occurring when using the method for the determination of Vitamin E in high concentration. As for the results of the collaborative trial, it was seen that in some cases (e.g. Vitamin D_3) the relative standard deviation for reproducibility RSDR % obtained during the trial was higher than 15%. This value was estimated to be satisfactory by the NRLs and the method fit for purpose.

The topic of high RSDR% occurring during the collaborative trial of multi-analyte methods was further discussed with the presentation of the results obtained during the collaborative study of the multi-analyte method for the determination of carotenoids in animal feed, also in the frame of the collaboration with CEN. RSDR% as high as 30% were obtained for some analytes in some matrices tested. The project leader however reported the outcome of the discussions held during the relevant CEN meetings: due to the multi-analyte character of the method and to the complexity introduced by the presence of the stereoisomers, at the levels of concentration addressed this RSDR% was still found acceptable.

Urea in feed

Background: urea (carbamide) is a feed additive authorised only in ruminants feed (Commission Implementing Regulation (EU) No 839/2012). However the official method for the determination of urea in premixtures, feedingstuff and water listed in Commission Regulation (EC) N0 152/2009 expresses a scope of 'feed'. The method is a spectrophotometry based method. Two NRLs reported that this official method is not fit for purpose for the determination of urea in pet food, while it may be still applicable to ruminant feed. In addition the Regulation does not contain full validation data of this method.

Other two methods are currently used in the NRLs and / or official control laboratories for the determination of urea in feed, namely an enzymatic method and a LC-MS/MS method. However full validation of these methods is not available and their description regarding the target matrices is not clear. The two NRLs abovementioned performed a comparison of these three methods and addressed a request to the European Commission and the EURL-FA to raise this issue of 'contradicting' or misleading scope of the official method.

Although the official method is not questioned regarding its application for ruminant feed, it was agreed that the EURL-FA would address the issue and perform further testing of the three methods in different feed including pet food and provide an opinion to DG SANTE regarding a potential revision of Commission Regulation (EC) N0 152/2009 e.g. limiting its scope to ruminant feed. In parallel and depending on the results obtained the EURL-FA agrees to amend their evaluation report (authorisation task) by recommending an alternative method e.g. LC-MS/MS and/or enzymatic based if proved suitable.

One NRL therefore presented the LC-MS/MS developed and implemented at their premises for the determination of urea in dry pet food and discussion took place. The LC-MS/MS method shows limitations at low mass fractions of urea in feed. Furthermore only one daughter ion at a m/z ratio of 44 and the parent ion (m/z 60) are used for the determination although these masses are rather non-specific.

As regards the spectrophotometric method and sample preparation, another NRL informed about a step of centrifugation they introduce before measuring by spectrophotometry in order to remove possible turbidity.

The following conclusions were drawn:

- A potential revision of the EURL-FA evaluation report would recommend (i) restricting the scope of the official spectrophotometric based method to ruminant feed only and within a specific working range e.g. 1% to 8% and (ii) the other suitable methods specifying for each the specific scope (matrix and working range)
- The EURL-FA Control will perform the tests on the three methods as described above in order to address the need of an analytical method for the determination of urea as "undesirable substance".
- VD-LUFA, attending the meeting as 'observer', indicated that currently 20 ring trials are on-going in Germany. VD-LUFA agreed to share the results with the EURL-FA when available.
- Based on all above the EURL will issue a recommendation to DG SANTE addressing the different points of urea as feed additive and urea as undesirable substance.

Revision of the Commission Regulation (EC) 152/2009

A representative from DG SANTE presented the main changes occurring for the revision of the Commission Regulation (EC) 152/2009. Details can be seen in the related slides. It should be noted that no decision is taken yet and all points raised in the presentation are still in discussion. The laying down principle is that this revision process should be the opportunity to change all the aspects that were not optimal in the current version. Annex I was recently updated. Annex II: for sample preparation the slurry method could be added to the dry grinding. Annex III: for urea, the suggestion (see paragraph above) is to continue using the photometry method and to centrifuge to remove turbidity; for the determination of total phosphorus the DG SANTE representative asked the workshop participants if the method currently described should be deleted or if a new fit for purpose method should just be added since many labs are still using the current method. Annex IV: the same is valid for some trace elements. Finally the revision of the method for the determination of diclazuril, triggered by the NRLs and following the study performed by the EURL, will be introduced.

Cascade approach as implemented in Regulation (EU) 2017/625

In addition an explanation of the new cascade approach in Regulation (EU) 2017/625 was given by the DG SANTE representative. The choice of the method for performing official controls should be performed following the cascade steps in descending order defined as follows:

1. Level 1 method: method complying with Union rules establishing those methods or the performance criteria for those methods,
2. Level 2 method: International or European (CEN) standards or EURL developed or recommended methods when validated in accordance with internationally accepted scientific protocols,
3. Level 3 method: National standards or NRL developed or recommended methods when validated in accordance with internationally accepted scientific protocols or methods developed and single or inter-laboratory validated in accordance with internationally accepted scientific protocols,
4. Level 4: in case of urgent need, NRL or suitable OCL method until the validation of an appropriate method in accordance with internationally accepted scientific protocols.

The presentation was illustrated by a real case study raised by one NRL regarding the interpretation of the registry entry on total Copper determination in compound feed and in feed materials that, based on the EURL-FA report, recommends two methods, namely the Atomic absorption spectrometry method described in Reg. 152/2009 and the ICP-AES based method described in the Authorisation Regulation 2018/1039. The correct interpretation is that both methods may be used since they are both enlisted.

Labelling and control

Another case study related to Methionine and MHA as challenges in official control due to their labelling in some cases, was clarified (methionine was displayed on the label as

native and feed additive in the analytical constituents). Another DG SANTE representative explained that (i) the labelling was not correct and (ii) it is up to the feed business operators to choose the coefficient they use.

Role of VDLUFA in Germany – their approach

A representative presented the role of VDLUFA in feed analysis. Regarding the cascade approach, when a VDLUFA method exists, this method has higher priority at national level compared to the recommended NRL methods. VDLUFA organises a survey which is an investigation (type of "large" PT) on the determination of several parameters (up to 120 parameters). This study engages a number of laboratories (official control or public). The representative also presented the topic of how the analytical tolerances is dealt within VDLUFA (the analytical tolerance topic was also tackled in the presentation of the EURL-FA Control activities in 2018).

Minutes:
Prepared by Ursula Vincent
JRC-Geel, 30 Nov 2018