European Commission

JRC TECHNICAL REPORTS

Proficiency of European official control laboratories in the determination of authorized carotenoids in animal feed

EURL for Feed Additives Control PT exercise 2015 and annual workshop

Ursula Vincent, Federica Serano, Christoph von Holst

2016

Proficiency of European official control laboratories in the determination of authorized carotenoids in animal feed

This publication is a Technical report by the Joint Research Centre, the European Commission's in-house science service. It aims to provide evidence-based scientific support to the European policy-making process. The scientific output expressed does not imply a policy position of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use which might be made of this publication.

JRC Science Hub
https://ec.europa.eu/jrc

JRC100872

EUR 27810

ISBN 978-92-79-57548-8 (PDF)

ISSN 1831-9424 (online)

doi:10.2787/403547 (online)

How to cite: Vincent U., Serano F., von Holst C., *Proficiency of European official control laboratories in the determination of authorized carotenoids in animal feed*; EUR 27810, Publications Office of the European Union, Luxembourg, 2016, ISBN 978-92-79-57548-8, doi:10.2787/403547

Table of contents

Acknowledgements 3
Abstract 4
1. Introduction 6
2. Scope 8
3. Time frame 8
4. Test item 8
4.1 Preparation 8
4.2 Homogeneity and stability 10
4.2.1 Homogeneity 10
4.2.2 Stability 10
4.3 Distribution 10
5. Instructions to participants 10
6. Reference values and their uncertainties 11
7. Evaluation of results and discussion 12
7.1 General observations 12
7.2 Scores and evaluation criteria 13
7.3 Laboratory results and scoring 14
7.3.1 z-scores 16
7.3.2 False positives and false negatives 16
7.4. Additional information extracted from the questionnaire 17
7.5. Analytical considerations and key information from the EURL-FA reports 17
8. Conclusions from the PT exercise 17
9. Workshop 18
Annex 11 19
References 20
List of abbreviations and definitions 22
List of figures 23
List of tables 24
Annexes 25

Acknowledgements

All laboratories participating in this exercise are kindly acknowledged.

Participating Institute	Country
Finnish Food Safety Authority Evira, Research and Laboratory Department, Chemistry and Toxicology Unit, Section for Composition and Quality Indicators	Finland
Service Commun des Laboratoires des ministères du budget, des comptes publics, de la fonction publique et de la réforme de l'état (MBCPFPRE), de l'économie, des finances et de l'industrie (MEFI), Laboratoire de RENNES; SCL-L35, section Additifs de l'alimentation animale	France
Federal Institute for Risk Assessment (BfR), National Reference Laboratory for Feed Additives, FG 82	Germany
National Food Chain Safety Office, Food and Feed Safety Directorate, Feed Investigation National reference Laboratory	Hungary
National Institute of Nutrition and Seafood Research (NIFES)	Norway
DSM Nutritional Products - Analytical Research Center	Switzerland
National Reference Laboratory of the Central Institute for Supervising and Testing in Agriculture, Ústřední kontrolní a zkušební ústav zemědělský, Praha - ÚKZÚZ	Czech Republic

Abstract

The European Union Reference Laboratory for Feed Additives (EURL-FA), hosted by the Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC), a Directorate General of the European Commission, has been mandated by the Directorate General for Health and Food Safety (DG SANTE) to organise a proficiency test (PT) among appointed National Reference Laboratories (NRLs) in the frame of its control activities (according to the Regulation (EC) No 882/2004 [1]). The aim of this PT, was to assess the capacity of the NRLs to correctly determine selected authorised carotenoids in feed matrices at authorised concentration levels as established by European legislation. Twenty-seven laboratories were therefore invited and eight laboratories from eight countries registered to the 2015 PT exercise. A Swiss expert laboratory representing a feed additive producer was also invited to take part in this 2015 PT exercise. Results of analysis were reported by seven laboratories representing seven countries. The test items used in this exercise were produced by the EURL-FA Control by spiking milled blank poultry and fish feed with selected commercialised additives. The first test item consisted of a blank poultry feed spiked with canthaxanthin E 161g[1] (MAT 1). The second test item was a blank fish feed spiked with red carotenoid-rich *Paracoccus carotinifaciens* (2a(ii)167) containing astaxanthin, canthaxanthin and adonirubin (MAT 2) and the third test item was the blank fish feed (MAT 3). Laboratories were informed of the carotenoid composition of the test materials MAT 1 and MAT 2. For MAT 3, laboratories had to screen for the presence of authorised carotenoids and to quantify the detected ones.

Six laboratories reported results for astaxanthin and canthaxanthin in poultry and fish feed, while only two laboratories reported results for adonirubin in fish feed. The laboratories also reported qualitative results as regards the presence or absence of the other authorised carotenoids in all five test items.

The assigned values (x_a) for the concentration of astaxanthin, canthaxanthin and adonirubin in the samples were established by measurements performed at the EURL-FA Control utilizing a single-laboratory validated analytical method [5] that demonstrated to be fit for the purpose in at least two external PT exercises [6][7]. As for the uncertainties for the assigned values (u_a), the intermediate precision established during the single-house validation of the analytical method utilized for the measurement of the assigned values was used as recommended in ISO 21748 [8]. Participants were invited to report the uncertainties of their measurements for information. This was done by four out of six participants.

Laboratory results were rated using z-scores in accordance with ISO 13528 [4]. The standard deviation for proficiency assessment (σ_p) for each assigned value was set beforehand applying the modified Horwitz equation [11] for astaxanthin, canthaxanthin and adonirubin. The z-scores obtained were considered satisfactory if their absolute values were equal to or below 2.

Sixty-seven % of the reporting laboratories obtained satisfactory results for astaxanthin while only 17% reported satisfactory results for canthaxanthin for both materials' types. For adonirubin, only two laboratories reported results; the z-score was satisfactory for one laboratory and questionable for the other. The results are summarised in the following Table 1. The laboratories also reported qualitative results as regards the presence of one or more of the other authorised carotenoids. On the whole, the rate of false positive results was 50% for adonirubin, 40% for lutein and 20% for astaxanthin dimethyl disuccinate and beta-carotene.

[1] The revised code of the feed additive is 2a161g as specified in Regulation (EU) 2015/1586 [10]

Table 1: Summary results of the proficiency test exercise. x_a is the assigned value for the analytes.

	Analyte	x_a mg kg^{-1}	Total number of z-scores	Number of satisfactory z-scores $\|z\|\leq 2$	Relative number of satisfactory results (%)
MAT 1 Poultry feed	Canthaxanthin	19.3	6	1	17
MAT 2 Fish feed	Astaxanthin	57.8	6	4	67
	Canthaxanthin	5.91	6	1	17
	Adonirubin	18.6	2	1	50

1. Introduction

Prior to their use within the European Union, feed additives need to be authorised according to Regulation (EC) No 1831/2003 of the European Parliament and of the Council [9]. The Commission Regulations authorising the specific feed additives (Annex 1) most often contain specific conditions of use such as a maximum content of the active substance in feed or specification of the composition of the feed additive, which in turn require the availability of suitable methods of analysis for official control on feedingstuffs. The applicant for authorisation is therefore obliged to submit appropriate methods of analysis suitable for official control purposes. It is the primary objective of the EURL-FA to establish, whether these methods are suitable for enforcement of legal limits. All feed additives authorised within the European Union are listed in the Register of Feed Additives[2] [12] maintained by the European Commission.

Feed additives are authorised via Commission Implementation Regulations, which contain key information such as (a) the link to the corresponding opinion of the European Food Safety Authority (EFSA), (b) the characterisation of the feed additive (c) the conditions of use and (d) a reference to the analytical method as evaluated by the EURL-FA. For each feed additive the EURL-FA publishes a report on its website[3] containing important information on the method protocol. A key component of the concept is that once the feed additive is authorised, official control laboratories need to apply the specific protocol included in the Regulation, when checking samples containing this feed additive for compliance with legal limits. The method protocols are available from the EURL-FA on request.

The following table (Table 2) summarises the link between the feed additive, the Regulation authorising the feed additive, the corresponding EURL-FA report and a short description of the analytical method. When looking for a protocol related to a specific method, the best way is to look at the Commission's feed additive register [12] which also contains a direct link to the Regulation of each feed additive. Identification of the corresponding EURL-FA report is then possible via the EURL-FA website or by contacting the EURL-FA.

Apart from the nutritional importance in human and animal health as metabolic precursors of vitamin A and antioxidants, carotenoids are used for the direct colouring of foodstuffs as well as for pigmentation of animal products via their addition to complete feedingstuffs. These feed additives are classified in the category "sensory additives" and functional group "colourants": substances which, when fed to animals, add colours to food of animal origin". For instance, astaxanthin and canthaxanthin are added to salmon and trout feed for flesh colouration, whereas lutein is widely used in poultry farming for egg yolk coloration. In addition, authorisation of these substances includes values for the maximum content in complete feedingstuffs as specified in respective Commission Regulations. Legal limits of carotenoids when utilised as feed additives are expressed in terms of the sum of the Z-and all-E-forms, consequently analytical methods that are fit for the intended purpose need to address the presence of the various E/Z isomers of the target carotenoid.

[2] Commission's website containing the systematically updated feed additive register: http://ec.europa.eu/food/food/animalnutrition/feedadditives/registeradditives_en.htm

[3] EURL-FA website for EURL-FA reports: https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports

Table 2: Link to regulatory aspects for the selected feed additives

Feed additive	Regulation (EC) No	EURL-FA report identification number	Summary of the method description as indicated in the EURL-FA report and included in the Regulation
Canthaxanthin (2a161g), produced by chemical synthesis	2015/1486 [10]	FAD 2008-48	For the quantification of canthaxanthin in the premixtures and feedingstuffs: Normal Phase High Performance Liquid Chromatography coupled to visible detection (NP-HPLC-VIS, 466 nm)
Red carotenoid rich Paracoccus carotinifaciens (2a(ii)167)	721/2008 [13]	FAD 2006-021	Normal phase High Performance Liquid Chromatography (HPLC) coupled to UV/visible detection for determination of astaxanthin, adonirubin and canthaxanthin in feedingstuffs

Enforcing the legislation and performing the compulsory monitoring requires the availability of reliable analytical methods. Whilst the above mentioned Regulations contain a short description of the analytical methods, the corresponding EURL-FA report give more detailed information. The following details of the methods are copied form these reports:

- EURL-FA report FAD 2008-48 for Canthaxanthin (2a161g): *For the determination of Canthaxanthin in premixtures and feedingstuffs, the Applicant submitted a normal phase high performance liquid chromatographic method with visible detection (NP-HPLC-VIS) [14]. The analytical method was single laboratory validated and further verified* [15]*. The method consists of an enzymatic digestion of the sample in order to release the active substance, followed by extraction with ethanol and dichloromethane. The extraction procedure differs in consideration of the nature of the sample and of the Canthaxanthin concentration declared. The extract is purified by open-column chromatography on silica gel. Finally, an aliquot is injected into an isocratic normal-phase HPLC system adjusted at 466 nm. The selected chromatographic conditions allow for a full resolution of the cis/trans isomers of Canthaxanthin, carotenes and other xanthophylls present in the feed. The active substance is expressed as the sum of the all-trans and cis isomers (total Canthaxanthin). The separated trans/cis isomers are individually quantified against a standard solution prepared with the all-trans Canthaxanthin. Furthermore, the quantification of the cis isomers of Canthaxanthin includes the use of experimentally determined relative response factors, in order to compensate for the different absorbance coefficients of the cis isomers compared to all-trans Canthaxanthin* [16]*.*
- EURL-FA report FAD 2006-021 for Red carotenoid rich Paracoccus carotinifaciens (2a(ii)167): *The proposed method [17] involves a multi-extraction procedure with mixtures of organic solvents. The first step is to wet the sample followed by extracting the sample with a mixture of tetrahydrofuran (THF) and methanol (MeOH) (20:1 v/v). After mixing for 3 to 5 minutes, n-hexane is added and the resulting mixture is further mixed and centrifuged at 1800 g for 10 minutes. The upper solvent layer containing the target analyte is then transferred to a volumetric flask. The whole procedure is successively repeated other two times and the upper organic layers resulting from each extraction step are combined with the first one. Once the three upper organic layers are combined, the volumetric flask is filled up to the mark with the appropriate volume of a mixture of n-hexane, THF and MeOH (40:20:1 v/v/v). An aliquot of this solution is then injected on the HPLC system. The HPLC analysis is performed on two serially connected 250 x 4.6mm Wakosil-II 5 SIL-100 columns using a mixture of n-hexane, THF and MeOH (40:20:1 v:v:v) as mobile phase, measuring the astaxanthin peak at a wavelength of 470 nm.*

As stated in Regulation No 882/2004 of the European Parliament and the Council [1], one of the core duties of the EURL-FA Control is to organise inter-laboratory comparisons (ILCs) for the benefit of National Reference Laboratories. In a continuous effort to be updated on the reliability of analytical results delivered by laboratories in charge of ensuring the official control of carotenoids in feed, the European Commission's Directorate-General for Health and Food Safety (DG SANTE) requested the EURL-FA Control to organise a PT for the network of National Reference Laboratories (NRLs) to assess their performance on the determination of carotenoids at additive level in compound feed. Another laboratory from an industrial carotenoids' producer and member of FEFANA[4] was also invited to take part in the exercise.

The current exercise is the first one organised by the EURL-FA control for the assessment of the determination of carotenoids at additive levels in compounds feed. JRC-IRMM is an ISO/IEC 17043:2010 [2] accredited PT provider and the provisions of the standard related to the operation, data evaluation and reporting of PTs were adhered to. .

This report is in compliance with the reporting requirements of ISO/IEC 17043:2010 [2] and summarises the outcome of the PT exercise. The report also gives an overview of the topics addressed during the annual workshop with the NRLs.

2. Scope

The scope of this PT was to assess the proficiency of the participating laboratories to correctly determine carotenoids in feed samples and report results in the specified units within a defined time frame. Participating laboratories were informed of the composition in carotenoids of two out the three test materials MAT 1, MAT 2 and MAT 3. Given the fact that MAT 1 and MAT 2 contained the target analytes at the authorised level, an additional aim of the PT exercise was also to assess the capability of the laboratories to deliver satisfactory results on samples that contain the target analytes at trace level. The laboratories were therefore also requested to screen for traces of authorised carotenoids in all three materials and to quantify their content when detected.

Statistical assessment of the proficiency of laboratories was evaluated by calculating z-scores according to ISO 13528 [4].

3. Time frame

The proficiency test was agreed upon by the EURL-FA Control and the Directorate General for Health and Food Safety (DG SANTE). Invitation letters were sent to the participants on 09th March 2015 (Annex 2). The samples were dispatched to the participants on 1st June 2015. The reporting deadline was 3rd July 2015.

4. Test item

4.1 Preparation

Blank poultry and fish feed (commercial laying hens feed and commercial fish compound feed) available at the EURL-FA Control were used for the preparation of the spiked materials. The main ingredients of the fish and poultry feed were respectively wheat, soya, pepper, 2.2% crude ash, 1.0% cellulose, 21.0% proteins, 0.6% phosphorus, 0.2% calcium, 2% fat, vitamins, oligo-elements, minerals, anti-oxidants, preservatives and maize, wheat, peas, soybean, chalk,

[4] FEFANA: EU Association of Specialty Feed Ingredients and their Mixtures; the EURL-FA Control collaborates with FEFANA on dedicated topics, including the determination of carotenoids in feed.

monocalcium phosphate, salts, aminoacids, 12% fat, vitamins, additives. The test items were first tested at the EURL-FA Control laboratories using High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-UV, [5]) to ensure that no contamination by any authorised carotenoid was present. The feed was ground and sieved to obtain particle sizes lower than 500 µm.

The following feed additives were used in this study, where the first name corresponds to the description in the respective Regulations authorising these feed additives, whilst the second name specifies the commercial name under which the products are placed on the market.

- For canthaxanthin E 161g: Lucantin® Red was used for the production of Mat 1
- For red carotenoid-rich *paracoccus carotinifaciens* 2a(ii) 167: Panaferd-AX was used for the production of MAT 2.

These products were selected for this study, since the applicants had sent corresponding samples to the EURL-FA, when submitting the request for application that led to the authorisation of these feed additives. Required test material was taken from these samples available in the EURL-FA sample repository. Lucantin® Red is a technical formulation containing at least 10 %[5] of canthaxanthin (active substance) produced by chemical synthesis. Panaferd-AX is a natural product containing the active substances astaxanthin at 2.1%, canthaxanthin at 0.2% and adonirubin at 0.7%.

The sample set consisted of three different test items. The concentrations of the selected carotenoids were set close to the maximum content as defined in the legislation (Annex 1). Test item 1 was prepared by spiking the blank poultry feed while test item 2 was prepared from the blank fish feed. Test item 1 (MAT 1) contained canthaxanthin at approximately 20 mg kg^{-1} and test item 2 (MAT 2) contained astaxanthin, canthaxanthin and adonirubin at approximately 58, 6 and 20 mg kg^{-1} respectively. Test item 3 (MAT 3) was a commercial fish feed but without any carotenoid (blank feed) addition.

Explanations regarding the labelling information: MAT 1 and MAT 2 contained feed additives with different content of the active substances astaxanthin, canthaxanthin and adonirubin and therefore the labelling conditions as specified in Regulation (EC) No 767/2009 apply [18]. The labels mentioned the nature of the feed additive contained in the feed materials as registered in the version of the European Union register of Feed additives released at the time of the dispatch of the test materials and were as follows:

- MAT 1: "Feed Material 2015 N°xxx, Canthaxanthin E161 g",
- MAT 2: "Feed Material 2015 N°xxx, RED CAROTENOID-RICH (PARACOCCUS CAROTINIFACIENS) 2a(ii) 167"[6],

MAT 3: "Feed Material 2015 N°xxx, potentially containing carotenoids".

The preparation of the test material Mat 1 and 2 required the mixing of two solid materials, namely the compound feed and the respective feed additives. Therefore a complex multi-step preparation procedure was applied, in order to obtain test material with sufficient homogeneity of the 20 g sub-samples. The required mass fraction of the feed additives in the feed was calculated taking into account (i) the concentration of the carotenoids in the feed additives and (ii) the target concentrations of these carotenoids in the feed. Then specific sieve fractions were prepared from the feed and feed additives and the materials were mixed applying a step-wise dilution technique. A detailed description of this procedure is given in Annex 3. Sub-samples of 20 g were prepared and filled into aluminium bags, sealed under vacuum, labelled and stored at -20 °C until further dispatch and/or analysis.

[5] Upon request for further information, the supplier declared 11% and provided a certificate of analysis specific to the sample utilized.
[6] Red Carotenoid-rich *Paracoccus Carotinifaciens*) 2a(ii) 167 contains 3 active carotenoid substances, viz astaxanthin, canthaxanthin and adonirubin.

4.2 Homogeneity and stability

4.2.1 Homogeneity

To assess the homogeneity of the test items produced, 10 bags of each test item were randomly selected. Two aliquots from each bag were extracted and further analysed in duplicate by HPLC-UV at the EURL-FA Control. One key aspect to be taken into account in the case of the feed additive canthaxanthin E161g is that it requires an enzymatic digestion to ensure sufficient extraction of the analyte from the sample. The individual results of the sub-sample duplicates were subjected to analysis of variances (ANOVA). The results obtained for each of the test items confirmed that the homogeneity of the test items was sufficient (Annex 4) to proceed with the PT exercise.

4.2.2 Stability

Experience at EURL-FA Control has shown that the test items prepared by spiking with carotenoid additives and kept at -20°C were stable for several weeks. It was therefore decided to proceed with the dispatch of the samples as such. During the term of the exercise, all remaining bags of each test item (more than three per test item) were kept at -20°C. At the end of the exercise, two bags of each test item were analysed in duplicate. Each replicate was injected twice. The values obtained for the concentration of each measurand were compared with those obtained during the homogeneity study. The results demonstrate that the test items were stable during the time frame of this PT exercise for the recommended storage temperature tested (Annex 5).

4.3 Distribution

All samples were dispatched to participants by IRMM on 1st June 2015. Each participant received:

a) One bag containing approximately 20 g of test item MAT 1a,

b) One bag containing approximately 20 g of test item MAT 1b,

c) One bag containing approximately 20 g of test item MAT 2a,

d) One bag containing approximately 20 g of test item MAT 2b,

e) One bag containing approximately 20 g of test item MAT 3,

f) An accompanying letter with instructions for sample handling and reporting (including the individual lab code) (Annex 6) and

g) A "Confirmation of receipt" form to be sent back to IRMM after receipt of the test items (Annex 6).

h) The reporting sheet to be used to report the results was sent in an electronic message on 23rd June 2015 to each participant (Annex 7).

5. Instructions to participants

Concrete instructions were given to all participants in a letter accompanying the test items and in the reporting sheet (Annex 6, Annex 7).

Laboratories were asked to perform the analysis on the five test items received, applying the method they would utilize to carry out an official control. Presence or absence of the carotenoids had to be reported as "detected" or "not detected". When a measurand was detected, participants were asked to report the content as the concentration of the detected carotenoid in mg kg^{-1} of feed given with 3 decimals, with the associated measurement uncertainty. Instructions on how to report the uncertainty was also provided in the reporting sheet. In addition, details on the analytical method (sample preparation, instrumental

technique, limits of detection and of quantification) used to perform the measurements were also to be reported together with explanations on how the reported uncertainty was evaluated (calculated). If a measurand was not analysed, the participating laboratory had to report "not analysed".

All results and additional information were to be reported in a special Excel® form developed at the EURL-FA Control. Each laboratory was assigned a unique code which was individually communicated in each instruction letter accompanying the test items (Annex 6).

6. Reference values and their uncertainties

The total content in astaxanthin, canthaxanthin in fish feed, canthaxanthin in poultry feed and adonirubin used to design the appropriate spiking of the material was set as the nominal value calculated from the formulation, following the IUPAC protocol [3] and ISO 13528 [4] and corrected for purity according to Equation (1).

$$x = \frac{m_{meas}}{M} \times p \times 10 \qquad \text{Eq. (1)}$$

where

x is the nominal concentration of the measurand astaxanthin, canthaxanthin, or adonirubin, in mg kg^{-1} of feed

m_{meas} is the weighed mass of the corresponding measurand feed additive, in mg

M is the mass of blank feed to be spiked, in g

p is the purity of the measurand standard substance as declared on the certificate of analysis provided by the supplier.

The assigned value (x_a) for the total content in astaxanthin, canthaxanthin in fish feed, canthaxanthin in poultry feed and adonirubin was the measured value at the EURL-FA Control obtained using a single-laboratory validated analytical method that demonstrated to be fit for the purpose in at least two independent and external PT exercises [6][7].

The standard error calculated from the intermediate precision established during the single-laboratory validation of the method used for the measurements was set as the associated standard uncertainty to the assigned values in accordance with ISO 21748 [8].

Table 3 displays the assigned values (x_a), the associated expanded uncertainties (U_a) and the standard deviations for PT assessment (σ_p).

Table 3: Nominal concentrations, Assigned concentrations (x_a) in mg kg^{-1} of feed, expressed as mean value ± expanded uncertainty (k=2) and the associated standard deviations for proficiency assessment, σ_p, in %.

Test item	Measurand	Nominal concentration mg kg^{-1}	$x_a \pm U_a$ (k=2) mg kg^{-1}	Standard deviation σ_p (%)
			Assigned	
MAT 1	Canthaxanthin	20.0	19.3 ± 0.35	10
MAT 2	Astaxanthin	56.0	57.8 ± 1.7	8.6
	Canthaxanthin	8.00	5.91 ± 0.36	12
	Adonirubin	18.7	18.6 ± 0.44	10

For all selected carotenoids, the target concentration was above 120 µg kg^{-1}; values for σ_p were therefore calculated applying the Horwitz equation (Equation (2)) [11].

$$RSD_{R_{Horwitz}} = \sigma_p = 2 \times c^{-0.15} \qquad \text{Eq. (2)}$$

where

$RSD_{RHorwitz}$ is the relative standard deviation predicted by Equation 2,

σ_p is the target standard deviation set for the PT exercise, and

C is the concentration ratio (e.g. 1=100 g/100 g or 0.001=1000 mg kg^{-1}).

All σ_p values are summarised in Table 3.

7. Evaluation of results and discussion

7.1 General observations

Out of 28 laboratories invited to participate in the exercise, only 8 laboratories from 8 countries registered. Most laboratories reported either not having sufficiently frequent requests to analyse carotenoids or not having an in-house method. Seven laboratories reported results; the eighth laboratory did not report results since their internal quality criteria were not met. Six laboratories performed the analysis for astaxanthin, canthaxanthin in fish feed and canthaxanthin in poultry feed and 2 laboratories performed the analysis for adonirubin. All laboratories except one responded comprehensively to the questionnaire included in the reporting form. All results were included in the final evaluation.

Figure 1 gives an overview of the geographical distribution of participating laboratories.

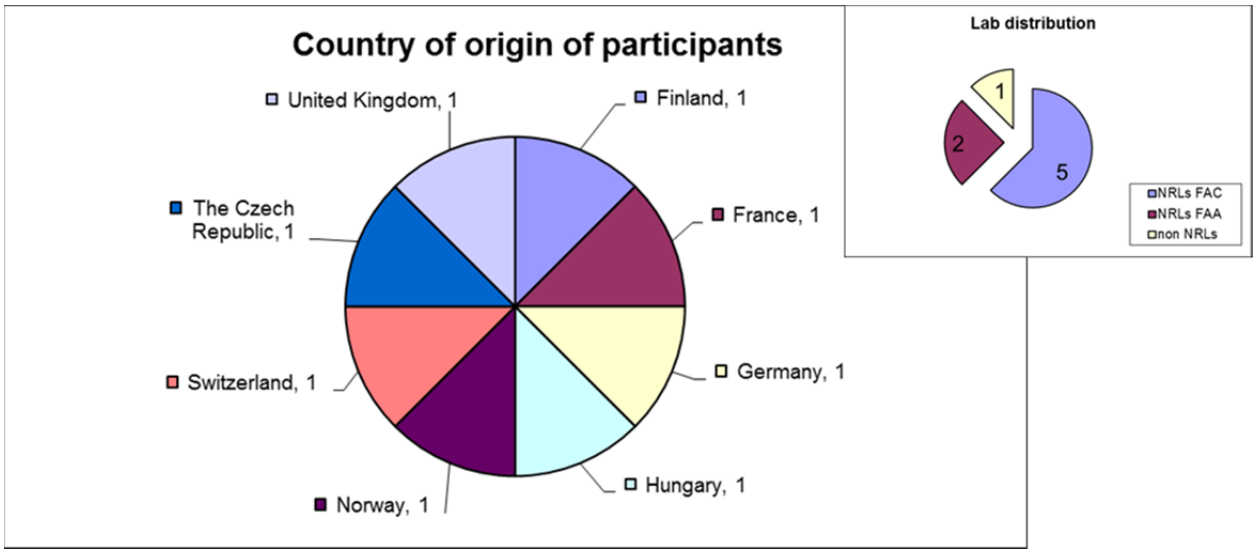


Figure 1: Country of origin of the participants – NRLs and non NRLs laboratories

7.2 Scores and evaluation criteria

Individual laboratory performance is expressed in terms of z-scores in accordance with ISO 13528 [4] following Equation (3).

$$z = \frac{x_{lab} - x_a}{\sigma_p} \qquad \text{Eq. (3)}$$

where

x_{lab} is the measurement result reported by a participant,

x_a is the reference value (assigned value),

σ_p is the standard deviation for proficiency assessment.

The assigned reference values (x_a) are summarised in Table 3.

The interpretation of the z-score is done as follows:

|z-score| ≤ 2 satisfactory performance

2 < |z-score| < 3 questionable performance

|z-score| ≥ 3 unsatisfactory performance

Figure 2 displays the distribution of the z-scores.

The z-score compares the participant's deviation from the assigned value with the target standard deviation for proficiency assessment (σ_p) used as common quality criterion. σ_p is defined by the PT organiser as the maximum acceptable standard uncertainty.

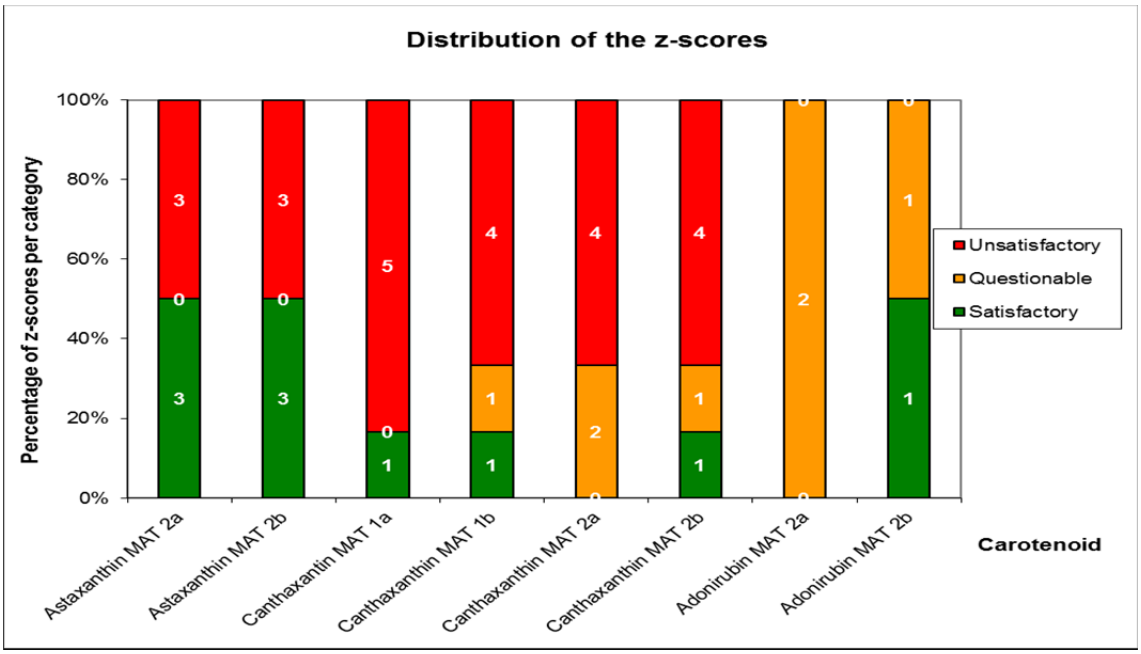


Figure 2: Complete distribution of the z-scores values;
the number of reporting laboratories for each category is given in the graphs

7.3 Laboratory results and scoring

The results reported by the participants for the total content of astaxanthin, canthaxanthin and adonirubin in the analysed test samples together with the related uncertainties are given in Annex 8.

Not all laboratories analysed all 4 measurands in the five test items and therefore they did not report results for one or more given measurand.

One participant commented that 20 g of test material was not sufficient seen the test portion size prescribed in their method. Additionally, if more than one method is to be applied on the same sample to determine different carotenoids, this amount of test material is too limited.

No laboratory reported results with a $|z| \leq 2$ for all 4 measurands present in the samples[7]. Moreover, four laboratories displayed questionable or unsatisfactory performance for all 4 measurands in all samples (Table 4). The technical reasons for such biased results should be further investigated by the relevant laboratories. This exercise will be supported by the EURL-FA Control.

[7] The samples contained only 3 different analytes but the 3 analytes were to be measured in 2 samples and 1 analyte in 2 samples.

Table 4: Laboratory performance per measurand.

	MAT 1a	MAT 1b	MAT 2a			MAT 2b		
	CXN	CXN	CXN	AXN	ADR	CXN	AXN	ADR
L01								
L02								
L03								
L04								
L05								
L06								
L07								

AXN: Astaxanthin; CXN: Canthaxanthin; ADR: Adonirubin; MAT: material; the white cells indicate "samples not analysed for the selected measurand" or not reported results; Green: $|z| \leq 2$, Yellow: $2 < |z| < 3$, Red: $|z| \geq 3$.

Furthermore, only two laboratories reported results for adonirubin. This is surprising, because Commission Regulation (EC) No 721/2008 [13] authorising RED CAROTENOID-RICH 2a(ii) 167 specifies adonirubin as one of the three active substances. In addition, the maximum content of 100 mg kg^{-1} is expressed as the sum of astaxanthin, adonirubin and canthaxanthin, thus requiring the measurement of this compound when checking for compliance with legal limits.

Some of the differences of concentration means between two samples containing the measurands at the same concentration were much higher than 15-20%[8] (Annex 8).

Additionally the robust mean calculated according to ISO 13528 [4] from the results reported indicate, for some measurands, significant differences compared to the assigned values of the target carotenoids present in the test items (Table 5).

Table 5: Statistical comparison of the robust mean with the assigned value for each measurand.

		x_a mg kg^{-1}	σ_p %	Robust mean mg kg^{-1}	Robust standard deviation mg kg^{-1}	Robust RSD %
MAT 1a	Canthaxanthin	19.3	10	11.5	4.35	38
MAT 1b	Canthaxanthin	19.3	10	12.7	3.29	26
MAT 2a	Astaxanthin	57.8	8.6	46.1	11.7	25
	Canthaxanthin	5.91	12	5.65	3.44	61
	Adonirubin[9]	18.6	10	13.3	0.160	1.2
MAT 2b	Astaxanthin			54.2	21.4	40
	Canthaxanthin	5.91	12	7.20	5.76	60
	Adonirubin[10]	18.6	10	15.0	2.34	16

S_R: standard deviation for reproducibility.

[8] Accepted difference between two replicate measurements in Regulation (EC)152/2009, Annex IV/A/DA/BA/EB – determination of vitamin A in feed [13]

[9] Only two laboratories reported values

7.3.1 z-scores

The results for astaxanthin, canthaxanthin and adonirubin and for each laboratory are summarised in Annex 9.

7.3.2 False positives and false negatives

Another important aspect for official control is the percentage of false positive and false negative rates of the method used, because the former performance characteristic addresses the specificity of the method, whereas the latter aspects is about the sensitivity of the method.
In this PT exercise, if a sample was analysed for a selected analyte and the determined concentration would be lower than the limit of detection, then the analyte should be reported as "not detected" and the reported result should be "lower than LOD". Likewise, if a sample was analysed for a selected analyte and the determined concentration would be lower than the limit of quantification, then the analyte should be reported as "detected" and the reported result should be "lower than LOQ". If an analyte present in the sample is reported as "not detected" (<LOD) or "detected" but with a concentration "lower than LOQ" (LOD<xxx<LOQ) and the method used claims to have an LOD or LOQ lower then analyte amount present in the sample the result is classified as false negative. Obviously if the method used is e.g. not sensitive enough for the scope of the analysis, the non-detection of the analyte is due to a lack of sensitivity.
In the questionnaire to the participants, information on the limits of detection (LOD) and quantification (LOQ) was requested. Based on this information (Annex 8), the false negative results were therefore also identified during the evaluation.

A result is considered as "false positive" if the laboratory detects and reports a numerical value (above the LOQ) for an analyte which was not present in the given sample.

Table 6 displays the percentages of false negatives and false positives in this study.

Table 6: False positive and false negative rates for targeted and screened carotenoids as reported by the participants.

	N	False positive rate* %	False negative rate %	
			True	Lack of sensitivity
Astaxanthin	6	-	-	-
Canthaxanthin	6	-	17 (not detected in MAT 2b)	-
Adonirubin	2	50 (detected in MAT 1)	-	-
Lutein	5	40 (detected in MAT 1 and MAT 2)	-	-
Astaxanthin dimethyldisuccinate	5	20 (detected in MAT 1, MAT 2 and MAT 3)	-	-
beta-carotene	5	20 (detected in MAT 1)	-	-

*N: number of reporting laboratories; * false positives for canthaxanthin are only for Mat 3, for astaxanthin for Mat 1 and MAT 3.*

7.4. Additional information extracted from the questionnaire

Additional information was gathered from the questionnaire filled in by the participants. A compilation of the method parameters used by the participants is given in Annex 10.

7.5. Analytical considerations and key information from the EURL-FA reports

Comparing both methods provided by the applicants for authorisation of the carotenoids feed additives, reveals significant differences of the protocols. In particular, the sample preparation is different, since the feed additive 2a161g requires enzymatic digestion, which is not the case for the feed additive 2a(ii)167. This is due to the fact that the first feed additive is a technical formulation applying a microencapsulation process and containing canthaxantin produced by chemical synthesis, whereas the latter one is a natural product. When canthaxantin is added to feed via these formulations, an additional enzymatic digestion step is required to measure the whole content of canthaxantin in feed. This means that if such a step is not applied, results of analysis may be systematically too low.

When analysing authorised feed additives it is always required to check whether the corresponding EURL-FA report contains additional requirements for the method protocol that need to be taken into account.

8. Conclusions from the PT exercise

This proficiency test exercise, dedicated to the National Reference Laboratories and Official Control Laboratories, is the first one of this kind organised by the EURL-FA Control targeting carotenoids at additive levels in compound feed materials. The participating laboratories were requested to screen for the presence or absence of the other carotenoids and, if detected, to report the content of all detected carotenoids in mg kg^{-1} of feed with the associated uncertainty value. Five test items were sent to the laboratories for analysis. Two test items contained 3 carotenoids (astaxanthin, canthaxanthin and adonirubin), two others contained 1 carotenoid (canthaxanthin) and the last test item was a blank. The proficiency of the laboratories in correctly determining the carotenoids was assessed by the calculation of the z-scores.

For authorised feed additives, official control requires the use of the recommended methods of analysis as provided in the EURL-FA reports. In particular, it is always required to check whether the corresponding EURL-FA report contains additional requirements for the method protocol that need to be taken into account. This is for instance the case for the feed additive canthaxanthin 2a161g, which requires an enzymatic digestion to ensure sufficient extraction of the analyte from the sample.

Proficiency test exercises shall be performed in official control conditions. As major conclusion, the PT exercise therefore demonstrated that this requirement was not fulfilled since the majority of the participating laboratories did not apply the recommended method(s). As first corrective action, training in how to select the method of analysis for carotenoids control in feed was provided by the EURL-FA in the frame of the annual workshop.

On the whole and more in detail, the proficiency of laboratories was not satisfactory; only 7 laboratories participated in the study and between 0% and 43 % of the laboratories reported satisfactory results, expressed as z-scores, depending on the target carotenoid and its concentration in one or the other feed material. The variability of the results obtained for a target carotenoid in two similar materials was high.

The laboratories also reported qualitative results as regards the presence of one or more of other authorised carotenoids. The rate of false positive results was 20% for astaxanthin dimethyl succinate and beta-carotene, 40% for lutein and 50% for adonirubin. It has to be noted that these percentages have to be considered with caution since they are calculated on a very low number of laboratories/results for a given measurand.

Carotenoids' analysis is a challenge due to the nature of the analytes and specifically to the process of their production as well as to the presence of the cis- and trans- forms while the trans- form is the one mainly used in the standards used for quantification.

Another key aspect is the need for harmonisation of the measurements and further monitoring. As regards harmonisation, the EURL-FA Control has engaged in the standardization of an analytical method for the determination of carotenoids in feed in the frame of its CEN[10] activities.

9. Workshop

The 4th workshop of the EURL-FA Control and the consortium of National Reference Laboratories (NRLs) was held at the JRC-IRMM on November 17-18 2015. A total of twenty-seven participants attended the event, representing 17 National Reference Laboratories (NRLs), 1 National Official Control Laboratory (OCL), 1 expert laboratory from an additive producer, DG Health and Consumers, and the EURL-FA.

This 4th workshop was the concluding event for the organisation of the first Proficiency Test (PT) exercise for the determination of authorised carotenoids at authorised level in animal feed. The results from the PT exercise were presented as well as "real-world" considerations on the properties of carotenoids and their impact on their analysis.

In addition this annual workshop also gave the opportunity to exchange with the NRLs on activities carried out in the field of analysis of feed additives, to report on problems encountered and on the usefulness of the network to help solving analytical issues. As an example, the results of the inter-comparison carried out to assess the fitness for purpose of a modified Community method for the determination of the coccidostat diclazuril in feed contributed to a discussion on the appropriateness of issuing a recommendation for revision of the Community method to DG SANTE.

The activities performed by the EURL-FA Control in 2014 were reviewed and the work program 2016-2017 submitted to DG SANTE for approval was presented. The NRLs were informed that one PT will be organised in 2016 for the determination of vitamins (A and potentially E) in animal feed and in 2017, 2 PTs will be organised, one on the determination of cobalt in animal feed in collaboration with the EURL for Heavy Metals and the second one for the determination of coccidiostats in feedingstuffs, upon request of many NRLs.

Training on selection of methods for feed additives analysis as well as on factorial design was provided.

The support to the European Committee for Standardization (CEN) was also summarised. Furthermore,

The launch of the web platform for discussion and exchange was highlighted with the presentation of the news groups created and NRLs were encouraged to contribute to the fora with their input to the defined topics or suggestions for new discussion topics. Finally, two potential training topics for the 2016 workshop were proposed by the EURL-FAC, *viz.*:

- Statistics in the analysis (LOD, LOQ) Matrix effects elimination during LC-MS/MS acquisition,
- Practical training for method development for coccidiostats in feed,

for which the NRLs are requested to agree or not until June 2016 and/or to propose new training needs.

The overall satisfaction survey for the organisation of the workshop was very good. The response rate for the survey was 75%; 91% of the answers were either good or excellent.

[10] CEN: Comité Européen de Normalisation – European Standardization Body

Annex 11 displays the survey addressed to the laboratories as well as the evaluation of the responses received.

References

[1] Commission Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules, *Official Journal of the European Union*, L 165 (2004) 1 – 141.

[2] ISO/IEC 17043:2010 - "Conformity assessment -- General requirements for proficiency testing", issued by ISO-Geneva (CH), International Organization for Standardization.

[3] Thompson M., Ellison S., Wood R., The international harmonized protocol for the proficiency testing of analytical chemistry laboratories. Pure Appl. Chem., Vol. 78, No. 1, pp. 145–196, 2006.

[4] ISO 13528:2015 - *"Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparisons"*, issued by ISO-Geneva (CH), International Organization for Standardization.

[5] Vincent U., Serano F., Determination of Carotenoids in animal compound feed by Reverse Phase High Performance Liquid Chromatography – UV detection (RP-HPLC-UV), Version: March 2015, Standard Operating Procedure EURL-FA Control 01. CIRCA-BC platform, Joint research Centre, EURL for Feed Additives Control, pp 1–17, 2015.

[6] Report on Proficiency test – "Collaborative Study on the determination of astaxanthin in fish feed – 2013", organised by DSM Nutritional Products, Switzerland, 2013

[7] Report on Proficiency test – "Collaborative Study on the determination of astaxanthin in fish feed – 2014", organised by DSM Nutritional Products, Switzerland, 2014

[8] ISO 21748:2010 - " Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation", issued by ISO-Geneva (CH), International Organization for Standardization.

[9] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, *Official Journal of the European Union*, L 268 (2003) 29 – 43.

[10] Commission Implementing Regulation (EU) 2015/1486 of 2 September 2015 concerning the authorisation of canthaxanthin as feed additive for certain categories of poultry, ornamental fish and ornamental birds, *Official Journal of the European Union*, L 229 (2015) 5 – 8.

[11] W. Horwitz, R. Albert, The Horwitz Ratio (HorRat): A Useful Index of Method Performance with Respect to Precision. J. AOAC Int., 89 (2006) 1095 – 1109.

[12] European Union Register of Feed Additives http://ec.europa.eu/food/food/animalnutrition/feedadditives/registeradditives_en.htm; http://ec.europa.eu/food/food/animalnutrition/feedadditives/docs/comm_register_feed_additives_1831-03.pdf, pursuant to Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, *Official Journal of the European Union*, L 268 (2003) 29 – 43.

[13] Commission Regulation (EC) No 721/2008 of 25 July 2008 concerning the authorisation of a preparation of red carotenoid-rich bacterium Paracoccus carotinifaciens as a feed additive, *Official Journal of the European Union*, L 198 (2008) 23 – 25.

[14] Technical dossier, Section II: 2.6.1 Method of analysis for the active substance, in EURL-FA Dossier No. FAD-2008-0048.

[15] Supplementary information: Canthaxanthin Premix Feed Verification.pdf, in EURL-FA Dossier No. FAD-2008-0048.

[16] Technical dossier, Section II: 2.6.1.d, in EURL-FA Dossier No. FAD-2008-0048.

[17] Method description: "Method of Analysis for Carotenoids in Fish Feeds", in EURL-FA Dossier No. FAD-2006-021.

[18] Regulation (EC) No 767/2009 of the European Parliament and of the Council of 13 July 2009 on the placing on the market and use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, Commission Directive 80/511/EEC, Council Directives 82/471/EEC, 83/228/EEC, 93/74/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC, *Official Journal of the European Union*, L 229 (2009) 1 – 28.

[19] Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, *Official Journal of the European Union*, L 54 (2009) 59 – 65.

List of abbreviations and definitions

EURL-FA: European Union Reference Laboratory for Feed Additives

EURL-FA Control: EURL-FAC: European Union Reference Laboratory for Feed Additives Control (branch)

IRMM: Institute for Reference Materials and Measurements

JRC: Joint Research Centre

DG SANTE: Directorate General for Health and Food Safety

NRL: National Reference Laboratory

OCL: Official Control Laboratory

CEN: Comité Européen de Normalisation – European Standardization Body

FEFANA: EU Association of Specialty Feed Ingredients and their Mixtures

PT: proficiency test

MAT: Material

HPLC: High Performance Liquid Chromatography

NP-HPLC: Normal Phase High Performance Liquid Chromatography

RP-HPLC: Reverse Phase High Performance Liquid Chromatography

VIS: visible detection

HPLC-UV: High Performance Liquid Chromatography coupled to spectrophotometric detection

LC-MS/MS: Liquid Chromatography tandem Mass Spectrometry

THF: tetrahydrofuran

MeOH: Methanol

ANOVA: Analysis of variances

σ_p: standard deviation for proficiency assessment

z: z-score

AXN: Astaxanthin

CXN: Canthaxanthin

ADR: Adonirubin

Rep: replicate

RSD: relative standard deviation

LOD: Limit of detection

LOQ: Limit of quantification

List of figures

Figure 1 Country of origin of the participants – NRLs and non NRLs laboratories
Figure 2 Complete distribution of the z-scores values

List of tables

Table 1 Summary results of the proficiency test exercise expressed as z-scores. xa is the assigned value for the analytes

Table 2 Link to regulatory aspects for the selected feed additives

Table 3 Nominal concentrations, Assigned concentrations (x_a) in mg kg^{-1} of feed, expressed as mean value ± expanded uncertainty (k=2) and the associated standard deviations for proficiency assessment, σ_p, in %

Table 4 Laboratory performance per measurand

Table 5 Statistical comparison of the robust mean with the assigned value for each measurand

Table 6 False positive and false negative rates for targeted and screened carotenoids as reported by the participants.

Annexes

Annex 1: Legal references of the authorisation of the feed additives included in the study and specified by the name and the unique code

Feed additive	Active substance(s)	Target animals	Legal reference	Comment	Maximum content in feed (mg kg^{-1})	Other provisions
Canthaxanthin E161 g	Canthaxanthin	Poultry other than laying hens	1.)	Valid until 21.9.2015	25	1)
Canthaxanthin E161 g	Canthaxanthin	Laying hens	1.)	Valid until 21.9.2015	8	1)
Canthaxanthin E161 g	Canthaxanthin	Salmon, trouts	1.)	Valid until 21.9.2015	25	2)
Canthaxanthin E161 g	Canthaxanthin	Salmon, trouts	1.)	Valid until 21.9.2015	25	2)
Canthaxanthin E161 g	Canthaxanthin	Pet and ornamental fish	1.)	Valid until 21.9.2015	-	-
Canthaxanthin 2a161g	Canthaxanthin	Chickens for fattening and minor poultry species for fattening.	2.)	Entering into force: 22.9.2015	25	1.)
Canthaxanthin 2a161g	Canthaxanthin	Laying poultry and poultry reared for laying.	2.)	Entering into force: 22.9.2015	8	1.)
Canthaxanthin 2a161g	Canthaxanthin	Ornamental fish and ornamental birds except ornamental breeder hens.	2.)	Entering into force: 22.9.2015	100	2.)
Canthaxanthin 2a161g	Canthaxanthin	Ornamental breeder hens.	2.)	Entering into force: 22.9.2015	8	2.)
Red carotenoidrich Paracoccus carotinifaciens, 2a(ii)167	Astaxanthin Adonirubin Canthaxanthin	Salmon, trout	3.)	Entering into force: 13.8.2008	100	3.)

Legal reference 1. Canthaxanthin was authorised under Directive 70/524/EEC without a time limit for poultry and with a time limit for ornamental birds and ornamental fish. That product was subsequently entered in the Register of feed additives as an existing product, in accordance with Article 10(1) of Regulation (EC) No 1831/2003.

Legal reference 2. Commission Implementing Regulation (EU) 2015/1486 of 2 September 2015 concerning the authorisation of canthaxanthin as feed additive for certain categories of poultry,

ornamental fish and ornamental birds. The end of period of authorisation is 23.9.2025. This Regulation substitutes the legal reference 1.

Legal reference 3. Commission Regulation (EC) No 721/2008 of 25 July 2008 concerning the authorisation of a preparation of red carotenoid-rich bacterium *Paracoccus carotinifaciens* as a feed additive. The end of period of authorisation is 15.8.2018.

Other provisions 1): The mixture of canthaxanthin with other carotenoids and xanthophylls is allowed provided that the total concentration of the mixture does not exceed 80 mg kg^{-1} in the complete feedingstuff.

Other provisions 2): The mixture of canthaxanthin with other carotenoids and xanthophylls is allowed provided that the total concentration of the mixture does not exceed 100 mg kg^{-1} in the complete feedingstuff.

Other provisions 3): a.) The maximum content is expressed as the sum of astaxanthin, adonirubin and canthaxanthin. b.) The mixture of the additive with astaxanthin or canthaxanthin is allowed provided that the total concentration of the sum of astaxanthin, adonirubin and cantaxanthin from other sources does not exceed 100 mg kg^{-1} in the complete feedingstuff.

Annex 2: Invitation letter sent to NRLs

Ref: Ares(2015)1078027

Dr Ursula Vincent
Project Leader European Reference Laboratory for Feed Additives (control)
(EURL-FA control)
Joint Research Centre
Food Safety and Quality
Institute for Reference Materials and Measurements (IRMM)

09 March 2015

Call for participation in an inter-comparison study for the determination of authorised carotenoids in feed at authorised levels

1. Introduction

Feed additives are authorised within the European Union according to Regulation (EC) No 1831/2003 (Community Register of Feed Additives pursuant to Regulation (EC) No 1831/2003) requiring various criteria to be fulfilled including the need of providing suitable methods of analysis for official control in feedingstuffs. Apart from the nutritional importance in human and animal health as metabolic precursors of vitamin A and antioxidants, carotenoids are used for the direct colouring of foodstuff as well as for pigmentation of animal products via their addition to complete feedingstuffs. These feed additives are classified in the category "sensory additives" and functional group "colourants: substances which, when fed to animals, add colours to food of animal origin". For instance, astaxanthin and canthaxanthin are added to salmon and trout feed for flesh colouration, whereas lutein is widely used in poultry farming for egg yolk coloration. In addition, authorisation of these substances includes target concentration limits in complete feedingstuffs as specified in respective Commission Regulations. For instance, astaxanthin dimethyl disuccinate is authorised by Commission Regulation (EC) No 393/2008 for salmon and trout, with a maximum level of this compound in feed at 138 mg kg^{-1}. Legal limits of carotenoids when utilised as feed additives are expressed in terms of the sum of the Z-and all-E-forms, consequently analytical methods that are fit for the intended purpose need to address the presence of the various E/Zisomers of the target carotenoid.

Enforcing the legislation and performing the compulsory monitoring requires the availability of reliable analytical methods. In a continuous effort to be updated on the reliability of analytical results delivered by laboratories in charge of ensuring the official control of coccidiostats in

feed, the European Commission's Directorate-General for Health and Food Safety requested the EURL-FA Control to organise an inter-comparison study for the determination of carotenoids in feed in 2015. This letter therefore constitutes the call for participation to this proficiency testing exercise targeting carotenoids in animal feed.

2. The organising team

The study will be conducted by the European Union Reference Laboratory for Feed Additives Control (EURL-FA Control), hosted at the European's Commission Joint Research Centre Institute for Reference Materials and Measurements (IRMM).

3. Objective

The major objective of this study is to assess the proficiency of the participating laboratories to correctly determine carotenoids potentially added to feed samples at levels authorised in the European legislation. This aim will be achieved by conducting an inter-comparison study in which the laboratories analyse feed samples applying their own analytical method and reporting the results to the organiser of the study. The evaluation of the results will show which laboratories deliver acceptable results. The proficiency test also includes evaluating the capability of the laboratories to carry out the requested analysis within a defined time frame.

4. Test material

Samples will be prepared containing typical compound feed fortified with selected authorised carotenoids (Regulation (EC) No 1831/2003, Community Register of Feed Additives pursuant to Regulation (EC) No 1831/2003) at authorised concentration levels. Each laboratory will have to quantitatively analyse 5 samples and to submit the results to the coordinator of the study.

Prior to sending out the samples to the participants the organising team will have demonstrated sufficient homogeneity of the test material by analysing randomly taken sub-samples.

5. General outline of the exercise

The participants are requested to report the results of the analyses together with the information about the analytical method applied. Each laboratory will be assigned a unique code and only the organiser of the study (EURL-FA Control) knows the key to this code. The EURL-FA Control will send out a report on the outcome of the study containing information about the score of the laboratories; the personal laboratory keys will be individually communicated to each participant.

Appropriate statistical tool for data evaluation will be used to investigate the proficiency of the laboratories. Statistical assessment of the proficiency of laboratories will be evaluated by calculating an individual dimensionless Z-score calculated according to ISO 13528.

$$z = \frac{(x - x_a)}{\sigma_p} \qquad \text{Eq. 1}$$

Where x = the value reported by the participant

x_a = the assigned value

σ_p = the target standard deviation

Participation in the inter-comparison study is free of charge.

The proficiency test is scheduled for the period of June 2015.

The exercise will be completed by the organisation of a workshop opened to all participants to the study. The target period for the workshop is November 2015. The workshop is free of charge. Travel and accommodation costs will be reimbursed for one representative of each National reference Laboratory for Feed Additives defined according to Regulation (EC) No 882/2004 and Regulation (EC) No 1831/2003.

6. Expression of interest

National Reference Laboratories for Feed Additives (Control) defined according to Regulation (EC) No 882/2004 shall participate in the proficiency exercise organised by the EURL-FA Control depending on their expertise. Other official control laboratories may also participate in the proficiency exercise if places are still available. The total number of participating laboratories may not exceed 30.

The laboratories are kindly asked to register for taking part in the exercise by email to the functional mailbox JRC-IRMM-EURL-FEED-ADDITIVES-CONTROL@ec.europa.eu by **27 March 2015** at the latest. Please clearly indicate:

- Your institution name
- The name and surname of the contact person
- The complete postal address where the samples should be dispatched (postal boxes are not accepted by the courier companies)
- A contact telephone number
- A contact fax number
- A contact email address

These details will only be used for the dispatch of the materials and for any further correspondence.

Important note: It is not compulsory to be able to determine ALL authorised carotenoids to take part in the study.

Best regards,

Dr Ursula VINCENT
Study coordinator, EURL-FA Control

Annex 3: Production of the test material

Particle size distribution of the feed additives used in the PT: In previous PT's the test materials were produced applying the slurry technique in which the target analytes were added to the feed after being dissolved in an appropriate solution. This procedure allowed for a very good homogeneity of the prepared test samples. However, this approach was not possible for the present study, since both feed additives were particle based products. One main challenge of this specific PT was therefore to prepare homogenous test materials by mixing two types of particles, namely the feed additives and the compound feed. In particular, the final number of particles from the feed additive in the feed per test item was identified as critical factor. In essence, if this number is very low, e.g. 10, the variation of this number between the individual test items will always be very high regardless of which mixing procedure is used. Therefore, prior to the production of the test samples, some simulations were performed, to estimate achievable homogeneity of the test items. For these simulations specific criteria were taken into account , i.e. an amount of 20 g feed per test item, the density of the feed additive, the concentration of the target carotenoid in the feed additive, the target concentration of the carotenoid in the feed and the particle size distribution of the feed additives used. The simulations were based on the principle that for each set of these criteria a nominal number of particles of the feed additive in test item containing 20 g of feed was calculated and by applying the Poisson distribution the corresponding relative standard deviation (RSD) was assessed indicating the best homogeneity that can be obtained under the conditions selected. An example is given in the following table for the feed additive "Lucantin Red".

Table 1: Achievable homogeneity for the feed additive Lucantin Red in 20 g test item depending on the corresponding particle size.
Constant parameters: Mass fraction of CXN in the feed additive (%): 10. Density of the feed additive: 0.7 g m^{-3}. Target concentration of CXN in feed: 20 mg kg^{-1}. Required mass of the feed additive for 20 g of the test item: 3.6 mg. RSD: Relative standard deviation estimated from the Poisson distribution.

Particle size of feed additive (µm)	850	425	250
Number of particles of the feed additive in 20 g of the test item	16	129	635
RSD (%)	25	8.8	4

This table shows that the majority of the particle size should be below 250 µm, to ensure that this error does not jeopardize the required homogeneity of the test items. A similar simulation was performed for Red carotenoid rich Paraccoccus carotinifaciens showing that this effect was less pronounced. This was mainly due to the fact that (1) the carotenoid concentration in the feed additive was lower compared to "Lucantin Red" and therefore the final feed samples had a higher content of the feed additive and (2) 98 % of all Red carotenoid rich *Paraccoccus carotinifaciens* particles had a particle size below 500 µm.

Procedure for the production of the test material: In the first step, both feed additives were sieved and the size fraction between 150 and 250 µm of these products were utilised for the preparation of the test material. For the production of a homogeneous mixture of the feed additive and the feed, the stepwise dilution technique was applied, where in each step the mass ratio of both components to be mixed was not above 10. The procedure is explained in more detail for the production of the test material containing Red carotenoid rich *Paraccoccus carotinifaciens*: taking into account (1) the carotenoid concentration in "Red carotenoid rich *Paraccoccus carotinifaciens*", (2) the target carotenoid concentration in the feed and (3) the total amount of feed required, the purpose of the exercise was to mix 318 mg of "Red

carotenoid rich *Paraccoccus carotinifaciens*" homogenously to feed to obtain a final amount of 1750 g test material. By applying the stepwise dilution technique 318 mg of "Red carotenoid rich *Paraccoccus carotinifaciens*" was mixed manually with 2.86 g of feed to obtain 3.18 g material. Then, the whole amount of this mixture was added to feed to get 31.8 g and mixed again. This step was repeated once again and mixed in a turbula mixer. In the last step 318 g of the mixture were added to 282 g feed to a get an amount of 600 g of material. This material was then split into three subsamples of 200 g. Each of these subsamples was mixed with 384 g feed and mixed again. Finally, the three test materials of 583 g were amalgamated to obtain the final amount of about 1750 g. The homogenisation of this material was achieved by splitting it into smaller portions using a commercial sample divider (Retsch PT 100, Hahn Germany) and pooling the portions again. This procedure was repeated three times. From the pooled and homogenised material after the third mixing step, 20 g of material were packed into sealed aluminium bags.

Annex 4: Homogeneity of the materials

10 bags of each test item were randomly selected (except for the blank material). Two aliquots from each bag were extracted and further analysed in duplicate by HPLC-UV according to the procedure implemented at the EURL-FA Control. The precision of the EURL-FA Control analytical method has been demonstrated to be suitable for the assessment of the homogeneity of the materials. The obtained concentrations were corrected for purity. The mean concentration values of the sub-sample duplicates were subjected to analysis of variances (ANOVA single factor – F-test). If the F-value obtained from the data set was lower than the F-critical, the material was considered adequately homogeneous for the related analyte.

All materials were considered suitable to undergo the collaborative trial.

Note: a colour code is applied in all tables of this annex; the RSD % given is calculated from the standard deviation of the analytical procedure, whereas the RSD% is calculated from the between-sample standard deviation.

Canthaxanthin - Poutry feed

Homogeneity study

Sacket	Subsample 1	Subsample 2
1	18.50	18.50
2	19.70	18.80
3	19.00	18.40
4	19.20	16.90
5	19.30	18.40
6	19.30	19.40
7	19.30	18.90
8	19.20	20.00
9	21.20	19.20
10	21.50	21.20

average: **19.30**

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	2	37.00	18.50	0.00
Row 2	2	38.50	19.25	0.40
Row 3	2	37.40	18.70	0.18
Row 4	2	36.10	18.05	2.65
Row 5	2	37.70	18.85	0.41
Row 6	2	38.70	19.35	0.00
Row 7	2	38.20	19.10	0.08
Row 8	2	39.20	19.60	0.32
Row 9	2	40.40	20.20	2.00
Row 10	2	42.70	21.35	0.05

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.58E+01	9	1.76E+00	**2.89**	0.06	**3.02**
Within Groups	6.09E+00	10	6.09E-01			
Total	2.19E+01	19				

Standard deviation of the analytical procedure (mg/kg): 0.78
RSD (%): 4.04
Between-sample standard deviation (mg/kg): 0.76
RSD (%): 3.930

Canthaxantin - Fish feed (Panaferd)

Homogeneity study

Sacket	Subsample 1	Subsample 2
1	1.29	1.23
2	1.30	1.28
3	1.28	1.21
4	1.18	1.20
5	1.19	1.34
6	1.20	1.21
7	1.10	1.17
8	1.17	1.27
9	1.25	1.29
10	1.30	1.26

average **5.91**

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	2	2.52	1.26	0.00
Row 2	2	2.58	1.29	0.00
Row 3	2	2.48	1.24	0.00
Row 4	2	2.38	1.19	0.00
Row 5	2	2.53	1.27	0.01
Row 6	2	2.41	1.21	0.00
Row 7	2	2.27	1.14	0.00
Row 8	2	2.44	1.22	0.01
Row 9	2	2.54	1.27	0.00
Row 10	2	2.56	1.28	0.00

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4.28E-02	9	4.75E-03	**1.94**	0.16	**3.02**
Within Groups	2.45E-02	10	2.45E-03			
Total	6.72E-02	19				

Standard deviation of the analytical procedure (mg/kg): 0.05
RSD (%): 4.00
Between-sample standard deviation (mg/kg): 0.03
RSD (%): 0.575

Astaxanthin - Fish feed (Panaferd)

Homogeneity study

Sacket	Subsample 1	Subsample 2
1	55.80	61.20
2	50.30	67.00
3	56.70	61.50
4	59.20	59.40
5	57.80	54.20
6	57.30	52.40
7	51.10	56.90
8	56.80	65.10
9	59.70	59.30
10	56.70	57.30

average **57.79**

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	2	2.00	117.00	58.50
Row 2	2	2.00	117.30	58.65
Row 3	2	2.00	118.20	59.10
Row 4	2	2.00	118.60	59.30
Row 5	2	2.00	112.00	56.00
Row 6	2	2.00	109.70	54.85
Row 7	2	2.00	108.00	54.00
Row 8	2	2.00	121.90	60.95
Row 9	2	2.00	119.00	59.50
Row 10	2	2.00	114.00	57.00

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9.00E+01	9	1.00E+01	**0.42**	0.89	**3.02**
Within Groups	2.36E+02	10	2.36E+01			
Total	3.26E+02	19				

Standard deviation of the analytical procedure (mg/kg): 4.85
RSD (%): 8.40
Between-sample standard deviation (mg/kg): **2.295** Ubb* (since MSBG<MSW)
RSD (%): **3.972**

Adonirubin - Fish feed (Panaferd)

Homogeneity study

Sacket	Subsample 1	Subsample 2
1	18.20	19.60
2	16.10	21.30
3	17.30	19.50
4	18.50	19.00
5	19.10	17.80
6	18.80	17.20
7	16.10	18.70
8	18.60	21.40
9	19.40	19.00
10	17.70	18.50

average **18.590**

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Row 1	2	37.80	18.90	0.98
Row 2	2	37.40	18.70	13.52
Row 3	2	36.80	18.40	2.42
Row 4	2	37.50	18.75	0.13
Row 5	2	36.90	18.45	0.85
Row 6	2	36.00	18.00	1.28
Row 7	2	34.80	17.40	3.38
Row 8	2	40.00	20.00	3.92
Row 9	2	38.40	19.20	0.08
Row 10	2	36.20	18.10	0.32

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9.108E+00	9	1.012E+00	**0.38**	0.921	**3.02**
Within Groups	2.687E+01	10	2.687E+00			
Total	3.598E+01	19				

Standard deviation of the analytical procedure (mg/kg): 1.64
RSD (%): 8.82
Between-sample standard deviation (mg/kg): **0.78** Ubb* (since MSBG<MSW)
RSD (%): **4.169**

Annex 5: Stability of the materials

The stability was evaluated on two series of measurements of each analyte. The first series was performed at T0, when the materials were produced and homogenised and the second after the completion of the proficiency exercise by all participants and reception of the results, namely at T18 weeks.

The remaining bags of each test item were kept at -20°C during the whole duration of the PT exercise. At the end of the exercise, two bags of each test item were analysed in duplicate using the analytical procedure implemented at the EURL-FA Control and each replicate was injected twice.

A one-tailed F-test showed that the variances of the two series were equal. The following t-test demonstrated that the mean value of the concentration obtained after 18 weeks was not significantly different to the mean value of the concentration at T0. The samples can therefore be considered as stable at the recommended storage temperature tested during the whole duration of the PT exercise.

Fish feed

	T0	T 18 weeks	T0	T 18 weeks	T0	T 18 weeks
	Astaxanthin		Adonirubin		Canthaxanthin	
	mg kg^{-1}		mg kg^{-1}		mg kg^{-1}	
Bag 233 - replicate 1 - average 2 injections	58.500	53.979	18.900	16.151	6.200	4.993
Bag 233 - replicate 2 - average 2 injections	58.650	55.196	18.700	17.411	6.350	5.401
Bag 254 - replicate 1 - average 2 injections	59.100	50.370	18.400	15.691	5.700	5.138
Bag 254 - replicate 2 - average 2 injections	59.300	52.740	18.750	15.686	5.900	5.283
F value (one tail)	0.0193		0.0517		0.4309	
t value (one tail)	0.0007		0.0006		0.0014	
degrees of freedom data set 1 = degrees of freedom of data set 2			= 3			
degrees of freedom total			= 6			
Fcrit (P=0.05)			= 9.277			
t_{10} at 95% (P=0.05)			= 1.94			

Poultry feed

	T0	T 18 weeks
	Canthaxanthin	
	mg kg^{-1}	
Bag 193 - replicate 1 - average 2 injections	18.500	19.238
Bag 193 - replicate 2 - average 2 injections	19.250	18.714
Bag 196 - replicate 1 - average 2 injections	18.700	17.092
Bag 196 - replicate 2 - average 2 injections	18.050	18.280
F value (one tail)	0.3445	
t value (one tail)	0.2964	

degrees of freedom data set 1 = degrees of freedom of data set 2	= 3
degrees of freedom total	= 6
Fcrit (P=0.05)	= 9.277
t_{10} at 95% (P=0.05)	= 1.94

Annex 6: Accompanying letter and receipt form

01 June 2015

Proficiency Test exercise 2015

Determination of authorised carotenoids in feed at authorised levels

INSTRUCTIONS

The materials you received should be analysed in routine conditions, i.e. utilizing your usual method for control.

Please check the content of the package, fill in and send back the 'List of Contents of the Package' form as specified below.

All materials have been grinded and homogenised; no further pre-treatment is necessary before the analysis.

Analyse all materials for the presence, the content and the correct labelling of the authorised carotenoid(s). The content should be reported as the concentration of the detected carotenoid in **mg kg^{-1}** of feed given with 3 decimals. When carotenoids are present, the concentration(s) are as defined in the Regulation (EC) No 1831/2003 for carotenoids authorized as feed additives.

Instructions on how to report your results and information on the method used will be sent to you in due time in the format of a digital form via an electronic message.

Please note that the ultimate deadline for reporting the results is **03 July 2015**.

Please remember that the major objective of this study is to assess the proficiency of the participating laboratories to correctly determine carotenoids potentially added to feed samples at levels authorised in the European legislation and to report results in the specified units and accompanied with a compliance statement on the analysed samples within a defined time frame.

Any deviation as regards the non-respect of the deadline and/or the specified units for reporting will lead to the exclusion of your results from the statistical evaluation.

Statistical evaluation

Appropriate statistical tool for data evaluation will be used to investigate the proficiency of the laboratories. Statistical assessment of the proficiency of laboratories will be evaluated by calculating an individual dimensionless Z-score calculated according to ISO 13528.

$$z = \frac{(x - x_a)}{\sigma_p} \qquad \text{Eq. 1}$$

Where x = the value reported by the participant

x_a = the assigned value

σ_p = the target standard deviation

In Autumn 2015, a draft report including the statistical evaluation of all valid participant results will be issued and distributed to all reporting participants. The report will be confidential. The list of participating laboratories will be included but any result will be strictly linked only to the laboratory code. Each participant will be able to retrieve its results and calculated z-score using its individual confidential laboratory code communicated on the list of contents of the package form included in this letter.

Finally, a concluding workshop opened to all participants to the study will be organised to close the exercise in November 2015 (target date: 17-18.11.2015, 1 day). Participation to the PT and to the workshop is free of charge. However, travel and accommodation costs will only be reimbursed for one representative of each National reference Laboratory for Feed Additives defined according to Regulation (EC) No 882/2004 and Regulation (EC) No 1831/2003.

If you have any question, please contact the EURL-FA (Control), jrc-irmm-eurl-feed-additives-control@ec.europa.eu.

Kind regards,

Dr Ursula VINCENT (Proficiency Test Coordinator)

On behalf of the EURL for Feed Additives

LIST OF CONTENTS OF THE PACKAGE[11]

ACKNOWLEDGEMENT OF RECEPTION

Dear «TITLE» «SURNAME»«SURNAME»,

please find below the list of contents for the proficiency test exercise related to the determination authorised carotenoids in feed at authorised levels, organised by the EURL-Feed Additives (Control), on behalf of DG SANTE (European Commission).
Please check that the sample codes of the samples you received correspond to those declared on this list. The samples should **be stored at -18°C / -20°C** upon reception and until one hour before the analysis, when they are left at room temperature until analysis.

Your laboratory code is given below. This code will be applied to your laboratory for the whole PT exercise.

Your laboratory code is: «SURNAME»

Number of samples in the package: 5

Sample code	Present Y/N
«SURNAME»	
«SURNAME»	
«SURNAME»	
«SURNAME»	
«SURNAME»	

Samples received on (dd/mm/yyyy) :
Content checked on (dd/mm/yyyy) :
Comments (if applicable) :

Date: Signature:

[11] Form to fill in, sign and send back to the EURL-Feed additives (control) by electronic mail (jrc-irmm-eurl-feed-additives-control@ec.europa.eu) or by fax (+32 14 571 787)

Annex 7: Reporting sheet

This information will assist you in reporting your results

This excel workbook contains four worksheets and should be sent back when filled in.
In all worksheets, the accessible areas for writing are the dedicated empty **couloured areas**.
In the worksheet "**Method questionnaire**", enter the information related to the method used for the identification and quantification (if relevant) of the carotenoids. Please do so comprehensively, for each analyte, in order to allow appropriate evaluation and relevant discussion of the results.
Please **use** the "comments" text box for any additional information you wish to provide and/or to specify an "**other**" answer. Please be precise for **each** of the carotenoids.
"**Report form**": please fill in the results from each of the 5 unknown samples sent to your laboratory. Remember that only the empty **coloured areas** of the sheets are reserved for inserting results and other requested information. Since the assay most probably gives you both qualitative results and a value for the concentration, we ask you to report the result as "Detected", "Not detected", "Not analysed", and to report the concentration you determined in **mg kg^{-1}**. Please note that:
- use the "**.**" as **decimal point** (and not the ",");
- an analyte for which the determined concentration would be lower than LOQ should be reported as "Detected" and for the concentration: "<**LOQ**";
- an analyte for which the determined concentration would be lower than LOD should be reported as "Not detected" and for the concentration: "<**LOD**".

As for the expanded uncertainty, it is obtained by multiplying the combined standard uncertainty by a coverage factor *k;* please report the expanded uncertainty with k = 2, to give a level of confidence of 95%. Please specify in the comments text box how the reported uncertainty was calculated. If you do not report an uncertainty, please fill in with "nr"; **do not leave any blank**.

Reporting the results

The analysis should be conducted within 1 month from the date of reception of the samples. The **deadline** for reporting the results is **03 July 2015**.
Please modify the name of the file by inserting the labcode. For instance: For laboratory with the labcode L04 the filename should be:

L04_PT_Carots_2015_Reporting_sheet.xls

What to report to the EURL-FA Control:
- the complete current file
- the signed and dated "r**esults to be faxed or emailed**" sheet

How to report to the EURL-FA control:
- by e-mail to jrc-irmm-eurl-feed-additives-control@ec.europa.eu
- by Fax: +32 14 571 787

In addition, please send to us the chromatograms from all analyses either by e-mail or by normal mail.
The information collected will be kept confidential and only used for discussion and exchange during the final workshop.
Thanks and kind regards,
Dr Ursula Vincent

Results reporting form for the determination of carotenoids in feedingstuffs

Name of laboratory:		
Lab code:		You will find the labcode on the document accompanying the shipment of the samples
	☐	I confirm that I have read the information in the worksheet "Important information"
	From	To
		Period during which the analysis was conducted (dd/mm/yyyy - dd/mm/yyyy)

	Result of analysis	**Result of analysis**	**Result of analysis**	**Result of analysis**	**Result of analysis**
Labcode					
Sample Code					
Astaxanthin					
concentration in mg kg^{-1}					
expanded uncertainty in mg kg^{-1}					
Canthaxanthin					
concentration in mg kg^{-1}					
expanded uncertainty in mg kg^{-1}					
Adonirubin					
concentration in mg kg^{-1}					
expanded uncertainty in mg kg^{-1}					
Other					
concentration in mg kg^{-1}					
expanded uncertainty in mg kg^{-1}					

Note: The major objective of this study is to assess the proficiency of the participating laboratories to correctly determine carotenoids potentially added to feed samples at levels authorised in the European legislation. The statistical evaluation of the results will therefore focus on the quantitative results delivered by the participating laboratories

Legend: BACARE = Ethyl ester of beta-apo-8'-carotenoic acid; AXN DMDS = Astaxanthin dimethyldisuccinate

Print this area for reporting the results

Date, sign and return to EURL-FA Control

by fax:+32 14 571 787
by email: jrc-irmm-eurl-feed-additives-control@ec.europa.eu

PT exercise 2015: CAROTENOIDS IN FEEDINGSTUFFS

Laboratory name: **0**

Laboratory code:

Sample code	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Astaxanthin	no result	0	no result	0	no result	0	no result	0	no result	0
		0		0		0		0		0
Canthaxanthin	no result	0	no result	0	no result	0	no result	0	no result	0
		0		0		0		0		0
Adonirubin	no result	0	no result	0	no result	0	no result	0	no result	0
		0		0		0		0		0
Other	no result	0	no result	0	no result	0	no result	0	no result	0
		0		0		0		0		0

---------- ----------

Signature (responsible of analysis): Date

Annex 8: Reported concentration results with associated uncertainties and reported limits of detection and quantification, in mg kg^{-1} of feed

1) spikes at additive levels

	Astaxanthin (MAT 2)					
$X_{ref} \pm \sigma_p$	57.785 ± 4.820					
	MAT 2a		MAT 2b			
LAB	x_{lab}	u_{lab}	x_{lab}	u_{lab}	LOD	LOQ
L01	59.608	6.557	57.395	6.314	0.2	5
L02	42.000	6.300	84.300	12.650	-	-
L03	42.600	1.000	42.100	1.000	0.5	1.6
L04	NA	NA	NA	NA	-	-
L05	53.823	3.230	57.821	3.470	-	1.4
L06	48.600	4.700	59.500	0.445	0.3	1
L07	27.900	NR	23.264	NR	-	-

	Canthaxanthin (MAT 2)					
$X_{ref} \pm \sigma_p$	5.910 ± 0.854					
	MAT 2a		MAT 2b			
LAB	x_{lab}	u_{lab}	x_{lab}	u_{lab}	LOD	LOQ
L01	NA	NA	NA	NA	-	-
L02	8.790	1.540	17.600	3.100	-	-
L03	4.200	0.200	4.100	0.200	0.5	1.7
L04	2.300	NR	2.320	NR	0.3	1
L05	8.959	0.540	9.598	0.575	-	1.2
L06	4.020	6.100	5.070	3.170	0.3	1
L07	LOQ	ND	0.000	ND	-	-

	Adonirubin (MAT 2)					
$X_{ref} \pm \sigma_p$	18.590 ± 2.246					
	MAT 2a		MAT 2b			
LAB	x_{lab}	u_{lab}	x_{lab}	u_{lab}	LOD	LOQ
L01	NA	NA	NA	NA	-	-
L02	NA	NA	NA	NA	-	-
L03	13.200	1.000	13.500	1.000	0.25	0.8
L04	NA	NA	NA	NA	-	-
L05	NA	NA	NA	NA	-	-
L06	13.400	2.000	16.400	2.650	-	-
L07	NA	NA	NA	NA	-	-

	Canthaxanthin (MAT 1)					
$X_{ref} \pm \sigma_p$	**19.295 ± 2.892**					
	MAT 1a		**MAT 1b**			
LAB	**x_{lab}**	**u_{lab}**	**x_{lab}**	**u_{lab}**	**LOD**	**LOQ**
L01	NA	NA	NA	NA	-	-
L02	4.650	0.815	11.700	2.050	-	-
L03	12.600	0.500	12.800	0.500	0.5	1.7
L04	9.320	NR	10.000	NR	0.3	1
L05	13.377	0.805	15.239	0.915	-	1.2
L06	16.800	1.485	17.000	0.405	0.3	1
L07	11.111	NR	9.726	NR	-	-

Figures 1-4 display the results as reported by the participants, in mg kg^{-1}; the error bars represent the reported uncertainties.

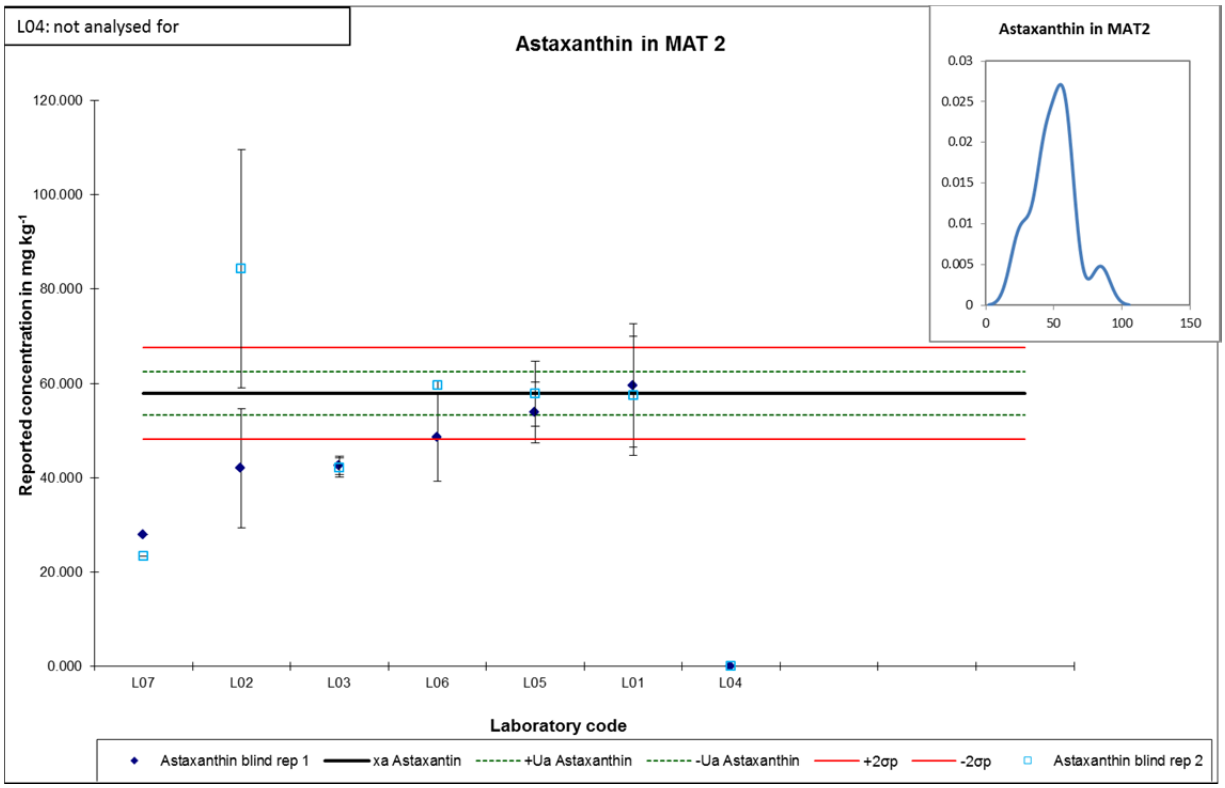


Figure 1: Reported concentrations for astaxanthin in MAT 2 – the error bars are the associated uncertainties as reported by the participants. The estimation of the concentration from the homogeneity study is displayed as indicative value without laboratory label.

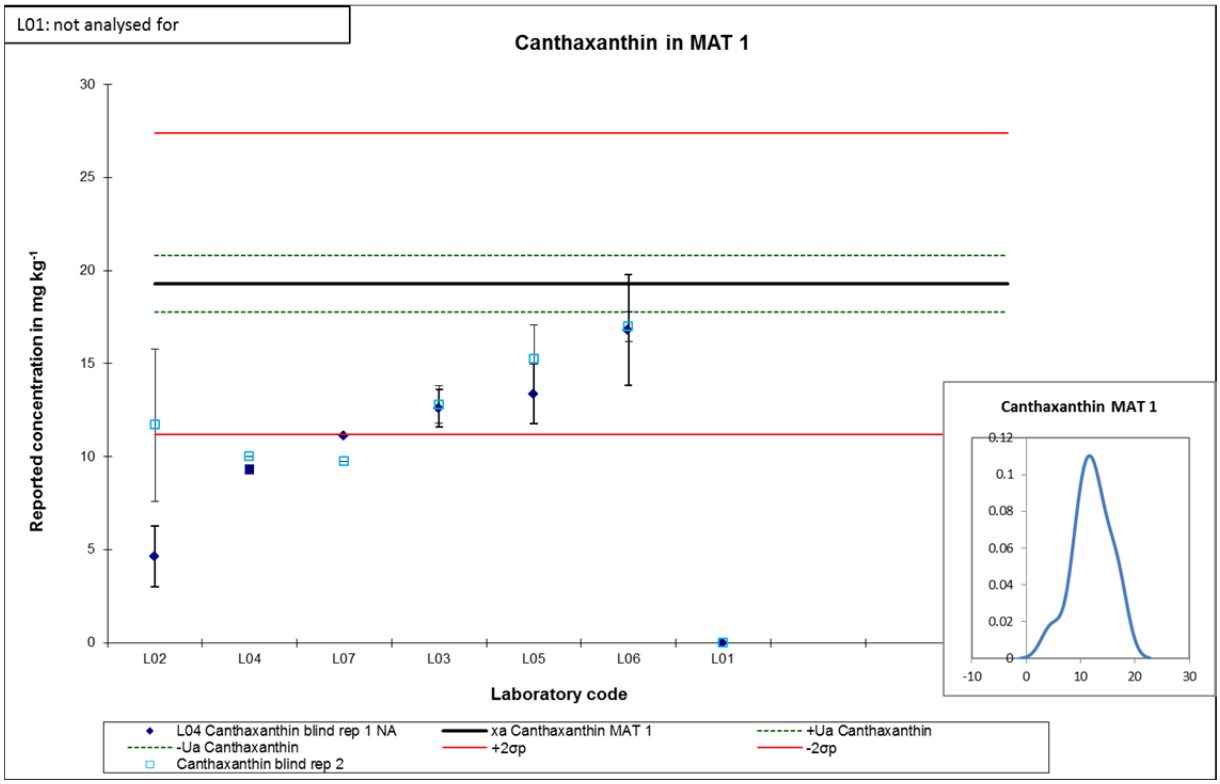


Figure 2: Reported concentrations for canthaxanthin in MAT 1 – the error bars are the associated uncertainties as reported by the participants. The estimation of the concentration from the homogeneity study is displayed as indicative value without laboratory label.

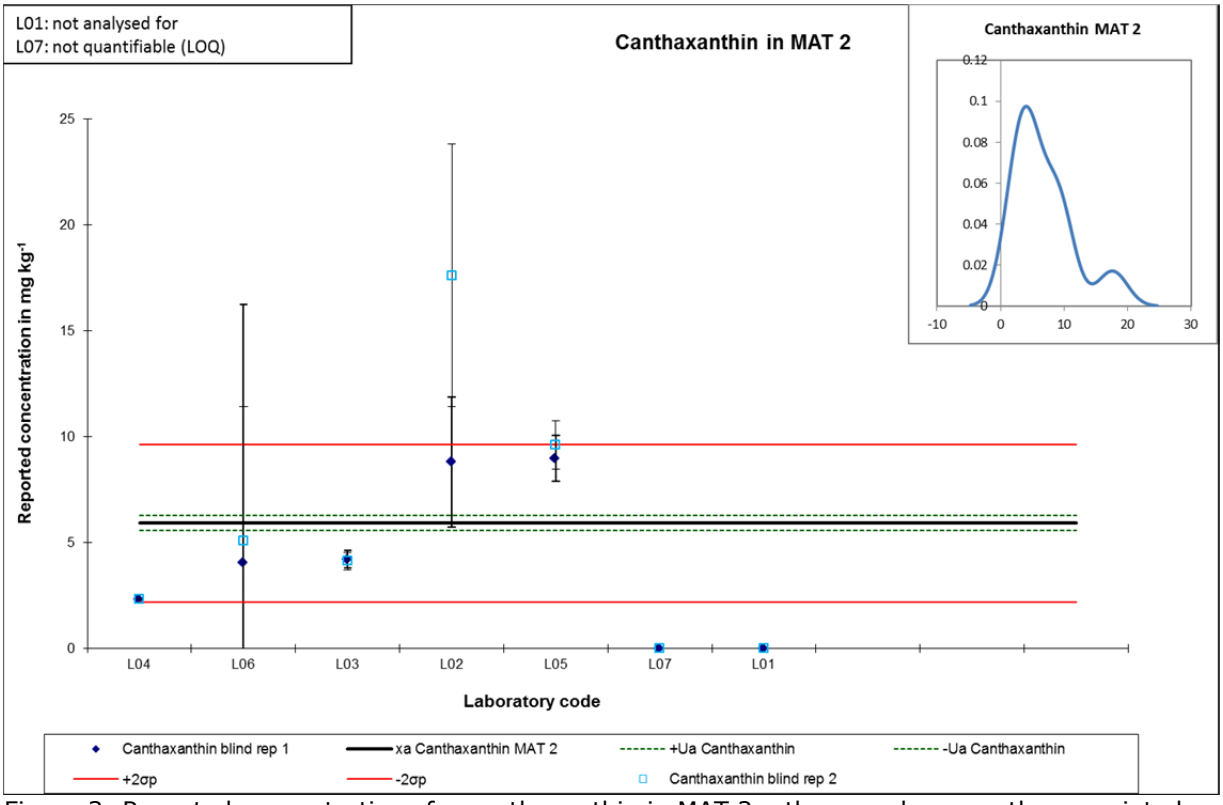


Figure 3: Reported concentrations for canthaxanthin in MAT 2 – the error bars are the associated uncertainties as reported by the participants. The estimation of the concentration from the homogeneity study is displayed as indicative value without laboratory label.

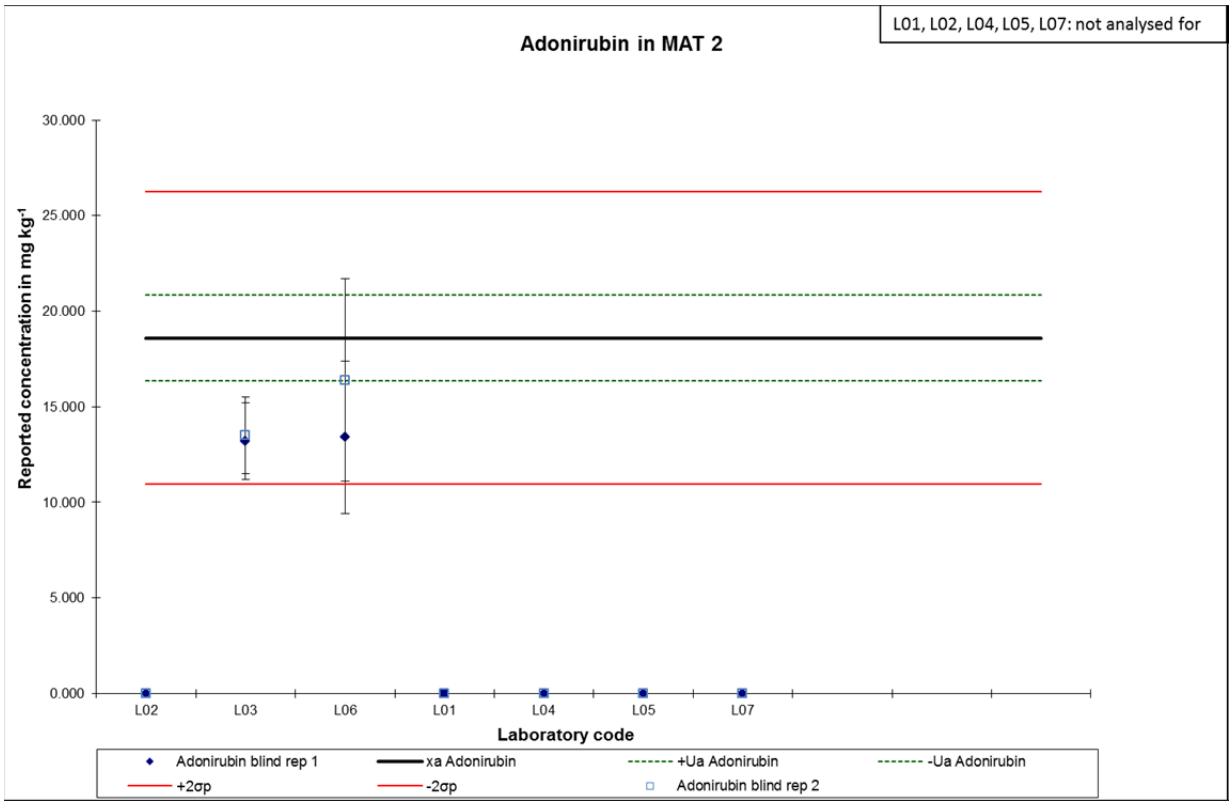


Figure 4: Reported concentrations for adonirubin in MAT 2 – the error bars are the associated uncertainties as reported by the participants. The estimation of the concentration from the homogeneity study is displayed as indicative value without laboratory label.

2) in the non-spiked materials

	Adonirubin MAT 1		Lutein MAT 1		Lutein MAT 2		BCAR MAT 1	
$X_{ref} \pm \sigma_p$	0.000 ± 0.000							
LAB	x_{lab}	u_{lab}	x_{lab}	u_{lab}	x_{lab}	u_{lab}	x_{lab}	u_{lab}
L02	-	-			7.760 9.660	1.360 1.690	18.300	3.200
L03	1.600	0.250	1.200	0.100				

BCAR: beta-carotene

	AXN DMDS MAT 1		AXN DMDS MAT 2		AXN DMDS MAT 3	
$X_{ref} \pm \sigma_p$	0.000 ± 0.000					
LAB	x_{lab}	u_{lab}	x_{lab}	u_{lab}	x_{lab}	u_{lab}
L01	D		D		D	

AXN DMDS: astaxanthin dimethyl disucinate; D: detected but no reported concentration

All laboratories correctly reported the absence of the spiked measurands in all materials except for the laboratories displayed in the table above in the indicated materials.

There were no other false positive reported in any of the three materials.

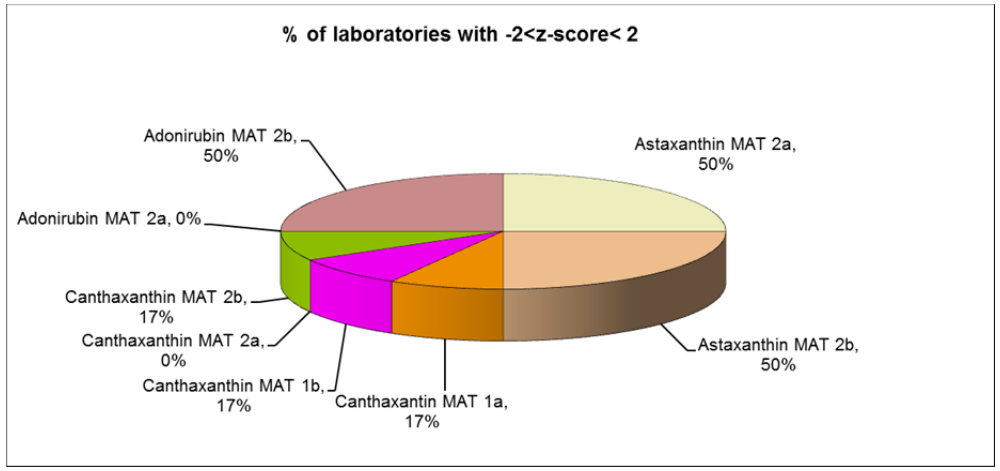
The limits of detection (LOD) and of quantification (LOQ), in mg kg^{-1}, as reported by the participants for the remaining carotenoids are displayed in the following tables.

	Citranaxanthin		Lutein		Zeaxanthin		Beta-carotene	
LAB	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**
L01	-	-	-	-	-	-	-	-
L02	-	-	-	-	-	-	-	-
L03	0.3	1.1	0.2	0.6	0.4	1.3	0.4	1.4
L04	-	-	-	-	-	-	-	-
L05	-	-	-	-	-	-	-	-
L06	-	-	-	-	-	-	-	-
L07	-	-	-	-	-	-	-	-

	Capsanthin		Astaxanthin DMDS		Ethyl ester of beta-apo-8'-carotenoic acid	
LAB	**LOD**	**LOQ**	**LOD**	**LOQ**	**LOD**	**LOQ**
L01	-	-	-	-	-	-
L02	-	-	-	-	-	-
L03	-	-	-	-	-	-
L04	-	-	-	-	-	-
L05	-	-	-	-	-	-
L06	-	-	-	-	-	-
L07	-	-	-	-	-	-

Annex 9: z- scores calculated for each reporting laboratory

	Z-scores							
LAB	Astaxanthin MAT 2a	Astaxanthin MAT 2b	Canthaxantin MAT 1a	Canthaxanthin MAT 1b	Canthaxanthin MAT 2a	Canthaxanthin MAT 2b	Adonirubin MAT 2a	Adonirubin MAT 2b
Target value	57.785	57.785	19.295	19.295	5.910	5.910	18.590	18.590
L01	0.36	-0.08	NA	NA	NA	NA	NA	NA
L02	-3.16	5.31	-7.45	-3.86	4.00	16.25	NA	NA
L03	-3.04	-3.14	-3.40	-3.30	-2.38	-2.52	-2.83	-2.67
L04	NA	NA	-5.07	-4.73	-5.02	-4.99	NA	NA
L05	-0.79	0.01	-3.01	-2.06	4.24	5.13	NA	NA
L06	-1.84	0.34	-1.27	-1.17	-2.63	-1.17	-2.72	-1.15
L07	-5.98	-6.91	-4.16	-4.87	ND	ND	NA	NA



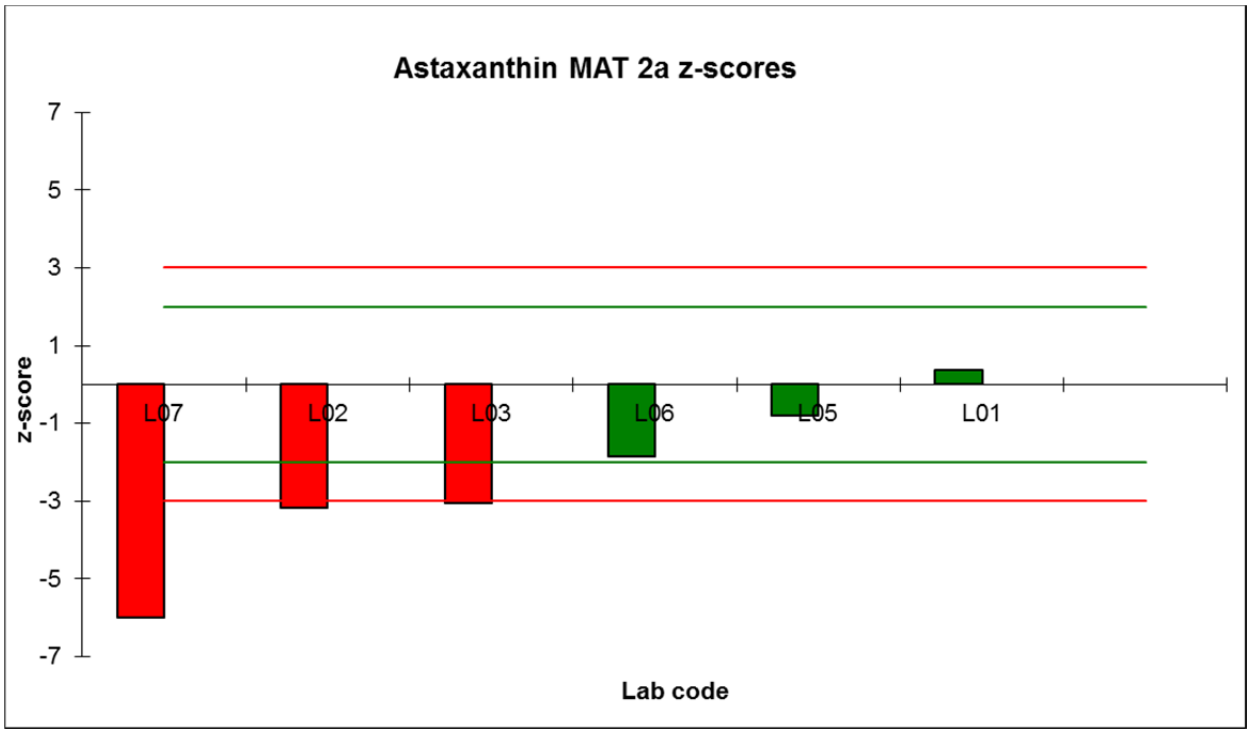


Figure 5: MAT 2: z-scores for the determination of astaxanthin (x_a: 56.010 mg kg^{-1}) (replicate 1) for the participating laboratories. The green line shows the limit for satisfactory ($|z| \leq 2$) and the red line for unsatisfactory performance ($|z| > 3$).

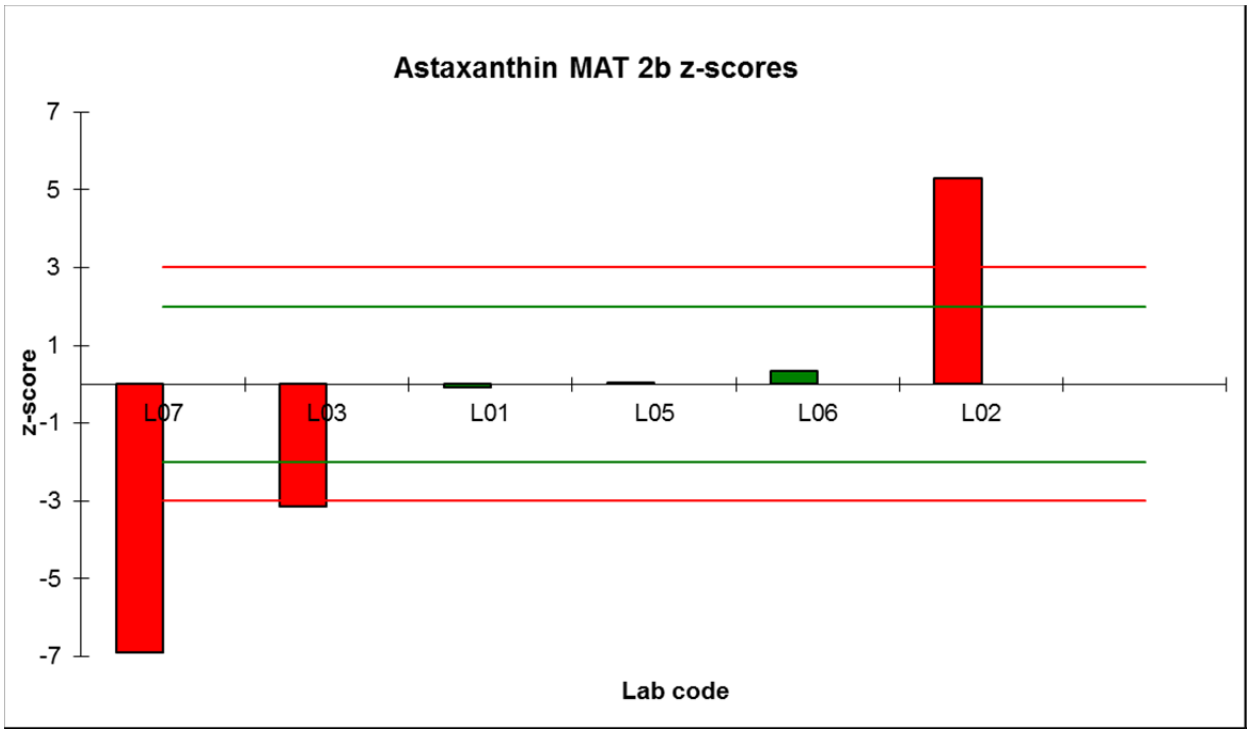


Figure 6: MAT 2: z-scores for the determination of astaxanthin (x_a: 56.010 mg kg^{-1}) (replicate 2) for the participating laboratories. The green line shows the limit for satisfactory ($|z| \leq 2$) and the red line for unsatisfactory performance ($|z| > 3$).

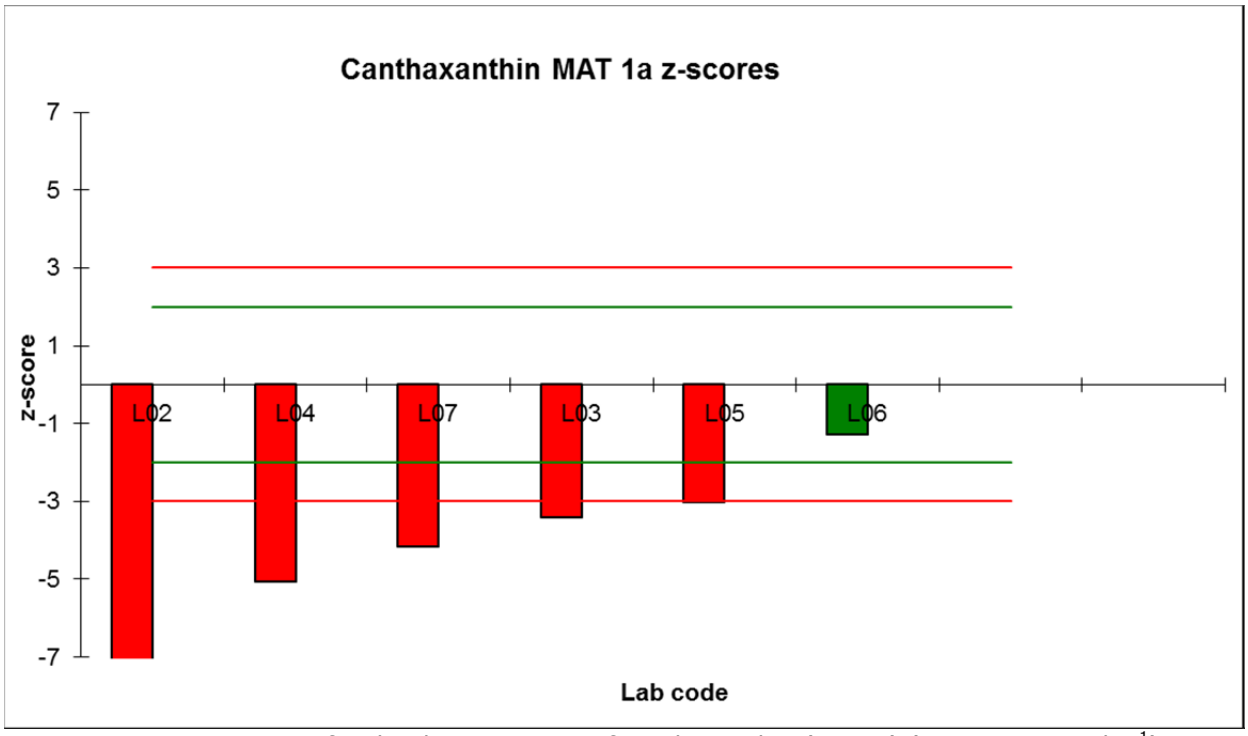


Figure 7: MAT 1: z-scores for the determination of canthaxanthin (MAT 1) (x_a: 19.996 mg kg^{-1}) (replicate 1) for the participating laboratories. The green line shows the limit for satisfactory ($|z| \leq 2$) and the red line for unsatisfactory performance ($|z| > 3$).

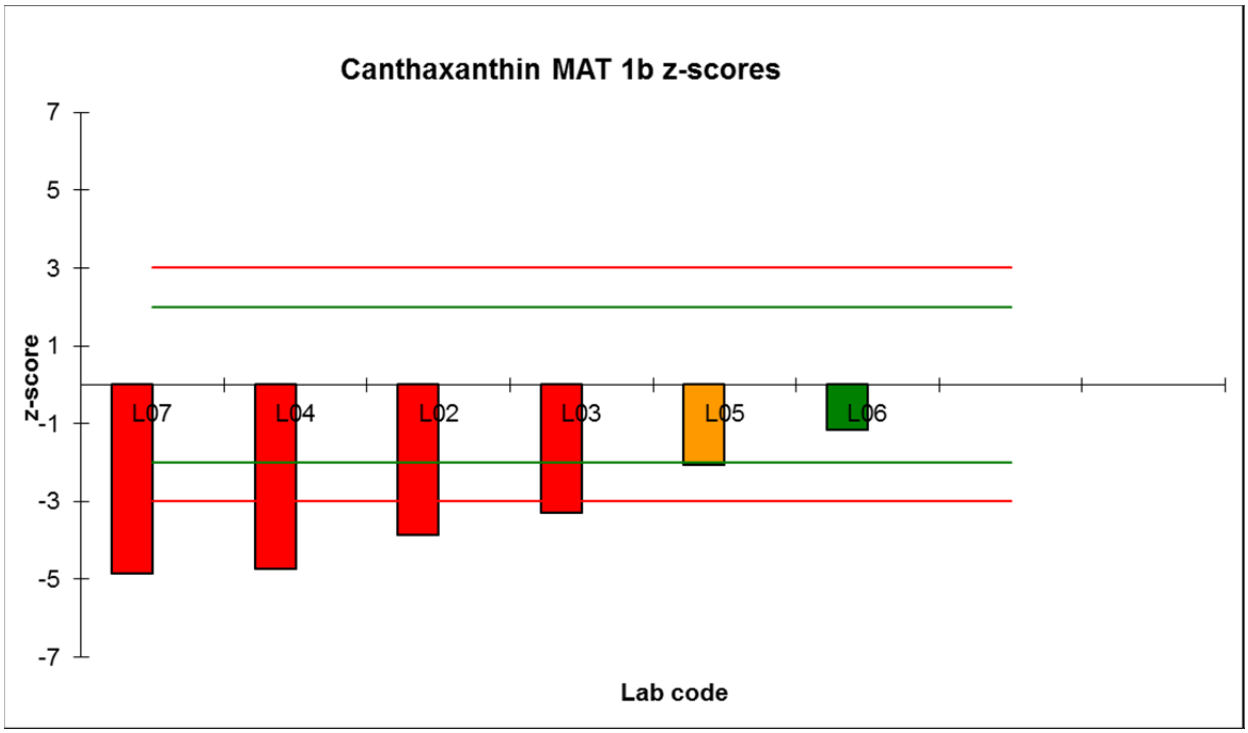


Figure 8: MAT 1: z-scores for the determination of canthaxanthin (MAT 1) (x_a: 19.996 mg kg^{-1}) (replicate 2) for the participating laboratories. The green line shows the limit for satisfactory ($|z| \leq 2$) and the red line for unsatisfactory performance ($|z| > 3$).

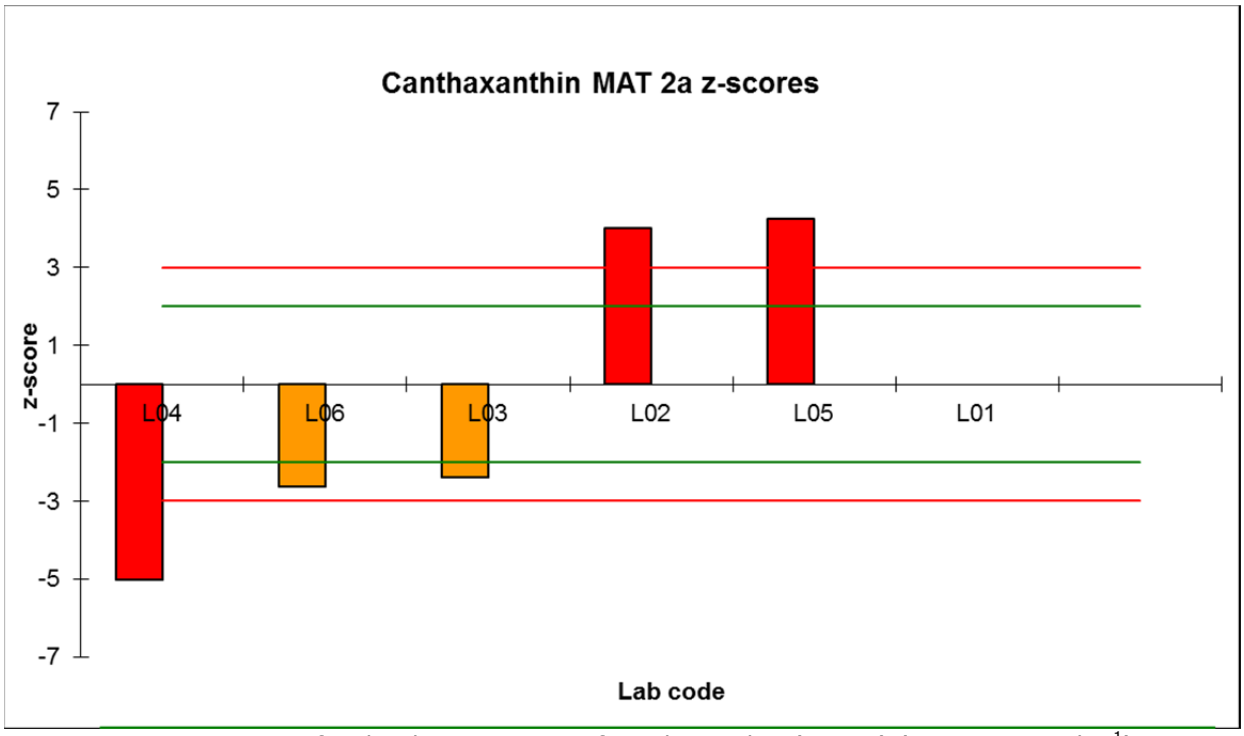


Figure 9: MAT 1 z-scores for the determination of canthaxanthin (MAT 2) (x_a: 8.001 mg kg^{-1}) (replicate 1) for the participating laboratories. The green line shows the limit for satisfactory ($|z| \leq 2$) and the red line for unsatisfactory performance ($|z| > 3$).

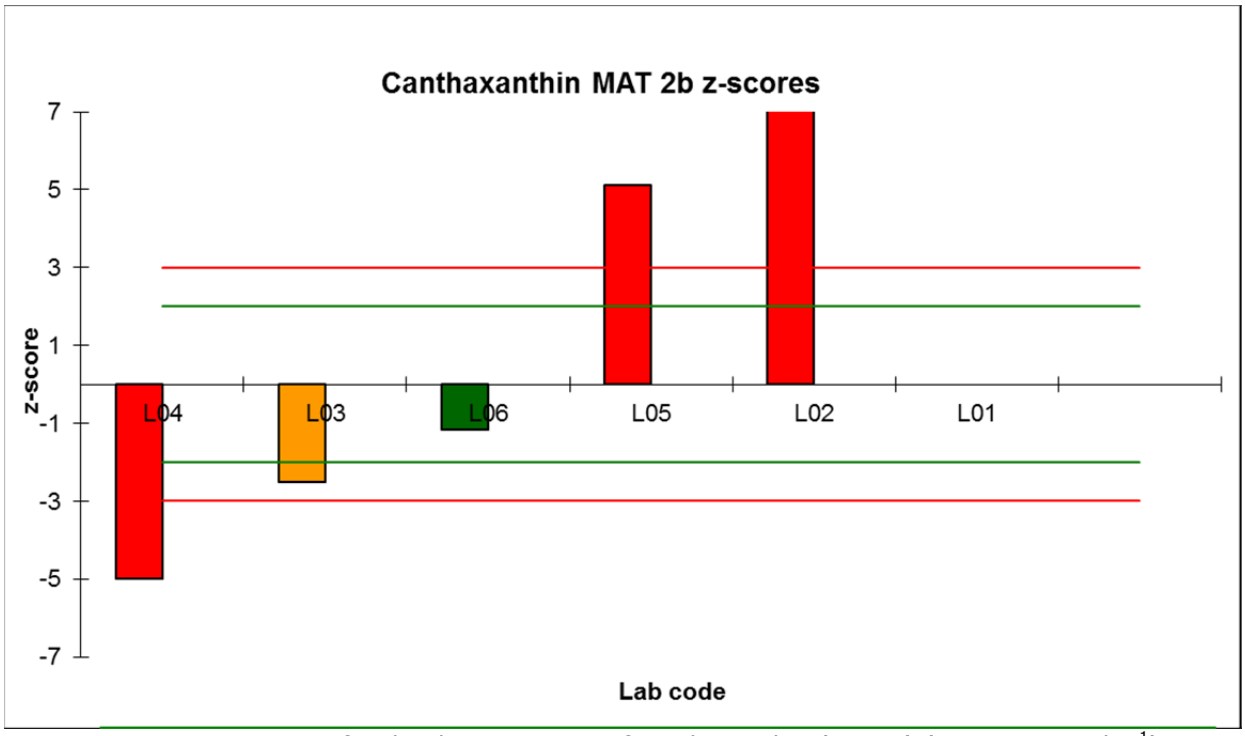


Figure 10: MAT 2: z-scores for the determination of canthaxanthin (MAT 2) (x_a: 8.001 mg kg^{-1}) (replicate 2) for the participating laboratories. The green line shows the limit for satisfactory ($|z| \leq 2$) and the red line for unsatisfactory performance ($|z| > 3$).

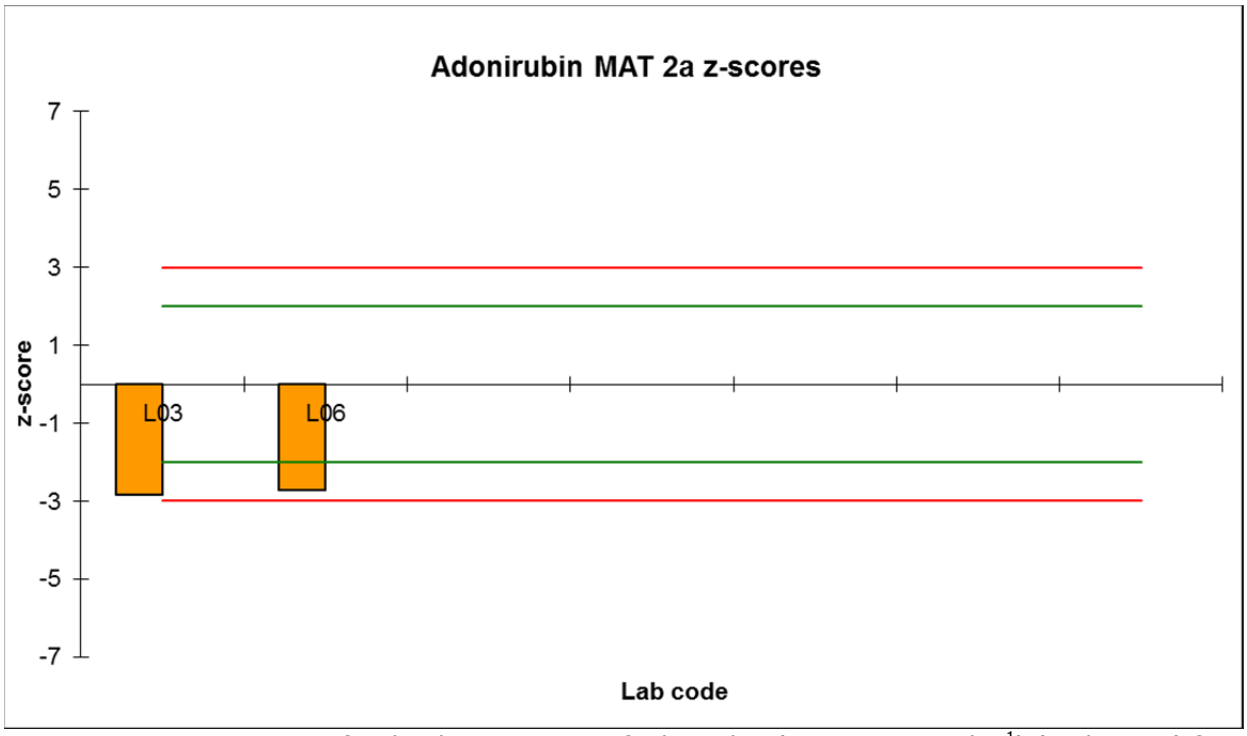


Figure 11: MAT 2: z-scores for the determination of adonirubin (x_a: 18.670 mg kg^{-1}) (replicate 1) for the participating laboratories. The green line shows the limit for satisfactory ($|z| \leq 2$) and the red line for unsatisfactory performance ($|z| > 3$).

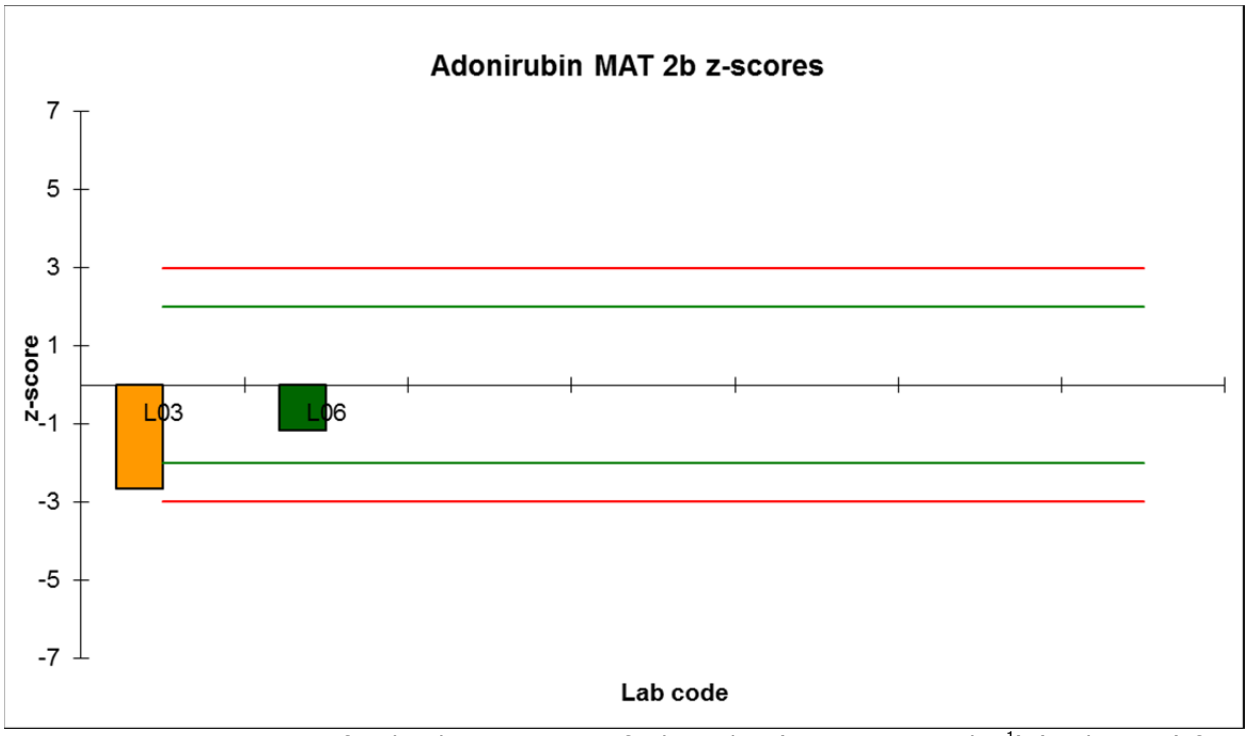


Figure 12: MAT 2: z-scores for the determination of adonirubin (x_a: 18.670 mg kg^{-1}) (replicate 2) for the participating laboratories. The green line shows the limit for satisfactory ($|z| \leq 2$) and the red line for unsatisfactory performance ($|z| > 3$).

Annex 10: Analytical methods used by participants

Methods are tabulated according to the information supplied by the participants.

	Laboratory number			
	Astaxanthin	Canthaxanthin	Adonirubin	Other
Is the method used accredited?				
Yes	L05	L05		
No	L01, L02, L03, L04, L06, L07	L02, L03, L04, L06, L07	L03, L06, L07	L01, L02, L03, L06, L07
Is it a				
multi-analyte method	L02, L03, L05, L06, L07	L02, L03, L04, L05, L06, L07	L03, L06, L07	L02, L03, L06, L07
single analyte method	L01			
HPLC method	L01, L02, L03, L05, L06, L07	L02, L03, L04, L05, L06, L07	L03, L06, L07	L02, L03, L06, L07
Other method				
Was the package under vacuum?				
Yes	L01, L02, L03, L05, L06, L07	L02, L03, L04, L05, L06, L07	L03, L06, L07	L02, L03, L06, L07
No				
Sample amount used for analysis (g)				
≥1 - <5	L01, L05, L07	L04, L05, L07	L07	L01, L02, L07
≥5 - <10	L02, L03	L02, L03	L03	L03
≥10	L06	L06	L06	L06
Digested with enzyme?				
Yes	L01, L03, L05, L06, L07	L03, L04, L05, L06, L07	L03, L06, L07	L01, L03, L06, L07
No	L02	L02		L02

	Laboratory number			
	Astaxanthin	Canthaxanthin	Adonirubin	Other
Enzyme used				
Protex 6L	L01, L05, L06	L05, L06	L06	L01, L06
Alcalase/2.59 U/ml	L03	L03	L03	L03
Protease from Bacillus	L07	L07	L07	L07
Amount of enzyme used				
100 mg	L01, L06	L06	L06	L01, L06
125 mg	L03	L03	L03	L03
100 µl	L05	L05		
0.035 mg (???)	L07	L07	L07	L07
Incubation time/temperature				
30 min/50°C	L01, L05, L06	L05, L06	L06	L01, L06
20 min/50°C	L03, L07	L03, L07	L03, L07	L03, L07
Digested in ultrasonic bath?				
Yes	L01, L06, L07	L04, L06, L07	L06, L07	L01, L06, L07
No				
Extracted using ASE?				
Yes	L03	L03	L03	L03
No				
Liquid-solid extraction?				
Yes	L02, L05	L02, L05		
No				L02
diluted?				
Yes	L01, L05, L06	L05, L06	L06	L01, L06
No	L02, L03, L07	L02, L03, L04, L07	L03, L07	L02, L03, L07

	Laboratory number			
	Astaxanthin	Canthaxanthin	Adonirubin	Other
Extraction solvents				
water, ethanol, dichloromethane	L01			L01
hexane, acetone	L02	L02		
Ether diethylique				L02
acetone	L03, L07	L03, L07	L03, L07	L03, L07
ethanol, dichloromethane	L05, L06	L05, L06	L06	L06
Clean-up				
SPE	L01, L05, L06	L05, L06	L06	L01, L06
Liquid-liquid extraction	L02	L02		L02
SPE sorbent used				
Silica gel 60	L01			L01
silica	L05	L05		
silicagel	L06	L06	L06	L06
Centrifugation?				
Yes	L03, L07	L03, L07	L03, L07	L03, L07
No				
Guard column?				
Yes	L01, L02, L03	L03 L02	L03	L01, L02, L03
No	L05, L06, L07	L04, L05, L06, L07	L06, L07	L06, L07

	Laboratory number			
	Astaxanthin	Canthaxanthin	Adonirubin	Other
HPLC stationary phase				
Si 60	L01			L01
C18 Symmetry 300 TM	L02	L02		
C30 YMC				L02
Supelcosil Suplex pkb-100	L03	L03	L03	L03
C18		L04		
Lichrosorb Si 60	L05	L05		
Lichrosorb	L06	L06	L06	L06
C18 RP amide	L07	L07	L07	L07
Elution programme?				
Isocratic	L01, L02, L05, L06	L02, L04, L05, L06	L06	L01, L06
Gradient	L03, L07	L03, L07	L03, L07	L02, L03, L07
Mobile phase components				
heptane acetone	L01			L01
acetonitrile, methanol, water, acid	L02	L02		
methanol, MTBE				L02
ACN/ TBME/Water+ BHT	L03	L03	L03	L03
methanol		L04		
acetone/ heptane 15/85	L05	L05		
heptane/ acetone 88/12	L06	L06	L06	L06

	Laboratory number			
	Astaxanthin	Canthaxanthin	Adonirubin	Other
Flow rate (ml min)				
≥0.2 - <0.5				
≥0.5 - <0.7	L03, L07	L03, L07	L03, L07	L03, L07
≥0.7 - <1				
≥1	L01, L02, L05, L06	L02, L04, L05, L06	L06	L01, L02, L06
HPLC injection volume (µl)				
≥5 - <10	L03	L03	L03	L03
≥10 - <25	L01, L02	L02		L01, L02
≥25 - <50	L05	L04, L05		
≥50 - <100	L07	L07	L07	L07
≥100	L06	L06	L06	L06
HPLC column temperature (°C)				
ambient*	L03, L05	L03, L04, L05	L03	L03
>ambient* - <30	L06, L07	L06, L07	L06, L07	L06, L07
≥30	L0, L02	L02		L01, L02,
Autosampler temperature (°C)				
<10				
≥10 - <15		L04		
≥15 - <ambient*	L01, L03, L06	L03, L06	L03, L06	L01, L03, L06
≥ambient* - <20	L02, L05, L07	L02, L05, L07	L07	L02, L07
≥20				
Off				
Peaks resolved				
Yes	L01, L02, L03, L05, L06, L07	L02, L03, L04, L05, L06, L07	L03, L06, L07	L01, L02, L07
No				L06
Instrument type?				
UV/Vis	L01, L02, L03, L05	L02, L03, L04, L05	L03, L06	L01, L02, L03, L06
DAD	L06, L07	L06, L07	L07	L07

	Laboratory number			
	Astaxanthin	Canthaxanthin	Adonirubin	Other
Selected wavelength				
470	L01, L05, L06	L05	L06	L01, L06
477	L02	L02		
450				L02
410	L03, L07	L03, L04, L07	L03, L07	L03, L07
466		L06		
Is the selected wavelength corresponding to the lambda max				
Yes	L06, L07*	L06, L07*	L07*	L07*
No	L01, L02, L03, L05	L02, L03, L04, L05	L03, L06	L01, L02, L03, L06
Calibration type?				
External calibration	L01, L02, L03, L05, L06, L07	L02, L03, L04, L05, L06, L07	L03	L01, L02, L03
Matrix matched calibration				
Standard addition calibration				
Internal standard used?				
Yes				
No	L01, L02, L03, L05, L06, L07	L02, L03, L04, L05, L06, L07	L03, L06, L07	L01, L02, L03, L06, L07
Calculations based on				
Area	L01, L02, L03, L05, L06, L07	L02, L03, L04, L05, L06, L07	L03, L06	L01, L02, L03, L06
Height				

* as reported but chromatograms seem to be at another lambda

Annex 11: Satisfaction survey performed at the workshop

Survey form template

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements (Geel)

To improve our services your input is appreciated!

4th EURL FA Workshop Control 17-18/11/2015

1. How would you rate the following information provided to you before the event?

	Excellent	Good	Fair	Poor	N/A*
Logistical information about the event (date, place, activities, programme)	☐	☐	☐	☐	☐
Information about the objectives and theme of the event	☐	☐	☐	☐	☐
Information about the contents of sessions / presentations	☐	☐	☐	☐	☐

* Not applicable

If poor indicate why: .....

.....

2. How would you rate the ...?

	Excellent	Good	Fair	Poor	N/A*
venue / facilities	☐	☐	☐	☐	☐
catering / meals	☐	☐	☐	☐	☐
hotel	☐	☐	☐	☐	☐
transport arrangements	☐	☐	☐	☐	☐
registration procedure for the event	☐	☐	☐	☐	☐
information provided during the event	☐	☐	☐	☐	☐
assistance provided by JRC staff	☐	☐	☐	☐	☐
social activities organised (dinner)	☐	☐	☐	☐	☐

* Not applicable

If poor indicate why: .....

.....

3. How would you rate the …?

	Excellent	Good	Fair	Poor	N/A*
length of the event	☐	☐	☐	☐	☐
division of time between presentations and discussions	☐	☐	☐	☐	☐

* Not applicable

If poor indicate why: .....

.....

4. Do you have any comments concerning the <u>organisation</u> of the event, or suggestions for improvement?

.....

.....

.....

5. Do you have any comments concerning the <u>content</u> of the event, or suggestions to improve events in the future?

.....

.....

.....

6. To what extent do you agree with the following statements?

	Strongly agree	Agree	Neutral	Disagree	Strongly disagree
The event has provided me with new information on the JRC.	☐	☐	☐	☐	☐
The event has provided me with networking opportunities.	☐	☐	☐	☐	☐
The event has improved my knowledge and expertise in my field of science and research.	☐	☐	☐	☐	☐

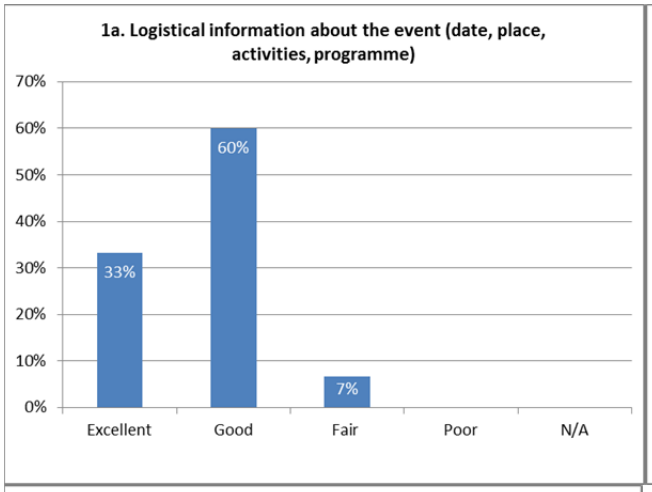
If you disagree/strongly disagree indicate why: .....

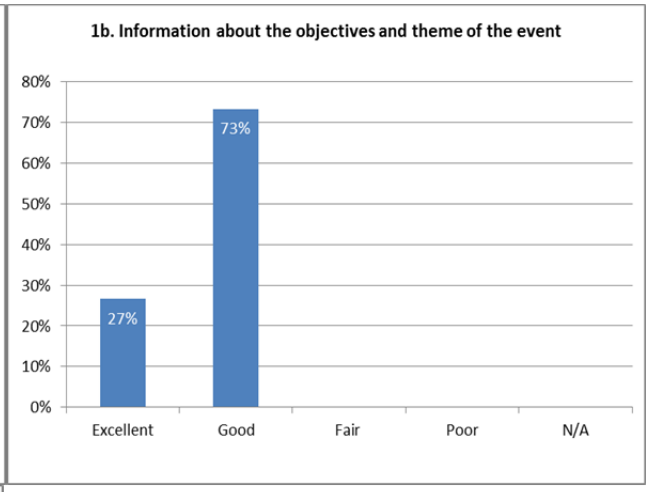
.....

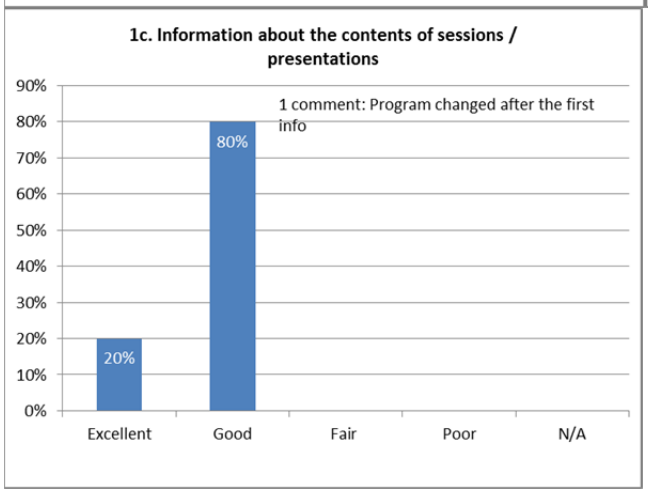
Please leave this evaluation at the secretariat desk - Thank you

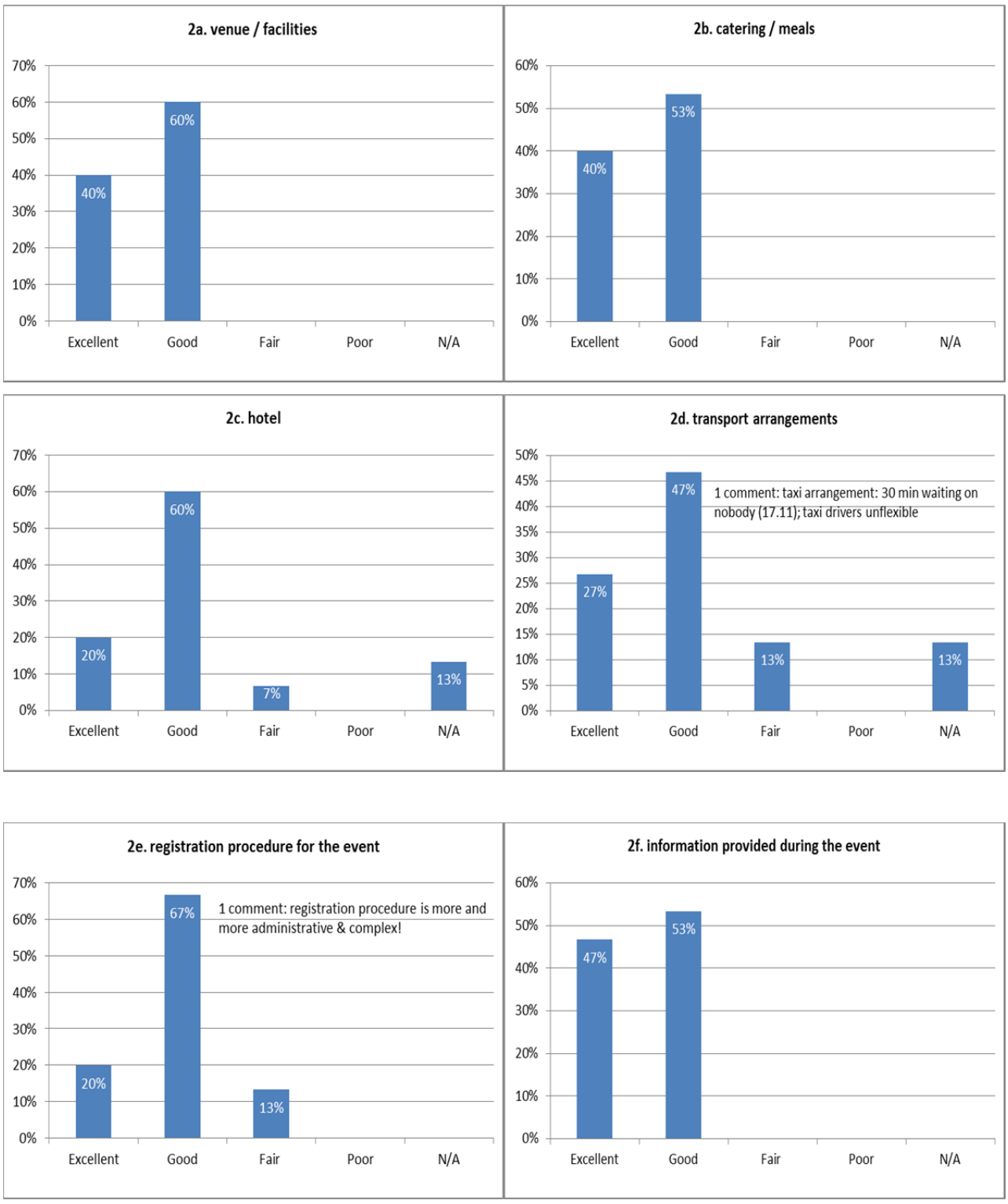
Evaluation

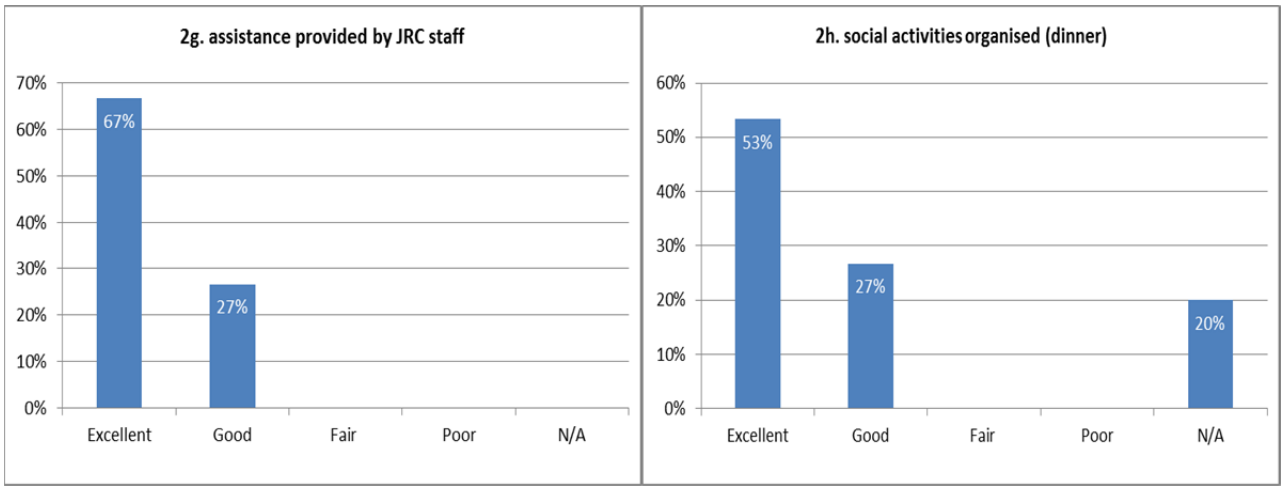
Number of eligible participants	20
Number of response forms received	15
Response rate	75%

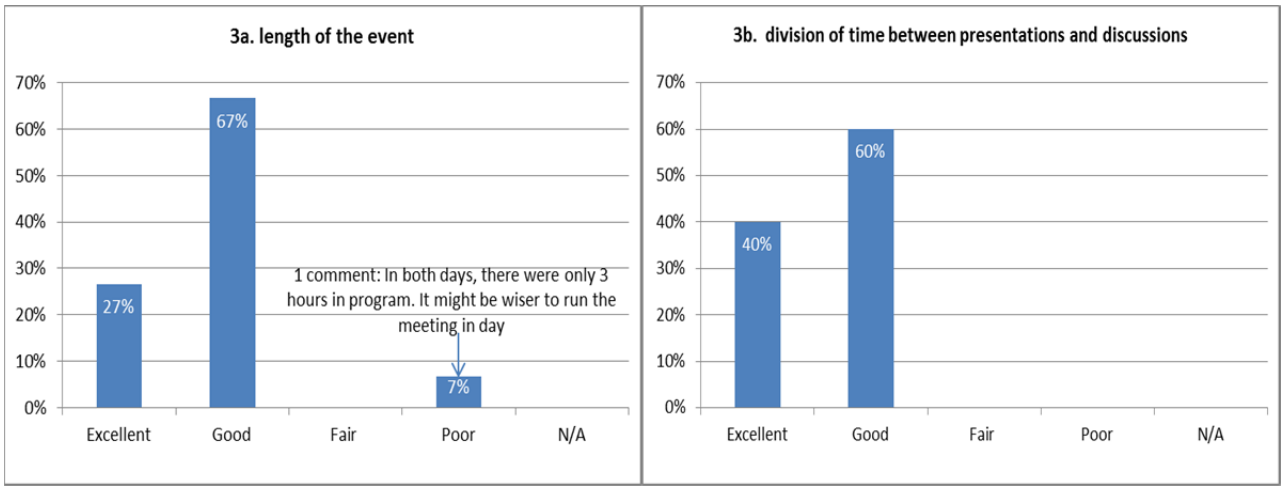







2a. venue / facilities
70%
60%
50%
40%
30%
20%
10%
0%
40%
60%
Excellent
Good
Fair
Poor
N/A
2b. catering / meals
60%
50%
40%
30%
20%
10%
0%
40%
53%
Excellent
Good
Fair
Poor
N/A
2c. hotel
70%
60%
50%
40%
30%
20%
10%
0%
20%
60%
7%
13%
Excellent
Good
Fair
Poor
N/A
2d. transport arrangements
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
27%
47%
13%
13%
1 comment: taxi arrangement: 30 min waiting on nobody (17.11); taxi drivers unflexible
Excellent
Good
Fair
Poor
N/A
2e. registration procedure for the event
70%
60%
50%
40%
30%
20%
10%
0%
20%
67%
13%
1 comment: registration procedure is more and more administrative & complex!
Excellent
Good
Fair
Poor
N/A
2f. information provided during the event
60%
50%
40%
30%
20%
10%
0%
47%
53%
Excellent
Good
Fair
Poor
N/A





4. Do you have any comments concerning the organisation of the event, or suggestions for improvement?

Comment 1: Please provide the presentation electronically (stick or platform)

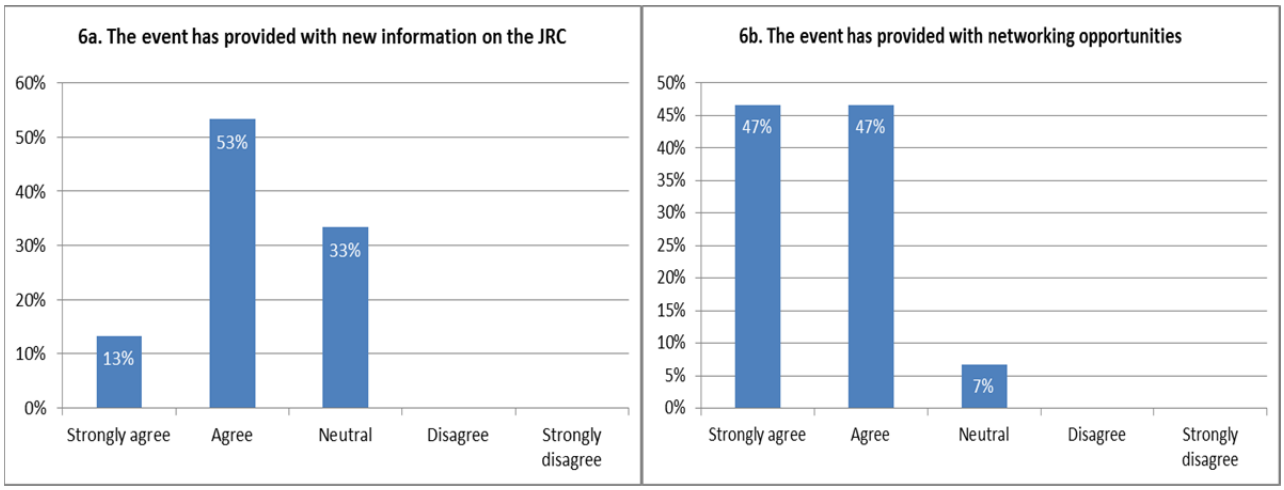
Comment 2: You have a good installation of sound. Micros, speakers, PLEASE USE THEM. Pocket micros for the speaker. Remark also valid for Authorisation workshop

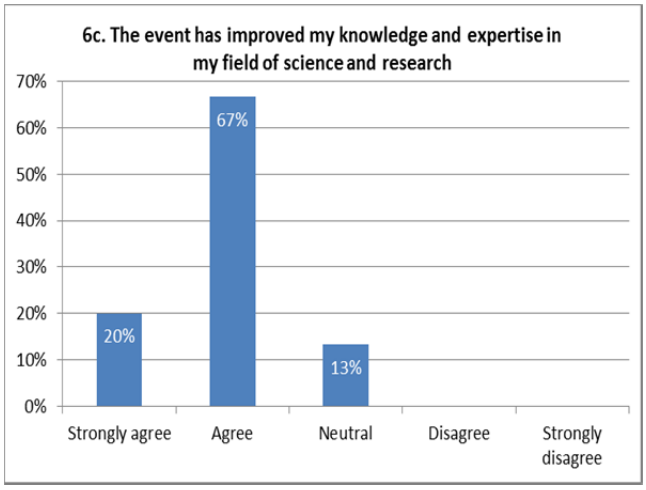
Comment 3: In both days, there were only 3 hours in program. It might be wiser to run the meeting in 1 day

5. Do you have any comments concerning the content of the event, or suggestions to improve events in the future?

Comment 1: points of discussion should be highlighted before the meeting in order to give the participants chance to collect information

Comment 2: Control part was a little disappointment because there was a lot of discussion about the carotenoids. And I'm working on the residue area.





The survey will be followed up by the EURL-FA Control and the comments assessed. However, the EURL-FA Control would appreciate clarification to be given, e.g. through the CIRCA-BC platform, regarding the comments to question 5. Indeed, the first draft programme of the workshop has been distributed in July 2015 and was regularly updated until 3 weeks before the workshop. The first draft already highlighted the schedule of the sessions as well as, the topics of discussion.

As regards comment 3 in question 4, the last year's survey underlined the preference of the majority of the participants for a two-half days' programme for the workshop.

How to obtain EU publications

Our publications are available from EU Bookshop (http://bookshop.europa.eu),
where you can place an order with the sales agent of your choice.

The Publications Office has a worldwide network of sales agents.
You can obtain their contact details by sending a fax to (352) 29 29-42758.

LA-NA-27810-EN-N

JRC Mission

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new methods, tools and standards, and sharing its know-how with the Member States, the scientific community and international partners.

Serving society
Stimulating innovation
Supporting legislation

Publications Office

doi:10.2787/403547

ISBN 978-92-79-57548-8