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Biogenic amines in krill meal: Liquid chromatographic determination with post-column derivatization and fluorescence detection

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Summary/recommendation:

Biogenic amines are formed by microbial decarboxylation of amino acids and represent a considerable toxicological risk in some food products.

This method is intended for quantification of histamine, cadaverine and putrescine in krill meal down to 10 mg/kg, which was determined to be a reasonable quantification limit. The method uses liquid chromatography with OPA (o-Phthaldialdehyde) as derivatization reagent followed by fluorescence detection. Recovery experiments showed that the recovery of the amines is good, between 88 and 108 %, for all concentration levels (approximately 2.40-120 mg/kg). The method is fit for purpose.

This report is a continuation of Nofima report 15/2015 “Histamine in fish: Liquid chromatographic determination with post-column derivatization and fluorescence detection”, and some chapters are equivalent to report 15/2015.

Summary/recommendation in Norwegian:

Biogene aminer dannes ved mikrobiell dekarboksylering av aminosyrer og representerer en betydelig toksikologisk fare i enkelte matvarer.

Denne metoden er ment for kvantifisering av histamin, cadaverin og putrescin i krillmel ned til 10 mg/kg, som ble bestemt til en fornuftig kvantifiseringsgrense. Metoden benytter væskekromatografi med OPA (o-ftaldialdehyd) som derivatiseringsreagens etterfulgt av fluorescensdeteksjon. Gjenvinningsforsøk viste at gjenvinningen av aminene er god, mellom 88 og 108 %, for alle konsentrasjonsnivåer (rundt 2,40-120 mg/kg). Metoden passer til formålet.

Denne rapporten er en fortsettelse av Nofima-rapport 15/2015 «Histamin i fisk: Væskekromatografisk bestemmelse med post-kolonne derivatisering og fluorescensdeteksjon», og noen kapitler er tilsvarende som i rapport 15/2015.

Table of Contents

1 Introduction

The aim of this project is to validate a method for determination of histamine, cadaverine and putrescine in krill meal. Tyramine was not validated because of lack of ring test participation. The method has previously been validated for histamine in fish, and this report is partly equivalent to the previous report (Nofima report 15/2015). The method uses liquid chromatography with OPA (O-Phthaldialdehyde) as a derivatization reagent followed by fluorescence detection.

Validating a method means investigating and establishing the method's quality parameters. The tested method parameters will include selectivity, linearity, precision, accuracy, measuring range, ruggedness, and uncertainty. Validation performed by one laboratory is called internal validation (NMKL 2009). Validation determines the suitability of an analysis for providing the desired information (Douglas A. Skoog 2004).

2 Theory

This chapter describes the method, the degree of validation and the validation points. The method description is attached in appendix 6.

2.1 Background and method principle

Biogenic amines are formed by microbial decarboxylation of amino acids. Histamine from histidine, cadaverine from lysine and putrescine from ornithine. Biogenic amines represent a considerable toxicological risk in some food products (Etienne 2006).

The biogenic amines are extracted from krill meal by homogenization with 0.6 M perchloric acid. The extract is measured by use of HPLC (high-performance liquid chromatography), and OPA as derivatization reagent. Fluorescence detection of OPA-derivates increases the sensitivity compared to UV-detection, and it is assumed to be less interferences. The derivatization is done post column, which decreases potential instability problems with OPA-derivates. This method also use internal standard for calculation, which decreases the contributions to the measurement uncertainty. Especially since the internal standard is added early, before the extraction.

The following eluents are used for the gradient in the chromatographic determination:

1. Sodium acetate buffer
2. Methanol
3. Acetonitrile/sodium acetate buffer

The flow rate is set to 1 ml/min and each injection takes 45 minutes. The column temperature is set to 35 °C and the chromatographic separation is performed on a Hypersil ODS (C18) column (15 cm × 4.6 mm). The excitation and emission wavelengths are set to 365 and 418 nm, respectively.

2.2 Degree of validation

The method has been internally developed and demands a full internal validation (NMKL 2009). As described, the method was validated for histamine in fish and the report was published in March 2015 (Nofima report 15/2015). The validation parameters from the previous validation work is included in this report where relevant. This includes selectivity, ruggedness and theoretical uncertainty calculations. The tested and evaluated method parameters for histamine, cadaverine and putrescine in krill meal will include linearity, precision, accuracy, measuring range, and experimental uncertainty.

2.3 Validation points

The following chapter is copied from the report of histamine in fish. The validation points that are evaluated are summarized in this chapter. The laboratory work and the results/discussion in connection to the validation points are described in chapter 3 and 4, respectively.

2.3.1 Selectivity

Selectivity is the recommended term for expressing whether a method can determine the requested analyte under certain conditions in the presence of other components with similar properties. In chromatographic methods, selectivity is based on the separation process, also called separation selectivity. The selectivity indicates how strongly the result is influenced by other compounds in the sample (Vessmann 2001).

2.3.2 Linearity

The linearity is investigated by regression analysis and the least squares method. By using the least squares method one will find the regression curve that best fits the data set, by looking at the square of the deviations between the observed point and the estimated curve. The generated curve is the one with the smallest possible area of the squares. The regression curve has the equation $y=mx+b$, where m is the slope and b is the y-intercept. The least squares method also returns the standard deviations of m and b (s_m and s_b), and the standard error of the estimate (s_y), which is a rough estimate on a typical standard deviation from the regression curve. It is assumed that any deviations from linearity are caused by deviations in the measurements, and that the concentrations are accurate. To determine how well the curve fits the dataset, the *F*-value from the *F*-distribution is calculated. The *F*-value is the relationship between the regression sum of squares and the residuals sum of squares. In an *F*-distribution it is assumed that the points in the data set are randomly scattered (non-linear). When the *F*-value is higher than the table values (*F*-critical) it means that with 95 % probability the points are not a random spread, but a linear regression is justified (Løvås 2005, Corporation 2013, College no date).

2.3.3 Precision

Precision describes the compliance between independent results achieved in exactly the same way under specific conditions. Precision must not be confused with accuracy, which describes how close the measurement is to the true or accepted value. Precision is usually expressed as the standard deviation of the results. The precision of the method can be determined as:

a) Repeatability: This means the analytical method should be used on identical samples in the same laboratory using the same equipment within a short period.

b) Reproducibility: This means the analytical method should be used on identical samples on different laboratories using different equipment (Douglas A. Skoog 2004, NMKL 2009).

Repeatability is often expressed as the repeatability limit (r), which is an expression for the absolute difference with 95 % confidence interval between two independent test results achieved under the requirements mention in paragraph a) in the section above (ISO 1994). r is calculated as shown in equation 2.1.

$$r = t \times \sqrt{2} \times S_r \tag{2.1}$$

t is the two-tailed Student t-value at 95 % confidence interval and S_r is the standard deviation of the repeatability. S_r is calculated by using equation 2.2.

$$S_r = \sqrt{\frac{\sum_{i=1}^{n}(x_i - y_i)^2}{2n}} \quad (2.2)$$

where x_i and y_i is the two measurements and n is the number of double test results (NMKL 2009).

Usually r is calculated by assuming that the degrees of freedom approach infinity and that t=1.96. By these conditions r is calculated as shown in equation 2.3.

$$r = 2.8 \times S_r \quad (2.3)$$

2.3.4 Accuracy

Interlaboratory study (ring test)

Accuracy describes the relationship between the true level of analyte in a sample and the result achieved by analysis. To evaluate the accuracy of a method one can use data from an interlaboratory study (ring test).

Nofima BioLab has participated in a few ring tests hosted by Lvu (Labor Vergleichs Untersuchung) and CHEK (Chemical Quality Assurance) where this method has been used by Biolab. Note that the other participants have used different methods.

To evaluate the results from the ring test one can calculate different sums/values that indicate how close the laboratory's result is in relationship to others. The ring test organizers often uses "z-score" (z) which is a normalized value that gives every result a score seen in context to the other values in the data set. z-score is calculated as shown in equation 2.4.

$$z = \frac{(X - X_{SLP})}{u_{SLP}} \quad (2.4)$$

X is the participant's result, X_{SLP} is the organizer's best estimate on the value of the sample and u_{SLP} is an estimate on the spread between the results expressed as the standard deviation for all the participant's results (ISO 2005, Thomson 2006).

By including the laboratory's own measurement uncertainty in the calculation, zeta-score (ζ) can be used instead, as shown in equation 2.5.

$$\zeta = \frac{(X - X_{SLP})}{\sqrt{u_X^2 + u_{SLP}^2}} \quad (2.5)$$

u_X is the laboratory's standard deviation. By using zeta-score it is important to be aware that a certain value can be caused by either a big deviation from the assigned value and great uncertainty, but also a small deviation from the assigned value and a proportionally small uncertainty. Based on this, IUPAC (International Union of Pure and Applied Chemistry) does not recommend the use of zeta-score unless it is reported together with z-score. The laboratory also need to know its own uncertainty (ISO 2005, Thomson 2006).

Another international accepted method for evaluating ring test results is E_n-value (error normalized-value) as shown in equation 2.6.

$$E_n\text{-value} = \frac{X - X_{SLP}}{\sqrt{(U_X)^2 + (U_{SLP})^2}} \quad (2.6)$$

U_X and U_{SLP} are the expanded measurement uncertainties for X and X_{SLP}. As for zeta-score the measurement uncertainty is included in the calculation, but opposed to z- and zeta-score, expanded uncertainty is used with a coverage factor of 2. Table 1 shows acceptable, suspicious and unacceptable values of the three scores/values (ISO 2005).

Table 1 Acceptable, suspicious and unacceptable values of z-score (z), zeta-score (ζ) and E_n-value (E_n).

Result	*z*	ζ	E_n
Acceptable	|0-2|	|0-2|	|0-1|
Suspicious	|2-3|	|2-3|	|1-2|
Unacceptable	≥ |3|	≥ |3|	≥ |2|

The narrower limits of acceptable values for E_n are due to the expanded measurement uncertainty. Some values in the suspicious area are normal. Statistically, 1 out of 20 scores are in this area (Thomson 2006).

Nofima BioLab uses E_n-value to evaluate ring tests. The standard deviation reported by the organizer is divided by the square root of the number of participants (n) to achieve a standard uncertainty for the X_{SLP}. The reason behind this calculation is to avoid that the spread of the entire population will make it too easy to achieve acceptable comparisons with the X_{SLP}-value. The calculation is shown in equation 2.7.

$$E_n\text{-value} = \frac{X - X_{SLP}}{\sqrt{(U_X)^2 + \left(\frac{U_{SLP}}{\sqrt{n}}\right)^2}} \quad (2.7)$$

Recovery/spiking

The data material from the ring tests is limited, and therefore accuracy has also been investigated by using recovery tests. Recovery (or recovery factor) is defined by IUPAC as, "Yield of a preconcentration or extraction stage of an analytical process for an analyte divided by amount of analyte in the original sample" (Burns 2002). In an extraction step, the analyte is transferred from a complex matrix to a simpler matrix in which the instrumental detection is done. Loss of analyte can be anticipated during the extraction, and recovery gives the method's efficiency. Recovery should, if possible, be compensated for. When using methods with addition of internal standard and a calibration curve instead of a standard curve, the appropriate term is "apparent recovery" (NMKL 2012).

Usually the recovery is determined during a method validation by spiking, which is adding a known quantity of the analyte to the sample, extract, measure and divide by the spiked value (NMKL 2012).

The recovery (R %) in a spiked blank sample can be calculated by using equation 2.8 (NMKL 2012).

$$R\ \% = \frac{Q_{A(extr)}}{Q_{A(add)}} \times 100 \quad (2.8)$$

$Q_{A(extr)}$ is the level of extracted (recovered) analyte, and $Q_{A(add)}$ is the added (spiked) analyte before the extraction.

If a blank sample is not available, and the spiked sample is a real sample, the recovery can be calculated by using equation 2.9. The original level of analyte must be determined (NMKL 2012).

$$R\,\% = \frac{Q_{Aextr(orig+add)} - Q_{A(orig)}}{Q_{A(add)}} \times 100 \quad (2.9)$$

$Q_{Aextr(org+add)}$ is the level of measured analyte in the spiked sample, and $Q_{A(orig)}$ is the level of measured analyte in the real sample before spiking.

The standard error of the recovery is calculated in absolute terms as the standard error of the mean (*SEM*) as shown in equation 2.10, and in relative terms as the standard uncertainty for the recovery (u_{rec}) as shown in equation 2.11 (NMKL 2012).

$$SEM = \frac{SD}{\sqrt{n}} \quad (2.10)$$

$$u_{rec} = \frac{\%\ RSD}{\sqrt{n}} \quad (2.11)$$

where *SD* and % *RSD* are the standard deviation and the relative standard deviation of the recovery, and *n* is the number of replicates (NMKL 2012).

It is important to not confuse recovery with bias (*b*). Incomplete recovery will lead to bias, (Linsinger 2008) but bias is a systematic analytical error that may or may not be significant. It is an estimate of a systematic measurement error. Bias should be identified and, if possible, eliminated, but bias should usually not be corrected for (NMKL 2012). A certified reference material (CRM) is usually required for the determination of bias, but if no CRMs are available the recovery can be used to calculate the bias (NMKL 2012). In both cases, bias can be calculated by equation 2.12 and relative bias (*b* %) by equation 2.13 (Linsinger 2008, NMKL 2012).

$$b = \frac{x_{meas}}{x_{ref}} \quad (2.12)$$

$$b\% = \left(\frac{x_{meas} - x_{ref}}{x_{ref}}\right) \times 100 \quad (2.13)$$

x_{meas} is the measured result while x_{ref} is the reference value, which can be a CRM, an accurately prepared sample (e.g., by spiking), well-designed intercomparisons or measurements with another method of demonstrated accuracy (Linsinger 2008).

To see if the recovery and the bias are statistically significant, a *t*-test is performed according to equation 2.14 (NMKL 2012).

$$t = \frac{|X - T|}{u} \times \sqrt{n} \quad (2.14)$$

X represents the extracted analyte, *T* represents the calculated level of analyte in the spiked sample, and *u* is the uncertainty of the method (a summary of different uncertainty sources, see chapter 2.3.7). If the bias is statistically significant, *t* is higher than t_{crit}. The value for t_{crit} (two-tailed, 95 % confidence, degrees of freedom = *n*–1) is found in a table of critical *t*-values (NMKL 2012).

The big advantage of using recovery experiments is that the matrix is representative for real samples. The biggest limitation is that the analyte in the real sample can be strongly bound physically or chemically to the matrix, which normally will not be the case for the added analyte. This could mean that one can achieve a high recovery factor for the added analyte, without reaching a complete determination of the naturally occurring analyte (NMKL 2012). Also, the form of the spike may present a problem as different compounds and grain sizes representing the analyte may behave differently in an analysis (Van Reeuwijk 1998). One may experience four different scenarios (NMKL 2012):

1. The native (original) analyte remains (i.e., is recovered) and the spike is partially lost, and one will achieve false bad recovery.
2. The native analyte is partially lost and the spike remains, and one will achieve false good recovery.
3. The native analyte and the spike remain, and one will achieve a true good recovery.
4. The native analyte is partially lost and the spike is proportionally lost, and one will achieve a true good recovery.

2.3.5 Measuring range

The measurement range for a method is defined as the range where the method is validated, and is the range where the method gives acceptable accuracy and precision. The measurement range is determined by the limit of detection (*LOD*) and the limit of quantification (*LOQ*) (NMKL 2009). The limit of detection is the lowest analyte concentration that can be detected with a certain degree of confidence and is commonly calculated by equation 2.15 (Armbruster, Tillman et al. 1994, NMKL 2009).

$$LOD = c \times SD_{blind} \tag{2.15}$$

SD_{blind} is the standard deviation for the blind samples' mean value, and c is a constant which is found in a table of critical t-values (degrees of freedom = n–1 and usually $\alpha = 0.01$). For $\alpha = 0.01$ and $n = 20$, $c = 3$ is often used (NMKL 2009).

The limit of quantification is the lowest analyte concentration that can be quantified with a given measurement uncertainty within a certain degree of confidence and is commonly calculated by equation 2.16 (Armbruster, Tillman et al. 1994, NMKL 2009).

$$LOQ = c \times SD_{blind} \tag{2.16}$$

Rigid rules for the limit of quantification cannot be given but should be evaluated in each case. $c = 6$ or 10 is often used (NMKL 2009).

In chromatographic methods, the standard deviation of the blind sample is often found by measuring the noise signal of a blank injection several times, and then calculating the standard deviation of the noise signal. The calculation of the *LOQ* is carried out according to equation 2.16.

2.3.6 Ruggedness

Ruggedness describes the analytical method's sensitivity to small differences in the experimental conditions (NMKL 2009). The method operates with specific amounts and volumes of sample and reagents, so that in the connection to this method it would be interesting to look at ruggedness as differences between laboratories using different equipment, also described as reproducibility (chapter 2.3.3). Due to lack of collaborative laboratories this was not investigated. Ruggedness associated with different chemicals, sample types and different day-to-day variations was covered by the recovery experiments, and will not be discussed any further.

2.3.7 Uncertainty

The method's uncertainty contributors are summed up in an Ishikawa (fishbone) diagram, and a theoretical calculation of the measurement uncertainty is carried out as described in Eurachem (1995) (Eurachem 1995).

The method's experimental measurement uncertainty (u_{SLP}) includes internal and external uncertainty elements and is calculated by equation 2.17.

$$u_{SLP} = \sqrt{u_{LAB}^2 + u_{LAB-\bar{X}}^2} \tag{2.17}$$

u_{LAB} is Nofima Biolab's internal standard deviation for the repeatability. This value is determined from differences between double measurements in common sample matrixes with results in the normal area.

$u_{LAB-\bar{X}}$ is Nofima BioLab's uncertainty for the deviations from the average results in the ring tests which is described in chapter 2.3.4. The uncertainty is calculated by equation 2.18.

$$u_{LAB-\bar{X}} = \sqrt{\frac{\sum(LAB-\bar{X})^2}{2d}} \tag{2.18}$$

d is the number of double measurements.

The method's total measurement uncertainty (*u*) is calculated by summarizing all measurable contributors to uncertainty: Ring tests, recovery and precision. The uncertainty is reported as expanded uncertainty (*U*) with a coverage factor (*k*) of 2 which correspond to 95 % confidence interval.

3 Experimental

The following chapter describes the laboratory work done in connection to the validation work.

3.1 Linearity

The linearity was checked by injection of histamine, cadaverine and putrescine standards of low concentration. The amount injected was plotted against the area of the amine peak and the internal standard peak, and a regression test was done.

The standards were prepared as shown in Table 2.

Table 2 The preparation of standard solutions containing histamine, cadaverine, putrescine and internal standard.

Conc., amine (free base) (mg/ml)	Conc., internal standard (mg/ml)	Amount injected (20 µl) of each compound (mg)	Amount injected (20 µl) of each compound (ng)
0,0001	0,0001	0,000002	2
0,0002	0,0002	0,000004	4
0,0003	0,0003	0,000006	6
0,0005	0,0005	0,00001	10
0,001	0,001	0,00002	20
0,003	0,003	0,00006	60
0,005	0,005	0,0001	100
0,01	0,01	0,0002	200

3.2 Precision

The precision of the method was calculated as the repeatability. The calculation was based on double measurements done in connection to the spiking, as described in chapter 3.3.

3.3 Accuracy

The recovery test was performed by spiking of histamine, cadaverine and putrescine in a krill meal sample (journal number 2015-2166-1). The amines were weighed as amine×2HCl and diluted to known concentration with 0.6 M perchloric acid (PCA). The sample matrix was also analyzed without addition to check what the original level of analyte was before spiking.

The preparation of the samples is shown in Table 3.

Table 3 The preparation of spiked samples of krill meal. The amines were weighed as amine×2HCl and corrected for molar weight and purity.

Spiked sample no.	Conc. of each amine in standard solution (mg/l)	Sample amount (krill meal) (g)	Added volume of standard solution (mL)	Conc. of each amine in spiked sample (mg/kg)
1	-	10	0	0
2	12.0	10	2	2.40
3	50.0	10	2	10.0
4	300	10	2	60.0
5	600	10	2	120

The analysis of the spiked and unspiked samples was performed as normal by following the method description. The number of replicates per spiking level was six.

3.4 Measuring range

Evaluations of the signal/noise ratio for real samples were performed and the linearity and spread in the lower level was evaluated. Blank samples were analyzed and *LOD* and *LOQ* were determined.

4 Results and discussion

4.1 Selectivity

The separation selectivity is good, the biogenic amines are well separated on the column. A standard solution with tyramine, putrescine, cadaverine, histamine and internal standard (1,6-Diaminohexane dihydrochloride) is shown in Figure 1.

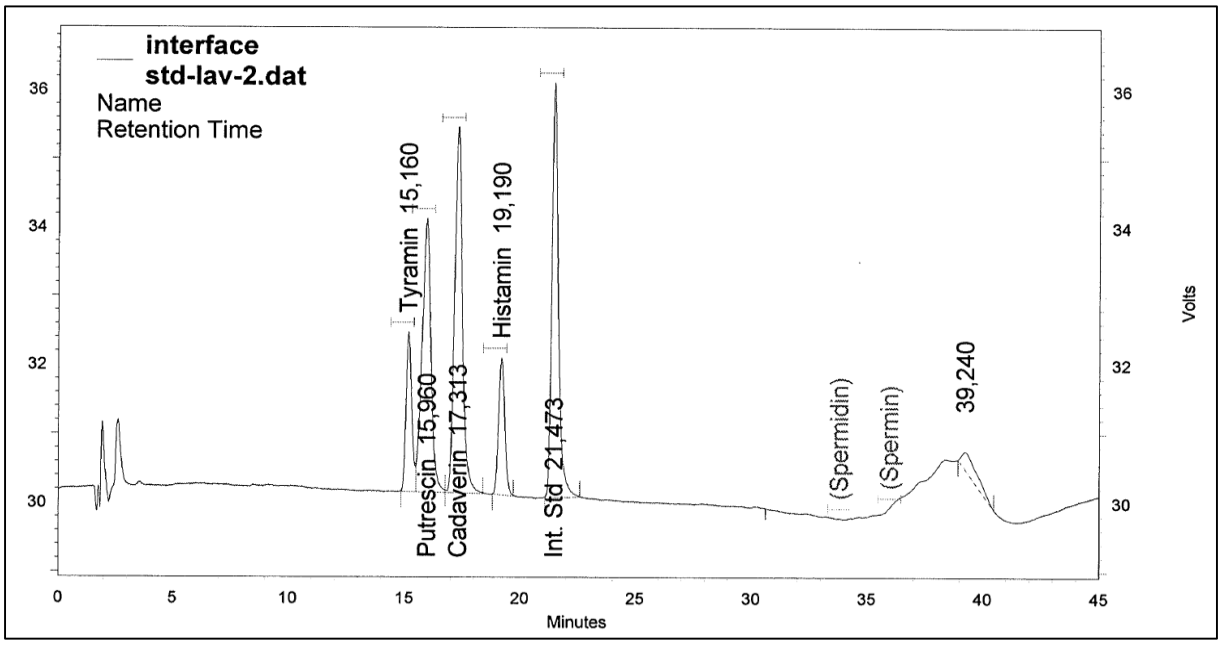

Figure 1 Chromatogram of the standard solution containing tyramine, putrescine, cadaverine, histamine and 1,6-Diaminohexane dihydrochloride (internal standard).

Histamine and cadaverine is baseline separated from other peaks in the chromatogram. Putrescine is unresolved from tyramine, which is usually present in the standard solution. Under normal conditions the peak resolution (R_S) should be ≥ 1. To keep the separation of the biogenic amines at an acceptable level, conditions described in the method description must be applied.

4.2 Linearity

The linearity of the injected standards versus the area of the amine peaks and the internal standard peak is shown in Figure 2-7. The data material is shown in appendix 1.

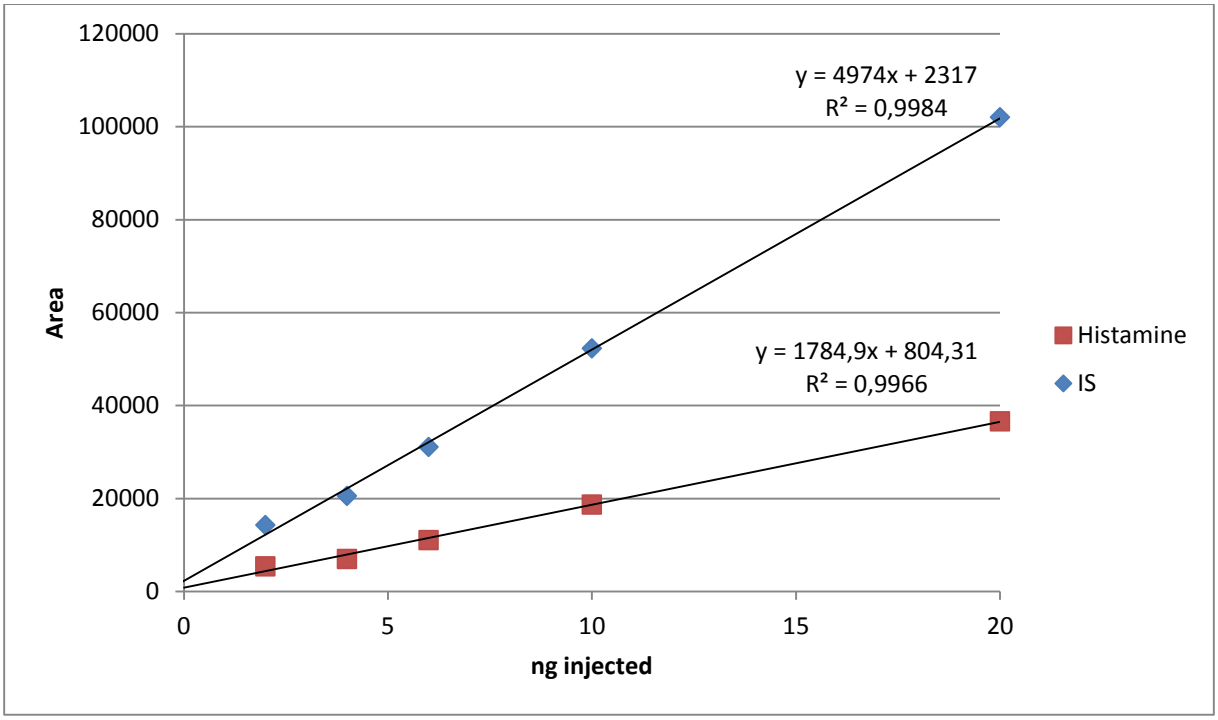

Figure 2 *The injected standard (2-20 ng) plotted against the area of the histamine peak and the area of the internal standard (IS) peak.*

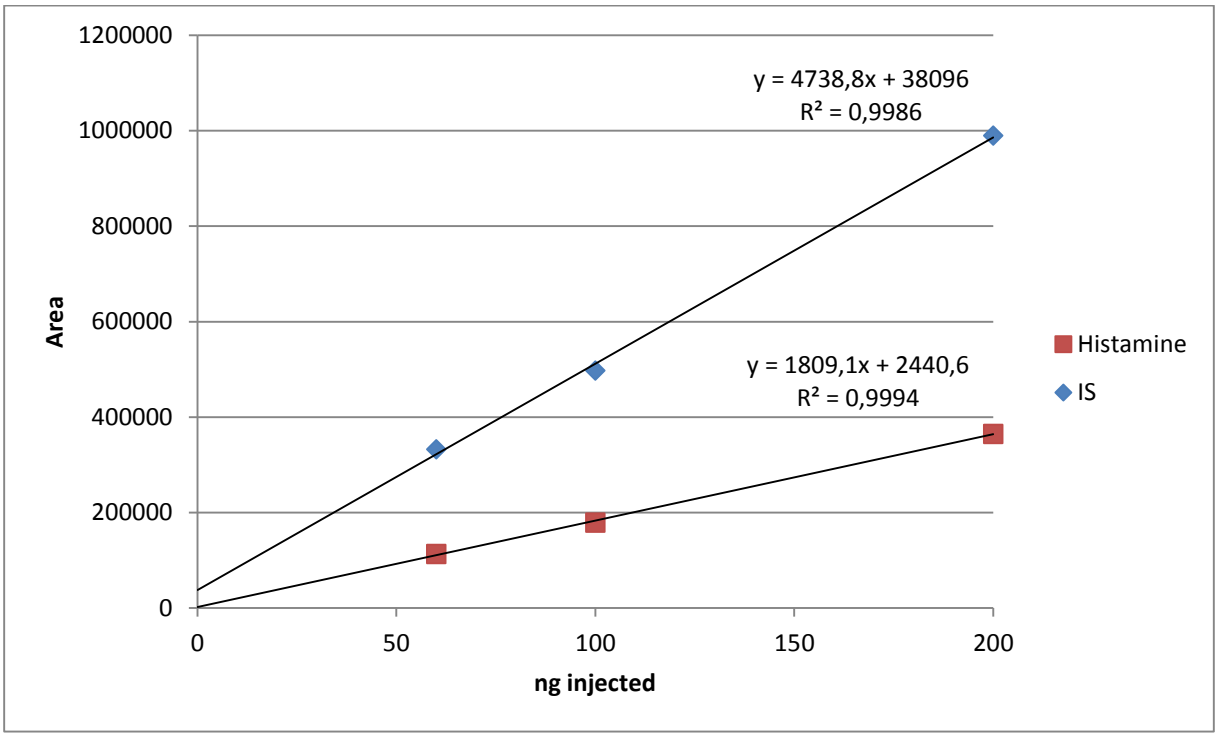

Figure 3 *The injected standard (60-200 ng) plotted against the area of the histamine peak and the area of the internal standard (IS) peak.*

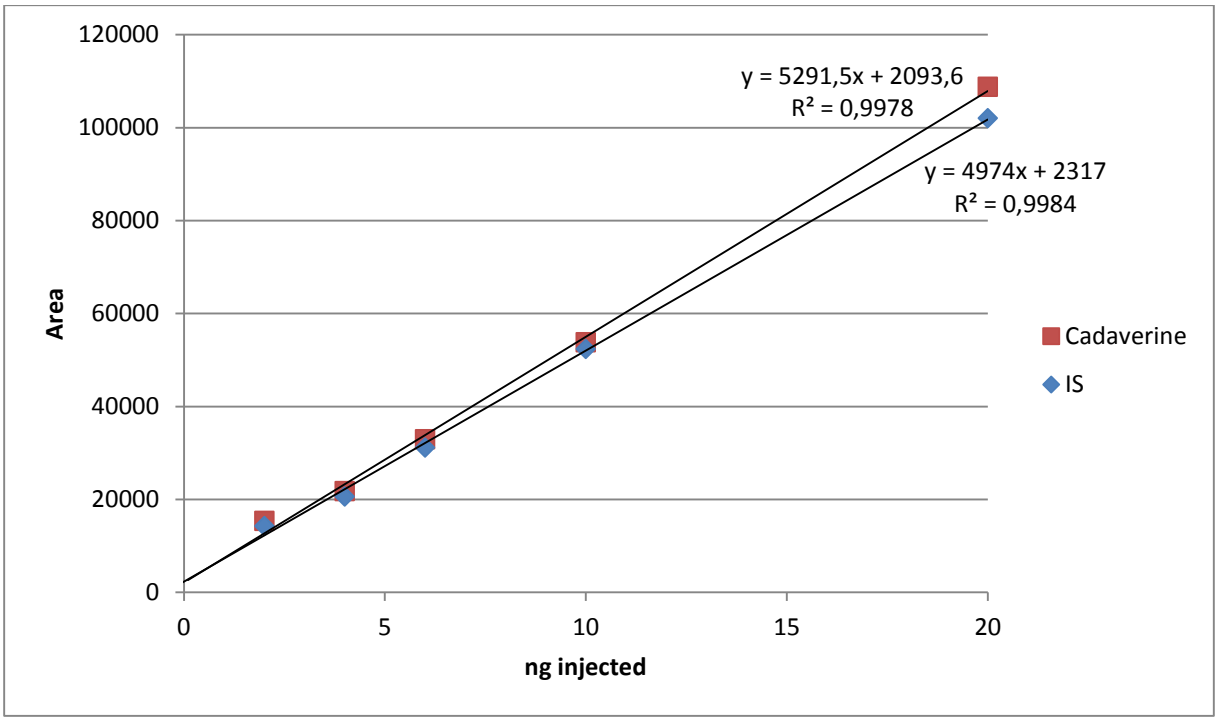

Figure 4 *The injected standard (2-20 ng) plotted against the area of the cadaverine peak and the area of the internal standard (IS) peak.*

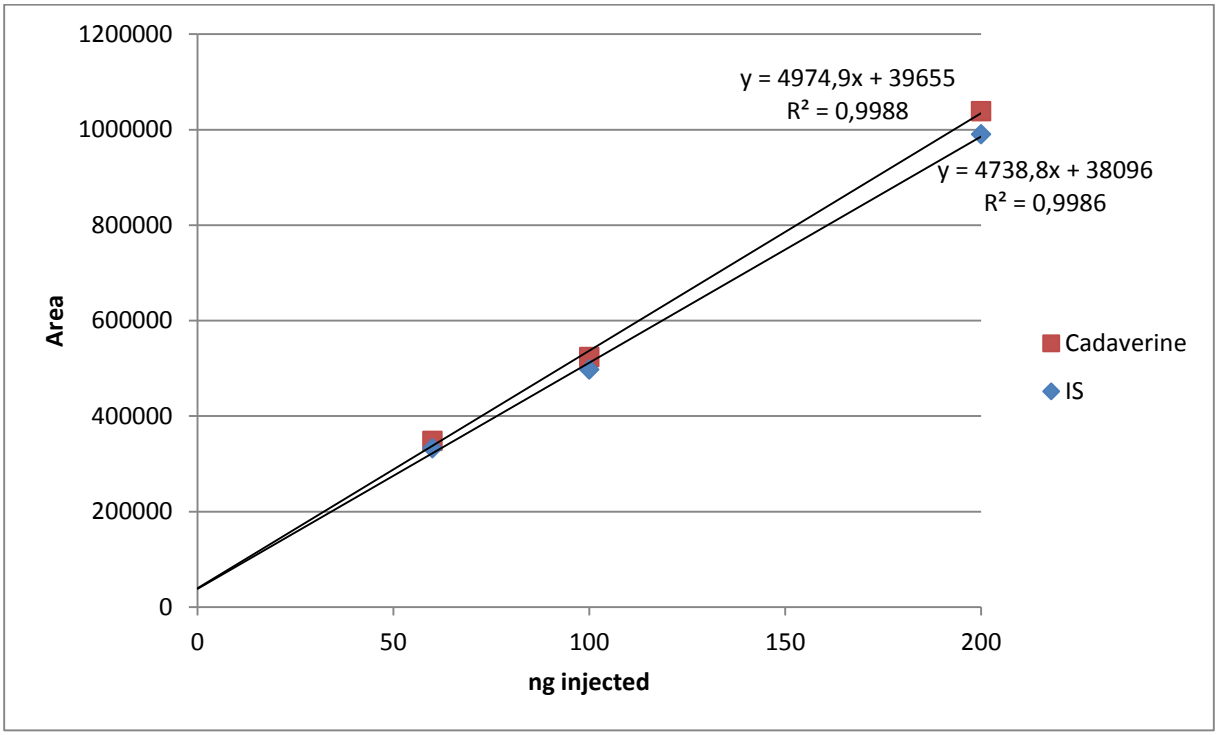

Figure 5 *The injected standard (60-200 ng) plotted against the area of the cadaverine peak and the area of the internal standard (IS) peak.*

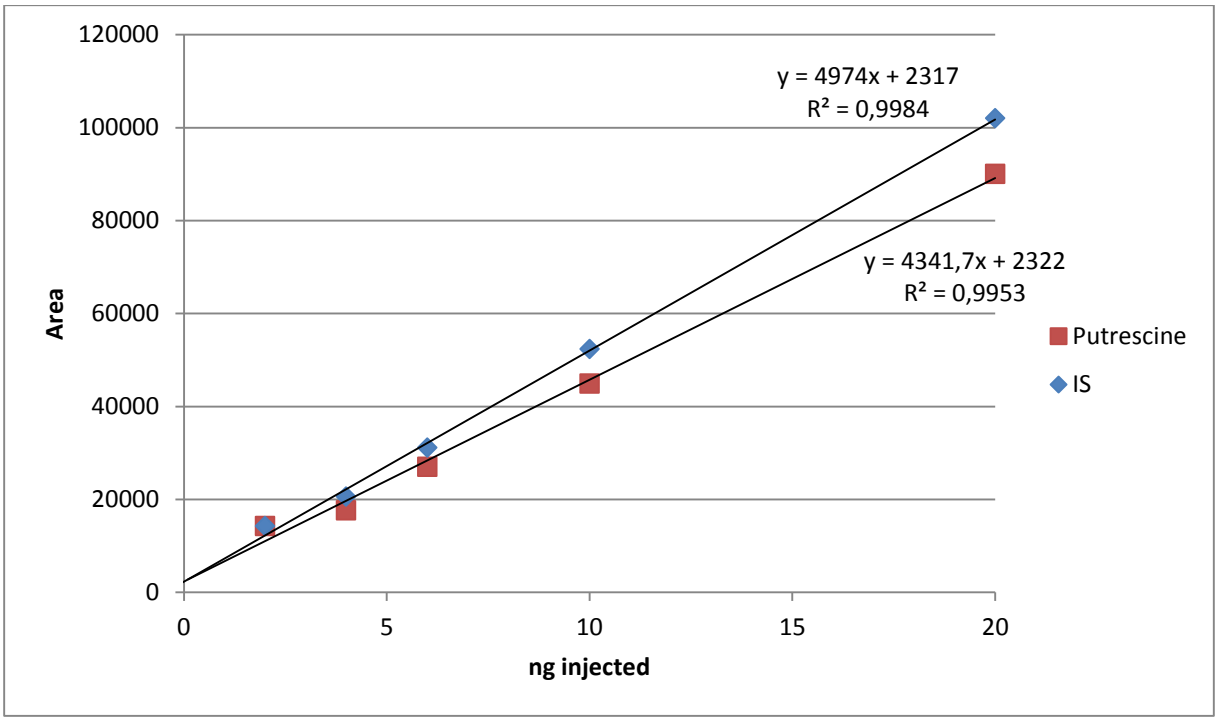

Figure 6 *The injected standard (2-20 ng) plotted against the area of the putrescine peak and the area of the internal standard (IS) peak.*

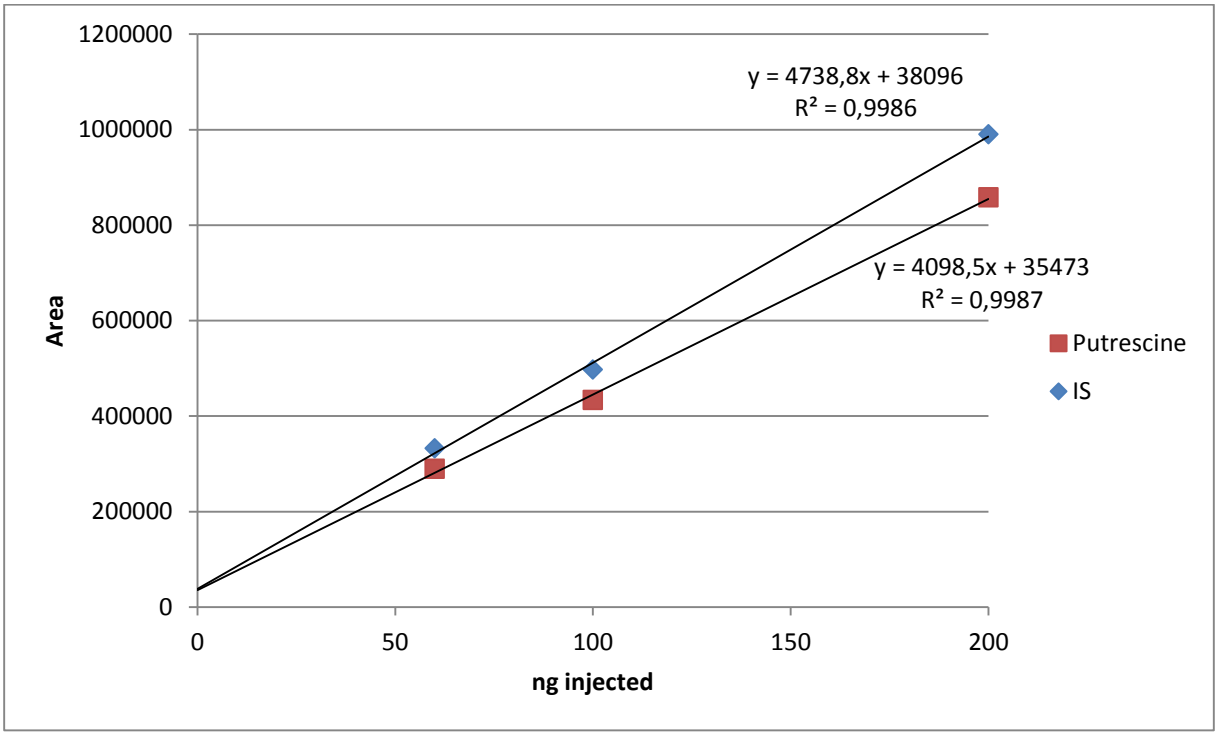

Figure 7 *The injected standard (60-200 ng) plotted against the area of the putrescine peak and the area of the internal standard (IS) peak.*

The response factors were calculated for the three amines at each concentration. The average response factor (RF) for histamine was 2.79 and the % *RSD* between the RFs (n=8) was 3.74 %. The average RF for cadaverine was 0.95 and the % RSD was 1.31 %. The average RF for putrescine was 1.13 and the % RSD was 4.87 %. The linearity of the calibration is good with R^2-values close to 1. The

F-values from the *F*-distribution are higher than the table values. This means, as mentioned in chapter 2.3.2, that the linear regression is justified. The illustrations show that there is a small deviation from linearity for the standard of 2 ng injected. This corresponds to an amine level between 3 and 4 mg/kg given a sample weight of 10 g, and is below what is set as the quantification limit of the method, see chapter 4.5.

4.3 Precision

The within laboratory precision calculated as the repeatability was based on the spiking results, where the results were treated as double measurements in the order they were analyzed. The calculation was done using equation 2.2 and 2.3, and is shown in appendix 2. The repeatability is shown in Table 4.

Table 4 The repeatability of histamine, cadaverine and putrescine calculated for the low (2.40-10.0 mg/kg) and high (60.0-120 mg/kg) concentration levels.

Repeatability	**Histamine**		**Cadaverine**		**Putrescine**	
Level	**Low**	**High**	**Low**	**High**	**Low**	**High**
r	0.526	2.19	0.241	2.81	0.157	3.08
***CV* %**	2.8	0.89	1.3	1.1	0.82	1.2

The precision of the results is good. The repeatability is below what is acceptable at the different concentration levels (NMKL 2009).

4.4 Accuracy

4.4.1 Ring tests

Nofima BioLab has participated in a few ring tests for histamine, cadaverine and putrescine by using this method. The ring tests have been organized by Lvu for all three amines and by CHEK for histamine. The sample matrixes have been fish paste and mackerel. The results of the ring tests are shown in Table 5-7. Calculations were done by using equation 2.4, 2.5 and 2.7.

Table 5 *The result of the ring tests for histamine analyzed by use of this method. The ring tests were organized by Lvu and CHEK and analyzed between 2011 and 2014. The z-score, the ζ-score and the E_n-value was calculated by use of equations 2.4, 2.5 and 2.7.*

Organizer	**Lvu**	**CHEK**	**Lvu**	**Lvu**	**Lvu**
Sample number	1	499	1 and 2	413-13	413-35
Sample type	Fish paste	Mackerel	Fish paste	Fish paste	Fish paste
Date	14/1/2011	25/1/2012	9/4/2012	22/10/2013	21/10/2014
Result, Nofima	137.0	75.00	130.5	59.95	137.5
u_{Nofima}	10.28	5.63	9.79	4.50	10.31
Mean value	136.4	73.00	156.0	60.80	145.1
Number of participants	24	14	29	18	27
u_{SLP}	12.78	6.13	26.22	5.48	26.10
z-score	0.05	0.33	-0.97	-0.16	-0.29
ζ-score	0.04	0.24	-0.91	-0.12	-0.27
***E_n*-value**	0.03	0.17	-1.17	-0.09	-0.33

Table 6 *The result of the ring tests for cadaverine analyzed by use of this method. The ring tests were organized by Lvu and analyzed between 2011 and 2014. The z-score, the ζ-score and the E_n-value was calculated by use of equations 2.4, 2.5 and 2.7.*

Organizer	**Lvu**	**Lvu**	**Lvu**	**Lvu**
Sample number	1	1 and 2	413-13	413-35
Sample type	Fish paste	Fish paste	Fish paste	Fish paste
Date	14/1/2011	9/4/2012	22/10/2013	21/10/2014
Result, Nofima	161.50	140.50	276.50	497.00
u_{Nofima}	12.11	10.54	20.74	37.28
Mean value	172.60	148.00	288.00	521.20
Number of participants	24	29	18	27
u_{SLP}	8.66	12.00	18.40	56.10
z-score	-1.28	-0.63	-0.63	-0.43
ζ-score	-0.75	-0.47	-0.41	-0.36
***E_n*-value**	-0.62	-0.45	-0.36	-0.38

Table 7 *The result of the ring tests for putrescine analyzed by use of this method. The ring tests were organized by Lvu and analyzed between 2011 and 2014. The z-score, the ζ-score and the E_n-value was calculated by use of equations 2.4, 2.5 and 2.7.*

Organizer	**Lvu**	**Lvu**	**Lvu**	**Lvu**
Sample number	1	1 and 2	413-13	413-35
Sample type	Fish paste	Fish paste	Fish paste	Fish paste
Date	14/1/2011	9/4/2012	22/10/2013	21/10/2014
Result, Nofima	64.90	89.50	110.50	196.50
u_{Nofima}	4.87	6.71	8.29	14.74
Mean value	66.10	93.20	111.00	191.40
Number of participants	20	24	15	22
u_{SLP}	8.61	6.49	8.66	28.00
z-score	-0.14	-0.57	-0.06	0.18
ζ-score	-0.12	-0.40	-0.04	0.16
***E_n*-value**	-0.13	-0.36	-0.04	0.18

The E_n-values are shown graphically in 8-10.

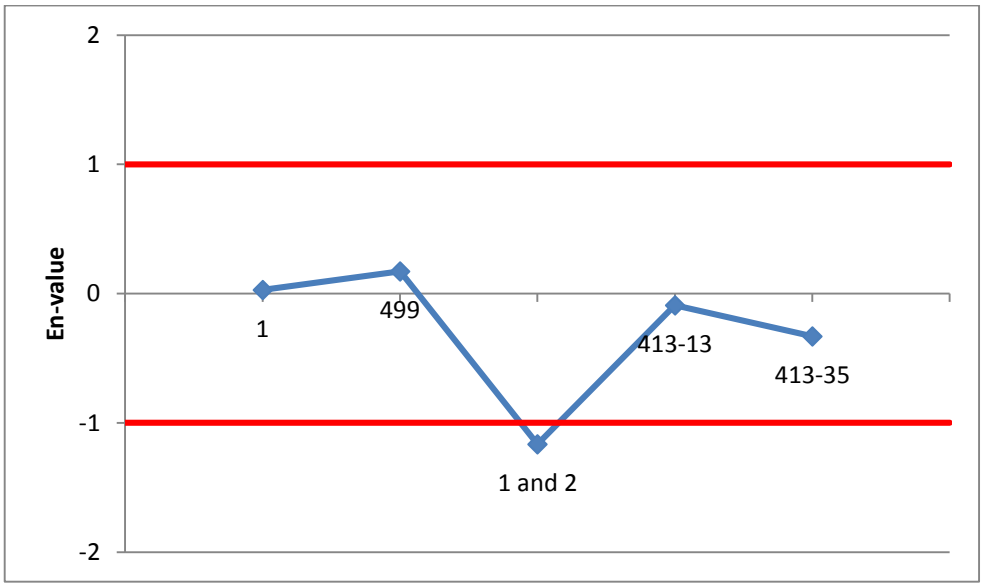

Figure 8 *The E_n-values for the five ring tests for histamine shown graphically.*

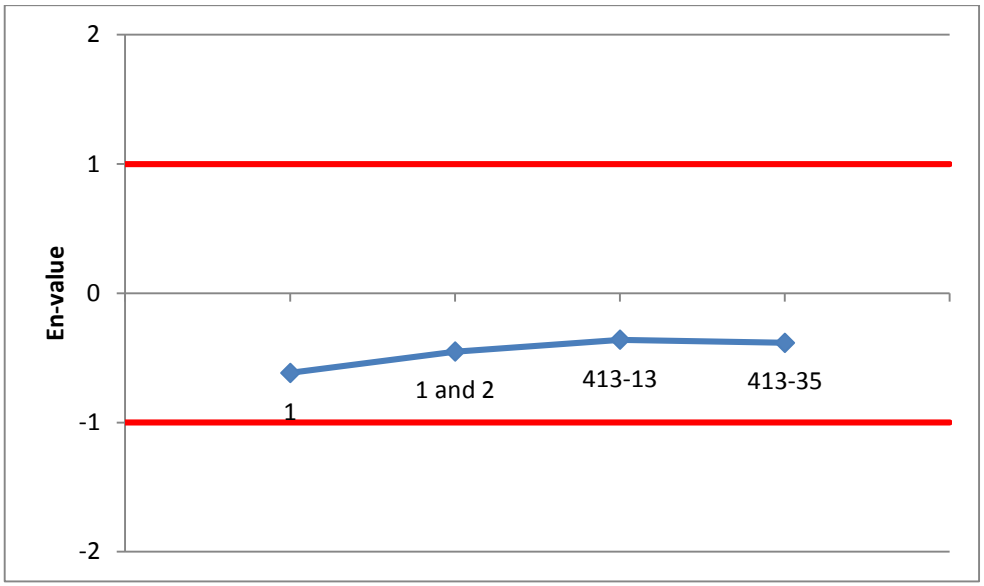

Figure 9 *The E_n-values for the five ring tests for cadaverine shown graphically.*

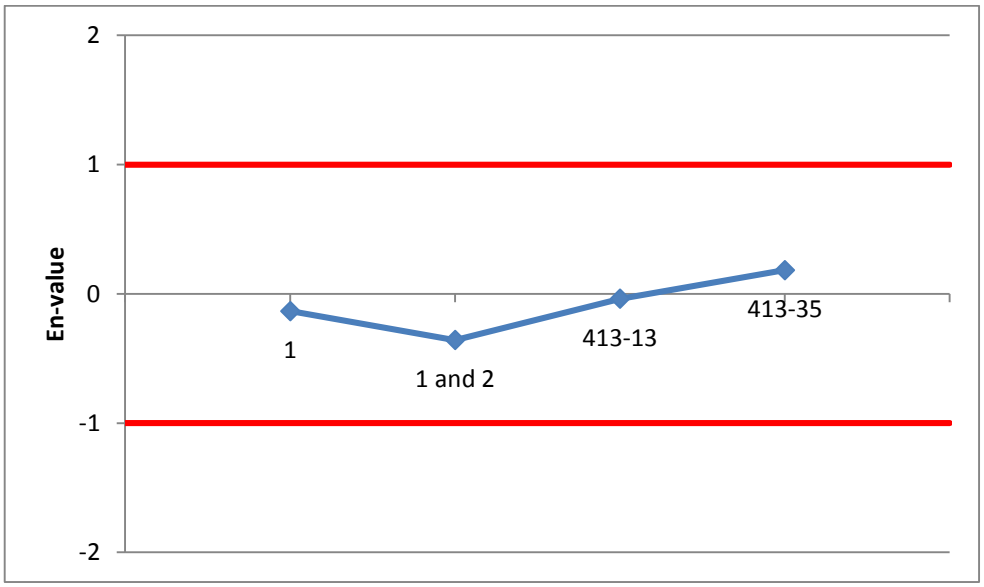

Figure 10 *The E_n-values for the five ring tests for putrescine shown graphically.*

The E_n-value for the ring tests are in the acceptable range, except from the sample analyzed 9/4/2012 for histamine which is in the suspicious range. The z-score and ζ-score for this sample is in the acceptable area. The value of u_{SLP} is high, which may indicate for example sample inhomogeneity. The ring test results are considered to be good, but it is important to notice that the data material is limited.

4.4.2 Recovery/spiking

The results of the recovery/spiking test are shown in Table 8-10. A complete overview of the results is shown in appendix 3.

Table 8 The results of the recovery/spiking test for histamine. The spiked concentrations are the amine levels calculated in Table 3.

Number of samples analyzed	Average result, spiked sample (mg/kg)	Spiked conc. (mg/kg)	Original level in sample matrix (mg/kg)	Recovery (%)
6	2.93	2.40	0.802	88
6	10.4	10.0	0.802	96
6	57.2	60.0	0.802	94
6	116	120	0.802	96

Table 9 The results of the recovery/spiking test for cadaverine. The spiked concentrations are the amine levels calculated in Table 3.

Number of samples analyzed	Average result, spiked sample (mg/kg)	Spiked conc. (mg/kg)	Original level in sample matrix (mg/kg)	Recovery (%)
6	3.04	2.40	0.667	93
6	10.4	10.0	0.667	96
6	59.1	60.0	0.667	97
6	118	120	0.667	97

Table 10 The results of the recovery/spiking test for putrescine. The spiked concentrations are the amine levels calculated in Table 3.

Number of samples analyzed	Average result, spiked sample (mg/kg)	Spiked conc. (mg/kg)	Original level in sample matrix (mg/kg)	Recovery (%)
6	3.38	2.40	1.03	108
6	10.1	10.0	1.03	93
6	59.1	60.0	1.03	97
6	119	120	1.03	98

The recovery lies between 88 and 108 %, which is considered to be very good for this concentration level. With the exception of 88 % for histamine and 108 % for putrescine at the lowest spiking level, the recovery lies between 93 and 98 %. Expected recovery for 100 mg/kg is 90-107 %, and 80-110 % for 1 to 10 mg/kg (NMKL 2012). The % *RSD* between the results of the spiked samples is low (between 0.71 and 4.5 % *RSD*), which indicates that the homogeneity of the spiked samples were good. The bias was calculated and a *t*-test was performed to check if the bias was significant and needed correction by using equation 2.13 and 2.14, respectively. The *t*-values lie between -0.01 and – 0.88 and the calculation showed that the bias is not significant and that correction for recovery is not necessary.

4.5 Measuring range

The signal/noise ratio between a blank injection and an injection of 2 ng free base of histamine is shown in Figure 11.

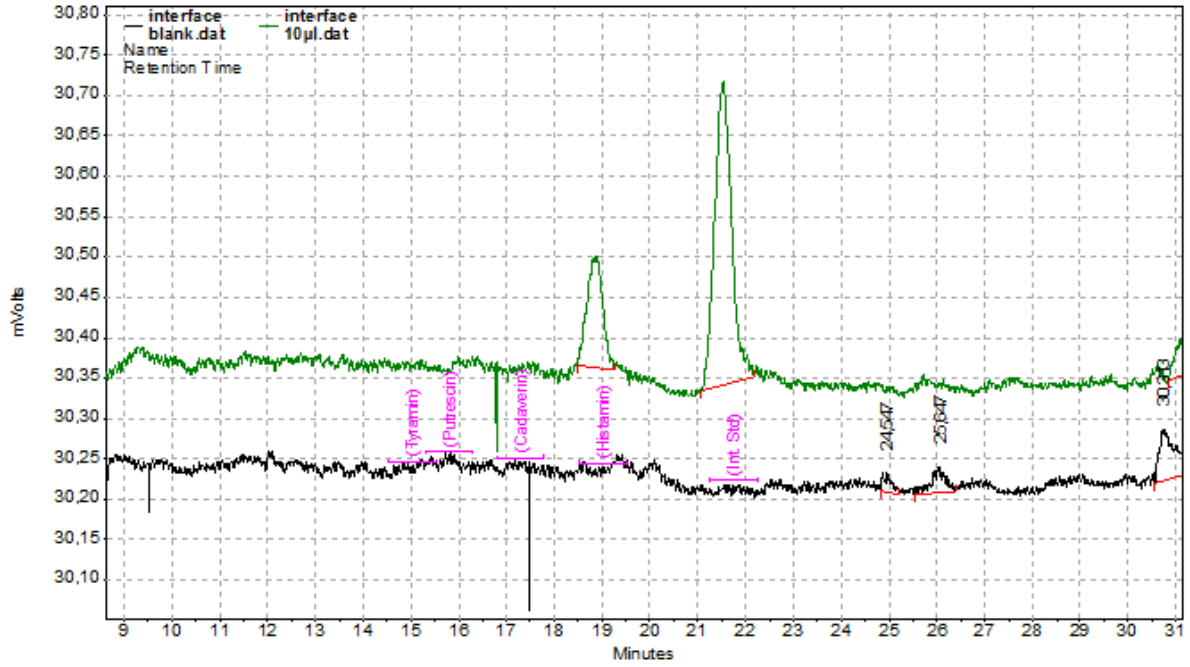

Figure 11 Overlay of a blank injection and an injection of 2 ng free base of histamine.

The noise signal was measured 16 times and the standard deviation (*SD*) of the signal was calculated to 0.013. This is shown in appendix 4. The *LOD* (3×*SD*) was calculated to 0.038 and the *LOQ* (10×*SD*) was calculated to 0.126 by using equation 2.15 and 2.16, respectively. 2 ng of free base injected gives a signal equal to 0.135, and hence the *LOQ* can be given as 2 ng histamine injected. This corresponds to between 3 and 4 mg/kg following the given procedure with 10 g sample weight. Since the spiking of krill meal with a spiked concentration of 2.40 mg/kg showed a somewhat poorer recovery for histamine and putrescine than for the rest of the spiked samples, it was chosen to set the *LOQ* to 10 mg/kg. Also, as described in chapter 4.2, the linearity deviates a bit for concentrations lower than 10 mg/kg, which indicates that this is a more reasonable *LOQ*.

4.6 Uncertainty

4.6.1 Theoretical uncertainty

The contributors to the method's measurement uncertainty are shown in the Ishikawa diagram in Figure12. The figure shows the uncertainty contributors for histamine, but the same contributors replies to cadaverine and putrescine.

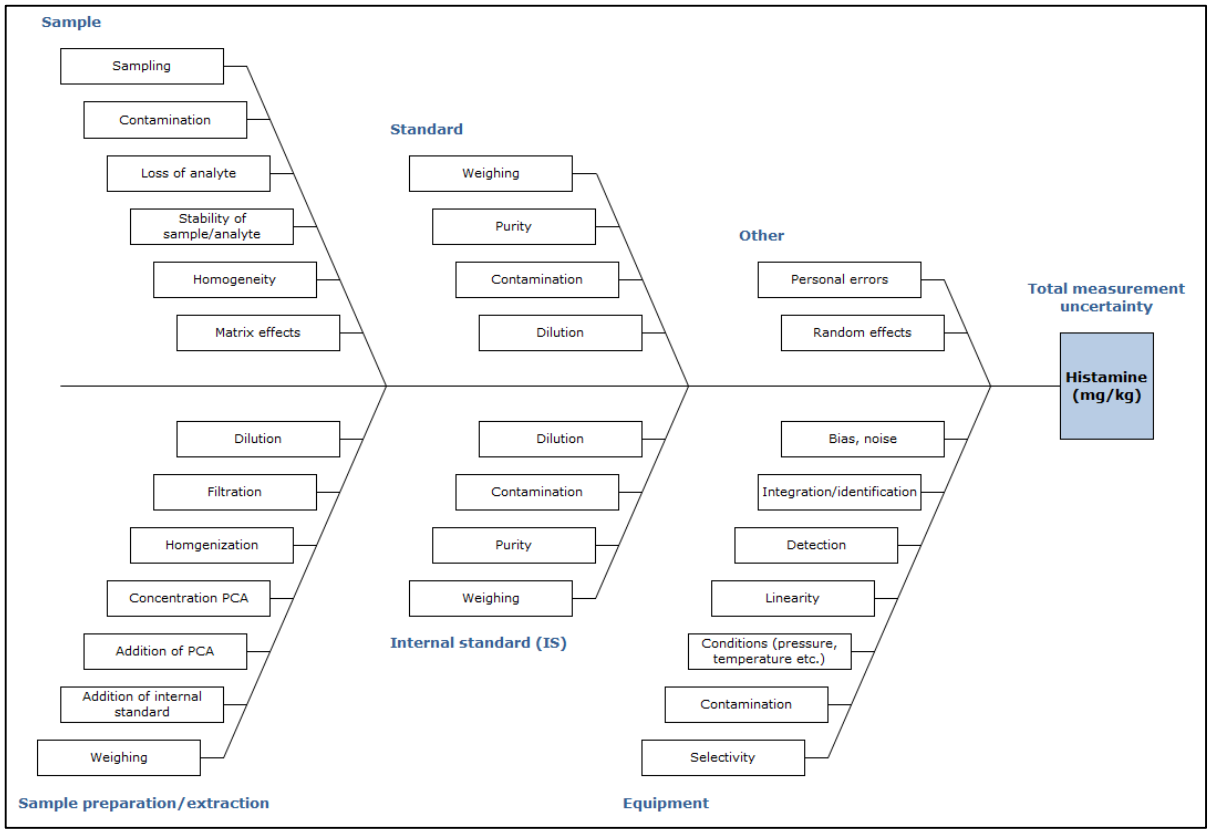

Figure 12 An Ishikawa diagram showing the contributors to the method's measurement uncertainty (shown for histamine).

The theoretical uncertainty was calculated by using the Eurachem spreadsheet method, and is shown in appendix 5 (Eurachem 1995). The theoretical uncertainty for a sample containing about 100 mg/kg of histamine was calculated to 3.01 % (expanded uncertainty).

Figure 11 shows the distribution of the theoretical uncertainty.

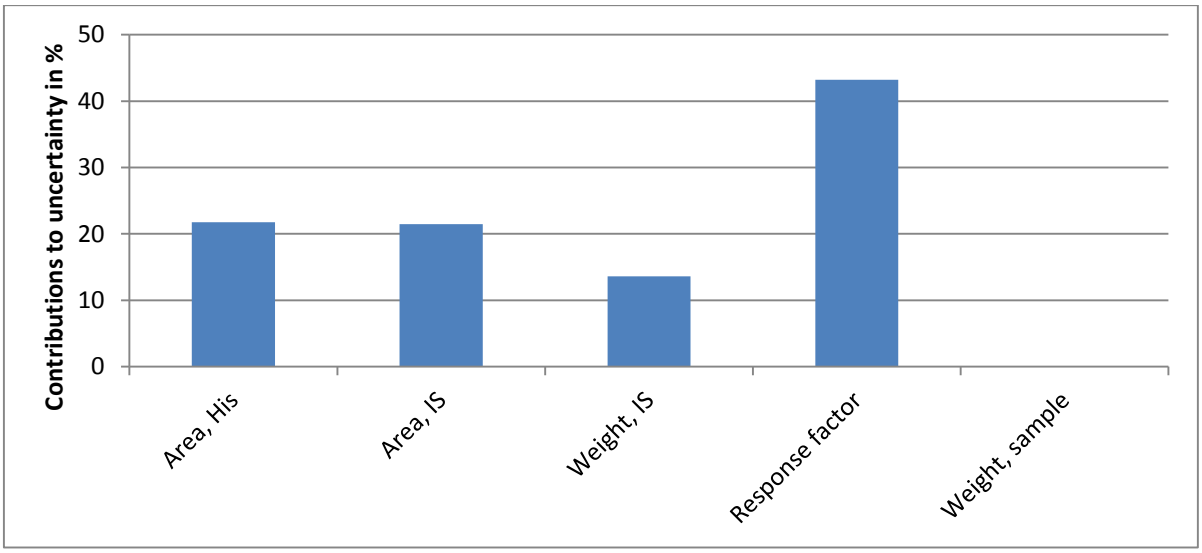

Figure 13 The different uncertainty contributors to the total theoretical measurement uncertainty of the method. The uncertainty was calculated using the Eurachem spreadsheet method.

The largest uncertainty contributor is the response factor, which is depending on both uncertainty in the areas of the histamine and internal standard peaks, and the concentrations of the standard solution and the internal standard solution. The uncertainty of the peak areas depends on several factors, like the detector response, the flow rate, the temperature in the column oven, fluctuations in the mobile phase, and integration (Barwick 1999). The uncertainty of the standard and internal standard solutions depend on the scale used for weighing the chemical, the purity of the compounds, and dilutions done by use of volumetric flasks and automatic pipettes. The peak areas of the injected sample are also large contributors to uncertainty, and so is addition of the internal standard solution. Weighing the sample contributes little. The theoretical uncertainty is low, but it is important to notice that the uncertainty only involves measureable contributors. Uncertainty associated with the sample, the sample preparation, other chromatographic conditions and personal errors are not taken into account.

4.6.2 Experimental uncertainty

The combined measurement uncertainty was based on the precision of the samples ($u_{precision}$), the ring test uncertainty (u_{SLP}), and the standard uncertainty for the recovery (u_{rec}).

The uncertainty of the precision was calculated in chapter 4.3 (reported as CV %).

The uncertainty based on the five ring tests (chapter 4.4.1) was calculated to 7.7 % *RSD* for histamine 5.0 % *RSD* for cadaverine and 2.8 % *RSD* for putrescine by using equation 2.17 and 2.18. The calculation is shown in appendix 5. If the deviating result for histamine of the ring test analyzed 9/4/2012 is omitted, the uncertainty is 3.7 % *RSD*.

The standard error of the mean (*SEM*) from the recovery test was calculated for all concentration levels and sample matrixes by using equation 2.10. The combined standard uncertainty for the recovery (u_{rec}) was calculated to 2.1 % for histamine, 1.5 % for cadaverine and 0.95 % for putrescine for all spiking levels by using equation 2.11.

The combined measurement uncertainty was calculated to the following for the low levels (2.40-10.0 mg/kg):

$$u_{histamine} = \sqrt{u_{precision}^2 + u_{SLP}^2 + u_{rec}^2} = \sqrt{2.8\%^2 + 7.7\%^2 + 2.1\%^2} = 8.5\%$$

$$u_{cadaverine} = \sqrt{u_{precision}^2 + u_{SLP}^2 + u_{rec}^2} = \sqrt{1.3\%^2 + 5.0\%^2 + 1.5\%^2} = 5.4\%$$

$$u_{putrescine} = \sqrt{u_{precision}^2 + u_{SLP}^2 + u_{rec}^2} = \sqrt{0.82\%^2 + 2.8\%^2 + 0.95\%^2} = 3.1\%$$

The combined measurement uncertainty was calculated to the following for the high levels (60.0-120 mg/kg):

$$u_{histamine} = \sqrt{u_{precision}^2 + u_{SLP}^2 + u_{rec}^2} = \sqrt{0.89\%^2 + 7.7\%^2 + 2.1\%^2} = 8.1\%$$

$$u_{cadaverine} = \sqrt{u_{precision}^2 + u_{SLP}^2 + u_{rec}^2} = \sqrt{1.1\%^2 + 5.0\%^2 + 1.5\%^2} = 5.3\%$$

$$u_{putrescine} = \sqrt{u_{precision}^2 + u_{SLP}^2 + u_{rec}^2} = \sqrt{1.2\%^2 + 2.8\%^2 + 0.95\%^2} = 3.2\%$$

This corresponds to an expanded uncertainty (± 2s) of 17 % for histamine, 11 % for cadaverine and 7 % for putrescine for all concentration levels (rounded up to the nearest whole number). If the deviating ring test for histamine is omitted, the expanded uncertainty is 11 % for the low level and 9 % for the high level.

The ring test organizers inform that the samples are prepared by spiking with amines. Since the recovery is excellent, the uncertainty connected to ring test results will probably decrease when more ring test samples have been analyzed and the data material is bigger.

5 Conclusion

The validation of the method has established important method parameters. A summary is shown in Table 11.

Table 11 A summary of the method parameters established in the validation.

Method parameter	Summary
Selectivity	Good, no interfering compounds in the chromatogram. Peak resolution (R_S) should be ≥ 1 for tyramine and putrescine.
Linearity	Good for the entire concentration range, R^2-values close to 1.
Precision	The repeatability lies between 0.82 and 2.8 CV % for all three amines over the entire concentration range. The precision is good.
Accuracy	Ring tests: Acceptable z-scores, zeta-scores and E_n-values with the exception of one ring test for histamine (suspicious range). The u_{SLP} for this ring test was high, which can indicate for example sample inhomogeneity. Recovery: Apparent recoveries between 88 and 108 % for all concentration levels. The recovery is good, and the bias is not significant (there is no need for correction of recovery). The apparent recovery for spiking concentrations between 10.0 and 120 mg/kg lies between 93 and 98 %.
Measuring range	The limit of quantification (*LOQ*) for the method is 10 mg/kg.
Uncertainty	Theoretical: 3.01 % expanded uncertainty. Highest contributions to theoretical uncertainty come from the peak areas and the preparation of the standard and internal standard solution. Experimental: Histamine: 17 % expanded uncertainty (±2s) for the entire concentration range. 11 % (low level) and 9 % (high level) expanded uncertainty if the deviating ring test result is omitted. Cadaverine: 11 % expanded uncertainty. Putrescine: 7 % expanded uncertainty.

The method is fit for purpose.

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APPENDIXES

Appendix 1 – Linearity

The linearity was checked by plotting ng of each compound injected against the area of the amine and internal standard peak.

Standard (ml)	IS (mg/ml)	Amine (mg/ml)	20 µl injected for each compound (ng)	IS, Area	Histamine, Area	Cadaverine, Area	Putrescine, Area
0.010	0.0001	0.0001	2	14292	5459	15356	14299
0.020	0.0002	0.0002	4	20609	7024	21774	17650
0.030	0.0003	0.0003	6	31163	11115	32920	27028
0.050	0.0005	0.0005	10	52372	18731	53855	44955
0.100	0.001	0.001	20	102057	36659	108807	90030
0.300	0.003	0.003	60	332739	113637	348070	289770
0.500	0.005	0.005	100	497540	179634	523258	433584
1.000	0.01	0.01	200	989987	365315	1038606	858530

Average RF	2.79	0.949	1.13
SD	0.10	0.012	0.055
% RSD	3.74	1.31	4.87

Least squares method

Statistics	Internal standard	Histamine	Cadaverine	Putrescine
Degrees of freedom (n-2)	6	6	6	6
Slope (m)	4949	1820	5192	4292
s_m	70	11	71	63
y-intercept (b)	6430	729	6938	6324
s_b	5764	936	5836	5147
R^2	1	1	1	1
s_y	12910	2096	13071	11527
F	4989	25597	5357	4707

Appendix 2 – Precision

Precision of the method was determined by treating the results of the spiking experiments as double measurements. The tables on this page shows the precision in the low and high concentration area for histamine.

Low concentration area:

Sample	Date	Result 1	Result 2	Diff.	Diff^2	Average	n
Krill meal	18/8/2015	2.9541	3.0941	-0.14	0.0196	3.02	1
Krill meal	18/8/2015	2.8487	2.9981	-0.15	0.0223	2.92	2
Krill meal	18/8/2015	2.9498	2.7127	0.24	0.0562	2.83	3
Krill meal	20/8/2015	10.4234	10.4945	-0.07	0.0051	10.46	4
Krill meal	20/8/2015	10.7037	10.1716	0.53	0.2831	10.44	5
Krill meal	20/8/2015	10.1463	10.3146	-0.17	0.0283	10.23	6

n= 6 SUM D^2= 0.415 Average= 6.65

Reproducibility			Repeatability	
Average:	6.65		**S_r**	0.186
Standard deviation:	4.081		**CV %**	2.8
			r = 2.8 * Sr	0.526

High concentration area:

Sample	Date	Result 1	Result 2	Diff.	Diff^2	Average	n
Krill meal	18/8/2015	57.5929	57.4106	0.18	0.0332	57.50	1
Krill meal	18/8/2015	56.5250	57.8820	-1.36	1.8414	57.20	2
Krill meal	18/8/2015	56.9586	56.8377	0.12	0.0146	56.90	3
Krill meal	19/8/2015	113.8917	115.9870	-2.10	4.3903	114.94	4
Krill meal	19/8/2015	114.7188	115.4228	-0.70	0.4956	115.07	5
Krill meal	19/8/2015	117.5245	116.8891	0.64	0.4037	117.21	6

n= 6 SUM D^2= 7.179 Average= 86.47

Reproducibility			Repeatability	
Average:	86.47		**S_r**	0.773
Standard deviation:	32.073		**CV %**	0.89
			r = 2.8 * Sr	2.188

The tables on this page shows the precision in the low and high concentration area for cadaverine.

Low concentration area:

Sample	Date	Result 1	Result 2	Diff.	Diff^2	Average	n
Krill meal	18/8/2015	3.0416	3.0311	0.01	0.0001	3.04	1
Krill meal	18/8/2015	2.9191	2.9854	-0.07	0.0044	2.95	2
Krill meal	18/8/2015	3.0442	3.1984	-0.15	0.0238	3.12	3
Krill meal	20/8/2015	10.5661	10.5495	0.02	0.0003	10.56	4
Krill meal	20/8/2015	10.5332	10.3192	0.21	0.0458	10.43	5
Krill meal	20/8/2015	10.1813	10.2942	-0.11	0.0127	10.24	6

n= 6 SUM D^2= 0.087 Average= 6.72

Reproducibility			Repeatability	
Average:	6.72		**S_r**	0.085
Standard deviation:	4.039		**CV %**	1.3
			r = 2.8 * Sr	0.241

High concentration area:

Sample	Date	Result 1	Result 2	Diff.	Diff^2	Average	n
Krill meal	18/8/2015	59.2462	59.1187	0.13	0.0163	59.18	1
Krill meal	18/8/2015	58.1804	59.7909	-1.61	2.5937	58.99	2
Krill meal	18/8/2015	58.9305	59.3492	-0.42	0.1753	59.14	3
Krill meal	19/8/2015	116.1503	117.3288	-1.18	1.3889	116.74	4
Krill meal	19/8/2015	119.2743	118.5183	0.76	0.5715	118.90	5
Krill meal	19/8/2015	118.5149	115.8553	2.66	7.0735	117.19	6

n= 6 SUM D^2= 11.819 Average= 88.35

Reproducibility			Repeatability	
Average:	88.35		**S_r**	0.992
Standard deviation:	32.052		**CV %**	1.1
			r = 2.8 * Sr	2.807

The tables on this page shows the precision in the low and high concentration area for putrescine.

Low concentration area:

Sample	Date	Result 1	Result 2	Diff.	Diff^2	Average	n
Krill meal	18/8/2015	3.4108	3.3495	0.06	0.0038	3.38	1
Krill meal	18/8/2015	3.3107	3.4292	-0.12	0.0140	3.37	2
Krill meal	18/8/2015	3.4235	3.3709	0.05	0.0028	3.40	3
Krill meal	20/8/2015	10.1749	10.1179	0.06	0.0032	10.15	4
Krill meal	20/8/2015	10.2097	10.0961	0.11	0.0129	10.15	5
Krill meal	20/8/2015	10.0267	10.0457	-0.02	0.0004	10.04	6

n= 6 SUM D^2= 0.037 Average= 6.75

Reproducibility			Repeatability	
Average:	6.75		**S_r**	0.056
Standard deviation:	3.686		**CV %**	0.82
			r = 2.8 * Sr	0.157

High concentration area:

Sample	Date	Result 1	Result 2	Diff.	Diff^2	Average	n
Krill meal	18/8/2015	58.7762	58.8977	-0.12	0.0148	58.84	1
Krill meal	18/8/2015	58.4744	59.9171	-1.44	2.0814	59.20	2
Krill meal	18/8/2015	58.9596	59.2979	-0.34	0.1144	59.13	3
Krill meal	19/8/2015	118.1984	117.9667	0.23	0.0537	118.08	4
Krill meal	19/8/2015	120.2156	120.3447	-0.13	0.0167	120.28	5
Krill meal	19/8/2015	118.9293	115.4699	3.46	11.9674	117.20	6

n= 6 SUM D^2= 14.248 Average= 88.79

Reproducibility			Repeatability	
Average:	88.79		**S_r**	1.090
Standard deviation:	32.587		**CV %**	1.2
			r = 2.8 * Sr	3.082

Appendix 3 – Spiking/recovery

The results of the analysis of spiked samples of krill meal are shown in the table below. The values in the brackets are the amount of each amine added to the samples.

Amine	Histamine					Cadaverine					Putrescine				
No.	Blank	"2.40"	"10.0"	"60.0"	"120"	Blank	"2.40"	"10.0"	"60.0"	"120"	Blank	"2.40"	"10.0"	"60.0"	"120"
1	0.9848	2.9541	10.4234	57.5929	113.8917	0.6697	3.0416	10.5661	59.2462	116.1503	1.1297	3.4108	10.1749	58.7762	118.1984
2	0.8613	3.0941	10.4945	57.4106	115.9870	0.5489	3.0311	10.5495	59.1187	117.3288	1.0516	3.3495	10.1179	58.8977	117.9667
3	0.7650	2.8487	10.7037	56.5250	114.7188	0.7011	2.9191	10.5332	58.1804	119.2743	1.0355	3.3107	10.2097	58.4744	120.2156
4	0.8511	2.9981	10.1716	57.8820	115.4228	0.7334	2.9854	10.3192	59.7909	118.5183	0.9543	3.4292	10.0961	59.9171	120.3447
5	0.7094	2.9498	10.1463	56.9586	117.5245	0.6396	3.0442	10.1813	58.9305	118.5149	0.9841	3.4235	10.0267	58.9596	118.9293
6	0.6428	2.7127	10.3146	56.8377	116.8891	0.7088	3.1984	10.2942	59.3492	115.8553	1.0500	3.3709	10.0457	59.2979	115.4699
Average	0.802	2.93	10.38	57.20	115.74	0.667	3.04	10.41	59.10	117.61	1.03	3.38	10.11	59.05	118.52
SD	0.12	0.13	0.21	0.51	1.35	0.07	0.09	0.16	0.54	1.39	0.06	0.05	0.07	0.50	1.79
% RSD	15.23	4.49	2.03	0.89	1.17	9.95	3.04	1.57	0.91	1.18	5.89	1.39	0.71	0.85	1.51
R %		**88**	**96**	**94**	**96**		**93**	**96**	**97**	**97**		**108**	**93**	**97**	**98**
SEM		0.05	0.09	0.21	0.55		0.04	0.07	0.22	0.57		0.02	0.03	0.20	0.73
u_{rec}		1.83	0.83	0.37	0.48		1.24	0.64	0.37	0.48		0.57	0.29	0.35	0.62
bias %		21.93	3.76	-4.66	-3.55		26.53	4.07	-1.50	-1.99		40.93	1.12	-1.58	-1.23
t		-0.04	-0.06	-0.52	-0.73		-0.01	-0.06	-0.35	-0.68		-0.02	-0.32	-0.69	-0.88
t_{crit}		2.571	2.571	2.571	2.571		2.571	2.571	2.571	2.571		2.571	2.571	2.571	2.571

Appendix 4 – LOD and LOQ

The measurement of the noise signal from a blank injection, and the calculation of the *LOD* and *LOQ* are shown in the table below. The calculation was perfomed for histamine.

No.	Noise signal (peaks)
1	30.21
2	30.19
3	30.18
4	30.20
5	30.20
6	30.18
7	30.20
8	30.19
9	30.21
10	30.19
11	30.20
12	30.18
13	30.20
14	30.21
15	30.17
16	30.18
SD	0.012604
***LOD* (3x*SD*)**	0.037812
***LOQ* (10x*SD*)**	0.126041

Appendix 5 – Uncertainty

Calculation of the theoretical uncertainty for histamine by using the spreadsheet method in Eurachem (1995) is shown in this appendix. The calculation of the uncertainty for the response factor and the internal standard solution is not shown, but was calculated using the same method. The standard deviations from these calculations are included in the table below. The calculation is shown for histamine in fish, but the same uncertainty contributors applies to other sample materials for all amines.

Symbol	A_{His}	A_{IS}	W_{IS}	RF_{His}	W_{sample}	1000
Value	240806	102887	0.250	3.192	20	1000.000
***SD*, $u(x_i)$**	1688	721	0.001	3.15E-02	1.22E-03	- (constant)

A_{His}	240806.000	242494.050	240806.000	240806.000	240806.000	240806.000	240806.000
A_{IS}	102887.000	102887.000	103608.238	102887.000	102887.000	102887.000	102887.000
W_{IS}	0.250	0.250	0.250	0.251	0.250	0.250	0.250
RF_{His}	3.192	3.1917	3.1917	3.1917	3.2232	3.1917	3.1917
W_{sample}	20.00000	20.00000	20.00000	20.00000	20.00000	20.00122	20.00000
1000	1000.000	1000.000	1000.000	1000.000	1000.000	1000.000	1000.000
Histamine	**93.3765**	**94.0311**	**92.7265**	**93.8939**	**94.2990**	**93.3708**	**93.3765**
$u(y,x_i)$		0.6545692	-0.6500126	0.5174251	0.9224858	-0.0056966	0.0000000
$u(y)^2$, $u(y,x_i)^2$	1.970E+00	4.285E-01	4.225E-01	2.677E-01	8.510E-01	3.245E-05	0.000E+00
Sum r_i, $u(y,x_i)^2/u(y)^2$	1	0.21752	0.21451	0.13592	0.43203	0.00002	0.00000
100 % Sum r_i, $u(y,x_i)^2/u(y)^2$	100	21.75239	21.45060	13.59223	43.20313	0.00165	0.00000

$u_c(y)$	**1.4035**

$u(y,x_i)/u(x_i)$		0.00	0.00	373.51	29.26	-4.67	0.00
	Histamine	A_{His}	A_{IS}	W_{IS}	RF_{His}	W_{sample}	1000
ABS(u(y,xi))	1.4034665	0.6545692	0.6500126	0.517425094	0.9224858	0.0056966	0.0000000

Expanded uncertainty, K=2	**2.8069**
RSD %, K=2	**3.01**

Uncertainty calculation based on ring test results is shown in the tables below.

Histamine:

Program	Sample	Nofima	"Average"	Diff.	Diff^2	Average	n
Lvu	1 Fish paste	137.0	136.4	0.60	0.3600	136.70	1
CHEK	499 Mackerel	75.00	73.00	2.00	4.0000	74.00	2
Lvu	1 and 2 Fish paste	130.5	156.0	-25.50	650.2500	143.25	3
Lvu	413-13 Fish paste	59.95	60.80	-0.85	0.7225	60.38	4
Lvu	413-53 Fish paste	137.5	145.1	-7.60	57.7600	141.30	5
	n=	5	SUM D^2=	713.093	Average=	111.13	

				Repeatability	
Average:		111.13		**S_r**	8.444
Nofima-"AVERAGE"	%CV_{Sr} =	7.60			
Nofima	%CV_{Sr} =	1.33			
$u_{(Nofima-AVERAGE)}$		8.44			
$u_{(Nofima)}$		1.48			
u_c		8.57			
U (+/- 2s)		17.15			
%RSD		7.7			
%RSD (+/- 2s)		**15.4**			

Cadaverine:

Program	Sample	Nofima	"Average"	Diff.	Diff^2	Average	n
Lvu	1 Fish paste	161.5000	172.6000	-11.10	123.2100	167.05	1
Lvu	1 and 2 Fish paste	140.5000	148.0000	-7.50	56.2500	144.25	2
Lvu	413-13 Fish paste	276.5000	288.0000	-11.50	132.2500	282.25	3
Lvu	413-53 Fish paste	497.0000	521.2000	-24.20	585.6400	509.10	4
	n=	4	SUM D^2=	897.350	Average=	220.53	

				Repeatability	
Average:		220.53		**S_r**	10.591
Nofima-"AVERAGE"	$\%CV_{Sr}$ =	4.80			
Nofima	$\%CV_{Sr}$ =	1.33			
$u_{(Nofima-AVERAGE)}$		10.59			
$u_{(Nofima)}$		2.94			
u_c		10.99			
U (+/- 2s)		21.98			
%RSD		4.98			
%RSD (+/- 2s)		**10.0**			

Putrescine:

Program	Sample	Nofima	"Average"	Diff.	Diff^2	Average	n
Lvu	1 Fish paste	64.9000	66.1000	-1.20	1.4400	65.50	1
Lvu	1 and 2 Fish paste	89.5000	93.2000	-3.70	13.6900	91.35	2
Lvu	413-13 Fish paste	110.5000	111.0000	-0.50	0.2500	110.75	3
Lvu	413-53 Fish paste	196.5000	191.4000	5.10	26.0100	193.95	4
	n=	4	SUM D^2=	41.390	Average=	92.31	

				Repeatability	
Average:		92.31		**S_r**	2.275
Nofima-"AVERAGE"	$\%CV_{Sr}$ =	2.46			
Nofima	$\%CV_{Sr}$ =	1.33			
$u_{(Nofima-AVERAGE)}$		2.27			
$u_{(Nofima)}$		1.23			
u_c		2.59			
U (+/- 2s)		5.17			
%RSD		2.80			
%RSD (+/- 2s)		**5.60**			

Appendix 6 – Method description

Biogenic amines in krill meal:
Liquid chromatographic determination with post-column derivatization and fluorescence detection

1. Scope and field of application

This method is a quantitative determination of biogenic amines in krill meal. The limit of quantitation is 10 mg/kg under the conditions described in this procedure.

2. Principle

The biogenic amines are extracted from a homogenized sample with 0.6 M perchloric acid. A specific amount of internal standard is added prior to homogenization.

Separation and detection of putrescine, cadaverine and histamine is performed in a HPLC system with the use of gradient elution, post-column derivatization with o-Phthaldialdehyde (OPA) and fluorescence detection with excitation wavelength at 365 nm and emission wavelength at 418 nm.

3. Equipment

3.1 Liquid chromatographic (LC) equipment capable of mixing four solvents in a quaternary pump system performing gradient elution
3.2 Auto sampler
3.3 Fluorescence detector
3.4 Extra pump for isocratic addition of OPA
3.5 Column oven, t=35 °C
3.6 HPLC column, Hypersil ODS 15 cm x 4.6 mm
3.7 Homogenizer, Ultra Turrax
3.8 Balance, 0.1 mg
3.9 Plastic beakers, 500 mL
3.10 Measuring flasks, 3000, 2000, 250 and 100 mL
3.11 Medicated cotton
3.12 Automatic pipette, 1-5 mL and 100-1000 µL
3.13 Reagent tubes, 10 mL
3.14 Disposable syringes, 2 mL
3.15 Syringe filters, hydrophilic 0.20 µm
3.16 Vortex mixer
3.17 Auto sampler vials, 1.5 mL
3.18 Water pressure pump
3.19 Filter glass ware assembly with 0.45 µm filter
3.20 Glass beakers, 100 mL, 2000 mL
3.21 pH-meter
3.22 Stirrer, magnetic
3.23 Glass bottle, opaque 1000 mL

4. Reagents

4.1 Sodium acetate trihydrate, p.a
4.2 1-octanesulfonic acid, sodium salt, HiPerSolv for HPLC.
4.3 Methanol, HPLC grade
4.4 o-Phthaldialdehyde (OPA), for fluorometry
4.5 Brij-35, polyoxyethylenelaurelether, 30 % w/v
4.6 2-Mercaptoethanol, 99 % p.a
4.7 Potassium hydroxide (KOH), p.a
4.8 Histamine di-hydrochloride, min. 99%.
4.9 Cadaverine di-hydrochloride, min. 98%
4.10 Putrescine di-hydrochloride, min. 98%
4.11 1,6-Diaminohexane dihydrochloride, min. 99%.
4.12 Perchloric acid, p.a
4.13 Acetic acid , p.a
4.14 Boric acid, p.a
4.15 Acetonitrile, HPLC grade.

5. Solutions

5.1 Eluent A: 2.5 M sodium acetate trihydrate/0.01 M 1-octanesulfonic acid
   a. Weigh 27.22 g sodium acetate trihydrate and 4.23 g 1-octanesulfonic acid sodium salt in a 2 liter glass beaker.
   b. Add 1800 mL distilled water.
   c. Adjust pH with the use of acetic acid to 4.50 ± 0.01.
   d. Transfer to a 2 liter measuring flask. Fill to mark with distilled water.
   e. Filter the solution through a 0.45 µm filter by the use of a water pressure pump.
   f. The solution is stored in a plastic flask at room temperature.

5.2 Eluent B: Methanol

5.3 Eluent C: 0.2 M sodium acetate trihydrate /10 M 1-octanesulfonic acid/acetonitrile
   a. Weigh 54.44 g sodium acetate trihydrate and 5.62 g 1-octanesulfonic acid in a 2 liter glass beaker
   b. Add 1800 mL distilled water
   c. Adjust pH with the use of acetic acid to 4.50 ± 0,01
   d. Transfer to a 2 liter measurement flask. Fill to mark with distilled water
   e. Filter the solution through a 0.45 µm filter by the use of a water pressure pump
   f. The solution is stored in a plastic flask at room temperature.
   g. Mix solution:acetonitrile in the ratio 10:3 prior to use.

5.4 Eluent D: Solution to flush the HPLC system after last injection: 100 mL methanol in 1000 mL measuring flask, fill to mark with distilled water.

5.5 1 M boric acid solution:
   a. Weigh 123.66 g boric acid into a 2 liter glass beaker.
   b. Add 1800 mL distilled water.
   c. Adjust pH in the solution to 10.00 ± 0.01 with KOH.
   d. Fill to mark with distilled water.

5.6 o-Phthaldialdehyd solution (OPA):
   a. Weigh 1 g OPA in a 100 mL beaker.
   b. Add 10 mL methanol and dissolve with magnetic stirring.
   c. Transfer the solution to an opaque bottle and add 1000 mL boric acid solution (1 M (5.5)), 3 mL Brij-35 and 3 mL 2-mercaptoethanol. Shake the solution and place the flask in the dark until the next day.

d. Filter the solution through a 0.45 µm filter by the use of a water pressure pump just prior to use.

5.7 0.6 M perchloric acid (PCA):
   a. Add 200 mL perchloric acid to a 3 liter measuring flask that contains approximately 2 liter distilled water. Fill to mark with distilled water

5.8 Amine-stock solution (100 mg/100 mL free base):
   a. Weigh 165.7 mg histamine x 2HCl
   b. Weigh 171.5 mg cadaverine x 2HCl
   c. Weigh 182.9 mg putrescine x 2HCl

   in a 100 mL measuring flask. Fill to mark with 0.6 M PCA (5.7).

5.9 Internal standard solution (100 mg/100 mL free base):
   a. Weigh 407.3 mg 1,6-Diaminohexane dihydrochloride into a 250 mL measuring flask.
   b. Fill to mark with 0.6 M PCA (5.7).

5.10 Standard-working solution (0.1 mg/100 mL):
   a. Add 0.1 mL of amine stock-solution and equal amount of internal standard solution into a 100 mL measuring flask.
   b. Fill to mark with 0.6 M PCA (5.7).

6. Procedure

6.1 Extraction:
   a. Weigh accurately approximately 10 g krill meal sample into a 250 mL suitable plastic beaker.
   b. Add 150 mL 0.6 M PCA and 250 µL internal standard solution (5.9) and homogenize with Ultra Turrax in 2 minutes.
   c. Filter the solution trough medicated cotton into a 250 mL measuring flask. Carefully rinse the beaker and cotton with distilled water and fill to mark.
   d. Filter approximately 4 mL of the sample solution trough a 0.20 µm syringe filter.
   e. Pipette the solution into an auto sampler vial. The sample is ready for injection into the HPLC system.

6.2 Analysis:
   a. Set the fluorescence detector's wavelength to ex. 365 nm and em. 418 nm
   b. Set the column oven to t=35 °C
   c. Start the pump that delivers the OPA reagent by use of a T-connection after (post) the column. OPA is mixed in excess in 1:1 ratio with the eluent flow. The "mixing-tubing" before detection is 1 meter. The flow is set to 1 mL/min.
   d. Start the HPLC pump. The flow is set to 1 mL/min.
   e. Program the number of samples/injections. Each injection takes 45 minutes. The injection volume is 20 µL.
   f. All eluent gradients are linear, see table A and figure A.
   g. The HPLC system is flushed with eluent D, 10 % v/v methanol solution after each completed series.

Table A Gradient profile

Step	Time (min.)	Eluent A	Eluent B	Eluent C
0		75	0	25
1	25	35	0	65
2	30	0	10	90
3	35	0	20	80
4	40	75	0	25
5	45	75	0	25

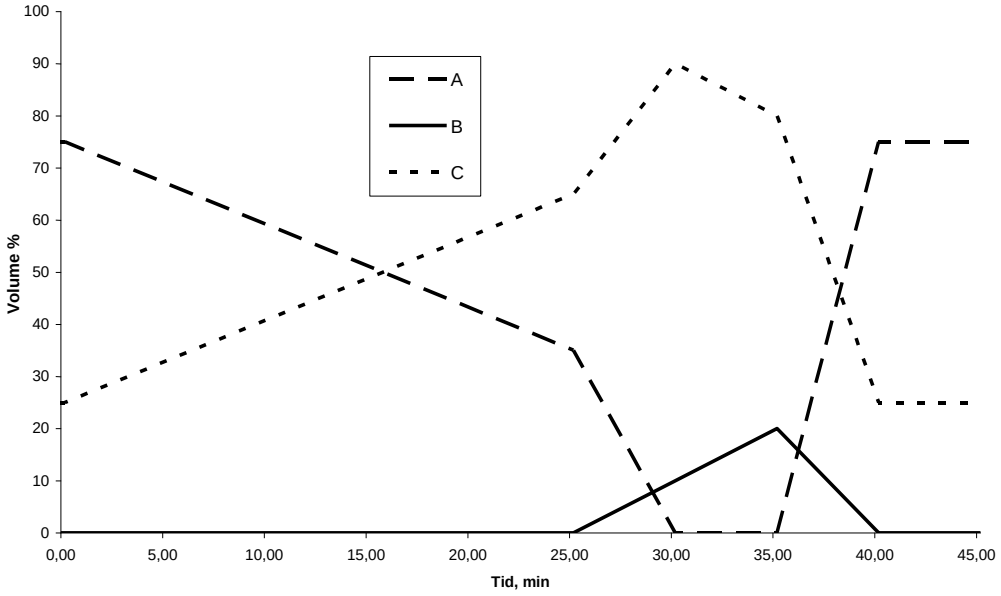

Figure A Gradient profile

7. Calculations

The response factor (RF) is calculated from analysis of standard-working solution, where the concentration of internal standard and amine standard are the same:

$$RF_{a\min e} = \frac{C_{a\min e} \times A_{i.s}}{C_{i.s} \times A_{a\min e}} = \frac{A_{i.s}}{A_{a\min e}} \qquad (7.1)$$

The concentration of amine in the sample is calculated from the results of sample solutions with added internal standard:

$$\text{Amine mg/kg} = \frac{A_{a\min e} \times W_{IS} \times RF_{a\min e}}{A_{i.s} \cdot W_{Sample}} \times 1000 \qquad (7.2)$$

A_{amine}	=	Amine peak area
$A_{i.s}$	=	Internal standard peak area
W_{IS}	=	0.25 mg, amount of internal standard added
W_{Sample}	=	Sample amount, g
RF_{amine}	=	Response factor amine
C_{amine}	=	Concentration in standard-working solution, 0.1 mg/100 mL
$C_{i.s}$	=	Concentration in standard-working solution, 0.1 mg/100 mL

Results should be rounded to the nearest whole number. Results below 10 mg/kg is reported as <10 mg/kg.

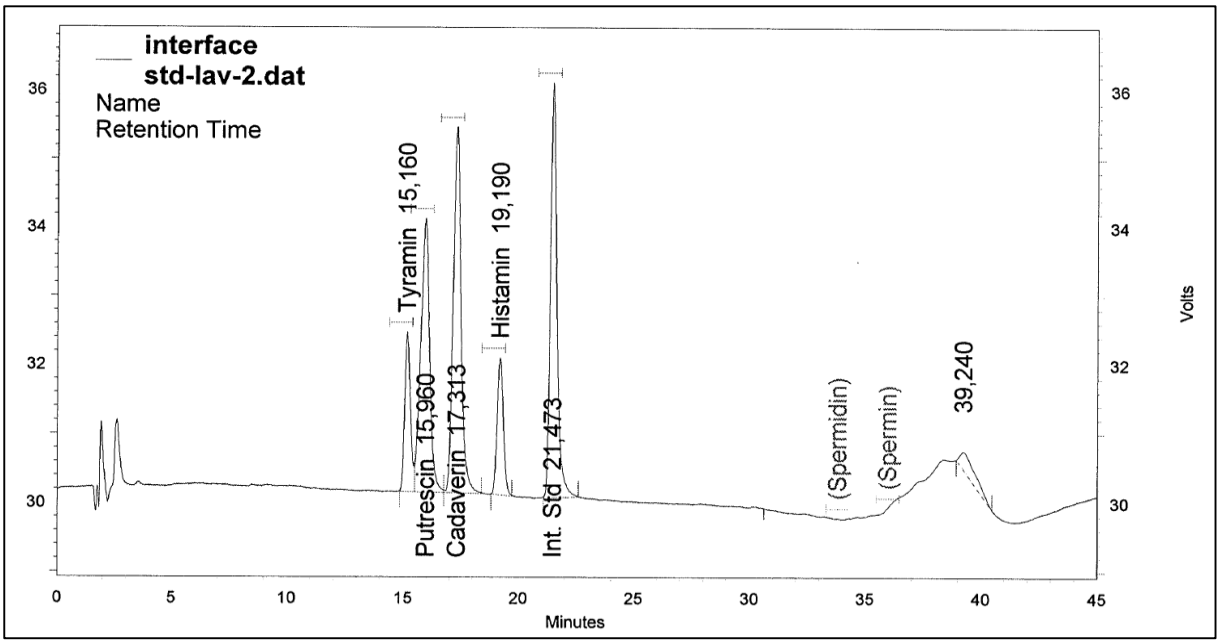

Figure B Example chromatogram of standard mixture. The standard also contains tyramine.

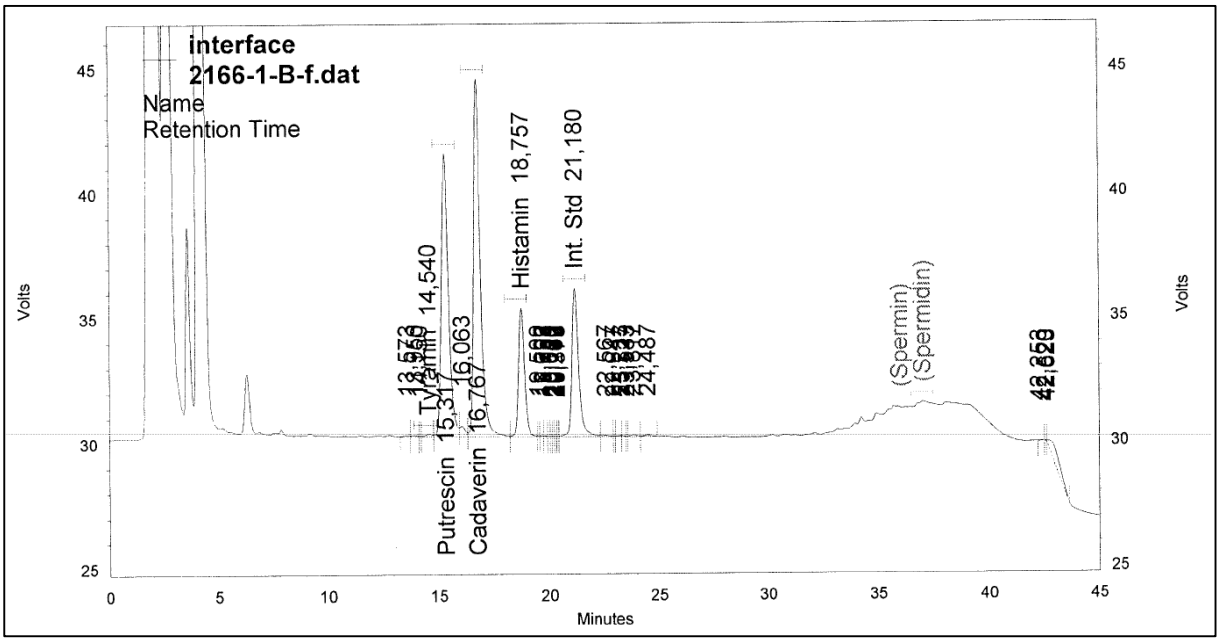

Figure C *Example chromatogram of a krill meal sample spiked with 60 mg/kg each of histamine, cadaverine and putrescine.*

Appendix

Durability of solutions:

Eluent A and C	14 days
1 M Boric acid solution	14 days
OPA reagent	24 hours
0,6 M PCA	1 month
Amine-stock solution	10 weeks at 4-6 °C
Internal standard solution	10 weeks at 4-6 °C
Standard-working solution	1 day
10% methanol/water solution	1 month

Storage:

1,6-Diaminohexane dihydrochloride, min. 99% is hygroscopic and must be kept in a desiccator. OPA reagent must be kept in the dark prior to filtration and use.

Uncertainty contributors:

Source	Contribution to uncertainty		
	Small	Medium	Large
1. Weighing, sample	X		
2. Extraction and filtration		X	
3. Dilution to 250 mL (measuring flask)	X		
4. Preparation of standard solution			X
5. Preparation of internal standard solution			X
6. Calculation of response factor			X
7. Adding internal standard, 250µL			X
8. Pipetting sample	X		
9. Post-column derivatization	X		

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