Development of a method for identification and quantification of polycyclic musks in sewage water

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Preface

This master thesis concludes my studies in chemistry at Norwegian University of Life Sciences (UMB), Department of Chemistry, Biotechnology and Food Science (IKBM). The laboratory work was carried out at IKBM mainly in 2012. The assignment was supervised by Prof. Roland Kallenborn (IKBM). I would like to thank my supervisor for the guidance, experimental training and useful discussions. I would also like to thank Dr. Dag Ekeberg (IKBM) for the help with the gas chromatography–mass spectrometry (GC-MS). Lastly I would like to thank friends and family for support.

Ås, 14.05.13

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Abstract

Polycyclic musks are a class of fragrances used as additives in personal care products. Because of their use patterns, the majority ends up in wastewater, and ultimately the environment through sewage effluents and use of sewage sludge. Polycyclic musks have a relatively low acute toxicity, but due to possible long-term toxicities to aquatic species and a potential for bioaccumulation, the fate and release of these compounds to the environment should be studied further

A method for the determination of polycyclic musks in sewage water, using solid phase extraction and GC-MS, was developed. The method was applied for identification and quantification of the polycyclic musks: Cashmeran (DPMI), Celestolide (ADBI), Phantolide (AHDI), Galaxolide (HHCB) and Tonalide (AHTN) in influent and effluent samples from a sewage treatment plant at Bekkelaget, Oslo.

HHCB and AHTN were detected in influent and effluent samples in maximum concentrations of 7040 and 1967 ng/l, and 486 and 85 ng/l, respectively, while DPMI, ADBI or AHDI were not detected in any of the sewage samples. LOD for the analyzed musks were determined to be in the low ng/l, and varied from 1,1 ng/l (AHTN) to 18,7 ng/l for DPMI.

The reported values of polycyclic musk in sewage indicate that use of these compounds have not been reduced in Norway in recent years, and may possibly have increased.

Sammendrag

Polysykliske muskstoffer er en klasse parfymestoffer som brukes som tilsetning i produkter til personlig pleie. På grunn av deres bruksmønstre ender det meste av disse stoffene opp i avløpsvann, og til slutt miljøet gjennom kloakkutslipp og bruk av slam fra renseanlegg. Polysykliske muskstoffer har relativt lav akutt giftighet, men på grunn av mulige langtidsvirkninger på vannlevende organismer og mulighet for bioakkumulering bør utslippene og skjebnene til disse forbindelsene studeres nærmere.

I dette arbeidet er det utviklet en fremgangsmåte for bestemmelse av polysykliske muskstoffer i avfallsvann ved hjelp av fastfase-ekstraksjon og GC-MS. Metoden ble brukt for identifisering og kvantifisering av de polysykliske musksstoffene Cashmeran (DPMI), Celestolide (ADBI), Phantolide (AHDI), Galaxolide (HHCB) og Tonalide (AHTN) i prøver av innløps- og utløpsvann fra et kloakkrenseanlegg på Bekkelaget i Oslo.

HHCB og AHTN ble påvist i innløps- og utløpsprøvene i maksimale konsentrasjoner på henholdsvis 7040 og 1967 ng/l og 486 og 85 ng/l, mens DPMI, ADBI og AHDI ikke ble funnet i noen av prøvene. Deteksjonsgrensene for de analyserte muskstoffene ble funnet å være i det nedre ng/l-området, og varierte fra 1,1 ng/l for AHTN til 18,7 ng/l for DPMI.

De rapporterte verdiene av polysykliske muskstoffer i kloakk tyder på at bruk av disse stoffene ikke er blitt redusert i Norge de siste årene, og kan ha økt.

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1 Introduction

Musk fragrances are an important ingredient in many personal care products. The use patterns cause a large amount to end up in waste water and sewage treatment plants (STP). And exposure of these compounds to the environment occurs mainly through sewage treatment plants effluents into rivers, lakes and seawater. Since musk compounds were for the first time discovered in the environment in the 1980s[1, 2] concerns have arisen over possible toxicity to the aquatic environment, particularly the long term effects.

The use of the so called nitro musks have diminished significantly in the EU, due to regulations. But the use of the polycyclic musks is still high and widespread, primarily HHCB, but also AHTN. These compounds are lipophilic and have been shown to bioaccumulate in aquatic biota and sludge. Because the long-term effects of these compounds are not known, it is important to monitor the amount of polycyclic musks released into the environment.

The purpose of this master thesis was the development of a method to identify and quantify polycyclic musk compounds in sewage samples, with the use of solid phase extraction for sample clean-up and final analysis on GC-MS, and to validate the method and use it to determine polycyclic musk in sewage samples.

2 Theory

2.1 Introduction to musks as a group of different chemicals

Musk fragrances are a group of compounds used as fragrances in perfumes. Their smell is often considered woody and is used as the "bass notes" in perfumes. Musk fragrance was originally obtained from the gland of the musk deer that lives in east Asia[3]. But due to high prices of natural musk, efforts were made to find cheaper synthetic alternatives. Today most musk is produced synthetically, but although trade in natural musk is restricted, the musk deer is still endangered.

The first synthetic musk was synthetized at the end of the 19th century. Since then, a variety of compounds with different structure and functional groups, that all have musky odor have been synthesized. Musk refers to a particular fragrance, and is not a chemical classification, so the chemical structure of musk compounds can vary. Synthetic musks are usually classified by chemical structure in three groups: nitromusks, polycyclic musks and macrocyclic musk.

Today musk fragrances are used extensively in detergents, perfumes, fabric softeners and other personal care products. This use pattern causes a large amount to end up in waste water, sewage treatment plants and ultimately in rivers, lakes, and oceans.

Human exposure to musk is mainly directly from products through the skin. But exposure is also indirectly through drinking water[4], inhalation[5] or from consumption of e.g. fish[6].

2.1.1 Nitro musks

Nitro musks are derivatives of nitrated benzenes. Examples of nitromusks are: musk Xylene, musk Ketone, musk Ambrette, musk Tibetene, musk Moskene, with musk Xylene and musk Ketone being the most important[3]. With the exception of musk Ambrette, which have been shown to be neurotoxic, the acute toxicity of nitromusks is relatively low, but they have a potential for bioaccumulation, and there have been concerns for long term toxic effects[7, 8].

In the 1980's nitro musks were for the first time identified in the environment and were shown to bio accumulate in aquatic organisms[1, 2]. Nitro musk have also been identified in humans[9]. Together with the concerns of toxicity, this led to a reduction in the use of nitro musks in many countries.

In the European union the use of musk Xylene and musk Ketone decreased from 174 and 124 tons in 1992, to 86 and 40 tons in 1998[3]. According to Norwegian national status on emissions from 2011, the annual discharge of musk Xylene, primarily through sewer discharge, have been reduced by 76% from 0,6 to 0,14tons from 1995 to 2009[10]. These reductions of the use of nitro musks in Europe have

gradually been replaced by polycyclic musks, but this shift to polycyclic musk have not been observed in the USA.

In Europe the use of nitro musks is regulated. Musk Ambrette was prohibited for use in 1995, and musk Moskene and musk Tibetene in 1998[3]. Musk Xylene and musk Ketone are still allowed, but use is restricted by EU regulation on cosmetic products[11].

2.1.2 Polycyclic musks

Polycyclic musks are a class of synthetic musk first synthesized in the 1950s[3], containing two or more structural rings. Compared to the nitro musks, they are more stable and not as susceptible to photo-degradation or reduction in alkaline environments, because of the lack of nitro groups. This stability makes them useful for use in detergents. Polycyclic musks are lipophilic and have been shown to bioaccumulate in animal and human tissue[12]. They also show a tendency to be adsorbed, and bioaccumulate in particulate matter, sewage sludge and sediments [13, 14]. The chemical structures of different polycyclic musks are shown in Table 2-1. The most used polycyclic musks are Tonalide (AHTN) and Galaxolide (HHCB), which contribute more than 90% of the total volume of polycyclic musks used[15]. Some relevant physic and chemical properties of these compounds are shown in Table 2-2.

One aspect of the polycyclic musks that is important to consider when discussing metabolism and toxicology is that they have different stereoisomers. This is important because possible toxic effects, uptake in organisms and biodegradation may be stereospecific. Galaxolide has four possible stereoisomers, but only two of those contribute to the musky smell[16]. Similarly AHTN has two possible enantiomers, but only S-AHTN gives a musky odor[17]. However the commercial production of these compounds gives racemic mixtures[17].

Table 2-1 Trade names and chemical structures of different polycyclic musks.

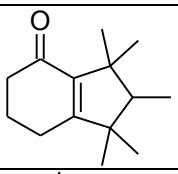
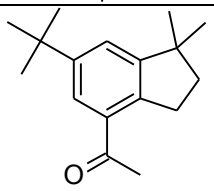
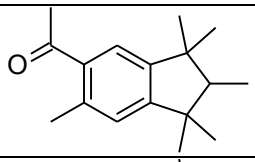
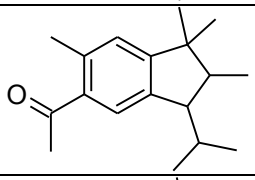
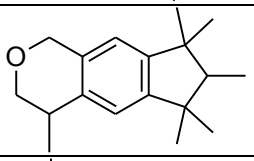
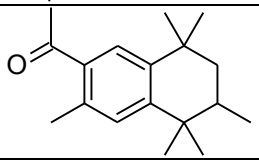
Trade name	Abbreviation	Cas no.	Structure	Mol. Form.
Cashmeran	DPMI	33704-61-9		$C_{14}H_{22}O$
Celestolide	ADBI	13171-00-1		$C_{17}H_{24}O$
Phantolide	AHDI	15323-35-0		$C_{17}H_{24}O$
Traseolide	ATII	68140-48-7		$C_{18}H_{26}O$
Galaxolide	HHCB	1222-05-5		$C_{18}H_{26}O$
Tonalide	AHTN	1506-02-1		$C_{18}H_{26}O$

Table 2-2 Physico-chemical properties of HHCB and AHTN. The values are obtained from European risk assessment reports for HHCB and AHTN[18, 19].

	HHCB	**AHTN**
Partition coefficient n-octanol/water, log Kow	5,3	5,4
Water solubility, mg/l	1,75	1,25
Vapour pressure, Pa	0,0727	0,0682

Of the polycyclic musks HHCB and AHTN are the most widespread, and as a result most studies on toxicity primarily focus on these two compounds.

Polycyclic musks are lipophilic and have been found in a variety of aquatic biota. These findings indicate that polycyclic musks have a potential for bioaccumulation, however as discussed in a By Dietrich[20], several different studies suggest that unlike for example PCBs there is no significant biomagnification of polycyclic musks between different trophic levels, and the concentrations found in aquatic organisms are a direct consequence of exposure to contaminated water. The toxicological threats to aquatic and sedimentary organisms are therefore biggest in water systems with relatively low flow, and high input of effluent from STPs.

The toxicity of polycyclic musks to aquatic species have been studied for algae, daphnia, fish and amphibians, and polycyclic musks have been found to be more toxic to aquatic species than the nitro musks[8, 20]. Based on the levels of polycyclic musk normally detected in sewage water, the risk of acute toxicity to aquatic life have been concluded to be small[20].

EU risk assessment reports have been made for HHCB and AHTN in 2008. The risk assessments conclude that for HHCB and AHTN "There is at present no need for further information and/or testing and no need for risk reduction measures beyond those which are being applied already."

In addition to acute toxic effects, the long term toxicity of polycyclic musks has also been studied, and they have been investigated for possible endocrine disruptive effects, but no firm conclusions have been made[7, 17, 20].

According to studies by RIFM (Research Institute for Fragrance Materials) and IFRA (International Fragrance Association) the total use of HHCB and AHTN in Europe has decreased from 2400 and 885 tons in 1992, to 1427 tons and 358 tons in 2000, respectively[15]. Other polycyclic musks are of less importance, in 2000 AHMI, AITI and ADBI together totaled only 30 tons[15]. A draft for a risk assessment report for HHCB by the US Environmental Protection Agency (EPA), report that the use of HHCB increased from 1275 ton in 2000 to 1700 ton in 2011, reflecting increased demand for fragranced products[21].

Because of their use in detergents and personal care products, a large proportion of the polycyclic musks used are discharged into waste water, and subsequently introduced to the environment through sewage water. Waste water is typically treated in sewage treatment plants, where a large part of the polycyclic musks are removed from the wastewater. The removal percentage can vary greatly between different STPs, but many studies show a removal of polycyclic musks by 80-90 %[18, 19]. The removal by STPs can be attributed to metabolism, chemical breakdown and adsorption to sewage sludge. A study by Kai Bester 2004[13] attributes the majority of this reduction to sorption to

sewage sludge, and less to metabolism and breakdown of the compounds. HHCB and AHTN both have polar transformation products with HHCB-lactone being the most important for HHCB[22].

The highest concentrations of polycyclic musks in the environment are found in wastewater and sewage sludge, but they have been identified in a variety of surface waters around the world, including rivers, lakes and oceans[23]. Because of their lipophilic nature they have also been identified in sediments from rivers and lakes[24]

2.1.3 Macrocyclic musks

The macrocyclic musks have traditionally been more expensive than nitro and polycyclic musks, explaining the low consumption of these compounds. However, their use is expected to increase as cheaper methods for synthesis are developed and the use of nitro musks and polycyclic musks is reduced. There are few studies on the environmental impact of these compounds, but they are thought to degrade more easily in the environment[3, 17].

2.2 Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is an analytical method that combines the strengths of GC and MS. It gives good identification of compounds in a sample because compounds are separated by chemical properties in the GC and by mass and fragmentation patterns in the MS. It is unlikely that compounds in the sample have the same retention time on the GC, and have the same masses and fragmentations in the MS.

The theory in this chapter is based upon from lecture notes[25, 26], and the books: “Mass spectroscopy, a foundation course” by K.Downard[27] and from Chromatography and separation science” by Satinder Ahuja[28].

2.2.1 Gas chromatography

A chromatographic system separates compounds based on different distribution of the compounds between two phases. One phase, the *stationary phase* is held stationary, while the other phase, the *mobile phase* is moved. Compounds are separated by having different distribution between the mobile and the stationary phase, expressed as the partition coefficient, k, given by Equation 1.

$$k = \frac{Consentration\ \ in\ phase\ 1}{Consentration\ in\ phase\ 2} \qquad (1)$$

Because one phase is continuously moving, the compounds are moved through the chromatographic system at different speeds and can be detected or collected separately.

Different types of chromatography are used, for analytical or preparative purposes, respectively. Common examples are: thin film chromatography, high pressure liquid chromatography (HPLC) and gas chromatography (GC).

In gas chromatography the mobile phase is an inert gas, called the *carrier gas*. Common carrier gases include helium, nitrogen, argon and hydrogen. The stationary phase is usually a liquid film, although gas-solid chromatography exists, that coats the inside of the column, or a solid support inside the column, whose purpose is to increase the area of contact between the two phases.

The column is held inside an oven, allowing for controlled temperature programs. Increasing the temperature of the column during a run may reduce retention times and increase resolution for slow-eluting compounds. It can also be necessary to increase the temperature in order to volatilize samples. Samples are injected at the head of the column, with a split/splitless system or directly on the column. A split/splitless system can split the injected sample, so that only a part of the injected sample is injected on the column; which is useful for concentrated samples. A normal injection volume on a capillary column is around 0,1-2 µl. At the end of the separation column there is a detector that detects compounds as they emerge, resulting in different peaks in a detector chromatogram.

The most common types of column used in analytical GCs are capillary columns with an inner diameter of 0.1-0.5 mm, and a length of 10-100m. The column is made of fused silica, and the stationary phase can either be a liquid or a solid, and are usually classified by polarity. Two types exist, WCOT (wall coated open tubular) where the stationary phase coats the wall of the column, and SCOT (support coated open tubular) where the stationary phase is adsorbed to a thin layer of solid support lining the column.

Ideally every compound in the mixture to be separated should be represented in the chromatogram as a narrow, symmetrical peak, however, in practice diffusion leads to a broadening of peaks, and insufficient separation give only partially resolved peaks. The effects of diffusion on column efficiency are given by the van Deemter equation, which describes the relationship between plate height, a measure of column efficiency, and the velocity of the mobile phase. The van Deemter equation is used to find the optimal velocity for a specific column.

Incomplete separation will result in valley or uneven-shaped peaks. The resolution of the separation can be calculated using Equation 2. where t2 and t1 are retention of the unresolved peaks, and W1 and W2 are peak width at half height.

$$Resolution = 2 \times \frac{t2 - t1}{W1 - W2} \quad (2)$$

In addition to diffusion, explained by the van Deemter equation, several other factors reduce the efficiency of the separation. They include damage to the column or solid phase, buildup of contamination on the column or bleeding of the column. Too high sample concentrations injected can also cause overloaded peaks and tailing.

2.2.2 Mass spectrometry

Mass spectrometry (MS) is a method for separation and detection and quantification of compounds in a sample. Mass spectrometers consist of three principal parts, an ion source, a mass analyzer and a detector. Samples are introduced to the ion source and given a charge. These ions are separated in the mass analyzer by electrical and magnetic fields, so that only ions of a particular mass/charge (m/z) ratio reach the detector at any given moment. The MS is operated under near vacuum.

The most common ion source used for GC-MS is electron ionization (EI). In EI-ion sources, a high-energy beam of electrons bombard molecules in the sample, knocking off electrons, forming radical cations. If the energy transferred to the molecules by the electrons is sufficiently large, the cations can dissociate into two smaller fragments, which are either a cation and a neutral radical, or a radical cation and a neutral molecule. The fragmentations given by EI give specific EI-spectra that are constant between different instruments as long as the energy used for ionization is identical. Usually 70 electron volts are applied. At this energy mostly single charged ions are formed. Databases containing EI-spectra are routinely used for identification. Because of the high degree of fragmentations, electron ionization is considered a strong ionization technique. Chemical ionization is an alternative to EI in which a neutral gas, often methane is introduced to the ionization chamber at much higher concentrations than the analyte. The gas is ionized and passes the charge to the analyte. This is a “softer” technique, which causes less fragmentation than EI.

The ions created in the ion source are accelerated by electrical fields into the mass analyzer, which separates them according to m/z. Common types of analyzers include “Time of flight” (TOF), magnetic and electric sector instruments, quadrupoles, and ion cyclotron resonance. Quadrupoles are commonly used in GC-MS. A quadrupole mass analyzer consists of four metallic rods arranged parallel to each other. A voltage consisting of a direct current component and an alternating current

is applied to each pair of rods opposite each other. Ions are passed through the quadrupole, between the rods. The voltages generate electric fields that affect the trajectories of the ions. By changing these fields, only ions of a certain m/z - ratio is allowed to pass through the quadruple to the detector. One of the advantages of quadrupoles over analyzers using magnetic fields is the ability to rapidly change the electric fields, enabling fast scanning of different m/z - ratios. The mass analyzer can either be run in full scan mode or in selected ion monitoring (SIM). In full scan, the quadrupole scans through a range of m/z-values, detecting all fragments, while In SIM mode only selected ion fragments are detected. The SIM mode is preferred for quantification, because more time is spent detecting each of the specific fragments, thereby increasing the sensitivity.

After the mass analyzer ions are passed to a detector. For scanning mass analyzers electron multipliers are common. Charged ions hitting the detector cause electrons to be emitted which in turn cause secondary emission, and so forth creating a cascade of electrons that create a detectable current.

2.3 Solid phase extraction

Solid phase extraction (SPE) is an extraction technique that removes compounds from a sample based on affinity for a solid phase that the sample moves through. Samples are transferred through the solid phase material, and either unwanted contaminants or target compounds are retained. Retained compounds can then be eluted from the column with an appropriate solvent. For a more targeted extraction, the solid phase is washed with a washing solution before target compounds are eluted.

The solid phase materials are usually bought pre-packed by the supplier in cartridges or columns, and are usually classified by their polarity:

- Reverse phase materials have non-polar hydrophobic groups and is used for extraction of non-polar compounds from polar liquids.
- Normal phase materials have polar groups and are used for extraction of compounds with polar functional groups from non-polar liquids.
- Ion exchange materials have charged groups that attract charged compounds.

By carefully choosing the solid phase material and solvents for washing or elution of the columns targeted extractions are possible

2.4 Sewage and Sewage treatment plants

2.4.1 Sewage composition

Sewage is a term that applies to all types of wastewater, but is often a combination of wastewater from multiple sources. Sewage water typically contains a large amount of solid particles, but the composition of sewage is highly dependent on the sources of the wastewater. Common sources are effluents from residential housing, industries and agriculture. In many places of the world surface runoff is also entering the sewage system, and the volumes of sewage water and concentrations can change throughout the year, depending on rainwater and snow melting.

Greywater is a term that refers specifically to residential wastewater from showers, laundry and sinks etc., but not wastewater from toilets.

2.4.2 Sewage treatment plant at Bekkelaget

The sewage treatment plant at Bekkelaget is located in Oslo, Norway and is one of two STPs that treat wastewater from Oslo. It receives an average of 1100 l/s, approximately 30% is industrial wastewater; the rest is a combination of wastewater from households and surface water[29].

The sewage entering the plant first undergoes pretreatment where sand and larger objects are removed. Solid sludge particles in the water are then removed in several large sedimentation tanks. Nitrogen is removed with activated sludge and phosphorous is removed by addition of iron sulphate. The activated sludge is then removed by sedimentation and the sewage undergoes a final filtration before being released at a depth of 50 m in the Oslo fjord.

The treatment efficiency for 2011 was 90% removal of phosphorous, 70% removal of nitrogen and 90% reduction of suspended solid particles [30]. It is assumed that the efficiencies for 2012 are comparable. The sludge removed by the sewage treatment plant is fermented at 55°C, and mainly used in agriculture as fertilizer and for improving soil[30].

3 Quality control in analytical organic chemistry

The practical value of a set of measurements is greatly dependent on the uncertainty associated with the results. The uncertainty of measurements is a combination of accuracy and precision, where accuracy is a measure of how close the measurements are to the true value, while precision is the relative spread of the measurements. The goal of quality control is to minimize systematic and random errors, and ensure repeatability, reproducibility and comparability of the results.

This chapter presents relevant methods and criteria for quality control. The theory in this chapter is based upon: "Quality Assurance and Quality Control in the Analytical Chemical Laboratory" by Piotr Konieczka and Jacek Namieśnik[31] and "Quality control in organic trace analysis" by Michael Oehme[32].

3.1 Quantification by the internal standard method

For analysis by GC-MS the internal standard method is the preferred method for quantification whenever a suitable internal standard can be obtained. The internal standard (ISTD) is a compound that is expected to behave similar to the analyte during analysis, and that can be quantified separately. The ISTD is added in equal amount to all samples, and serves as a reference for the detector response. A reference is necessary because conditions during sample pretreatment and during injection vary from sample to sample, and the absolute detector signal of the analyte will not be constant, even for identical samples. However the analyte signal relative to the ISTD signal, "the relative response of the analyte" remains constant.

A relative response factor, f_i, is calculated from standards with known amounts on analyte and ISTD using equation 3, where $Amount_{ISTD}$ is the amount of ISTD in the samples, $Amount_{analyte}$ is the amount of analyte in the samples, $Area_{analyte}$ is the area of the analyte peak in the chromatogram and $Area_{ISTD}$ is the area of the ISTD peak in the chromatogram.

$$f_i = \frac{\mathrm{Amount_{ISTD}}}{\mathrm{Amount_{analyte}}} \times \frac{\mathrm{Area_{analyte}}}{\mathrm{Area_{ISTD}}} \qquad (3)$$

The relative response factor is usually estimated from a linear regression over several measuring points, where f_i is the regression coefficient. The y-intercept of the regression is usually expected to

be close to zero, and may then be omitted from calculation of unknown concentrations, but if the y-intercept is large it should be included in calculations.

The amount of analyte in samples is calculated using Equation 4, with the response factor and y-intercept from the calibration.

$$Amount_{analyte} = \left(\frac{Amount_{ISTD} \times Area_{analyte}}{Area_{ISTD}} - \text{y-intercept}\right)\frac{1}{f_i} \qquad (4)$$

There are two requirements for choosing a compound for use as an internal standard. The compound should have chemical properties similar to the analyte, but be fully separable from them, and the compound must not be naturally present in the analyzed samples. For MS analysis, deuterated analyte standards fulfill these requirements. They have similar chemical properties, but are easily separated by their mass by the MS and they are not present in environmental samples.

Quantification with internal standard has several advantages:

- The method is more robust towards variable method conditions and matrix effects, because a change that increases the analyte signal is likely to increase the signal of the internal standard as well.
- It is not necessary to know the exact volume of the sample extract injected on the GC-MS.
- If the internal standard is added early in the analysis, the internal standard compensates for losses of analyte during sample preparation.
- If the internal standard is introduced to the samples early in the analysis, the loss of ISTD during sample clean up (recovery) can be calculated, and used as a quality control for every sample.

3.2 Calculating the recovery of the internal standard

The amount of internal standard remaining after sample preparation is a good indicator on the efficiency of the clean-up, and the stability of the method. This value is called the recovery of the internal standard, and is usually calculated as a percentage. According to state of the art quantification in organic trace analysis, the recovery of the internal standard should be higher than 60-80 % [32].

Determining the recovery of the ISTD is done by adding a separate compound called the recovery standard (RSTD) to samples immediately before injection. The recovery of the ISTD (rec. ISTD) is then calculated relative to this. A specific relative response factor (f_r) and y-intercept must be calculated for the ISTD relative to the RSTD. The recovery of ISTD is then calculated using Equation 5. Where $Amount_{RSTD}$ is the amount of RSTD added to samples, $Amount_{analyte}$ is the amount of analyte detected in the samples, $Added_{ISTD}$ is the amount of ISTD added to the samples, $Area_{analyte}$is the area of the analyte peak in the chromatogram, $Area_{ISTD}$ is the area of the ISTD peak in the chromatogram.

$$rec.ISTD = \left(\frac{Amount_{RSTD} \times Area_{ISTD}}{Area_{RSTD}} - \text{y-intercept}\right)\frac{100}{f_i \times added_{ISTD}} \qquad (5)$$

3.3 Linearity of the method

When discussing the linearity of the method, the focus is on the linearity of the GC-MS response. This is usually linear over a wide range of concentrations. It is nonetheless important to verify that the instrument is in fact linear over the range of concentration worked with.

Calibration of the detector response is done with several analyte standards at different concentrations, and internal standard concentrations kept constant. If the response is linear, regression with the method of least squares is performed over the data points, and the coefficient of regression is used as a relative response factor for the analyte, when calculating analyte concentrations with Equation 4.

Residual plots from the regression are a useful way of presenting data so that lack of, or deviation from linearity can be detected, for example outliers, or deviations from linearity at both ends of the detectors linear range.

3.4 Blank samples

A blank sample is a sample that does not contain the analyte. An ideal blank has an identical matrix to the regular samples, but without the analyte. When such a sample is unobtainable a blank sample lacking the matrix is used. A method blank is a blank sample that has undergone the full method. The purpose of blank samples is to estimate the background noise and to identify contaminants.

3.5 Limit of detection and limit of quantification

The limit of detection (LOD) is the lowest concentration of analyte that can be distinguished from the background noise with statistical significance. The limit of quantification (LOQ) is the lowest concentration where quantification with a determined certainty is possibly. There are many approaches for estimating the LOD and LOQ, but both of these values are related to the background noise of the chromatogram.

The background noise of a chromatogram is often displayed as a signal-to-noise ratio (S/N). This is calculated as the height of the signal peak above mean baseline noise, divided by the variance [33].

An S/N ratio of 3 is a common threshold for LOD. LOD for a given peak can be calculated using Equation(6 6, where S/N is the S/N-ratio of the peak and Concentration is the concentration of analyte in the sample.

$$LOD = \frac{3}{\mathrm{S/N}} \times Concentration \qquad (6)$$

This method assumes that the S/N of the analyte peak is linear down to the LOD. This may not always hold true, and LOD should be estimated from samples with a low analyte concentration, close to the values of LOD. For a robust estimate, it should be estimated from multiple samples, and with different methods.

Limit of quantification can either be related to the noise in the chromatogram analogous to determination of LOD. But it can also be determined experimentally, by determining the precision of the method at various concentration levels.

3.6 Retention time

The retention time of compounds is used to locate compound peaks in the chromatogram, this value should be constant between GC-runs, however it is expected that differences in matrix can cause shifts in retention time. However, the retention time relative to other peaks should remain constant, and can be used for identification if necessary.

3.7 Ion-ratio

When analyzing samples with selected ion monitoring (SIM), usually two ion-fragments are detected for each compound. The primary ion is used for quantification, and the secondary ion is used as a confirmation ion. The ratio of the quantification ion peak to the secondary ion peak should remain constant between samples, and should not deviate from reference values by more than 20 % for quantification[32]. Large differences can be an indication of background noise or interfering compounds.

3.8 Chromatographic separation

The GC should ideally achieve baseline separations for all the peaks to be quantified, so that no overlaps with neighboring peaks occur. In practice however, baseline separation is often not achieved and quantification must be done with partial separation. It is important to note that this practice increases the uncertainty of the measurements.

4 Method development

The method used in this thesis can be summarized in four steps: 1) filtration of the samples, 2)cleanup and concentration with SPE-columns, 3) further concentration of SPE-extracts, and 4) analysis of the concentrated samples on GC-MS. This general method for analyzing musk in water matrixes is well documented[17]. The specific columns used in this work, OASIS HLB-columns have also been used previously for extracting musk from aquatic samples[4]. It was however necessary to fit the general method to the equipment available, and to the matrix of the sewage samples. This chapter describes parts of the development of the method, which are relevant for thediscussion of the method. The full method, after these improvements is described in Chapter 5.

During method development greywater was used as a substitute for sewage samples. The greywater analyzed during method development was collected at a small-scale sewage treatment plant at UMB used for experimental purposes. The plant receives greywater and blackwater separately from approximately 50 residential apartments. Sampling was done from untreated greywater and frozen or used immediately. The matrix of the greywater was assumed to be similar to that found in sewage water, but levels of musk in the samples was expected to possibly deviate from sewage samples, because of the relatively low number of households and because the inhabitants, mostly students, are not a representative selection of the entire population.

Because of the distribution and use of polucuclic musks, contamination of the sample can be problem that should be considered.

Method blanks were made as described in Chapter 5.

4.1 Drying of solid phase extraction columns

The OASIS HLB-columns used for solid phase extraction, which will be described in Chapter 5.4.3, is a reverse phase sorbent, where HLB stands for “hydrophilic lipophilic balance”. The sorbent used is a copolymer composed of the hydrophilic N-vinylpyrrolidone and the lipophilic divinylbenzene. According to Waters, the sorbent used in Oasis HLB columns give good retention of compounds, even if the sorbent dries out[34]. It was theorized that this property could be utilized to remove water from the column, and by extension the samples, by drying the sorbent with air before elution, without loss of analyte.

Without drying the column, elution of the columns introduces water to the samples that then have to be removed by an additional step in the analysis. This possibly lowers the recovery and increases

the chances of contaminating the samples. Drying the column bed before elution would effectively remove water from the samples.

Initially, SPE columns were dried before elution by drawing air through the column with a water-jet pump for approximately 45 minutes, as done in a master thesis from 2008[35].This succeeded in removing the water from the column. However this process was later abandoned because the extraction of the paper filters discussed in chapter 4.3.1 introduced additional water to the samples, and a step to remove water from the sample would anyway have to be added in the method.

4.2 Filtration of samples

Loading sewage water directly on the Oasis column without any filtering was tried, but clogged the column. For filtration of the samples, one type of disposable in-line filters was tested: Millex® syringe glass fiber filters from EMD Millipore, US, with diameter: 25mm and pore size: 2µm. Inline filters have several advantages over filtration with an open funnel: the ability to connect the filters directly to the SPE-column simplifies the lab-work, and reduces the chances of contaminating the samples.

The filters where tested by filtrating greywater samples. These tests showed that the filters had too little capacity, and got clogged. This could possibly have been rectified by using a filter with larger capacity, a filter with larger pore size or an additional pre-filter.

It was however decided to filter sewage samples with paper filters, in open funnels, as described in chapter 0, because these paper filter were readily available, and proved to give sufficient filtration of the samples before loading onto the SPE-columns. The use of paper filters gave additional problems with analyte retention in the filters, discussed in Chapter 4.3.1.

4.3 Method development for improving recovery

A preliminary analysis of a method blank and a greywater sample gave low ISTD recoveries: 11,6 and 27,7%. This was unexpected, and showed that modifications to the method were necessary.

Table 4-1 list ISTD recoveries of samples analyzed during method development that contributed to important changes to the method, and illustrate important points of interest. Sample numbers are included for easy reference. The results show a trend of increasing recovery. This increase is the result of modifications to the methods throughout the method development. Some of the changes and improvements to the method and the practical lab work were planned, others were results of increased experience with the equipment used, most importantly the use of rotary evaporator and N_2 stream for concentration of the samples. It is likely that both the planned changes and increased experience contributed to the increase in recovery seen in Table 4-1. For many of the changes, only a single sample was analyzed.

The factors mentioned above led to highly variable method conditions and high uncertainties, which implicates that the preliminary results during method development, shown in Table 4-1, must be discussed with care, and they illustrate the improvements to the method.

Because the conditions during analysis were subject to changes, describing the exact conditions for the analysis of all the samples in Table 4-1 would be extensive. Instead the major differences between the analyses of the samples are described.

The most important changes included the filtration step of the method. Cleanup with SPE-column, concentration of SPE-extracts with a combination of rotary evaporator and N_2 and the analysis on the GC-MS are with only small changes identical to the methods to be described in Chapter 5.

During method development the old TCN-standard listed in Table 5-2 Table 5-2was used as RSTD. The results of the calibration of this standard with AHTN-D3 are shown in Chapter 6.2.

4.3.1 Retention in paper filters:

Two analyzed samples, a method blank and a grey water sample, (samples 053001 and 053004 respectively) gave low recoveries of ISTD: 12% and 28% respectively.

The reason for these low recoveries where hypothesized to be that the ISTD was retained in the filtrating step. The filters used for filtration were held in a sintered glass funnel, and retention could be in the sintered glass funnel, the filter, retained sewage particles or most likely a combination of all three. A sintered glass funnel is usually considered as inert and should not adsorb the ISTD. However, the sintered glass funnel used had previously been used to filter sewage samples and if inadequately cleaned, sewage sludge particles could have been trapped in the sintered glass disc.

In order to test for retention during filtration, two parallel methods blanks (sample 060110 and 060101), with and without filtration were analyzed, and gave recoveries of 11 % and 66%

respectively, proving that retention of ISTD during filtration was a major cause for the low recoveries observed.

Retention in the sintered disc was confirmed by filtering a method blank using only the sintered disc and extracting the sintered disc with acetone (sample 61310). This resulted in a recovery of the ISTD of 45%. Although the retention in a separate confirmed clean sintered glass disc was not tested, it is safe to assume that the greater part of the recovery observed in sample 661310 was due to ISTD adsorbed to sewage particles trapped in the sintered glass, and not the sintered glass itself.

The analysis of sample 661310 also showed that the ISTD retained by the sintered glass disc was readily extracted by acetone. In order to avoid issues with the reproducibility of the method it was decided to use a glass funnel without a sintered glass disc. Using this funnel a method blank was filtrated (sample 061402), and the filter extracted with acetone. Recovery of ISTD in the filter extract was 28 %. This confirmed that a significant amount of musk was absorbed by the paper filter. Because of the musk retention in the paper filters and by the sewage particles, it was decided to extract filters with acetone and add the extracts to the SPE-columns after loading of sewage samples, as given in the final method described in Chapter 0. The extraction of the paper filter was tested by analyzing a method blank (062503), which gave a recovery of 61%.

The extraction of the filters added an extra step to the analysis, increasing possible contamination. Inert fiberglass filters would possibly give less retention than the paper filters used, but because of retention to solid sewage particles trapped by the filter, filters would still need to be extracted if the total amount of musk in the samples was wanted, and not just the amount in the aqueous partition.

4.4 Final changes to the method

An influent sample from the STP at Bekkelaget was analyzed (062204), and gave a recovery of the ISTD of 61,4 %.

Sewage water matrix devoid of musk for use as blanks was not readily available. As a substitute, sewage water was filtrated and extracted with the SPE-columns to remove musk compounds. A filtrated and extracted greywater sample was analysed (062203) and showed no presence of polycyclic musks, indicating good efficiency of the Oasis SPE-columns. A problem with using extracted samples as blanks is that a large portion of the matrix is removed by filtration and extraction. However, the results indicate that it may be a feasible method for making blank samples that includes the aqueous part of the matrix.

Extracted greywater or sewage water samples were not used for method blanks in this thesis because of time constraints, and because usage of extracted samples as blanks would require knowledge of the repeatability of the complete removal of polycyclic musks from the samples, which was not sufficiently, proven.

For all the samples mentioned above iso-octane was used as solvent for spiking solutions, including addition of ISTD to samples. Musks have limited solubility in water, and to increase the solubility of the standards, new solutions were made with acetone as solvent, rather than iso-octane. Using these new standards a spiked method blank (62706) and a greywater sample (62707) were analyzed. These tests showed recoveries of 68 and 86%. Acetone is a more volatile solvent than iso-octane, and evaporation from standards is a problem. By using acetone, thedecrease in precision is sacrificed for the better accuracy achieved by better solubility.

A summary of the observations and conclusions made due to the changes are shown in Table 4-1.

Table 4-1 Sample numbers, method development samples, recoveries of ISTD and observations and conclusions that gave progress to the method development. Sample numbers are included for easy reference. There is significant uncertainties associated with the calculated recoveries, and the values are mainly relevant in the context of their discussion in the text.

Sample number	**Description of sample and analysis**	**ISTD Recovery %**	**Observation/conclusion**
053001	Method blank	12	ISTD possibly retained by sintered glass funnel.
053004	Greywater sample	28	
060101	Method blank without filtering	66	ISTD confirmed retained by sintered glass funnel.
060110	Method blank with filtering	11	
061310	Filtration of method blank with sintered funnel, followed by extraction of the funnel	45	ISTD readily extracted from sintered glass funnel. ISTD assumed to be retained on sewage particles trapped in sintered glass funnel.
061402	Filtration of method blank with paper filter, followed by extraction of the filter	28	A significant amount of ISTD was adso0rbed by the paper filter.
062203	Extracted greywater	51	Extraction of greywater is a viable option for making blank samples with the aqueous part of the matrix.
062204	Sewage sample from STP at Bekkelaget	61	The final changes to the method gave consistent recoveries of ISTD.
062503	Method blank	61	
062706	Method blank (Spiked)	68	
062707	Greywater	86	

5 Methods and materials

There are considerable differences in the methods and materials used in method development and the final analysis.

Most of the methods chemicals and equipment described in this chapter were also used during development of the cleanup method, but because changes concerning the practical lab work where made throughout the development, the specific methods and materials used are described separately in Chapter 0.

All glassware was washed three times with acetone and allowed to dry under a fume hood before use. The volumetric flasks used in the analysis were of A quality.

5.1 Chemicals

Deionized water used throughout the analysis.

Table 5-1 Chemicals used in the analysis

	Cas nr.	**Purity**	**Supplier**
Acetone	67-64-1	puriss. p.a ≥99,5 % (GC) ACS reagent, reag. Ph Eur	Sigma-Aldrich Norway AS
N-hexane	110-54-3	puriss. p.a ≥99 % (GC) ACS reagent, reag. Ph Eur	Sigma-Aldrich Norway AS
Methanol	67-56-1	Chromasolv, for HPLC ≥99,9 %	Sigma-Aldrich Norway AS
Iso-octane	540-84-1	≥99,5 % (GC) ACS reagent, reag. Ph Eur.	Merck KGaA, 64271 Darmstadt, Germany

5.2 Analytical standards

Crystalline standards of musk compounds (Table 5-2 Table 5-2) were used during method development. For the final quantification of sewage samples from Bekkelaget, analytical standards AHTN and HHCB of a higher purity were obtained. The crystalline standards of DPMI, ADBI, AHDI and ATTI were used throughout the lab work.

Two separate analytical standards of 1,2,3,4-tetrachloronaphtalene (TCN) were used in the lab work (Table 5.2). An old TCN-standard with expiration date in 2003 was employed for the development of

the method, and a new crystalline standard of TCN was used for the final analysis of sewage samples. The separate TCN standards gave different response factors, and separate calibrations were made for each. Analytical standards were stored at approximately -8 °C.

Table 5-2 Analytical standards

Compound	**CAS**	**Supplier**	**Purity (%)**
DPMI (cashmeran) (crystalline standard)	33704-61-9	LGC Standards GmbH, Germany	89,5
ADBI (celestolide) (crystalline standard)	13171-00-1	"	99,8
AHDI (phantolide) (crystalline standard)	15323-35-0	"	93,1
ATTI (traseolide) (crystaline standard)	68140-48-7	"	83,2
HHCB (galaxolide) (crystalline standard)	1222-05-5	"	53,5
AHTN (Tonalide) (crystalline standard)	1506-02-1	"	97,9
HHCB (Galaxolide) (100µg/ml)	1222-05-5	"	76
AHTN (Tonalide) (100µg/ml)	1506-02-1	"	99
AHTN-D3 (100µg/ml)	Unknown	Dr. Ehrenstorfer Augsburg, Germany	99,5 (isotopic purity: 99%)
TCN (1,2,3,4-tetrachloronaphthalene) expiry date: 2003	20020-02-4	Unknown	Unknown
TCN (1,2,3,4-tetrachloronaphthalene)	20020-02-4	Sigma-Aldrich, Schnelldorf, Germany	Unknown

Working standards with iso-octane as a solvent were made from all the analytical standards in Table 5-2 Analytical standards Table 5-2. In addition, working solutions with acetone as a solvent where made for the high purity standards of HHCB and AHTN and for AHTN-D3. The acetone working standards where used for adding standards to water and sewage samples.

A 100 µl Hamilton syringe obtained from Hamilton Co. Nevada was used for making working standards for HHCB and AHTN from the higher purity standards, and disposable glass-micropipettes with a micropipette controller were used for making standards, and making standard additions to samples: BLAUBRAND intraMARK micropipettes with a volume of 10, 25, 50, 100, 200µl were from Brand Gmbh + CO. KG, Postfach 1155 97861, Wertheim Germany.

Biohit Eline autopipettes, from Biolite, Finland were used for the making of calibrations with the standards of lower purity, as well as during method development.

The stability of the primary standards were checked gravimetrically when the standards where used.

5.3 Sampling of sewage samples

The sewage treatment plant at Bekkelaget routinely collects 24 hour batch samples of unprocessed sewage water (influent) and processed sewage water (effluent). 500 ml samples were collected from these batch samples on 08.05.2012 and 09.05.2012. The samples were collected and frozen approximately -8 °C.

5.4 Sample cleanup

The method for cleanup of sewage samples is summarized in Figure 5-1.

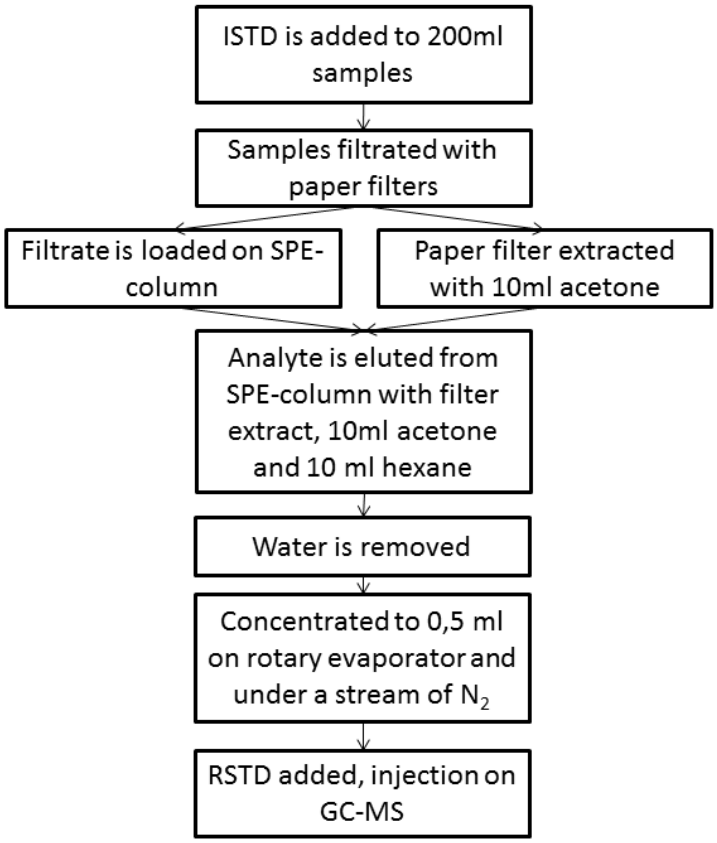


Figure 5-1 Overview of the method, showing the major steps in the analysis. Each step is described in more detail below.

5.4.1 Sample preparation

Frozen sewage samples were thawed at room temperature and transferred to 200ml volumetric flasks. Method blanks were made with 200ml water. 125 ng ISTD in 25µl acetone was added under magnetic stirring to all samples.

5.4.2 Filtration

Samples were filtered using qualitative paper filters with particle retention of 17-30 µm and a diameter of 130mm, from VWR international, Leuven, Belgium. The filters were held in a glass funnel, and the samples were drawn through the filter using a water jet vacuum pump.

The used paper filters were extracted using approximately 10ml of acetone added to the filter. The acetone was drawn through the filter with vacuum.

5.4.3 Solid phase extraction

Filtered samples were rinsed with Oasis HLB 6cc solid phase extraction columns packed with 500 mg of sorbent with particle size 60µm and pore size 80Å obtained from Waters Corporation, Milford, Massachusetts, USA. The columns were placed in an Agilent Sampling 20 position vacuum manifold, and vacuum was made by a water jet pump. The columns were prepared with 10ml of methanol followed by 10ml of water, and were not allowed to dry before samples were loaded. The preparation procedure for the OASIS columns was adopted from an earlier master thesis from 2008[35]. The filtered samples were added to the columns at a rate of approximately 10ml/min.

Elution of the SPE-columns was done with a total of 20 ml acetone and 10ml hexane. The 10 ml of acetone used for extracting the used paper filters were added to the columns, followed by 10ml acetone and finally 10 ml hexane. The procedure for elution of the columns were adopted from an article by William D. Wombacher and Keri C. Hornbuckle [4].

Any water present in the sample formed a water phase that was removed with a 25ml glass pipette.

For selected samples a breakthrough test of the SPE-column was made by extracting filtered sewage samples with two SPE-columns connected in a series, and eluting the columns separately.

5.4.4 Concentration

The samples were concentrated down to 2-3ml using a Heidolph VV2000 rotary evaporator at 55°C, and a pressure of 550-600mBar. The samples were then further concentrated under a gentle stream of N_2 to approximately 0,5ml. Samples were transferred to 1,5 ml GC vials, and 50 ng of recovery

standard (TCN) was added. Vials and crimp lids with septums were obtained from VWR international, Leuven, Belgium.

5.5 GC-MS analysis

GC-MS analysis was performed on a Perkin Elmer Autosystem XL-GC Turbomass MS, Modell 640E806241 equipped with a WCOT VF-5ms fused silica column, length: 25m, ID: 0,25mm, df: 0,25µm, from Crawford Scientific™ Ltd, Lanarkshire, Scotland, UK. Carrier gas was helium of grade 5.5, obtained from Oslo, Norway.

The temperature program was adopted with small changes from an article by Gatermann et al. 1999[36]. The program used was: 70°C [2min], 6°C/min -> 250°C. After every run the column was cleaned with 45°C/min -> 300[0]. GC flow: 15ml/min, pressure: 60 psi.

The autosampler had a sampling rate of 1,56250 pts/s, and the needle was cleaned with 2 pre injection solvent washes, 2 pre injection sample washes and 2 post injection solvent washes. 1µl aliquot of the samples was injected with splitless injection.

The MS instrument was a quadrupole with an EI ion source. Filament voltage: 70V.

EI-MS spectra were made for all analyzed compounds by analyzing pure standards in full scan mode from 50 to 350 m/z. Full scans were also used to identify compound peaks in the chromatogram and determine retention times of compounds.

Selected ion monitoring (SIM) was used for quantification. A SIM program was made that included two ions for each analyzed compound, one quantification ion and one secondary ion chosen from the full scan EI-spectra. The SIM program used is shown in Table 6-1. Compound peaks in the chromatograms where integrated by “QuanLynx v4.1” with no smoothing of the peaks. In addition all integrated chromatograms were controlled manually. Linear regression of calibration data was done using method of least squares with no weighting of the curve.

6 Results

6.1 Identification and integration of chromatogram peaks

Pure standards of musk compounds and internal- and recovery standard were analyzed on the GC-MS in full scan mode. The identity of peaks in the full scan chromatogram was determined by comparing their EI-specta with spectra from the NIST mass spectral library [37]. Retention times, quantification and secondary ions, and reference ion ratios for the analyzed compounds where determined from the analysis of pure standards. The results are shown in Table 6-1. A chromatogram of the analyzed musks is shown in Figure 6-1.

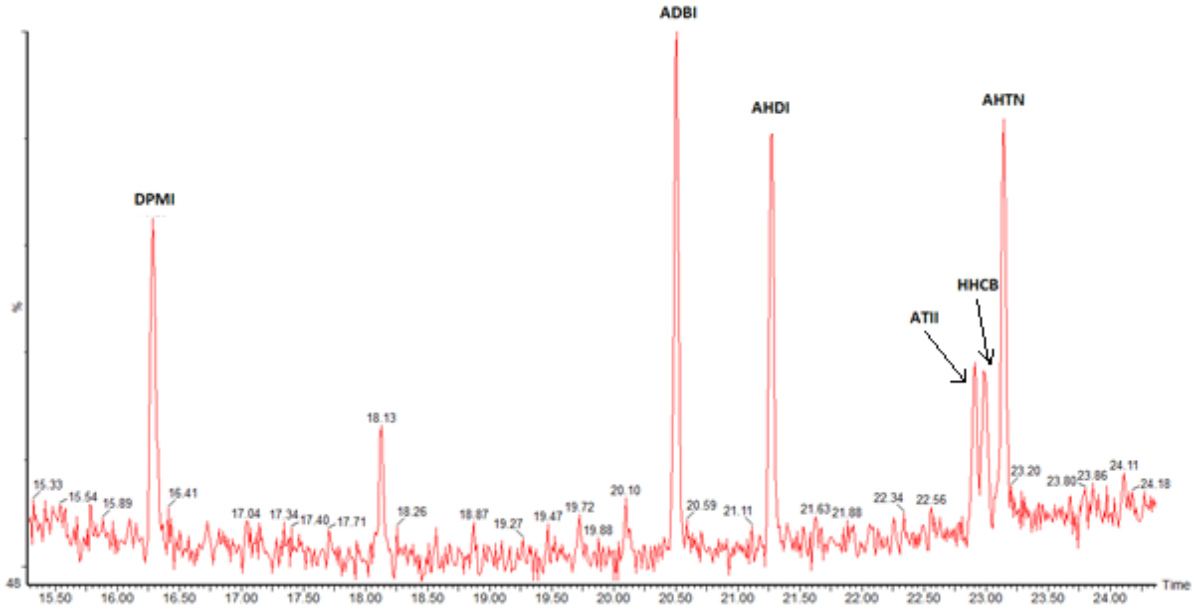


Figure 6-1 Full scan chromatogram of the analyzed musks. The retention times shown in the chromatogram deviates from the values reported in Table 6-1, because approx. 5 cm of the head of the column was cut, after this chromatogram was made.

Quantification- and secondary ions were chosen based on intensity of the fragments and their m/z-ratios. Peak identities were confirmed by calculating percent deviation of peak ion-ratios with reference ion-ratios (Table 6-1).

Table 6-1, Ion ratios, quantification ion, secondary ions and retention times were derived from analyses of pure standards. The quantification ion and secondary ions were included in the SIM-program used for quantification.

Compound	Reference Ion-ratio	Quantification ion	Secondary ion	Retention time (min)
DPMI (Cashmeran)	1,84	191	206	15,62
ADBI (Celestolide)	2,88	229	244	19,79
AHMI (Phantolide)	4,90	229	244	20,59
ATII (Traseolide)*	Unknown	215	173	Unknown
HHCB (Galaxolide)	2,42	243	213	22,28
AHTN (Tonalide)	4,25	243	258	22,45
AHTN-D3	3,81	246	261	22,41
TCN	1,25	266	264	24,68

*The quantification ion and secondary ion for ATII was not included in the SIM program.

The different analyzed compounds have distinguishable EI-spectras, shown in Appendix 1, but because of the similarities in molecular structure for the polycyclic musks several of the analyzed musks share base peaks. This is a problem when the same compounds are not fully separated by the GC-MS.

Table 6-1 show that DPMI, AHTN -D3 and TCN were all easily separated from the other standards by the GC-MS. ADBI and AHMI share the same quantification ion and secondary ion, but shows a difference of 50 seconds in retention time.

AHTN and HHCB share the same quantification ion and the peaks were not fully resolved. The resolution for the separation was calculated for pure standards using Equation 2, and gave a resolution of 1,1. This resolution theoretically gives a peak overlap of less than 2%[28], however, the chromatograms for analyzed sewage samples shoved potentially larger overlap of the peaks, illustrated in *Figure 6-2*.

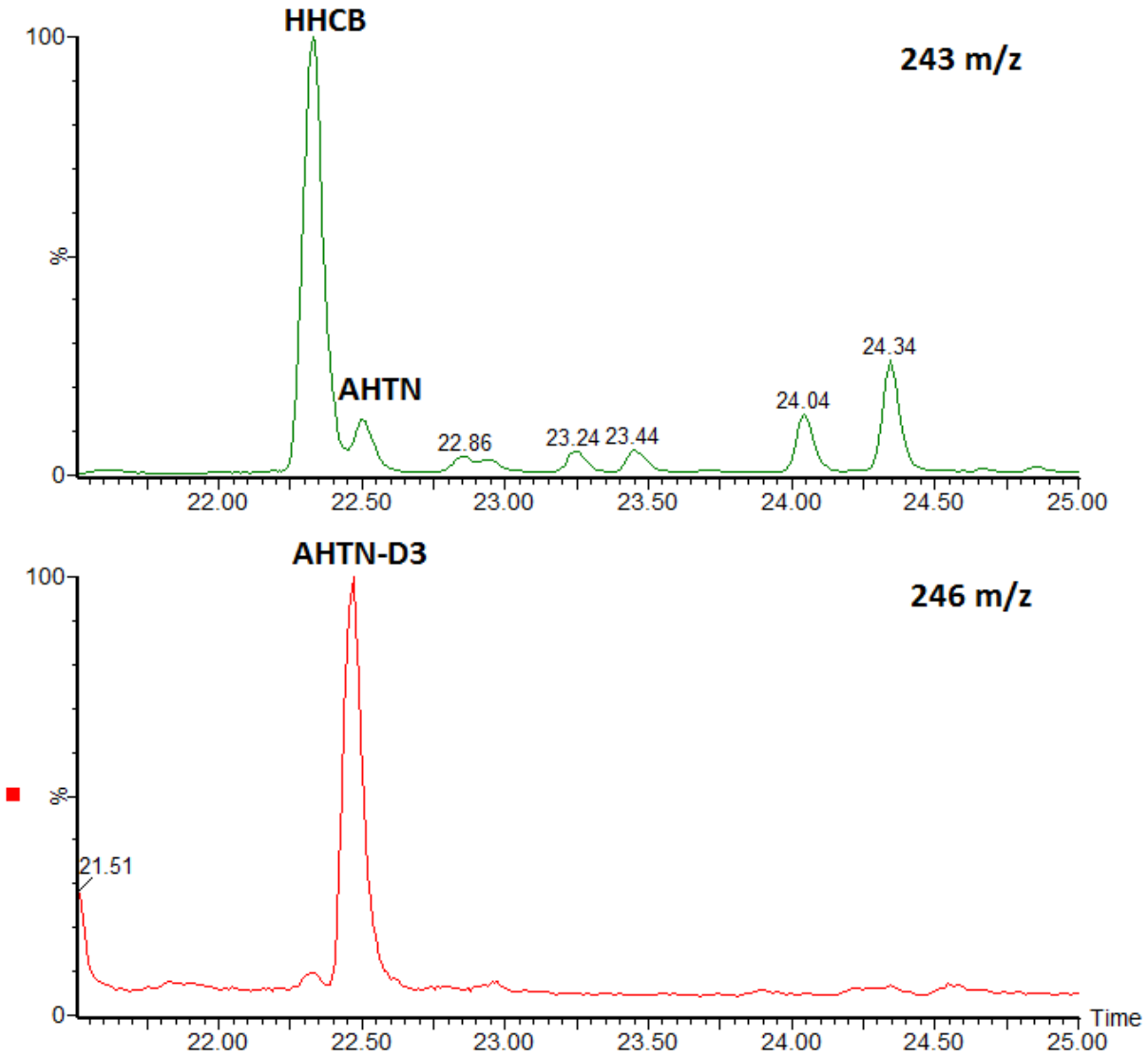


Figure 6-2 Chromatogram of the quantification ion of HHCB and AHTN (243 m/Z) and for AHTN-D3 (256 m/z.), for influent sample 1.

ATII and HHCB were only partially resolved in the chromatogram. The analyzed ion fragments for ATII are not present in large amounts in the full EI-spectra for HHCB, but a preliminary analysis of sewage water showed large amounts of HHCB, which was assumed to mask any ATII that may have been present in the sample. Because of problems with identification and low levels expected in the sewage samples it was not included in further analysis, as discussed in Chapter 7.1.

6.2 Calibration of detector response

As described in Chapter 5.2, for many of the compounds analyzed, multiple analytical standards were used. Data and results from the different calibrations are described below, and coefficient of regression, relative response factor, y-intercept and the range of concentrations used for the calibrations are shown in Table 6-2. The linear regression and plots of residuals are shown in APPENDIX 2.

Calibrations of DPMI, ADBI and AHDI were made from 5 standards samples containing 25, 50, 100, 200 and 400 ng, respectively of all three compounds. 125 ng of ISTD was added to all samples. The calibration of DPMI gave a y-intercept that deviated significantly from 0, while y-intercepts for ADBI and AHDI were close to 0. All the calibrations gave coefficients of regressions close to 1.

As described in Chapter 5.2, two separate standards of HHCB and AHTN were obtained, standards of higher purity and standards of lower purity. The standard of higher purity was used for quantification.

Calibration with the HHCB standards of high purity was done over 4 data points, with the amount of HHCB in the calibration samples distributed exponentially from 200 to 4000ng. 125ng of ISTD was added to all the samples. Calibrations of the HHCB standards of lower purity were done over 5 data points, with the amount of HHCB in the calibration samples equally distributed from 240 to 19200ng. 118ng of ISTD was added to all the samples. The two calibrations gave similar relative response factors and y-intercepts. The calibrations gave coefficients of regression close to 1.

Calibration with the AHTN standards of high purity was done over 4 data points, with the amount of AHTN in the calibration samples distributed exponentially from 100 to 2000ng. 125ng of ISTD was added to all the samples. Calibrations of the AHTN standards of lower purity were done over 5 data points, with the amount of AHTN in the calibration samples equally distributed from 10 to 400ng. 118ng of ISTD was added to all the samples. The two calibrations gave different relative response factors and both y-intercepts deviated significantly from 0. Both calibrations gave coefficients of regression close to 1.

As described in Chapter 5.2, two separate standards of TCN were used as recovery standard: a new TCN-standard was used for the analysis of sewage samples, and an old standard solution was used during method development.

Separate calibrations were made for each standard. Calibration with the new TCN-standard was done over 5 data points, and the amount of AHTN-D3 in the calibration samples were distributed

exponentially from 25 to 500ng, and 50ng of TCN was added to the samples. Calibration with the old TCN-standard was also done over 5 data points, and the amount of AHTN-D3 in the calibration samples were distributed equally from 50 to 150ng, with 25ng of TCN added to the samples. The results of the calibrations are shown in Table 6-2.

Table 6-2 Data and results from the calibration of HHCB, AHTN and AHTN-D3, using all the different analytical standards Table 6-2 shows that there are large variations in the y-intercepts for the calibrations of the different standards. Comparing y-intercepts and the range of the standard samples used for calibration indicate a pattern where deviations of y-intercept from 0, are correlated with concentrations in the standard samples.

Table 6-2 Data and results from the calibration of HHCB, AHTN and AHTN-D3, using all the different analytical standards. The ranges covered by the calibrations are shown as ng added to the samples, and as concentrations equivalent to water samples analyzed.

Analytical standard	**Coefficient of determination R^2**	**Relative response factor**	**Y-intercept**	**Range of the standard samples for calibration (ng)**	**Range of the standard samples for calibration ng/l**
HHCB (Galaxolide), higher purity standard	0,999	0,773	101,9	200 - 4 000	1 000 - 20 000
HHCB (Galaxolide), low quality standard	0,998	0,767	92,8	240 - 19 200	1 200 - 96 000
AHTN (Tonalide), higher purity standard	0,999	1,133	48,6	100 - 2 000	500 - 10 000
AHTN (Tonalide), low quality standard	0,998	1,364	20,7	10 - 400	50 - 2 000
AHTN-D3 (Internal standard)(new TCN standard)	0,992	0,620	-8,6	25 - 500	125 - 2 500
AHTN-D3 (Internal standard)(old TCN standard)	<1	0,824	-2,1	50 - 150	250 - 750
DPMI (Cashmeran)	0,994	2,039	-29,4	25 - 400	125 - 2 000
ADBI (Celestolide)	0,999	1,873	-1,4	25 - 400	125 - 2 000
AHDI (Phantolide)	0,998	1,912	2,3	25 - 400	125 - 2 000

6.3 Limit of detection and limit of quantification

6.3.1 Limit of detection

Limits of detection (LOD) were calculated with Equation 6, an analyte peak from a single standard for each compound. The estimates are shown in Table 6-3. The concentration of the standards, and therefore the amount of compound injected on the GC varied greatly for the different injections. Table 6-3 lists the concentration of samples used for determining LOD.

The estimates for LOD listed in Table 6-3 are more correctly described as the instrument detection limits (IDL). Due to matrix effects method detection limits (MDL) may be higher. But specific MDL where not estimated, and the estimates in Table 6-3 are therefore used as LOD.

Table 6-3 Estimated values of limit of detection (LOD). The estimated detected limits and the sample concentrations are reported as ng/l of analyzed water samples.

Compound	LOD ng/l	Concentration of samples used to estimate LOD (ng/l)
DPMI (Cashmeran)	18,7	57
ADBI (Celestolide)	12,1	57
AHMI (Phantolide)	11,2	57
ATII (Traseolide)	3,2	600
HHCB (Galaxolide)	1,1	25
AHTN-D3	3,1	250

6.3.2 Limit of quantification

The calibrations described in Chapter 6.2 gave large y-intercepts for several of the analyzed compounds. In addition, the calibrations were based on standard samples with a relative long span of concentrations. For these reasons, the calibration data were unsuited for estimating limit of quantification (LOQ). discussed in Chapter 7.2. Instead, LOQ was set as the lower end of the linear range established by the calibrations used for quantification (Chapter 6.2).

Table 6-4 Limits of quantification for the different analyzed compounds. The estimated detected limits and the sample concentrations are reported as ng/l of analyzed water samples.

Compound	**LOQ (ng/l)**
DPMI (Cashmeran)	125
ADBI (Celestolide)	125
AHMI (Phantolide)	125
ATII (Traseolide)*	1 000
AHTN	500
AHTN-D3	125

6.4 Method blanks

Two method blanks were analyzed, with and without the addition of ISTD. RSTD was added to all blanks. The levels of musk in the method blank without ISTD were below the detection limit, while in the blank with ISTD added the HHCB level was detected with a S/N of 4. The recovery of internal standard in the blanks is shown in Table 6-5.

In addition to the method blanks described above, 3 method blanks were spiked with HHCB and AHTN in increased amounts for the intended purpose of detecting lack of linearity in the recoveries. However, no calibrations of HHCB or AHTN were made, and the recoveries in the analyzed samples could not be calculated. The recoveries of ISTD are however still relevant. A breakthrough test was made for the spiked blank with the largest concentration. The test showed considerable breakthrough. Recoveries of ISTD for method blanks and the breakthrough sample are shown in Table 6-5.

Table 6-5, Recoveries calculated for method blanks. Levels of musk in the blanks were below LOD.

Sample	**ISTD rec %**	**Spiked concentration of HHCB ng/l**	**Spiked concentration of AHTN ng/l**
Method blank	n/a		
Method blank + ISTD	79,3		
spiked blank 1	73,0	500	250
spiked blank 2	81,2	2 000	1 000
spiked blank 3	60,9	20 000	10 000
breaktrough of spiked blank 3	41,5		

6.5 Analysed sewage samples

Results from the analysis of wastewater sample from the STP at Bekkelaget, recovery of ISTD and concentrations of HHCB and AHTN both in influent and effluent samples, as well as breakthrough of the influent samples are shown in Table 6-6. The data show that HHCB, and to a lesser degree AHTN are the major polycyclic musks present in sewage water. DPMI, ADBI or AHDI were not detected in any of the analyzed sewage samples.

Ion-ratios for HHCB were within 20% of the reference values shown in Table 6-1. For AHTN, deviations from reference values were 35-41% for effluent samples and 20 and 23 % for influent samples.

Table 6-6 *Results from the analysis of sewage samples from the sewage treatment plant at Bekkelaget. Samples are listed with the date of sampling, and numbering if more than one sample was taken. Concentrations of HHCB and AHTN were calculated from the amount in 200ml samples. DPMI, ADBI or AHMI were not detected in any of the sewage samples.*

Sample	**IS rec %**	**HHCB (ng/l)**	**AHTN (ng/l)**
Bekkelaget effluent sample 08.05.2012 (1)	107	1843	67*
Bekkelaget effluent sample 08.05.2012 (2)	89	1967	85*
Bekkelaget effluent 09.05.2012	97	1858	64*
Bekkelaget influent 08.05.2012	94	7040	486*
Bekkelaget influent 09.05.2012	97	5653	365*
Breakthrough test of Bekkelaget influent 08.05.2012	not detected	HHCB identified** S/N = 11	
Breakthrough test of Bekkelaget influent 09.05.2012	not detected	HHCB identified** S/N =16	

**Signal was outside the linear range of the calibration used.
*Because ISTD was not detected, the amount of HHCB in the breakthrough samples could not be calculated.

The removal percentage of HHCB and AHTN by the sewage treatment plant (STP) was calculated to be 70% for HHCB and 83% for AHTN. The removal percentages were calculated from the average concentration of analyte in influent and effluent samples in Table 6-6, without regard of the day of sampling. The percent reduction of concentration through the STP was calculated with Equation 7.

$$Reduction\ \% = 100 * \left(1 - \frac{average\ concentration\ in\ outlet\ samples}{average\ concentration\ in\ inlet\ samples}\right) \qquad (7)$$

7 Discussion

7.1 Identification

All the analyzed compounds were unambiguously identified from full scans of standard solutions, and the analysis on the GC was sufficient for separating all the analyzed compounds. However, some of the peaks were only partially separated, and this lead to problems when analyzing sewage samples, as discussed below.

Early analyses showed that ATII and HHCB were only partially separated on the GC, and because of the large amounts of HHCB expected in the samples from the STP at Bekkelaget and the low levels of ATII expected, it was deemed unlikely that ATII could be detected in the sewage samples. It was therefore not included in the final analysis. This is in accordance with an earlier study that analyzed sewage sludge from Bekkelaget, and reported the amount of ATII to be between 1,6 be 1,7 % of the amount of HHCB[38].

HHCB and AHTN peaks were not fully separated in the chromatogram, and the same quantification ion was used (Table 6-1). The resolution for the separation was calculated to be 1,1 (Chapter 6.1). For the analysis of sewage samples, the large differences in concentrations of HHCB and AHTN in the samples, and therefore peak area, together with tailing of the HHCB peak, illustrated with Figure 6-2, result in an increased uncertainty for the integration of the AHTN peak. This problem was observed for both the influent and effluent samples. Choosing a different quantification ion for AHTN could have reduced this problem, but no ideal alternative was found. Choosing an ion fragment with lower intensity, for example m/z: 159, 187 or 201 as quantification ion for AHTN would reduce the interference from HHCB, but would increase the LOD for AHTN. A better solution would be to improve the separation of HHCB and AHTN, and reduce tailing of HHCB, by optimizing the GC-program.

The ion ratios for AHTN in sewage samples (Chapter 6.5) deviated significantly from the reference value, but seemed to be relatively constant between samples. The deviations were most likely caused by the partially resolved HHCB and AHTN peaks discussed above.

7.2 Calibration

It was assumed that the calibrations done with analytical standards of different purity would give similar relative response factors and y-intercepts. All the calibrations gave coefficients of determination close to 1, indicating good linearity; however, several of the calibrations gave y-intercepts that deviated significantly from 0, as shown in Table 6-2. In GC-MS with SIM-mode there is usually little background noise and the linear range is long. The y-intercept is therefore expected to be close to 0, and the y-intercept can then be related to the LOD.

As the calibrations were done with analysis of a single parallel at each concentration, and with relative large concentrations, it is possible that the large deviations of y-intercept from 0 are due to random variations in sample concentrations. However, the similar relative response factors and the high y-intercepts for the two calibrations of HHCB, suggest that this is not the case. It is instead suggested that these large deviations are a result of the high concentrations in the standard samples used for making the calibrations (where also the lowest concentrations were relatively high), and a possibly nonlinear response. Because of the high concentrations, even a small deviation from linearity would give a large change in the y-intercept. The trend described in Chapter 6.2, that the largest y-intercepts where observed for the calibrations with the highest standard concentrations, seems to confirm this theory. The two calibrations of AHTN gave different relative response factors and y-intercepts. As the concentration ranges used for making the calibrations of AHTN are different, this seems to support the above argument.

From the arguments above it is concluded that the detector responses may deviate from linearity. Since the y-intercept is assumed not to relate directly to background noise, the y-intercept is included when calculating the amount of analyte in unknown samples, using Equation 4. However, as shown in Table 6-2, all the calibrations gave coefficients of regression close to 1, indicating linearity. Quantification within the range of responses covered by the calibrations should therefore not be problematic.

7.3 Sewage samples and blanks

The only contamination observed in the method blanks were small amounts of HHCB detected in one of the method blanks. The amount was below the LOQ, but based on calculated S/N-ratios the amount of HHCB was close to the LOD. The low contaminations observed are not expected to influence the results.

The recoveries of ISTD observed for the method blanks and spiked samples, shown in Table 6-5, varied from 60,9 to 81,2 %. The lowest recovery was observed for the sample spiked with 4000 ng of HHCB and 2000 ng of AHTN. A breakthrough test of the spiked blank gave an ISTD recovery of 40%, indicating that the low recovery may be due to overload of the SPE-column.

The ISTD was not identified in the breakthrough tests of influent sewage samples, but HHCB was identified in small amounts in both of the tests. These results were low compared to the large amounts of ISTD identified in the breakthrough test of the spiked blank sample. As discussed above, the high recovery of ISTD observed in the breakthrough test, may be due to overload of the column, and the differences between the breakthrough test can therefore be caused by the lower amounts of analyte in the sewage samples. However, the difference may also be attested to matrix effects in the sewage samples, as described below.

The analyses of sewage samples gave good recoveries of ISTD, which were consistently higher than for the analyzed method blanks and spiked blanks. Polycyclic musks are known to adsorb to sewage sludge, as was also shown during method development (Chapter 4.3). The increased recoveries observed for the sewage samples are therefore attested to matrix effects, namely adsorption to sewage particles trapped in the paper filter, as described during method development, and also adsorption to sewage particles trapped by the SPE-column.

The day-to-day variations of musk concentrations in sewage water are assumed to be small. The different levels of musk observed for influent and effluent samples respectively, are attributed to the precision of the method. But the data are too small to accurately estimate the precision.

The AHTN response in the influent and effluent samples were below the linear range established by the calibrations, and therefore below the determined LOQ. For this reason the uncertainties of the reported values are significant. However, the signals detected in the influent samples are only slightly below the range of the calibration. DPMI, ADBI or AHDI was not identified in the sewage samples.

7.4 Limit of detection and limit of quantification

The estimated limits of detections, shown in Table 6-3, for the different compounds vary from 18,7 ng/l for DPMI, to 1,1 ng/l for AHTN-D3. Because the concentrations of the samples used for making the estimations were orders of magnitude above the estimated LODs, and the separate LODs were estimated from a single parallel, there is considerable uncertainty with these estimates.

Table 7-2 lists LODs reported in two other comparable studies. Both of these reported LODs lower than those reported in this thesis. The LOD reported for HHCB and AHTN in this thesis are sufficiently low, because of high concentration in the substrate.

Levels of DPMI, ADBI, AHDI and ATII detected in STPs in Sweden from 2008 and 2010 are shown in Table 7-1. The levels reported are probably relevant also for Norway, and clearly indicate that the LODs reported for ADBI and AHDI in this thesis are insufficient for identification of these compounds. Table 7-1 shows that large maximum concentrations of DPMI in influent and effluent samples was found, and based on this, DPMI would be expected to be identified in the sewage samples from Bekkelaget. As the determined LODs are instrument detection limits (Chapter 6.3.1), it is possible that matrix effects interfere with the detection of DPMI, ADBI and AHDI. However a report on musk in sewage sludge in the Nordic countries show overall low concentrations of DPMI[38], indicating that the large concentrations of DPMI shown in Table 7-1 are not necessarily relevant for comparison with the concentrations measured in wastewater from the STP at Bekkelaget.

Table 7-1 Minimum and maximum values of DPMI, ADBI, AHDI and ATII detected in influent and effluent samples from STPs in Sweden from 2008 and 2010[39].

	Influents ng/l min-max	**Effluent ng/l min-max**
DPMI	1 - 150	1 - 308
ADBI	nd - 17	nd - 4
AHDI	nd - 2	nd - 10
ATII	nd - 10	nd - 12

Because of concerns with the linearity of the detector response, discussed in Chapter7.2, LOQ was defined as the lower end of the calibrations. This gave LOQ of 125 (DPMI, ADBI, AHDI and AHTN-D3), 500 ng/l (AHTN) and 1271 (HHCB) for the different musks, as shown in Table 6-4. These values are for some of the compounds orders of magnitude above the LODs, and are most likely conservative estimates. With analysis of standard solutions and samples at lower concentrations, the LOQ could likely be set lower.

7.5 Comparison of the results of the analysis with results from other studies

A report from the Norwegian Climate and Pollution Agency (KLIF) from 2011[40], analyzed HHCB and AHTN in effluent sewage samples from the STP at Bekkelaget. Sampling was done autumn 2010. The results are shown in Table 7-2. The reported concentrations correspond well to the concentrations in effluent reported in this thesis, with the exception that concentrations of HHCB were found to be somewhat larger.

The concentration of HHCB in the influent and effluent samples from Bekkelaget found in this thesis are large compared to concentrations in influent and effluent reported earlier, shown in Table 7-2. Only the report from USA shows higher concentrations. The concentrations of AHTN found in influent are similar to the levels reported in Germany and USA, but higher than those for China, while the Influent concentrations of AHTN are low compared to the other studies shown in Table 7-2. This might be due to high removal percentages of AHTN, discussed below, or an effect of the high uncertainty related to the reported AHTN concentrations. Usage patterns of musks are expected to be comparable for northern European countries[15], and that the levels of HHCB found in this thesis are high compared to the levels in sewage waters from Germany and Sweden are intriguing, and indicate that usage of HHCB has not been reduced in recent years. The fact that the reported HHCB concentrations in sewage effluent are larger than those found in effluent from Bekkelaget in 2010, may rather suggest an increased usage of polycyclic musks.

Table 7-2, Selected studies on the concentration of HHCB and AHTN in influents and effluents from different STPs, as well as reported LODs.

	HHCB Influents ng/l	**AHTN Influents ng/l**	**HHCB Effluent ng/l**	**AHTN Effluent ng/l**	**HHCB LOD ng/l**	**AHTN LOD ng/l**
This thesis	5653-7040	365-486*	1843-1967	64-85*	3,1	1,1
Bekkelaget, Norway 2011[40]			719-1259	66,7-87,0		
Sweden 2003[41]			157-423	42-104	0,5	0,5
Germany 2004[13]	1941	583	669	212		
USA 2007[42]	1780-12700	304-2590	2360-3730	495-807		
China 2009[43]	1251,4-3003,8	111,9-286,3	492, 8-1258,3	47,3-190,9	0,4	0,4

*Below LOQ.

The calculated 70 % removal of HHCB is lower than expected, based on several studies summarized in a 2008 EU risk assessment report[18], that report removal percentages for HHCB about 80-90 %, although lower removal percentages are also reported. Because of the uncertainty of the quantification of AHTN, and particularly the levels in the effluent samples, the calculated removal percentage is also uncertain. However the calculated removal for AHTN-D3 of 83 % was as expected based on several studies summarized in the 2008 EU risk assessment report[19]

The large removal percentages for polycyclic musks in STPs are primarily explained by their adsorption to sewage particles and the subsequent removal of sludge from wastewater[13]. As the sludge removed by the STPs are commonly used for improving soil in agriculture, it is important to monitor pollutants in the sludge. The levels of polycyclic musk in the dewatered sludge were not studied in this thesis. However, polycyclic musk in dewatered sludge from multiple STPs in Norway was analyzed in the earlier citied study from 2011[40]. This study concluded that based on predicted no-effect concentrations (PNEC) for soil for HHCB[18] and AHTN[19], the risk to the terrestrial environment from the application of HHCB and AHTN was low[40]. The same report from 2011 also conclude that for HHCB and AHTN "the concentrations present in undiluted effluent are below that required to pose a risk to the environment."[40] based on comparison with PNEC values[18, 19].

A limitation of the use of PNEC-values for risk assessment is that the estimates are based on experiments where organisms are subjected to the effects of a single pollutant, which does not account for the combined and synergistic effects of the mixture of different pollutants that is present in the environment. Long term toxic effects may also not be detected. As the result from this thesis indicates that the use of HHCB may have increased since 2010, it is important to continue monitoring the release of polycyclic musks to the environment, and study the fate of these compounds.

8 Conclusion

The developed method was sufficient for quantifying HHCB in influent and effluent sewage samples from Bekkelaget. Quantification of AHTN was uncertain because of partial separation from HHCB, because of shared ion fragments and the large discrepancies in concentration. The method was deemed insufficient for detecting ATII in the sewage samples.

HHCB and AHTN were detected in all the influent and effluent sewage samples from Bekkelaget, with HHCB being found in the largest concentrations. DPMI, ADBI and AHDI were not detected in any of the sewage samples. Measurements of influent and effluent showed a reduction of 70 % for HHCB and 83 % for AHTN through the STP, this is in agreement with results from earlier studies.

Compared to separate studies, the observed concentrations of polycyclic musk in influent and effluent were surprisingly large, and the concentrations of HHCB in wastewater from Bekkelaget seem to have increased since 2010. For these reasons it is important to continue monitoring the release of polycyclic musks to the environment, and study the fate of these compounds.

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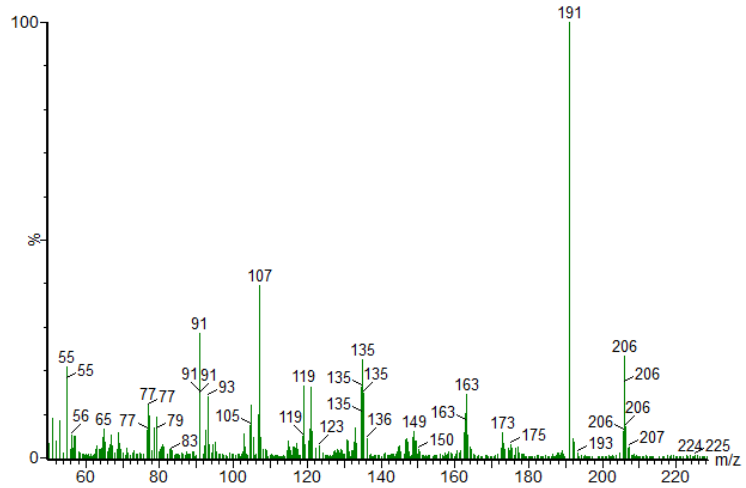
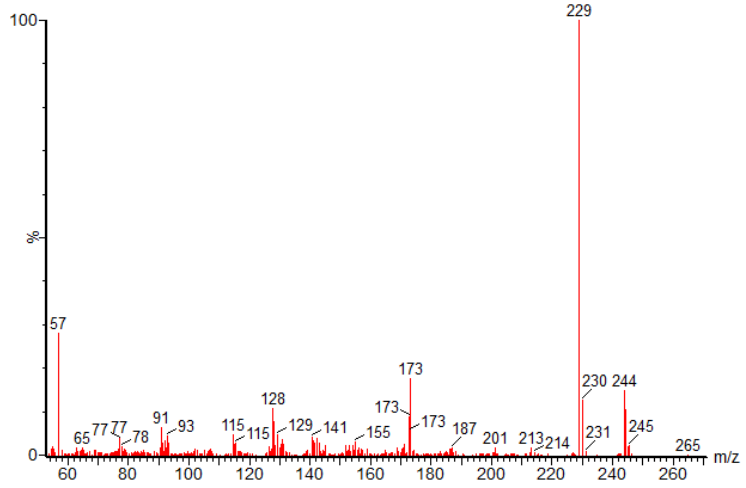
APPENDIX 1 – Mass spectra

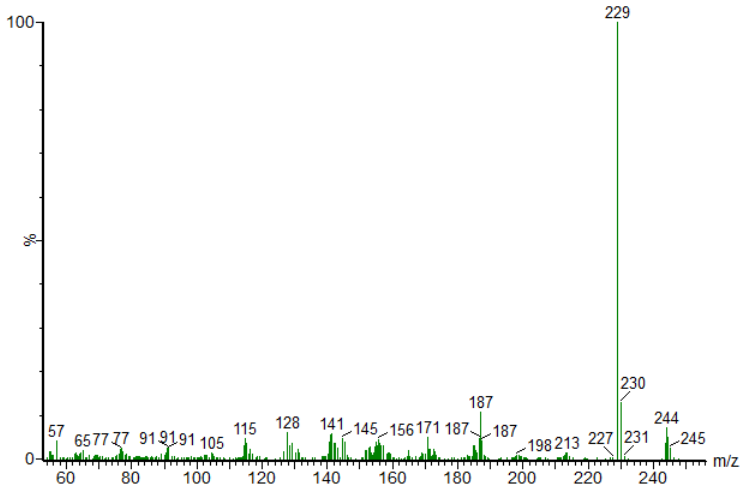
Full scan EI-spectra of all the analyzed compounds..



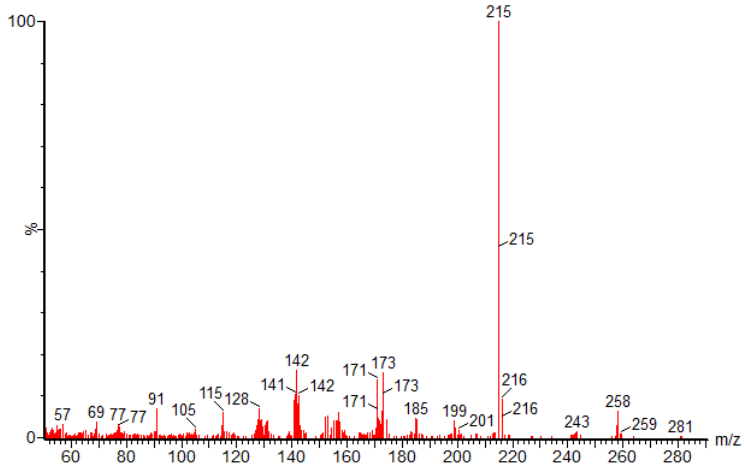
Full scan EI-spectra of DPMI.



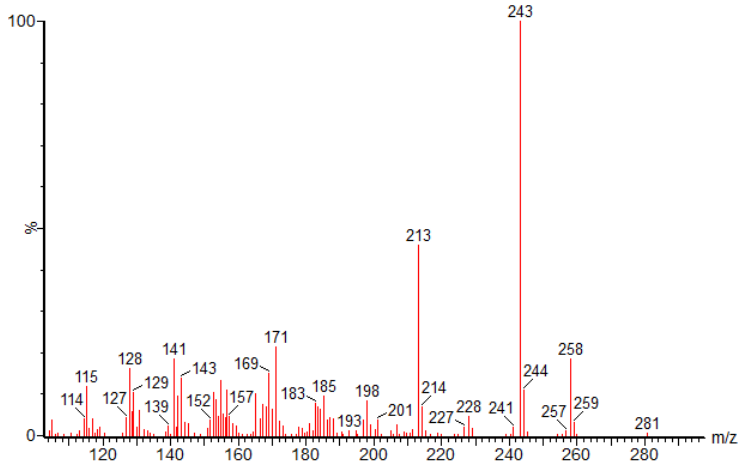
Full scan EI-spectra of ADBI.



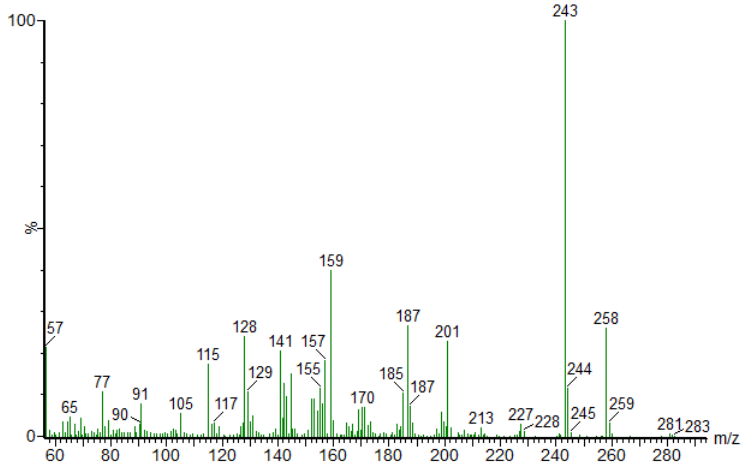
Full scan EI-spectra of AHDI



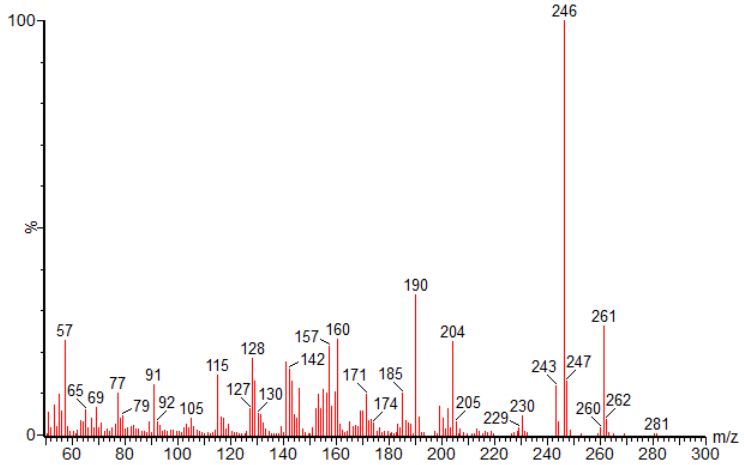
Full scan EI-spectra of ATII



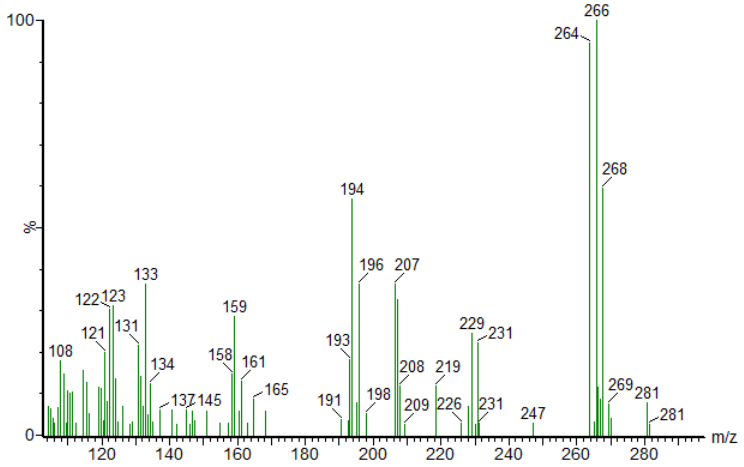
Full scan EI-spectra of HHCB



Full scan EI-spectra of AHTN



Full scan EI-spectra of AHTN-D3

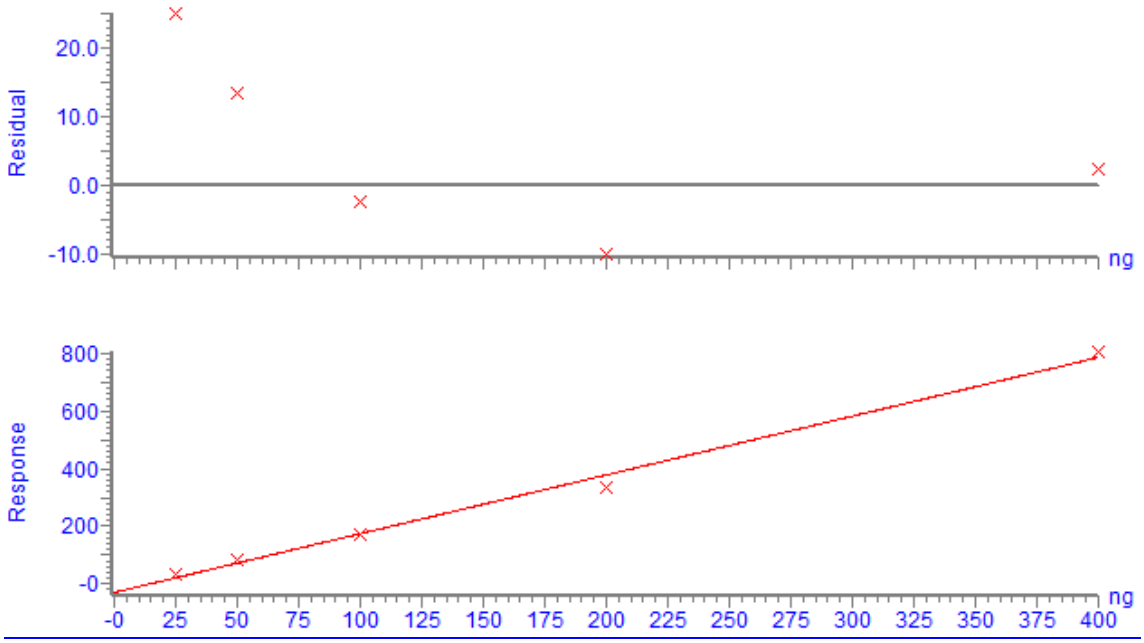


Full scan EI-spectra of 1,2,3,4-tetrachloronaphtalene (TCN)

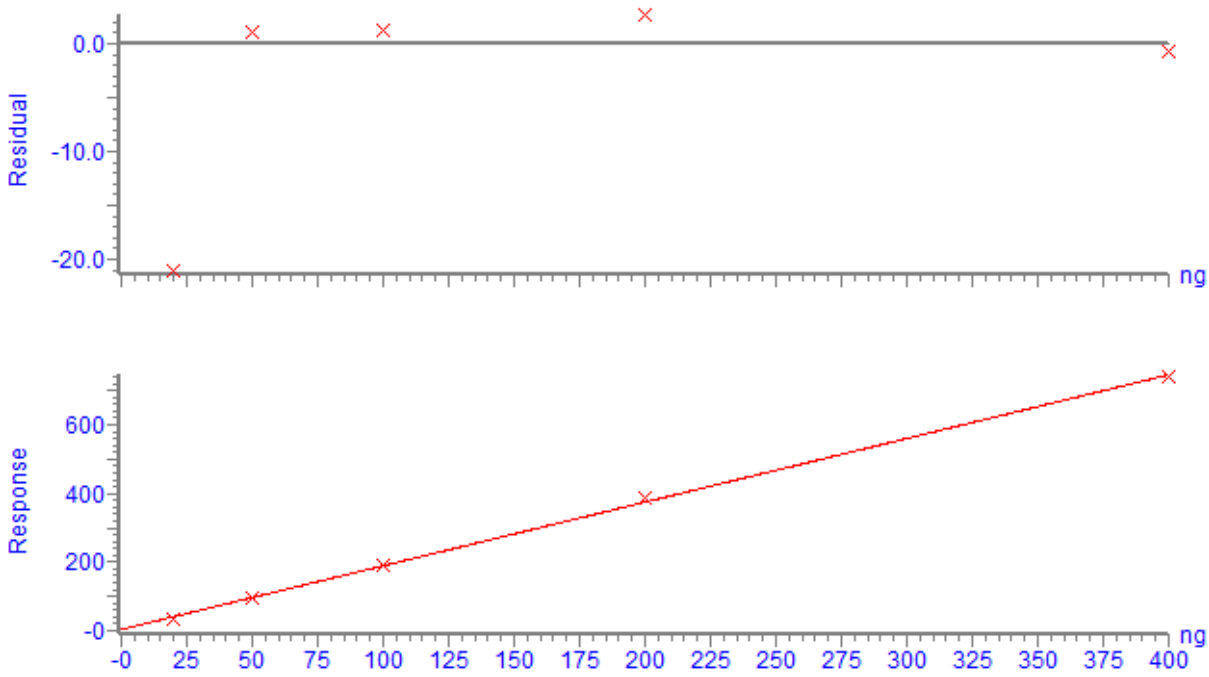
APPENDIX 2 – Calibration data

Calibration data, with linear regression and plot of residuals, for the calibration of all the standards.

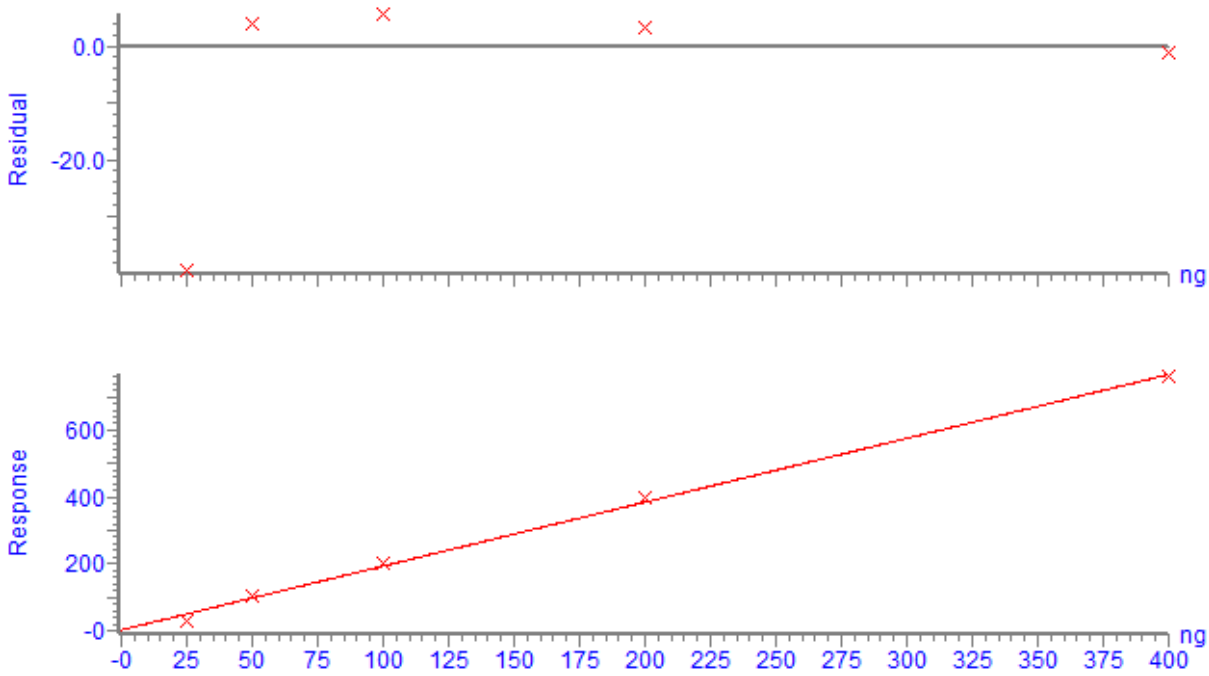
Compound name: Cashmeran
Correlation coefficient: r = 0.996885, r^2 = 0.993780
Calibration curve: 2.0385 * x + -29.4144
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None



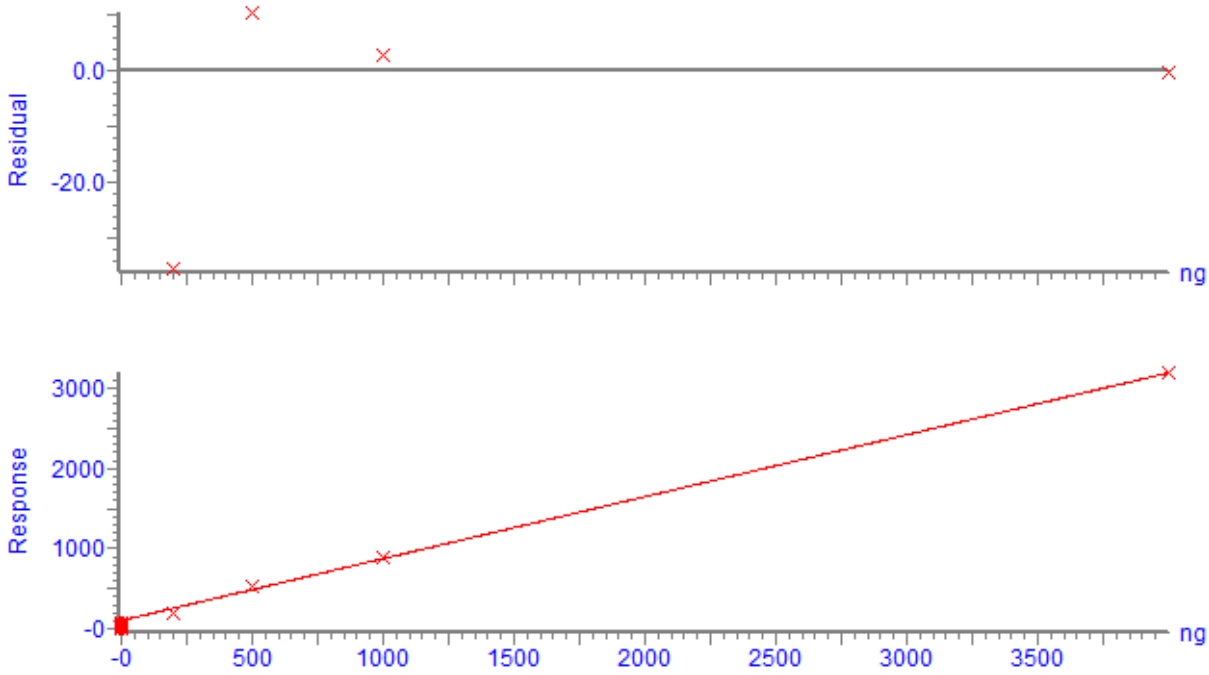
Compound name: Celestolide
Correlation coefficient: r = 0.999702, r^2 = 0.999404
Calibration curve: 1.85542 * x + 3.83707
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None



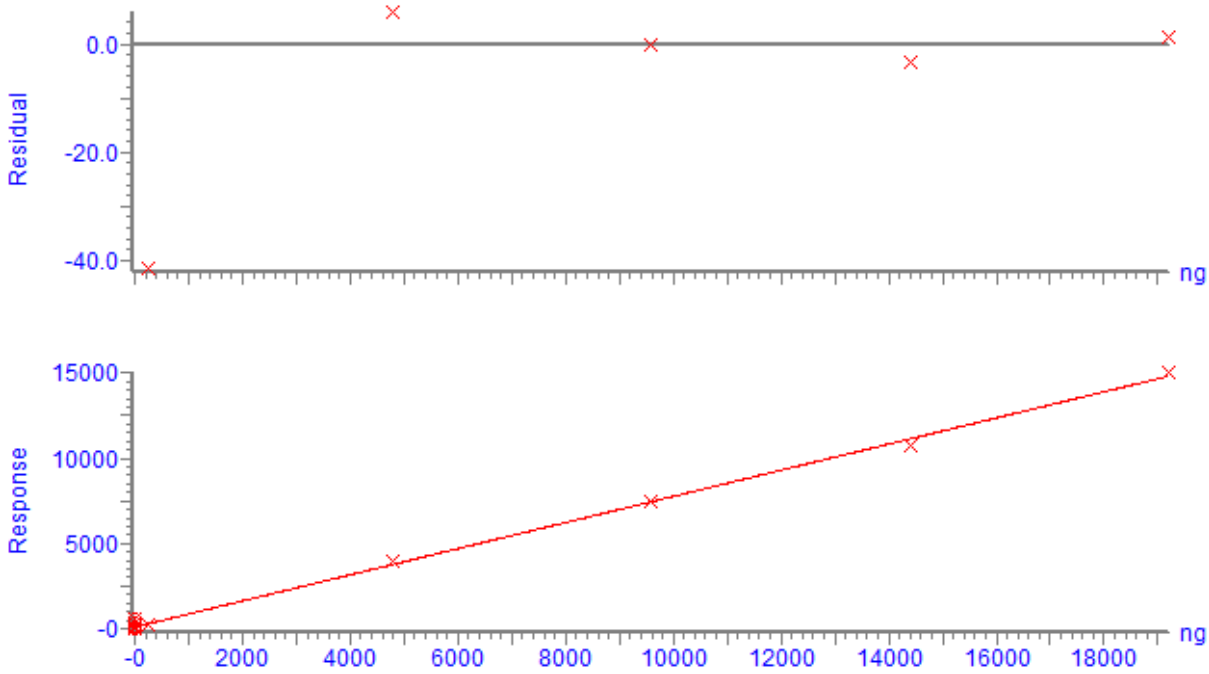
Compound name: Phantolide
Correlation coefficient: r = 0.998943, r^2 = 0.997886
Calibration curve: 1.91145 * x + 2.29961
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None



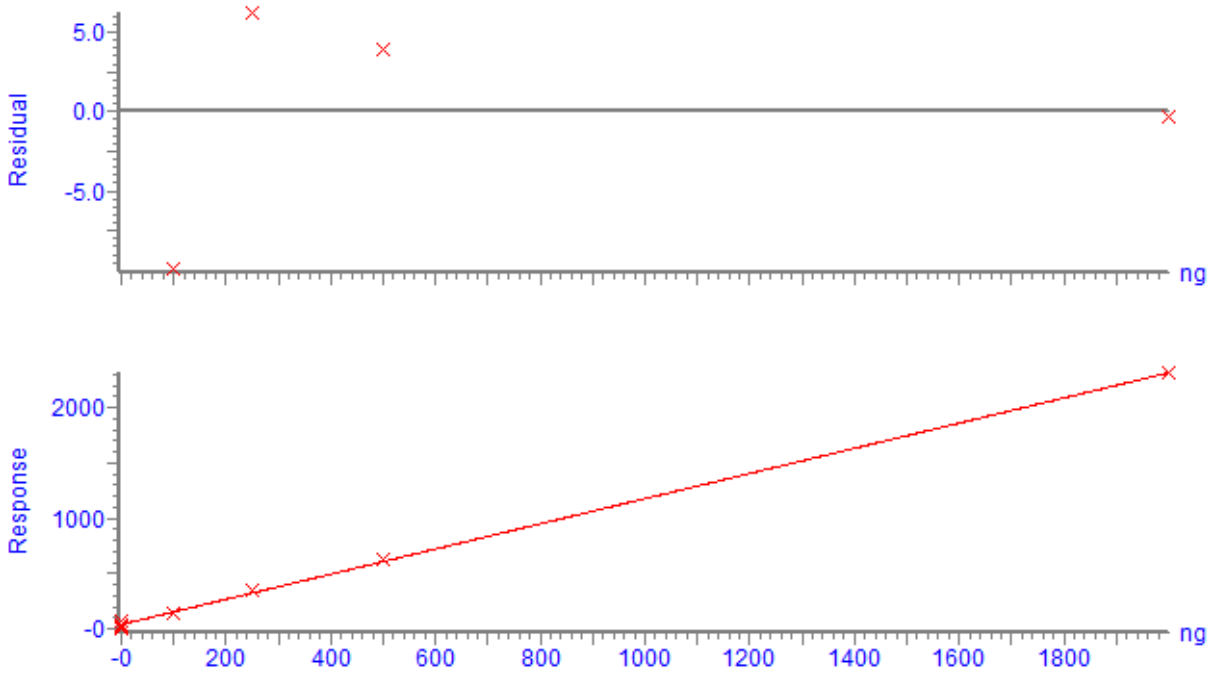
Compound name: Galaxolide
Correlation coefficient: r = 0.999529, r^2 = 0.999058
Calibration curve: 0.772851 * x + 101.898
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None



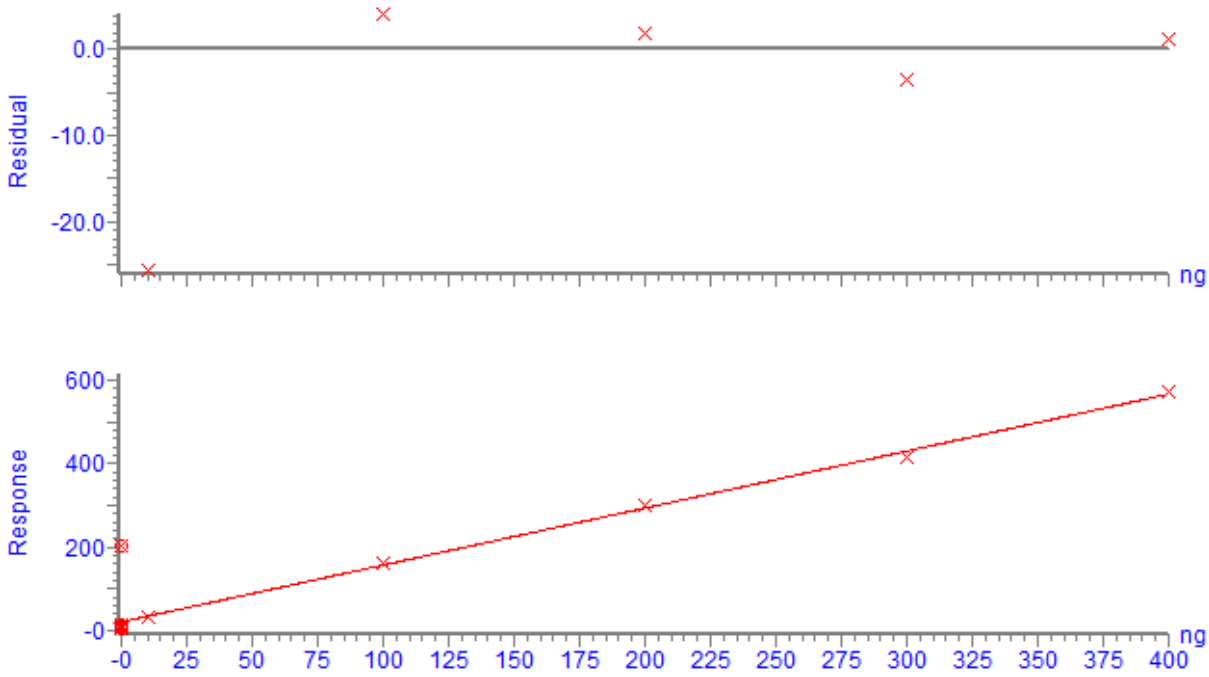
Compound name: Galaxolide
Correlation coefficient: r = 0.999140, r^2 = 0.998281
Calibration curve: 0.767085 * x + 92.7657
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None



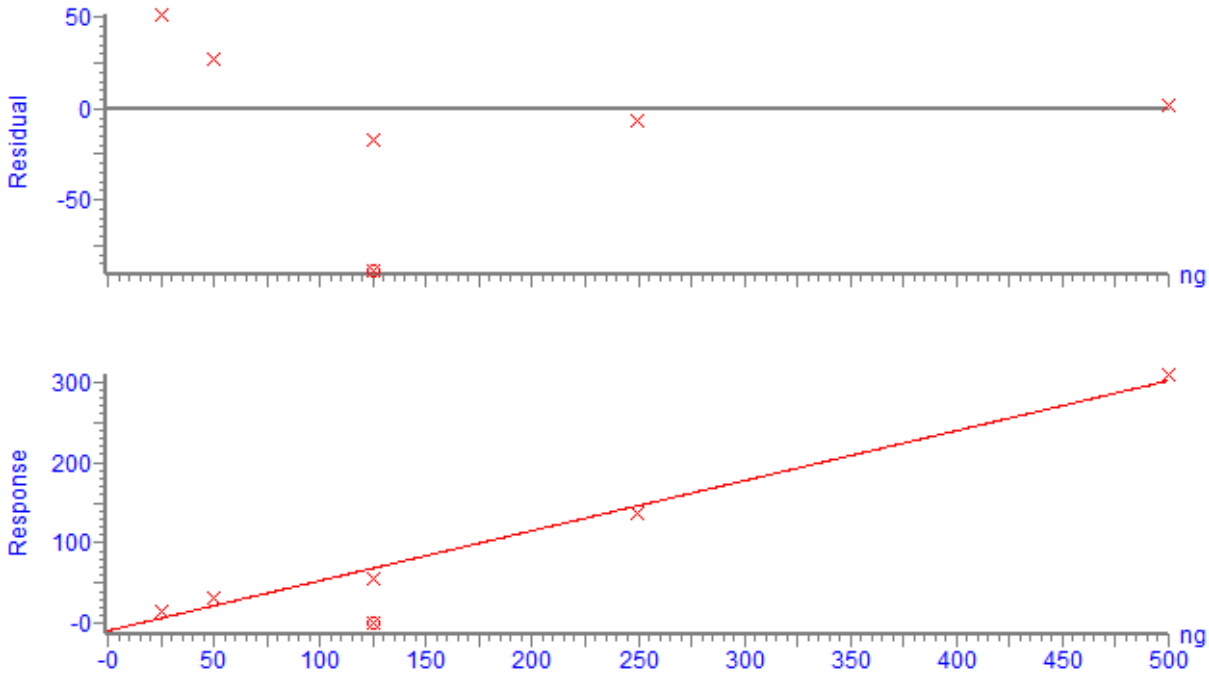
Compound name: Tonalide
Correlation coefficient: r = 0.999596, r^2 = 0.999193
Calibration curve: 1.13935 * x + 38.421
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None



Compound name: Tonalide
Correlation coefficient: r = 0.999106, r^2 = 0.998214
Calibration curve: 1.36367 * x + 20.6963
Response type: Internal Std (Ref 1), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None



Compound name: AHTN-D3
Correlation coefficient: r = 0.995970, r^2 = 0.991955
Calibration curve: 0.620152 * x + -8.57861
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None



Compound name: AHTN-D3
Correlation coefficient: r = 0.999831, r^2 = 0.999663
Calibration curve: 0.824354 * x + -2.11207
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None