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POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) IN ANIMAL FEEDS, ANIMAL FATS, VEGETABLE OILS/FATS, FATTY ACIDS

Part 1 limits

Part 2 analytical methods

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SUMMARY

Following the contamination of palm oil with diesel oil, temporary standards have been set up in Belgium for polycyclic aromatic hydrocarbons (PAHs) in fats and fatty acids destined for animal feed. Subsequently, a working group was set up consisting of a number of Belgian and Dutch experts, analysts as well as toxicologists, in order to achieve a better norm and to formulate uniform analytical methods. As far as the limit is concerned, an important basic principle is dealing with the so-called TEF-values, with which, analogous to the dioxins, the content of toxicologically relevant PAHs is brought back to one substance, i.e. benzo(a)pyrene. As limit for PAHs in fats and their derived products destined for animal feed, **50 µg BaPEQ/kg** was chosen, with an action limit of **15 µg BaPEQ/kg**. Ideally, this limit should be based on either toxicological knowledge or ALARA principles. Unfortunately, there is a lack of information with regard to the transfer of PAHs from animal feed to for instance milk, even though it is expected that the major part will be metabolized. Whether in the case of cows this results in harmful metabolites is not known.

The limit includes the following compounds on the basis of their toxicity:

- benzo(a)pyrene
- dibenzo(a,h)anthracene
- benzo(a)anthracene
- benzo(b)fluoranthene
- benzo(k)fluoranthene
- indeno(1,2,3-c.d)pyrene
- chrysene
- acenaphtylene
- fluoranthene
- acenaphtene
- phenanthrene
- pyrene

Proposals with regard to the limit, the analytical methods to be applied and the manner of reporting are described in this report. For this purpose, the working group has studied a number of methods of analysis and has presented proposals with regard to uniform analytical methods. In the meantime, these analytical methods have been tested by several laboratories in a small ring-trial.

The analysis by means of HPLC with detection via fluorescence is not suitable for the determination of all selected compounds. In particular the volatile compounds cannot be determined quantitatively and consequently it is possible that an underestimation is made of the actual value. A result obtained with HPLC/FL should be considered indicative. As recent data have shown that PAHs are also formed during different kinds of drying processes and consequently can occur in other animal feed raw materials such as dried grass, lucerne, citrus pulp and copra, it is to be expected that in the short run a limit will also be required for these kinds of products.

PART 1 LIMITS

1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds consisting of two or more aromatic six-ring structures. PAHs can originate among other things during combustion processes when organic compounds are heated to a high temperature. During the combustion of organic material, carbon radicals, hydrogen radicals and CH radicals are released. These free radicals can polymerize and form stable polycyclic aromatic hydrocarbons. This formation can also take place at lower temperatures (100-150°C), but then the reaction speed is considerably reduced. The molecules that are formed at these low temperatures also contain substituted compounds, among others methyl and ethyl substituted PAHs. In fossil fuels, the content of substituted PAHs is often higher than the content of PAHs themselves.

The extent of emission to the environment is, for the greater part, determined by exhaust fumes of cars and smoke gasses of different industries and waste processing systems. After deposition, the PAHs strongly combine with organic material that is located in the soil. Physical properties such as degree of acidity and temperature determine the length of stay and degradability of the PAHs in the soil. Depending on the environment in which they are located, PAHs can be chlorinated, nitrated or alkylated to the relevant metabolites. As yet there has not been much research with regard to the different metabolites in soil, agricultural products and, for instance, animal feeds. In particular the relationship of these metabolites and the original substances is not known. Consequently, it is not known whether reported contents of non-substituted PAHs are representative of the total PAH content.

PAHs have a slight systemic toxicity. However, many PAHs are carcinogenic; the carcinogenic potency only becomes manifest after bio-transformation in the body to so-called diol-epoxides. According to recent experiments (RIVM 1999), the additional risk of cancer in case of lifelong exposure to 5 ng BaP (benzo[a]pyrene) per kg of body weight (bw) per day is 1 per 10⁶. The current exposure of the population is estimated to be 2.7 ng BaP/kg bw/day, and for all PAHs based on BaPEQ 7.3 ng/kg bw/day (RIVM 1999).

1.1 Sources/avoidability

The main sources are, among others, products that have been contaminated as a result of direct emission. The deposition on grass as well as on large-leaved crops (spinach, cauliflower, etc.) is especially important in the agricultural field. As far as animal feed is concerned, the cause of the danger is mainly found in those raw materials that are dried, such as pieces of grass, citrus pulp, copra, etc. Another possible source is the use of technical fats which, as a result of a treatment, may be contaminated with PAHs.

The background content of PAHs in animal products can, among other things, be pushed back by using raw materials that are unsuspected as well as refraining from recycled materials, or by well monitoring these streams.

Production processes should also be monitored with regard to critical points. For instance, in case of drying processes natural gas should be used as fuel.

1.2 Transfer from animal feed to animals, humans and environment

There is no information concerning the transfer of PAHs from animal feed to animal products. The available information with regard to kinetics and metabolism of PAHs in farm mammals is extremely scarce and purely qualitative. PAHs are well absorbed in the gastrointestinal tract; in test animals the absorption is > 50%. Even though PAHs are rather lipophilic (so a certain accumulation might be expected), they are relatively quickly metabolised to hydroxylated products and conjugates that are excreted via urine and/or faeces. In 1998, the WHO concluded that the turnover of PAHs in the body is fast and that no accumulation occurs.

In the framework of the current problem, it seems to be advisable to study the transfer of PAHs, especially in the case of lactating cows. Therefore, cows have to be fed contaminated materials, after which the contents in milk can be determined by means of a recently developed very sensitive GC/MS method. Moreover, the metabolization of a number of PAHs will be examined by means of liver microsomes (or liver slices), in order to check whether harmful metabolites can be formed, which possibly will end up in the milk.

1.3 Estimation of exposure and its concomitant risks

With reference to the batches of palm oil fatty acids contaminated with PAHs that were released by Belgium and that have been processed to animal feed in the Netherlands which were subsequently fed to dairy cattle, RIKILT and RIVM made an estimation of the exposure and the risk.

1.3.1 Exposure estimation

Assuming that:

- 1 a milk producing cow consumes 10 kg of feed per day (*realistic estimation based on expert judgement*);
- 2 this feed contains 1% mixed fatty acids, i.e. 100 grams (*realistic estimation based on expert judgement*);
- 3 these mixed fatty acids contain 50 µg BaPEQ per kg (*the proposed limit*); then that cow ingests 5 µg BaPEQ per day.

Further assuming that:

- 4 after oral ingestion by the cow, the biological availability of PAHs is 100% (*realistic estimation based on expert judgement*);
- 5 within a couple of days, the cow develops a steady state situation (*expert judgement*);
- 6 the BaPEQ ingested by the cow is not metabolised and/or excreted, but fully transferred to the milk (*"worst case" estimation*);
- 7 the cow produces 1 kg of milkfat per day (*realistic estimation based on expert judgement*); then the content in the milkfat is 5 µg BaPEQ per kg milkfat.

The milkfat consumption of the average Dutch person is 18.45 grams/day, or 0.325 g/kg bw/day. Therefore, the ingestion of BaPEQ as a result of the consumption of contaminated milk

and milk products is $18.4 \times 5 = 92$ ng/day or 1.4 ng/kg bw/day or 28% of the "TDI", as described above.

It should be emphasised that especially assumption 6 concerns a "worst case" scenario. By using the MilCons-model (Freijer et al 1999) and with the help of PBPK-modelling which allows modelling the transfer of orally ingested lipophilic substances to milk, assuming that the biological half-life amounts to 2 hours, it turns out that if a cow ingests 5 µg BaPEQ per day, a steady state situation is reached after approx. 3 months. In that steady state situation, the excretion via the milkfat is 2.4 µg/kg, in the initial period until the steady state situation, the excretion via milkfat is lower. A shorter half-life leads to a lower steady state, and therefore also to a lower excretion via the milk. Consequently, according to this model, the ingestion of the average consumer via the milkfat will amount to a maximum of 0.8 ng BaPEQ/kg bw/day (i.e. in the steady state situation).

1.3.2 Risk estimation

In case of lifelong average consumption of contaminated milk and milk products, the ("worst case" for the average consumer) estimated ingestion of 1.4 ng/kg bw/day of BaPEQ as a result of consuming contaminated milk and milk products, leads to an additional risk of cancer of 0.3 per 10^6 (i.e. 1.4/5 per 10^6). In case of lifelong average consumption, the more realistic estimation based on the Milcons-model leads to an additional risk of cancer of 0.2 per 10^6 (i.e. 0.8/5 per 10^6).

The actual risk will be lower, because:

- it may be assumed that the exposure of the cattle only concerns a limited period;
- after the exposure is ended, cattle that have consumed PAH-contaminated feed for some time, will have metabolised and excreted these PAHs within a couple of days so subsequently the animal products, including the milk, are no longer contaminated.

Therefore, the real exposure of the consumer will be limited to at the most some weeks. Under those circumstances, the contribution of this consumption to the occurrence of cancer can be considered negligible.

2 LIMIT FOR POLYCYCLIC AROMATIC HYDROCARBONS IN ANIMAL FEEDS (application of the TEF principle)

The Belgian government has introduced provisional limits of 10 and 50 µg/kg for benzo(a)pyrene and total PAHs respectively. The question arises whether, based on the present toxicological knowledge, different limits could be set. Since in this case, as in the case of dioxins, it involves a complex group of compounds, there also is the question whether it is possible to apply a TEQ-principle. By reason of the study in question, a provisional norm of 50 µg BAPEQ/kg is proposed for the carcinogenic PAHs in animal feeds and animal feed raw materials, with an action limit of 15 µg BAPEQ/kg. The proposed action limit and max. limit assume the upper bound principle.

2.1 Limits for benzo(a)pyrene and other PAHs

When setting toxicology based limits for substances in animal feeds or animal feed components, it is essential to verify to what extent these substances may result in harmful effects in the target animal or subsequently via the residues in the consumer. In the case of PAHs, of course the

question is whether carcinogenic effects are relevant to the target animal, in view of their relatively short life span.

It seems more important to verify whether the exposure to PAHs ultimately leads to unacceptable residues in animal products such as milk, eggs and meat. Unfortunately, little is known about this subject. However, PAH's have been detected in animal products and consequently it cannot be excluded that these are transmitted via the animal feed. In the case of PAH's, as opposed to dioxins, it is a matter of metabolism, which probably already largely takes place in the intestinal wall and in the liver. This leads to metabolites that are partly responsible for the toxicity of the mentioned substances. These reactive metabolites will rather quickly bind with proteins and DNA or are degraded into non-toxic compounds. However, in particular a number of diol-compounds turns out to still be toxic, also due to the fact that they can be further activated into reactive metabolites. Also, in the intestine conjugates can again be split up into the free diols. In view of their increased polarity, conjugates probably can be excreted rather soon and therefore will not accumulate. Little is known about the possible toxicity of the so-called covalent bound metabolites of PAHs. However, in practice it is assumed that they are not toxic. A similar problem applies to numerous other substances, among which the mycotoxin aflatoxin B₁.

Presently there are no limits for PAHs. This is also due to the fact that so far published carcinogenicity studies are not suitable for a quantitative risk evaluation. For that reason, the Dutch RIVM has recently carried out an oral chronic study with benzo(a)pyrene in rats. A provisional limit, achieved by linear extrapolation and based on 1 additional case of cancer per million people at lifelong exposure amounts to 5 ng/kg body weight per day.

On the basis of this exposure limit, residue limits could be determined, but this has not yet been done. However, in case of fats and oils intended for human consumption, preliminary norms of 1, 5 and 25 µg/kg of product are used for benzo(a)pyrene, heavy PAHs and total PAHs, respectively. Regarding the strong degradation of these substances, for animal feeds and animal feed raw materials, a provisional limit of 50 µg/kg could be proposed. Moreover, one could work with an action limit of 15 µg/kg. If the action limit is exceeded, a closer investigation is started to determine the source of the contamination. To further support the limit, research should be carried out with regard to the behaviour of PAHs and toxicologically relevant metabolites in agricultural animals.

2.2 TEF values for PAHs

Regulations with regard to permissible residue levels are usually based on individual substances. This especially causes problems with complex mixtures of substances with similar toxic effects. A well-known example are the dioxins, 17 of which are toxicologically important. Moreover, there are also a number of polychlorobiphenyls (PCBs) which can also bind to the Ah-receptor and, consequently, have an effect similar to the dioxins. In order to deal with this problem, the so-called TEQ-principle was chosen, with which the toxicity of a group of dioxins is related to that of the most toxic one, the 2,3,7,8-tetrachloro-p-dioxin, or TCDD. For that purpose, international commissions have determined so-called toxic equivalence factors, or TEF values, for individual dioxins and PCBs, the most recent review of which has taken place under auspices of the WHO. Consequently, the mentioned TEF-values are often called WHO-TEFs. During the analysis, the

contents of the individual dioxins are multiplied with the TEF value and ultimately a content in pg BaPEQ/kg of product of fat is found.

The TEF values are determined by weighing all kinds of possible effects; of course the effects in humans and subsequently in animals are more important than for instance the effects on cultured cells.

Similar to dioxins, a number of polycyclic aromatic hydrocarbons (PAHs) is able to bind to the Ah-receptor and, on the basis of that property, could be assigned a dioxin-TEF. However, in practice the differences between dioxins and PAHs are too big. Dioxins are degraded very poorly and consequently tend to accumulate in fatty tissue. Also, the recently revised limit for TCDD is no longer based on the cancer causing properties, but on the effects of the immune system and the endometriosis observed in monkeys. On the other hand, the carcinogenicity of a number of PAHs has been clearly demonstrated as well as DNA-damaging properties that emerged as a result of numerous genotoxicity tests. Therefore, a different set of TEF values should be used for PAHs, based on these carcinogenic properties and effects in the mentioned tests. As benzo(a)pyrene seems to be the most toxic congener, it is obvious that this substance should be used as standard and to determine a TEF of 1 for it.

For analytical purposes, application of the TEQ-principle for this group of substances means that in principle all toxicologically relevant substances should be analysed. Possibly, based on practice, it can be decided to not include congeners that are difficult to analyse and that do not actually contribute to the total content (in BAPEQs) in the analysis. At this moment, benz(a)anthracene, benzo(a)pyrene, dibenz(a,h)anthracene are classified by the IARC (1983) as *probably* carcinogenic to humans (group 2A) and benzo(b)fluoranthene, benzo(j)fluoranthene, benzo(k)fluoranthene and indeno(1,2,3-c,d)pyrene as *possibly* carcinogenic to humans (group 2B). In the meantime, chrysene should also be added to that list, whereas fluoranthene is suspect (IPCS, 1998). Naphtalene, anthracene, benzo(g,h,i)perylene and fluorene do not seem to be carcinogenic, the remaining substances (acenaphtene, acenaphtylene, phenanthrene and pyrene) remain suspect based on positive genotoxicity tests. Possibly, the purity of the examined substances plays an important role therein.

However, a great advantage of the use of TEF-values as opposed to the current practice is that the non-relevant PAHs become considerably less important. Thus the proposed limits of 10 and 50 µg/kg of product for benzo(a)pyrene and total PAHs respectively, do not take into account the toxicity of the mentioned substances since, in practice, the carcinogenic PAHs often contribute only a small part to the total.

Although in the past several proposals have been made with regard to TEF values for PAHs (Nisbet and Lagoy 1992, EPA 1993 and Kalberlah 1995), so far they have not been used generally (appendix 1). A recent publication of Collins et al. (1998) supports most of these values. An important difference between the propositions of Nisbet and Lagoy on the one side and the EPA and Kalberlah on the other side, is the assignment of low TEF-values to a number of PAHs, for which the carcinogenicity has not been proven. This particularly concerns anthracene, benzo(g,h,i)perylene, naphtalene and fluorene. It is obvious that TEF-values are not assigned to these substances. The TEF value for dibenzo(a,h)anthracene, as proposed by Nisbet and Lagoy is rather high and should have to be lowered to 1. Based on these considerations, Table 1 presents a proposition. Table 2 shows a calculation example for contaminated palm oil.

It seems to be well advised to obtain international consensus for these values in the future, preferably by establishing an international commission. In order to prevent misunderstandings, it also seems desirable to distinguish between different kinds of TEF values, whether or not based on substance group or toxic effect, for instance dioxin-TEF (or Ah-TEF), PAH-TEF (or carcinogenicity-TEF).

2.3 Upper and Lower bound principle

After the analysis has been performed, the data interpretation is as follows:

The analysis provides contents for each compound (i.e. of the twelve selected compounds), expressed in $\mu\text{g}/\text{kg}$ fat or product. Then, on the basis of toxicity factors, a total content is calculated, expressed in μg BAPEQ/kg fat or product and subsequently this is tested against the action limit and the max. limit.

The "Upper bound" principle is used for the interpretation of analytical results. This means that for congeners that have not been detected, a value equal to the detection limit is filled in, which, of course, is then again multiplied with the corresponding TEF value. In other words, non-identified compounds contribute to the total content. When using the "Lower bound" principle, non-identified compounds are not included. This means that "zero" is filled in for non-detected congeners. Consequently, per definition the "Lower bound" principle indicates a lower value or, if all congeners are identified, a value equal to the one when using the "Upper bound" principle. This has been illustrated in the calculation example in Table 2.

Table 1. List of proposed TEF values for polycyclic aromatic hydrocarbons

Substance	Proposed
	TEF
Benzo(a)pyrene	1
Dibenzo(a,h)anthracene	1
Benzo(a)anthracene	0,1
Benzo(b)fluoranthene	0,1
benzo(k)fluoranthene	0,1
indeno(1,2,3-c,d)pyrene	0,1
chrysene	0,01
acenaphtylene	0,01
fluoranthene	0,01
acenaphtene	0,001
phenanthrene	0,001
pyrene	0,001

Table 2. Calculation example based on a contaminated palm acid sample

Substance	TEF	content (µg/kg)	Content (µg BAPEQ/kg) lower bound	Content (µg BAPEQ/kg) upper bound
dibenzo(a,h)anthracene	1	<1,0	0	1
benzo(a)pyrene	1	11	11	11
benzo(a)anthracene	0,1	98	9,8	9,8
benzo(b)fluoranthene	0,1	<1,0	0	0,1
benzo(k)fluoranthene	0,1	<1,0	0	0,1
indeno(1,2,3-c,d)pyrene	0,1	<1,0	0	0,1
chrysene	0,01	26	0,26	0,26
acenaphtene	0,001	<1,0	0	0,001
acenaphtylene	0,01	<1,0	0	0,01
fluoranthene	0,01	93	0,93	0,01
phenanthrene	0,001	510	0,51	0,51
pyrene	0,001	235	0,24	0,24
Total		973	21,9	23,1

Annex A. List of proposed TEF values for polycyclic aromatic hydrocarbons

Substance	Nisbet and Lagoy (1992) TEF	EPA (1993) TEF	Kalberlah (1995) TEF
dibenzo(a,h)anthracene	5	1	1
benzo(a)pyrene	1	1	1
benzo(a)anthracene	0,1	0,1	0,1
benzo(b)fluoranthene	0,1	0,1	0,1
benzo(k)fluoranthene	0,1	0,01	0,1
indeno(1,2,3-c,d)pyrene	0,1	0,1	0,1
anthracene	0,01		
benzo(g,h,i)perylene	0,01		
chrysene	0,01	0,001	0,01
acenaphtene	0,001		0,001
acenaphtylene	0,001	n.e.	0,01
fluoranthene	0,001	n.e.	0,01
fluorene	0,001		
naphtalene	0,001		
phenanthrene	0,001	n.e.	0,001
pyrene	0,001	n.e.	0,001

n.e.: not evaluated

PART 2 ANALYTICAL METHODS

1 OBJECTIVE AND RANGE OF APPLICATION

The method described applies to the determination of the content of polycyclic aromatic hydrocarbons (PAHs) in animal feeds, animal fats, plant oils/fats, fatty acids, and such.

When determining PAH, the following compounds are determined:

- benzo(a)pyrene
- dibenzo(a,h)anthracene
- benzo(a)anthracene
- benzo(b)fluoranthene
- benzo(k)fluoranthene
- indeno(1,2,3-c,d)pyrene
- chrysene
- acenaphtylene
- fluoranthene
- acenaphtene
- phenanthrene
- pyrene

After the contents of the individual compounds have been calculated in $\mu\text{g}/\text{kg}$ of product, the sum parameter is calculated in $\mu\text{g BaPEQ}/\text{kg}$ of product. For this purpose, with the help of the Toxic Equivalent Factors (TEF), the contents of the individual compounds are converted to the content of the most toxic component, benzo(a)pyrene and subsequently these values are summated. In the case of products with a fat percentage $<2\%$, the content is expressed on product basis and in case of fat percentages $>2\%$ on fat basis. Prior to starting the research, on the basis of the measured fat percentage or on the basis of the product knowledge, it should be determined to which category the product belongs.

Possibly better: Animal products, as well as animal and plant fats and fatty acids are reported on fat basis. Other animal feed raw materials and complete animal feeds on product basis. Animal products with a fat percentage lower than 2% are an exception - they should also be reported on product basis.

2 PRINCIPLE

All sample types, with the exception of fatty acids, are saponified with ethanolic KOH and then extracted with cyclohexane. Fatty acids are extracted via a caffeine-formic acid complex. The extracts obtained via a silicagel clean-up are measured by means of GC-MS or HPLC/Fluorescence. Prior to applying the chosen method for analysing "real" samples, it should be validated. Annex D provides a description of the validation procedure.

Remark 1:

When using an extremely sensitive analysis system (for instance high resolution mass spectrometer or a Time of Flight mass spectrometer) the quantity of sample being processed and the used chemicals internal standards etc. can be reduced by a factor of 20.

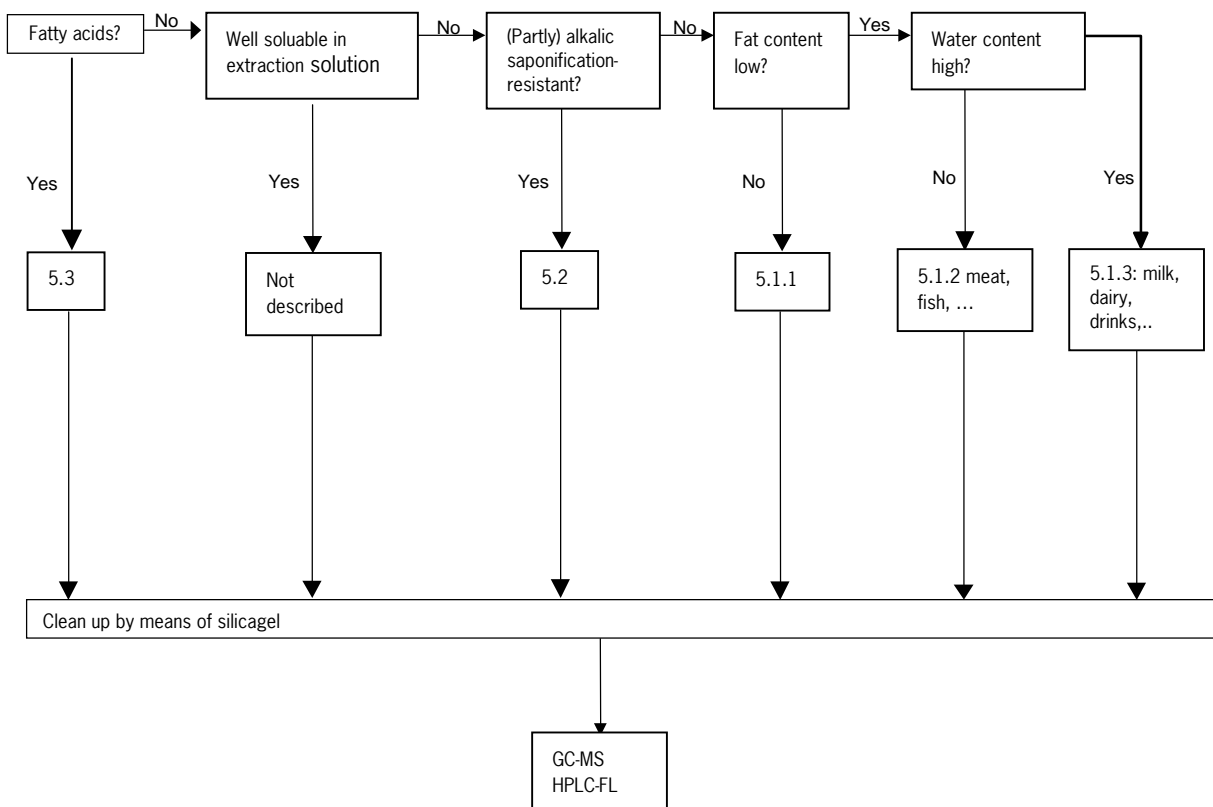
Remark 2:

The analysis by HPLC/Fluorescence is not suitable to determine all selected compounds. Acenaphthylene does not show any fluorescence and therefore is not measured. Especially for the volatile compounds such as acenaphthene, losses occur during the clean-up for which,, in contrast to the GC-MS analysis, no corrections can be made. This results in a serious underestimation of the real value.

Consequently, results obtained by means of HPLC/FL should be considered as indicative. Therefore, samples with a content close to the action limit should be reanalysed by means of GC-MS.

2.1 Clean-up/flow schedule

The following schedule indicates which type of sample can be investigated by which method:



3 EQUIPMENT, REAGENTS AND MATERIALS

3.1 Reagents

3.1.1 Reagents for the extraction and clean-up

- Acetone, p.a.
- Acetonitril, HPLC grade
- Cyclohexane distilled or equivalent
Add approximately 50 g potassium hydroxide to 3 l cyclohexane p.a. and distil with 10% first fractions and 10% last fractions
- Ethanol 96%, distilled or equivalent
Add approximately 50 g potassium hydroxide to 3 l technically pure ethanol and distil with 10% first fractions and 10% last fractions
- Ethanolic potassium hydroxide solution 2 M
Dissolve 112 g potassium hydroxide in 100 ml water and dilute this to 1 l with distilled ethanol. This solution should be prepared fresh daily.
- Ethanolic potassium hydroxide solution 1 M
Dissolve 56 g potassium hydroxide in 100 ml water and dilute this to 1 l with distilled ethanol. This solution should be prepared fresh daily.
- Potassium hydroxide p.a.
- Silicagel, ICN 63- 200 mesh, Active 60A (ICN Biomedicals).
- Demineralised water, or at least of the same purity
- Nitrogen, pure
- Formic acid
Make a 40% solution and a 90% solution in demiwat.
- Sodiumchloride
Make a 2% solution in demiwat
- Caffeine-formic acid solution
150g caffeine dissolved in 1 litre with 90% formic acid solution.
- Heated sodium sulphate

3.1.2 Reagents for the HPLC determination

- Milli Q water: Resistance: > 10 mΩ cm.
- Acetonitril, Uvasol
- Acetonitril, Pestiscan
- PAH standard solutions in acetonitril.

Stock solutions:

Preferably certified standards should be used.

For the following PAHs, assuming pure (min. 98%) solid product, a stock solution of approx. 100 µg/ml is prepared gravimetrically in acetonitril:

benzo(a)pyrene
dibenzo(a,h)anthracene
benzo(a)anthracene
benzo(b)fluoranthene

benzo(k)fluoranthene
indeno(1,2,3-c,d)pyrene
chrysene
acenaphtylene
fluoranthene
acenaphtene
phenanthrene
pyrene

A certified mixing standard SRM 1647D can be obtained. This mixing standard consists of the 16 EPA compounds in a concentration of 1-20 µg/ml in acetonitrile. Only the above-mentioned compounds of this mixing standard are used for quantification. The concentration in this mixing standard in microgram/ml is as follows:

fluoranthene	7.78
pyrene	8.38
B(a)A	3.94
Chrysene	3.69
B(b)F	4.16
B(k)F	4.70
B(a)P	4.92
dibenz(a,h)A	3.64
B(ghi)P	3.76
I[1,2,3-cd]P	4.37

-accurately weigh approximately 0.8 gram in a 25 ml volumetric flask and fill it to the line. Calculate the concentration for each PAH (by means of the certificate) and then dilute the solution obtained as follows: 0.5 ml; 1.0 ml; 2.0 ml; 3.0 ml; 4.0 ml; 5.0 ml to 50 ml ACN

Remark:

In many cases, the above-mentioned series of dilutions will be sufficient. Depending on the sensitivity of the detector and the desired analysis range, other dilution series can be made.

3.1.3 Reagents for the GC-MS determination

Remark:

The procedure described below starts from solid products where the stock solutions are self-made. However, already prepared and certified PAH mixtures are commercially available.

- Standard solutions for GC-MS determination

Stock solutions:

Native PAHs : (non-deuterated standards)

Preferably certified standards should be used.

Internal standards:

Of the following isotope-labelled PAHs, assuming pure (min. 98%) solid product, a stock solution of approx. 100 µg/ml is prepared gravimetrically in n-nonane or a different alkane or toluene.

D8-acenaphthylene
D10-acenaphthene
D10-phenanthrene
D10-fluoranthene
D10-pyrene
D12-benzo(a)anthracene
D12-chrysene
D12-benzo(b)fluoranthene
D12-benzo(k)fluoranthene
D12-benzo(a)pyrene
D12-indeno(1,2,3,c,d)pyrene
D14-dibenzo(a,h)anthracene

▪ *Recovery standard:*

Of D10-anthracene or a different isotope-labelled PAH, assuming pure (min. 98%) solid product, a working solution of approx. 100 µg/ml is directly prepared gravimetrically in n-nonane or a different alkane or toluene.

Remark:

With regard to the perishability of the standards, nonane is the best choice of solvent. However, in case of large-volume injection, the use of more volatile alkanes may be the obvious choice.

The stock solutions are stable for a maximum of 2 years and should be kept cool at approx. 4°C in a dark place.

Working solutions:

• *Internal standard working solution (IS dosage standard):*

By mixing the individual main standard solutions of the deuterium-labelled PAHs, an internal standard working solution with a concentration of approximately 10 µg/ml of each of the internal standards in n-nonane or a different alkane or toluene, is prepared gravimetrically.

• *Standard working solutions to check response linearity:*

Starting from the main standard solutions of the native and deuterium-marked PAHs, at least 5 standard working solutions are prepared in n-nonane, or a different alkane or toluene, which contain PAHs to be analysed in increasing concentrations from 0.02 to 5 µg/ml and the internal standards in a constant concentration of approximately 1 µg/ml.

• *Standard working solution for GC-MS calibration:*

To determine the relative response factors one uses a standard working solution in n-nonane or a different alkane or toluene, containing the PAHs to be analysed and the deuterium-labelled PAHs (internal standards and 'recovery' standards) in a concentration of approximately 1 µg/ml.

4 SAMPLE RECEIPT AND SAMPLE PREPARATION

The sample should be very homogeneous prior to taking sub-samples. PAHs can be extracted from wet and dried samples, however, storage, homogenizing and extraction are simpler with dried samples.

The drying of samples at room temperature or higher temperatures, as well as freeze drying, may change the concentration, for instance as a result of contamination or loss of components caused by evaporation (Law *et al.*, 1994).

4.1 Determination of fats (if applicable)

PAH-data are usually not expressed on fat basis. Nevertheless, the determination of fat in the tissues may be interesting, for instance in order to characterise the tissue. The fat content is best determined on a separate sub-sample. The total fat content can be determined with the Bligh and Dyer (1959) method, or an equivalent method.

According to the Bligh and Dyer fat determination, the fat, free as well as bound, is extracted from the sample with a system of chloroform, methanol and water.

The content can also be determined according to NEN 3148 (1965), NEN-ISO 6492 (1999) (Please also see www.nni.nl <http://www.nni.nl> normshop, ics code diervoeders 65.120.)

4.2 Determination of the percentage of dry substance

It sometimes may be useful to express PAH-data on the basis of dry weight. This may also take place on a separate sub-sample of the homogenate that is dried to a constant mass at 105°C. The percentage of dry substance can be determined according to NEN 3327 (1966), NEN 5397 (1982), NEN-EN-ISO 6865 (2001).

(Please also see www.nni.nl <http://www.nni.nl> norm shop, ics code diervoeders 65.120.)

4.3 Adding the internal standards

Prior to extraction, a known quantity of the internal standard mixture should be added to the samples that are measured by means of GC-MS. Add 40 µl of the internal standard working solution (IS dosage standard) with a concentration of 10 µg/ml (3.1.3) to 10 grams of the sample.

5 EXTRACTION

5.1 Extraction of foodstuffs that are not or only partly soluble in the extraction solvent, but that are homogeneously saponifiable by means of alkali

- Direct saponification with ethanolic KOH (oil, fat, chocolate, etc.).
- Weigh 10 grams of sample in a 500 ml Erlenmeyer flask.
- Reflux during 2 hours with 100 ml 2 molar ethanolic KOH (ethanol/water, 9/1,v/v).

- Add 200 ml cyclohexane through the cooler and let the solution boil for another 10 minutes. The boilerplate can already be turned off.
- Add 200 ml 1 molar ethanolic KOH through the cooler.
- Rinse the neck of the Erlenmeyer flask with several millilitres water and close it with a glass plug.
- Let the solution sit for 1 night in order to obtain a good separation between the water layer and the cyclohexane layer.
- Take 100 ml of the cyclohexane layer and evaporate to approx. 2 ml (see remark 11.4).
- In the same manner, prepare at least one blank chemical.

Clean the extract by means of silicagel column chromatography (6).

5.1.1 Adaptation for matrices with a lower fat content such as meat, fish, etc.

- Weigh 10 grams of sample in a 300 ml Erlenmeyer flask.
- Reflux during 2 hours with 50 ml 2 molar ethanolic KOH (ethanol/water, 9/1,v/v).
- Add 100 ml cyclohexane through the cooler and let the solution boil for another 10 minutes. The boilerplate can already be turned off.
- Add 100 ml water through the cooler.
- Rinse the neck of the Erlenmeyer flask with several millilitres water and close it with a glass plug.
- Let the solution sit for 1 night in order to obtain a good separation between the water layer and the cyclohexane layer.
- Take 50 ml of the cyclohexane layer and evaporate to approx. 2 ml (see remark 11.4).
- In the same manner, prepare at least one blank chemical.

Clean the extract by means of silicagel column chromatography (6).

5.1.2 Adaptation for matrices with a low fat content and a high water content such as milk, dairy products, drinks, (liquid) smoke aromas, etc.

- Weigh 20 grams of sample in a 300 ml Erlenmeyer flask.
- Add 6.7 g KOH pellets, dissolve.
- Add 100 ml ethanol and mix.
- Reflux during 2 hours.
- Add 100 ml cyclohexane through the cooler and let the solution boil for another 10 minutes. The boilerplate can already be turned off.
- Add 100 ml water through the cooler.
- Rinse the neck of the Erlenmeyer flask with several millilitres water and close it with a glass plug.
- Let the solution sit for 1 night in order to obtain a good separation between the water layer and the cyclohexane layer.
- Take 50 ml of the cyclohexane layer and evaporate to approx. 2 ml (see remark 11.4).
- In the same manner, prepare at least one blank chemical.

Clean the extract by means of silicagel column chromatography (6).

5.2 Extraction of foodstuffs that are not soluble in the extraction solvent and that are partly resistant to alkaline saponification

- Weigh approx. 0.1 g sample in a previously extracted soxhlet extraction thimble.
- Extract the sample in a soxhlet extractor during 4 hours with 200 ml acetone in a 300 ml Erlenmeyer flask containing a few boiling chips. During the extraction, wrap the Erlenmeyer flask in aluminium foil (see Remarks, 13.2).
- Then carefully evaporate the acetone (boiling delay), with the help of a rotation film evaporator at approx. 40° C. No acetone may be smelled anymore.
- Add 100 ml 0.5 M ethanolic KOH to the residue and let it all saponify in 2 hours while refluxing.
- Continue the procedure as described under 5.1.2.

5.3 Extraction of fatty acids by means of complexation with caffeine and formic acid/extraction with cyclohexane

Remark:

For the method described below it is recommended to perform the first steps up to "collect the" for the benefit of a faster separation in a slightly raised temperature. Maintaining the temperature (40° C) is simple by means of (a tray of) warm water.

- Weigh 10 g of oil or fat in a 100 ml Erlenmeyer flask.
- Transfer the sample to a 250 ml separatory funnel with the help of 40 ml cyclohexane.
- Shake until a homogenous solution is obtained.
- Rinse the neck of the separatory funnel with several millilitres of cyclohexane.
- Shake the cyclohexane three times with 20 ml formic acid (90%).
- Vigorously shake the cyclohexane during two minutes with 20 ml 90% caffeine/formic acid solution.
- Repeat this step with 12 ml 90% caffeine/formic acid solution.
- Collect the caffeine/formic acid fractions in a 1000 ml separatory funnel.
- Add 300 ml of a 2% NaCl solution and shake vigorously.
- Extract the water layer twice with 50 ml cyclohexane during the two minutes.
- Collect the cyclohexane in a 250 ml separatory funnel.
- Wash the cyclohexane twice with 30 ml formic acid (40%).
- Carefully add 2.5 g sodium sulphat to the cyclohexane.
- Decant the cyclohexane in a 250 ml round bottom flask/KD-tube.
- Rinse with 2 x 15 ml cyclohexane.
- Concentrate the cyclohexane and evaporate the cyclohexane to approx. 2 ml (see remark 11.4).
- In the same manner, prepare at least one blank chemical.

Clean the extract by means of silicagel column chromatography (6).

6 CLEAN-UP

6.1 Preparation of silicagel

The silicagel is deactivated with 15% water.

Pipette 15 ml distilled water in a dry, clean bottle with screw cap. Add 85 g fresh silicagel (dried one night at 130°C). Close the bottle and shake till all lumps have disappeared. In order to obtain an even distribution of the water, the bottle is being rolled on a roller bank for 2 hours at 100 rpm.

6.2 Column chromatography with regard to silicagel

See remark 11.2.

Weigh 10 g deactivated silicagel in a beaker and add 20 ml hexane. Stir this mixture till there are no more air bubbles.

Pour the mixture into a chromatographic column and rinse the walls with a little hexane. Let the hexane run off until just above the silicagel surface. Transfer the concentrated extract obtained by the procedures described in paragraph 5 by means of a Pasteur-pipette to the column and rinse the Erlenmeyer flask (or K.D. tube) three times, each time with 2 ml hexane. Let this settle until the column is just not standing clear of the liquid. Elute with 120 ml hexane/dichloromethane (80:20 v/v).

Collect the eluate in the original vessel. Perform this column cleaning in a darkened fume hood (see remark 11.2). Concentrate the eluate until several millilitres (see remark 11.4).

7 ANALYSIS BY HPLC

7.1 HPLC analysis principle

The analysis takes place by means of fluid chromatography and fluorescence detection. The separation occurs on a C-18 column and water-acetonitril gradient elution. The identification is based on retention time.

7.2 Equipment and materials for the HPLC analysis

- HPLC equipment suitable for gradient elution and supplied with a programmable fluorescence detector, an auto sampler and a PC with control and data processing software.
- HPLC column; Vydac 201 TP 5 μ m, 4.6 x 25 cm or equivalent (the gradient setting is reflected in Annex A).

7.3 HPLC procedure

7.3.1 Sample preparation

Let the remaining solvent of the cleaned extract obtained in 6.2 evaporate in air in the dark during 1 night, or carefully remove the remaining solvent with a small nitrogen stream.

Redissolve the residue in 1.0 ml acetonitril. The extract can be kept in the dark for several days. Prior to injecting a series of samples, first a gradient is ran without injection in order to stabilise the column.

Then a suitable standard solution is injected at least two times with the wavelengths set as follows (Excitation 298 nM, Emission 439 nM) (survey run). On the basis of these two survey runs, the switching times of the fluorescence detector are set. Annex A1

reflects the optimal wavelengths.

The blanks are measured as the first samples of a analysis series. Subsequently, five standard calibration solutions are injected and the calibration curve is determined (see 7.3.2).

If the calibration curve is linear (see 9.7) and the chromatogrammes meet the criteria with regard to the resolution (the peak separation of the least separated peaks should be minimally 0.6 and the asymmetry factor of all analytic peaks should be in the 0.9-1.5 range), the sample extracts are measured. Dilute the sample if necessary to obtain a concentration which falls within the range of the calibration curve. Of all solutions, standards, blanks and sample extracts 20 µl is injected.

7.3.2 Calibration

The quantitative determination of the compounds mentioned in paragraph 1 takes place in accordance with the external standard method, meaning that each component is quantified in relation to the calibration line.

At least in the beginning of each series, the calibration standards (3.2.2) are measured. Furthermore, a calibration solution is injected around a well determined number of extracts (for instance 5). The peak area of each relevant PAH-compound is measured. These benchmark figures are averaged for each analyte and this value is used for the calculation.

7.3.3 Analysis

If there is not sufficient separation obtained to measure the different peaks at optimal wavelength settings, the extract can be measured twice.

Programme A is ran at analysis 1 and programme B at analysis 2. The settings for both series of the fluorescence detector are reflected in Annex B.

7.3.4 Identification

The identification takes place by comparing the retention times of the peaks in the sample with those of the standard.

7.4 Calculation

The content of each component is calculated as follows:

$$G = \frac{C_{st} * (H_m - H_{bl}) * E}{H_{st} * W * V}$$

- G = content of the separate PAHs (ng/g),
- C_{st} = concentration of the standard (ng/ml),
- V = dilution of the standard,
- H_{st} = surface PAH peak of the standard (mV),
- H_m = surface PAH peak in sample (mV),
- H_{bl} = surface PAH peak in blank (mV),
- W = weighed mass (g),

E = final volume (ml).

8 ANALYSIS BY GC-MS

8.1 GC-MS principle

The analysis takes place by means of a gas chromatograph (GC), equipped with a mass spectrometric detector (MS), in accordance with the 'selected ion monitoring' (SIM) method. Alternatively, provided that there is sufficient detectability and adaptation of the concentrations of calibration and dosage standards given below, it is possible to work in "full scan" modus starting from the extracted ion chromatogrammes.

The identification of a polyaromate to be determined is based on the comparison of the retention time in the specific ion chromatogramme of sample and calibration solution. The quantitative determination goes in accordance with the internal standard method, whereby known quantities of deuterium-labelled components are added to the sample as internal standards prior to the extraction. For each of the polyaromates to be analysed, the equivalent deuterium-labelled compound is added (isotope dilution). Contents are calculated using the integrated peak areas and/or peak heights of the most characteristic ions of the native compounds and the internal standards.

The quantitative determination in accordance with the internal standard method allows to automatically and accurately correct for the losses that may occur in the following steps of the analysis: extraction, purification, evaporation and injection .

This can be achieved by adding a so-called recovery-standard, for instance D10-anthracene, or a different non-coeluting compound. The quality criteria as established in the European directives EEG 93/256 are also taken into consideration.

8.2 Equipment and materials for the GC-MS analysis

- Syringes of 50-250 µl to add the internal standard and recovery standard.
- GC-MS consisting of a capillary gaschromatograph, an auto-sampler, a mass spectrometer and a PC with control and data processing software.
Eventually the GC is equipped with a PTV or on column large-volume injector.
- Fused silica GC-column with apolar stationary phase, for instance DB5-ms, 30 m x 0.25 mm x 0.25 µm, or equivalent.

Remark:

On an apolar phase, benzo(b)fluoranthene and benzo(j)fluoranthene on the one hand and dibenzo(a,c)anthracene and dibenzo(a,h)anthracene on the other hand, co-elute.

A semipolar column can be used alternatively (for instance a 60 m x 0.25 mm x 0.25 µm DB-1701, or equivalent). In that case, benzo(j)fluoranthene co-elutes with benzo(k)fluoranthene, which allows an interferencefree determination of benzo(b)fluoranthene. Based on the simultaneous analysis of real samples on both columns, it can be inferred that benzo(j)fluoranthene is continuously present in a concentration which amounts to approximately 40% of the concentration of benzo(b)fluoranthene.

8.3 GC-MS procedure

8.3.1 Sample preparation GC-MS analysis

Procedure for final concentrating and adding of the recovery standard:

- Transfer the extract (6.2) which has been cleaned and reduced to some ml's to an amber-coloured sample flask in which, eventually, previously 1 ml n-nonane or toluene has been brought as 'keeper' (rinse the tube afterwards);
- Evaporate the extract to a final volume of approximately 1 ml;
- With the help of a syringe, add a known quantity of the recovery standard work solution to the final extract, such that the concentration amounts to approx. 1 µg/ml.

The extract can be kept in the dark for several days.

Remark:

In case of large volume injection, the above operations are not applicable and an adjusted quantity of recovery standard is added in relation with the injection volume (typically 1 ng recovery standard is injected).

8.3.2 Analysis (GC-MS)

Of the extracts obtained under 8.4.1 and of the standard work solution for GC-MS calibration, standard 1 µl is injected in the gas chromatograph splitless or on-column. Alternatively, a large-column injection with a PTV injector or an on-column injector with solvent vapour exit can be applied. Normally, the chromatographic separation of the components takes place on an apolar column with chemically bound phase. The detection of the components takes place by means of a low resolution mass spectrometer in the SIM-mode, with selection and registration of the molecular ion of the PAH to be analysed, the deuterium-labelled internal standards and the recovery standard. Alternatively the full scan mode can be used, with extraction of the ion chromatogrammes that are specific for the PAHs. In view of the reduced detectability in full scan modus, the concentrations of the calibration standard, internal standards and recovery standard can be increased by a factor 10.

The mass spectrometer is set to maximal response for the ions 131, 219, 264 and 414 with the help of the reference gas PFTBA, or equivalent.

The typical GC-MS working conditions for PAH analysis are reflected in Annex C.

Remark:

If a signal is observed that is bigger than the highest concentration of the (linear) range (see below), then the extract should be diluted.

8.3.3 Calibration (GC-MS)

The quantitative determination of the different PAHs takes place according to the so-called internal standard method. In this case each component is quantified in relation to the deuterium-labelled equivalent compound that was added to the sample at the beginning of the extraction or at the beginning of the clean-up.

At least in the beginning and at the end of each series of analyses, and further after a well determined number of extracts (for instance 5), the calibration solution is injected. Of each PAH, native or labelled, the peak area in the ion chromatogramme of the characteristic ion is measured for the calibration solutions as well as the sample preparations. Subsequently, relative response factors for each component to be analysed are determined from the relation of the surfaces of the native components and the equivalent internal standards (see also 7.2). For the internal standards, relative response factors are determined in relation to the recovery standard.

8.3.4 Identification (GC-MS)

The presence of native PAHs in the samples is confirmed on the basis of the following data and criteria:

- the registration of a peak at the characteristic m/z, with a peak height greater than 3 times the noise level;
- the retention time of the observed peak in the ion chromatogramme of the native PAH, whereby a difference of maximally 10 sec as compared to the retention time for the deuterium-labelled PAH is used (deuterium-labelled PAHs elute approx. 5 sec earlier than the native PAHs).

The identification of internal standards is also based on the characteristic m/z and the signal/noise relationship, and furthermore on the elution order such as experimentally determined. Of the identified peaks, the area or, alternatively, the peak height is determined.

The characteristic m/zs of the native and labelled PAHs are reflected in Annex C.

8.4 Calculations (GC-MS)

Internal calibration

Relative response factors

Based on the integrated peak areas of the native PAH component and the equivalent internal standard in the respective ion chromatogrammes of the calibration solution, for each PAH component the relative response factor (RRF) is calculated as follows:

$$RRF_i = \frac{A_i \cdot C_{IS}}{A_{IS} \cdot C_i}$$

with

RRF_i = relative response factor of PAH component i

A_i = peak surface of PAH component i at injection of the calibration solution

C_i = concentration (in ng/μl) of PAH component i in the calibration solution

C_{is} = concentration (in ng/μl) of the equivalent internal standard in the calibration solution

A_{is} = peak area (peak height) of the equivalent internal standard at injection of the calibration solution

In order to determine the PAH content in a sample, it is preferred to use the average RRF values obtained starting from two injections of the calibration solution, i.e. the last one preceding and the first one following the sample analysis.

Content of the PAH components in the sample

Using the integrated peak areas (peak heights) of the PAH component and the equivalent internal standard in the respective ion chromatogrammes of the sample preparation, and taking into consideration the relative response factor of the PAH component considered, the concentration of the PAH component in the sample can be calculated as follows:

$$C_i = \frac{A_i \cdot g_{IS}}{A_{IS} \cdot \langle RRF_i \rangle \cdot G \cdot 1000}$$

with

C_i = the content in mg/kg of PAH component in the sample

A_i = peak area of the PAH component i at injection of the preparation

A_{IS} = peak area of the equivalent internal standard at injection of the preparation

g_{IS} = quantity in ng of the equivalent internal standard added to the sample

$\langle RRF_i \rangle$ = the average relative response factor for PAH component i starting from two injections of the calibration solution, preceding and following the sample preparation

G = weighed mass (g)

Remark:

As mentioned above, on apolar columns benzo(j)fluoranthene only coelutes with benzo(b)fluoranthene and thus both compounds are measured together. One reports benzo(b)fluoranthene+benzo(j)fluoranthene.

9 QUALITY CONTROL (GC-MS and/or HPLC/FL)

A number of quality controls are described below. For each part it is indicated for which analysis technique these controls are relevant.

9.1 Response linearity (GC-MS)

Starting from minimally 5 standard solutions with different concentrations of PAH compounds, and in case of internal quantification with a definite concentration of internal standards, and starting from ten times the minimum detectable quantity (see below), the linearity of the detector response is verified.

In case of internal quantification, the relation of the detector response of the PAH component and the equivalent internal standard is plotted in relation to the relationship between the concentration of the PAH components and the internal standard.

For each PAH compound, a straight line should be obtained, the variation coefficient V_{xo} (see ISO-8466-1990:1) of which is smaller, or the determination coefficient of which is larger, than an assumed value (for instance $V_{xo} < 15\%$, $r > 0.99$).

In addition, one should plot C_i/A_i in relation to C_i or $(A_i \cdot C_{IS})/(A_{IS} \cdot C_i)$ in relation to C_i . The linear range is defined as being the area for which the deviation of C_i/A_i or $(A_i \cdot C_{IS})/(A_{IS} \cdot C_i)$ with regard to the average value is maximally 15%.

A control of the linearity is carried out at each serious instrumental intervention.

Remark:

When the peak area for a certain component in an injected sample extract is higher than the highest surface that was obtained during the most recent linearity test or during the positioning of the calibration straight line, a re-analysis should take place on the original extract after dilution.

9.2 Specificity (GC-MS)

In case of GC-MS analysis, the column quality is verified by means of the separation of the critical pair benzo(b)fluoranthene and benzo(k)fluoranthene in the chromatogramme of the calibration solution. When using a 30 m apolar column, the gas chromatographic separation percentage ($100 \times \text{height valley/height highest peak}$) should be smaller than 60%.

9.3 Sensitivity (GC-MS and HPLC/FL)

The minimum detectable quantity is a measure for the sensitivity of the apparatus. On the basis of signal-noise relationship registered for the PAH compounds in the chromatogramme of the calibration standard solution, the sensitivity of the apparatus can be verified. This should be such that the requested reporting limit can be obtained without problems. This should be checked for each series.

9.4 Response factors and relative response factors (GC-MS)

In case of internal calibration, relative response factors are determined on the basis of a calibration solution from the central region of the linear range. Within one and the same analysis series, it should be verified to what extent the relative response factors obtained for two consecutive injections of the calibration solution, with intermediate injection of at least one sample extract, deviate. For each PAH, the relationship RRF_1/RRF_2 should be between 0.85 and 1.15. Preferably, the average of 2 relative response factors is used for the calculation of the contents of an intermediate series of samples.

9.5 Blank (GC-MS and HPLC/FL)

In each analysis series at least one procedure blank is determined. Here the entire analysis procedure is followed, however, without intake of samples. The registered chromatogramme should be free of interfering peaks larger than 10% of the peaks registered for the sample with the exception of sample values smaller than 5 times the requested reporting limit, as a result of which interfering peaks should not be larger than half of the requested reporting limit.

9.6 Control sample (GC-MS and HPLC/FL)

A control sample is taken in on a regular basis.

Of at least 3 PAHs spread over the entire retention time area, the contents are registered in control cards, together with the sum of the content of all PAHs. The registered values should comply with the criteria concerning the control cards.

Remark:

For purposes of validation, in relation to the matrix a certified reference material is used (unless this would not be available). In order to verify the correctness and reproducibility of the daily analyses, it is acceptable to use a spiked sample or, in the worst case, an independent control standard.

9.7 Recovery efficiency of the internal standards (GC-MS)

If the internal quantification method is used, then, on the basis of the signal registered for the internal standards and the recovery standard, for each sample the recovery efficiency of the internal standards can be determined:

$$R\% = \frac{A_{IS} \cdot g_{RS} \cdot 100}{A_{RS} \cdot g_{IS} \cdot RRF_{IS}}$$

with

R% = recovery efficiency (in %)

A_{IS} = peak area of the internal standard at injection of the preparation

A_{RS} = peak area of the recovery standard at injection of the preparation

g_{RS} = quantity (in ng) of the recovery standard added to the preparation

g_{IS} = quantity (in ng) of the internal standard added to the sample

$\langle RRF_{IS} \rangle$ = average relative response factor of the internal standard in relation to the recovery standard, starting from two injections of the calibration solution, preceding and following the sample preparation

Justified quantification is only allowed if the recovery efficiency of the separate internal standards is minimally 50%.

10 REPORTING

In addition to the contents for the individual congeners in $\mu\text{g}/\text{kg}$, the sum-parameter is also calculated in μg BAPEQ/kg of fat or product. For this purpose the contents of the individual congeners are converted to the content of the most toxic component, benzo(a)pyrene, by means of the Toxic Equivalency Factors (TEF) (see table 1 part 1) and subsequently the sum of these values is found. If the action limit of 15 $\mu\text{g}/\text{kg}$ is exceeded, a closer investigation is started with regard to the source of the contamination. If the 50 μg BaPEQ/kg limit is exceeded, the product may not be used for the preparation of animal feed.

11 GENERAL REMARKS

- 11.1 Residues of reagents, reaction products and solvents need to be transported in special containers in accordance with the location regulations.
PAHs are suspected to possess carcinogenic properties. Skin contact should be avoided with regard to solid substances, extracts and solutions of PAHs.
- 11.2 Due to the fact that PAHs degrade rapidly under the influence of UV light, all samples, eventual solid standard substances, standard solutions and extracts should be kept in the dark to the extent possible.
During work on a table, sunlight should be kept out. During saponification and the Soxhlet extraction the Erlenmeyer flasks should be wrapped in aluminium foil.
The silicagel cleaning is a critical step in the analysis. PAHs adsorbed to silicagel are extremely photo-oxidation sensitive. Therefore, the column cleaning should take place rapidly and in a darkened fume hood.
- 11.3 The mixing-standards calibration solutions can be kept for approximately 1 year, provided that they are well closed and kept in the dark.
- 11.4 If so desired, the evaporation of the cyclohexane can also take place with the help of Kuderna-Danish equipment.
- 11.5 The PAH analysis is sensitive to contamination. Soxhlet thimbles are previously extracted with acetone, dried and then stored dustfree. Glasswool and boiling chips can be cleaned simultaneously in this manner.
- 11.6 Glasswool should be carefully rinsed with a suitable solvent (sufficient pure diethylether or acetone). Special attention should be given to the danger of contamination from rotation film evaporators and reflux coolers. Be careful when using pipetting balloons/pipettors and compressed air (blow-drying glasswork).
- 11.7 Always perform (sufficient) chemical blanks.
- 11.8 For two reasons, solvents for the HPLC analysis should be well degassed, for instance by means of continuous degassing of the solvent reservoirs with helium before and during use or vacuum degassing: to guarantee optimal functioning of the high pressure pump(s), and to avoid 'quenching' effects of fluorescence signals (for instance of pyrene) by means of oxygen. Polytetrafluorethylene (PTFE) pipes should not be used downstream from the solvent tanks because this material is not oxygen-proof. Stainless steel or polyetheretherketone (PEEK) is recommended.
In case of preparing fatty acids, it is recommended to keep the fluid, the sample, as well as the glasswork warm until the complexation. Keeping warm is not a requirement, however, it can be useful for the purpose of a faster separation. Keeping warm can be accomplished simply by means of (a container with) warm water.

LITERATURE

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Annex A HPLC gradient setting to determine PAHs (Gradient programme)

Step	Time	Flow	%A Water	%B ACN
0	0	1.00	50.0	50.0
1	2.0	1.00	50.0	50.0
2	30.0	1.00	0.0	100.0
3	15.0	1.00	0.0	100.0
4	5.0	1.00	50.0	50.0
5	5.0	1.00	50.0	50.0

Annex A1: optimised excitation and emission wavelengths for PAHs^{*1}

PAK	λ_{ex} nm	λ_{em} nm
acenaphthene	292	324
Fluoranthene	360	460
Pyrene	336	376
Benz(a)anthracene	288	390
Chrysene	268	383
Benzo(b)fluoranthene	300	436
Benzo(k)fluoranthene	308	414
Benzo(a)pyrene	296	408
Benzo(g,h,i)perylene	300	410
Dibenzo(a,h)anthracene	297	398
Indeno(1,2,3,c,d)pyrene	302	506

*1 Derived from NEN 5771

Annex B HPLC settings to determine PAHs when analysis twice
(setting of the detectors)

Prog	Time in min.	λ_{em}	λ_{ex}
1	t ₁	280	334
	t ₂	280	340
	t ₃	292	366
	t ₄	360	460
	t ₅	288	390
	t ₆	305	430
	t ₇		
2	t ₁	280	334
	t ₂	280	340
	t ₃	253	402
	t ₄	336	376
	t ₅	288	390
	t ₆	295	427
	t ₇	302	506

Annex C Typical GC-MS working conditions to determine PAHs

Column specifications : DB-5MS or equivalent, 30 m x 0.25 mm x 0.25 µm

GC settings

Carrier gas and pressure : Helium, 75 kPa
 Injection modus : Splitless (purge on after 1 min.)
 Split vent : 30 ml/min
 Septum purge : 1 ml/min
 Injection volume : 1 µl
 Injection temperature : 300°C
 interface temperature : 275°C

MS settings

Source temperature: 250°C
 Electrons energy: 70m eV
 SIM-ions: see Table 1

Temperature programming GC-oven

125°C : isotherm during 1 min
 125°C → 205°C : 20°C/min
 205°C → 305°C : 10°C/min
 305°C : isotherm during 15 min
 total duration : 30 min

m/z values:

<i>PAH-component</i>	<i>m/z</i>
Acenaftylene	152
Acenaftene	154
Fluoranthene	202
Pyrene	202
Benzo(a)anthracene	228
Chrysene	228
Benzo(b)fluoranthene	252
Benzo(k)fluoranthene	252
Benzo(a)pyrene	252
Indeno(1,2,3,c,d)pyrene	276
Dibenzo(a,h)anthracene	278
Benzo(g,h,i)perylene	276
d8-acenaftylene	160
d10-acenaftene	164
D10-fluoranthene	212
D10-pyrene	212
D12-benzo(a)anthracene	240
D12-chrysene	240
D12-benzo(b)fluoranthene	264
D12-benzo(k)fluoranthene	264
D12-benzo(a)pyrene	264
D12-indeno(1,2,3,c,d)pyrene	288
D14-dibenzo(a,h)anthracene	292
D12-benzo(g,h,i)perylene	288

Annex D

Validation scheme

For the purpose of validation, the following characteristic features should be determined per type of matrix (animal fatty acids, animal feed):

Specificity

The discriminating ability of the method to determine analytes and related products should be checked. Representative blank samples should be analysed in order to verify the prevention of possible interferences and the effect of interfering substances. For this purpose a number of blank samples should be analysed and it should be checked whether interferences are detected in the area where the analytes to be determined are expected. Additionally, representative blank samples should be spiked with a relevant concentration of one or more substances that might interfere with the identification and/or quantification.

Accuracy

In order to determine the correctness, one should use certified reference material, of which the content of the components to be determined is as close as possible to the limit value concerned. This sample should be analysed six times.

The correctness is calculated by means of the following formula:

$$J (\%) = 100 \times X/W$$

Whereby: X is the average of the 6 analyses;
W is the true value.

If no (C)RM is available, the recovery percentage should be determined with regard to samples with added standards.

Recovery

If no (C)RM is available, the recognition percentage should be determined per type of matrix on the basis of charged samples on three levels.

The following formula is used:

$$R (\%) = (X - X_{bl})/Z \times 100$$

Whereby: Z is the added quantity of analyte
 X_{bl} is the average content of the analyte in the blank sample.

Precision

Repeatability: the degree of conformity between consecutive results that are obtained with the same method in identical analysis material and under repeatability circumstances. The

repeatability is calculated on the basis of at least 6 analyses performed out on the same sample material. The criteria for the repeatability (intra-laboratory relative standard deviation VC %) amount to half of those obtained with the Horowitz comparison for the inter-laboratory variation coefficient.

Intra-laboratory reproducibility: the degree of conformity between consecutive results that are obtained with the same method in identical analysis material and under reproducibility circumstances. A measure for the intra-laboratory reproducibility is the relative standard deviation (RSD %) of variation coefficient (VC%), calculated on the basis of the Horowitz comparison:

$$\text{VC \%} = 2^{(1-0.5 \log C)}$$

Whereby: C, the content, is expressed as a power of ten.

In relation to the contents to be detected, the following VC % values should be found:

1	µg/kg	→	30 %
100	µg/kg	→	23 %
200	µg/kg	→	21 %
1000	µg/kg	→	16 %

Limit of Detection (LOD)

The charging of a blank sample at the expected LOD-level should result in a signal/noise relationship of at least 3/1.

Limit of Quantification (LOQ)

The charging of a blank sample at the expected LOD-level should result in a signal/noise relationship of at least 6/1.

Linearity

The range of the calibration curve should be chosen such that it is representative for the concentrations to be expected of the analytes to be distinguished in the samples.

At least 5 concentration levels (including 0) should be used when determining the curve. The curve may not forcibly go through the zero point.

The mathematical connection of the curve and the 'goodness-of-fit' of the values with regard to the curve should be described.

It is allowed to use a 1-point calibration under the condition that the curve goes through the zero point (not forced) and the connection is linear.

