UMWELTPROBENBANK
DES BUNDES

Guideline for Sampling and Sample Processing

Roe Deer (*Capreolus capreolus*)

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Guidelines for Sampling, Transport, Storage and Chemical Characterization of Environmental and Human Samples

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1 German Environmental Specimen Bank

The German Environmental Specimen Bank (ESB) is an instrument for environmental monitoring of the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU) subject to specialist and administrative coordination by the Federal Environment Agency (UBA). The ESB collects ecologically representative environmental and human samples and stores and investigates them for environmentally relevant substances.

Specific operating procedures as well as the conception of the ESB are the basis of the program. (Umweltbundesamt 2008, 2014)

The long-term storage is carried out under conditions which, as much as possible, exclude a change in state or a loss of chemical characteristics over a period of several decades. The archive therefore provides samples for retrospective investigations of substances for which the potential risk for the environment or human health is not yet known.

Comprehensive information on the ESB is available at www.umweltprobenbank.de.

2 Objective of this Guideline

Sampling is the first and most important step to safeguard the quality of samples and data. It is the result of science-based, standardized methods to avoid contamination and inhibit loss of chemical information. The need for an exceptionally high level of quality assurance results from the extraordinary value of the samples as archive material. Representativeness and reproducibility of the samples are the basis for spatial and temporal comparison.

The current guideline is an update of the Tarricone *et al*. (2012) version.

Transport, further sample treatment and storage as well as chemical analysis have to be carried out according to the current guidelines of the ESB.

3 Function of the Specimen Type

The European roe deer (*Capreolus capreolus)* has been investigated as a subject for environmental monitoring since the beginning of the 1970s and has been emphasized as a qualified bioindicator (Holm *et al*., 1990, Hecht 2001, Tataruch 2001, Kierdorf *et al*. 2008, Pokorny *et al*. 2009, Durkalec *et al*. 2015, Bakowska *et al.* 2016).

As selective herbivores, roe deer take the position of first-order consumers in terrestrial ecosystems. Sufficient knowledge concerning food consumption and nutrition of roe deer is available due to several research studies (Hespeler 1999, 2016).

The following criteria underline the appropriateness of the use of roe deer as an indicator organism:

- Wide geographic distribution. It is spread from South-Western Europe through Central and Northern Europe to Russia (Ural Mountains) and Asia Minor (Turkey, Caucasian region, Iran, Hespeler 2016) Other subspecies substitute the species in Siberia and Eastern Asia.
- Most commonly distributed, free-living, tall herbivore in Europe, within Germany alone more than 1,000,000 deer are shot every year (Deutscher Jadgschutzverband = German Hunting Association)[1].
- Distributed throughout almost all terrestrial ecosystems in Central Europe (Hespeler 2016).
- Considerable location loyalty (Debeffe *et al*. 2014, Hespeler 2016), between 10 – 40 ha for variable territory size, depending on game density, age, sex, gender ratio, regional habitat structure, food supply and season, therefore the activity range is appropriately limited.
- Physiologically and eco-physiologically well-investigated species (Frölich *et al*. 2001, Gehrke 2001, Wisser *et al*. 2001).
- Comprehensive experience concerning the accumulation behavior of wild roe deer for elements and organic substances, as well as radionuclides.
- Popular for human nourishment.

Within the ESB program, the roe deer represents the level of consumers in terrestrial ecosystems.

4 Target Compartments

ESB sampling aims at the liver of the roe deer as the target compartment:

- Most substances are best traceable in the liver. This applies to all thus-far-investigated Chlorinated Hydrocarbons and for elements such as Cr, Mo, Mn, Cu and Fe. The elements Cd, Pb, V, Zn and Ca accumulate more in the kidneys than in the liver. Hg, Al and Mg presumably present equal concentrations in both organs.
- Substances are very homogeneously distributed.
- With approx. 300 – 500 g, the liver provides a sufficiently large sample amount, approx. four to five times the amount from kidneys.
- Liver fat contents are subject to less seasonal variation than those in the kidneys.
- Removal of the liver does not decrease the value of the venison.
- The liver is located in the abdomen of the roe deer, which is not damaged by a precise heart shot. The risk of unwanted contamination by the bullet can be drastically reduced by exclusively using roe deer with undamaged abdomens for pollutant studies.

5 Predefinitions for the Sampling

5.1 Selection and Definition of Sampling Sites

As a consequence of the deer's mobility (10 – 40 ha activity range), sampling sites are relatively large. Roe deer are shot during a normal hunt by authorized hunters. In Germany, hunting is organized in a district system. Each hunting district tenant holds the right to hunt in his own district, therefore hunting districts are the most important organizational subdivision. Roe deer density determines the number of districts per sampling region. These are included in the area-related sampling scheme, in order to assure long-term sample consistency.

In case of an extremely low roe deer density and/ or in small-sized sampling regions, it may become necessary to define the whole investigated area as the sampling site.

5.2 Selection of Individuals and Sample Size

For statistical reasons, the storage of the livers of at least 10 animals (yearlings, of both sexes) is required per sampling site and sampling period.

Yearlings are closely territorially bound by the doe. At the sampling date they are older than 12 months and have been exposed to the pollution over the course of an entire year.

Exclusively livers of healthy animals are collected. These must not exhibit any variations of the normal condition of venison and organs. Sicknesses, depending on their kind and manifestation, alter the physiology of the organism. Sick animals canbe identified prior to shooting by conspicuous appearance (shaggy coat, body mass losses) and/or abnormal behavior.
Therefore, sampling is to be carried out by experienced hunters, who are able to distinguish these criteria and guarantee adherence to the sampling guideline. Subsequent to shooting and gutting, other manifestations of sickness are detectable, e.g. deviation from the normal size, shape, and color of organs, deposits at organs, increased body fluids, abnormal smell, or extreme ecto- or endoparasite infestation. Livers from non-healthy animals are not used for ESB storage.

5.3 Sampling Period and Frequency

The yearlings are shot from early May until the start of the rutting season (depending on weather conditions, about mid-July). Sampling can be conducted yearly, without serious consequences for the natural populations to be expected.

5.4 Area Related Sampling Scheme

Based on the sampling guidelines, specific definitions for the individual sampling areas and sites must be made and documented in an area-related sampling scheme. These include, but are not limited to:

- location and demarcation of the sampling sites,
- required sample size,
- time frame for sampling,
- appropriate authorities.

Here it is important to consider how to ensure a long-term sampling continuity. If changes are made, the document must be updated.

6 Sampling Procedure

All data collected in the course of sampling and throughout the biometric sample description are documented in the respective specimen data sheets (see appendix).

For each roe deer, a separate record (specimen data sheet 2) containing the following information is created by the hunter:

- date and time of the kill,
- name of the hunter,
- number of the proof of origin to identify the shot animal,
- chronological sampling procedure,
- position of the bullet hole, exit wound and bullet type,
- description of the health status of the animal.

Thereby a deviation from the guideline can be identified for each individual.

Preparation for roe deer sampling requires comprehensive organization. First, with the help of local contact persons, reliable hunters are selected. Preceding initial sampling, these particular hunters must be briefed on all steps in the course of sampling. Instruction thereby focuses on the selection of animals complying with the guidelines, a contamination-free kill and proper sample packaging. At the start of each sampling interval the necessary packaging materials must be compiled and handed over to the hunters.

It is also important to always stay in contact with the local contact persons in order to be able to quickly react if problems arise and to be able to comlete the sampling including pickup of the samples with the time period allotted in the guideline.

6.1 Required Equipment and Cleaning Procedures

Field work:

- 15 specimen data sheets 2,
- 15 instructions for sampling procedure,
- 15 plastic bags,
- 15 linen bags,
- cooling devices (at least -15°C) for liver transport and intermediate storage.

For the sampling of a roe deer the hunter needs to obtain the following materials/documents in advance:

- one specimen data sheet 2,
- one instruction for sampling procedure,
- one plastic bag for packaging the liver.

For packaging the roe deer livers, only plastic materials that are suitable for cryogenic storage conditions (up to -200°C) are be used. They must not contain any additives and should be non-reactive to lipophilic substances in the sample (suitable plastic bags are made of fluorinated ethylene propylene [FEP]).

Laboratory:

- specimen data sheet 3 for storage condition,
- clean bench with particle and activated carbon filtration,
- stainless steel containers with lid and fastener,
- scale (reading 1 g) to determine liver weight,
- waterproof pen to label the stainless steel containers,
- laboratory clothing and disposable gloves,
- cooling device for the storage of the samples in the gas phase above liquid nitrogen (LIN),
- protective clothing for liquid nitrogen handling.

Sample containers and all equipment are cleaned in a laboratory washer using a chlorine-free powerful washing agent in a first step. After cold and hot (90 – 95°C) rinsing, neutralization using 30 % phosphorus acid in warm water is performed, followed by hot and cold rinsing with deionized water. After this procedure the containers are dried in a cabinet dryer at 130° C (± 10°) for a minimum of one hour (sterilization). The containers remain in the closed cabinet dryer while they are left to cool. Sterilization is not applied to synthetic materials

6.2 Sampling Technique

The roe deer are killed by means of an aimed bullet shot during stand hunting or stalking. Organs of roe deer shot in the course of a drive hunt are not suitable as sample specimen. Pre-mortal stress caused by hounding or not-directly-lethal shots may influence the pollution concentration within the organs. The abdomen of the animal should not be injured by the shot. Neck shots and precise chamber shots result in the lowest bullet contamination hazards, and are therefore the preferable manner of shooting. In the specimen data sheet for sample description (specimen data sheet 2), the exact position of the bullet's point of entry and exit must be recorded.

During the sampling process, extremely close attention must be paid to avoid contact of the samples with hair, plants, soil particles, etc. Sampling should be carried out as follows:

- the roe deer must be gutted within a timeframe of 30 minutes following the kill,
- the uninjured liver must be placed in the plastic bag which is placed into the portable linen bag,
- alternatively, the whole corpse can be immediately transported to the collection point, where the liver is removed and subsequently placed inside the plastic bag. In this case the Order on Meat Hygiene (VO (EG) 583/2004) must be followed,
- the specimen data sheet for sample description must be filled out and placed inside the repective linen bag,
- livers need to be deep-frozen within the portable bag, together with the respective specimen data sheet 2 within 24 hours at the latest (min. -15°C).

The interim storage of the samples in the deep-freezer must not exceed 4 weeks.

If necessary, sample collectors are equipped with a deep-freezer. Transport from the sample collection point to the laboratory is conducted via portable deep freezers at a minimum temperature of -15° C or inside portable Dewars in the gas phase above liquid nitrogen. The data of the steps "sample pickup and verification", "storage" and "transfer" are to be entered into specimen data sheet 3.

The transferal into labeled (container ID, sample ID) stainless steel containers will take place under clean air conditions in the laboratory as follows:

- under clean air conditions the liver samples are weighed inside a clean stainless steel container without their plastic bags,
- afterwards they are transferred in the pre-cooled stainless steel containers designated for storage,
- subsequently, the containers will be stored in the gas phase above liquid nitrogen,
- the ID of the container will be recorded together with the weight of the liver and the storage date and time on the specimen data sheet 3.

7 Biometric Sample Characterization

During sampling, data concerning weight, state of heath, and infestation with parasites of roe deer are collected. All data gained in course of the sampling are to be documented in the respective specimen data sheet. The determination of the liver weight is performed as described in Chap. 6.2. For a better interpretation of the values of the chemical analysis, additional data of the roe deer's browsing behavior is collected. Thus every five years a browsing survey is performed in the sampling areas. The respective instruction can be found in the appendix ('browsing survey').

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Checklist to Prepare and Conduct the Sampling

Specimen Type:	**Roe deer (*Capreolus capreolus*)**
Target Compartment	liver
Individual Specimens	yearlings (both sexes)
Random Sample Number	at least 10 animals
Sample Quantity for the ESB	2,200 g liver
Sampling Period	early May, to the beginning of the rutting season (ca. mid-July)
Sampling Frequency	annually
Equipment Required for Field Work	• specimen data sheet for sample description • instruction for the sampling procedure • PTFE-bags and portable linen bags
Sample Packing until Further Processing	• plastic bags for interim deep freeze storage • stainless steel containers with lids and fasteners
Transport and Interim Storage	• cooling device (at least -15°C) at the collection point • cooling device (at least -15°C) for transport from the collection point to the laboratory • cooling device (dewar) for the storage of the samples in the gas phase above liquid nitrogen (LIN)
Required Equipment for Laboratory Work	• specimen data sheet for storage condition • clean bench with particle and activated carbon filtration • weighing pans • scale (reading 1 g) • laboratory clothing and disposable gloves • waterproof pen to label the stainless steel containers • protective clothing for liquid nitrogen handling
Sample Characterization	• sex • body weight [kg] • state of health • infestation with parasites • liver weight (reading 1 g)

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 1: Sampling Location

Roe deer (*Capreolus capreolus*)

Identification:

__ __ __ __ **/ X /** __ __ __ __ **/** __ __ __ __ __ **/** __

	Specimen Type
	Specimen Condition
	Collection Date (MM/YY)
	Sampling Area (SA)
	Sampling Region (SR)
	Sampling Site (SS)
	Additional information

Sampling Site (plaintext) _____

Sampling Point (number) _____

Sampling Point (plaintext) _____

Sampling Leader _____

Remarks _____

_____

_____

_____

_____

_____

Notes _____

_____

_____

_____

_____

_____

_____

_____

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 2: Sample Description

Roe deer (*Capreolus capreolus*)

Identification: **Sampling Site:**

__ __ __ __ **/ X /** __ __ __ __ **/** __ __ __ __ __ **/** __ _____

No. of Proof of Origin __ __ __ __ District/Division: _____

Name of the Hunter: _____

Date of Kill: __ __ . __ __ . __ __

Time: shot: __ __ : __ __ perished: __ __ : __ __

gutting: __ __ : __ __ storage: (deep freezer) __ __ : __ __

Bullet Type: _____ ☐ lead ☐ no lead

Shooting Distance: __ __ __ m

Injured Organs: ☐ lung ☐ spleen ☐ gut

☐ none ☐ heart ☐ rumen

☐ liver ☐ other: _____

Bullet Hole: ☐ heart shot ☐chamber shot (Entry/Exit) ☐ neck shot (Entry/Exit) ☐ gut shot

(please mark the bullet's point of entry and exit)

Sex: ☐ male ☐ female

Weight: __ __ kg ☐ with head ☐ without head entered in ISESB * _ _ kg

(gutted, with skin) *not filled out by hunter

State of Health. ☐ healthy ☐ sick ☐ emaciated

Injuries: _____

Parasitic Infestation:

Ectoparasites: ☐ none ☐ scarce ☐ average ☐ extreme

Endoparasites: ☐ none ☐ scarce ☐ average ☐ extreme

Organs infected: _____

Date, Signature of the Hunter

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 3: Storage

Roe deer (*Capreolus capreolus*)

These fields are filled out by Trier University

Identification:

__ __ __ __ **/ X /** __ __ __ __ **/** __ __ __ __ **/** __

Sample:: __ __ __ District/Division: _____

Date of sample pickup and verification: __ __ . __ __ . __ __

Signature: _____

Date of storage above liquid nitrogen: __ __ . __ __ . __ __

Signature: _____

Date of transfer to stainless steel container: __ __ . __ __ . __ __

Liver Weight: __ __ __ g

Number of stainless steel container: __ __ __ __ __ __ __

Signature _____

GERMAN ENVIRONMENTAL SPECIMEN BANK

Sampling Protocol

Roe deer (*Capreolus capreolus*)

Sampling Area: _____ Identification: __ __ __ __ __

Underlying Version of the Sampling Guideline __ __ . __ __ . __ __

Underlying Version of the Sampling Scheme __ __ . __ __ . __ __

1. Objective of the Sampling: _____

_____

2. Actual Timeframe of the Sampling:

Start		End		Sample no.		Sampling leader	Remarks
date	time	date	time	from	to		

3. Participants: internal _____

_____

external _____

4. Checklist Referring to Sampling Scheme and Sampling Guideline: ☒ as prescribed

- ☐ 4.1 Sampling Period
- ☐ 4.2 Sampling Site and Sampling Point (selection/definition)
- ☐ 4.3 Selection of the Individual Specimens
- ☐ 4.4 Technical Preparations
- ☐ 4.5 Cleaning Procedure for the Packages
- ☐ 4.6 Sampling Technique/ Method of Capture
- ☐ 4.7 Sample Amount
- ☐ 4.8 Data Collection
- ☐ 4.9 Transport and Interim Storage

Number, kind and reason for deviation (clear text):

_____

_____

_____

_____

Remarks: _____

_____

_____ __ __ . __ __ . __ __ _____

Recorder Date Signature

Browsing Survey

In order to describe the roe deer's habitat quality, every five years a mapping of deer browsing activity needs to be carried out within the perimeters of the sampling areas. Browsing intensity determination should be scheduled / undertaken almost at the end of the vegetation period. At this point in time it is possible to record the herbaceous and woody plants, which have been browsed during summer, as well as the woody plants used as a food resource over the course of the winter. Mast years of forest trees and the composition of additional winter feed must also be recorded.

Among the cited methods for characterizing roe deer browsing behavior (Zai 1964, Klötzli 1965, Al-Kittani 1973, Voser-Huber & Niervergelt 1975, Petrak 1982, 1987, Guthörl 1990), the methods suggested by Klötzli (1965) and Guthörl (1990) were primarily considered. The investigation should be carried out by a skilled person who, ideally, supervises all sampling areas.

The browsing survey, whose results are documented in the respective specimen data sheets, should be carried out as follows:

Forest Territories:

In the first step, partial habitats important for roe deer within the sampling area and potential browsing grounds are selected. Potential browsing grounds are all sites where forest floor flora and forest regeneration develop due to a sufficient incidence of light (e.g. clear-felled areas, areas of seedling brush through thicket stage, natural regeneration sites, windthrow sites, pipeline routes, forest edges and waysides and the like). Information on preferential browsing grounds should be gathered by local hunters, foresters or other persons familiar with the area. Thereafter, a representative number of mapping area plots within the sampling region is selected, on which the actual browsing intensity should be estimated. The number and size of mapping plots selected depend on the respective forest structure.

If browsing mappings are to be completed on larger sites, the area will be divided into five units. The first and fifth unit cover the marginal zones, the third one, the center. Minimum size of each recorded site is 25 m². To determine the **grazing offer** (occurrence of plants) on each recorded site, a vegetation analysis is performed according to the method by Schmidt (1974), which is a refinement of the method by Braun-Blanquet (1964).

Smaller mapping plots will not be subdivided. It must be considered that the estimations of the cover values (species: area ratio) in the case of phytosociological methods do not represent harvest estimations. The cover value is defined as the area which would be covered if all above-ground parts of the recorded plants were projected vertically onto the ground. The estimation of the plant species cover value (Tab. 1) is performed for a stratum of up to 1.5 m height (grazing height of the roe deer).

Tab. 1: Scale for the estimation of cover values (Schmidt 1974)

< 1	up to 1	up to 2	up to 3	up to 8	up to 10
up to 15	up to 20	up to 25	up to 30	up to 40	up to 50
up to 60	up to 70	up to 80	up to 90	up to 100	

In contrast to the browsing investigations conducted for forest concerns, which aim at gathering information about of the state of forest rejuvenation and therefore only consider the young forest trees (Schwab 1999), investigations concerning the grazing behavior include all existing plants. For each plant species on the recorded site, the browsing intensity is determined. When doing so, it is important to differentiate between different browsing animal species (Reimoser and Reimoser 1998). While it is easy to differentiate between the feeding marks of ruminants and rodents (e.g. jack rabbits), it is normally not possible to distinguish between the different ruminant species by their feeding marks (Petrak 1982). The bite patterns caused by ruminants are uneven and fibered, but not smooth like those produced by the jack rabbit (Bang and Dahlström 2000). The specific grazing species can be identified by the different browsing height, which coresponds to body height, the appearance of browsing marks and unambiguous characteristics, such as droppings and footprints. However, it must be kept in mind that individuals can considerably enlarge their upwards grasp by standing on their hind legs. Additionally a closed snow cover, provided that it bears the burden of the animals, can also increase upwards browsing range (Petrak 1982).

Tab. 2: Browsing intensity classes for gramineae, herbs, and young trees (Klötzli 1965)

Browsing intensity class	Browsing intensity	Gramineae and herbs	Shrubs or young trees
0	none		
1	low	1-5% of the plant bitten to a minor degree	only about 1-5 browsing marks per plant
2	moderate	6-20% of the plant bitten to a minor degree, plant inhibited in growth	6-20 browsing marks per plant, no inhibition of growth
3	severe	20-50% bitten in a striking degree, e.g. sprouts decapitated, growth often stopped	> 20 browsing marks per plant, plant inhibited in growth (browsing of the top shoot will be especially noted)
4	extreme	> 50% bitten in a striking degree, plants often ± completely destroyed	> 20 browsing marks per plant, plant without noteworthy sprout growth in the present vegetation period

A restriction of the browsing survey to a vegetation height of 1.5 m may lead to an underestimation of the browsing degree caused by the roe deer.

For one particular site, the browsing degree for each plant species is assessed according to a five-class scale of browsing intensity (Tab. 2) developed by Klötzli (1965). By averaging the values of the browsing intensity ascertained from the recorded sites / grazing grounds, the browsing intensity (= browsing rate) of the sampling area is calculated.
Depending on frequency and intensity of browsing, the plant species are divided into popularity groups (Tab. 3). The five-class popularity scale developed by Klötzli (1965), has been adapted to the situation found in the investigated ESB areas and expanded by four additional classes. The measure of browsing frequency is the continuity of browsing.

Tab. 3: Definition of the popularity groups (modified according to Klötzli 1965)

Popularity rate	Definition of the popularity rate	Browsing continuity	Average browsing intensity
0	± Never browsed	< 1%	Little
1	Occasionally slightly browsed	1-40%	Little
2	Often slightly browsed	41-70%	Little
3	Regular slightly browsed	71-100%	Little
	Occasionally moderately browsed	1-40%	Little to moderate
4	Often moderately browsed	41-70%	Little to moderate
	Occasionally moderately to severely browsed	1-40%	Moderate to severe
5	Regularly moderately browsed	71-100%	Little to moderate
6	Often moderately to severely browsed	41-70%	Moderate to severe
	Occasionally severely browsed	1-40%	Severe to extreme
7	Regularly moderately to severely browsed	71-100%	Moderate to severe
8	Often severely browsed	41-70%	Severe to extreme
	Regularly severely browsed	71-100%	Severe to extreme

The browsing continuity for each species is calculated as follows:

$$\frac{\text{No. of surveys in which the species was found browsed}}{\text{No. of surveys in which the species occurred}} \times 100$$

In order to derive the popularity rate, three browsing continuity categories (browsing continuity 1–40% = occasionally browsed, browsing continuity 41–70% = often browsed, browsing continuity 71–100% = regularly browsed) are combined with the average browsing intensity. An estimation for the proportion of feed grazed from an individual plant species is based on the browsing intensity class determined for the respective plant species, multiplied by the cover values of the recorded sites. This provides an indication for browsing stress on the recorded sites / grazing grounds. By averaging the values of browsing stress ascertained from the recorded sites / grazing grounds, the browsing stress for sampling is calculated.

Field areas:

In field areas, the grazing offer on the utilized agricultural area must be registered and recorded in the respective specimen data sheet. It is important to distinguish between fallow land, grassland, and the main field crop species. Groves and adjacent edges must also be recorded. On fallow lands, phytosociological surveys as well as browsing surveys should be performed according to the method described above.