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Improved radiocarbon dating of contaminated protein-containing archaeological samples via cross-flow nanofiltrated amino acids
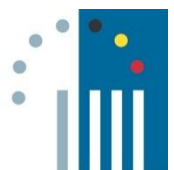
Thesis submitted in fulfillment of the requirements
for the degree of Doctor (PhD) in Applied Biological Sciences

This is a joint research project of the following partners

Dutch translation of the title:

Verbeterde radiokoolstofdatering van gecontamineerde proteïnbevattende archeologische stalen via cross-flow nanogefiltreerde aminozuren.

To refer to this thesis:

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Last but not least, I dedicate this thesis to my partner Susana and my two daughters, Emmanuelle and Carla. They helped me through the hard times and made me aware that there is more in life than work

List of Abbreviations

^{12}C	Carbon-12
^{13}C	Carbon-13
^{14}C	Carbon-14
CO_2	Carbon dioxide
N	Nitrogen
n	Neutron
p	Proton
β^-	Beta particle
e^-	Electron
ν^-	Anti-neutrino
t	The elapsed time or radiocarbon age
A_0	^{14}C activity at t = 0 or activity of a modern sample
A	Activity after time t or activity of the sample with unknown age
$T_{1/2}$	Half-life
δ	Delta value
‰	Per mil
A_n	Normalized activity of the sample
A_m	Measured activity of the sample
Δt	Age difference
AD	Anno domino
NBS	National Bureau of Standards
ln	Natural logarithm
A_{0n}	Normalized ^{14}C activity at t = 0
A_n	Normalized activity after time t
BP	Before present
BC	Before Christ
calAD	Calibrated ^{14}C date in AD
calBC	Calibrated ^{14}C date in BC
calBP	Calibrated ^{14}C date in BP
Intcal09	Internation radiocarbon calibration curve 2009
%	Per cent
AMS	Accelerator Mass Spectrometry
GPC	Gas proportional counting
LSC	Liquid scintillation counting
HSs	Humic substances
Wt%	Weight percent

C:N ratio	Carbon to nitrogen ratio
MW	Molecular weight
Da	Dalton
HPLC	High performance liquid chromatography
HS	Humic substance
NaOH	Sodium hydroxide
HA	Humic acid
nm	Nanometer
CuO	Cupper oxide
Ag	silver
HCl	Hydrochloric acid
UV	Ultra violet
a.u.	Arbitrary unit
hr	Hour
PMT	Photomultiplier tube
V	Volt
mg	Milligram
min	Minutes
σ	Standard deviation
MWCO	Molecular weight cut-off
RO	Reverse osmosis
μm	Micrometer
M	Molar
ml	Millilitre
PDA	Photo diode array
VIS	Visible
pMC	Percent modern carbon
°C	Degree celcius
VPDB	Vienna Pee Dee Belemnite
Air	Ambient Inhalable Air
IAEA	International atomic energy agency
EA	Element analyser
HT	High temperature
Fo	Fluorescence

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CHAPTER 1   : Background and research objectives

1.1 BACKGROUND

1.1.1 RADIOCARBON DATING

1.1.1.1 Introduction

Radiocarbon dating provides a means for dating objects independently of stratigraphic or typological relationships and made possible a worldwide chronology, thus transforming archaeological investigation (Bar Yosef 2000; Taylor et al. 1994). Radiocarbon dating provides the most consistent technique for dating materials and events that occurred during the last 50,000 years on the surface of the Earth. Moreover, radiocarbon dating is also of significant use in other fields than archaeology, including environmental studies, ecology, geology, climatology, hydrology, meteorology, and oceanography (Bowman 1990; Lowe 1997).

1.1.1.2 Definition

Radiocarbon or ^{14}C is the radioactive isotope of carbon. It is the basis for radiocarbon dating and is useful for dating materials that contain carbon younger than 50,000 years (Fallon 2011).

Radiocarbon dating is a radiometric technique based on measuring the relative amount of radiocarbon in matter containing carbon as a component (Goffer 2007).

Carbon has three naturally occurring isotopes, i.e. atoms of the same atomic number but different atomic weights namely carbon-12 (^{12}C), carbon-13 (^{13}C) and carbon-14 (^{14}C). They do not occur equally. Carbon consists of ca. 98.9% of ^{12}C, 1.11% of ^{13}C and 1 part per trillion of ^{14}C respectively. Unlike ^{12}C and ^{13}C, ^{14}C is unstable and therefore radioactive (Bowman 1990).

Carbon-14 has a half-life of 5,730 years. The half-life is the time taken for an amount of a radioactive isotope to decay to half its original value. Because this decay is constant it can be used as 'clock' to measure elapsed time assuming the starting amount is known (Fallon 2011).

1.1.1.3   Production and decay

The really uncommon characteristic of ^{14}C is that it is being formed continuously. This happens in the upper Earth's atmosphere by the interaction of neutrons produced by cosmic rays with nitrogen-14 atoms. After formation, the ^{14}C atoms rapidly react with oxygen to form $^{14}CO_2$, which is chemically not distinguishable from $^{12}CO_2$ and $^{13}CO_2$. The ^{14}C production process can be written as follows:

$$^{14}_{7}N + n \rightarrow ^{14}_{6}C + p$$
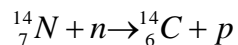
Whereby:
n: neutron
p: proton.

The $^{14}CO_2$ then joins the Earth's carbon cycle (Figure 1.1). Plants incorporate $^{14}CO_2$ during photosynthesis and organisms that eat plant material take up this ^{14}C. Hence ^{14}C gets incorporated in components of terrestrial and aquatic ecosystem (e.g. wood, peat, shell, bones corals, etc.). When a living organism dies, it does no longer exchange CO_2 with the atmosphere (i.e. it incorporates no longer $^{14}CO_2$). This starts the radioactive decay 'clock' since radiocarbon undergoes radioactive decay, the dead remains start to lose ^{14}C by emitting ß$^-$ particles, which is the result of the conversion of a neutron to a proton, converting ^{14}C back to its original ^{14}N (Bowman 1990; Fallon 2011).
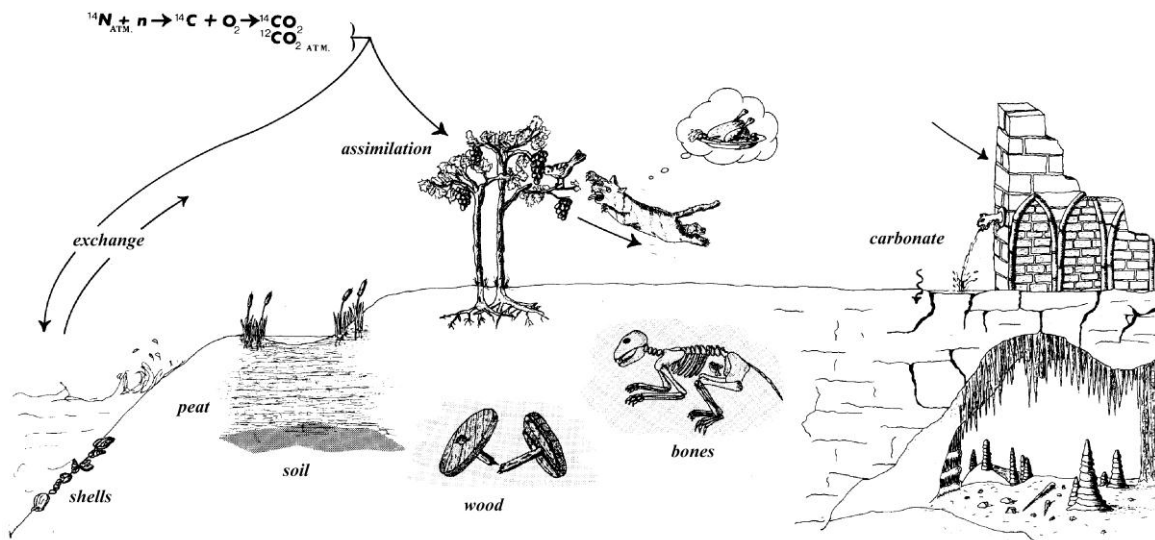
Figure 1.1   : Incorporation of ^{14}C into components of terrestrial and aquatic ecosystems (A. Terfve © KIK-IRPA)

The ^{14}C decay reaction is as follows:

$$^{14}_{6}C \rightarrow\,^{14}_{7}N + e^{-} + v^{-}$$
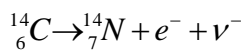
Whereby:
e⁻: electron
v^{-}: anti-neutrino

This loss, which is regulated only by the radioactive decay law (see further) results in a continuous reduction in the total number of ^{14}C atoms, as well as in the relative amount of ^{14}C atoms to those of stable ^{12}C in the dead tissues. Therefore, the age of dead remains, or of any carbon-containing matter, can be determined by measuring the relative amount of ^{14}C they contain. (Goffer 2007).

1.1.1.4   History of radiocarbon dating

The radiocarbon dating method was developed around 1950 by W.F. Libby (1908-1980), who received the Nobel Prize in chemistry in 1960 for this important discovery (Libby 1955; Berger 1983).
His first publication showed the comparisons between known age samples and radiocarbon age (Libby et al. 1949; Libby 1952).

1.1.1.5   Radiocarbon age calculation

The ^{14}C level decreases at a rate that is determined by the law of radioactive decay (Bowman 1990; Van Strydonck 2012):

$$A = A_0 . e^{-\lambda.t}$$

$$t = \frac{1}{\lambda} . \ln \frac{A_0}{A}$$

with $\dfrac{1}{/} = \dfrac{T_{1/2}}{\ln 2}$

Whereby

λ = constant equal to the reciprocal of the meanlife τ

t = the elapsed time or radiocarbon age;

A_0 = ^{14}C activity at t = 0 or activity of a modern sample;

A = activity after time t or activity of the sample with unknown age;

$T_{1/2}$ = half-life.

1.1.1.6   The Libby half-life time

When the first date-lists were published by Arnold and Libby they used the revised half-life of 5568±30 years instead of the previously used half-life of 5720±47 years (Arnold and Libby 1951) (Figure 1.2). This revised value would, in their opinion, resolve some of the problems that arose when new sets of samples were analysed. Unfortunately this result was wrong by 3% and it would take until 1962 before the exact half-life of 5730±40 years was determined (Godwin 1962).

Meanwhile, the 'BP' (Before Present) term was introduced, using 1950 as the 'zero year' in the radiocarbon time scale. So 1000 BP would correspond to a calendar date of 1950-1000 = 950 AD (anno domino). It is clear that by using the wrong half-life this results in a wrong age determination. But by 1962 the number of radiocarbon measurements produced and published were already so important that changing the half-life in future calculations would cause a tremendous chaos. So it was decided to maintain the wrong, so-called, Libby half-life in the calculations (Van Strydonck 2012).
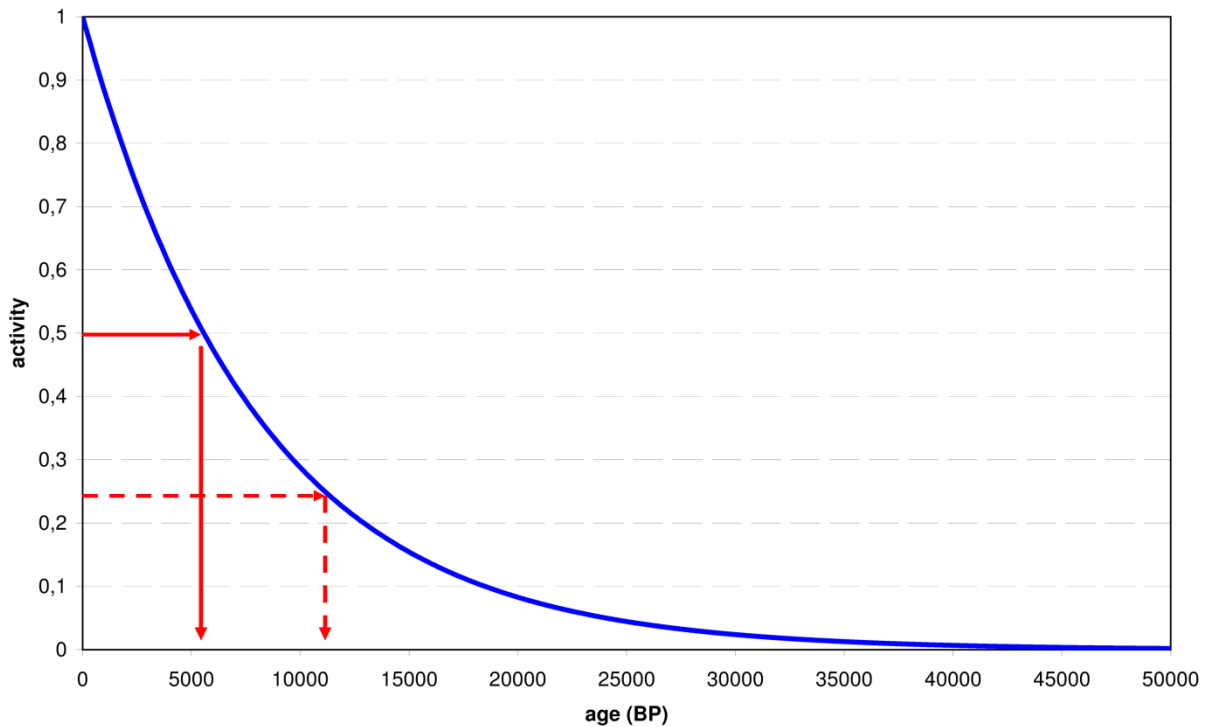
Figure 1.2   : Radioactive decay using the 'Libby half-life' of 5568±30 years. This means that after: 5568 years only 50% of the original radiocarbon remains, after 11136 years only 25%, etc. After 10 half-lives (ca. 50,000 year) the remaining ^{14}C is so low that accurate measurements are almost impossible.

1.1.1.7   Isotopic fractionation

Although ^{12}C, ^{13}C and ^{14}C are all carbon isotopes and chemically indistinguishable, there will be in any biological pathway a tendency for the lightest isotope ^{12}C to be preferentially taken up. Similarly ^{13}C will be taken up in preference to ^{14}C. Growing plants and animals, which are still exchanging with the biosphere are therefore expected to have a lower ^{14}C level than the atmosphere. This differential uptake is referred to as fractionation, and needs to be taken into account if useful radiocarbon results are to emerge. Fortunately, the fact that carbon has three isotopes of which two are stable enables a correction for fractionation to be applied.

The isotopic fractionation between the isotopes ^{14}C and ^{12}C is twice that between the isotopes ^{13}C and ^{12}C (Craig 1954, Bowman 1990; Van Strydonck 2012), so that by measuring the latter the first can be calculated..

The relative ^{13}C content in a sample can be expressed in $\delta^{13}C$ values:

$$\delta^{13}C(^o/_{oo}) = \frac{(^{13}C/_{12}C)_{sample} - (^{13}C/_{12}C)_{std.}}{(^{13}C/_{12}C)_{std.}}.1000$$

The $^{13}C/^{12}C$ ratio represents the carbon isotope ratio of a sample (*sample*) and the Vienna Pee Dee-Belemnite standard (*std*), respectively. Table 1.1 represents some $\delta^{13}C$ average values of different materials.

Table 1.1   : Average $\delta^{13}C$ values for some natural materials (Bowman 1990; Van Strydonck 1992).

Material	$\delta^{13}C$ (‰)
Wood (C3 type plants like leaf trees)	-25 to -30
Plants from arid environments (C4 type plants)	-13 to -10
Charcoal	-25 to -30
Peat	-30
Bone collagen	-19 (depending on the type of diet)
Freshwater plants	-16
Marine plants	-12
Atmospheric CO_2	-8
Marine carbonate (shells)	0

As for the measurement relative to a standard, the agreed value to which the $\delta^{13}C$ for radiocarbon dating must be corrected is -25‰. This is approximately the value for wood, although any other value could be chosen if it was universally used.

The formula for the normalized (fractionation corrected) activity (A_n) becomes:

$$A_n = A_m.\left[1 - \frac{2.(25 + \delta^{13}C)}{1000}\right]$$

Where:

A_n: normalized activity of the sample

A_m : measured activity of the sample

This formula can be simplified to give an approximate age difference, which is the error in the radiocarbon measurement ($\Delta t = t_n - t_m$) when no correction for isotopic fractionation is applied would then be:

$\Delta t = (\delta^{13}C + 25).16 \ years$

This means an age correction of about 16 years for every 1‰ difference from -25‰. If the $\delta^{13}C$ is larger than -25‰, the corrected age is older than the measured age. In the early years of radiocarbon dating this correction was not applied, but nowadays it is done automatically by the dating laboratories (Bowman 1990; Van Strydonck 2012).

1.1.1.8   The conventional and the calibrated radiocarbon date

Definition of a radiocarbon date

The information held in a sample by radiocarbon is comparing its present radiocarbon concentration to the radiocarbon concentration in the atmosphere, resulting in a conventional radiocarbon date of the sample (van der Plicht 2012).
Although almost all radiocarbon laboratories used the same formula to calculate the radiocarbon age, it took the radiocarbon community until 1977 before a formal definition of a radiocarbon date was given (Stuiver and Polach 1977).
Because of the problems inherent in definition of the present day activity of the atmosphere, wood from 1890 AD was used as the modern radiocarbon standard, extrapolated for decay to 1950 AD. Later this was replaced by an artificial NBS (National Bureau of Standards) oxalic acid standard. By definition, a conventional radiocarbon date is a radiometric age wherein the radiocarbon content of a sample is compared to that of the NBS oxalic acid standard and normalized for isotopic fractionation (see above). The Libby half-life is used in the age calculation and the result is expressed in BP ± 1 standard deviation (σ) (Van Strydonck 2012).

$$ t = 8033 . \ln \frac{A_{0n}}{A_n} $$

Whereby
t = radiocarbon age in BP;
A_{0n} = normalized ^{14}C activity at t = 0 or normalized activity of a modern sample e.g. oxalic acid;
A_n = normalized activity after time t or normalized activity of the sample with unknown age;
8033 = $T_{1/2}$/ln2 and half-life $T_{1/2}$ = 5568 years

Calibration of a Radiocarbon date

Past variations in the natural, atmospheric ^{14}C concentration (De Vries 1958) caused that radiocarbon years are different (and vary) from calendar years. This problem was solved by defining the **radiocarbon timescale**, which appears to be elastic, but this can not be taken literally. We now know that 5,000 ^{14}C years ago (BP) corresponds with roughly 5,000 BC or 7,000 calendar years ago (van der Plicht 2012) (Figure 1.3). It must be clear that it was a historical mistake to call a radiocarbon measurement an "age". Nowadays, **BP** stands for a radiocarbon measurement performed in agreement with the adopted definition (Van Strydonck 2012).

As a consequence, radiocarbon dates need to be calibrated into calendar years. Only then the method can be considered 'absolute' (e.g. Van der Plicht and Mook 1989). This calibration by the means of a calibration curve has been named the "*second radiocarbon revolution*" by Renfrew (1999).

This calibration (Figure 1.3) curve is drawn by comparison of radiocarbon dates with absolute chronologies obtained mainly from dendrochronology, varve counting and coral dating. The transformation or calibration of a radiocarbon date can be performed by means of computer programs. The most popular are OxCal (Bronk 1995; Bronk 2001; Bronk 2013), Calib (Stuiver and Reimer 1995; Reimer 2013). The obtained calendar age is expressed as calAD, calBC or calBP (= calendar years before 1950AD) (Figure 1.4) (Van Strydonck 2012).
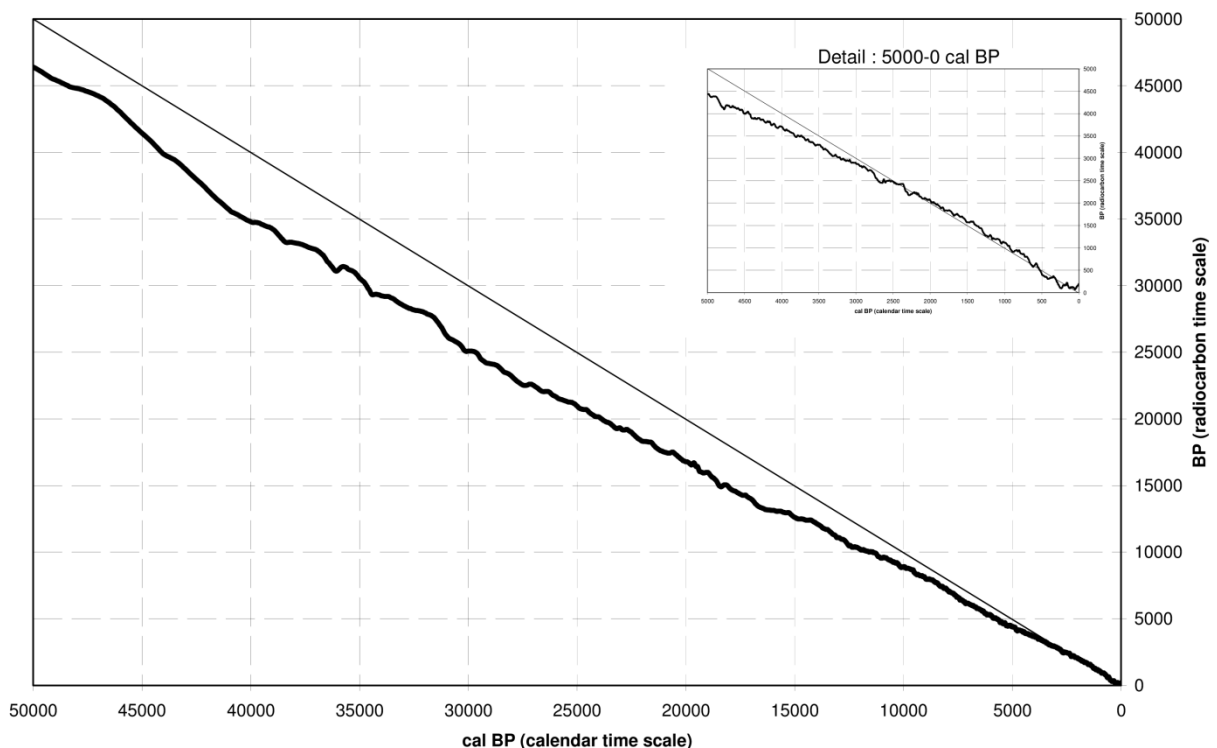
Figure 1.3   : Relationship between the radiocarbon time scale in BP and astronomical time scale in cal BP (calendar years before 1 950 AD). The curve depicted here is the IntCal09 curve.
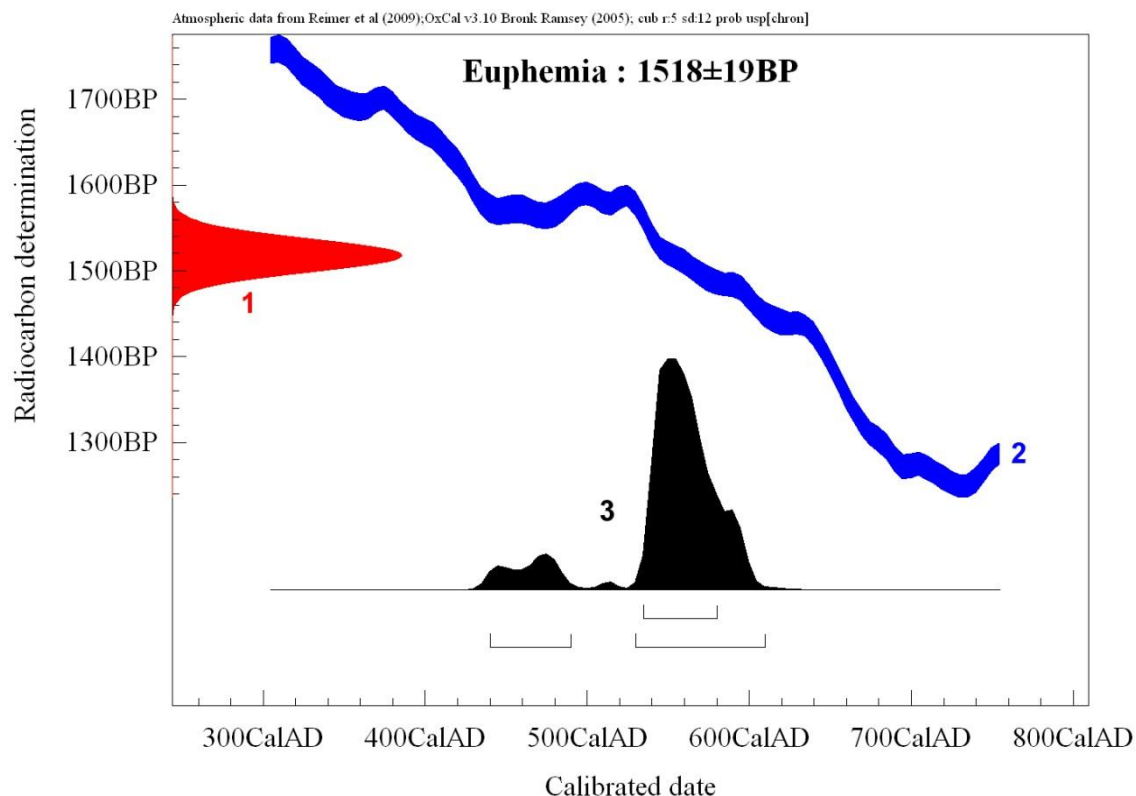
Figure 1.4   : Calibration of a radiocarbon date by the OxCal program
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2: The calibration curve [IntCal09]
3: The probability distribution after calibration indicates that within the range of 1 standard deviation (68.2% probability) the real age is delimited between 535-580 calAD and within the range of 2 standard deviations (95.4%) for 10.3% between 440-490 calAD and for 85.1% between 530-610 calAD.

1.1.1.9 Measuring ^{14}C

There are 2 methods of measuring ^{14}C: by conventional radiocarbon dating, which detects one of the ^{14}C decay, or by Accelerator Mass Spectrometry (AMS) which directly analyses the number, or a proportion of the number of ^{14}C atoms relative to ^{13}C or ^{12}C atoms in the sample. Although the principles of conventional and AMS dating are different, both produce ^{14}C results that are interpretable in the same way (Bowman 1990).

Conventional radiocarbon dating

The nucleus of a ^{14}C atom is unstable and when it decays to nitrogen (^{14}N) a ß⁻ particle is emitted. A ß⁻ particle is an electron resulting from the radioactive decay of a nucleus.

The ß⁻ particle can be detected easily because it is electrically charged. The amount of ß⁻ particles discharged in unit time by radiocarbon decay in a sample of known weight can be counted.

The detection and counting of ß⁻ particles can be done by:

- Gas proportional counting (GPC) is a technique where the carbon sample is first converted to gas (carbon dioxide or methane or acetylene) before measurement in gas proportional counters takes place. A proportional counter uses a combination of the mechanisms of a Geiger-Muller tube and an ionisation chamber. The principle is based on the distance that decay particles travel in matter related to their charge and mass and on the density of the matter that the decay particles are interacting with. (Van Strydonck 1992 ; Knoll 2010).
- Liquid scintillation counting (LSC) is another radiocarbon dating technique that became popular since the 1960s. In this method, the sample is in liquid form and a scintillator is added. This scintillator produces photons when it interacts with a ß⁻ particle. A vial with a sample is passed between two photomultipliers, and only when both devices register photons that a count is made (Bowman 1990).

The conventional radiocarbon decay counting technique generally provides reliable results, but it has some limitations: a relatively large sample (several grams but often more) is required to obtain reliable data, and a long (usually several hours) period for e counting the ß⁻ radiation emitted by the disintegrating radiocarbon.

AMS dating

A more recently developed technique, known as the Accelerator Mass Spectrometry (AMS) radiocarbon dating technique, is based on counting the relative amount of radiocarbon to stable carbon isotopes (^{12}C and ^{13}C) of a sample, in a mass spectrometer.
The principle of AMS is based on mass spectrometry: when a magnetich field is applied to a moving charged particle, the particle is deflected from the straight path along which it was travelling. If charged particles of different mass, but the same velocity, are subject to the same magnetic field, the heavier particles are deflected the least and detectors at different angles of deflection then receive particles of different mass. However, with AMS charged particles are subjected to large voltage differences so that they travel at very high speeds. This enables various devices to be used to discriminate against the much more abundant elements, such as ^{14}N, and molecules, such as ^{13}CH, which would otherwise swamp the ^{14}C signal (Bowman 1990).

The AMS technique requires only very small samples (several milligrams) and the time necessary to measure the amount of ^{14}C in each sample is much less than that required for counting disintegration events in the conventional technique (Goffer 2007).

The importance of using small samples for AMS makes it possible to radiocarbon date valuable objects e.g. art, textiles or parchments etc. (Goffer 2007).

AMS also enables compound-specific dating such as single amino acids of bone collagen that are isolated by High Performance Liquid Chromatgraphy and then ^{14}C dated (Gillespie and Hedges 1983; Gillespie et al. 1984; Stafford et al.1987; Stafford et al.1988; Van Klinken and Mook 1990; Stafford et al.1991; Van Klinken 1991; Tripp et al. 2006).

High precision AMS measurements are performed on 0.5 - 1 mg Carbon. However, AMS measurements can be achieved at a μg level but with a lower precision (Santos et al. 2007; Delqué-Kolic et al. 2013).

1.1.2 SAMPLE CONTAMINATION, QUALITY CONTROL AND PRE-TREATMENT

1.1.2.1 Sample contamination

It is fundamental in radiocarbon dating that no process other than radioactive decay has altered the ^{14}C level in a sample since its removal from the biosphere. Any addition of carbon-containing material is contamination, and it must be removed before the dating process begins otherwise a false result will be obtained (Bowman 1990).

Protein-containing materials from archaeological and geological sites are susceptible to chemical and environmental processes, which can result in degradation (diagenetic alteration or breakdown) and introduction of exogenous carbon-containing compounds (contamination). In particular, humic substances (HSs) from the soil may interact with the material of interest and cause major problems for ^{14}C dating (Stafford et al. 1988; Van Klinken and Hedges 1995; Van Klinken 1999; Van Strydonck et al. 2005).

Bones are one of the most preferable materials for paleodietary and archaeological chronology studies because they are often directly related to the prehistoric event. The collagen fraction of bones is usually used for stable isotope and ^{14}C analyses.

Wool, hair and silk are gaining more attention as suitable radiocarbon dating material due to their short lifespan (Taylor et al. 1995; Geyh 2001; Van Strydonck et al. 2004).

For these reasons, contaminated wool, hair, silk and bone collagen will be studied in this PhD as a means to detect and remove exogenous carbon contamination.

1.1.2.2   Quality control

Quality control of sample material (e.g. collagen) is receiving considerable attention in order to obtain more reliable ^{14}C dates (DeNiro 1985; Van Klinken 1999; Van Strydonck et al. 2005). This study focus on the quality control of protein-containing material.

Bone Collagen

Extraction of bone collagen for ^{14}C dating is labour-intensive and time-consuming and sometimes results in very low protein recovery used for ^{14}C dating. In addition, attempts to sample bones for direct dating can result in needless destruction of archaeologically significant material where the collagen does not survive (Hublin et al. 2008).
Therefore, some authors proposed pre-screening techniques to determine the protein preservation before extracting the collagen in the laboratory:

- Correlation between microstructural characteristics, especially porosity, and the protein content have been demonstrated by Nielsen-Marsh and Hedges (1999, 2000), Turner et al. (2002), Tripp et al. (2010);
- Following Brock et al. (2010, 2012) measuring the %N of the whole bone might be useful to predict if the bone would yield sufficient collagen for ^{14}C dating.

The preservation state of the bone collagen can be evaluated by different quality indicators. The C:N ratio reflects contamination and/or degradation (De Niro 1985; Schoeninger et al. 1989; Ambrose 1990). Ambrose (1993) recommends as additional criteria the determination of the weight percentage of extractable collagen (collagen %) from whole bone. A minimal weight percentage of 1% collagen should indicate good preservation state, while van Klinken (1999) sets the threshold at 0.5%. The percentage of carbon (%C) and nitrogen (%N) also provide information about the preservation state of the collagen extract. According to Ambrose (1990), well-preserved archaeological collagen should have carbon and nitrogen contents greater than 13.1 and 4.8 wt % respectively. Higher values than those found in fresh collagen (43%C and 16%N, respectively) suggest contamination (van Klinken 1999).
Other authors also recommend amino acid analysis to judge the collagen quality (Stafford et al. 1991, Bocherens et al. 1994). A more detailed option, however less studied, is the amino acid racemization, in particular aspartic acid (D/L-asp) has often been investigated in this context (e.g. Bada et al. 1999; El Mansouri et al. 1996; Poinar et al. 1996; Tuross 2002). After death and during the inhumation period, the D-form of this amino acid emerges from the L-form. That racemization is a function of collagen degradation rather than of time and temperature has only been demonstrated by Collins et al. (1999).

The improvement of bone collagen quality via contaminant removal by applying new techniques can, hence, be verified by analysing the parameters mentioned above.

Hair, wool and silk

Hair and wool consist mainly of the protein keratin which is a α-helix structure with cystine, leucine, glutamic acid, arginine and serine as the most abundant amino acids (Sibley and Jakes 1984). The most significant of the amino acids is cystine because it is the source of primary valence inter-and intramolecular disulphide crosslinks. Other intermolecular forces which associate one polymer chain to another are hydrogen bonds and ion-ion salts (Morton and Hearle 1975).

Silk protein (fibroin) is a polypeptide polymer of 15 amino acids, the largest percentage in the fibre consists of the amino acids glycine, alanine, and serine. These substances are small in size, with no large side groups. Therefore, the polypeptide chains can pack together closely as β-sheets (Sibley and Jakes 1984).

For archaeological human and animal analyses, also called "wool and hair isotopic analysis", the C:N ratio can be used to indicate if hair keratin is contaminated or not. Analyses of modern human hair defines a C:N range for uncontaminated archaeological hair between 2.9 and 3.8 (O'Connell and Hedges 1999a; O'Connell and Hedges 1999b; O'Connell et al. 2001).

Taylor et al. (1995) conducted ^{14}C, C:N and amino acid composition analyses of archaeological hair. The Glycine/Glutamine and Glycine/Aspartic acid ratios indicate well-preserved chemical structures of the hair. Comparing C:N ratios of archaeological samples in this study with modern samples (O'Connell and Hedges 1999a; O'Connell and Hedges 1999b; O'Connell et al. 2001) and due to reasonable ^{14}C dates on archaeological samples a C:N range of 2.9 - 3.8 can be defined as a reliability range for uncontaminated hair.

No quality indicators were available in the literature for archaeological dyed wool and silk and one aim of this PhD is to develop a quality control tool-kit to define the sample quality of archaeological dyed wool and silk because mordanting and dyeing may alter the %C, %N and C:N ratio.

1.1.2.3   Pre-treatment

Bone collagen

Collagen is composed of a triple helix, which generally consists of two identical chains (α1) and an additional chain that differs slightly in its chemical composition (α2). The amino acid composition of collagen is atypical for proteins, particularly with respect to its high hydroxyproline content. The most common motifs in the amino acid sequence of collagen are glycine-proline-X and glycine-X-hydroxyproline, where X is any amino acid other than glycine, proline or hydroxyproline (Szpak 2011).

The collagen fraction from a bone is obtained by dissolving the demineralized bone in hot acidic water (Longin 1971). However, this treatment will also lead to the presence of other hot water soluble components in the collagen fraction such as HSs (Van Klinken and Mook 1990). Adding an alkaline step helps to remove basic soluble organics such as humic acids, but not completely (Van Klinken and Hedges 1995; Arslanov et al. 1993).

Ultrafiltration of bone collagen, dissolved as gelatin (Molecular weight (MW) ~100 000 Da), has received considerable attention as a means to obtain more reliable ^{14}C dates and stable isotope signatures (Brown et al. 1998; Bronk et al. 2004; Higham et al. 2006; Mellars 2006). This should be an effective method for the removal of low molecular weight contaminants from bone collagen but it does not remove high molecular weight contaminants, such as cross-linked humic–collagen complexes (Brock et al. 2007). Moreover, comparative dating studies have raised the question of whether this cleaning step itself may introduce contamination with carbon from the filters used (Bronk et al. 2004; Brock et al. 2007; Hüls et al. 2007; Hüls et al. 2009).

Separation, isolation and ^{14}C dating of individual amino acids hydrolysed from bone collagen by preparative high performance liquid chromatography (HPLC) is a good strategy for dealing with contamination in proteins but it is a time-consuming and labour-intensive technique (Gillespie and Hedges 1983; Gillespie et al. 1984; Stafford et al. 1987; Stafford et al. 1988; Van Klinken and Mook 1990; Stafford et al. 1991; Van Klinken 1991; Tripp et al. 2006; McCullagh et al. 2010; Marom et al. 2012; Marom et al. 2013). Most previous studies focused on Hydroxyproline because it constitutes about 10% of bone collagen and it is not present in significant quantities elsewhere in nature. However, some of these methods introduced extraneous carbon into the ^{14}C-dated fractions since column bleeding is a common phenomenon in chromatography. Therefore, sample blanks should be measured. McCullagh et al. (2010) and Marom et al. (2012, 2013) showed the potential of individual amino acid dating.

Hair, wool and silk

Wool, silk and hair are pre-treated with solvents followed by acid-base-acid treatment for stable isotopes and ^{14}C analyses, but this pre-treatment may not be adequate for removing all contaminants (Kim et al. 2008).

1.2 RESEARCH OBJECTIVES AND THESIS OUTLINE

^{14}C dating of contaminated protein-containing material e.g. bone collagen, hair, wool and silk usually results in unreliable results if conventional pre-treatment methods are used.

The objective of this thesis was to develop a method to detect ^{14}C contamination in protein-containing archaeological material and develop a method to improve sample quality of contaminated samples to obtain more accurate ^{14}C dates. Quality control is gaining more attention in the ^{14}C community; hence a quality control system was introduced.

This thesis has the following specific objectives:

(1) To develop a protocol to detect ^{14}C contamination in protein-containing material for archaeological use, e.g. bone collagen, wool, hair and silk;

(2) To develop a new nanofiltration method to improve the sample quality of contaminated samples, i.e. remove contaminants, and to obtain more accurate ^{14}C dates;

(3) To develop a quality control tool-kit to check for sample quality improvement;

(4) Application of the new method on protein-containing samples from a well-documented archaeological site.

Chapter 1 aims providing background information for radiocarbon dating, sample contamination, sample quality control and sample pre-treatment protocols for bone collagen, wool, hair and silk of archaeological origin.

Chapter 2 describes a non-destructive fluorescence spectroscopy method developed to monitor the presence of humic substances, one of the major ^{14}C contaminants in wool, hair, silk and collagen.

Chapter 3 focuses on the development of a new nanofiltration method in order to improve bone collagen quality for ^{14}C AMS dating. Two nanofiltration types were tested: dead-end and cross-flow filtration.

In **Chapter 4** the cross-flow nanofiltration technique was applied in combination with a quality control procedure on real archaeological samples. Quality control parameters for protein-containing material were analysed before and after cross-flow nanofiltration of hydrolysed protein-containing archaeological samples..

Chapter 5 demonstrates the applicability of the cross-flow nanofiltration method by means of a case study of two skeletons and their gars of two bishops, with known age of death.

Finally, the main findings and conclusions from this study and future research are summarized in **Chapter 6**.

CHAPTER 2   : Monitoring the presence of humic substances in wool and silk by the use of non-destructive fluorescence spectroscopy: quality control for ^{14}C dating of wool and silk

2.1   ABSTRACT

Radiocarbon dating of degraded wool and silk still provides ^{14}C results of questionable reliability. In most cases, degraded wool/silk contains humic substances (HSs). Thus, a non-destructive fluorescence spectroscopy method, using a fiber-optic probe, was developed to monitor the presence of HSs in degraded wool and silk.

This method can provide information about the presence of HSs before and after pre-treatment and about the ^{14}C age reliability.

Modern silk and wool were contaminated with HSs and alkali treated during different durations. Fluorescence spectroscopy was used to monitor the successs of the alkali-treatment of HSs removal indicating that a NaOH-wash is not sufficient in full removal of HSs.

As a result we suggest to consider wool/silk samples with an assumed HSs contamination with care for ^{14}C dating. C

2.2   INTRODUCTION

Wool and silk are proteinic fibers. Wool is an animal fiber that consists mainly of keratin. Raw silk is obtained from the cocoons of the larvae of the mulberry silkworm *Bombyx mori* and consists of the proteins fibroin and sericin. In order to make silk fabric soft and glassy, a degumming process is necessary to remove sericin, leaving only fibroin in silk to manufacture textiles (Sashina et al. 2006).

Wool and silk are gaining more attention as suitable ^{14}C dating material due to their short lifespan, potentially presenting the true age of an object is made of these materials (Van Strydonck et al. 2004). However, wool and silk are susceptible to rapid decomposition and are rarely excavated in archaeological sites. Archaeological wool and silk fibers can be degraded by microorganisms (Janaway 1985; Gillard et al. 1994). Moreover; humidity and heat accelerate the degradation of these textiles (Sibley and Jakes 1984). Only if the growth of microorganism is hindered in a special environment can the textile survive (Sibley and Jakes 1984; Cronyn 2001). Such special environments include peat bogs, the lake bottoms, deserts, the salt mines, and permafrost soils. In such cases, the activity of the microorganisms is slowed down due to anaerobic

conditions, the absence of available water, and the presence of metals such as copper (Kars and Smit 2003).

Degraded wool and silk may contain contaminants such as mold, fungus, dirt, humic substances (HSs), or other carbon-containing materials (Kim et al. 2008), which may affect the ^{14}C date. Conventional pre-treatment methods (solvent treatment followed by acid-base-acid treatment) for ^{14}C analyses may not be adequate for removing all contaminants (Kim et al. 2008). HS contamination of wool or silk is a major problem in obtaining reliable ^{14}C dates. Thus, HSs can be classified according to the ease in which they are soluble in alkaline or acidic solutions (Head 1987):

1. humic acid is the fraction extracted by alkaline solution that becomes insoluble after acidification;
2. fulvic acids are soluble both in acid and alkaline solutions;
3. the residue insoluble in acid and alkaline solutions has been termed "humin."

Quality control of sample material (e.g. charcoal, collagen) is receiving considerable attention in order to obtain more reliable ^{14}C dates (DeNiro 1985; Alon et al. 2002; Van Strydonck et al. 2005). A fluorescence spectroscopy method is developed in this study for screening textiles for humic substance presence in order to obtain reliable ^{14}C dates.

Fluorescence spectroscopy should be ideal for independently detecting HSs present in archaeological wool and silk samples. Fluorescence is the result of a three-stage process (excitation, excited-state lifetime, emission) that occurs in certain molecules, generally polyaromatic hydrocarbons or heterocycles, called fluorophores (Lakowicz 1999). HSs are thought to be complex aromatic macromolecules with amino acids, amino sugars, peptides, and aliphatic compounds involved in linkages between the aromatic groups (Stevenson 1982). Thus, HSs have fluorescent properties. Fluorescence spectroscopy is a common technique in soil science by analyzing HSs in solution (Bachelier 1980-1981; Simpson et al.1997). The technique is applied on solid material (e.g. wool or silk fabric/fiber) using a fiber-optic probe to detect HSs.

This technique has the advantage over solution studies in avoiding the formation of any new fluorophores and/or the destruction of others during protein hydrolysis or solubilization. For example, some of the fluorophores easily detected in solid-state studies on wool keratin or silk fibroin are barely detectable after protein hydrolysis or solubilization (Millington et al. 2002). These chemical treatments are necessary in order to measure the fluorescence of a solution. Fluorescence spectroscopy is favoured over other techniques because it is non-destructive technique. The fiber-optic probe takes light to the sample via an optical light guide (excitation). The sample absorbs this light

and emits light back via an optical light guide to the spectrofluorometer (fluorescence) (Figure2.1).
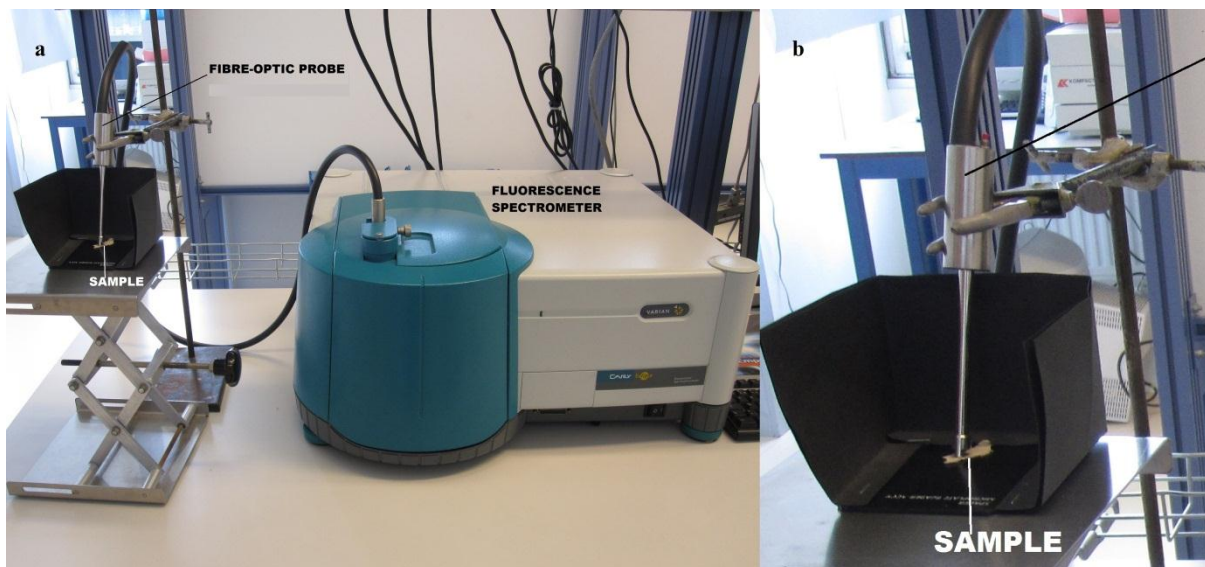
Figure 2.1   : a) Varian Cary Eclipse Fluorescence Spectrophotometer with a fiber-optic probe and b) Fiber-optic probe on textile sample.

In this study, a qualitative/semiquantitative fluorescence spectroscopy technique is developed that distinguishes the (naturally dyed) textile or fiber from the HSs by choosing the appropriate excitation and emission wavelengths (Bachelier 1980-1981; Simpson et al.1997; Clarke 2002).

2.3   MATERIALS AND METHODS

2.3.1   Sample selection

Modern, undyed silk fabric was acquired from a textile shop (La Fourmi, Brussels, Belgium). The modern wool fibers were delivered by a Belgian farmer (Galle, Emelgem). The undyed silk fabric and wool were analyzed using spectrofluorescence spectroscopy as a means to obtain reference spectra, free of humic substances (HSs).

Commercially available HSs were used: humic acid depur, called "HA Roth" in this paper (Carl Roth, Karlsruhe, Germany), humic acid practical grade, called "HA MP Biomedicals" (MP biomedicals, Brussels, Belgium) and humic acid sodium salt, "HA Sigma" (Sigma-Aldrich, Bornem, Belgium) . These commercially prepared HSs were analyzed using fluorescence spectroscopy in order to determine similarities and/or differences in their spectra.

Archaeological samples, chosen to represent various ages and preservation environments, were obtained from sites listed in Table 2.1, and analyzed with fluorescence spectrometry before ^{14}C pre-treatment and ^{14}C dating. All the archaeological samples were naturally dyed fabrics. Some of these samples were examined with fluorescence spectroscopy before and after ^{14}C pre-treatment in order to determine the extent to which HSs were removed by the pre-treatment.

Table 2.1   : Archaeological samples, chosen to represent various ages and preservation environments, were obtained from different sites.

Sample name	Archaeological site	Country	Material type	Preservation environment	Presumed historical date
Mainz 1	Mainz	Germany	Wool	Waterlogged soil	5 BC (Roman)
Mainz 2	Mainz	Germany	Wool	Waterlogged soil	5 BC (Roman)
Mainz 3	Mainz	Germany	Wool	Waterlogged soil	5 BC (Roman)
Mainz 4	Mainz	Germany	Wool	Waterlogged soil	5 BC (Roman)
Beerlegem	Beerlegem	Belgium	Wool	Presence of metal	AD 600-650 (Merovingian)
Ieper	Ieper	Belgium	Wool	Waterlogged soil	13th century
OS2562	Oudenburg	Belgium	Wool	well	Late Roman
OS24909	Oudenburg	Belgium	Wool	well	Late Roman
1924-01	Unknown	Afghanistan	Wool	sand	AD 1000-1200
Bourelet	Unknown	Egypt	Wool	sand	AD 200-450 (Coptic)
1923-02	Unknown	Egypt	Silk	sand	AD 700-1000 (Coptic)
Hallstat 79429	Hallstat	Austria	Wool	Salt mine	800-400 BC

2.3.2   Sample preparation

Modern silk fabric and modern wool fibers were washed separately:

1. With a 1%Neutral, non-ionic soap solution (ETS-René Dejonghe, Ghent, Belgium) with a maximum temperature of 80°C for the wool and 60°C for the silk to remove chemical products applied on the wool or silk by the manufacturer to make the textile shiny;

2. With Milli-Q water and;

3. Finally dried at 60°C.

This washing procedure has no influence on the amino acid composition of the silk and wool. This was internally tested and confirmed by the textile laboratory in the Royal Institute of Cultural Heritage (Brussels, Belgium).

About 650 mg of each HS was dissolved in 125 ml Milli-Q (Millipore) water. The pH of the HS solution was ca. 5. To this solution, we added ca. 450 mg of silk and slowly agitated the mixture at room temperature for different durations (3 hr, 64 hr and 188 hr). About 500 mg of wool was added to the HS solution and the mixture was slowly agitated for 120 hr or 200 hr at 80°C. Wool has greater stability in acid environment due to its disulphide bonds. Therefore, a temperature of 80°C was used to accelerate wool degradation.

The wool or silk was then removed from the HS solution, rinsed several times with Milli-Q water and finally washed with MilliQ-water for 15 min in an ultrasonic bath before drying at 40°C. Samples names consist of material type (wool or silk), brand of HS and agitation duration (e.g. Silk HA Roth 3 hr).

The wool and silk contaminated with HS were analyzed with fluorescence spectroscopy in order to register reference spectra. The HS-contaminated wool (fibers) and silk (fabric and yarn) were pre-treated with 1%NaOH for different durations to remove the HSs, and finally analyzed with spectrofluorescence to determine the degree of HS removal.

2.3.3   Fluorescence spectroscopy

Analyses were made in natural atmosphere at room temperature using a Varian Cary Eclipse Fluorescence Spectrophotometer. Non-destructive analysis of the textile fibers

was carried out using a fiber-optic probe. Spectra were acquired in the excitation wavelength range of 340-475 or 400-475 nanometer (nm) and 509 nm as the emission wavelength. The following wavelengths were chosen: the main excitation bands of humic acids at 465, 480 and 490 nm (Simpson et al.1996); the principal emission bands of humic acids in the wavelength range of 509-515 nm (Bachelier 1980-1981).

No interfering fluorescence, originated from the natural organic dyes, are observed at these wavelengths (Clarke 2002). Synthetic organic dyes were introduced in the mid-19th century, with mauveïne being the first synthetic organic dye produced in 1856 (Holme 2006; Herbst et al. 1997). Therefore, all archaeological dyed textiles samples manufactured before 1856 can be analyzed with our method.

Excitation was produced by a 15W Xenon pulse lamp. The excitation and emission band width were set at 10 nm. Scan control (medium) consisted of 600 nm min^{-1}, average time of 0.1 s, and data interval of 1 nm. The software automatically chose the excitation and emission filter during analysis. Fluorescence intensity was measured in arbitrary units (au). The fluorescence spectrophotometer also has room light immunity that excludes fluorescence contributed by ambient light.

2.3.4   ¹⁴C dating

Archaeological wool and silk samples were pre-treated as follows:
1. Extraction in an ultrasonic bath for 15 min with hexane (twice);
2. Rinsing with acetone;
3. Extraction in an ultrasonic bath for 15 min with acetone (twice);
4. Rinsing with acetone;
5. Extraction in an ultrasonic bath for 15 min with ethanol (twice);
6. Rinsing with Milli-Q water;
7. Extraction in an ultrasonic bath for 15 min with Milli-Q water;
8. Rinsing with Milli-Q water;
9. 15 min in cold 1%NaOH;
10. Rinsing with Milli-Q water;
11. 15 min in cold 1% HCl;
12. Rinsing with Milli-Q water;
13. Drying of sample at 40°C.

All chemical products were purchased from Merck (Belgium).

The solvent pre-treatment started with the most apolar solvent (hexane) and ended with the most polar solvent (ethanol). Rinsing with acetone is necessary after hexane and acetone extraction and with Milli-Q water after ethanol extraction in order to remove organic solvent remains. The duration of the NaOH step depends on the quality of the sample. This step was stopped when the sample started to fall apart or eventually started dissolving. The wool samples from Ieper and Beerlegem dissolved completely in the NaOH-step due their degraded state. They could only be pre-treated with the solvents.

CO_2 was obtained by sample combustion in the presence of CuO and Ag. Graphitisation was done with H_2 over a Fe catalyst and between 1 and 2 mg graphite was prepared. Targets were prepared at the Royal Institute for Cultural Heritage in Brussels (Belgium) (Van Strydonck et al. 1990). [14]C dates were measured on the AMS at the Leibniz Labor für Altersbestimmung und Isotopenforschung in Kiel, Germany (Nadeau et al. 1998). [14]C calibrations were performed using OxCal 3 (Bronk Ramsey 1995, 2001) and IntCal09 calibration curve date (Reimer et al. 2009).

2.4   RESULTS AND DISCUSSION

2.4.1   Fluorescence spectroscopy

The uncontaminated wool in this study shows two excitation band centres (Figure 2.2):
- at 380 nm. This fluorescence has previously been assigned to N-formyl kynurenine which is a known oxidation product of the amino acid tryptophan (Smith 1995).
- at 430nm. Previous work has shown that oxidised wool contains β-carboline fluorophores derived from tryptophan in exactly this position (Smith et al. 1994)
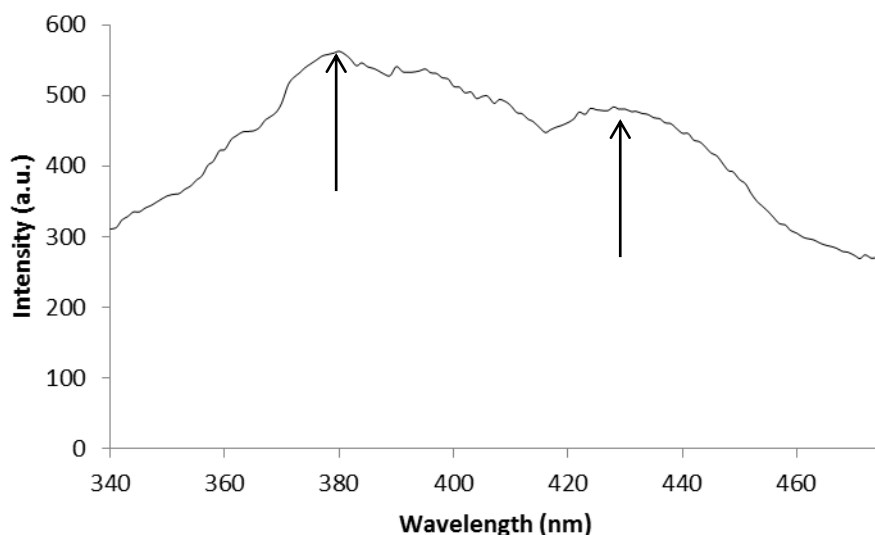
Figure 2.2   : Excitation spectrum between 340 and 475 nm of uncontaminated wool fibers. Excitation band centers are observed at 380 and 430 nm, indicated by an arrow.
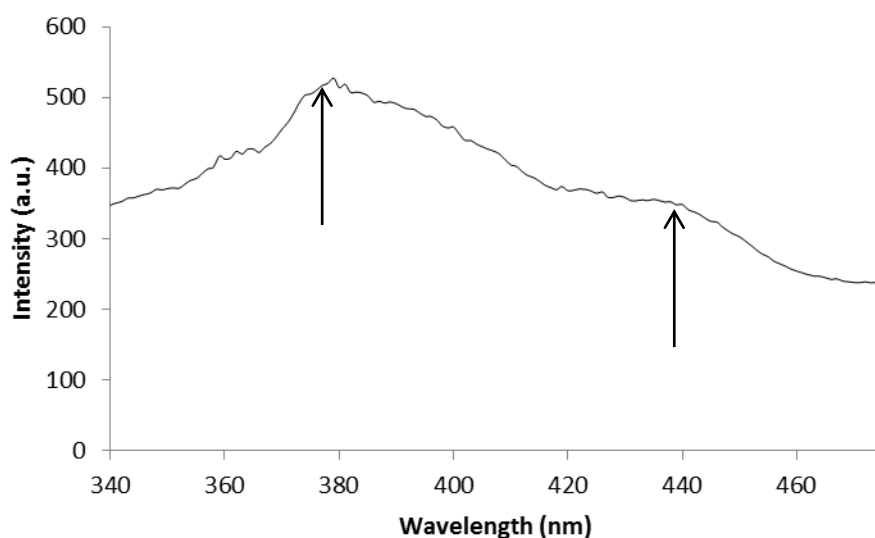
Figure 2.3   : Excitation spectrum between 340 and 475 nm of uncontaminated silk fabric. Excitation band centers are observed at 378 and 440 nm, indicated by an arrow.

The uncontaminated silk fabric has excitation band centres at 378 and 440 nm (Figure 2.3). These positions were observed in silk fabric exposed to UV light. Untreated silk has very little visible fluorescence, but after exposure to UV light a new feature develops which is very similar in position and appearance to the fluorescence in reduced wool. It is likely that both features are due to protein oxidation products that are nonreducible (Millington and Kirschenbaum 2002). All analyzed HSs show a clear fluorescence increase between excitation wavelength 430 and 475 nm (Figure 2.4).
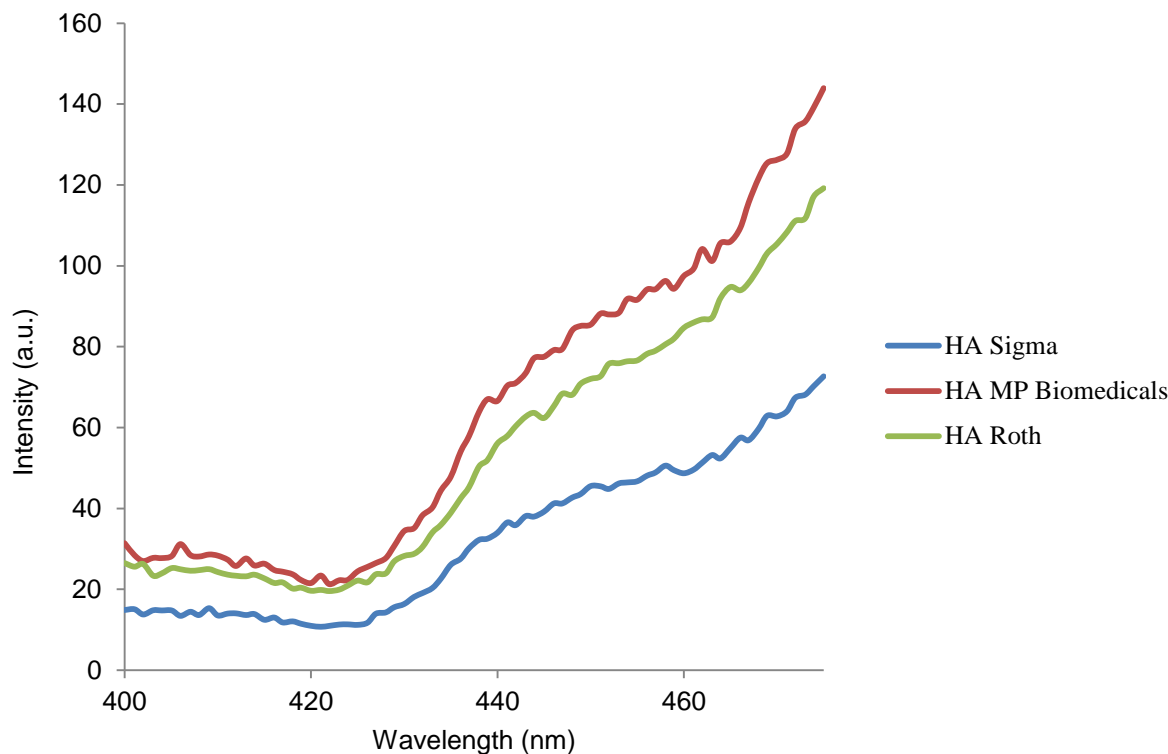
Figure 2.4   : Excitation spectrum between 400 and 475 nm of the humic substance HA Roth, HA Sigma, and HA MP Biomedicals.

Further analyses started at an excitation wavelength of 400 nm instead of 340nm in order to detect mainly the HS and minimize the fluorescence interference of the textile (Table 2.2), and to make the analysis faster and more straightforward.

Table 2.2   : Excitation and emission band centres (nm) of different materials (Bachelier 1980-1981, Millington et al. 2002, Simpson et al. 1996)

	Excitation band center (nm)	Emission band center (nm)
Wool	375	450
Oxidised wool	430	500
Silk after UV light	370	440
Humic acids	465, 480 and 490	509-515

If we compare the spectra of the HS-contaminated silk samples (Silk HA Roth 64 hr (Figure 2.5a) and silk HA Roth 188 hr (Figure 2.5b) with those of HS (Figure 2.4) and uncontaminated silk (Figure 2.3), we observe a linear increase in fluorescence between 460 and 475 nm in both contaminated samples. This is not the case for uncontaminated silk (Figure 2.3) but it is for HA Roth (Figure 2.4). This fluorescence can be explained by

the presence of HSs in the fiber (Table 2.2). Silk HA Roth 64hr (Figure 2.5a) was still well-preserved and a little contaminated with HS, indicated by the light brown coloured silk fabric. The uncontaminated silk fabric was white. Silk HA Roth 188 hr (Figure 2.5b) started falling apart, indicating degradation, and probably consequently fluorophore degradati of the silk. The sik fabric was dark brown, indicating a large HS contamination The observed fluorescence between 440 and 475 nm can be assigned to the fluorophores of the silk and of the HS. Depending on the preservation state of the silk fabric, either the silk fluorophores or the HS fluorophores are mainly detected in a particular wavelength range. The fluorescence of Silk HA Roth 64hr (Figure 2.5a) can be mainly assigned to the fluorophores of the silk between 440 and 460 nm and to HS fluorophores between 460 and 475 nm. The fluorescence of Silk HA Roth 188 hr (Figure 2.5b) is mainly coming from the fluorophores of the HS between 440 and 475nm.
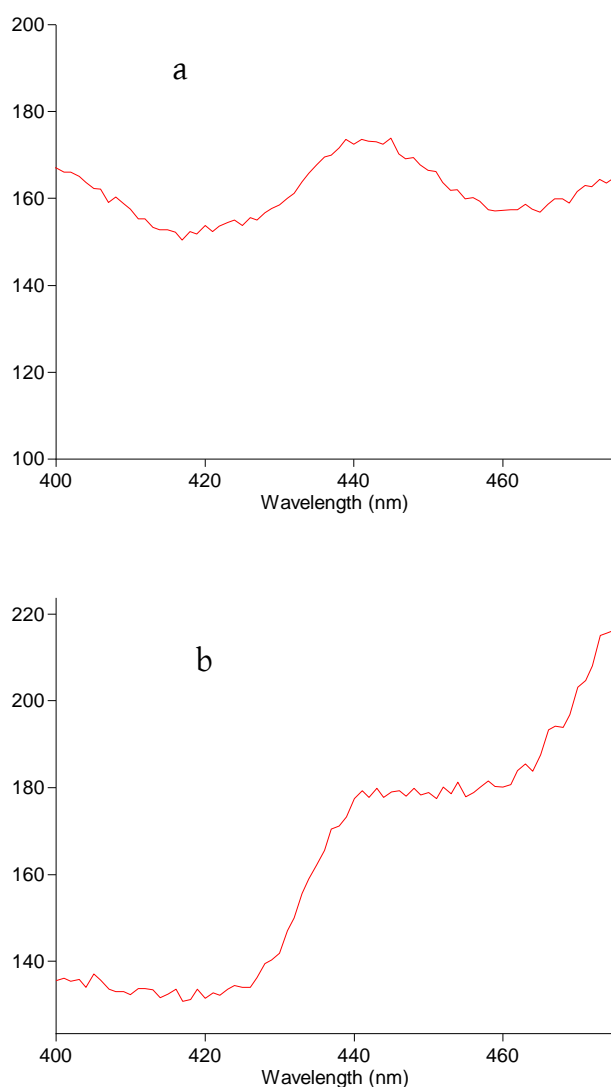
Figure 2.5   : Excitation spectrum of modern silk fabric immersed in mixture of humic substance (Roth) and Milli-Q water during 64 hr (a) and 188hr (b).

Therefore, the slope was calculated using a linear fit (least squares) to the curve between 465 and 475 nm (Table 2.3). All analyzed HSs (HA Roth, HA MP Biomedicals and HA Sigma) have a positive slope. Uncontaminated wool and silk have both a negative slope, while HS contaminated samples (Silk HA Roth 3 hr, Silk HA Roth 64 hr, Silk HA Roth 188 hr, Wool HA Roth 120 hr, and Wool HA Roth 200 hr) have a positive slope. Thus, the slope can be used as a qualitative indicator for the presence of HS: a negative slope indicating HS absence and a positive slope indicating HS presence.

Table 2.3   : Spectrofluorescence slope values of analyzed HSs and HS-contaminated silk or wool samples, applied PMT detector voltage. The slope was calculated using a linear fit (least squares) to the curve between 465 and 475 nm.

Sample name	Slope	PMT detector voltage (V)
HA Roth	+2.63	950
HA MP Biomedicals	+3.60	950
HA Sigma	+1.73	950
Non-contaminated Silk	-0.67	650
Silk HA Roth 3 hr	+0.77	650
Silk HA Roth 64 hr	+0.76	650
Silk HA Roth 188 hr	+3.05	800
Silk HA Aldrich 64 hr	+0.01	650
Non-contaminated Wool	-0.22	750
Wool HA Roth 120 hr	+0.20	750
Wool HA Roth 200 hr	+0.77	1000

The chosen photomultiplier tube (PMT) detector voltage influences the slope value. If a higher voltage was applied on the same sample, it resulted in a higher slope. This can be explained by the disproportionate fluorescence intensity increase when a higher PMT voltage is applied (Lawaetz and Stedmon 2009). A fluorescence intensity calibration is necessary to compare slope values measured with different PMT voltages. Lawaetz and Stedmon (2009) developed a fluorescence intensity calibration using the Raman scatter peak of water for liquid samples. Research is ongoing to develop a fluorescence intensity calibration method for solid samples using a fiber-optic probe.

For wool/silk samples in a degraded state and a positive slope value a higher PMT voltage was necessary. Possible explanation is mineralization of the fibers. This can be defined as the combination and/or replacement of the organic matrix, including the fluorophores, of the fiber with an inorganic one (Gillard et al. 1994). Another possibility could be protein degradation (fluorophores are part of the protein) caused by microbial attack or by microenvironmental conditions (Sibley et al. 1984).

If we want to compare slopes of the same sample before and after ^{14}C pre-treatment, the same voltage must be applied. In this case, the slope can be used as a semiquantitative indicator for HS presence. In case of a significant HSs removal due to the NaOH wash the slope between 465 and 475 should decrease. This HS removal is shown in Table 2.4 where the slope value of the contaminated test samples decreases as a function of the NaOH wash duration. However, no complete HS removal was obtained.

In case of a significant HSs removal due to the NaOH wash the slope between 465 and 475 should decrease

Table 2.4   : Spectrofluorescence slope value of Silk HA Roth 188 hr (fabric and yarn) and of Wool HA Roth 200 hr (fibers) as a function of NaOH wash duration (min). The slope was calculated using a linear fit (least squares) to the curve between 465 and 475 nm. PMT voltage was 800V for the silk samples and 1000V for the wool samples

	Slope value as a function of NaOH-wash duration (min)					
Sample name	0	15	30	45	60	90
Silk HA Roth 188 hr – *Fabric*	3.05	2.61	1.69	1.27	0.53	0.49
Wool HA Roth 200 hr - *Fibers*	0.77	0.67	0.05	n.a.	n.a.	n.a.

Table 2.5 demonstrates a dependency of the NaOH-treatment duration on the sample type e.g. HS-contaminated silk fabric can be pre-treated longer than HS contaminated silk yarns taken from fabric. HS-contaminated wool fibers are especially susceptible to NaOH: ca. 85% of the sample is lost after 30 min of treatment. It is really exceptional that archaeological samples can be fully treated with 1%NaOH during 15 min in our laboratory.

Table 2.5   : Weight (mg) of Silk HA Roth 188 hr (fabric and yarn) and of Wool HA Roth 200 hr (fibers) as a function of NaOH wash duration (min).

	Weight (mg) as a function of NaOH-wash duration (min)					
Sample name	0	15	30	45	60	90
Silk HA Roth 188 hr – *Fabric*	4.78	4.21	3.8	3.65	3.25	2.93
Silk HA Roth 188 hr –*Yarn*	5.82	4.31	1.62	n.a.	n.a.	n.a.
Wool HA Roth 200 hr - *Fibers*	41.2	29.1	6.4	n.a.	n.a.	n.a.

The slopes of different unknown samples contaminated with HSs can not be compared even with the same PMT voltage since the chemical composition of the HSs is unknown and fluorescence depends on the amount of fluorophores (polyaromatic hydrocarbons or heterocycles) present in the HS. These assessments are supported by the analyses of 3 HSs: HA Roth, HA Biomedicals, and HA Sigma. These samples were analyzed applying the same voltage (950V). The calculated fluorescence slope is different for the three HSs (Table 2.3).

The analyzed archaeological samples (Table 2.1) can be divided into 2 groups:
1. The first group showing an excitation spectrum comparable to the spectrum of sample Silk HA Roth 188 hr (Figure 2.5b): Mainz 1, Mainz 2, Mainz 3, Mainz 4, Beerlegem, Ieper, OS2562 and OS24909. This is an indication for HS presence;
2. the second group has an excitation spectrum matching the spectrum of the uncontaminated silk (Figure 2.3): 1924-01, Bourelet, 1923-02 and Hallstat 79429. This indicates HS absence.

The slope of these archaeological samples (Table 2.1) was calculated (Table 2.6). Analytical precision for the slope was greater than 0.30 (pooled standard deviation), as determined by multiple measurements of four samples (Silk HA Roth 3hr, Beerlegem, Hallstat 79429 and Mainz 2) at 750V. Positive and negative slopes indicate the presence and absence of HSs, respectively. Thus, without a proper cleaning for HSs removal less reliable ^{14}C dates for archaeological silk or wool samples with a positive fluorescence slope can be expected unless *in situ* humification has occurred.

The samples Mainz 1, Mainz 2, Mainz 3, Mainz 4, Beerlegem, Ieper, OS2562 and OS24909 show a positive slope and consequently HS presence in the textile. The samples 1924-01, Bourelet, 1923-02 and Hallstat 79429 have a negative slope that indicates HS absence in the textile.

Table 2.6   : Spectrofluorescence slope values of analyzed archaeological samples, applied PMT detector voltagen and difference of slope values before and after ^{14}C pre-treatment. The slope was calculated using a linear fit (least squares) to the curve between 465 and 475 nm.

Sample name	Slope	PMT detector Voltage (V)	Slope difference between untreated and ^{14}C pre-treated sample
Mainz 1 untreated	+4.37	950	
Mainz 3 untreated	+3.98	950	
Mainz 2 untreated	+5.44	950	
Mainz 2 after ^{14}C pre-treatment	+2.97	950	2.47
Mainz 4 untreated	+3.73	950	
Mainz 4 after ^{14}C pre-treatment	+2.45	950	1.28
Beerlegem untreated	+13.54	950	
Beerlegem after ^{14}C pre-treatment	+9.89	950	3.65
Ieper untreated	+4.51	950	
Ieper after ^{14}C pre-treatment	+3.00	950	1.51
OS2562 untreated	+2.15	950	
OS24909 untreated	+1.08	950	
1924-01 untreated	–1.16	650	
Bourelet untreated	–0.53	800	
1923-02 untreated	–1.03	650	
Hallstat 79429 untreated	–3.69	750	

Four archaeological samples (Mainz 2, Mainz 4, Beerlegem and Ieper) were analyzed before and after ^{14}C pre-treatment applying the same voltage (950V). The slope difference of the uncontaminated and pre-treated textiles was calculated (Table 2.6). A slope decrease for the 4 samples is noted, meaning that the amount of HSs decreases after pre-treatment. However, the slope for the 4 samples remains positive, which means that there were still HSs present in the four samples after ^{14}C pre-treatment.

The samples Mainz 2 and Mainz 4 underwent the full pre-treatment including the NaOH wash. Partial dissolution of the fulvic acid likely occurred during the 15-min sample treatment with Milli-Q water in the ultrasonic bath. The NaOH-step should be able to remove fulvic and humic acids because fulvic acids are soluble in acid and alkaline solutions and humic acid is the fraction extracted by alkaline solution (Head 1987). However, the NaOH wash is not sufficient to remove all HSs as indicated by a positive fluorescence slope after ^{14}C pre-treatment. A harsher NaOH wash results in completely dissolving of the textile-humic mixture, more quickly than releasing HSs from the contaminated textile. Therefore, most of the protein-humic linkages are at least similarly strong as the forces that keep the α-helices (wool) or β-sheets (silk) together (and thus soluble). Van Klinken and Hedges (1995) noted this phenomenon for collagen-

humic mixtures. In the case of wool, alkalis act simultaneously on 3 bond types of the keratin: the peptide bond, the S-bridges and the salt bonds (Sibley and Jakes 1984) causing a fast decomposition of wool. The fibroin silk fibers are even susceptible to decomposition by alkalis and acids: alkalis only breaks the bond between the last amino acid and the rest of the polymer, while acids break the bonds between the amino acids on random places in the polymer (Tímar-Balázsy and Eastop 1998).

The samples Beerlegem and Ieper could not be pre-treated with NaOH due to sample dissolving in NaOH. However, the slope decrease can be assigned to dissolution of fulvic acids during the 15-min sample treatment with Milli-Q water in the ultrasonic bath, since fulvic acids are soluble in acid and alkaline solutions (Head 1987).

2.4.2   AMS dating

Two groups of textiles were obtained after fluorescence analyses (Table 2.4):
1. The first group containing HSs: Mainz 1, Mainz 2, Mainz 3, Mainz 4, Beerlegem and Ieper;
2. The second group where no HSs were detected: samples 1924-01, Bourelet, 1923-02, Hallstat 79429.

The ^{14}C dates of the Mainz samples (Table 2.7) are older than the expected archaeological date for the textiles based on the dated coins and the typochronologically dated Italic Samian pottery found in association with the dated textiles (ca. 5BC) (Böhme-Schönberger and Mitschke 2005) . Moreover, all ^{14}C dates for the Mainz samples are very close to each other. This seems to indicate a uniform HS contamination, even after pre-treatment. The average ^{14}C date of the four Mainz samples is 2116±13BP (χ^2-Test: df=3 T=4.2(5% 7.8)). The difference of the average ^{14}C date and the presumed archaeological (5BC) date is 105 (68.2%) 175; 50 (1.3%) 70, 80 (94.1%) 200 at 95,4% probability.

Table 2.7   : ^{14}C age and calibrated age and presumed historical data of archaeological textile samples.

Sample name	Archaeological site	Lab code	^{14}C age (BP)	Calibrated age (2σ)	Presumed historical date
Mainz 1	Mainz	KIA-41534	2120±25	340 (1.1%) 320BC 210 (94.3%) 50BC	5 BC
Mainz 2	Mainz	KIA-41535	2075±25	180 (94.5%) 30BC	5 BC
Mainz 3	Mainz	KIA-41536	2145±25	360 (23.7%) 290BC 230 (71.7%) 90BC	5 BC
Mainz 4	Mainz	KIA-41537	2125±25	350 (4.6%) 320BC 210 (90.8%) 50BC	5 BC
Beerlegem	Beerlegem	KIA-42365	1705±30	AD 250 (95.4%) 410	AD 587
Ieper	Ieper	KIA-43347	750±25	AD 1220 (95.4%) 1285	13th century AD
1924-01	Unknown	KIA-40874	980±20	AD 1010 (54.5%) 1060 AD 1080 (40.9%) 1160	AD1000-1200
Bourelet	Unknown	KIA-39433	1675±30	AD 250 (11.8%) 300 AD 320 (83.6%) 430	AD 200-450
1923-02	Unknown	KIA-42114	1090±30	AD 890 (95.4%) 1020	AD 700-1000

From Table 2.7 above, the following conclusions can be made: 1) The difference is provoked by HS contamination of the textiles and the ^{14}C age is older than the expected archaeological date. 2) But the presence of HSs is not always an indication for ^{14}C contamination. *In situ* humification of the material is not influencing the ^{14}C date. An archaeological hypothesis is that the textile fragments were used to fortify the bog ground when the Romans built their camp. Therefore, the use of older, worn out textiles is assumed and can also explain the calculated difference.

The wool of Ieper has been excavated from a watercourse. Two archaeological hypotheses can be proposed: 1) Textile manufacturing started in the mid-13th century at that site (Haneca et al. 2009). It is possible that the excavated wool was deposed in the watercourse during that period. In this case, the ^{14}C date is in agreement with this archaeological date (Table 2.7). However, fluorescence spectroscopy proved the

presence of HS. *In situ* humification of the material can explain the presence and the agreement between the ^{14}C and the archaeological date. 2) Nevertheless, it is also possible that the textile deposit occurred when a new port was built in AD 1290 (dendrochronological date of the felling of an oak tree used in the construction, with bark still present, Haneca et al. 2009). Then, the ^{14}C date is a bit too old. This can be due to HS contamination and may explain the difference of the ^{14}C date and the dendrochronological date (AD 1290) that is between 5 and 70 years (95.4% probability).

^{14}C dating of wool or silk, showing HS presence by fluorescence analysis, does not aid archaeologists in clarifying the archaeological context where different archaeological considerations (e.g. in Mainz and Ieper) can be made.

The ^{14}C date of Beerlegem (AD 250-410 with 95.4% probability) is older than the archaeological date (late 6th century - first half of the 7th century) (Table 2.7). The older ^{14}C date confirms the HS contamination of the textile analyzed by spectrofluorometry. The archaeological evidences and information suggest the unreliability of the ^{14}C date:

1. The textile was found in a burial chamber. The features of the grave goods (glass beakers, an urn, fragments of a bronze dish, beads, a golden ring, a bronze decorative disc, a silver necklace with a bone pendant) date this grave to the first half of the 7th century AD and indicate that a wealthy woman was buried in this chamber (Roosens 1959).
2. The felling date of a bottom plank of the grave was AD 587 ± 10, obtained by dendrochronology (Roosens 1977).
3. The study of textile fragments resulted in spinning and weaving techniques with a great diversity and demonstrated a high development degree of the Merovingian textile technique in Belgium (Lefève 1959).
4. The Merovingians arrived in Belgium in the second half of the 5th Century (Wightman 1985).

The samples Beerlegem and Ieper could not be pre-treated with NaOH to remove humic acids from the fiber. ^{14}C dating of these texiles can provide more unreliable results than textiles pre-treated with a NaOH wash. The fluorescence analysis of Mainz 2 and Mainz 4 before and after ^{14}C pre-treatment showed that the solvent pre-treatment with NaOH and HCl wash is not sufficient to remove all the HS in the textile sample (Table 2.6).

Therefore, ^{14}C dates made on HSs contaminated wool/silk should be interpreted with care.

The ^{14}C dates of the samples 1924-01, Bourelet, 1923-02 and Hallstat 79429 (Grömer 2005), without detected HSs contamiantion, are in agreement with the determined archaeological dates (Table 2.7).

2.5 CONCLUSION

Fluorescence spectrometry is a quick and non-destructive pre-screening method to detect the presence of contaminant HSs in naturally (un)dyed wool and silk and gives information about the reliability of the ^{14}C dates. However, one must carefully consider wool/silk samples wherein HSs are detected with fluorescence analyses for ^{14}C dating, because the conventional solvent pre-treatment method with NaOH wash is in most cases not sufficient to remove all HS contaminants. As a result, unreliable ^{14}C dates can be provided.

Compound-specific ^{14}C dating of individual amino acids (Van Klinken et al. 1990, Tripp et al. 2006, McCullagh et al. 2010) of the silk/wool protein can be a potential method for dating proteinic textiles showing HS contamination. However, the conventional pre-treatment method yields reliable ^{14}C dates of proteinic textiles, wherein no HSs were observed with spectrofluorescence.

CHAPTER 3   Development of a nanofiltration method for bone collagen ^{14}C AMS dating

3.1 ABSTRACT

Radiocarbon dating of bones is usually performed on the collagen fraction. However, this collagen can contain exogenous molecules, including humic substances (HSs) and/or other soil components that may have a different age than the bone. Incomplete removal can result in biased ^{14}C dates. Ultrafiltration of collagen, dissolved as gelatin (molecular weight (MW) ~100,000 Dalton), has received considerable attention to obtain more reliable dates. Ultrafiltration is an effective method of removal of low-molecular weight contaminants from bone collagen but it does not remove high-molecular weight contaminants, such as cross-linked humic collagen complexes. However, comparative dating studies have raised the question whether this cleaning step itself may introduce contamination with carbon from the filters used.

In this study, a nanofiltration method was developed using a ceramic filter to avoid a possible extraneous carbon contamination introduced by the filter. This method should be applicable to various protein materials such as collagen, silk, wool, leather and should be able to remove low-molecular and high molecular weight HSs. In this study bone collagen was hot acid hydrolysed to amino acids and nanofiltrated. A filter with a molecular weight cutoff (MWCO) of 450 Dalton was chosen in order to collect the amino acids in the permeate and the HSs in the retentate.

Two pilot studies were set up. Two nanofiltration types were tested in pilot study 1: dead-end and cross-flow filtration. Humic substance (HS)-solutions with fossil carbon and modern hydrolysed collagen contaminated with HSs were filtrated and analyzed with spectrofluorescence to determine the HS removal. Cross-flow nanofiltration showed the most efficient HS removal. A second pilot study based upon these results was set up wherein only cross-flow filtration was performed. ^{14}C measurements of the permeates of hydrolysed modern collagen contaminated with fossil HSs demonstrate a significant but incomplete removal of HSs (between 63 and 85%).

3.2 INTRODUCTION

Bones from archaeological and geological sites are susceptible to chemical and environmental processes which can result in bone degradation and introduction of exogenous carbon-containing compounds, in particular HSs from the soil interacting with the bone collagen (Arslanov and Svezehentsev 1993; Van Klinken and Hedges 1995).

The collagen fraction of the bones is usually used for ^{14}C dating. This fraction is obtained by dissolving the demineralized bone in hot acidic water (Longin 1974). This treatment will also lead to the presence of other hot water soluble components in the collagen such as HSs and degraded protein fragments (Van Klinken and Mook 1990). Adding an alkaline step helps to remove base soluble organics such as humic acid (Van Klinken and Hedges 1995).

It has been suggested that ultrafiltration is an effective method to remove low-molecular weight contaminants and degraded proteins from the high-molecular weight collagen protein (Brown et al. 1988; Bronk et al. 2004). Ultrafiltration is a membrane process whose nature lies between nanofiltration and microfiltration. The pore sizes of the membranes used range from 0.05 μm (on the microfiltration side) to 2 nm (on the nanofiltration side). Ultrafiltration is typically used to retain macromolecules and colloids from a solution, the lower limit being solutes. A low pressure between 1 - 10 bar is applied and separation is based on particle size. Ultrafiltration clean-up of bone collagen has gained recent attention.Redating of previously dated bones using ultrafiltration yielded significant older and more consistent ^{14}C ages (Bronk et al. 2004; Higham et al. 2006) Nevertheless, other dating studies proved the introduction of contamination via the filters (Bronk et al. 2004; Brock et al. 2007; Hüls et al. 2007; Hüls et al. 2009).

Compound-specific ^{14}C dating of individual amino acids upon collagen hydrolysis and obtained by preparative High Performance Liquid Chromatography (HPLC), has been performed in the past (Gillespie and Hedges 1983; Gillespie et al. 1984; Stafford et al.1987; Stafford et al.1988; Van Klinken and Mook 1990; Stafford et al.1991; Van Klinken 1991; Tripp et al. 2006). Some of these methods introduced extraneous carbon into the ^{14}C dated fractions and sample blanks were not always reported. However, since column bleeding is a common phenomenon in chromatography sample blanks should be measured. McCullagh et al. (2010) showed the potential of individual amino acid dating. The drawback of the technique is that it is time consuming.

Nanofiltration is more straightforward and less labour intensive than individual amino acid isolation by HPLC. Nanofiltration is an intermediate filtration process between reverse osmosis (RO) and ultrafiltration (UF) that rejects molecules having a size of

about one nanometer (Eriksson 1988). The pore sizes of the membranes used range from 0.5 to 2 nm and corresponds with MWCO of 300-500 Dalton (Mulder 1996). A high pressure between 10-50 bar is applied and separation is based on differences in solubility and diffusivity (Mulder 1996). It has been introduced since 1980s, mainly used for softening water and removing organics. It has found many applications in various water purification and treatment as well as product separation processes because of its two remarkable features: one is the MWCO which ranges from 200 to 2000 Da; the other is the separation of electrolytes due to the membrane materials containing charged groups (Schafer et al. 2004; Wang et al. 2009).

In this study a nanofiltration method was developed using a ceramic filter as a means to avoid extraneous carbon contamination possibly introduced by typical ultrafilters. This method should be applicable on various protein materials e.g. collagen, silk, wool, leather. Hot acid hydrolysis releases the amino acids of the protein material. It should be able to remove low-molecular and high molecular weight HSs, depending on the choice of MWCO membrane cut-off and the fraction (permeate/filtrate or retentate). Here a filter membrane with a cutoff of 450 Dalton was used in order to collect the amino acids in the permeate (MW of amino acids varies between 75.07 and 204.23 Dalton (Asquith 1977)) and the humic substances (HSs) in the retentate (MW of HSs varies between 1000 and 300000 Dalton) (Stevenson 1982).

3.3   MATERIALS AND METHODS

3.3.1   Sample selection

An archaeological human bone of the site *Punta de Los Gavilanes* (Murcia, Spain) was selected for pilot study 1. A modern bovine bone was chosen for pilot study 2.

A commercially available HS was used (humic acid depur, referred as HA Roth in this paper (Carl Roth, Karlsruhe, Germany) to artificially contaminate the bone material in pilot study 1 and 2.

3.3.2   Sample preparation

Two pilot studies were set up. An overview of the analyzed sample types for both pilot studies is listed in Table 3.1.

Two nanofiltration systems were tested in pilot study 1: dead-end and cross-flow filtration. The samples prepared for pilot study 1 were analysed with spectrofluorescence before and after nanofiltration to determine the best filtration technique to remove HSs (Boudin et al. 2011). Based upon the results of these analyses it was decided to set up pilot study 2 wherein the samples were cross-flow nanofiltrated on a downscaled installation and ^{14}C dated to verify the HS removal and the filter efficiency (Figure 3.1).

Table 3.1   : An overview of the analyzed samples, sample names and definition, used for pilot studies 1 and 2.

Pilot study 1 : Spectrofluorescence analysis

Sample name	Sample definition
HA Roth_H_2O	HA Roth dissolved in RO water
HA Roth_ H_2O_cross-flow	Cross-flow permeate of HA Roth_H2O
HA Roth_ H_2O_dead-end	Dead-end permeate of HA Roth_H2O
HA Roth_NaOH	HA Roth dissolved in NaOH
HA Roth_ NaOH_cross-flow	Cross-flow permeate of HA Roth_NaOH
HA Roth_ NaOH_ dead-end	Dead-end permeate of HA Roth_NaOH
AA_collagen_ HA Roth	Amino acids after hydrolysis of collagen/HA Roth mixture (before nanofiltration)
AA_collagen_ HA Roth_ cross-flow	Cross-flow permeate of amino acids after hydrolysis of collagen/HA Roth mixture
AA_ collagen_ HA Roth_ dead-end	Dead-end permeate of amino acids after hydrolysis of collagen/HA Roth mixture

Pilot study 2 : [14]C analysis

Sample name	Sample definition
HA Roth_ fulvic acids	HA Roth dissolved in Milli-Q water, filtrated over a 0.7 µm glass filter and freeze-dried
Collagen	Sample Collagen extracted from a modern bovine bone via the Longin method
Collagen_NaOH	Sample Collagen extracted from a modern bovine bone via the Longin method plus a NaOH-wash
Collagen_HA Roth	Sample Collagen contaminated with HA Roth
Collagen_ NaOH_HA Roth	Sample Collagen_NaOH contaminated with HA Roth
AA_Collagen_cross-flow	Cross-flow permeate of amino acids after hydrolysis of sample Collagen
AA_Collagen_NaOH_cross-flow	Cross-flow permeate of amino acids after hydrolysis of sample Collagen_NaOH
AA_Collagen_HA Roth_cross-flow_1	First collected fraction of cross-flow permeate of amino acids after hydrolysis of sample Collagen_HA Roth
AA_Collagen_HA Roth_cross-flow_2	Second collected fraction of cross-flow permeate of amino acids after hydrolysis of sample Collagen_HA Roth
AA_Collagen_NaOH_ HA Roth_cross-flow	Cross-flow permeate of amino acids after hydrolysis of sample Collagen_ NaOH_HA Roth

Collagen was extracted from the bones following the Longin method (Longin 1971). For pilot study 1 and for one bone of pilot study 2 a NaOH-wash was introduced between the demineralization and hydrolisation step. After hydrolysation, the samples were freeze-dried.

Aliquots of HA Roth, of about 650 mg each, were dissolved in 1000 ml reverse osmosis (RO) water and 0.125 %NaOH for pilot study 1. These HS-mixture contained fulvic acids when dissolved in RO water (pH = 7.15), and fulvic and humic acids when NaOH was the solvent.

An aliquot of about 650 mg HA Roth was also dissolved in 125 ml Milli-Q water and slowly agitated at room temperature during 24 hours. The mixture was filtrated over a 0.7 µm glass filter (Millipore), then freeze-dried and finally ^{14}C dated in pilot study 2. This sample was named HA Roth_fulvic acids.
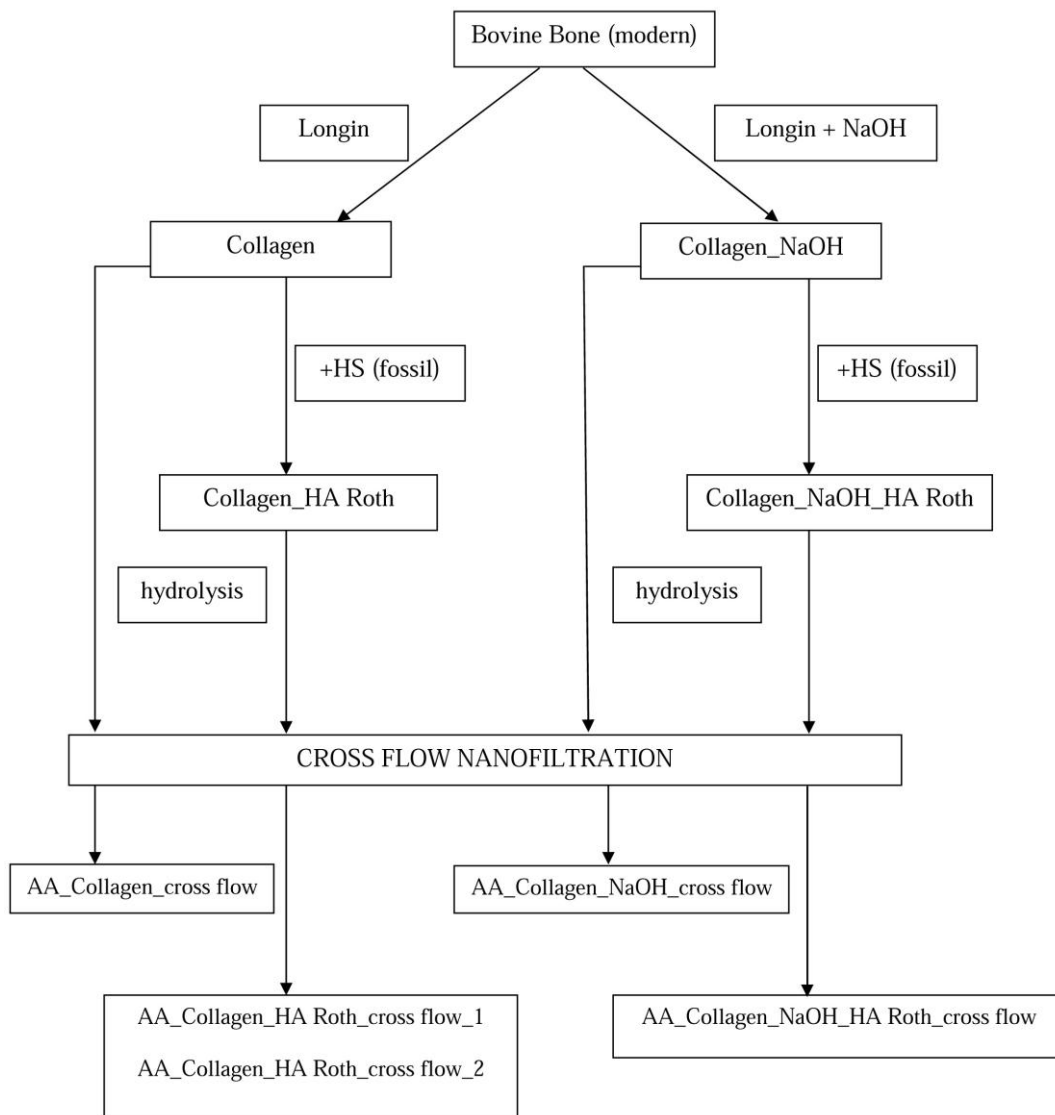
Figure 3.1   : Flowchart of sample preparation for pilot study 2

Aliquots of HA Roth, of about 650 mg each, were dissolved in 125 ml RO water. These HS-solutions were added to about 200 - 750 mg of collagen and slowly agitated at room temperature during 24 hours. The mixture was filtrated over a 0.7 mm glass filter (Millipore) and then freeze-dried.

In a next step, the collagen-HS mixtures were hydrolysed into amino acids. Hydrolysis was carried out for approximately 70 – 200 mg aliquots of collagen-HS using 6M HCl in a sealed tube in a nitrogen atmosphere at 105°C for 24 hours. HCl was removed using a solvent evaporator (Genevac EZ-2, Genevac, UK). The dry hydrolysate was diluted with RO water to 1000 ml for pilot study 1 and to 300 ml for pilot study 2.

Aliquots of HA Roth, of about 5 - 10 mg each, were dissolved in 10 ml Milli-Q water for chromatographic analysis with HPLC-PDA to register a reference chromatogram and retention times for non-hydrolysed fulvic acids.

Hydrolysis of HA Roth was carried out on circa 10 mg HA Roth. Fifty µl Milli-Q water was added to the dry hydrolysate before injection and then analysed with High performance liquid chromatography Photo diode array detector (HPLC-PDA) to verify if HS remained intact after hydrolysis or not.

3.3.3   Nanofiltration

Nanofiltration is a pressure driven membrane process and is characterised by a membrane pore size between 0.5 and 2 nm and operating pressures between 5 and 40 bar.

In general nanofiltration has two distinct properties:

- The pore size of the membrane corresponds to a MWCO value of approximately 300-500 Da. Therefore, the separation of components with these molecular weights from higher molecular weight components can be accomplished.
- Nanofiltration membranes have a slightly charged surface. Because the dimensions of the pores are less than one order of magnitude larger than the size of ions, charge interaction plays a dominant role. This effect can be used to separate ions with different valences (Rautenbach and Gröschl 1990).

Two nanofiltration types were tested: dead-end filtration and cross-flow filtration.

Dead-end filtration is the simplest filtration (Figure 3.2). Here all the feed is forced through the membrane, so that contamination in the retentate, e.g. > 450 Da fraction increases, and consequently the quality of the permeate decreases with time (Mulder 1996). During cross-flow filtration the feed flow travels tangentially across the surface of the filter (Figure 3.2). The principle advantage of this filtration is that the filter cake

(which can block the filter) is substantially washed away during the filtration process, increasing the length of time that a filter unit can be operational (Koros et al. 1996).
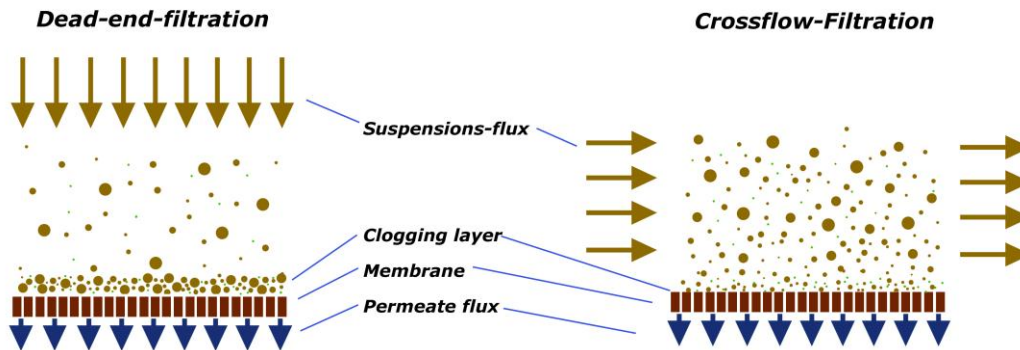
Figure 3.2   : Scheme of dead-end and cross-flow filtration.

Two home-made filtration installations were set up using a stainless steel centrifugal pump (Speck Pumps, Belgium), Swagelock accessories (tubings, fittings, flexibles) and a ceramic filter with a 450 Dalton cut off (Inopor, Germany) (Figure 3.3).

A pressure of 10 bar (nitrogen) was applied in dead-end and cross-flow mode.

The centrifugal pump is only required in cross-flow mode as a means to obtain a tangential feed flow.

Cross-flow velocity was 3.0 m/s for pilot study 1 and 2.

A filter with a cutoff of 450 Dalton was used in order to collect the amino acids in the permeate (MW of amino acids varies between 75.07 and 204.23 Dalton) and the HSs in the retentate (MW of HSs varies between 1000 and 300000Dalton) (Stevenson 1982).

The filtration setup can easily be rebuilt from dead-end operation into cross-flow mode and vice versa.

A minimum feed volume must remain in the filtration installation to prevent the pump from stopping in cross-flow mode. This volume was 800 ml for the installation used in pilot study 1 and 100 ml in pilot study 2.

The feed volume for pilot study 1 was 1000 ml and 200 ml for pilot study 2.

The volume of permeate collected for analysis in this study was 200 ml for pilot study 1 and 100 ml for pilot study 2.

To verify the filter reproducibility, two consecutive permeate fractions of sample AA_Collagen_HA Roth were collected during cross-flow filtration and referred to as Collagen_HA Roth_cross-flow_1 and Collagen_HA Roth_cross-flow_2 in this paper (Table 3.1 and Figure 3.1).

Cleaning of the filtration installation was done after every filtration by consecutively rinsing with RO-water, then with 1%NaOH and finally with Milli-Q water. The cleaning

efficiency was tested by measuring the flux after every rinsing step. The flux obtained after the NaOH step was equal as the flux of Milli-Q water before performing the filtrations.
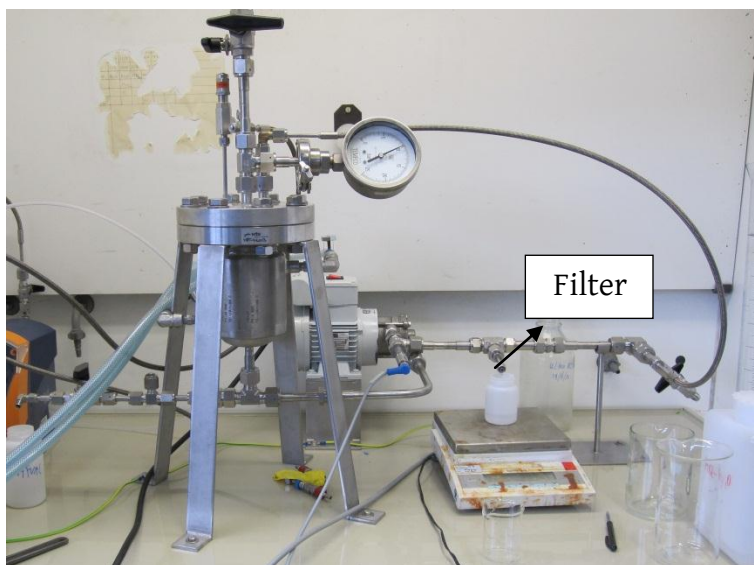
Figure 3.3   : Filtration setup in cross-flow mode used for pilot study 2.

3.3.4   Fluorescence spectroscopy

Fluorescence spectroscopic measurements (Varian Cary Eclipse, Belgium) of solutions produced during pilot study 1 were performed using quartz cells (Table 3.1). A scan speed of 600 nm.min^{-1} was used with a slit width opening of 10 nm. Spectra for HSs were acquired in the excitation wavelength 340-475 nm and an emission wavelength of 509 nm (Bachelier 1980-1981; Bloom and Leenheer 1989; Simpson et al. 1997).

Three aromatic amino acids with fluorescent properties (tryptophan, tyrosine and phenylalanine) are not detected with the chosen excitation and emission wavelength (Lakowicz 1999).

Fluorescence spectra for blanks (RO water) were also obtained for Raman spectral overlap. Since all the spectra were recorded on the same instrument using the same experimental parameters, a comparative discussion of the spectra is acceptable, although no corrections for fluctuation of instrumental factors and for scattering effects (e.g. primary and secondary inner filter effects) were applied to the data. The software automatically chose the excitation and emission filter during analysis. Fluorescence intensity was measured in arbitrary units (a.u.).

3.3.5   High perf ormance liquid chromatography Photo diode array detector (HPLC-PDA)

Chromatography was performed on a Waters HPLC system consisting of a multi-solvent pump 625, a 600E controller and a PDA detector, all controlled by Empower 1 PC software.

Humic substance and amino acid analyses were done using the method described in Smith et al. (2009), except that detection in this study was performed with PDA instead of an Isotope Ratio Mass Spectrometer.

In this study scanning was done between 200 and 800 nm; chromatograms were registered at 210 nm. UV/VIS absorption intensity was measured in absorption units (a.u.).

3.3.6 ^{14}C AMS dating

^{14}C dating was performed on samples of pilot study 2 (Table 3.1 and Figure 3.1).

The permeates of the hydrolysed collagen-HS mixture and of the hydrolysed collagen, thus amino acids alone, were freeze dried, dissolved in 2 ml Milli-Q water, transferred into quartz tubes with CuO and Ag and dried in a desiccator before combustion to CO_2. Graphitisation of CO_2 was carried out using H_2 over a Fe catalyst. Targets were prepared at the Royal Institute for Cultural Heritage in Brussels (Belgium) (Van Strydonck and Van der Borg 1990-1991) and ^{14}C concentrations were measured on the AMS at the Leibniz Labor für Altersbestimmung und Isotopenforschung in Kiel (Germany) (Nadeau et al. 1998). ^{14}C results are expressed in pMC (percent modern carbon) and indicate the percent of modern (1950) carbon corrected for fractionation using the δ^{13}C measurement.

The HS removal via cross-flow filtration can be determined by calculating the percent C contamination using the ^{14}C results.

The percent C contamination was calculated as follows:

Non-filtrated collagen-HS-mixtures (Collagen_HA Roth and Collagen_NaOH_HA Roth)

$$^{14}C_{\text{collagen\_(NaOH)\_HA Roth}} = {}^{14}C_{\text{HA oth\_fulvic acids}} \times x + (1 - x) \times {}^{14}C_{\text{collagen\_(NaOH)}}$$

Hydrolysed, cross-flow filtrated samples (AA_Collagen_HA Roth_cross-flow and AA_Collagen_NaOH_HA Roth_cross-flow)

$$^{14}C_{\text{AA\_Collagen\_(NaOH)\_HA Roth\_cross-flow}} = {}^{14}C_{\text{HA Roth\_fulvic acids}} \times x + (1 - x) \times {}^{14}C_{\text{AA\_Collagen\_(NaOH)\_cross-flow}}$$

Percent C contamination is x × 100.

The ^{14}C concentrations of the filtrated amino acids of collagen (AA_collagen_cross-flow and AA_collagen_NaOH_cross-flow) were used to calculate the percent C contamination in the hydrolysed, cross-flow filtrated samples.

The ceramic filter used has a cutoff of 450 Dalton, which means that theoretically 90% of 450 Dalton (and larger) molecules should be retained by the filter.

The percent contamination removal can be calculated using the following assumptions:

1. HSs remain intact after hydrolysis,
2. all HSs are larger than 1000 Dalton,
3. the ratio amino acids/HSs is equal in the hydrolysed and the non-hydrolysed solution.

$$\% \text{ Removal} = \frac{(\%C \text{ contamination }_{\text{collagen-HS mixture}} - \%C \text{ contamination amino acids after cross-flow})}{\%C \text{ contamination }_{\text{collagen-HS mixture}}} \times 100$$

Wherein collagen-HS mixture refers to sample Collagen_HA Roth or Collagen_NaOH_ HA Roth, and amino acids after cross-flow filtration refers to sample AA_Collagen_HA Roth_cross-flow or AA_Collagen_NaOH_HA Roth_cross-flow.

3.4   RESULTS AND DISCUSSION

3.4.1   Pilot Study 1 - Fluorescence spectroscopy

The flux is proportional to the driving force which is pressure in the case of nanofiltration. A lower flux can be the consequence of concentration polarisation. However, it should be mentioned that fouling is the dominating factor in flux decline in the case of nanofiltration (Mulder 1996).

The fluxes of HA Roth_H2O_cross-flow, HA Roth_ NaOH_cross-flow and AA_collagen_HA Roth_cross-flow are in the same magnitude order of the flux of RO water after cross-flow filtration indicating minor contribution of fouling and concentration polarisation.

Nevertheless, the fluxes observed during dead-end filtration are lower compared to the fluxes of cross-flow nanofiltration for the same samples (see Table 3.2) indicating concentration polarisation.

The decrease in fluorescence intensity after cross-flow and dead-end filtration compared to the feed solution, observed in all the samples of pilot study 1, indicates HS removal (Table 3.2). However, cross-flow filtration seems to retain more of the HSs compared to dead-end filtration mode as the measured fluorescence intensity is closer to the values for the blank (RO water) (Table 3.1 and 3.2).

As a result, cross-flow nanofiltration was used in pilot study 2.

Table 3.2   : Flux (kg/m^2.h), fluorescence excitation range (nm), minimum and maximum fluorescence intensity (arbitrary unit) of the samples before and after nanofiltration in pilot study 1.

Sample name	Flux (kg/m².h)	Fluorescence (excitation range - nm)	Minimum and maximum Fluorescence intensity (arbitrary unit)
HA Roth_H$_2$O		340 to 390	200 – 400
HA Roth_ H$_2$O_cross-flow	110	340 to390	2,5 – 6
HA Roth_ H$_2$O_dead-end	75	340 to 390	25 – 50
HA Roth_NaOH		415 to 470	200 – 600
HA Roth_ NaOH_cross-flow	125	340 to 390	8 – 13
HA Roth_ NaOH_ dead-end	55	340 to 390	210 -250
AA_collagen_ HA Roth		340 to 390	60 – 170
AA_collagen_ HA Roth_ cross-flow	70	340 to 390	4 – 19
AA_ collagen_ HA Roth_ dead-end	30	340 to 390	15 – 60
RO water		340 to 390	1 - 2
RO water_cross flow	100	n.a.	n.a.

3.4.2   Pilot Study 2 - ^{14}C AMS dating

The percent C contamination in the non-filtrated, non-hydrolysed collagen-HS samples, Collagen_HA Roth and Collagen_NaOH_HA Roth, were 7.44 % and 24.90 % respectively (Tables 3.1 and 3.3).

The ^{14}C concentrations of the hydrolysed, cross-flow filtrated samples are shown in Table 3.3. Measured pMC-values of the uncontaminated, filtrated amino acids of collagen (AA_collagen_cross-flow and AA_collagen_NaOH_cross-flow) demonstrate a ^{14}C decrease of 1.90 pMC for AA_collagen_cross-flow and 1.61 pMC for AA_collagen_

NaOH_cross-flow compared to their original ^{14}C-values of non-hydrolysed collagen. A contamination may have occurred during hydrolysis and/or filtration. It is possible that the extraneous carbon was introduced due to a dirty filter or remaining carbon in the pump system from previous experiments. Some solution always remains in the pump and is not removed with the rinsing step. These two contamination phenomena are highly possible because the tests were performed at The Flemish Institute for Technological Research where the filtration setup is mainly used for organic compounds. However, partly contamination probably occurred (circa 0.002%) when diluting the samples with RO water to the necessary volume to perform cross-flow filtration because total organic carbon analyses of the RO water revealed values of 2 ppm (parts per million).

Two samples of the same feed, respectively AA_Collagen_HA Roth_cross-flow_1 and AA_Collagen_HA Roth_cross-flow_2, were collected and ^{14}C dated. Percent C contamination after cross-flow filtration was 1.53% and 2.73%, respectively, clearly demonstrating HS removal compared to starting contamination of 7.44 %C (measured in the non-filtrated, non-hydrolysed collagen-HS sample (Collagen_HA Roth)). Increased percent C contamination can be observed between the first collected fraction (AA_Collagen_ HA Roth_cross-flow_1) and the second collected fraction (AA_Collagen_HA Roth_cross-flow_2). A possible explanation is the formation of a clogging layer of HSs on the filter followed by HSs of the clogging layer that pass the filter and consequently cause an increase in contamination in fraction AA_Collagen_HA Roth_cross-flow_2. If this is the case, a removal of the retentate and performing the cleaning step are required in order to perform a second filtration.

Further analyses should clarify these hypothesis.

The percent C contamination of the non-filtrated, non-hydrolysed sample Collagen_NaOH_HA Roth showed a decrease from 24.90% to 3.70% after cross-flow filtration of the hydrolysate (AA_Collagen_NaOH_ HA Roth_cross-flow). This demonstrated a significant, but incomplete, HS removal of 85 %.

Table 3.3   : ¹⁴C AMS (percent modern carbon, pMC) results of samples prepared for pilot study 2 and percent C contamination of the collagen-HS mixture and the hydrolysed collagen-HS mixture after cross-flow filtration.

Sample name	LAB-code	¹⁴C (pMC)	Stdev ¹⁴C (pMC)	%C contamination
Humic substances				
HA Roth_ fulvic acids	KIA-42747	0.28	0.17	
Collagen				
Collagen	KIA-42745	105.41	0.32	
Collagen_NaOH	KIA-42744	104.64	0.29	
Collagen_HA Roth	KIA-42746	97.59	0.30	**7.44**
Collagen_ NaOH_HA Roth	KIA-42748	78.68	0.27	**24.90**
Amino acids after cross-flow filtration				
AA_Collagen_cross-flow	KIA-43046	103.51	0.30	**1.80**
AA_Collagen_NaOH_cross-flow	KIA-43047	103.03	0.37	**1.54**
AA_Collagen_HA Roth_cross-flow_1	KIA-43049	101.93	0.30	**1.53**
AA_Collagen_HA Roth_cross-flow_2	KIA-43050	100.69	0.36	**2.73**
AA_Collagen_NaOH_ HA Roth_cross-flow	KIA-43048	99.25	0.40	**3.70**

The percent contamination removal of sample AA_Collagen_NaOH_HA Roth_cross-flow amounts to 85% which is close to the theoretically expected 90%. The results of AA_Collagen_HA Roth_cross-flow_1 and AA_Collagen_HA Roth_cross-flow_2 are more disturbing. The percent removal of both samples, respectively 79% and 63% (Table 3.4), shows a considerable difference with the theoretically expected value of 90%. This difference was probably provoked by not emptying the pump between every filtration indicated by the decreasing percent contamination removal after every filtration (first 85%, then 79% and finally 63%). Some solution always remains in the pump and is not removed with the rinsing step. This phenomenon was observed during later experiments, described in Chapter 4.

Table 3.4   : Percent contamination removal of the hydrolysed, cross-flow filtrated collagen-HS mixtures.

Sample name	%Contamination removal
AA_Collagen_HA Roth_cross-flow_1	79
AA_Collagen_HA Roth_cross-flow_2	63
AA_Collagen_NaOH_HA Roth_cross-flow	85

3.4.3   High performance liquid chromatography Photo Diode Array detector (HPLC-PDA)

The chromatograms of non-hydrolysed and hydrolysed HA Roth are shown in Figure 3.4 and 3.5. The HS compound present at retention time 110 minutes in non-hydrolysed HA Roth (Figure 3.4) disappears after hydrolysis but new compounds appear in the chromatogram of hydrolysed HA Roth, respectively at retention time 78 minutes, 87 minutes and 91 minutes (Figure 3.5). If we assume that these three compounds released by hydrolysis may be smaller than 450 Dalton and pass the filter, this phenomenon could explain:

1. the "too old" ^{14}C dates of samples AA_Collagen_HA Roth_cross-flow_1, AA_Collagen_HA Roth_cross-flow_2 and AA_Collagen_NaOH_HA Roth_cross-flow,

2. the "too low" % removal for the samples AA_Collagen_HA Roth_ cross-flow_1 and AA_Collagen_HA Roth_cross-flow_2 (Table 3.4).

If this assumption is correct, the calculated percent removal (Table 3.4) is only an estimation of the real percent removal.

MW determination of hydrolysed HA Roth should clarify if compounds smaller than 450 Dalton are released and pass the filter. If this is correct, a filter with a cutoff 200 Dalton can be used instead of 450 Dalton.
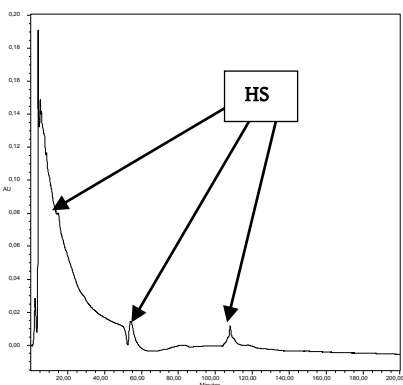
Figure 3.4   : Chromatogram of non-hydrolysed HA Roth
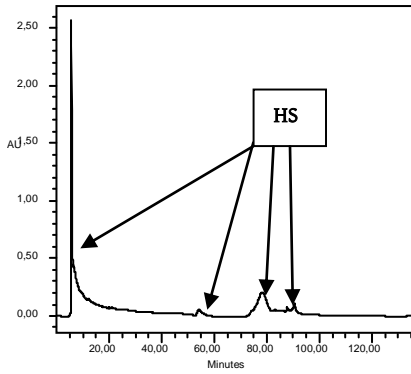
Figure 3.5   : Chromatogram of hydrolysed HA Roth

3.5   CONCLUSION

The nanofiltration methods described in this paper showed some advantages over ultrafiltration. There is no risk of carbon contamination coming from the filter because the filter material is ceramic. It is suitable for all types of proteinaceous material, not only collagen. Unlike ultrafiltration where only low-molecular HSs are eliminated, it should remove low-molecular and high-molecular weight HSs.

Spectofluorescence analyses indicated that cross-flow filtration was a more efficient technique than dead-end filtration to remove HSs.

Cross-flow filtration has the disadvantage that a minimum feed volume must remain in the installation during filtration to prevent the pump from stopping. This means that not all the feed volume can be filtered, which causes sample loss. Consequently, the sample size needed for ^{14}C dating increases.

[14]C AMS dating of cross-flow filtrated hydrolysed collagen-HS mixtures, i.e. amino acids-HS mixtures, demonstrated a significant but yet incomplete removal of HSs (between 63 and 85%). Hot acid hydrolysis of HS may form new chemical compounds, as demonstrated by HPLC analysis. It is possible that these compounds are smaller than the filter cutoff of 450 Dalton and pass through the filter. Further research should clarify this issue.

Further cross-flow filtrations using a filter with a 200 Dalton cutoff will be carried out to improve HSs removal and verified by ^{14}C analyses.

CHAPTER 4   Improved radiocarbon dating for contaminated archaeological bone collagen, silk, wool and hair samples via cross-flow nanofiltrated amino acids

ABSTRACT

Radiocarbon dating and stable isotope analyses of bone collagen, wool, hair and silk contaminated with extraneous carbon(e.g. humic substances (HS)) does not yield reliable results if these materials are pre-treated using conventional methods.

A cross-flow nanofiltration method was developed that can be applied to various protein materials like collagen, hair, silk, wool and leather, and should be able to remove low-molecular and high-molecular weight contaminants. To avoid extraneous carbon contamination via the filter a ceramic filter (molecular weight cut-off of 200 Dalton) was used. As such, amino acids, released by hot acid hydrolysis of the protein material, were collected in the permeate and contaminants in the retentate (> 200 Dalton).

^{14}C dating results for various contaminated archaeological samples were compared for bulk material (pre-treated with the conventional methods) and for cross-flow nanofiltrated amino acids (permeate) originating from the same samples. Contamination and quality control of ^{14}C dates of bulk and permeate samples were obtained by measuring C:N ratios, fluorescence spectra, $\delta^{13}C$ and $\delta^{15}N$ values of the samples. Cross-flow nanofiltration decreases the C:N ratio which means that contaminants have been removed.

Cross-flow nanofiltration clearly improved sample quality and ^{14}C results. It is a quick and non-labor intensive technique and can easily be implemented in any ^{14}C and stable isotope laboratory for routine sample pre-treatment analyses.

4.1   INTRODUCTION

Protein containing materials from archaeological and geological sites are susceptible to chemical and environmental processes which can result in degradation (diagenetic alteration/breakdown) and introduction of exogenous carbon-containing compounds (contamination). In particular, humic substances (HSs) from the soil may interact with the material of interest and cause major problems for any isotopic application such as

[14]C dating (Stafford et al. 1988; Van Klinken and Hedges 1995; Van Klinken 1999; Van Strydonck et al. 2005; Boudin et al. 2011).

Bones are one of the most preferable materials for paleodietary and archaeological chronology studies since they are often directly related to the prehistoric event. The collagen fraction of bones is usually used for stable isotope and ^{14}C analyses. Ultrafiltration of bone collagen, dissolved as gelatin (Molecular weight (MW) ~100,000 D), has received considerable attention as a means to obtain more reliable ^{14}C dates and stable isotope signatures (Brown et al. 1988; Bronk Ramsey et al. 2004; Higham et al. 2006; Mellars 2006). This is an effective method for removal of low-molecular weight contaminants from bone collagen but it does not remove high-molecular weight contaminants, such as cross-linked humic-collagen complexes (Brock et al. 2007). Moreover, comparative dating studies have raised the question whether this cleaning step itself may introduce contamination with carbon from the filters used (Bronk Ramsey et al. 2004; Brock et al. 2007; Hüls et al. 2007; Hüls et al. 2009).

Wool, hair and silk are gaining more attention as suitable radiocarbon dating material due to their short lifespan (Taylor et al. 1995; Geyh 2001; Van Strydonck et al. 2004).

Wool, silk and hair are pre-treated with solvents, followed by an acid-base-acid treatment for stable isotopes and ^{14}C analyses. However, this pre-treatment may not be adequate for removing all contaminants (Kim et al. 2008; Boudin et al. 2011).

Separation, isolation and ^{14}C dating of individual amino acids hydrolysed from bone collagen by preparative high performance liquid chromatography (HPLC) is a good strategy for dealing with contamination in proteins but it is a time consuming and labour intensive technique. (Gillespie and Hedges 1983; Gillespie et al. 1984; Stafford et al. 1987;Stafford et al.1988; Van Klinken and Mook 1990; Stafford et al. 1991; Van Klinken 1991; Tripp et al. 2006; McCullagh et al. 2010)

Most previous studies focused on the amino acid hydroxyproline because it constitutes about 10% of bone collagen and it is not present in significant quantities elsewhere in nature (Ward and Courts 1997). However, some of these methods introduced extraneous carbon into the ^{14}C dated fractions since column bleeding is a common phenomenon in chromatography. Therefore, sample blanks should be measured. Nevertheless, McCullagh et al. and Marom et al. showed the potential of individual amino acid dating (McCullagh et al. 2010; Marom et al. 2012).

Another option is nanofiltration of hydrolysed proteins, i.e. amino acids, which is more straightforward and less labour-intensive than individual amino acid isolation by HPLC. Another advantage is the possibility of using a ceramic filter to avoid exogenous carbon contamination via the filter.

In a previous study a filter membrane with a molecular weight cut-off (MWCO) of 450 Dalton was used to collect the amino acids in the permeate and the HSs in the retentate (Boudin et al. 2013). That study also showed that cross-flow nanofiltration is more efficient in retaining HSs than dead-end nanofiltration. However, hot acid hydrolysis of

contaminant HSs also causes formation of low molecular weight contaminant compounds which passed the 450 Dalton ceramic filter (Boudin et al. 2013a).

In this study, cross-flow nanofiltration with a ceramic filter membrane with a MWCO of 200 Dalton was used, which should improve the retention of contaminants. The efficiency of the cross-flow nanofiltration was verified by comparing:

1. C:N ratio of the bulk sample (before nanofiltration) with C:N ratio of the cross-flow nanofiltrated amino acids (referred to as permeate in this paper).
2. ^{14}C date of permeate with the presumed historical date.

4.2   MATERIALS AND METHODS

4.2.1   Sample selection

Protein-containing samples from different material types (wool, hair, silk and bone collagen), selected to represent various ages and preservation conditions, were obtained from sites listed in Table 4.1. Different material types and preservation conditions were selected to test the robustness of the cross-flow nanofiltration method to improve ^{14}C dating.

Table 4.1   : List of archaeological samples, chosen to represent various potential ages and preservation conditions

Sample name		Archaeological site	Country	Presumed historical date
	COLLAGEN			
	Bone type			
MOO304	Human	Moorsel	Belgium	AD 900-1150
A243 nr.819	Animal	Antwerp	Belgium	AD 700-1000
Gent V1B-S12-S4	Human	Ghent	Belgium	AD 900-1000
Leffinge D50	Animal	Leffinge	Belgium	AD 900-1050
Andorra UE1311	Human	Canillo	Andorra	AD 700-1100
Italy 1	Human	Falacrinae	Italy	300-100 BC
Entrée 4	Animal	Tiène des Maulins	Belgium	Circa 40000 BC
Basel 11-2/20/22	Animal	Basel	Belgium	4000-3700 BC

Continued Table 4.1

Sample name		Archaeological site	Country	Presumed historical date
Grijpskerke	Animal	Grijpskerke	The Netherlands	250-12 BC
Beerse 199	Human	Beerse	Belgium	AD 700-1000
Baldwin femur	Human	Tournai	Belgium	AD 1068
Baldwin skull	Human	Tournai	Belgium	AD 1068
Beerse 58	Human	Beerse	Belgium	AD 700-1300
Radbot skull	Human	Tournai	Belgium	AD 1098
KK 316 skull	Human	Kruishoutem	Belgium	AD 900-1400
KK 316 sponge	Human	Kruishoutem	Belgium	AD900-1400
SILK				
Fuji Silk		unknown	unknown	Modern
Crepeline		unknown	unknown	Modern
DM 1923-01		unknown	Egypt	AD 600-900
Baldwin 43		Tournai	Belgium	AD 1068
Baldwin 46		Tournai	Belgium	AD 1068
KERATIN (HAIR AND WOOL)				
	Material type			
North Ronaldsay sheep	Hair	North Ronaldsay	Orkney Islands	Modern
BM53912	Wool	unknown	Egypt	AD 100-400
Beerlegem	Wool	Beerlegem	Belgium	AD 587
OS2562	Wool	Oudenburg	Belgium	AD 319-380
E29479	Wool	unknown	Egypt	AD 400-700
Emelgem sheep	Hair	Emelgem	Belgium	Modern
Mainz 2	Wool	Mainz	Germany	5 BC (Roman)
Mainz 5	Wool	Mainz	Germany	5 BC (Roman)
Radbot hair	Hair	Tournai	Belgium	AD 1098
Ieper	Wool	Ieper	Belgium	AD 1200-1300

Lacking references from the literature, modern undyed, mordanted, non-mordanted and naturally dyed silk (Bombyx mori) samples were obtained from the Textile laboratory at the Royal Institute for Cultural Heritage (Brussels, Belgium) and used to establish a C:N range for silk samples as a quality.

4.2.2   Sample preparation

4.2.2.1   For bulk analyses

Collagen was extracted from the bones following the Longin method (Longin 1971). A NaOH-wash was introduced between the demineralization and hydrolisation step.

Wool, silk and hair samples were pre-treated with hexane, acetone, ethanol, Milli-Q water (Merck Millipore, Belgium), 1%NaOH and 1% HCl as described in detail in Boudin et al. for bulk ^{14}C and stable isotope analysis (Boudin et al. 2011). The samples 'OS2562', 'Baldwin 43' and 'Baldwin 46', 'Ieper' and 'Beerlegem' could only be pre-treated with solvents as they dissolved completely during the NaOH step.

4.2.2.2   Cross-flow nanofiltrated amino acid analyses

Pre-treated bulk samples underwent the following steps:

1. hydrolysis was carried out on ca 15 - 50 mg material using 2 ml 6M HCl in a sealed tube under nitrogen atmosphere at 110°C for 24 hours;
2. the hydrolysate was filtrated over a 0.7 μm glass fiber filter (Millipore APFF03700);
3. the filtrate was diluted till 100 ml with Milli-Q water;
4. cross-flow nanofiltration;
5. freeze drying of the permeate;
6. freeze dried permeate was dissolved in 1 ml Milli-Q water;
7. 50 μl solution was transferred in a tin-cup and dried at 50°C for stable isotope and C:N analysis;
8. the remaining solution was transferred into a quartz-tube with CuO and Ag and dried in a desiccator for combustion, then for graphite production and subsequent ^{14}C analysis.
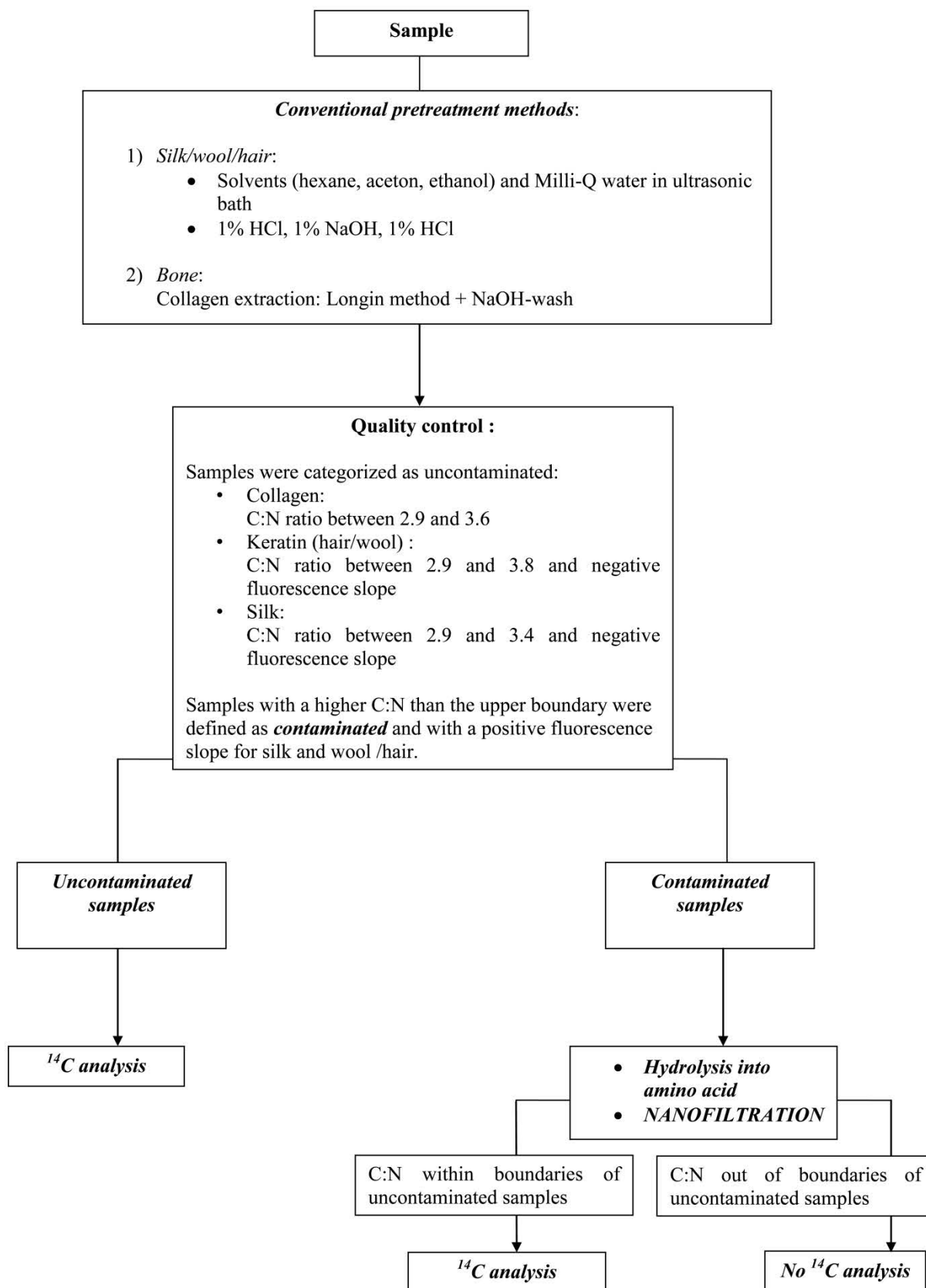
Figure 4.1   : Workflow for archaeological wool, silk, hair and bone collagen for ^{14}C dating.

4.2.3   Sample categorisation: uncontaminated or contaminated

Spectrofluorescence analyses and C:N ratio determinations were carried out on pre-treated bulk silk, wool and hair samples, listed in Table 3.1, to classify the samples as uncontaminated or contaminated.

The C:N ratio of the bone collagen samples was measured to categorize the collagen sample as uncontaminated or contaminated (Table 4.1).

The applied criteria to define uncontaminated archaeological samples were (Figure 4.1):

1. collagen: C:N ratio between 2.9 and 3.6 (De Niro 1985; Ambrose 1990);

2. wool and hair: C:N between 2.9 and 3.8 and a negative fluorescence slope (O'Connell and Hedges 1999a, 1999b, 2001; Boudin et al. 2011);

3. silk: C:N between 2.9 and 3.4 and a negative fluorescence slope (Boudin et al. 2011). This C:N range was based on our own analyses.

Samples not fulfilling these conditions (i.e. higher C:N, positive fluorescence slope) were defined as contaminated. A higher C:N is the result of introduction of exogenous carbon-containing compounds (i.e. contamination).

4.2.4   Design

This study was designed to be undertaken in 3 phases:

1. stable isotope (δ^{15}N and δ^{13}C values) analyses were carried out on a series of uncontaminated archaeological samples in order to determine if contamination and/or isotope fractionation occurred during nanofiltration. These phenomena, contamination and fractionation, can influence and alter the stable isotope values.

2. a series of uncontaminated archaeological samples were analyzed for ^{14}C to verify if contamination occurred during cross-flow nanofiltration.

3. stable isotope (δ^{15}N and δ^{13}C values), C:N ratio determinations and ^{14}C analyses of contaminated archaeological bulk samples and their permeate were performed. Comparing the bulk C:N with the C:N of the permeate provides information concerning the efficiency and specificity of the cross-flow nanofiltration and the obtained sample quality after nanofiltration. A C:N decrease indicates contaminant removal and if the C:N falls within the boundaries of uncontaminated samples, a more accurate ^{14}C date should be obtained.

4.2.5   Methods

4.2.5.1   Fluorescence spectroscopy

Non-destructive fluorescence spectroscopy (Cary Eclipse, Varian, Belgium) analyses of the textile samples were carried out using a fiber-optic probe as described in 2.3.3. Therefore, the slope was calculated using a linear fit (least squares) to the curve between 465 and 475 nm. The fluorescence slope can be used as a qualitative indicator for the presence of HS:

1. a negative slope indicates absence of HSs and consequently an uncontaminated sample; and

   a positive slope indicates the presence of HS and thus sample contamination.

The method is described in detail in Boudin et al. (2011).

4.2.5.2   Cross-flow nanofiltration

An in-house developed filtration installation was set up using a stainless steel centrifugal pump (NPY-2251-MK, Speck Pumps, Belgium), Swagelock accessories (tubings, fittings, flexibles, Belgian Fluid System Technologies, Belgium) and a ceramic filter with a 200 Da MWCO (Inopor, Germany)). A pressure of 10 Bar (nitrogen) was applied in cross-flow mode. The centrifugal pump is required in cross-flow mode as a means to obtain a tangential feed flow.

Used cross-flow velocity was 6.5 m/s.

A filter with a MWCO of 200 Da was used in order to selectively collect amino acids in the permeate (MW of amino acids varies between 75.07 and 204.23 Da) and the HSs (MW of HSs varies between ca. 1000 and 300,000 Da) in the retentate (Stevenson 1982).

The feed volume was 100 ml. The collected permeate volume amounted to 80-90 ml because a minimum feed volume must remain in the filtration installation to avoid the pump stopping in cross-flow mode. This volume was minimum 10 ml.

Cleaning of the filtration installation was carried out after every filtration sequence by consecutively rinsing with Milli-Q water (200 ml), 1%NaOH (300 ml) and finally three times with Milli-Q water (300, 200 and 200 ml). The pump was then disconnected from the system because some liquid always remains in the pump. The pump was emptied and rinsed several times with Milli-Q water, emptied again and finally connected again to the filtration setup. Finally, the filtration system was flushed (dried) with nitrogen gas.

4.2.5.3   Stable isotopes (δ^{13}C and δ^{15}N) and C:N ratio

Stable Isotope ratios (δ^{13}C and δ^{15}N) values, C:N ratios were determined on the pre-treated bulk samples and the permeate.

Carbon and nitrogen stable isotope compositions were measured as the ratios of the heavier isotope to the lighter isotopes (^{13}C/^{12}C or ^{15}N/^{14}N) and are reported in delta (δ) notation as parts per thousand (‰), where δ^{13}C or δ^{15}N = ([Rsample/Rstandard] - 1) × 1000, and R is ^{13}C/^{12}C or ^{15}N/^{14}N, relative to internationally defined standards for carbon (Vienna Pee Dee Belemnite, VPDB) and nitrogen (Ambient Inhalable Reservoir, AIR).

Analyses were performed in duplicate on a Thermo Flash EA/HT elemental analyser, coupled to a Thermo DeltaV Advantage Isotope Ratio Mass Spectrometer via ConfloIV interface (all supplied by ThermoFisher Scientific, Bremen, Germany). Standards used were IAEA-N1, IAEA-C6, and internally calibrated acetanilide.

The analytical precision was smaller than 0.25‰ for both δ^{13}C and δ^{15}N values, based on multiple measurements.

4.2.5.4 ^{14}C dating

The necessary sample size for a ^{14}C analysis of cross-flow nanofiltrated amino acids must be ca. 20% higher than for a bulk ^{14}C analysis. The losses occur during hydrolysis (ca. 10%) and another 10% due to the minimum feed volume necessary in the filtration setup to avoid the pump stopping.

Dried samples were transferred into quartz tubes with CuO and Ag and combusted to CO_2. Graphitization of CO_2 was carried out using H_2 over a Fe catalyst. Targets were prepared at the Royal Institute for Cultural Heritage in Brussels (Belgium) and ^{14}C concentrations were measured with Accelerated Mass Spectrometry (AMS) at the Leibniz Labor für Altersbestimmung und Isotopenforschung in Kiel (Germany) (Van Strydonck and van der Borg 1990-1991; Nadeau et al.1998). The ^{14}C results are expressed in pMC (percentage modern carbon) and indicate the percent of modern (1950) carbon corrected for fractionation using the δ^{13}C measurement. Calibrations of ^{14}C dates and χ^2-test (weighted mean) calculations were performed using OxCal 3 and the IntCal09 calibration curve data (Bronk Ramsey 1995; 2001; Reimer et al. 2009).

A χ^2-test was done to obtain a weighted mean (average ^{14}C date) (Shennan 1988). An error message was generated if the confidence limits dropped below 5%. The value given for T is the χ^2-value calculated and the value given in brackets is the level above which T it should not rise (the degrees of freedom are given by df).

4.3 RESULTS AND DISCUSSION

4.3.1 C:N ratio of silk

Stable isotope values and C:N ratios of modern undyed, mordanted, non-mordanted and naturally dyed silk (all Bombyx mori) are listed in Table 4.2.

The lowest C:N ratio was 2.9 and the highest 3.4. In this study archaeological silk samples, which fell in this range, were defined as uncontaminated. Silk samples with C:N ratios higher than 3.4 were classified as contaminated.

The mordanting and dyeing process did not change the isotopic signature or the C:N ratio (Table 4.2).

Table 4.2   : Stable isotope (δ^{13}C and δ^{15}N values) and atomic C:N ratio analyses of modern undyed, mordanted, non-mordanted and naturally dyed silk (all Bombyx mori).

Sample name	δ^{13}C (‰)	δ^{15}N (‰)	atomic C:N
Fuji silk fabric	–26.0	3.9	3.2
Fuji silk 19 fabric	–26.1	4.3	3.4
Silk michelle fabric	–25.3	5.0	3.2
Silk crepeline fabric	–25.9	2.4	3.1
Silk yarn A untreated	–23.8	6.0	3.1
Silk yarn B untreated	–24.8	4.3	3.1
Silk yarn B indigo	–24.1	4.4	3.0
Silk yarn B redwood-alum mordant	–24.2	4.4	3.0
Silk yarn C cochinea-alum mordant	–24.0	5.4	3.0
Silk yarn C indigo on silk -unmordanted	–24.1	5.1	3.0
Silk yarn C mordanted (alum+ cream of tartar)	–24.1	5.4	3.0
Silk yarn C dyer's greenweed-alum mordanted (with potash)	–24.7	6.0	3.1
Fabric untreated silk	–26.2	2.3	2.9
Fabric alum mordant silk-dyer's greenweed	–26.2	2.3	3.1
Average			*3.1*
Standard deviation			*0.1*

4.3.2 Phase 1 analyses: Stable isotope analysis of uncontaminated samples

The bulk and permeate stable isotope values of uncontaminated samples were in good agreement (Table 4.3). These results indicate that no isotope fractionation and/or contamination occurred during filtration.

Table 4.3 : Stable isotope analysis (δ^{13}C and δ^{15}N values) and atomic C:N ratio of uncontaminated wool, hair, silk and bone collagen samples before (bulk) and after (permeate) cross-flow nanofiltration. Fluorescence slope values for bulk silk, hair and wool samples.

Sample name	δ^{13}C (‰)	δ^{15}N (‰)	atomic C:N	Fluorescence Slope
		COLLAGEN		
MOO304				
bulk	–20.3	10.2	3.2	
permeate	–20.5	10.2	3.2	
A243 nr 819				
bulk	–22.2	6.5	3.3	
permeate	–22.3	6.4	3.1	
Gent V1B-S12-S4				
bulk	–20.1	9.6	3.1	
permeate	–20.0	9.8	3.1	
Leffinge D50				
bulk	–21.5	6.4	3.1	
permeate	–21.4	6.4	3.1	
Andorra UE1311				
bulk	–18.8	9.2	3.1	
permeate	–18.6	9.2	3.1	
Italy 1				
bulk	–19.1	9.6	3.2	
permeate	–18.7	9.5	3.1	
Entrée 4				
bulk	–20.5	4.8	3.2	
permeate	–20.4	4.7	3.3	
Basel 11-2/20/22				
bulk	–19.8	6.6	3.2	
permeate	–19.7	6.6	3.2	

Continued Table 4.3

Sample name	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	atomic C:N	Fluorescence Slope
Grijpskerke (oviscapra)				
bulk	-21.6	11.6	3.3	
permeate	-22.3	11.3	3.2	
		SILK		
Fuji Silk				
bulk	-26.0	3.9	3.1	-0.67
permeate	-25.9	3.9	3.1	
Crepeline				
bulk	-25.9	2.4	3.1	-0.11
permeate	-25.7	2.4	3.1	
		KERATIN (HAIR AND WOOL)		
Emelgem sheep				
bulk	-26.5	8.1	3.7	-0.22
permeate	-26.5	7.7	3.4	
North Ronaldsay sheep				
bulk	-16.9	9.8	3.5	-0.06
permeate	-16.4	9.8	3.5	
E29479				
bulk	-16.3	9.3	3.7	-3.09
permeate	-16.4	9.5	3.6	

4.3.3   Phase 2 analyses: ^{14}C analysis of uncontaminated samples

There is a good agreement between the ^{14}C date of the bulk and of the permeate of the uncontaminated samples. Their weighted mean (average ^{14}C date) are calculated applying the χ^2-test. The weighted mean of every uncontaminated sample was in agreement with the presumed historical date. (Tables 4.1 and 4.4). This indicates that no ^{14}C contamination occurred during filtration.

Table 4.4 : Laboratory code, radiocarbon ages (BP), calibrated ages (2 σ), χ^2-test and atomic C:N ratio of uncontaminated wool, hair, silk and bone collagen samples before (bulk) and after (permeate) cross-flow nanofiltration. Fluorescence slope values for bulk silk, hair and wool samples. (n.a.: not analysed)

Sample name	Lab-code	^{14}C age (BP)	Calibrated age (2 σ)	χ^2-test (Pass/Fail)	atomic C:N	Fluorescence Slope
			COLLAGEN			
MOO304						
bulk	KIA-44329	945±25	AD1020 (95.4%) 1160		3.2	
permeate	KIA-45494	950±25	AD1020 (95.4%) 1160		3.2	
Average (weighted)		948±18	AD1020 (95.4%) 1160	Pass		
Grijpskerke (oviscapra)						
bulk	KIA-39616	2200±25	370 (95.4%) 190BC		3.3	
permeate	KIA-46102	2195±25	370 (95.4%) 180BC		3.2	
Average (weighted)		2198±18	360BC (95.4%) 190BC	Pass		
A243 nr 819						
bulk	KIA-42342	1265±30	AD660 (93.1%) 830		3.3	
			AD840 (2.3%) 860			
permeate	KIA-46382	1345±30	AD640 (85.2%) 720		3.1	
			AD740 (10.2%) 770			
Average (weighted)		1305±21	AD660 (95.4%) 780	Pass		
Beerse nr.199						
bulk	KIA-41892	1125±25	AD860 (95.4%) 990		3.4	
permeate	KIA-46806	1060±25	AD890 (13.1%) 920		n.a.	
			AD940 (82.3%) 1030			
Average (weighted)		1093±18	AD890 (95.4%) 995	Pass		
			SILK			
DM1923-01						
bulk	KIA-42113	1220±35	AD680 (95.4%) 890		3.1	-0.66
permeate	KIA-46101	1180±25	AD770 (90.8%) 900		n.a.	
			AD920 (4.6%) 950			
Average (weighted)		1194±25	AD720 (1.7%) 740	Pass		
			AD770 (93.7%) 900			

Continued Table 4.4

Sample name	Lab-code	^{14}C age (BP)	Calibrated age (2 σ)	χ^2-test (Pass/Fail)	atomic C:N	Fluorescence Slope
			KERATIN (HAIR AND WOOL)			
E29479						
bulk	KIA-42363	1555±30	AD420 (95.4%) 580		3.6	-3.09
permeate	KIA-46192	1475±30	AD540 (95.4%) 645		3.6	
Average (weighted)		1515±30	AD430 (95.4%) 620	Pass		
BM53912						
bulk	KIA-45788	1815±25	AD120 (94.2%) 260		n.a.	n.a.
			AD300 (1.2%) 320			
permeate	KIA-46194	1810±20	AD130 (95.4%) 260		n.a.	
Average (weighted)		1812±19	AD130 (95.4%) 250	Pass		

4.3.4 Phase 3 analyses: Stable isotope analysis and ^{14}C analysis of contaminated samples

^{14}C analyses were carried out on a series of contaminated samples (Tables 4.5, 4.6 and 4.7). The permeate C:N ratio was always lower than the bulk C:N ratio, which indicates contaminant removal and thus improved sample quality for ^{14}C dating after cross-flow nanofiltration. However, not all permeate C:N ratios were within the C:N boundaries of uncontaminated samples and thus unreliable ^{14}C data are expected.

4.3.4.1 Contaminated wool and hair

Table 4.5 lists the results of contaminated wool and hair samples. The bulk C:N ratios of the wool and hair samples indicate contamination, except for 'Radbot hair'. The positive fluorescence slope of all samples confirms this and signifies that the samples are contaminated with HSs. The HS contamination is also proven by ^{13}C enrichment of the permeate relative to the bulk, except for 'Beerlegem', because peat HSs have δ^{13}C values around -27 ‰ which is more negative than wool and hair δ^{13}C values. However, ^{15}N enrichment occurred for 'Beerlegem' after cross-flow nanofiltration. This could also be due to HS contamination, as confirmed by the positive fluorescence slope. Indeed the

$\delta^{15}N$ values of peat HS are around 0‰ and thus more negative than wool and hair $\delta^{15}N$ value (Kract and Gleixner 2000; Francioso et al. 2005). Therefore, the ^{14}C dates of all the bulk samples, except for 'Radbot hair', are considered as unreliable. This is also supported by the presumed historical dates, which are not in agreement with the calibrated ^{14}C dates (Table 4.5).

Table 4.5 : Laboratory code, radiocarbon ages (BP), calibrated ages (2), presumed historical date, stable isotope values (^{13}C and ^{15}N) and atomic C:N ratio of contaminated wool and hair samples before (bulk) and after (permeate) cross-flow nanofiltration. Fluorescence slope values for bulk hair and wool samples. (n.a.: not analysed)

Sample name	Lab-code	^{14}C age (BP)	Calibrated age (2 σ)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	atomic C:N	Fluorescence Slope	Presumed historical date
Beerlegem								
bulk	KIA-42365	1705±30	AD250 (95.4%) 410	-23.2	4.5	3.9	9.89	AD587
permeate	KIA-46083	1510±25	AD430 (10.3%) 490	-23.3	5.6	3.4		
			AD530 (85.1%) 620					
OS2562								
bulk	n.a.	n.a.		n.a.	n.a.	n.a.	2.15	AD319-380
permeate	KIA-46100	1750±25	AD230 (95.4%) 390	n.a.	n.a.	n.a.		
Mainz 2								
bulk	KIA-41535	2075±25	180 (95.4%) 30BC	-23.4	5.3	4.0	2.97	5BC
permeate	KIA-46193	2005±20	50BC (95.4%) 55AD	-21.9	5.1	3.5		
Mainz 5								
bulk	KIA-37616	2080±30	200 (94.3%) 20BC	-24.5	8.8	4.0	1.98	5BC
			10BC (1.1%) AD					
permeate	KIA-47417	1915±35	AD (92.8%) 180	-22.7	8.5	3.5		
			AD 190 (2.6%) 220					
Radbot hair								
bulk	KIA-46953	1025±25	AD 970 (95.4%) 1040	-21.6	12.8	3.8	0.25	AD1098
permeate	KIA-46955	1040±20	AD 970 (95.4%) 1025	-20.3	12.8	3.4		
Average (weighted)		1034±16	AD 985 (95.4%) 1025					
Ieper								AD1200-1300
bulk	KIA-43347	750±25	AD 1220 (95.4%) 1285	-26.1	12.2	43.3	3.00	
permeate	n.a.	n.a.		-24.2	n.a.	4.1		

The ^{14}C dates of the permeate of 'Beerlegem', 'OS2562', 'Mainz 2' and 'Mainz 4' are considered as reliable because the permeate C:N ratio falls within the specified C:N boundaries for uncontaminated wool and hair. The calibrated ^{14}C dates also correspond with the presumed historical dates (Table 4.5) (Roosens 1977; Bohme-Schonberger and Mitschke 2005, Vanhoutte et al. 2009).

The permeate C:N ratio of 'Ieper' was still out of the C:N range of uncontaminated samples (Table 4.5) and should result in an unreliable ^{14}C date. However, the permeate of 'Ieper' could not be ^{14}C dated because the carbon yield after combustion was too low to graphitize.

The bulk C:N ratio of 'Radbot hair' (=3.8) indicates an uncontaminated sample, while the positive fluorescence slope indicates HS contamination. Moreover, HS contamination is also proven by the ^{13}C enrichment of permeate relative to the bulk sample. The ^{14}C dates of the bulk and the permeate are in accord and an average can be calculated (Table 4.5). The possible HS contamination can be explained by *in situ* humification where hair organic matter (predominantly keratin) generates HSs through the Maillard reaction (Maillard 1913; Van Klinken and Hedges 1995). The average ^{14}C date is too old compared to the historical date (1098AD) but stable isotope analysis suggests that freshwater fish was an important part of this individual's diet (Brulet et al. 2012). A reservoir effect for freshwater organisms could thus explain the anomaly in the ^{14}C date and will be discussed further in this paper using the results of bone collagen, extracted from the skull of the 'Radbot' sample (Table 4.5).

4.3.4.2 Contaminated silk

Two contaminated silk samples were analyzed (Table 4.6). The C:N ratio and positive fluorescence slope indicate contaminated bulk samples and the fluorescence slope suggests HS contamination. The HS contamination is also confirmed by ^{13}C enrichment of permeate compared to the bulk for both samples.

The bulk ^{14}C dates of both samples are therefore considered unreliable.

The permeate ^{14}C dates of both silk samples are considered as reliable because the C:N ratio of the permeate falls within the specified C:N boundaries for uncontaminated silk. However, the bulk and permeate ^{14}C dates of 'Baldwin 43' are statistically not different, applying the χ^2-test (χ^2-Test: df=1 T=0.5(5% 3.8)). The possible HS contamination can be explained by *in situ* humification.

The permeate ^{14}C dates of both silk samples are too old compared to the presumed historical date (AD 1068) (Brulet et al. 2012). However, previous research has shown that

in several cases (parts of) textiles were re-used and this may explain the older ^{14}C dates of the permeate compared with the presumed historical date (Bénazeth et al. 2006).

Table 4.6 : Laboratory code, radiocarbon ages (BP), calibrated ages (2 σ), presumed historical date, stable isotope values (δ^{13}C and δ^{15}N) and atomic C:N ratio of contaminated silk samples before (bulk) and after (permeate) cross-flow nanofiltration. Fluorescence slope values for bulk silk samples. (n.a.: not analysed)

Sample name	Lab-code	^{14}C age (BP)	Calibrated age (2 σ)	δ^{13}C (‰)	δ^{15}N (‰)	atomic C:N	Fluorescence Slope	Presumed historical date
Baldwin 46								
bulk	KIA-47415	1260±30	AD 660 (91.9%) 830	-24.9	6.3	3.6	2.62	AD1068
			AD 840 (3.5%) 870					
permeate	KIA-47418	1175±30	AD 770 (83.1%) 900	-24.1	6.1	2.9		
			AD 910 (12.3%) 970					
Baldwin 43								
bulk	KIA-47414	1210±30	AD 690 (12.2%) 750	-24.5	6.8	3.7	2.07	AD1068A
			AD 760 (83.2%) 900					
permeate	KIA-47811	1180±30	AD 770 (86.0%) 900	-23.5	6.2	2.9		
			AD910 (9.4%) 970					

4.3.4.3   Contaminated bone collagen

Table 4.7 depicts the results of contaminated bone collagen. The bulk C:N ratio of the bone collagen samples indicates contamination, except for 'Radbot skull fragment B' and 'KK316 skull'. These two samples yielded uncontaminated collagen and were used as a reference sample to evaluate the isotopic data of the permeates of 'Radbot skull fragment A' and 'KK316 sponge extraction B'.

The bulk ^{14}C results of the contaminated bone collagen samples are considered as unreliable due to an elevated C:N ratio, not falling in the specified C:N range for uncontaminated bone collagen (Table 4.7).

Table 4.7   : Laboratory code, radiocarbon ages (BP), calibrated ages (2 σ), presumed historical date, stable isotope values (δ^{13}C and δ^{15}N) and atomic C:N ratio of contaminated bone collagen samples before (bulk) and after (permeate) cross-flow nanofiltration. Bone samples yielding uncontaminated collagen and used as a reference sample to evaluate the isotopic data of bulk and permeate of contaminated collagen, extracted from the same skeleton, are depicted in bold italic. (n.a.: not analysed)

Sample name	Lab-code	^{14}C age (BP)	Calibrated age (2 σ)	δ^{13}C (‰)	δ^{15}N (‰)	atomic C:N	Presumed historical date
Radbot skull fragment A							
bulk	KIA-46954	1165±25	AD 770 (77.4%) 900	-20.4	13.7	3.7	AD1098
			AD 910 (18.0%) 970				
permeate	KIA-46957	1025±25	AD 970 (95.4%) 1040	-19.9	14.2	3.4	
Radbot skull fragment B							
bulk	*KIA-47968*	*995±25*	*AD 980 (70.4%) 1050*	*-20.4*	*14.5*	*3.6*	AD1098
			AD1080 (25.0%) 1160				
KK 316 sponge extraction A							
bulk	KIA-47810	525±35	AD 1310 (20.1%) 1360	-22.3	11.4	4.4	AD900-1400
			AD 1380 (75.3%) 1450				
permeate	KIA-47815	490±25	AD1410 (95.4%) 1445	-21.6	11.3	3.7	
KK 316 sponge extraction B							
bulk	n.a.	n.a.	n.a.	-21.2	11.7	3.7	AD900-1400
permeate	KIA-48201	610±30	AD 1290 (95.4%) 1410	-20.8	11.6	3.3	
KK 316 skull							
bulk	*KIA-47809*	*655±35*	*AD 1270 (45.7%) 1330*	*-20.8*	*11.5*	*3.2*	
			AD 1340 (49.7%) 1400				
Baldwin femur							
bulk	KIA-47421	1510±30	AD 430 (14.9%) 490	-19.7	15.0	3.9	AD1068
			AD 500 (80.5%) 630				
permeate	KIA-47423	1080±30	AD 890 (95.4%) 1020	-20.4	15.0	3.4	
Baldwin skull							
bulk	KIA-47420	1185±30	AD 720 (1.9%) 740	-19.5	16.6	4.8	AD1068
			AD 770 (87.7%) 900				
			AD 910 (5.8%) 950				
permeate	KIA-47422	930±30	AD 1020 (95.4%) 1170	-19.2	16.5	3.9	
Beerse 58							
bulk	KIA-41894	1065±25	AD 890 (16.1%) 920	-21.5	11.1	4	AD700-1300
			AD 940 (79.3%) 1020				
permeate	KIA-47035	800±20	AD1210 (95.4%) 1270	n.a.	n.a.	3.2	

Radbot

Cross-flow nanofiltration of the hydrolysate of 'Radbot skull fragment A' resulted in a C:N decrease (Table 4.7). A more accurate ^{14}C date should be obtained with this C:N ratio. Good agreement between the ^{14}C date of the permeate of the contaminated collagen of 'Radbot skull fragment A' and the bulk ^{14}C date of the uncontaminated collagen of reference sample 'Radbot skull fragment B' was observed (a weighted mean can be calculated: 1010±18BP, χ2-Test: df=1 T=0.7(5% 3.8)). The calibrated ^{14}C date is too old, however, compared with the presumed historical date (AD 1098) (Brulet et al. 2012). Stable isotope (δ^{13}C and δ^{15}N) ratios indicate that freshwater fish was part of the diet, which provokes a reservoir effect on the ^{14}C date, explaining the too old ^{14}C date (Table 4.7):

- the elevated δ^{15}N value indicates aquatic (marine or freshwater) food consumption but cannot distinguish marine from freshwater; the negative δ^{13}C value indicates freshwater fish consumption mixed with terrestrial food while marine food have a more positive δ^{13}C value and thus can be excluded here (Lanting and van der Plicht 1996; Lanting and van der Plicht 1998; Cook et al. 2001; Cook et al. 2002;); stable isotope comparison of 'Radbot' with the Belgian medieval human bones indicates a difference in diet between 'Radbot' and the Belgian medieval individuals because freshwater fish was a part of Radbot's diet (Figure 4.2).
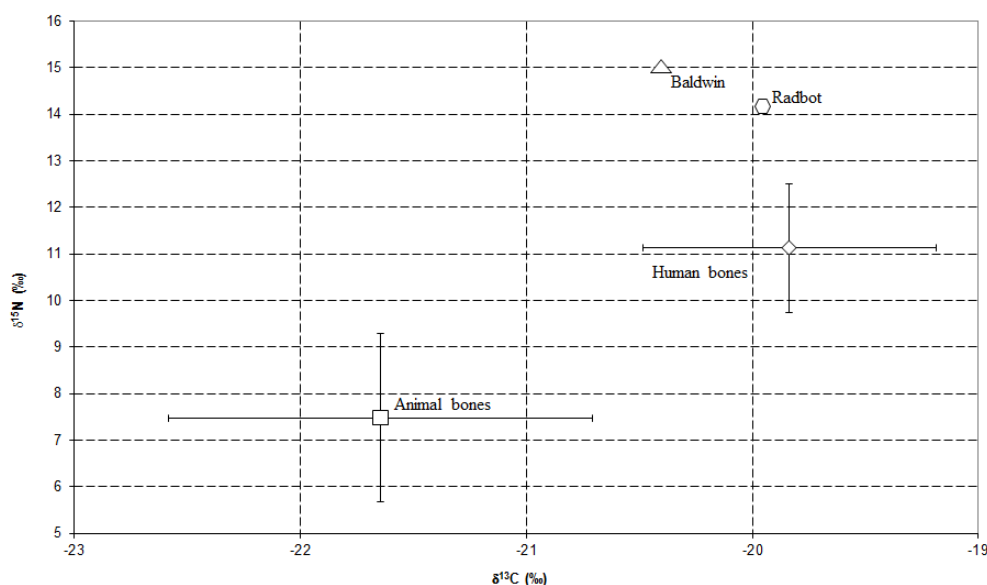
Figure 4.2 : Stable isotope data (δ^{13}C and δ^{15}N) from Radbot and Baldwin compared with (unpublished) measurements from medieval animal (n=115) and human (n=253) bones (n: number of analyzed samples).

KK316

Two collagen extractions were carried out on selected spongeous bone parts of skeleton 'KK316'. The collagen of sample 'KK316 sponge extraction A' is more contaminated, shown by a higher C:N ratio than for the collagen of sample 'KK316 sponge extraction B' (Table 4.7).

Cross-flow nanofiltration of the hydrolysates of both collagen samples resulted in a decrease in C:N ratio.

However, the permeate C:N ratio of sample 'KK316 sponge extraction A' falls outside the C:N boundaries of uncontaminated collagen. The contaminant is probably HSs, indicated by higher δ^{13}C value of the than of the bulk sample. The permeate δ^{13}C is, however, still more negative than the bulk δ^{13}C value of the uncontaminated collagen of reference sample 'KK316 skull', indicating that not all the HSs were removed during nanofiltration. This, therefore, also resulted in an unreliable ^{14}C date which is younger than the reliable bulk ^{14}C date of 'KK316 skull'. Maybe double cross-flow nanofiltration might be the solution to obtain a lower C:N ratio and consequently a more accurate ^{14}C date.

The permeate C:N ratio of sample 'KK316 sponge extraction B' is within the C:N boundaries of uncontaminated collagen. The δ^{13}C value and ^{14}C date of permeate are in good agreement with δ^{13}C and ^{14}C bulk values of reference sample 'KK316 skull'. The calibrated ^{14}C date is also in agreement with the presumed historical date (AD 900-1400) (Vermeulen et al. 1993).

Baldwin

Cross-flow nanofiltration of the hydrolysates of the samples 'Baldwin femur' and 'Baldwin skull" resulted in a C:N decrease comparedwith the bulk C:N.

The permeate C:N ratio of the 'Baldwin skull' is out of the C:N range for uncontaminated bone collagen and consequently an unreliable ^{14}C date is obtained. The permeate C:N ratio of 'Baldwin femur' falls within this C:N range and results in a reliable ^{14}C date. However, the calibrated ^{14}C date is too old relative to the presumed historical date (AD 1068) (Brulet et al. 2012). However, stable isotope values indicate that freshwater fish was part of the diet, which provokes a reservoir effect on the ^{14}C date (Table 4.7 and Figure 4.2).

Beerse 58

The sample 'Beerse 58' showed after nanofiltration a C:N ratio within the range for uncontaminated collagen. The obtained ^{14}C date of the permeate of 'Beerse 58' is in agreement with the presumed historical date (AD 700-1300) (Table 4.7).

4.4   CONCLUSION

Cross-flow nanofiltration of hydrolysed contaminated protein-containing samples e.g. bone collagen, hair, wool and silk, decreases the C:N ratio which indicates contaminant removal and improvement of the sample quality for ^{14}C dating. If the C:N ratio of the permeate falls within the boundaries of uncontaminated samples, more accurate ^{14}C dates are obtained.

In some cases, single cross-flow filtration may not be sufficient to obtain a C:N ratio within the boundaries of uncontaminated samples. Double cross-flow nanofiltration might be the solution and will be further investigated.

Cross-flow nanofiltration is a quick and non-labor intensive technique and can easily be implemented in any ^{14}C laboratory for routine sample pre-treatment analyses.

CHAPTER 5   An archaeological mystery revealed by radiocarbon dating of cross-flow nanofiltrated amino acids derived from bone collagen, silk and hair: The case study of the bishops Baldwin I and Radbot II from Noyon-Tournai.

5.1 ABSTRACT

Excavations in the cathedral of Tournai revealed two sepultures which were assigned by the excavators to bishops because of the special location in the cathedral.

One burial was assigned to Baldwin I, who died in AD 1068, because:

1. a ring with the inscription "BAL" was found;
2. a funeral stone with text was present on top of the grave mentioning the name "Baldewinus".

The second burial probably belongs to Radbot II who was the successor of Baldwin I and died in AD 1098.

Both burials contained textiles (silk), the skeleton, and a wooden pastoral staff. Human hair was still present on the skull of what was presumed to be Radbot II.

All the protein-containing materials were degraded and/or contaminated. Standard sample pre-treatment methods were not able to remove all the contaminants demonstrated by quality assessment.

Single and double cross-flow nanofiltration of the hydrolysed protein-containing materials were performed. The sample quality for ^{14}C dating was improved and ^{14}C data revealed interesting and surprising results:

1. The ^{14}C dates of the wooden pastoral staff and permeate 'Femur' confirm that the skeleton and tomb may belong to bishop Baldwin I.
2. The ^{14}C dates of 'Hair ' and permeate 'Skull' indicate that the skeleton may indeed belong to bishop Radbot II. The younger ^{14}C dates of the wooden pastoral staff and silk samples indicate a post-burial disturbance of the site burial during the 12th -13th century.

5.2   INTRODUCTION

Tournai Cathedral is one of Belgium's heritage sites and was listed in 2000 as a UNESCO World Heritage site. A team of CRAN (*Centre de recherche d'archéologie nationale*) of Université Catholique de Louvain (Belgium) excavated in the cathedral and its surroundings between 1996 and 2010 in order to understand the configuration of the monuments that preceded the present cathedral (Brulet et al. 2012a). The 12th century cathedral was preceded by a fairly large 11th century cathedral, a smaller Carolingian church and by a basilica from the beginning of the Early Middle Ages.

The complete plan of the 11th century cathedral could be reconstructed; it was the first cathedral whose existence was attested in the written sources. In that church, a flight of stairs gave access to the split-level choir. At the foot of the second flight of steps, the intact tombs of two 11th century bishops were excavated: Baldwin I (year of death: AD 1068) and probably Radbot II (year of death: AD 1098) (Brulet et al. 2012 a, b). This was quite surprising because normally the bishops of Noyon-Tournai were buried in Noyon (France) before the autonomy of the diocese of Tournai in AD 1146. Both tombs contained textiles (silk), the skeleton, a wooden pastoral staff and human hair was still present on the skull of Radbot II. The wooden pastoral staff is a symbol of the pastoral care of the bishop for the faithful and was added to the tomb of bishops during the middle ages (den Hartog 2012).

The aim of this research was to verify whether the skeletons could be assigned to Baldwin I and Radbot II. This study presents the results of the analysis of protein-containing samples after standard pre-treatment as well as after hydrolysis into amino acids and cross-flow nanofiltration. Cross-flow nanofiltration of the hydrolysed protein leads to more accurate ^{14}C results, as described in Boudin et al. (2013b).

5.3   MATERIALS AND METHODS

5.3.1   Sample selection

Table 5.1 lists the bone, hair and silk samples for analyses derived from the burials of Baldwin I and Radbot II, chosen to represent different preservation states. The table also refers to our sampling of the wooden pastoral staff, probably a branch or young tree which excludes an old-wood effect.

Table 5.1   : Samples derived from the burials of Baldwin I and Radbot II , chosen to represent different preservation states, in order to determine if the skeletons can belong to Baldwin I and Radbot II.

	Sample name	
Analysed material	Baldwin (AD 1068)	Radbot II (AD 1098)
HAIR (Keratin)		Hair
BONE COLLAGEN	Skull Femur	Skull fragment A Skull fragment B Bone 80 Tibia
SILK	43A 43B 46	20 22 49
WOOD	Wooden pastoral staff species: Prunus	Wooden pastoral staff species: probably Ligustrum vulgare

5.3.2   Sample preparation

5.3.2.1   Bulk analyses

Collagen was extracted from the bones following the Longin method (Longin 1971). A 1 %NaOH-wash was introduced between the demineralization and hydrolisation step for 15 minutes.

The hair sample was pre-treated with hexane, acetone, ethanol, Milli-Q water (Merck Millipore, Belgium), 1%NaOH and 1% HCl as described in detail in Boudin et al. (2011) for bulk ^{14}C and stable isotope analysis.

All silk samples could only be pre-treated with hexane, acetone, ethanol, Milli-Q water (Merck Millipore, Belgium) as they dissolved completely during the NaOH step.

Extracted bone collagen, pre-treated hair and silk samples will be referred as *bulk* in this paper.

The wood samples underwent AAA (acid-alkali-acid) treatment by using 1% HCl and 1%NaOH in 1 hour washes at 90°C.

5.3.2.2   Treatment for cross-flow nanofiltrated amino acid analyses

The in house developed filtration installation and the used protocol are described in 4.2.5.2. and in Boudin et al. (2013b).

A ceramic filter with a cutoff of 200 Dalton was used in order to selectively collect amino acids in the permeate of hydrolysed protein samples and the HSs in the retentate. This filter should theoretically retain 90% of > 200 Dalton sized molecules by a single cross-flow nanofiltration. Performing a second cross-flow nanofiltration of permeate (double cross-flow nanofiltration) should increase the retention of contaminants. Consequently, a better C:N ratio should be obtained and a more accurate ^{14}C date.

For cross-flow nanofiltrated amino acid analyses the pre-treated bulk samples underwent the steps described in 4.2.2.2.

5.3.3   Sample quality assessment

5.3.3.1   Stable isotopes ($\delta^{13}C$ and $\delta^{15}N$), %C, %N and C:N ratio

Stable Isotopes ($\delta^{13}C$ and $\delta^{15}N$), C:N ratio analyses were done on the pre-treated bulk samples and the permeate of the hydrolysed protein sample as described in 4.2.5.3. %C and %N were determined from the pre-treated bulk samples.

5.3.3.2   Fluorescence spectroscopy

Non-destructive fluorescence spectroscopy (Cary Eclipse, Varian, Belgium) analyses of the textile samples were carried out using a fiber optic probe as described in 2.3.3.
The fluorescence slope can be used as a qualitative indicator for HS presence:

1. Negative slopes indicate HS absence and consequently an uncontaminated sample;
2. Positive slopes indicate HS presence indicating, sample contamination.

The method is described in detail in Boudin et al. (2011).

5.3.3.3   Classification of uncontaminated vs. contaminated

Spectrofluorescence analyses and C:N ratio determinations were obtained on pre-treated bulk silk and hair samples to classify the samples as uncontaminated or contaminated.
The C:N ratio of the bone collagen samples was used to classify the collagen samples as uncontaminated or contaminated.
The applied criteria to define uncontaminated archaeological samples were as follows:
1. Collagen: C:N ratio between 2.9 and 3.6 (De Niro 1985; Ambrose 1990);
2. Wool and hair: C:N between 2.9 and 3.8 and a negative fluorescence slope (Boudin et al. 2011; O'Connell and Hedges 1999a; O'Connell and Hedges 1999b; O'Connell et al. 2001);
3. Silk: C:N between 2.9 and 3.4 and a negative fluorescence slope (Boudin et al. 2011; Boudin et al. 2013).

Samples not fulfilling these conditions were defined as contaminated. A higher C:N is the result of introduction of exogenous carbon-containing compounds (i.e. contamination).

5.3.3.4   Classification of well-preserved vs. poorly preserved (degraded)

The amount of carbon and nitrogen present in the bulk sample (bone gelatin, hair or silk) in relation to the bulk weight was determined and will be referred to as carbon and nitrogen weight proportion in percent (%C and %N). These two quality indicators provide information on protein degradation. A higher %C than the average %C of modern bulk may indicate contamination.

The %C and %N of modern un-dyed, mordanted, non-mordanted and naturally dyed silk (n=14, all Bombyx mori) were determined, as there were no data available in the literature and were 44.3±2.6 and 16.7±0.7 respectively.

Ambrose (1990) cites a collagen weight %C and %N range for modern bone and tooth between 15.3% to 47% and 5.5 to 17.3% for C and N, respectively.

Hair protein contains circa 45 %C and 15 %N (Benfer et al. 1978).

e amount of extracted collagen (gelatin) in relation to the weight of the whole bone sample will be referred to as collagen weight proportion in percent (%Collagen). The %Collagen is a quality indicator for collagen preservation. The threshold used in our laboratory is 2% and bones containing less than 2%Collagen are considered as poorly preserved.

5.3.4 ^{14}C dating

Samples (e.g. collagen, silk and hair) were prepared for ^{14}C dating as described in 4.2.5.4.

5.4   RESULTS AND DISCUSSION

5.4.1   Baldwin I

Table 5.2 lists the results of the analyses of the samples from the burial of Baldwin I.

Table 5.2   : Laboratory codes , ^{14}C ages (BP), calibrated ages (2σ), stable isotopes (δ^{13}C and δ^{15}N) , C:N before (bulk) and after cross-flow nanofiltration (permeate), %C, %N, %Collagen and fluorescence slope (Fo Slope) values of samples derived from Baldwin I's burial' (presumed historical date : AD 1068).

Sample name	Lab-code	^{14}C age (BP)	Calibrated age (2σ)	δ^{13}C (‰)	δ^{15}N (‰)	atomic C:N	%C	%N	Collagen yield (%)	Fo Slope
			BONE COLLAGEN							
Skull										
bulk	KIA-47420	1185±30	AD 720 (1.9%) 740 AD 770 (87.7%) 900 AD 910 (5.8%) 950	-19.5	16.6	4.8	14.0	3.4	0.55	
permeate	KIA-47422	930±30	AD 1020(95.4%)1170	-19.2	16.4	3.9				
Femur										
bulk	KIA-47421	1510±30	AD 430 (14.9%) 490 AD 500 (80.5%) 630	-19.7	15.0	3.8	15.2	4.7	0.71	
permeate	KIA-47423	1080±30	AD890 (95.4%) 1020	-20.4	15.0	3.4				
			SILK							
43A										
bulk		n.a.		-25.9	7.5	3.8	45.2	13.9		2.33
permeate	KIA-47419	1305±30	AD 650 (95.4%) 780	-24.9	6.6	3.0				
43B										
bulk	KIA-47414	1210±30	AD 690 (12.2%) 750 760AD(83.2%)900AD	-24.5	6.8	3.7	45.6	14.4		2.07
permeate	KIA-47811	1180±30	AD 770 (86.0%) 900 AD 910 (9.4%) 970	-23.5	6.2	2.9				
46										
bulk	KIA-47415	1260±30	660 (91.9%) 830AD AD 840 (3.5%) 870	-24.9	6.3	3.6	42.4	13.9		2.62
permeate	KIA-47418	1175±30	AD 770 (83.1%) 900 AD 910 (12.3%) 970	-24.1	6.1	2.9				
			WOOD							
Wooden pastoral staff	KIA-47416	1010±30	AD 960 (95.4%) 1160							

5.4.1.1   Bone collagen

The %C, %N and %Collagen of the bulk (collagen) samples indicate degradation (diagenetic alteration or breakdown) while introduction of exogenous carbon-containing compounds (contamination) is observed from their C:N ratio (Table 5.2).

The permeate C:N ratio of sample 'Skull' was not within the C:N boundaries of uncontaminated samples, hence an unreliable ^{14}C date is obtained (Table 5.2). On the other hand, the permeate C:N of 'Femur' falls with the desired C:N range and results in a more accurate ^{14}C date (Table 5.2).

However, the calibrated ^{14}C date (AD 890 (95.4%) 1020) of sample 'Femur' is too old compared to the presumed historical date (AD 1068) (Brulet et al. 2012b). The stable isotope values (δ^{13}C and δ^{15}N) indicate that freshwater fish was part of the diet, which provokes a reservoir effect on the ^{14}C date, explaining the too old ^{14}C date (Table 5.2):

1. Elevated δ^{15}N indicates aquatic (marine or freshwater) food consumption but can not distinguish marine from freshwater;
2. The δ^{13}C indicates freshwater fish consumption mixed with terrestrial food while marine foods have a more positive δ^{13}C and thus can be excluded here (Lanting and van der Plicht 1996; Lanting and van der Plicht 1998; Cook et al. 2001; Cook et al. 2002).

Stable isotope values of 'Femur' permeate compared to the average values of Belgian medieval human bones indicates a diet enriched in freshwater fish above that of individuals who likely ate a mainly vegetable and terrestrial animal diet. In Figure 5.1, average δ^{13}C and δ^{15}N for animal and human bones from a Belgian medieval context (Ervynck et al. 2014) which indicate human diet unaffected by fish consumption. Elevated ^{15}N values for both Baldwin I and Radbot II samples seem to indicate a significant contribution of freshwater fish to their diet (Figure 5.1).

Strikingly, stable isotopes of permeate of Baldwin I's femur are almost equal to Waldetrudis' stable isotopes (Table 5.3 and Figure 5.1). Waldetrudis, and her husband, Vincentius, are known as 7th century AD promoters of the Christian faith and became saints shortly after their death (Deveseleer 1999; Deveseleer 2001). The published ^{14}C date for Vincentius coincides with the historical context for this saint (Deveseleer 1999; Deveseleer 2001, Van Strydonck et al. 2009) and his stable isotope values indicate a mixed diet of terrestrial plant and animal products (Müldner 2009), while the ^{14}C date for Waldetrudis, who is known to have died around the same date as her husband, was

significantly older than expected (5th–6th century AD) (Van Strydonck et al. 2009) but which could be explained by a freshwater reservoir effect suggested by the stable isotope values (Table 5.3 and Figure 5.1). At first sight, the difference in diet between wife and husband might seem surprising, but historical records indicate that both persons ended their lives staying in 2 (separate) monasteries (Deveseleer 1999; Deveseleer 2001), in which food habits or rules could well have been rather different (Van Strydonck et al. 2009).

Tournai is located next to the river Scheldt and Soignies, the city of Vincentius and Waldetrudis, is situated on the river Senne. The Senne is an affluent of the river Dyle, while the Dyle is an affluent of the Scheldt. It is therefore highly probable that a similar reservoir effect was present in these two rivers. A reservoir correction was done on the ^{14}C date of the permeate of Baldwin I's femur applying a simple linear relationship between δ^{13}C or δ^{15}N and the calculated age offset between the ^{14}C dates of Vincentius and Waldetridus (Table 5.3) (Cook et al. 2001). For the permeate from Baldwin I's femur the corrected ^{14}C age determined with δ^{13}C is in accord with the corrected ^{14}C age determined with δ^{15}N and their calibrated ages correspond with the presumed historical date (AD 1068) (Table 5.3).
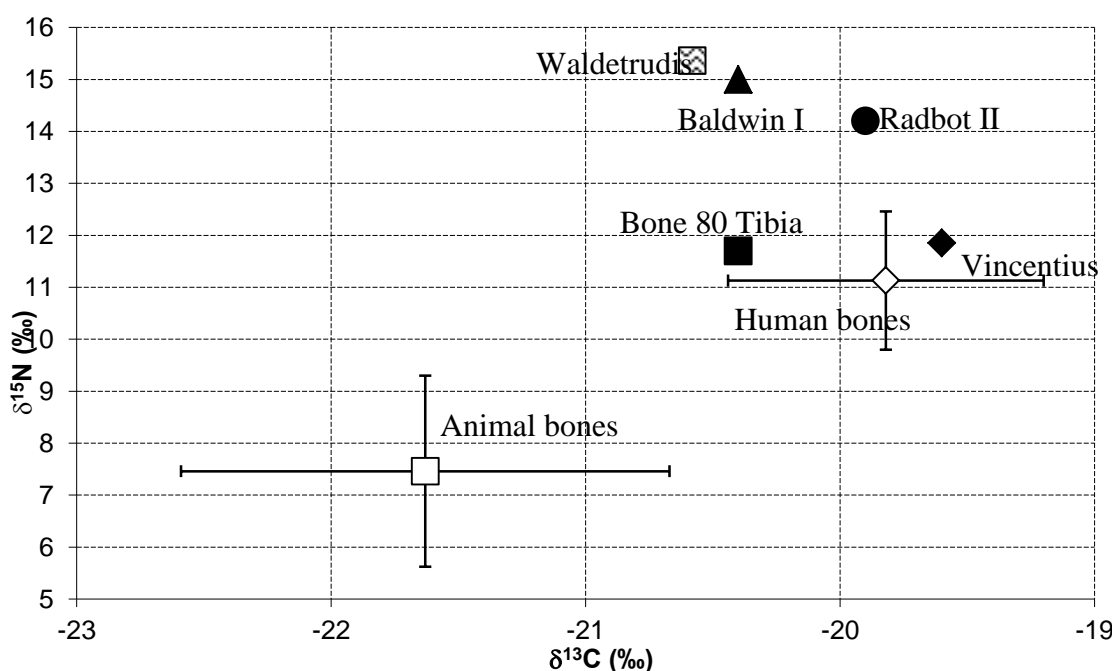
Figure 5.1   : Stable isotope data (δ^{13}C and δ^{15}N) from Radbot II, Baldwin I, Bone 80 Tibia derived from Radbot II's burial and Vincentius, Waldetrudis compared with measurements from medieval animal (n=107) and human (n=234) bones (n: number of analyzed samples) (Ervynck et al. 2014).

Table 5.3   : Laboratory codes , uncorrected and freshwater reservoir corrected ^{14}C ages of bone samples from Baldwin I and Radbot II (BP), calibrated ages (2σ), stable isotopes (δ^{13}C and δ^{15}N) , C:N, age offset (BP) in bone from Vincentius and Waldetrudis.

Sample name	Lab-code	δ^{13}C (‰)	δ^{15}N (‰)	atomic C:N	^{14}C age (BP)	age offset (BP)	corrected ^{14}C age (BP) with δ^{15}N	Calibrated age (2σ)	corrected ^{14}C age (BP) with δ^{13}C	Calibrated age (2σ)
Vincentius	KIA-10575	-19.6	11.9	3.3	1385±35					
Waldetrudis	UtC-9694	-20.6	15.4	n.a.	1530±40	145±53				
Radbot II skull fragment A permeate	KIA-46957	-19.9	14.2	3.4	1025±25		891±59	AD 1020 (95.4%) 1260	885±59	AD 1020 (95.4%) 1260
Radbot II skull fragment B bulk	KIA-47968	-20.4	14.5	3.6	995±25		858±59	AD 1030 (95.4%) 1280	851±59	AD 1020 (95.4%) 1260
Baldwin I femur permeate	KIA-47423	-20.4	15.0	3.4	1080±30		938±61	AD 980 (95.4%) 1220	936±61	AD 990 (95.4%) 1230

5.4.1.2   Silk

Three archaeological silk samples were analyzed (Table 5.2).

The %C, %N of the archaeological bulk silk samples correspond to the values of modern analysed silk samples. This indicates good preservation of the archaeological silk samples.

However, the C:N ratio and positive fluorescence slope indicate contaminated bulk samples and the fluorescence slope suggests HS contamination. The bulk ^{14}C dates of these samples are therefore considered unreliable.

The permeate ^{14}C dates of the silk samples are considered as reliable because the C:N of the permeate falls within the specified C:N boundaries for uncontaminated silk. However, the bulk and permeate ^{14}C date of '43 B' are not statistically different, applying the χ^2-test (χ^2-Test: df=1 T=0.5(5% 3.8)). The possible HS contamination can be explained by *in situ* humification.

The permeate ^{14}C dates of the silk samples '43A', '43B' and '46' are too old compared to the presumed historical date (AD 1068) for Baldwin I's death (Brulet et al. 2012b).

A possible explanation may be re-use of older (parts of) textiles as suggested by Bénazeth and Van Strydonck 2006 .

5.4.1.3   Wood

The ^{14}C date of the wooden pastoral staff, probably a branch or young tree which excludes an old-wood effect, is in agreement with the presumed historical date (AD 1068).

5.4.1.4   Conclusion for Baldwin I

The ^{14}C dates of the wooden pastoral staff and permeate of 'Femur' indicate that the burial can be assigned to Baldwin I, supported by the associated grave goods. The latter were a ring with the inscription "BAL" and a funeral stone with text was present on top of the grave. The name "Baldewinus" and his burial date, 28 april AD 1068 , are mentioned (Brulet et al. 2012b). An average of ^{14}C date of the wooden pastoral staff (KIA-47416: 1010±30BP, Table 5.2) and corrected ^{14}C date of 'femur' permeate (938±61BP or 936±61BP, Table 5.4) can be calculated: Baldwin I : 996±27BP (95.4% probability: AD 970 - 1170; χ^2-Test: df=1 T=1.1(5% 3.8)) and is in perfect agreement with presumed historical date (AD 1068).

5.4.2   Radbot II

Table 5.4 summarizes the results of the analyses of the samples from the burial of the results of Radbot II.

Table 5.4   : Laboratory codes, ^{14}C ages (BP), calibrated ages (2σ), stable isotopes (δ^{13}C and δ^{15}N) , C:N before (bulk) and after cross-flow nanofiltration (permeate), %C, %N, %Collagen and fluorescence slope (Fo slope) values of samples derived from Radbot II his burial' (presumed historical date : AD 1098).

Sample name	Lab-code	^{14}C age (BP)	Calibrated age (2 σ)	δ^{13}C (‰)	δ^{15}N (‰)	atomic C:N	%C	%N	Collagen yield (%)	Fo Slope
			HAIR (Keratin)							
Hair										
bulk	KIA-46953	1025±25	AD 970 (95.4%) 1040	-21.6	12.8	3.8	40.8	12.8		0.25
permeate	KIA-46955	1040±20	AD 970 (95.4%) 1025	-20.3	12.8	3.4				
Average (weighted)		1034±16	AD 985 (95.4%) 1025							
			BONE COLLAGEN							
Skull fragment A										
bulk	KIA-46954	1165±25	AD 770 (77.4%) 900 AD 910 (18.0%) 970	-20.4	13.7	3.7	11.3	3.5	1.70	
permeate	KIA-46957	1025±25	AD 970 (95.4%) 1040	-19.9	14.2	3.4				
Skull fragment B										
bulk	KIA-47968	995±25	AD 970 (95.4%) 1040 AD 1080(25.0%) 1160	-20.4	14.5	3.6	33.5	10.6	1.23	
Average (weighted) of KIA-46957 and KIA-47968		1010±18	AD 985 (95.4%) 1035							
Bone 80 Tibia										
bulk	KIA-46959	980±25	AD 990 (48.6%) 1060 AD 1070(46.8%) 1160	-20.4	11.7	3.1	41.4	15.6	8	

Continued Table 5.4

Sample name	Lab-code	^{14}C age (BP)	Calibrated age (2 σ)	δ^{13}C (‰)	δ^{15}N (‰)	atomic C:N	%C	%N	Collagen yield (%)	Fo Slope
			SILK							
20										
bulk	KIA-46960	900±25	AD 1040(95.4%) 1220	-20.7	8.1	4.4	41.1	10.9		2.07
permeate single										
filtration	KIA-46979	845±25	AD 1150(95.4%) 1260	-19.5	7.2	3.8				
permeate double										
filtration	KIA-47574	775±25	AD 1215(95.4%) 1280	-19.4	7.3	3.6				
22										
bulk	KIA-46958	890±35	AD 1030(95.4%) 1220	-20.6	8.1	4.1	41.9	11.8		1.49
permeate single										
filtration	KIA-46970	875±25	AD 1040(17.8%) 1100	-20	7.8	3.6				
			AD 1120(77.6%) 1230							
49										
bulk	KIA-46952	910±25	AD 1030(95.4%) 1210	-20.6	7.5	4.5	41.7	10.8		1.95
permeate single										
filtration	KIA-46956	815±25	AD 1175(95.4%) 1270	-20.4	7	3.6				
			WOOD							
Wooden pastoral staff	KIA-46574	815±25	AD 1175(95.4%) 1270	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	KIA-47969	850±35	AD 1040 (8.8%) 1090	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			AD 1120 (2.6%) 1140							
			AD 1150(84.0%) 1270							
Weighted mean		827±20	AD 1170(95.4%) 1260							

5.4.2.1   Hair

The %C and %N of the archeological 'hair' sample correspond with the percentages of well-preserved hair protein 45%C and 15%N respectively (Benfer et al. 1978).

The bulk C:N of 'Hair' (=3.8) indicates an uncontaminated sample, while the positive fluorescence slope indicates HS contamination.

The ^{14}C dates of the bulk and permeate 'Hair' samples match and an average can be calculated (Table 5.4). The possible HS contamination can be explained by *in situ* humification where hair organic matter (predominantly keratin) generates HS through the Maillard reaction (Maillard 1913; Gillespie and Hedges 1983). However, the average

^{14}C date is too old compared to the historical date (AD 1098). Stable isotope analysis suggests that freshwater fish could have been an important part of this individual's diet (Figure 5.1). A reservoir effect for freshwater organisms could thus explain the anomaly in the ^{14}C date and will be discussed further using the results of bone collagen, extracted from the skull of the 'Radbot II' sample (Table 5.4).

5.4.2.2   Bone collagen

The %C, %N and %Collagen of the collagen of 'Skull fragment A' indicate degradation while introduction of exogenous carbon-containing compounds (contamination) is observed from its C:N ratio (Table 5.4).

The collagen of 'Skull fragment B' is quite well-preserved, shown by %C and %N and uncontaminated shown by its C:N which is at the top of the acceptable C:N range for uncontaminated collagen. The collagen of 'Bone 80 Tibia' is very well-preserved as indicated by %C, %N and %Collagen and uncontaminated, proven by its C:N.

Cross-flow nanofiltration of the hydrolysate of 'Skull fragment A' resulted in a C:N decrease (Table 5.4) and a more accurate ^{14}C date should be obtained. A good agreement between the ^{14}C date of the permeate of the contaminated collagen of 'Skull fragment A' and the bulk ^{14}C date of the uncontaminated collagen of reference sample 'Skull fragment B' is observed and a weighted mean can be calculated: 1010±18BP, χ^2-Test: df=1 T=0.7(5% 3.8)). But the calibrated ^{14}C date (AD 985 - 1035, 95.4% probability) is too old compared to the presumed historical date (AD 1098) (Brulet et al. 2012b). The stable isotope values indicate that freshwater fish was part of the diet which provokes a reservoir effect on the ^{14}C date, explaining the too old ^{14}C date (Figure 5.1 and Table 5.4). The same reservoir correction is applied on permeate of 'Skull fragment A' and on the bulk of 'Skull fragment B' as with 'Baldwin I' (Table 5.3). The corrected ^{14}C ages for both samples determined with δ^{13}C and δ^{15}N are now in accord with each other and the calibrated ages correspond with the presumed historical date (AD 1098) (Table 5.3).

The calibrated aged of 'bone 80 Tibia' is also in agreement with the presumed historical date (AD 1098) (Table 5.4). However, this bone seem to come from another individual, as indicated by:

1. Aberrant stable isotopes compared to permeate of 'Skull fragment A'. Stable isotope values of 'bone 80 Tibia' fall into the cluster of Belgian medieval humans indicating a mixed diet of terrestrial plant and animal products and no fish consumption (Figure 5.1 and Table 5.4).
2. Collagen yield (8%) is much higher than from the skull (1.23-1.70%) (Table 5.4).
3. 'Bone 80 Tibia' was visually very different than all the other bones from Radbot II.

4.   Bone 80 was very hard (only possible to cut it with a microdrill) due to better preservation , e.g. higher collagen content and thus a better preservation of collagen-apatite-composite. All the other bones were very fragile (breakable manually).

We therefore speculate that 'Bone 80 Tibia' was added to the burial and this practice was not uncommon in relic shrines (Van Strydonck et al. 2006; Van Strydonck et al. 2009).

5.4.2.3   Silk

Three archaeological silk samples were analyzed (Table 5.4).

The %C, %N of the bulk archaeological silk samples correspond with the values of the modern analysed silk samples. This corroborates the good preservation of the archaeological silk samples. However, C:N ratio and positive fluorescence slope indicate contaminated bulk samples and the fluorescence slope suggests HS contamination. The bulk ^{14}C dates of these samples are therefore considered unreliable.

The permeate C:N ratio of all the silk samples was still not within the C:N boundaries of uncontaminated samples; even not after double cross-flow nanofiltration for sample '20' (Table 5.4). Thus, all the ^{14}C dates should be treated with extreme caution.

Increasing the number of cross-flow nanofiltrations results in a lower C:N for sample '20' and thus improved sample quality for ^{14}C dating (Table 5.4). The C:N decrease between bulk, permeate of single cross-flow nanofiltration and permeate of double cross-flow nanofiltration corresponds with a decreasing ^{14}C date. The ^{14}C date of the permeate after double cross-flow filtration can not be considered as reliable due to its still too high C:N,.unless this C:N value is caused by *in situ* humification. If *in situ* humification is not the case, introduction of exogenous carbon-contaminants explain this C:N value. In the latter case, the ^{14}C date of the permeate after double cross-flow filtration should be considered as a *terminus post quem* date.

The bulk and permeate ^{14}C dates of sample '22' are statistically not different, applying the χ^2-test (χ^2-Test: df=1 T=0.1(5% 3.8)) despite the C:N decrease and thus better ^{14}C sample quality after filtration. The removed contamination is therefore possibly HS, which were formed by *in situ* humification.

For sample '49' the permeate ^{14}C date is younger than the bulk ^{14}C date which indicates contamination by older exogenous carbon. Hereby, the less reliable ^{14}C date of the permeate due to their C:N can be considered as terminus post quem date. Unless the C:N values, out of C:N range for uncontaminated silk, is provoked by *in situ* humification.

None of the silk dates is in agreement with the presumed historical date of AD 1098.

5.4.2.4   Wood

The calibrated ^{14}C date from the weighted mean of the wooden pastoral staff was AD 1170-1260 (95.4% probability; Table 5.4), is not in agreement with the presumed historical date of Radbot II's death (AD 1098). The wooden pastoral staff was a small branch or young tree which excludes an old-wood effect.

5.4.2.5   Conclusion for Radbot II

The ^{14}C dates of the bulk and permeate of 'Hair', ^{14}C date of permeate 'Skull fragment A' and ^{14}C date of bulk 'Skull fragment B' indicate that the skeleton can be assigned to Radbot II.

If we assume that the C:N ratios of the silk samples are caused by *in situ* humification, then we observe a good agreement between wood ^{14}C date and 2 of the 3 silk ^{14}C dates (Figure 5.2).

Atmospheric data from Reimer et al (2009);OxCal v3.10 Bronk Ramsey (2005); cub r:5 sd:12 prob usp[chron]

KIA-47457 Silk 20 permeate double filtration 775±25BP

KIA-46970 Silk 22 permeate single filtration 875±25BP

KIA-46956 Silk 49 permeate single filtration 815±25BP

Wooden staff weighted mean 827±20BP

800CalAD   1000CalAD   1200CalAD   1400CalAD

Calibrated date

AD 1242 – 1255

Figure 5.2   : Calibrated ^{14}C ages of weighted mean of wooden pastoral staff and of the permeates of silk samples derived from the burial of Radbot II in case of *in situ* humification.

But if C:N ratios, out of the expected range for uncontaminated samples, of the silk samples are caused by younger exogenous carbon younger ^{14}C dates for the silk samples are expected.

The wood ^{14}C date and the silk ^{14}C dates indicate that perhaps a "disturbance" occurred between AD 1170 and 1260 (which is the calibrated ^{14}C date for the wooden pastoral staff):

- Was Radbot II re-buried there in the 13th century? or
- Were the clothes and the wooden pastoral staff added to the burial in the 13th century?

It is, though, an interesting coincidence that the ^{14}C dates correspond well with the construction of the new choir which began in AD 1242 and ended in AD 1255, shown by the black vertical line in Figure 5.2) and several arguments support the possibility of a 13th century activity in the burial site:

1. Bishop Gautier de Marvis (AD 1219 - 1252) had the original Romanesque choir demolished in the 13th century, in order to replace it with a Gothic choir of larger dimensions, inspired by the likes of Amiens Cathedral (Icomos 2000).
2. Both episcopal burials were explored in the two ancient choirs of the new cathedral and were situated in the 11th cathedral, in the narthex and at the bottom of the stairs which leads to the choir of the current cathedral (Brulet et al. 2012b).
3. The two burials were placed in the axe of the building. Two blue stone plates without inscription marked the location of the tombs. The concrete floor which enclosed them was restorated after the insertion of these funerary stone plates (Brulet et al. 2012b).

This 13th century activity in the grave was really unexpected as the cathedral, constructed during the 12th century, is located 2.5m higher than the eleventh century cathedral. The ancient structures were completely buried by a layer of rubble and also the episcopal tombs. But the concrete floor of the roman church was not conserved at this location which can indicate the presence of an element that localized these episcopal tombs (Brulet et al. 2012b).

5.5 CONCLUSION

The degraded bone collagen and silk material derived from Baldwin I and Radbot II were contaminated. Carbon, nitrogen and fluorescence spectroscopy on pre-treated "bulk" samples indicated that standard sample pre-treatment methods were insufficient to remove all the contamination for ^{14}C dating. Similar results from analyses of "permeate" samples treated by cross-flow nanofiltration of hydrolysed contaminated protein-containing samples indicate that it is a viable technique to improve sample quality for ^{14}C dating. However, single cross-flow nanofiltration may not be sufficient to obtain a C:N ratio within the boundaries of uncontaminated sample. Double or triple cross-flow nanofiltration may be a solution as shown by silk sample '20' of Radbot II but requires further investigation.

Regarding Baldwin I, the ^{14}C date of the wooden pastoral staff, the for freshwater reservoir corrected ^{14}C date of permeate 'Femur', the excavated ring with the inscription 'BAL' and the funeral stone with the inscription "Baldewinus" indicate that the skeleton and tomb belong to bishop Baldwin I. The older permeate ^{14}C dates of the silk samples indicate re-use.

For Radbot II, the for freshwater reservoir corrected ^{14}C dates of 'Skull' indicate that the skeleton may belong to bishop Radbot II. The younger ^{14}C dates of the wooden pastoral staff and silk samples indicate a post-burial disturbance of the site burial during the 12th -13th century, which is supported by several historical arguments. For example, a bone was also added to this burial, which was a practice not uncommon in relic shrines.

CHAPTER 6   Conclusions and future research perspectives

Carbon-14 dating of contaminated protein-containing material (e.g. bone collagen, hair, wool and silk) may result in unreliable ^{14}C dates if conventional pre-treatment methods are used. Quality control is gaining more attention in the ^{14}C community. Hence, the objectives of this thesis were to develop an integrated method to screen for presence of contamination and to increase sample quality of contaminated protein-containing archaeological samples in order to obtain more accurate ^{14}C dates.

Contaminated test samples were selected from archaeological sites where the presumed historical date was known by a dendrochronological date, a dated coin, a stylistic date, a funeral stone with inscripted name, written historical sources and typrochonological dated pottery.

6.1   CONCLUSIONS

The workflow developed during this study for ^{14}C dating of archaeological wool, silk, hair and bone collagen is depicted in Figure 6.1. All protein-containing samples were pre-treated with standard methods before the quality assessment tests. Samples categorized as uncontaminated could be ^{14}C dated after pre-treatment with the standard protocols. Hence, contaminated samples were treated with cross-flow nanofiltration and subsequently quality control parameters were measured on permeate to verify whether permeate fulfils the criteria for uncontaminated samples or not. Contaminated permeate samples were rejected for ^{14}C dating while uncontaminated underwent ^{14}C analysis. (Figure 6.1)

6.1.1 Sample quality assessment

6.1.1.1 Monitoring the presence of humic substances in wool and silk by the use of non-destructive fluorescence spectroscopy

Fluorescence spectrometry is a quick and non-destructive method to detect the presence of contaminant humic substances (HSs) in naturally (un)dyed wool and silk. It provides information on the reliability of eventual ^{14}C dates.

A positive fluorescence slope, calculated using a linear fit (least squares) of the fluorescence intensity measured between the excitation wavelengths 460 and 475nm, indicates presence of HSs while a negative slope is an indication of absence of HSs. Wool and silk samples wherein no HSs were detected can be pre-treated with the conventional methods and reliable ^{14}C results are obtained. However, wool and silk samples contaminated with HSs will be subjected to cross-flow nanofiltration (Figure 6.1)

6.1.1.2 C:N ratio determination as a means to identify ^{14}C contamination

The C:N ratio of pre-treated wool, hair, silk and bone (collagen) samples was measured to categorize the collagen sample as uncontaminated or contaminated (Figure 6.1).

The applied criteria to define uncontaminated archaeological samples were (Figure 6.1):
- collagen: C:N ratio between 2.9 and 3.6 (De Niro 1985; Ambrose 1990);
- wool and hair: C:N between 2.9 and 3.8 (O'Connell and Hedges 1999a, 1999b, 2001);
- silk: C:N between 2.9 and 3.4 . This C:N range was based on own analyses.

Samples with a higher C:N ratio were defined as contaminated.

Uncontaminated samples can undergo directly ^{14}C analyses and reliable ^{14}C dates should be obtained. However, cross-flow nanofiltration must be carried out on contaminated samples and subsequently the C:N ratio of the permeate must be checked. If the C:N ratio of the permeate falls within the C:N range of uncontaminated samples, the permeate can be ^{14}C dated resulting in a more accurate ^{14}C date (Figure 6.1).

6.1.2   Initial development of a 450 Dalton nanofiltration method for bone collagen used for ^{14}C dating

The nanofiltration methods described in this thesis showed some advantages over ultrafiltration. There is no risk of carbon contamination coming from the filter because the filter material is ceramic. It is suitable for all types of protein-containing material, not only collagen. Unlike ultrafiltration where only low-molecular HSs are eliminated, nanofiltration should remove low-molecular and high-molecular weight HSs. Fluorescence analyses indicated that cross-flow filtration was a more efficient technique than dead-end filtration to remove HSs. Cross-flow filtration has the disadvantage that a minimum feed volume must remain in the installation during filtration to prevent the pump from stopping. This means that not all the feed volume can be filtered, which causes sample loss. Consequently, the sample size needed for ^{14}C dating increases. However, ^{14}C dating of cross-flow filtrated hydrolysed collagen-HS mixtures, i.e. amino acids-HS mixtures, demonstrated a significant but yet incomplete removal of HSs. Hot acid hydrolysis of HS may form new chemical compounds, as demonstrated by HPLC analysis. It is possible that these compounds are smaller than the filter cut off of 450 Dalton and pass through the filter and, hence, causes contamination.

6.1.3   Improved radiocarbon dating for contaminated archaeological bone collagen, silk, wool and hair samples via 200 Dalton cross-flow nanofiltrated amino acids

A membrane with a molecular weight cut-off of 200 Dalton was used, instead of 450 Dalton, as a means to retain more carbon-containing contaminants e.g. the new formed chemical compounds after hot acid hydrolysis and obtain only amino acids from the protein-containing archaeological material.

Cross-flow nanofiltration was used because of the better retention of contaminants compared to dead-end nanofiltration. The efficiency of the cross-flow nanofiltration was verified by comparing:

1. C:N ratio of the bulk sample (before nanofiltration) with C:N ratio of the cross-flow nanofiltrated amino acids (permeate) to indicate the quality sample improvement.
2. the ^{14}C date of permeate with the presumed historical date.

Cross-flow nanofiltration, with a filter cut-off of 200 Dalton, of hydrolysed contaminated archaeological protein-containing samples, e.g. bone collagen, hair, wool and silk,

decreases the C:N ratio, which indicates contaminant removal and improvement of sample quality for ^{14}C dating. If the C:N ratio of the permeate falls within the boundaries of uncontaminated samples, more accurate ^{14}C dates were obtained.

Cross-flow nanofiltration is a quick and non-labour intensive technique and can easily be implemented in any ^{14}C laboratory for routine sample pre-treatment analyses.
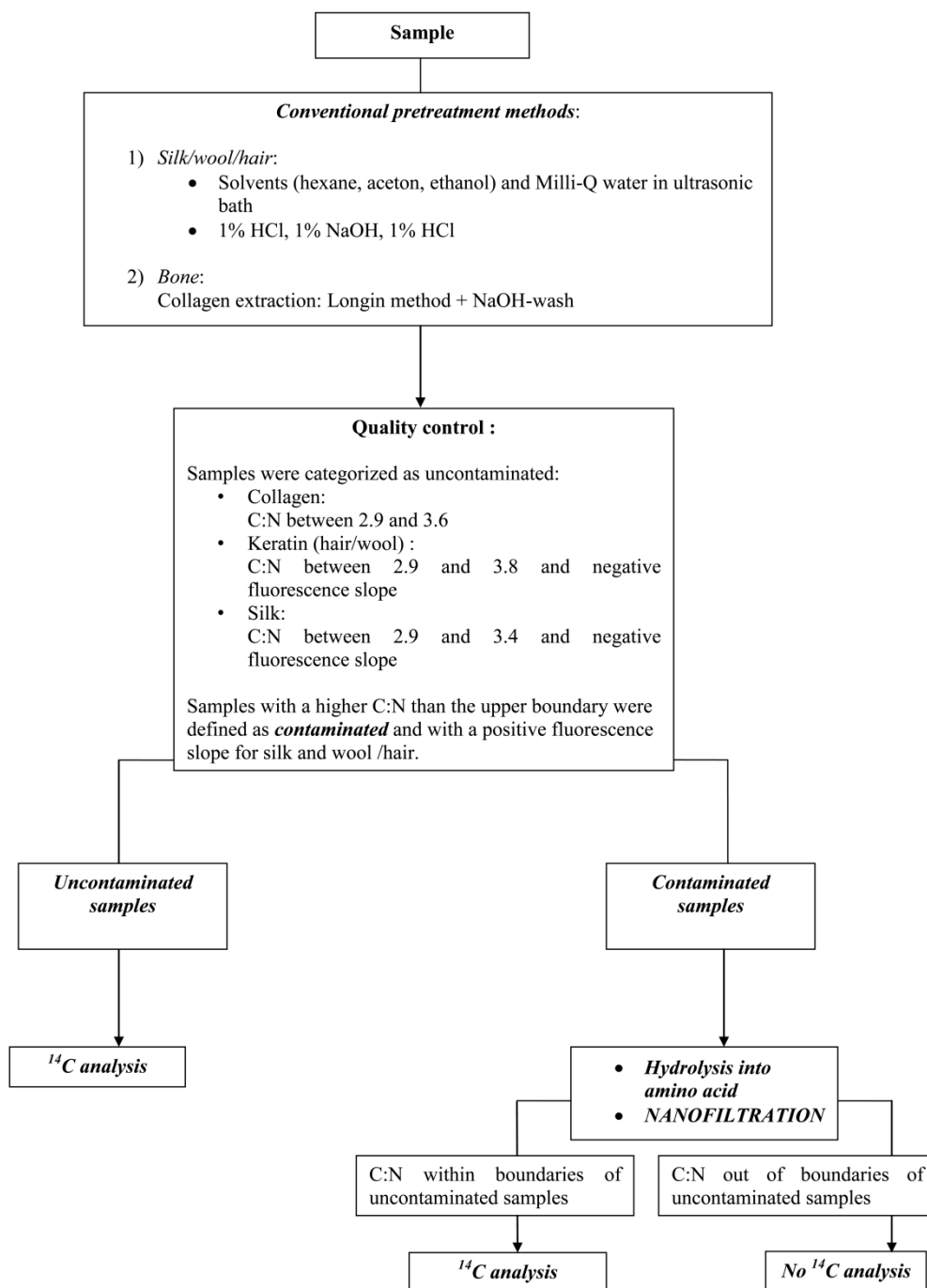
Figure 6.1   : Workflow for archaeological wool, silk, hair and bone collagen for ^{14}C dating.

6.1.4   Application of the developed cross-flow nanofiltration method on a well-documented archaeological site: The case study of the bishops Baldwin I and Radbot II from Noyon-Tournai

The degraded bone collagen and silk material derived from Baldwin I and Radbot II were contaminated based on C:N ratio and spectrofluorescence analyses. Carbon, nitrogen and fluorescence spectroscopy data on pre-treated samples indicated that conventional sample pre-treatment methods were insufficient to remove all contamination for ^{14}C dating. Results from analyses of permeate samples treated by cross-flow nanofiltration of hydrolysed contaminated protein-containing samples indicated that sample quality was improved and became suitable for ^{14}C dating, supported by the decrease in C:N ratio after cross-flow nanofiltration and good agreement of the ^{14}C dates with the presumed historical dates. However, single cross-flow nanofiltration may not always be sufficient to obtain a C:N ratio within the boundaries of uncontaminated samples. Double cross-flow nanofiltration may be a solution as shown by silk sample '20' of Radbot II, but requires further investigation.

For Baldwin I, The ^{14}C date of the wooden pastoral staff, the for freshwater reservoir corrected ^{14}C date of 'Femur', the excavated ring with the inscription 'BAL' and the funeral stone with the inscription "Baldewinus" indicate that the skeleton and tomb belong to bishop Baldwin I. Surprisingly, the older ^{14}C dates of the silk samples indicate that the cloths of Baldwin I used for burial were re-used from an older bishop.

Regarding Radbot II, the for freshwater reservoir corrected ^{14}C dates of 'Skull' indicate that the skeleton could belong to bishop Radbot II. Surprisingly, the younger ^{14}C dates of the wooden pastoral staff and silk samples indicate a post-burial disturbance of the burial site during the 12th -13th century, which is supported by several historical arguments. For example, a bone was also added to this burial, which was a practice not uncommon in relic shrines.

6.2   FUTURE RESEARCH PERSPECTIVES

- Fluorescence spectroscopy for
  1) Solid samples (wool and silk): The fluorescence slope, as described in Chapter 2, can be used as a qualitative indicator for the presence or absence of HSs in wool or silk samples. Future research will be carried out to develop a fluorescence intensity calibration method for wool and silk samples as a means to quantify HS contamination in archaeological wool and silk.
  2) Liquid samples: the fluorescence method, as described in Chapter 3, was used to detect and semi-quantify HSs in the hydrolysate of collagen contaminated with HS before and after nanofiltration. Future study will focus on developing a fluorescence intensity calibration method for hydrolysed collagen samples in order to quantify the HS contamination in the hydrolysed bone collagen. However, the method will also be tested for other sample matrices e.g. NaOH-extracts of charcoal and soils. Charcoal and soils are subjected to acid-alkali-acid treatment before ^{14}C analysis in order to remove exogenous carbonates (by acid wash) and humic acids (by alkali wash). Analyses of NaOH-extracts of charcoal and soils can provide information about the quantity of HS, and thus contamination, present in charcoal and soils. HSs may alter and in most cases probably cause younger ^{14}C dates by HS intrusion of upper layers into the to be dated material.
- Shampoos and dye products, produced from petroleum (hence absence of ^{14}C), make ^{14}C dates of human hair older. Accurate ^{14}C dates are necessary for forensic investigations. But standard pre-treatment laboratory protocols are unable to remove the introduced older ^{14}C of the shampoos and dye products. The developed cross-flow nanofiltration method may be able to remove these contaminants and yield reliable ^{14}C results.
- A new case study will be set up to further corroborate the developed methodology. Remainder of a wooden box was excavated in the Sint-Rumbold's cathedral (Mechelen, Belgium). The box contained relics: two skulls, a femur and some bone fragments. The skulls were dressed in textiles (mostly silk and some linen). The workflow for these protein-containing samples will be like depicted in Figure 6.1.
- In the case study of Baldwin I and Radbot II (Chapter 5) one sample was single and double cross-flow nanofiltrated. Double cross-flow nanofiltration resulted in better sample quality, shown by the C:N ratio, than single cross-flow nanofiltration. Therefore, single, double and triple cross-flow nanofiltration could be carried out on contaminated archaeological samples with a well-known presumed historical date. The sample quality will be checked by C:N analyses.

- Ultrafiltration of bone collagen is a commonly used technique in ^{14}C laboratories. This technique removes the low-molecular contaminants and not the high-molecular contaminants e.g. if the MWCO of the filter is 10 kDalton, all the contaminants < 10kDalton will be removed but not the HSs cross-linked with the collagen (> 10kDalton). Although the nanofiltration method developed in this study retains all compounds after hydrolysis > 200 Dalton (MWCO of filter) there is still a risk that extraneous carbon-containing compounds < 200 Dalton passes the filter and contaminate the permeate used for ^{14}C dating.

  Ultrafiltration requires less time than nanofiltration where hydrolysis into amino acids is necessary, which takes 24 hours. However, the current used ultra-filters are also the middle point of discussion as they may introduce carbon from the filter into the sample and alter the ^{14}C date. The filters are made of cellulose (modern carbon contamination) or polyethersulfone (fossil carbon contamination). Therefore, ceramic filters of 40 kDalton will be used in the in-house built filtration set-up and the retentate, which should only contain the bone collagen, will be ^{14}C dated.

  However, a comparative study between ultrafiltration and cross-flow nanofiltration should be carried out on contaminated bone collagen samples with a well-known presumed historical date derived from archaeological sites with different preservation environment. This study may clarify which filtration technique is the most suited for contaminated bone collagen depending on the preservation environment.

Summary

Carbon-14 (^{14}C) dating of contaminated protein-containing material e.g. bone collagen, hair, wool and silk usually results in unreliable results if conventional pre-treatment methods are used. Therefore, the objective of this PhD thesis was to develop a method to detect ^{14}C contamination in protein-containing archaeological material and develop a method to improve sample quality of contaminated samples to obtain more accurate ^{14}C dates. Quality control is gaining more attention in the ^{14}C community; hence a quality control system was introduced.

Contaminated test samples were selected from archaeological sites where the presumed historical date was known by a dendrochronological date, a dated coin, a stylistic date, a funeral stone with inscripted name, written historical sources or typrochonological dated pottery.

A non-destructive fluorescence spectroscopy method was developed to monitor the presence of humic substances, one of the major ^{14}C contaminants in archaeological material, in wool, hair and silk. A positive fluorescence slope, calculated using a linear fit (least squares) of the fluorescence intensity measured between the excitation wavelengths 460 and 475nm, indicates presence of humic substances in the sample, while a negative slope is an indication of absence of humic substances

All protein-containing samples were pre-treated with standard methods before subjected to the quality assessment tests. The quality control was checked by analyzing the C:N ratio for bone collagen, silk, wool and hair.

The applied C:N ratios to define uncontaminated archaeological samples were:

1. collagen C:N ratio between 2.9 and 3.6;
2. wool and hair C:N ratio between 2.9 and 3.8;
3. silk: C:N ratio between 2.9 and 3.4 .

Samples with a higher C:N ratio were defined as contaminated.

Uncontaminated samples can be used directly ^{14}C analyses and reliable ^{14}C dates should be obtained. However, contaminated samples need more clean-up to improve the sample quality. Nanofiltration is an option because it is suitable for all types of protein-

containing material and should remove low-molecular and high-molecular weight contaminants unlike ultrafiltration that only suits for bone collagen and only eliminates low-molecular contaminants.

A new nanofiltration method to improve quality for ^{14}C dating was developed for bone collagen as test material. First, two nanofiltration types were tested: dead-end and cross-flow filtration using a ceramic filter with a molecular weight cut-off of 450 Dalton. Nanofiltration should remove low-and high-molecular weight humic substances. Fluorescence analyses indicated that cross-flow filtration was a more efficient technique than dead-end filtration to remove humic substances. However, ^{14}C dating of cross-flow filtrated hydrolysed artificial collagen-humic substance mixtures, demonstrated a significant but yet incomplete removal of humic material. Hot acid hydrolysis of humic substances may form new chemical compounds, as demonstrated by HPLC analysis. It is possible that these compounds are smaller than the filter cut-off of 450 Dalton and pass through the filter and, hence, causes contamination.

Second, cross-flow nanofiltration technique was applied in combination with a quality control procedure on real contaminated archaeological samples. However, a membrane with a molecular weight cut-off of 200 Dalton was used as a means to retain more ^{14}C-containing contaminants, i.e. chemical compounds formed after hot acid hydrolysis and obtain only amino acids from the protein-containing archaeological material. The efficiency of the cross-flow nanofiltration was verified by comparing:

1. C:N ratio of the bulk sample (before nanofiltration) with C:N ratio of the cross-flow nanofiltrated amino acids (permeate) to indicate the quality sample improvement;
2. compare the ^{14}C date of permeate with the presumed historical date.

Cross-flow nanofiltration, with a filter cut off of 200 Dalton, of hydrolysed contaminated archaeological protein-containing samples, e.g. bone collagen, hair, wool and silk, decreases the C:N ratio, which indicates contaminant removal and improvement of sample quality for ^{14}C dating. If the C:N ratio of the permeate falls within the boundaries of uncontaminated samples more accurate ^{14}C dates were obtained.

Finally the applicability of the cross-flow nanofiltration method was demonstrated on contaminated protein-containing samples by means of a case study of two skeletons and their gars of two bishops from Noyon-Tournai. One burial was assigned to Baldwin I, who died in AD 1068, because:

1. a ring with the inscription "BAL" was found;
2. a funeral stone with text was present on top of the grave mentioning the name "Baldewinus".

The second burial probably belongs to Radbot II who was the successor of Baldwin I and died in AD 1098.

However, single cross-flow nanofiltration may not always be sufficient to obtain a C:N ratio within the quality boundaries for uncontaminated samples. Double cross-flow

nanofiltration may be a solution as shown by silk sample '20' of Radbot II, but requires further investigation.

This study resulted in a workflow for ^{14}C dating of archaeological wool, silk, hair and bone collagen. All protein-containing samples are pre-treated with standard methods before quality assessment tests. Samples categorized as uncontaminated can be ^{14}C dated after pre-treatment with the standard protocols. Hence, contaminated samples are treated with cross-flow nanofiltration and subsequently quality control parameters are measured on permeate to verify whether permeate fulfills the criteria for uncontaminated samples or not. Contaminated permeate samples are rejected for ^{14}C dating while uncontaminated undergo ^{14}C analysis.

Samenvatting

Radiokoolstofdatering (^{14}C) van gecontamineerd proteïnbevattend materiaal zoals beendercollageen, haar, wol en zijde levert onbetrouwbare resultaten op indien ze behandeld zijn met de standaard voorbehandelingsmethodes. Daarom was het doel van dit proefschrift om een methode te ontwikkelen om radiokoolstofcontaminatie in proteïnbevattende materialen te detecteren en om een methode te ontwikkelen om de staalkwaliteit van gecontamineerde stalen te verbeteren zodat meer accurate radiokoolstofdateringen worden verkregen. Kwaliteitscontrole krijgt meer en meer aandacht in de radiokoolstofgemeenschap en bijgevolg werd een kwaliteitscontrolesysteem geïntroduceerd.

Gecontamineerde teststalen werden geselecteerd van archeologische sites waarvan de vermoedelijke historische datum was gekend door middel van een dendrochronologische datering, een muntstuk met datum, een stilistische datering, een grafsteen met een naam als inscriptie, geschreven historische bronnen of typochronologische potscherfdateringen.

Een niet-destructieve spectrofluorescentiemethode werd ontwikkeld om de aanwezigheid van humussubstanties, een van de belangrijkste radiokoolstofdateringscontaminanten in archeologisch materiaal, in wol, haar en zijde te monitoren. Een positieve fluorescentierichtingscoëfficiënt, berekend door middel van een lineaire regressie van de fluorescentieintensiteit gemeten tussen de excitatiegolflengtes 460 en 475nm, wijst op de aanwezigheid van humussubstanties in het staal terwijl een negatieve fluorescentierichtingscoëfficiënt een indicatie is van afwezigheid van humussubstanties.

Alle proteïnbevattende stalen werden voorbehandeld met de standaardmethodes vooraleer onderworpen te worden aan de kwaliteitsevaluatietesten. De kwaliteitscontrole werd geverifieerd door C:N ratio analyses van beendercollageen, zijde, wol en haar

De toegepaste C:N ratio criteria om ongecontamineerde archeologische stalen te definiëren zijn:

1. Collageen: C:N ratio tussen 2.9 en 3.6;
2. Wol en haar: C:N ratio tussen 2.9 en 3.8;
3. Zijde: C:N ratio tussen 2.9 en 3.4.

Stalen met een hogere C:N ratio werden gedefinieerd als gecontamineerd.

Niet-gecontamineerde stalen kunnen onmiddellijk geanalyseerd worden op ^{14}C en betrouwbare resultaten zouden verkregen moeten worden. Daarentegen, moeten gecontamineerde stalen meer voorbehandeld worden om de staalkwaliteit te verbeteren. Nanofiltratie is een optie omdat het geschikt is voor alle types van proteïnbevattend materiaal en het zowel laagmoleculaire als hoogmoleculaire contaminanten zou moeten verwijderen, niet zoals ultrafiltratie dat enkel geschikt is voor beendercollageen en alleen de laagmoleculaire contaminanten elimineert.

Een nieuwe nanofiltratiemethode voor beendercollageen werd ontwikkeld om de kwaliteit voor ^{14}C AMS datering te verbeteren. Eerst werden twee nanofiltratietypes getest: dead-end en cross-flow nanofiltratie door middel van het gebruik van een keramische filter met een molecular weight cut-off van 450 Dalton. Nanofiltratie zou laagmoleculair-en hoogmoleculair-gewicht humussubstanties moeten verwijderen. Fluorescentieanalyses duidden aan dat cross-flow nanofiltratie efficiënter was dan dead-end filtratie om humussubstanties te verwijderen. Niettemin, ^{14}C datering van gehydrolyzeerde, met cross-flow genanofiltreerde artificiële collageen-humussubstantie mengels toonden een significante maar onvolledige verwijdering van de humussubstanties aan. Zuurhydrolyse van humussubstanties kan nieuwe chemische verbindingen vormen, zoals aangetoond met HPLC analyses. Het is mogelijk dat deze verbindingen kleiner zijn dan de filter cut off van 450 Dalton en de filter passeren, en bijgevolg contaminatie veroorzaken.

Daarna werd cross-flow nanofiltratie toegepast op echte archeologische stalen in combinatie met kwaliteitscontroleprocedures. Er werd echter een membraan met moleculair weight cut off van 200 Dalton gebruikt om meer koolstofhoudende contaminanten te weerhouden zoals de nieuwgevormde chemische verbindingen na zuurhydrolyse en zodoende alleen de aminozuren afkomstig van het proteinhoudend archeologisch materiaal door de filter te laten.

De efficiëntie van de cross-flow nanofiltratie werd gecontroleerd door vergelijking van de:

1. C:N ratio van het bulkstaal (voor nanofiltratie) met de C:N ratio van de met cross-flow nanogefiltreerde aminozuren (permeaat) om de staalkwaliteitsverbetering na te gaan;
2. Radiokoolstofdatering met de veronderstelde historische datering.

Cross-flow nanofiltratie, met een filter cut off van 200 Dalton, van gehydrolyzeerd gecontamineerd archeologisch proteinbevattend materiaal, zoals beendercollageen, haar, wol en zijde, doet de C:N ratio afnemen, wat wijst op contaminatieverwijdering en bijgevolg staalkwaliteitsverbetering voor ^{14}C datering. Indien de C:N ratio van het

permeaat in het C:N bereik valt van ongecontamineerde stalen, werden accuratere radiokoolstofdateringen verkregen.

Finaal wordt de toepasbaarheid van de cross-flow nanofiltratiemethode op gecontamineerd proteinhoudende stalen aangetoond door middel van een case study van twee skeletten en de gewaden van bisschoppen van Noyon-Tournai. Een graf werd toegewezen aan Baldwin I, die overleed in AD 1068, omdat:

1. een ring met de inscriptie "BAL" werd gevonden;
2. een grafsteen met tekst was aanwezig op het graf waarin de naam "Baldewinus" vermeld staat.

Het tweede graf behoort waarschijnlijk toe aan Radbot II die de opvolger was van Baldwin I en overleed in AD 1098.

Daarentegen kan een enkele cross-flow nanofiltratie niet altijd voldoende zijn om een C:N ratio te verkrijgen die in het C:N bereik valt van ongecontamineerde stalen. Een dubbele cross-flow nanofiltratie kan de oplossing zijn zoals aangetoond door het zijdestaal '20' van Radbot II maar verder onderzoek is vereist.

Dit onderzoek leidde tot een werkschema voor ^{14}C datering van archeologische wol, zijde, haar en beendercollageen. Alle proteinbevattende stalen worden voorbehandeld met de standaardmethodes voor de kwaliteitscontroletesten. Stalen gecategoriseerd als niet-gecontamineerd kunnen onmiddelijk gedateerd worden met ^{14}C na voorbehandeling met de standaardprotocols. Niettemin, gecontamineerde stalen moeten behandeld worden met cross-flow nanofiltratie en daarna wordt de kwaliteit van het permeaat geanalyseerd om na te gaan of het permeat voldoet aan de criteria van ongecontamineerde stalen of niet. Gecontamineerde permeaatstalen worden weerhouden van radiokoolstofdatering terwijl niet-gecontamineerde stalen geanalyseerd worden met ^{14}C.

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Curriculum Vitae

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PROFESSIONAL EXPERIENCE

2010 – Present: PhD candidate in Applied Biological Science (Thesis: Improved radiocarbon dating of contaminated protein-containing archaeological samples via cross-flow nanofiltrated amino acids), Faculty of Bioscience Engineering, Ghent University, Gent Belgium and Radiocarbon dating laboratory, Royal Institute for Cultural Heritage, Brussels Belgium (PhD defence is expected in June 2014)

2006 - 2010:	Research assistant (Food crust project and provision of services), Radiocarbon dating laboratory, Royal Institute for Cultural Heritage, Brussels Belgium
July 2001 - 2005:	Research assistant (Diet study via stable isotope analyses of bone collagen and provision of services), Radiocarbon dating laboratory, Royal Institute for Cultural Heritage, Brussels Belgium
2001 (January-June):	Chemist (Accreditation of pesticides via Gas chromatographe coupled to mass spectrometer), Fytolab, Faculty of Bioscience Engineering, Ghent University, Ghent Belgium
1999 - 2001:	Research assistant (Optimalisation of restoration mortar), Monument laboratory, Royal Institute for Cultural Heritage, Brussels Belgium
1998 (July-December):	Chemist analyst:

1. Quality control (Conforma NV, Destelbergen, Belgium)
2. Determination of sugar content (Suikergroep NV, Moerbeke, Belgium)
3. Production responsable (Dacor NV, Zwijnaarde, Belgium)
4. Air analyses (Labo Van Vooren, Zelzate, Belgium)

1998 (January-June):	Chemical analyst – Léonardo internship (Fate pollutants in the subsoil) Laboratório Nacional de Engenharia Civil (LNEC),Lisbon, Portugal
1997:	Labourer, Ghent Port Side, Gent, Belgium

EDUCATION

1996 – 1997:	Master in the Industrial Engineer Science: Chemistry (Thesis: Implementation and evaluation of an Ion Trap Detector in an existing chromatographical system), KaHo

Sint-Lieven, Gent, Belgium

1992 – 1996:	Master in the Industrial Engineer Science: Biochemistry (Thesis: Determination of the Potassium, Calcium, Sodium and Magnesium concentration in meat by Capillary Ion Analysis – Eramus-student: University of Girona, Spain) , KaHo Sint-Lieven, Gent, Belgium
1989 – 1992:	Sciences B (secondary school) Instituut H. Kindsheid, Ardooie, Belgium

CONFERENCES

Lunula: Contact day about the archaeology of the metal ages, Brussel, Belgium, February 2002.

Lunula: Contact day about the archaeology of the metal ages, Brussel, Belgium, February 2003.

International Symposium, Insular Vertebrate Evolution, The Paleontological approach, Palma de Mallorca, Spain, 16-19 September 2003. Presentation : Humans and Myotragus: the issue of sample integrity in radiocarbon dating.

VOBOV: Archaeological contact day, Ghent, Belgium, 26 November 2005.

TRACE 2006: Annual conference of the association oftree-ring research, Brussels, Belgium, 20-22 April 2006: Presentation: Calibration of radiocarbon dates.

VOBOV: Archaeological contact day, Mechelen, Belgium, 28 April 2007:

Radiocarbon and Archaeology 5th International Symposium, Zurich, Switzerland, 26-28 March 2008:

11th International AMS-conference, Rome, Italy, 14-19 September 2008.

Annual Meeting BASIS: Benelux Association of Stable Isotope Scientists. Brugge, Belgium, 2-3 april 2009.

20th International Radiocarbon conference, Big Island, Hawai, USA, 31 May-5 June 2009. Poster: Fish Reservoir Effect on Charred Food Residue [14]C Dates: Are Stable Isotope Analyses the Solution?

12th International AMS conference, Wellington, New Zealand, 20-25 March 2012. Presentation: Development of a nanofiltration method for bone collagen ^{14}C AMS dating.

21st International Radiocarbon Conference, Paris, France, 9-13 July 2012. Presentation: Improved radiocarbon dating for contaminated archaeological bone collagen, silk, wool and hair samples via cross-flow nanofiltrated amino acids.

7th International Radiocarbon and Archaeology Symposium, Gent, Belgium, 8-12 April 2013. Posters: 1) The atomic C:N ratio of wool and silk : a sample quality indicator for ^{14}C dating and 2) An archaeological mystery revealed by radiocarbon dating of cross-flow nanofiltrated amino acids: The case study of the bishops Baldwin and Radbot II from Noyon-Tournai.

ORGANISATION OF SCIENTIFIC CONFERENCES

Chronology and Evolution in the Mesolithic of N(W) Europe, Brussels, Belgium. 30 May–1 June 2007. Co-organizer.

7th International Radiocarbon and Archaeology Symposium, Gent, Belgium, 8-12 April 2013. Co-organizer

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