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PRETREATMENT PROTOCOLS PERFORMED AT THE ROYAL INSTITUTE FOR CULTURAL HERITAGE (RICH) PRIOR TO AMS ^{14}C MEASUREMENTS

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ABSTRACT. The Royal Institute for Cultural Heritage (RICH) radiocarbon (^{14}C) laboratory in Brussels, Belgium, has acquired experience for pretreating samples with 60 years of involvement in ^{14}C dating, and the implementation of routine protocols. These procedures as applied to wood, seeds, charred materials, bones, ivory, textiles (silk, wool, cotton, linen), paper, shells, cremated bones, mortars, lead carbonates, sediments, etc. are described in detail in this paper. They are evaluated against reference materials.

KEYWORDS: pretreatment, radiocarbon AMS dating.

INTRODUCTION

The Royal Institute for Cultural Heritage (RICH) radiocarbon (^{14}C) dating laboratory in Brussels, Belgium, reached its 30th anniversary of graphitizing samples for accelerator mass spectrometry (AMS) ^{14}C dating in 2019 (Boudin et al. 2015). RICH expertise in ^{14}C dating began in the 1960s with a gas proportional counter which was replaced by liquid scintillation counting (LSC) in 1992. A manual graphitization line for AMS samples was built in 1989. In 2008 due to the huge demand of AMS dating a second manual graphitization line for AMS was set up and LSC analyses were discontinued. The graphite samples were sent abroad to AMS facilities for measurements. Since 2013, AMS ^{14}C dating is performed directly at RICH thanks to a mini carbon dating system (MICADAS) (Boudin et al. 2015) developed at the Swiss Federal Institute of Technology in Zürich (ETH), Switzerland (Synal et al. 2007). In 2016, an automated graphitization system (AGE) (Nemec et al. 2010; Wacker et al. 2010) was installed at RICH (Boudin et al. 2019) to fulfil the high demand of AMS analyses. Over almost 60 years, various pretreatments have been performed and improved on samples before radiocarbon dating. Here, a summary of the routine pretreatment practices at the laboratory is presented.

ANALYZED MATERIAL

The samples undergoing ^{14}C dating at RICH can be categorized into 6 groups:

- Protein-containing materials
  - Collagen (e.g. bone, tooth, antler, ivory, narwhal tooth)
  - Keratin (e.g. hair, wool, rhinoceros horn)
  - Fibroin (silk)
  - Leather
- Charcoal and charred materials (e.g. wood, seeds, carbonized pottery residues);
- Cellulose-containing materials (e.g. wood, seeds, plant remains, plant fibers (textiles), and paper);
- Carbonate-containing materials (e.g. shells, cremated bones, bone apatite, mortars, lead carbonate);

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- Sediments;
- Chitin-containing materials (e.g. insect remains).

PRETREATMENT PROTOCOLS

To eliminate exogenous carbon atoms susceptible to alter the date, different pretreatment protocols are followed. These procedures will be described below according to the type of samples and the method used. The general protocols are explained, and they can be adjusted depending on the specific features of the samples such as the state of preservation and the burial environment. Pretreatment is performed in glass containers which are baked at 450°C during 1 hr to remove organic contaminants. Acidic and alkali solutions are commonly used to eliminate mineral and organic contaminants possibly present within the samples. Table 1 gives an overview of the treatments carried out at RICH. The samples are thoroughly rinsed with ultrapure Milli-Q™ deionized water in between each treatment.

AAA

The AAA pretreatment, also called ABA (acid-base-acid), involves a sequential washing with diluted acid, diluted alkali, and diluted acid (Olson and Broecker 1958) and it is commonly used on most types of organic matter (Santos and Ormsby 2013). At RICH, it is carried out on charcoal, coal (used as a background sample), wood, peats, seeds, sediments, plant remains (e.g. papyrus, nut shells) and food crust from pottery sherds. The first acid wash is used to remove sediment carbonates and other contaminants such as fulvic acid, the alkali solution to extract organic contaminants (including humic acids) and the final acid wash removes atmospheric CO_2 which could be absorbed in the alkaline step and neutralizes any NaOH remaining. The temperature and length of pretreatment vary depending on the sample. For specific samples, the treatment needs to be adapted (Bonneau et al. 2017). The general procedure followed at RICH consists of submerging a known quantity of sample in 0.3 M HCl at ~90°C during 1 hr, then in 0.25 M NaOH during 1 hr at ~90°C and finally again in 0.3 M HCl during 1 hr at ~90°C. In between each step, the sample is rinsed thoroughly with Milli-Q™ water. If the sample weight is very low (less than 3 mg) or it is not well-preserved the alkali treatment is first performed at room temperature. If the sample does not disaggregate, the temperature can be increased up to 90°C, and vice-versa, if the sample starts to disaggregate at 90°C, the heat should be stopped. If possible, the treatment is performed for a total of 60 min.

If after pretreatment the wood samples are still dark due to the presence of humic substances, bleaching is applied as described in Table 1. The bleaching consists of introducing the sample into a 1.6 M KOH solution for 1 hr at boiling point. The sample is then rinsed thoroughly with Milli-Q® water and introduced into a solution made of 1.2 M $NaClO_2$ in Milli-Q® with additional HCl at boiling point until the sample becomes white. If the solution becomes transparent, it is replaced. Finally, the sample is rinsed thoroughly with Milli-Q® water.

Collagen Extraction

This procedure is performed on bones (not incinerated nor burnt), teeth, ivory, and antler. The Longin method (Longin 1971) is used with additional steps. Before starting the procedure for collagen extraction, it is necessary to clean mechanically the samples with a Dremel® rotary tool equipped with a diamond cut-off wheel. It involves removing the surface to eliminate the porous parts, which can be source of contamination. For bones, the cortical part is

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Table 1 Summary of pretreatments performed at RICH depending on sample types.

Sample	Acid (HCl)	Acid (CH_3COOH)	Base (NaOH)	Acid (HCl)	Base (KOH)	Bleach ($NaClO_2$)
Wood, seeds, plant remains, charcoal, coal, carbonized pottery residues, sediments	0.3 M, 90°C, 1 hr	—	0.25 M, 90°C, 1 hr	0.3 M, 90°C, 1 hr	—	—
Collagen (bone, tooth, antler, ivory)	2.4 M, RT, 15 min	—	0.25 M, RT, 15 min	0.3 M, RT, 5 min	—	—
Hair (soil rich in humus substances)	—	—	0.25 M, at least 15 min	0.3 M, RT, 15 min	—	—
Insect remains	0.3, RT, 24 hr	—	—	—	—	—
Shells	0.3 or 2.4 M, RT, few min	—	—	—	—	—
Cremated bones	2.4 M, RT, few min	0.17 M, RT, 24 hr	—	—	—	—
Bone apatite	—	0.17 M, RT, 24 hr	—	—	—	—
Linen, cotton (plant fibers)	—	—	—	—	1.6 M, 90°C, 1 hr	1.2 M, 90°C, 1 hr

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preferably selected because it is often less porous (less contaminant can be absorbed). For teeth, only the part where dentine is present is taken for collagen extraction. Only the dentine part of the tooth contains collagen whereas enamel does not.

Between 0.5 and 1 g of sample is required which is broken into pieces measuring a few millimeters in length and width to increase the treatment surface and to fit inside round-bottom plastic tubes (16 × 100 mm) in order to use Ezee™ syringe filters (polypropylene with a polyethylene filter and a 60–90 μm pore size) for the demineralization process. Beforehand, the Ezee™ syringe filters are cleaned three times with Milli-Q™ water. The pieces are immersed in a 2.4 M HCl solution for 15 min and the HCl is removed using Ezee™ syringe filters. Then Milli-Q™ water is added and removed using Ezee™ syringe filters, this is repeated three times to rinse the sample thoroughly. This step also helps in eliminating some organic contaminants (like fulvic acids) and breaking some collagen hydrogen bonds for the further solubilization in water (Longin 1971). To eliminate any other contaminants such as humic acids (Arslanov and Svezhentsev 1993), the sample pieces are placed into a 0.25 M NaOH solution for 15 min and again rinsed with Milli-Q™ water and Ezee™ syringe filters. The pieces are then submerged for a second time in HCl at lower concentration (0.3 M) for 5 min to remove atmospheric CO_2 (which could have been absorbed during the previous step), and to neutralize the base if still present, and then rinsed with Milli-Q™ water. The treated bone fragments are transferred into a Duran® glass tube containing a pH3 HCl solution and left at 90°C for 10 hr. Afterwards, the solution is filtered with a Büchner funnel and a Millipore® glass fiber filter (7 μm pore size, i.e., about 525 kDa threshold) and freeze dried overnight.

The C:N ratio, the collagen yield and the carbon and nitrogen contents are checked, as well as the color and texture of the extracted collagen, to control the collagen quality (Van Klinken 1999). Commonly, well-preserved collagen is white and fluffy while degraded collagen appears brown in color and crystalline (Boudin et al. 2017). For modern bones, the percentage of collagen is around 20 %wt and after burial, the collagen content drops. At RICH, bones containing less than 2% collagen are considered as poorly preserved. Nevertheless, we date all collagen samples having a C:N ratio between 2.9 and 3.6 as C:N is used to indicate contamination (Boudin et al. 2017). The degree of preservation is also followed by the carbon and nitrogen contents. They should range between 15.3 to 47 %wt and 5.5 to 17.3 %wt respectively (Ambrose 1990). All these parameters are taken into account to ensure a reliable date. This method has shown good agreement in inter-laboratory AMS ^{14}C dating (Kuzmin et al. 2018) and for example, in dating skeletons from the same layers (Lull et al. 2016).

Solvent Procedure

Solvents are used in the case of animal textile (wool, silk), leather, hair, rhino horn and when chemical contaminants such as consolidants and preservatives are present on objects. It is necessary to start by the most apolar solvent. The *modus operandi* consists of immersing the sample placed in a beaker into an ultrasonic bath during 15 min in n-hexane (twice), then in acetone (twice), then in absolute ethanol (twice) and finally, once in Milli-Q™ water. The sample is finally dried in the oven at 50°C. If the solution is still opaque after a step, it is repeated until the solution is limpid.

In the case of consolidants, it is better to know the composition to find out which solvent would be the best to dissolve it. The customer may know which product was used or it can be

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determined using Fourier transformed infrared spectroscopy or py-GCMS. In the case of bees wax or unknown varnish, chloroform (stabilized with 0.6% ethanol) is used for 30 min in ultrasonic bath, which can be repeated for 15 min if the solution is opaque, followed by the solvent procedure (as explained previously). When the consolidate is unknown, toluene can also be used twice for 15 min in ultrasonic bath also followed by the solvent procedure.

Further treatment is sometimes required, for samples such as hair (if there is enough material), if the soil matrix contained a lot of organic compounds (e.g. humic acids). A treatment with 0.25 M NaOH (15 min) followed by 0.3 M HCl (15 min) at room temperature can be applied.

As for collagen, following pretreatment, the C:N and the carbon and nitrogen contents are measured to test for remaining contamination and the preservation state. For uncontaminated wool and hair, the C:N ratio is between 3.4 and 3.8 and the carbon and nitrogen contents are respectively around 45 %wt and 15 %wt (Benfer et al. 1978; Boudin et al. 2016). The silk C:N ratio ranges between 2.9 and 3.4 and the carbon and nitrogen contents between 44.3 ± 2.6 %wt and 16.7 ± 0.7 %wt, respectively (Boudin et al. 2014). The reliability of the method was shown by the good agreement of the ^{14}C dates with the presumed historical dates on archaeological silk and wool (Boudin et al. 2016).

In the case of felt-tip pen applied on fragile pieces, the labels are removed with a scalpel and the samples are placed in acetone for 15 min in ultrasonic bath. The treatment is repeated if the liquid is not limpid. The sample is then rinsed with ethanol and with Milli-Q™ water.

Nanofiltration

^{14}C dating of bones is usually performed on the collagen fraction. However, this collagen can contain exogenous molecules, including humic substances 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 ~100 kDalton), has received considerable attention to obtain more reliable dates (Brown et al. 1988; Ramsey et al. 2004). Ultrafiltration is an effective method of removal of low-molecular (<10–15 kDalton depending on the chosen molecular weight cut off of the filter) weight contaminants from bone collagen but it does not remove high-molecular weight contaminants (>10–15 kDalton), 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 (Hüls et al. 2007, 2009). A nanofiltration method was developed at RICH using a ceramic filter to avoid a possible extraneous carbon contamination introduced by the filter. This method is applicable to various contaminated protein materials e.g. collagen, silk, wool, hair. Protein material is considered contaminated if they don't fall within the following C:N range after the pretreatments explained previously (collagen extraction for bone and solvent procedure and AAA for hair, silk and wool):

- Collagen: C:N ratio between 2.9 and 3.6 (DeNiro 1985; Ambrose 1990);
- Wool: C:N between 3.4 and 3.8 (Boudin et al. 2016);
- Silk: C:N between 2.9 and 3.4 (Boudin et al. 2016).

The first step of this method is releasing the amino acids by hot acid hydrolysis of the contaminated protein. Hereafter, the cross-flow nanofiltration is performed and the

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molecular weight cut off of the ceramic filter is 368 Dalton. That enables the separation of the amino acids, all smaller than 368 Dalton, and the contaminants that are larger than 368 Dalton e.g. humic substances. The amino acids are collected in the filtrate (permeate) and the contaminants are collected in the retentate. Finally, the collected amino acids after nanofiltration are radiocarbon dated. To verify if the sample quality is improved after cross-flow nanofiltration C:N analysis of the filtrate (permeate) is performed. If the C:N ratio falls within the C:N ranges as described above, the sample is considered uncontaminated and a more accurate ^{14}C date is obtained. The method is described in detail in Boudin et al. (2013, 2017).

Shells (Marine, Freshwater, and Land Snails)

The pretreatment consists of removing the surface with HCl 0.3 or 2.4 M during a few minutes, the time and concentration depending on sample size, weight and state. It is necessary to assess the shell species since it has been claimed, for example, that some burrowing mollusks living in calcareous rocks can absorb old carbon as their local environment has a lower ^{14}C activity than the surrounding ocean, but in most cases, the ^{14}C activity in shells is close to the activity of the water where the shells live (Mangerud 1972). Most of dating problems that arise from shell dating are mostly linked to hard water effect in marine or lacustrine environment for aquatic shells and from ingestion of limestone for terrestrial gastropods (Goodfriend and Stipp 1983; Douka et al. 2010b). Moreover, diagenetic processes can lead to the replacement of the original crystal structure of shell, which often involves carbonate dissolution, recrystallization, and carbon exchange with the burial environment (Brock et al. 2010; Douka et al. 2010a). X-ray diffraction can be performed to assess the calcium carbonate polymorph (calcite and/or aragonite) and whether recrystallization has occurred. Shells made of aragonite can undergo recrystallization processes to form calcite. Knowing the composition of the shell species and determining if recrystallization happened can help prevent dating problematic shells.

Cremated Bones

Cremated bones no longer contain collagen due to the high temperature of burning, since organic matter disappears completely at temperatures higher than 600°C. The mineral part of the bone is dated with the few remaining carbonates. These were certainly subjected to carbon substitution with the wood during the cremation (Van Strydonck et al. 2010). However, it has been proven that reliable dates can be measured from cremated bones, since in most cases the wood has a similar age to the cremated individual (Major et al. 2019).

For the pretreatment, it is first necessary to clean the samples with a soft or steel brush to remove any visible contaminants such as sediments present on the surface. Charred parts (black in color) are also removed if present. The sample is then weighed. If it weighs more than 1.5 g, the sample may be placed in a 2.4 M HCl solution between 1 and 10 min. This step is carried out to remove the surface of the bone where it is more likely that carbon substitution happens (Van Strydonck et al. 2009). It also helps remove sediments trapped in the bone. The duration of this first step depends on the sample state. The sample is removed from the solution when approximately one third of its volume is dissolved (the sample is weighed before and after this step). If the sample weighs around 1 g but exhibits a high density, it is possible to place it for less than a minute in the 2.4 M HCl solution or in a 0.3 M HCl solution. After this step, the sample is thoroughly rinsed with Milli-Q™

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water and dried at 90°C for 30 min to 1 hr (or overnight at lower temperature). When dry, the sample is weighed and reduced to a fine powder in a ceramic mortar. Then, it is placed in a 0.17 M acetic acid solution for 24 hr, this treatment is performed in order to remove the secondary carbonates possibly present. It is again thoroughly rinsed with Milli-Q™ water and dried between 70 and 100°C during ~1 hr (or overnight). During the rinsing step, around 15% of the sample weight is lost (Rose et al. 2019). Therefore, if the sample weighs less than 1 g, it will be ground after the acetic acid treatment to avoid mass loss. The results of radiocarbon dating using this method are in good agreement with the ones from other laboratories (Naysmith et al. 2007; Rose et al. 2019).

Bone Apatite

Certain burial environments are not suitable for collagen preservation. In these cases, the apatite part of the bone can be dated. Arid and semi-arid environments are the best conditions for the preservation of apatite (Zazzo and Saliège 2011). The pretreatment performed at RICH consists of grinding the bone (with mortar and pestle) and immersing the bone sample (after mechanically cleaning the surface as explained in the collagen extraction section) into a 0.17 M acetic acid solution at room temperature during 24 hr.

Chitin-Containing Materials

For insects remains, they are placed for 15 min in a beaker containing acetone, in ultrasonic bath and rinsed thoroughly with Milli-Q™ water. Afterwards, they are submerged by a 0.3 M HCl solution for 24 hr at room temperature and rinsed again with Milli-Q™ water. This method was adapted from the one proposed by Tripp et al. (2004).

Mortar and Lime Burial

In the case of mortars and lime burials, the whitest and softest pieces are carefully chosen to avoid sand, agglomerates and unburnt limestone. The selected pieces are then crushed with a stainless-steel pestle in a ceramic mortar to obtain a fine powder. This process breaks up the porous, soft mortar carbonate while leaving the harder limestone particles intact. The powder is placed in a metal sifter for sieving (succession of 0.5, 0.25, 0.18, 0.1 mm and a collector) and placed in the shaker (EML200 – Haver and Boeker) at intensity 10 for 10 min. The smallest grains of mortar carbonate fragments pass through the coarser sieves and are thus separated from the larger aggregate grains, including the calcite crystals of the unburned limestone. Therefore, the grain size fraction < 0.1 mm is used for dating.

CO_2 EXTRACTION

For organic samples excluding sediments, the CO_2 extraction is performed thanks to an elemental analyser (Elementar Vario Isotope Select) before graphitization on the AGE. The samples, wrapped in a tin capsule, are dropped into the combustion column and burnt at 800 °C in the presence of O_2 (the column contains CuO and Ag to increase the temperature and catch sulphur) in addition to helium acting as a carrier gas. The gases are then sent to a reduction column with copper to remove the excess oxygen. They pass through a third column containing Sicapent® which absorbs water. The combustion gases (CO_2 and N_2) are then directed to the separation column, where the individual gases are detected by measuring the thermoconductivity.

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Sediments and inorganic samples are processed manually: sealed tube for combustion and graphitization on the manual line. The combustion of sediments is performed in quartz tubes containing copper oxide and a silver wire (to remove any sulfur compounds). These quartz tubes with copper oxide and a silver wire are heated beforehand at 850°C for 8 hr under ambient atmosphere to remove possible contaminants. Then, the sample is introduced into the quartz tube which is evacuated and sealed and the combustion is performed during 1 hr at 1000°C in a tube furnace.

For cremated bones and shells, the pretreated sample reacts *in vacuo* with phosphoric acid in excess to extract the CO_2. For cremated bones only, the CO_2 released is then transferred into a quartz tube with CuO and Ag and heated for 1 hr at 1000°C. This last step is necessary as silver helps in removing sulphur which may inhibit the graphitization.

For the moment, the lead carbonate and mortar samples are not subjected to chemical pretreatment; the CO_2 is directly extracted from selected part of the samples. For lead carbonates, the protocol follows the method developed by Beck et al. (2018, 2019) consisting of chemical decomposition in a quartz tube (without copper oxide) at 400°C for 2 hr.

For mortars and lime burials, the CO_2 fractions are obtained by the titration method or sequential dissolution (Van Strydonck et al. 1982, 2011; Hayen et al. 2017). The powdered mortar sample is kept in suspension while 2 mL of 0.1 M HCl are added for the first four fractions then different amounts of ~2.2 M HCl are added for the remaining fractions. The method is described in detail by Van Strydonck et al. (2015).

GRAPHITIZATION

Organic samples (except sediments) are graphitized on the AGE (Nemec et al. 2010; Wacker et al. 2010; Boudin et al. 2019) which is linked to the Elementar Vario Isotope Select. Sediments, and inorganic carbon-containing material (mortars, cremated bones, shells, bone apatite, etc.) are graphitized on the manual line according to Van Strydonck and Van der Borg (1990). Typically, graphite targets contain 1 mg C, but samples as small as 0.2 mg C can be routinely dated.

PRETREATMENT QUALITY ASSESMENT

The quality control of the pretreatments and the dating is assessed thanks to background/blank samples and international standard samples. The standard samples used at RICH are IAEA-C5 (wood), FIRI D (wood), VIRI T (peat), VIRI O (cellulose), VIRI R (murex shell), TIRI A (barley mash) and FIRI H (wood), some dating results obtained on these samples are presented in Table 2. The results are in good agreement with the consensus values either for 1 or 0.2 mg of graphite.

For the AGE graphitization line, the background samples are an interglacial wood (in house) and a cervical vertebra from a *Bison priscus* (Krasny Yar outcrop, Siberia). The bison bone is used for dating of bones older than 5000 years old. For the manual graphitization line, the IAEA-C1 Carrara is used for inorganic samples and an in-house coal (anthracite) for organic samples. The coal is not processed in the AGE because its combustion is difficult and could cause cross-contamination. Table 3 lists the dating results obtained on these background samples.

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Table 2 ^{14}C analyses of international standards at RICH. Chemical pretreatment was the same whatever the combustion and graphitization way. AGE: samples were combusted and graphitized on the automated graphitization system and manual: samples were combusted and graphitized with the manual line.

Standards	Graphitization line—graphite weight	Consensus value (pMC)	Average measurement value ($F^{14}C$)	n
IAEA-C5 (wood)	AGE—1 mg	0.2305	0.2301 ± 0.0012	38
	AGE—0.2 mg		0.2304 ± 0.0016	10
	Manual—1 mg		0.2301 ± 0.0011	9
FIRI D (wood)	AGE—1 mg	0.5705	0.5700 ± 0.0020	39
	AGE—0.2 mg		0.5700 ± 0.0021	10
	Manual—1 mg		0.5708 ± 0.0020	13
VIRI T (peat)	Manual—1 mg	0.6582	0.6589 ± 0.0028	24
VIRI O (cellulose)	Manual—1 mg	0.9846	0.9826 ± 0.0037	7
FIRI H (wood)	Manual—1 mg	0.7574	0.7560 ± 0.0030	16
VIRI R (murex shell)	Manual—1 mg	0.7334	0.7360 ± 0.0028	5
TIRI A (barley mash)	Manual—1 mg	1.1635	1.1639 ± 0.0043	4

Table 3 ^{14}C analyses of background samples at RICH. AGE is for samples graphitized on the AGE and manual for samples graphitized with the manual line.

Background samples	Graphitization line—graphite weight	Average measurement value ($F^{14}C$)	n
Interglacial wood	AGE—1 mg	0.0022 ± 0.0002	35
	AGE—0.2 mg	0.0053 ± 0.0002	10
Bison bone	AGE—1 mg	0.0038 ± 0.0002	6
Coal	Manual—1 mg	0.0020 ± 0.0001	12
IAEA-C1 (Carrara)	Manual—1 mg	0.0014 ± 0.0002	9

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